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# Diversity of the O-superfamily conotoxins from *Conus miles*

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**Abstract:** Conopeptides display prominent features of hypervariability and high selectivity of large gene families that mediate interactions between organisms. Remarkable sequence diversity of O-superfamily conotoxins was found in a worm-hunting cone snail *Conus miles*. Five novel cDNA sequences encoding O-superfamily precursor peptides were identified in *C. miles* native to Hainan by RT-PCR and 3′-RACE. They share the common cysteine pattern of the O-superfamily conotoxin (C–C–CC–C–C, with three disulfide bridges). The predicted peptides consist of 27–33 amino acids. We then performed a phylogenetic analysis of the new and published homologue sequences from *C. miles* and the other *Conus* species. Sequence divergence (%) and residue substitutions to view evolutionary relationships of the precursors' signal, propeptide, and mature toxin regions were analyzed. Percentage divergence of the amino acid sequences of the prepro region exhibited high conservation, whereas the sequences of the mature peptides ranged from almost identical with to highly divergent from inter- and intra-species. Despite the O-superfamily being a large and diverse group of peptides, widely distributed in the venom ducts of all major feeding types of *Conus* and discovered in several *Conus* species, it was for the first time that the newly found five O-superfamily peptides in this research came from the vermivorous *C. miles*. So far, conotoxins of the O-superfamily whose properties have been characterized are from piscivorous and molluscivorous *Conus* species, and their amino acid sequences and mode of action have been discussed in detail. The elucidated cDNAs of the five toxins are new and of importance and should attract the interest of researchers in the field, which would pave the way for a better understanding of the relationship of their structure and function. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

**Keywords:** *Conus miles* native to Hainan; O-superfamily conotoxins; cDNA cloning; sequence diversity

## INTRODUCTION

The conotoxins isolated from predatory tropical sea snails called *cone snails* (*Conus*) are an important group of venoms that are a rich pharmaceutical treasure as research tools and drug leads. These peptides exhibit a high degree of selectivity and potency for different ion channels and their subtypes, making them invaluable tools for unraveling the secrets of the nervous system [20,4]. Furthermore, several conotoxin molecules have profound applications in drug discovery, with some of them currently undergoing clinical trials [18,30]. Natural prey of cones consist of polychaete worms, other gastropods, pelecypods, octopuses, and small fish [22]. The composition of cone snail venom is extremely complex. It has been estimated that the venom of a single *Conus* species may contain between 50 and 200 different toxin components. Although there may be over 50 000 unique peptide sequences present in the approximately 500 cone species, these sequences may be generally grouped into A-, O-, T-, M-, P-, S-, and I-superfamilies [24]. Some other superfamilies not assigned thus far include conopressins, contryphans, conantokins, and contulakins, all of them being either linear or containing only one disulfide bond [32]. Members of each superfamily have in common a highly conserved signal sequence in their precursors, and family members within a superfamily have a characteristic arrangement of cysteine residues in the mature peptides. The superfamily may be subdivided into several families with distinct pharmacological activities (indicated within brackets in the following): A-superfamily ($\alpha$-, $\alpha$A-,$\kappa$A-, and $\rho$-conotoxins); M-superfamily (μ- and $\psi$-conotoxins); O-superfamily ($\omega$-, μO, $\delta$-, and $\kappa$-conotoxins); P-superfamily (spastic conotoxins); S-superfamily ($\sigma$-conotoxin); T-superfamily ($\tau$- and $\chi$-conotoxins); and I-superfamily (excitatory peptides) [14,36]. This conservation allows direct identification of new peptides belonging to a particular superfamily by cDNA cloning and PCR amplification using primers designed according to the signal sequence of the subfamily or superfamily genes [16,35].

The most extensively studied superfamilies are the A-, M-, and O-superfamilies. At the present time, the O-superfamily appears most diverse in terms of pharmacological function. Family members include $\omega$-conotoxins that block voltage-sensitive calcium channels, $\delta$-conotoxins that delay the inactivation of voltage-sensitive sodium channels, μO-conotoxins that block voltage-gated sodium channels (VGSCs), and $\kappa$-conotoxins that block voltage-gated potassium channels (VGKCs). They share a cysteine pattern (C–C–CC–C–C, with three disulfide bridges) common to O-superfamily conotoxins. For

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most fish- and mollusc-hunting *Conus* species, these three superfamilies (A, M, and O) account for the major proportion of venom peptides. The function of peptides of the other superfamilies in cone venoms is much less well-defined [15]. In spite of O-superfamily being a large and diverse group of peptides, widely distributed in the venom ducts of all major feeding types of conus and discovered in several conus species, no O-superfamily peptides from the worm-hunting cone snail *Conus miles* have been found till now. In this work, we used RT-PCR and 3′-RACE first to identify the O-superfamily peptides from *Conus miles* native to Hainan, and then to view the evolutionary relationships of the precursors' different regions by sequence divergence (%) and residue substitutions. This report provides a clear roadmap for a systematic exploration of O-superfamily conotoxins from *C. miles*.

## MATERIALS AND METHODS

### *Conus Miles* Specimens and Preparation of Total RNA

Specimens of *Conus miles* were collected from the South China Sea of Hainan Province. Live snails were frozen and stored at −80 °C. Their venom ducts were removed and snap-frozen in liquid nitrogen. Venom duct mRNA was prepared from 15 specimens. Venom duct tissue (∼50 mg) was ground into fine powder and homogenized. Total RNA was extracted from ducts and purified using the RNA isolation kit (Shanghai Huasun) according to the instruction manual. The details of total RNA isolation have been described earlier [38].

### cDNA Cloning and Sequencing

cDNA was prepared by the reverse transcription of RNA isolated from the *C. miles* venom duct. Approximately 10 μg of total RNA was transcribed into cDNA using AMV Transcriptase with a universal oligo (dT) 15 primer, or rapid amplification of cDNA 3′ ends (3′-RACE) primer-containing adapter primer, 5′-adapter (dT)15–3′ (Invitrtogen Co.). The resulting cDNA served as a template for PCR.

RT-PCR and 3′-RACE were performed using primers on conserved elements in the untranslated regions (UTRs) and adapter of O-superfamily conopeptides as follows: forward primer 1: 5′CATCGTCAAGATGAAACTGACGTG3′ (24 bp); reverse primer 2: 5′CACAGGTATGGATGACTCAG 3′ (21 bp); reverse primer 3: 5′GGCCACGCGTCGACTAGTAC3′ (3′-RACE adapter, 20 bp). The reverse primer 3 was a shorter version of the adapter primer without the poly dT tail. Conditions for RT-PCR (primer 1 and 2) and 3′-RACE (primer 1 and 3) were 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and a final step of 72 °C for 2 min. Amplification products were purified after separation on a 2.0% agarose gel by the Wizard DNA Clean-Up System (Promega Co.). The resulting PCR fragments were ligated directly into a T-overhang vector (pGEM-T Easy, Promega Co.). Plasmids containing inserts of approximately 300–1000 bp in size were sequenced by the dideoxy chain termination method on an ABI Model 3130 automated sequencer (Applied Biosystems). At least ten clones from each reaction were sequenced. Sequences were edited and assembled using an identity cutoff of 96% to avoid erroneous inclusion of PCR-generated mutants.

### Sequence Diversity Analysis

Sequences were edited to discard vector and adaptor regions, and were analyzed using the DNA sequencing software DNA Club. Sequence alignments were constructed using the DNA Star software. Divergence was calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign, as well as by analysis of the residue substitution rates (the number of residue substitutions) by the Jotun Hein method. The above sequence diversity analyses were specific to precursors' signal, propeptide, and mature toxin regions, respectively. The five novel O-superfamily cDNA sequences from *C. miles* have been deposited in the GenBank Nucleotide Sequence Database.

### Nomenclature

For this work, we employ a special nomenclature. We have abbreviated the 5-sequences obtained from clones as follows: the first two letters (Mi) represent *C. miles*; the next letter and number indicates the clone name in our experiment; the last letter is the clone character (N for nucleotide sequence, P for peptide precurser and M for mature peptide). Thus, the sequence MiK41N, MiK41P and MiK41M correspond, respectively, to the nucleotide sequence, peptide precursor, and mature peptide of the toxin MiK41 from *C. miles*.

## RESULTS

### Gene Cloning and Sequencing of Five Novel Conotoxins from *C. Miles*

The cDNAs of two novel O-superfamily conotoxins MiK41 and MiK42 were cloned using RT-PCR with primer 1 and 2. The 304 bp cDNA of MiK41P encoded a precursor including a signal sequence of 26 amino acids, a pro region of 20 residues, and a *C*-terminal toxin region of 28 amino acids that are predicted to be post-translationally processed (Figure 1(A)). Conotoxin MiK41 is separated from the pro region by the proteolytic site −K. For MiK42P encoded by a 335 bp cDNA, the signal sequence is 25 amino acids, the pro region of 26 amino acids and the *C*-terminal toxin region comprising 31 amino acids that are predicted to be post-translationally processed (Figure 1(B)). Conotoxin MiK42 is separated from the pro region by the proteolytic site – KR. Their precursors (MiK41P and MiK42P) have been deposited in the GenBank with accession numbers DQ141149 and DQ141150, respectively.

Three cDNAs of the novel O-superfamily conotoxins, MiEr95, MiEr93 and MiEr92, were cloned by 3′-RACE with primer 1 and 3. For MiEr95P encoded by a 390 bp cDNA, the signal sequence is 21 amino acids, the pro region is 21 amino acids and the *C*-terminal



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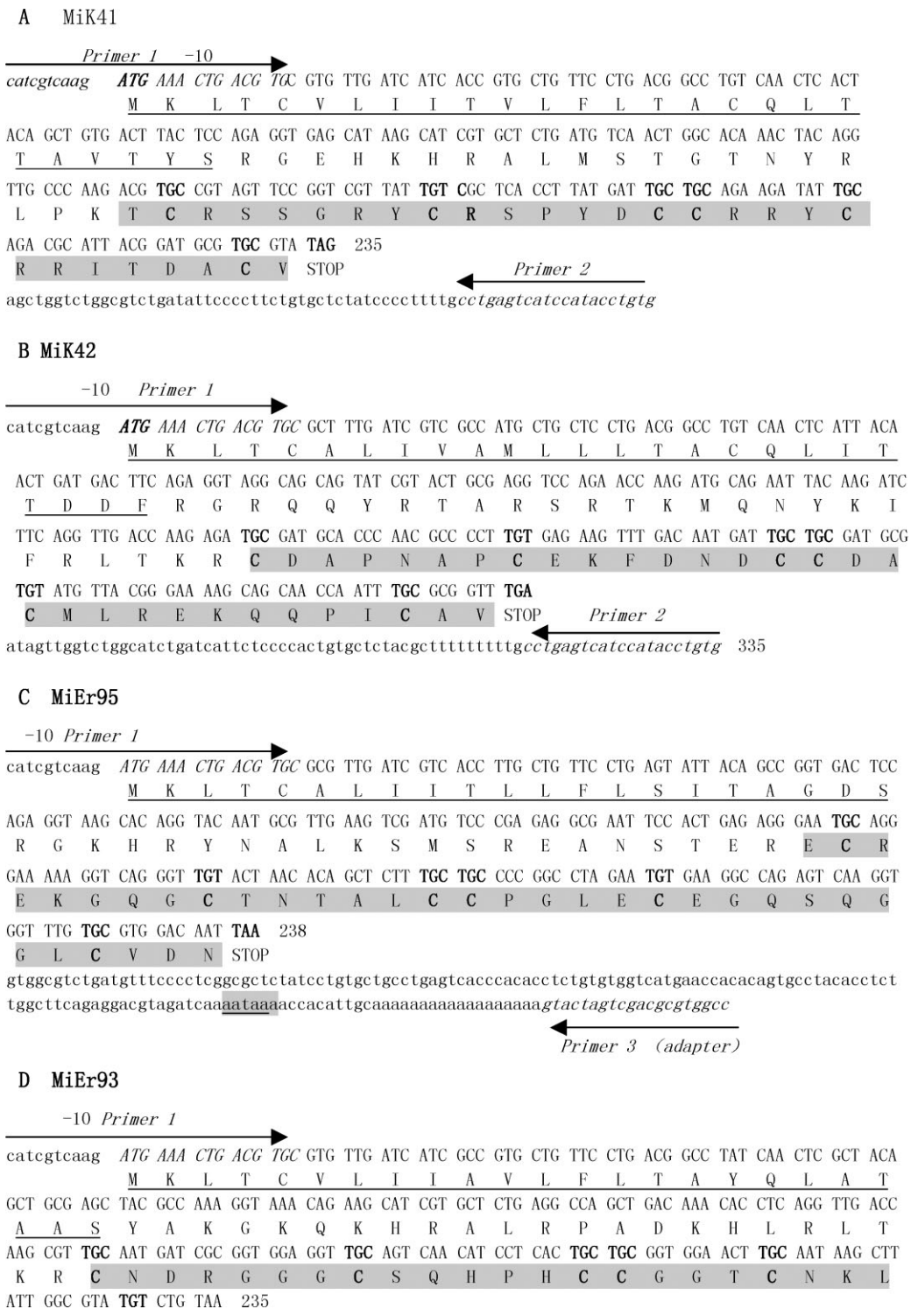


**Figure 1** The cDNA and the deduced sequences of five new O-superfamily conotoxins from *C. miles* native to Hainan, MiK41(A), MiK42(B), MiEr95(C), MiEr93(D), MiEr92(E). The untranslational regions are written in small letters and the signal peptide is underlined. The mature peptide and polyadenylation signal 'aataaa' are shaded. Primers for RT-PCR and 3′-RACE are denoted by italics and indicated with arrows. GenBank accession number of the five cDNAs are MiK41P DQ141149; MiK42P DQ141150; MiEr95P DQ141168; MiEr93P DQ141169; and MiEr92P DQ141170.

toxin region comprises 33 amino acids (Figure 1(C)). The generation of the mature toxin requires proteolytic cleavage (–R) of the *N*-terminal prepro region of the precursor. MiEr93N with 402 bp encoded a precursor including a signal peptide of 24 residues, a propeptide of 23 residues and the *C*-terminal toxin region comprising 27 amino acids (Figure 1(D)). The generation of the mature toxin requires proteolytic



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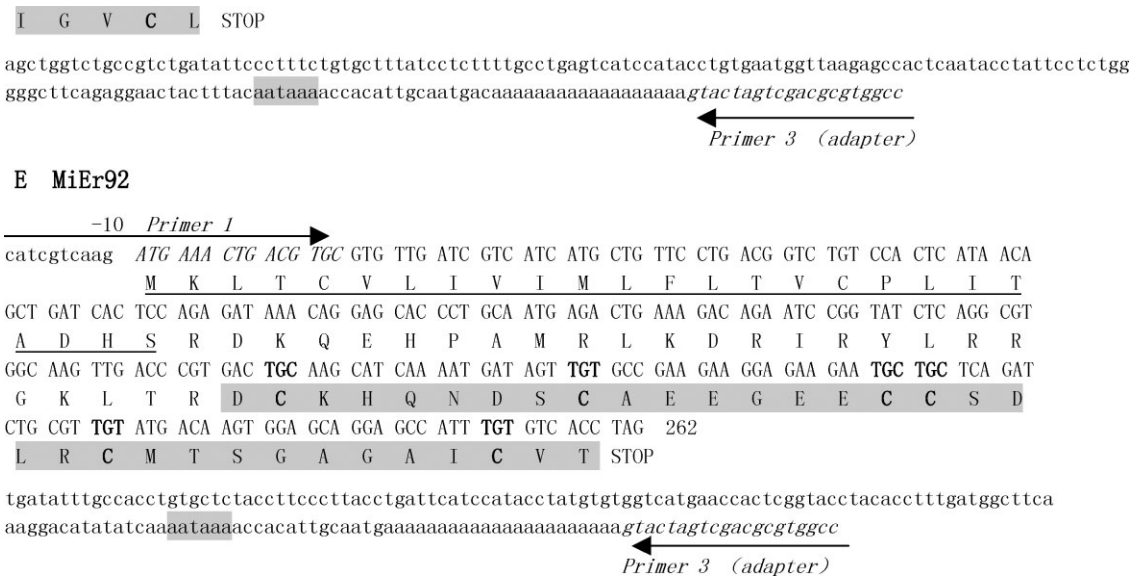


**Figure 1** (*Continued*).

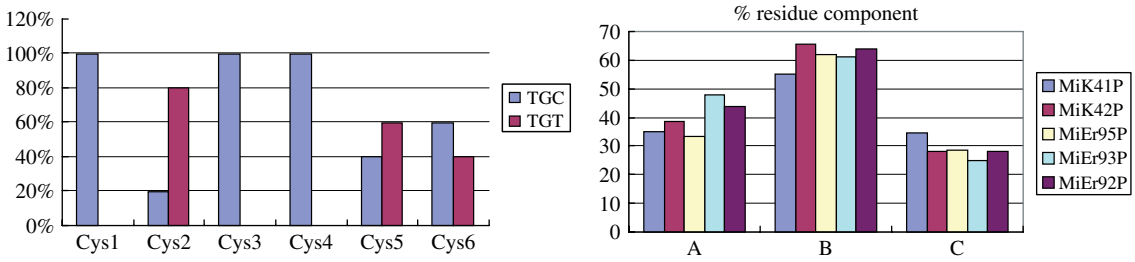


**Figure 2** Cysteine codon conservation within the hypervariable regions (left) and characteristics of the residue component (right) of the five novel conopeptides from *C. miles*. Right panel: (A), percentage of basic residues (R,K,H) in propeptides; (B), percentage of hydrophilic residues in propeptides; (C), percentage of hydrophilic residues in signal.

cleavage (–KR) of the *N*-terminal prepro region of the precursor. MiEr92N with 416 bp encoded a precursor including a signal peptide of 25 residues, a propeptide of 25 residues and the *C*-terminal toxin region comprising 33 amino acids (Figure 1(E)). The generation of the mature toxin requires proteolytic cleavage (–R) of the *N*-terminal prepro region of the precursor. The GenBank accession number of MiEr95P, MiEr93P and MiEr92P are DQ141168, DQ141169, and DQ141170, respectively. There are polyadenylation signal 'aataaa' for PolyA tail addition of all the 3′-UTR of MiEr95N, MiEr93N, and MiEr92N, which were cloned by 3′-RACE (Figure 1(C)–(D)).

## Sequence Analysis of the Five New O-Superfamily Conotoxins from *C. Miles*

In this study, the five newly found *O*-superfamily conotoxins from *C. miles* have the characteristic four-module organization, including a signal sequence, a 'pro' region, the toxin-encoding region followed by an uninterrupted stop codons- and a 3′-untranslated region (3′-UTR). The length range of the 3′- UTR in this study is not large, 152–167 bp, which varies with different peptides (Figure 1). Fragment of RT-PCR with pair primer1–2 of MiK41N and MiK42N is located before polyA, so their 3′-UTR excludes the complete polyA tail. All the five conotoxins share a cysteine pattern ($C^1$–$C^2$–$C^3C^4$–$C^5$–$C^6$, with three disulfide bridges) common to the O-superfamily conotoxins. The generation of the mature toxin requires proteolytic cleavage (–X (K)R or – XK) of the *N*-terminal prepro region of the precursor. It was striking to note that cysteine codons within the hypervariable mature domain are hyperconserved in accordance with Figure 2 (left). A conserved arrangement of cysteine residues generally implies a conserved disulfide configuration. Three cysteines in these peptides exhibit a position-specific codon conservation, the preferred codon triplet for cysteine 1, 3, and 4 being TGC, whereas the triplet TGT encodes cysteine 2 except in MiEr93 (TGC). Cys 5 and Cys 6 displayed no bias with TGC and TGT. Cysteine codon conservancy in this study differs slightly from that shown in the work of Refs 6 and 5.

Notably, propeptide regions consist of a high percentage of hydrophilic residues (55–66%), and most



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residues are basic amino acids (R, K, H) with 33–48%, which might facilitate the precursor processing into mature peptides by the proteolytic function, while the signal regions mainly consist of hydrophobic residues with percentage as low as 25–35% of hydrophilic amino acids, which might facilitate toxins to go through cytoplasm membrane for excretion (Figure 2, right). On the other hand, this study confirms that the striking variability of the mature peptide, i.e. of the amino acids between the cysteine residues, results in a wide diversity of biological activities even within a conopeptide superfamily. The five novel conopeptides in this work consistently have six amino acids between Cys 1 and Cys 2, the same as in the previously identified O-superfamily conotoxins, as verified by us. Fewer residues (4–6) exist between Cys 2 and Cys 3. The least number of residues of 2–4 amino acids are present between Cys 4 and Cys 5. There are 6–9 residues between Cys 5 and Cys 6 from vermivorous *C. miles* of this work, which are different from conotoxins with 4–5 residues of molluscivorous and piscivorous cone snails, e.g. interval residue number of $C^1$–$C^2$, $C^2$–$C^3$, $C^4$–$C^5$, $C^5$–$C^6$ of μO-MrVIA and μO-MrVIB from molluscivorous *C. marmoreus* is 6–9–4–4 [21]; of $\delta$-PVIA and $\delta$-SVIE from piscivorous *C. purpurascens* and *C. striatus*, respectively, is 6–6–3–4 [26,3]; of $\kappa$-Conotoxin PVIIA from piscivorous *C. purpurascens* is 6–6–3–5 [28]. Those of the piscivorous $\omega$-conotoxin GVIA from *C. geographus*, MVIIA and MVIIC from *C. magus*, and CVID from *C. catus* are 6–6–2–5; 6–6–3–4; 6–5–3–5; and 6–6–3–6, respectively [32].

In view of the specific residues in the mature region, it is especially striking that there are 7 basic arginines of 28 residues with high 25% in MiK41M; 6 glycines appear in 27 residues of MiEr93M; and about 20% of acidic aspartic acids and glutamic acids exist in both MiK42M and MiEr92M. The relationship between the high ratio of some specific residues in the toxin region and their bioactivities remains intriguing. However, it is noteworthy that conotoxin structure analysis would be beneficial for function research of the corresponding gene families, even though their potential biological activity is not clear so far.

## DISCUSSION

We used the NCBI database to search for genes and peptides homologous with the newly found O-superfamily conotoxins from *C. miles*. The amino acid sequences of 16 conotoxins had been described (Figure 3). In the present study, sequence diversity analysis displayed cDNA clones encoding five novel precursor peptides belonging to the MKLT family of four-loop scaffold conopeptides [5]. This group of peptides is characterized by a specific cysteine framework (C–C–CC–C–C), designated as pattern VI/VII. They share a high conservancy in the signal sequence of the prepropeptide region and high intra- and interspecies variability of the sequence of the mature peptides particularly of their inter-cysteine regions.

Notably, there are two conopeptides (MgJr93, MgJ42) that consist of exactly the same precursor except for one residue difference both in signals of MgJ42P(F) and MgJr93P (S) and in the mature region of MgJ42P(T)

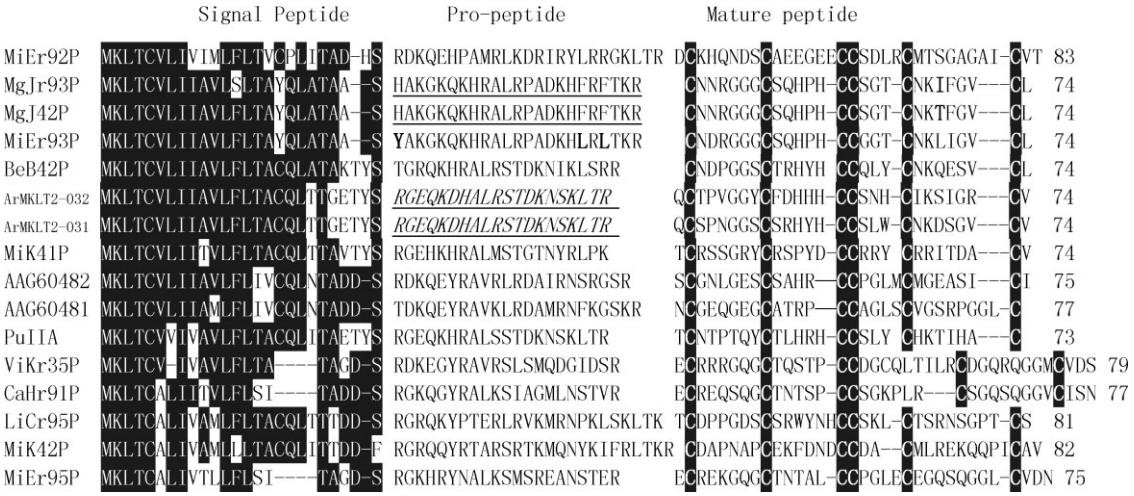


**Figure 3** Comparing five conotoxin prepropeptide sequences newly found from *C. miles* with other homologous O-superfamily conotoxins (MKLTC-clade). The highly conservative amino acids of signal sequences and the cysteine residues of the toxin regions appear in bold print with a black background. The GenBank accession numbers of the previously identified O-superfamily conotoxins are: MiK41P AAZ83750, BeB42P AAZ83782, ArMKLT2-031 AAG60488, AAG60485 ArMKLT2-032, ArMKLT2-0321 AAG60481, ArMKLT2-0322 AAG60482, MiEr92P AAZ83769, MiEr93P AAZ83768, MgJr93P AAZ83772, MgJ42P AAZ83752, PuIIA AAD33586, MiK42P AAZ83751, MiEr95P AAZ83767, LiCr95P AAZ83766, CaHr91P AAZ83770, ViKr35P AAZ83778, MiK41P AAZ83750, MiEr93P AAZ83768, and LiCr95P AAZ83766.



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and MgJr93P (I). Signal and pro regions of the two peptides (ArMKLT2-031 and ArMKLT2-032) are exactly the same with different mature peptides (Figure 3). These might be induced by the allelic selection of toxins in predatory cone snails [8]. So the percentage divergences between MgJr93, MgJ42, and MiEr93 are very low: 0–4.1 for signal, 0–13.7 for pro region, 3.7–15.9 for mature peptides (Tables 1–3). No signal and pro region divergence (0%) between ArMKLT2-031 and ArMKLT2-032 (Tables 1 and 2) is found; in contrast, mature region divergence between the two is as high as 62.6% (Table 3). The divergence of the mature toxin region is the highest (37.3–100%) compared with the signal region (0–79.9%) and the pro region (22–100%). Quite a number of signal and pro regions between any pair of precursors are very different from each other with 100% divergence (Tables 2 and 3). For the toxin region, the identity mainly originates from the conserved disulfide pattern. The divergence (D) relationships between sequence pairs of the three regions are D mature peptide region > D pro region > D signal (Tables 1–3). So it indicates that the O-superfamily conotoxins from *C. miles* have a prominent characteristic of diversity.

Previous authors have suggested that conotoxins undergo accelerated evolution, and their mature domain undergoes accelerated mutation [9,5]. Residue substitution analysis in different regions of the conopeptides in the present work confirms that this hypothesis holds also for the MKLT family of O-superfamily conopeptides found in our data set (Figure 4). Residue substitutions give rise to sequence

**Table 1** Sequence divergence (%) between the signal sequences of the 16 O-superfamily conopeptides

| 1 MiEr92s | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 MgJr93s | 58.4 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 3 MgJ42s | 49.4 | 4.1 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 4 MiEr93s | 49.4 | 4.1 | 0 | — | — | — | — | — | — | — | — | — | — | — | — |
| 5 ViKr35s | 28.7 | 36 | 28.7 | 28.7 | — | — | — | — | — | — | — | — | — | — | — |
| 6 CaHr91s | 41.3 | 58.4 | 49.4 | 49.4 | 36 | — | — | — | — | — | — | — | — | — | — |
| 7 MiEr95s | 41.3 | 79.9 | 68.5 | 68.5 | 28.7 | 15.1 | — | — | — | — | — | — | — | — | — |
| 8 AAG60482s | 33.4 | 41.3 | 33.9 | 33.9 | 22 | 20.9 | 41.3 | — | — | — | — | — | — | — | — |
| 9 AAG60481s | 27.6 | 49.4 | 41.3 | 41.3 | 28.7 | 27.1 | 41.3 | 4 | — | — | — | — | — | — | — |
| 10 BeB42s | 39.5 | 33.9 | 27.1 | 27.1 | 22 | 27.1 | 41.3 | 27.6 | 33.4 | — | — | — | — | — | — |
| 11 LiCr95s | 33.4 | 41.3 | 33.9 | 33.9 | 22 | 33.9 | 33.9 | 33.4 | 27.6 | 33.4 | — | — | — | — | — |
| 12 MiK42s | 39.5 | 49.4 | 49.4 | 49.4 | 36 | 49.4 | 49.4 | 46.2 | 39.5 | 53.5 | 12.6 | — | — | — | — |
| 13ArMKLT2-032s | 46.2 | 27.1 | 20.9 | 20.9 | 22 | 41.3 | 58.4 | 27.6 | 33.4 | 12.1 | 27.6 | 46.2 | — | — | — |
| 14ArMKLT2-031s | 46.2 | 27.1 | 20.9 | 20.9 | 22 | 41.3 | 58.4 | 27.6 | 33.4 | 12.1 | 27.6 | 46.2 | 0 | — | — |
| 15 MiK41s | 39.5 | 27.1 | 20.9 | 20.9 | 15.9 | 33.9 | 49.4 | 22.3 | 27.6 | 12.1 | 33.4 | 46.2 | 12.1 | 12.1 | — |
| 16 PuIIAs | 33.4 | 41.3 | 33.9 | 33.9 | 10.2 | 49.4 | 49.4 | 33.4 | 39.5 | 21.3 | 33.4 | 39.5 | 16.6 | 16.6 | 16.6 |

**Table 2** Sequence divergence (%) between the pro regions of the 16 O-superfamily conopeptides

| 1 MiEr92 pro | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 MgJr93 pro | 100 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 3 MgJ42 pro | 100 | 0 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 4 MiEr93 pro | 100 | 13.7 | 13.7 | — | — | — | — | — | — | — | — | — | — | — | — |
| 5 ViKr35 pro | 100 | 100 | 100 | 100 | — | — | — | — | — | — | — | — | — | — | — |
| 6 CaHr91 pro | 100 | 100 | 100 | 92.9 | 100 | — | — | — | — | — | — | — | — | — | — |
| 7 MiEr95 pro | 100 | 100 | 100 | 100 | 100 | 58.4 | — | — | — | — | — | — | — | — | — |
| 8 AAG60482 pro | 64 | 100 | 100 | 100 | 79.9 | 100 | 100 | — | — | — | — | — | — | — | — |
| 9 AAG60481 pro | 85.8 | 100 | 100 | 100 | 92.9 | 100 | 100 | 25.7 | — | — | — | — | — | — | — |
| 10 BeB42 pro | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | — | — | — | — | — | — |
| 11 LiCr95 pro | 99.2 | 100 | 100 | 100 | 100 | 100 | 100 | 99.2 | 100 | 100 | — | — | — | — | — |
| 12 MiK42 pro | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 53.5 | — | — | — | — |
| 13 ArMKLT2-032 pro | 100 | 92.9 | 92.9 | 79.9 | 100 | 100 | 100 | 100 | 100 | 73.7 | 100 | 100 | — | — | — |
| 14 ArMKLT2-031 pro | 100 | 92.9 | 92.9 | 79.9 | 100 | 100 | 100 | 100 | 100 | 73.7 | 100 | 100 | 0 | — | — |
| 15 MiK41 pro | 100 | 68.5 | 68.5 | 58.4 | 100 | 100 | 100 | 100 | 100 | 73.7 | 100 | 100 | 41.3 | 41.3 | — |
| 16 PuIIA pro | 100 | 62.6 | 62.6 | 52.8 | 100 | 86.4 | 100 | 100 | 100 | 52.8 | 100 | 100 | 22 | 22 | 28.7 |



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**Table 3** Sequence divergence (%) between the mature toxin regions of the 16 O-superfamily conopeptides

| 1 MiEr92m | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 MgJr93m | 100 | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 3 MgJ42m | 100 | 3.7 | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 4 MiEr93m | 100 | 15.9 | 15.9 | — | — | — | — | — | — | — | — | — | — | — | — |
| 5 ViKr35m | 100 | 100 | 100 | 100 | — | — | — | — | — | — | — | — | — | — | — |
| 6 CaHr91m | 100 | 100 | 100 | 100 | 60 | — | — | — | — | — | — | — | — | — | — |
| 7 MiEr95m | 100 | 100 | 100 | 100 | 47.5 | 37.3 | — | — | — | — | — | — | — | — | — |
| 8 AAG60482m | 100 | 100 | 100 | 100 | 100 | 100 | 100 | — | — | — | — | — | — | — | — |
| 9 AAG60481m | 100 | 100 | 100 | 100 | 71.4 | 63.7 | 63.7 | 89.9 | — | — | — | — | — | — | — |
| 10 BeB42m | 100 | 62.6 | 62.6 | 55.1 | 100 | 100 | 100 | 100 | 100 | — | — | — | — | — | — |
| 11 LiCr95m | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | — | — | — | — | — |
| 12 MiK42m | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | — | — | — | — |
| 13 ArMKLT2-032m | 100 | 79.9 | 79.9 | 79.9 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | — | — | — |
| 14 ArMKLT2-031m | 100 | 55.1 | 55.1 | 62.6 | 100 | 100 | 100 | 100 | 100 | 48.2 | 84.9 | 100 | 62.6 | — | — |
| 15 MiK41m | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | — |
| 16 PuIIAm | 100 | 100 | 96 | 100 | 100 | 100 | 100 | 100 | 100 | 75 | 100 | 100 | 96 | 100 | 100 |

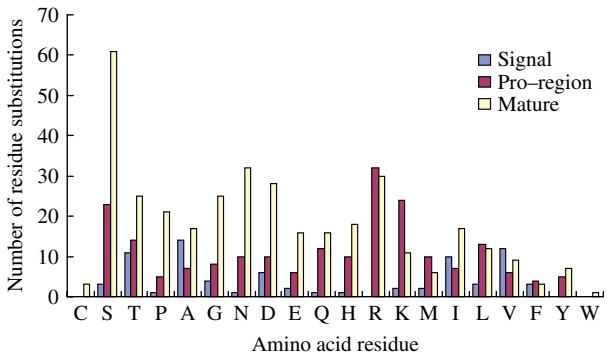


**Figure 4** Different apparent residue substitutions in the different domains of current alignment families for conopeptide precursor.

differences in the current alignment, which may be useful in identifying unusual patterns of substitutions and give the current phylogenetic tree relationships. Substitutions involving smaller distances are chosen over alternative residue substitutions, which would involve greater distances. As seen in Figure 4, the number of residue substitutions in the mature peptide region is significantly higher than that observed for the signal domain, with the propeptide region exhibiting an intermediate value. Examination of the mature peptide domain showed that substitutions between Ser (S) and many other residues including T (Thr), G (Gly), R (Arg), P (Pro), A (Ala), N (Asn), D (Asp) are typically very common; that also happened between R and S, H (His), K (Lys); between N and S, P, D; as well as between T and S, I (Ile), whereas substitutions between C (Cys), F (Phe), W (Trp) and the other residues are rare. As for the propeptide region, substitutions between K and R are the most common, followed by that between S and T, K and that between L (Leu) and I, whereas no substitutions happened between C, W and the other residues. Substitutions between P, F and the rest are also rare. As for the signal domain, most residue substitutions are very rare except for the few between T and A, I; and V (Val) and I. There are no substitutions of the four residues C, R, Y(Tyr), and W. Marked deviations from typical patterns like C and R, K may signal residues of structural interest. Therefore cysteine codons within the hypervariable mature domain are 'hyperconserved'. High frequency of conserved arginine and lysine residues appear in propeptide domain that forms part of the propeptide cleavage site. It may be the reason for the highest residue substitutions that happened between K and R, and low in the signal domain. Moreover, the substitution rates for the mature domains are almost certainly underestimated owing to the occurrence of multiple substitutions per site (apparent homoplasy) in the mature region, as shown in Figure 4. The difference in residue substitutions between different regions in these conopeptide precursors is also reflected in their degree of divergence, as demonstrated by the sequence divergence (%) shown in Tables 1–3.

Currently, lethal toxins produced by cone snails are in great demand for neuroscience research and are being developed as potent drugs. Conotoxins produced by *C. miles* are not an exception. There have been no conopeptide genes discovered from *C. miles* before this work using the NCBI database to search for homologous peptides. We do not know these conotoxins' biological activities now. However, previous research demonstrated that O-superfamily conotoxins are each typically selectively targeted to a diverse set of different molecular isoforms within voltage-gated calcium, sodium and potassium channels (Table 4), which are widely used in neuroscience as pharmacological tools for relative ion channel researches, some having been through extensive human clinical trials.



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**Table 4** Properties of characterized O-superfamily conotoxins ($C^1$–$C^2$–$C^3C^4$–$C^5$–$C^6$)

| Class | Source | Name | Sequence(*) | Mode of action (Target) | Ref. |
|---|---|---|---|---|---|
| ω | ***Piscivorous*** | | | | |
| | *C. geographus* | GVIB | CKSOGSSCSOTSYN---CCR-S-CNOYTKRCYG | N/P/Q-type $Ca^{2+}$ channels | [12], [24], [31] |
| | | GVIC | CKSOGSSCSOTSYN---CCR-S-CNOYTKRC | | |
| | | GVIIA | CKSOGTOCSRGMRD---CCT-S-CLLYSNKCRRY | | |
| | | GVIIB | CKSOGTOCSRGMRD---CCT-S-CLSYSNKCRRY | | |
| | | GVIA | CKSOGSSCSOTSYN---CCR-S-CNOYTKRCY | N-type $Ca^{2+}$channels | |
| | *C. magus* | MVIIA | CKGKGAKCSRLMYD---CCTGS-CR--SGKC | | |
| | | MVIIC | CKGKGAPCRKTMYD---CCSGS-CNR—RGRC | P/Q-type $Ca^{2+}$ channels | |
| | | MVIIB | CKGKGASCHRTSYD---CCTGS-CNR--GKC | N/P/Q-type $Ca^{2+}$ channels | [1], [23], [28], [31] |
| | | MVIID | CQGRGASCRKTMYN---CCSGS-CNR--GRC | | |
| | *C. catus* | CVIB | CKGKGASCRKTMYD---CCRGS-CR--SGRC | | |
| | | CVIC | CKGKGQSCSKLMYD---CCTGS-CSR-RGKC | | |
| | | CVIA | CKSTGASCRRTSYD---CCTGS-CR--SGRC | N-type $Ca^{2+}$ channels | |
| | | CVID | CKGKGAKCSKLMYD---CCSGS-CSGTVGRC | | |
| | *C. consors* | CnVIIA | CKGKGAOCTRLMYD---CCHGS-CSSSKGRC | | |
| | *C. tulipa* | TVIA | CLSOGSSCSOTSYN---CCR-S-CNOYSRKCR | | |
| | *C. striatus* | SVIA | CRSSGSOCGVTSI----CC-GR-C--YRGRCT | N/P/Q-type $Ca^{2+}$ channels | |
| | | SVIB | CKLKGQSCRKTSYD---CCSGS-CGR-SGKC | P/Q-type $Ca^{2+}$ channels | |
| | *C. radiatus* | RVIA | CKPOGSOCRVSSYN---CCS-S-CKSYNKKC | | |
| | ***Molluscivorous*** | | | | |
| | *C. pennaceus* | PnVIA | G--CLEVDYFCGIPFANNGLCCSGN-CVFV--CTPQ | L-type $Ca^{2+}$ channels | [1], [16] |
| | | PnVIB | DDDCEPPGNFCGMIKIGPP-CCSGW-CFFA--CA | | |
| | *C. textile* | TxVIIA | ---CKQADEPCDVFSLD---CCTGI-C--LGVCMW | | |
| μ 0 | *C. marmoreus* | MrVIA | --ACRKKWEYCIVPIIGFIYCCPGLICGPFV-CV | Diverse. $Na^+$ a nd $Ca^{2+}$ channels | [6,9,20, 32,38] |
| | | MrVIB | --ACSKKWEYCIVPILGFVYCCPGLICGPFV-CV | | |
| δ | *C. textile* | TxVIA | --WCKQSGEMCNVLDQN---CCDGY-CIVFV-CT | $Na^+$ channels | [10] [23] [31] |
| | | TxVIB | --WCKQSGEMCNLLDQN---CCDGY-CIVLV-CY | | |
| | | TxVIIA | ---CGGYSTYC-γV-DSγ---CCSDN-CVRSY-CTLF | | |
| | *C. gloriamaris* | GmVIA | VKPCRKEGQLCDPIFQN---CCRGWNC-VLF-CV | | [11,26] |
| | *C. negropuntatus* | NgVIA | -SKCFSOGTFCGIKOGL---CCSVR-CFSLF-CISFE | | [1] [23] |
| | *C. amadis* | Am2766 | ---CKQAGESCDI---FSQNCCVGT-CAFI--CIE | | |
| | ***Piscivorous*** | | | | |
| | *C. purpurascens* | PVIA | EACYAOGTFCGIKOGL---CCSEF-CLPGV-CFG | Diverse, Nav1.2 | [18], [36] |
| | *C. striatus* | SVIE | DGCSSGGTFCGIHPGL---CCSEF-CFLW--CITFID | Diverse, $Na^+$ channels | |
| | *C. magus* | MVIA | DGCYNAGTFCGIROGL---CCSEF-CFLW--CITFVDS | $Na^+$ channels | [1] |
| | *C. catus* | CVIE | YGCSNAGAFCGIHPGL---CCSEL-CLVW--CT | | |
| | *C. ermineus* | EVIA | DDCIKOYGFCSLPILKNGLCCSGA-C-VGV-CADL | Nav1.2a, Nav1.3 and Nav1.6, $Na^+$ channels | [2] |
| κ | *C. purpurascens* | PVIIA | --CRIONQKCFQHLDD---CCSRK-CNRFNKCV | Shaker potassium channel | [27,32] |

[a] O = hydroxyproline.

Conotoxins that target $Ca^{2+}$ channels have proven to be valuable not only as research tools but also as new therapeutics for the treatment of chronic pain. For example, in *C. magus* venom, the ω-conotoxin MVIIA is highly specific for N-type calcium channels (Cav2.2), while another peptide from the same venom, ω-conotoxin MVIIC, preferentially targets P/Q channels (Cav2.1) [25,13]. The molluscan L-type channel blocker ω-TxVIIA is unusual in that it may bind to the L-type channel at a site similar to that occupied by low molecular weight L-type blockers such as dihydropyridines, phenylalkylamines, and benzothiazepines, with Phe11 and Trp26 playing key roles [17,1]. Calcium channels are current or potential targets for therapeutics directed against intractable pain, ischemic brain damage, migraine, and some forms of epilepsy and ataxia [23]. In December 2004, ω-conotoxin MVIIA has been approved by the FDA of USA for AIDS and cancer pain medication [31]. It has been assigned the generic name ziconotide and the commercial name Prialt® by its developers, Elan Pharmaceuticals. ω-CVID, isolated and characterized from *C. catus* is being explored as an antinociceptive agent in Phase II clinical trials [29,18] (Table 4). A significant advantage of the ω-conotoxins over opiods such as morphine is that neither tolerance nor addiction develops with chronic administration.

Two distinct peptides (MrVIA and MrVIB) that belong to μO-class of O-superfamily from molluscivorous *C. marmoreus* were previously well characterized, which have diverse modes of action, e.g. affecting both sodium and calcium currents in molluscan neurons,



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being inhibitors of tetrodotoxin (TTX)-sensitive and TTX-resistant sodium channels in mammalian sensory neurons [7,10,21,33,39].

δ-Conotoxins target VGSCs, causing delayed inactivation. δ-TxVIA from snail-hunting *C. textile* prolongs $Na^+$ currents in molluscan neuronal membranes, but in vertebrate systems it binds to $Na^+$ channels without any toxic effects [11]. δ-PVIA from the fish-hunting *C. purpurascens* elicits excitatory symptoms in mice and fish, but is inactive in molluscs even at 100-fold higher doses [26]. δ-PVIA slows fast inactivation of Nav1.2-mediated currents expressed in Xenopus, as well as of $Na^+$ currents recorded from hippocampal neurons in culture [34]. δ-EVIA from fish-hunting *C. ermineus* [2] affects several rat neuronal $Na^+$ channel subtypes (Nav1.2a, Nav1.3 and Nav1.6) but not muscle subtypes (Nav1.4 and Nav1.5). δ-GmVIA from snail-hunting *C. gloriamaris* causes action potential broadening in Aplysia neurons [12,27]. Conotoxins δ-PVIA and δ-SVIE had comparable effects in delaying inactivation of $Na^+$ currents in dissociated sympathetic neurons from frog, but δ-SVIE was irreversible [37]. δ-SVIE was shown recently to interact with a conserved hydrophobic triad located in the domain-4 voltage sensor of the sodium channels [19].

$K^+$ channels are important not only for the repolarization phase of action potentials, but also in setting the resting membrane potential, and have a variety of specialized purposes in a wide range of cell types. The κ-conotoxin class is a member of the O-superfamily targeting $K^+$ channels (Table 4). κ-Ctx PVIIA from *C. purpurascens* was the first conotoxin used to block VGKC. This peptide inhibits the Shaker potassium channel and, together with other well-studied VGKC inhibitors such as charybdotoxin and the agitoxins from scorpion venoms, is useful for probing potassium channel structures [28,33].

Each *Conus* species has more than 100 peptides in its venom. So most *C. miles* conopeptides need to be explored further. For the first time, in this paper, we have shown from the sequence information about the five new O-superfamily peptides from *C. miles* that they are structurally related to the well-characterized conopeptides. This approach may help in designing and synthesizing peptides acting on particular targets such as receptors or ion channels. At times, this is also the only successful way, since identification of a peptide with specific pharmacological activity in a conus venom itself often fails either because of the limited amount of venom available or because of the chance that the peptide may be expressed only at low rate or not at all. So cDNA cloning and PCR amplification according to signal conservancy is an effective way for identification of new peptides [16,35]. For the neurobiologist, this will provide a tool kit to probe the different voltage-gated ion channels and define their functional biology in nervous systems. The diversity analysis is attractive in that it provides explanations for both the large number of toxin variants in conus venoms and the large number of *Conus* species in shallow marine ecosystems. The high intra-species diversity of the current alignment peptides implies hypermutation acting on particular toxin gene sequences. The mechanisms triggering these processes are largely unknown. Studies at the inter- and intra-species level may also provide clues for a better understanding of the phylogeny of these peptides.

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