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Novel α-conotoxins identified by gene sequencing from cone snails native to Hainan, and their sequence diversity

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Abstract: Conotoxins (CTX) from the venom of marine cone snails (genus *Conus*) represent large families of proteins, which show a similar precursor organization with surprisingly conserved signal sequence of the precursor peptides, but highly diverse pharmacological activities. By using the conserved sequences found within the genes that encode the α-conotoxin precursors, a technique based on RT-PCR was used to identify, respectively, two novel peptides (LiC22, LeD2) from the two worm-hunting *Conus* species *Conus lividus*, and *Conus litteratus*, and one novel peptide (TeA21) from the snail-hunting *Conus* species *Conus textile*, all native to Hainan in China. The three peptides share an α4/7 subfamily α-conotoxins common cysteine pattern (CCX_4CX_7C, two disulfide bonds), which are competitive antagonists of nicotinic acetylcholine receptor (nAChRs). The cDNA of LiC22N encodes a precursor of 40 residues, including a propeptide of 19 residues and a mature peptide of 21 residues. The cDNA of LeD2N encodes a precursor of 41 residues, including a propeptide of 21 residues and a mature peptide of 16 residues with three additional Gly residues. The cDNA of TeA21N encodes a precursor of 38 residues, including a propeptide of 20 residues and a mature peptide of 17 residues with an additional residue Gly. The additional residue Gly of LeD2N and TeA21N is a prerequisite for the amidation of the preceding *C*-terminal Cys. All three sequences are processed at the common signal site -X-Arg- immediately before the mature peptide sequences. The properties of the α4/7 conotoxins known so far were discussed in detail. Phylogenetic analysis of the new conotoxins in the present study and the published homologue of α4/7 conotoxins from the other *Conus* species were performed systematically. Patterns of sequence divergence for the three regions of signal, proregion, and mature peptides, both nucleotide acids and residue substitutions in DNA and peptide levels, as well as Cys codon usage were analyzed, which suggest how these separate branches originated. Percent identities of the DNA and amino acid sequences of the signal region exhibited high conservation, whereas the sequences of the mature peptides ranged from almost identical to highly divergent between inter- and intra-species. Notably, the diversity of the proregion was also high, with an intermediate percentage of divergence between that observed in the signal and in the toxin regions. The data presented are new and are of importance, and should attract the interest of researchers in this field. The elucidated cDNAs of these toxins will facilitate a better understanding of the relationship of their structure and function, as well as the process of their evolutionary relationships. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: α-conotoxins; RT-PCR; sequence diversity; *Conus lividus*; *Conus litteratus and Conus textile* native to Hainan

INTRODUCTION

The venom of carnivorous marine cone snails (genus *Conus*) provides a valuable source of biologically active peptides. Genus *Conus* is perhaps the most successful genus of marine invertebrates with over 500 species, all of which are venomous [1]. These toxins are composed of a complex mixture of disulfide-rich ion channel neurotoxins, commonly known as conotoxins (CTX), many of which target both the central and peripheral nervous systems. This diverse class of molecules provides a source of potent ion channel ligands that are highly selective for different classes of ion channel receptors and transporters. Their high degree of potency and selectivity makes them excellent tools for studying the nervous system [2]. *Conus* peptides have attracted extensive attention because of their potential to be developed as new research tools in the field of neuroscience and as novel medications in clinical use for pain, epilepsy, and other neuropathic disorders. Several are being directly developed as diagnostic and therapeutic agents [3–6]. The synthetic version of peptide MVIIA from *Conus magus* has been approved for treatment of chronic pain in humans, including chronic, intractable pain suffered by people with cancer, AIDS, injury, failed back-surgery, or certain nervous system disorders [7,8]. 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. Thus, the total number of conotoxins among the entire genus may consist of over 50 000 distinct neurologically active peptides [9].

Generally, conotoxins consist of 8–40 amino acid residues and, although small, they contain many of the structural elements present in larger proteins, including α-helices, β-sheets, and β-turns, and hence they

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are often referred to as mini proteins [10,11]. Many *Conus* peptide precursors have been elucidated via cDNA cloning. Each *Conus* peptide is initially translated as a larger prepropeptide precursor; a mature peptide of 8–40 amino acids is generally processed from a 60 to 90 amino acid precursor, with a single copy of the toxin located at the *C*-terminus. Conotoxins have been divided into superfamilies, determined by their disulfide bond framework, and further subdivided into classes according to their mode of action. Seven superfamilies of conotoxins are well characterized, namely, A-superfamily consisting of several distinct pharmacological families such as α-, αA-, κA-, and ρ-CTXs; O-superfamily consisting of ω-, κ-, δ-, μO-, and γ-CTXs; M-superfamily (μ- and ψ-CTXs); T-superfamily (τ- and χ-CTXs); P-superfamily (spastics CTXs); S-superfamily (σ-CTXs); and I-superfamily (excitatory peptides). Some other superfamilies that are not yet assigned include conopressins, contryphans, conantokins, and contulakins; all of them are either linear or contain only one disulfide bond [12,13]. Peptides within one superfamily, even from different *Conus* species, share a rather conserved pattern of disulfide bonds and a highly conserved signal sequence [14–17]. This conservancy 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 [18,19].

The A-superfamily of *Conus* peptides share two common cysteine patterns, one is α-, ρ-CTXs (CC–C–C; disulfide framework 1–3, 2–4; two disulfide bonds), the other pattern is αA-, κA-(CC–C–C–C–C; disulfide framework 1–5, 2–3, 4–6; three disulfide bonds). Although these are characteristic structural features, peptides of A-superfamily have different targets: α-, αA-CTXs block nicotinic acetyl choline receptor (nAChR); κA-CTXs block voltage-gated potassium channels (VGKC); ρ-CTXs block α_1-adrenoreceptors [20–22]. Besides the isolation and structural elucidation of these peptides from crude *Conus* venoms, new conopeptides belonging to the A-superfamily have been identified by cDNA cloning [23,24].

Perhaps the most conserved feature of cone snail venom is the α-conotoxins; these are a series of structurally and functionally related peptides that target nAChRs. Every venom examined thus far has its own distinct complement of nicotinic receptor antagonists, suggesting that, within the genus, there are literally thousands of novel peptides that act on nAChRs [25]. nAChRs are widely distributed in both the central and peripheral nervous system. A major advancement in recent years in the neuropharmacology of nAChRs has been the ability to more readily characterize particular neuronal subtypes by using specific conotoxins [26]. The carnivorous marine snails of the genus *Conus* are a rich source of peptides targeted to nAChRs. Without doubt, additional *Conus* peptides with novel nicotinic subtype specificity will be discovered in the coming years [27–29].

In this study we report that three α-conotoxins, LiC22, LeD2, and TeA21, were, respectively, identified by gene sequencing with RT-PCR from *Conus lividus, Conus litteratus,* and *Conus textile*, which are native to Hainan in China. They share an α4/7 subfamily α-conotoxins common cysteine pattern (CCX_4-CX_7-C, two disulfide bonds), which are competitive antagonists of nAChRs. Phylogenetic analysis of the new conotoxins in the present study and the published homologue α-conotoxins sequences from the other *Conus* species was performed systematically. Patterns of sequence divergence for three regions of signal, proregion, and mature peptides, both nucleotide acids and residue substitutions in DNA and peptide levels, as well as Cys codon usage were analyzed, which suggest how these separate branches originated.

MATERIALS AND METHODS

Cone Snail Tissues and Reagents

Specimens of the three *Conus* species (*Conus lividus, Conus litteratus, Conus textile*) were collected from the South China Sea off Hainan Province (Figure 1). Living snails were frozen and stored at −80 °C. Their venom ducts were dissected, then immediately frozen in liquid nitrogen.

Restriction enzymes T4 DNA Ligase, Taq DNA polymerase, X-gal (5-bromo -4-chloro-3-indolyl -β-D-galactoside), and IPTG (isopropyl -thio-β-D-galactoside) were purchased from SABC. pGEM-T easy vector system and gel purification kit Wizard DNA Clean-Up System for PCR product were from Promega. AMV Transcriptase system was from Invitrogen. Total RNA Isolation Kit was from Shanghai Huasun Co. The other reagents were of analytical grade and were also from SABC.

Preparation of Total RNAs

Venom duct tissue (~30 mg) frozen in liquid nitrogen was ground into fine powder and homogenized. By using a Shanghai Huasun Reagent kit, the total RNA extraction was carried out according to the instruction manual. The details of total RNA isolation was has been described previously [30].

Conotoxin cDNA Isolation and Sequencing

About 5 μg each of total RNA of venom ducts from the *C. lividus, C. litteratus,* and *C. textile*, respectively, were taken to convert the mRNA into cDNA using AMV Transcriptase with a universal oligo$(dT)_{15}$ primer for PCR amplification of the genes encoding α-conotoxins, 5′ forward primer 1 (5′ TCT G ATG GCA GGA ATG ACG CAG 3′), and primer 2 (5′ TCG TGG TTC AGA GGG TCC TGG 3′) based on the conserved propeptide sequence and the 3 end untranslated region (3′-UTR) of the α-conotoxin family.

PCR amplification was performed with a cycling protocol composed of an initial denaturation at 94 °C for 3 min, 35

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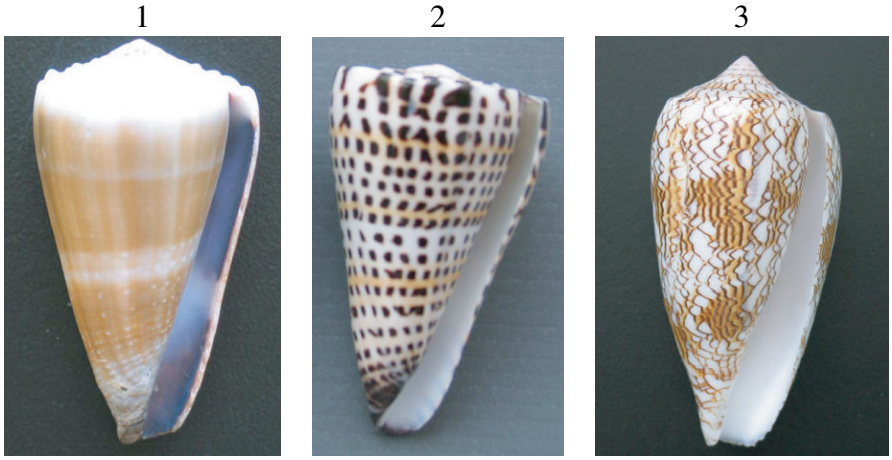

Figure 1 Conus species native to Hainan, which were analyzed for α-conotoxins. Shells of three species are shown: left to right, 1, *C. lividus*; 2, *C. litteratus*; 3, *C. textile*.

cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and terminated with a final extension at 72 °C for 2 min. PCR products were analyzed by electrophoresis on 2% agarose gel. The PCR product band was excised from the gel and purified with the Wizard DNA Clean-Up System (Promega). The purified PCR products were inserted into the pGEM-T Easy vector via TA cloning. Transformed colonies were screened with white–blue identification for sequence analysis. Positive transformed colonies with conopeptide precursors cDNA inserts were sequenced and analyzed using the ABI Model 3130 automated sequencer (Applied Biosystems).

Sequence Analysis of the α-Conotoxin Family and Nomenclature

cDNA sequences were analyzed applying the DNA sequencing softwares DNA star and DNA club. The three novel cDNA sequences have been deposited in the GenBank Nucleotide Sequence Database under the accession numbers DQ141133–DQ141135. For this research we have employed a special nomenclature. The three sequences obtained from clones are abbreviated as follows: first two letters 'Li' represent *C. lividus*, 'Le' represent *C. litteratus*, 'Te' represent *C. textile*; the letter and number that follows the first two letters indicate the clone name in our experiment, the last letter is the clone character (N for nucleotide sequence, P for peptide precursor, and M for mature peptide). Thus, the abbreviation LiC22N, LiC22P, and LiC22M correspond to the nucleotide sequence, peptide precursor, and mature peptide of toxin LiC22 from *C. lividus*, respectively.

RESULTS

cDNA Cloning of Three Novel α-Conotoxins

PCR amplifications of *C. lividus*, *C. litteratus*, and *C. textile* venom duct cDNAs using primer 1 and primer 2 produced three amplicons approximately 150 bp in length, respectively. Primer 1 was based on the consensus sequence of the 5 end of propeptide conotoxins in the NCBI database and primer 2 on a previously unrecognized highly conserved region in the 3′-UTR of α-conotoxins. The resultant PCR products were cloned, and multiple clones were isolated and sequenced. One insert LiC22N 148 bp and two inserts LeD2N and TeA21N 151 bp in length were further characterized. They separately encoded three proteins having lengths of 40, 41, and 38 amino acids, with a structure that is typical of α-conotoxin propeptide (Figure 2), and all of them displayed the characteristic structural organization of conotoxins [23], including a propeptide sequence at the *N*-terminus, followed by the mature toxin region and a closing 3′-UTR. All predicted mature peptides exhibited the common cysteine pattern of α-conotoxins (CC–C–C).

GenBank accession number of 148 bp LiC22N from *C. lividus* was DQ141133. The conotoxin precursor LiC22P contained an *N*-terminal propeptide having a length of 19 amino acids and was marked by a predominance of basic residues. Conotoxin LiC22 is separated from the proregion by the proteolytic site-XR, which would yield a peptide with a length of 21 amino acids (Figure 2(a)). GenBank accession number of 151 bp LeD2N from *C. litteratus* was DQ141134. An *N*-terminal propeptide of the conotoxin precursor LeD2P consisted of 21 amino acid residues. Conotoxin LeD2 is separated from the proregion by the proteolytic site -XR, which would yield a peptide with a length of 20 amino acids, further truncated to a length of 16 amino acids by removal of the *C*-terminal glycine during *C*-terminal amidation (Figure 2(b)). GenBank accession number of 151 bp TeA21N from *C. textile* was DQ141135. The conotoxin precursor TeA21P contained an *N*-terminal propeptide having a length of 20 amino acids and marked by a predominance of basic residues too. Conotoxin TeA21 is separated from the proregion by the proteolytic site -XRR, which would yield a peptide with a length of 18 amino acids, further truncated to a length of 17 amino acids by removal of the *C*-terminal

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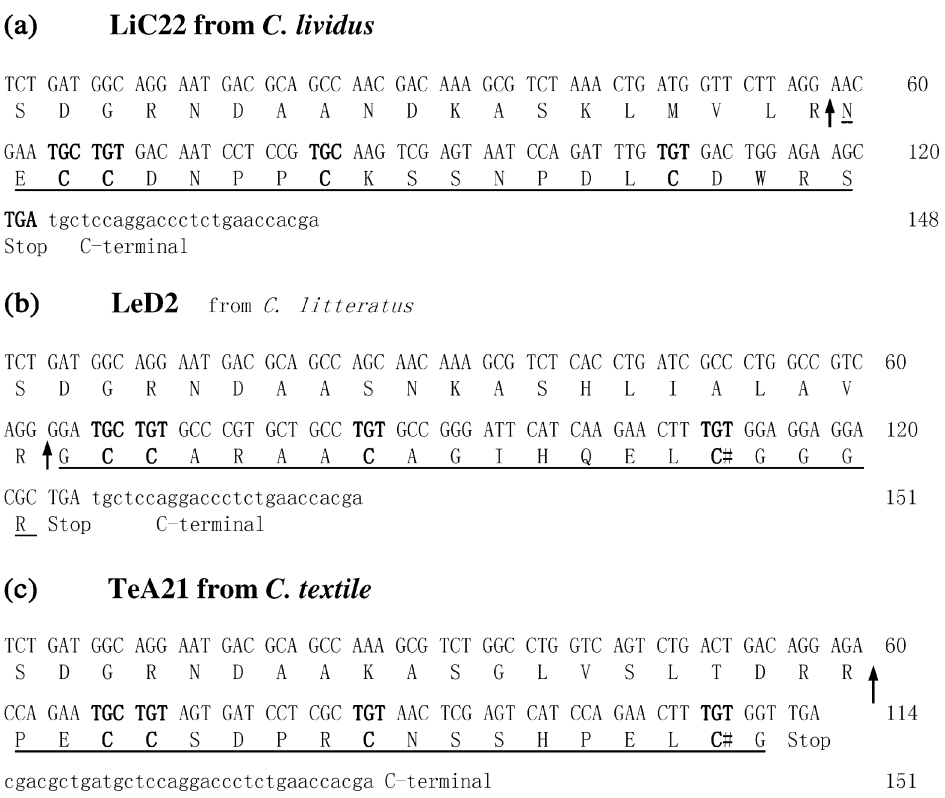

Figure 2 Nucleotide sequence and deduced amino acid sequence for (a) LiC22, (b) LeD2, (c) TeA21. The toxin regions are underlined. Nucleotides in the untranslated region are in small letters. The putative proteolytic processing sites R are indicated by arrows. The glycine following the *C*-terminal cysteine in the mature toxin is presumed to be processed to a *C*-terminal amide, which are indicated by #. The Cysteine and stop codons are indicated in bold print. Their GenBank accession numbers are LiC22P, DQ141133; LeD2P, DQ141134; and TeA21P, DQ141135, respectively.

glycine during *C*-terminal amidation (Figure 2(c)). Since members of each conopeptide gene family share a highly conserved signal and propeptide sequence and a constant arrangement of the cysteines in the sequence of the mature toxin, conopeptides identified in this work were designated to represent members of the α-conotoxin family.

DISCUSSION

Comparison of Properties of Predicted Novel Toxins LiC22, LeD2 and TeA21 with Previously Characterized α-Conotoxins

Different molecular forms of the α-conotoxin family are targeted to different molecular subtypes of nicotinic receptors, which are known to be antagonists at nAChRs. The nAChRs in the CNS are involved in a variety of normal physiological functions, including cognition, reward, motor activity, and analgesia. They are implicated in the pathophysiology and treatment of disease states, including chronic pain syndromes, Parkinson's, and Alzheimer's. Twelve neuronal nAChR subunits have been cloned in vertebrates (α2–10, and β2–4) [25]. Different combinations of subunits produce distinct nAChR subtypes. Homologous subunits are organized around a central cation channel that is gated by the endogenous ligand acetylcholine and the tobacco-plant toxin nicotine. On the basis of the intercysteine spacing, i.e. in a family of peptides with a number of amino acids in the first loop and mature toxins in the second loop, α-conotoxins can be divided into six groups: (i) the α4/7 subfamily (consensus sequence -CCX4CX7C-, (ii) the α3/5 subfamily (-CCX3CX5C-), (iii) the α4/3 subfamily -CCX4CX3C-), (iv) the α4/6 subfamily (-CCX4CX6C-), (v) the α4/4 subfamily (-CCX4CX4C-), and (vi) other α-conotoxins, e.g. Ca1.1 belong to α4/5 with little information [23]. The predicted mature peptides of the three newly found conotoxins in this study (LC22M, LeD2M, and TeA21M) are composed of 16–21 amino acid residues, with 4 and 7 residues, respectively, between cysteine residues in the mature toxins, which belong to the α4/7 subfamily (Figure 2).

The α3/5 subfamily is much more narrowly distributed; it is only found in fish-hunting *Conus* [23]. The α3/5 subfamily α-conotoxins predominantly target the muscle nAChRs ($\alpha 1\beta 1\delta\gamma$), e.g. α-Ctx GI, GIA, and GII from *C. geographus*; SI, SIA, and SII from *C. striatus*; CnIA and CnIB from *C. consors*; and MI from *C. magus*. To date, they have consistent features of muscle-specific nAChRs subtypes targets, reflecting

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extensive homology among the α3/5 subfamily [9]. So far there are only three peptides (α-RgIA, α-ImI and α-ImII) that belong to α4/3 as reported in the literature. All are from worm-hunting snails and have a common feature of potently inhibiting α7 or α9α10 receptors; thus the native targets of these peptides may be related to α7 and/or α9α10 receptors from vertebrates. No α4/3 peptides have been reported in fish- or mollusk-hunting cones. An intriguing possibility is that α4/3 toxins are a specialization of worm-hunting snails and evolved to target receptors specific to the prey, predators, and/or competitors of these snails [31]. One unique α-Ctx AuIB from molluscivorous *C. aulicus* displays a 4/6-loop framework with neuronal specific α3β4 targets [32]. Another one, α-Conotoxin BuIA from the fish-eating snail *Conus bullatus*, displays unusual 4/4 spacing, which potently blocks numerous rat nAChR subtypes, with highest potency for α3- and chimeric α6-containing nAChRs, and blocks α6/α3β2 nAChRs with a 40 000-fold lower IC50 than α4β2 nAChRs [33].

The α4/7 branch of the α-conotoxin family appears to be a stem group found in many different *Conus* species [27], which predominantly target neuronal subtypes of nicotinic receptors. The α4/7 subfamily has high specificity for neuronal nAChRs and is among the most ubiquitous nAChR antagonists present in the venoms of fish, mollusc, and worm-hunting cone snails. α-Ctx EI is an exception and is characterized by α4/7 loop structure with muscle nAChRs (α1β1δγ) [9]. All known neuronal α4/7 conotoxins contain a conserved serine residue in position 1, loop 1 (See Table 1), and a conserved proline residue in loop 1. A novel α-conotoxin, PeIA, cloned from *C. pergrandis*, with an unknown feeding habit, discriminates between rat α9α10 and α7 nicotinic cholinergic receptors [25]. Five α-Ctxs, GIC, GID, MII, MII[E11A], and PIA, isolated from piscivorous *C. geographus, C. magus*, and *C. purpurascens*, respectively, target the α3β2 or α6/β2 nAChRs, in which MII[E11A] and PIA can discriminate between the closely related α3 and α6 subunits (Table 1). Nine α–Ctxs, OmIA, PnIA/PnIB/[A10L]-PnIA, AuIA/AuIB/AuIC, EpI, and Vc1.1 from molluscivorous *C. omaria, C. pennaceus, C. pennaceus, C. aulicus, C. episcopatus, C. victoriae*, respectively, target the α3β2, α3β4, α7, or α3α7β4/α3α5β4 nAChRs. α-Ctx Vc1.1 shows promise as a lead molecule for developing drugs that alleviate pain. Five α-Ctxs, PnIA[N11S], TeA21 (in this paper), Tx1, Tx2, Mr1.1, come from three molluscivorous species *C. pennaceus, C. textile*, and *C. marmoreus*, respectively; their nAChRs targets are unknown. To date, there are 12α-Ctxs founded in six vermivorous species, *C. anemone, C. lividus, C. quercinus, C. litteratus, C. leopardus*, and *C.miles* (including LiC22 and LeD2 in this work), of which it is only known that AnIB targets α3β2/α7, the targets of the others are unknown (Table 1). So, the venom from vermivorous cone snails would be the major source of α4/7 conotoxins; however, most of them remain a matter of intrigue. α-Ctxs LiC22 and LeD2, studied in the present research, do not contain a conserved serine residue in position 1 or a conserved proline residue in position 3, both in loop 1, which are all known to neuronal α4/7 conotoxins, but molluscivorous α-Ctxs TeA21 do contain them. Their nAChR subtypes of the molecular target remain to be identified. On the other hand, this study confirms that the striking variability of the mature peptide, i.e. of the amino acids between and outside the cysteine residues, results in a wide diversity of biological activities even within a conopeptide subfamily.

Observation of *C*-terminal amidation of posttranslational modifications was very common in α4/7 conotoxins. There are 4 peptides (PeIA, Tx2, LiC22, and qc1.1; 13% of Table 1) without *C*-terminal amidation. The rest (87% of Table 1) are truncated to be mature toxins by removal of the *C*-terminal glycine during *C*-terminal amidation, which are indicated by* (Table 1). Four specific posttranslational modifications were observed in eight peptides of the α4/7 conotoxins: proline hydroxylation (EI, Vc1.1), sulfated tyrosine (ψ) (PnIB, [A10L]-PnIA, EpI, AnIB, AnIA), gamma-carboxyglutamic acid (Gla, γ) (GID,Vc1.1), and *C*-terminal carboxylate (GID). Most Pro residues were found to be unhydroxylated (Table 1). GIC, PeIA, and most vermivorous α4/7 conotoxins were identified by gene cloning. We note, however, that the peptides have not yet been isolated from the venom of cone snails, and it is possible that the native venom-derived peptides have posttranslational modifications that are not evident from inspection of the genetic sequence. Previous research showed that synthetic versions of the putative CTxs have highly selective targets to nAChRs subtypes. Determination of the posttranslational modifications of the three newly found toxins in this study will require purification of the native forms of peptides. Thus, *C. lividus, C. litteratus*, and *C. textile* represent a new source of useful pharmacological probes to characterize nAChRs. The discovery of newer α4/7 conotoxins that target neuronal nAChRs will provide the opportunity for computational modeling of receptor–ligand interactions. The nAChR homology models derived from the three-dimensional structure of acetylcholine-binding protein give further insight into the precise interactions of α-conotoxins with nAChR subtypes.

Phylogenetic Analysis of α4/7 Subfamily Precursors of α-Conotoxins

By using the NCBI database to search for homologous genes and peptides with the newly found conotoxins from *C. lividus, C. litteratus*, and *C. textile*, 18 of the α-conotoxin precursors had been described (Figure 3). There are three precursors from molluscivorous *C. textile* (Tx1, Tx2, and TeA21P), one from molluscivorous

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Table 1 Properties of Characterized α4/7 Conotoxins

Name (a)	Source	Sequence (b)	Mode of action (Target)	Ref.
PeIA, *C. pergrandis* (c)		GCCSHPACSVNHPELC	α9α10 and α7	[25]
EI, *C.ermineus*, Piscivorous		RD**O**CCYHPTCNMSNPQIC*	Muscle α1β1$\delta\gamma$	[9]
Piscivorous			Neuronal	
GIC	*C. geographus*	GCCSHPACAGNNQHIC*	α3/β2 α6/β2 > α4/β2 α3/β4	[34]
GID	*C. geographus*	IRDγCCSNPACRVNNOHVC^	α3β2	[9]
MII	*C. magus*	GCCSNPVCHLEHSNLC*	α3β2 α6/β2 > α4/β2 α3/β4	[35]
MII[E11A]	*C. magus*	GCCSNPVCHLAHSNLC*	α6/β2 > α3/β2	—
PIA	*C. purpurascens*	RDPCCSNPVCTVHNPQIC*	α6/β2 > α3/β2 > α3/β4	[36]
Molluscivorous				
OmIA	*C. omaria*	GCCSHPACNVNNPHICG*	α3/β2 > α7 > α6/β2	[37]
PnIA	*C. pennaceus*	GCCSLPPCAANNPDYC*	α3β2 > α7 > a3/b4	[38]
PnIA[N11S],	*C. pennaceus*	GCCSLPPCAASNPDYC*	Unknown (d)	—
PnIB	*C. pennaceus*	GCCSLPPCALSNPDΨC*	α7 > α3β2	—
[A10L]-PnIA	*C. pennaceus*	GCCSLPPCALNNPDΨC*	α7	[39]
AuIA	*C. aulicus*	GCCSYPPCFATNSDYC*	α3β4(weak)	[9,32]
AuIB	*C. aulicus*	GCCSHPACFATNPD-C*	α3β4	—
AuIC	*C. aulicus*	GCCSYPPCFATNSGYC*	α3β4(weak)	—
EpI	*C. episcopatus*	GCCSDPRCNMNNPDΨC*	α3β2/α3β4	—
Vc1.1	*C. victoriae*	GCCSD**O**RCNYDHPγIC*	α3α7β4/α3α5β4	[40]
TeA21, Tx1,	*C. textile*	PECCSDPRCNSSHPELC*	Unknown (d)	This work; [41]
Tx2,	*C. textile*	PECCSHPACNVDHPEICR	—	[41]
Mr1.1	*C. marmoreus*	GCCSHPACSVNNPDIC*	—	GenBank AY580325
Vermivorous				
AnIB	*C. anemone*	GGCCSHPACAANNQDΨC*	α3β2/α7	[38]
AnIA	*C. anemone*	CCSHPACAANNQDΨC*	Unknown (d)	[39]
AnIC	*C. anemone*	GGCCSHPACFASNPDΨC*	—	[38]
LiC22	*C. lividus*	NECCDNPPCKSSNPDLCDWRS	—	This work
QC1.3	*C. quercinus*			GenBank AY588 972
qc1.1	*C. quercinus*	DECCPDPPCKASNPDLCDWRS	—	GenBank AY580 319
lt1b	*C. litteratus*	GCCARAACAGIHQELC*	—	GenBank DQ345365
LeD2	*C. litteratus*			This work
lp1.1	*C. leopardus*			GenBank AY580 321
lp1.2	*C. leopardus*	GCCSHPACSVNNPYFC*	—	GenBank AY580 322
lp1.3	*C. leopardus*	CCSNPACGAGHPEICA*	—	GenBank AY580 323
Mi1.1	*C. miles*	GCCSNPPCYANNQAYCN*	—	GenBank AY588 975

(a): The first capital letter indicates the species origin.
(b): O, hydroxyproline; γ,γ-carboxyglutamate; ^, *C*-terminal carboxylate; Ψ, sulfated tyrosine; * , *C*-terminal carboxamide.
(c): Unknown feeding habit.
(d): Unknown = mode of action and classes have not been formally identified.

C. victoriae (Vc1.1), one from molluscivorous *C. marmoreus* (Mr1.1), three precursors from vermivorous *C. leopardus* (lp1.1, lp1.2, and lp1.3), two from vermivorous *C. quercinus* (QC1.3 and qc1.1), two from vermivorous *C. litteratus* (lt1b and LeD2P), two from vermivorous *C. miles* and *C. lividus* (Mi1.1 and LiC22P, respectively), one from C. *pergrandis* with unknown feeding habits (PeIA), and three from piscivorous *C. purpurascens*, *C. magus*, and *C. geographus* (PIA, MII, and GIC respectively) (Table 1). There are 11 precursors among them with complete signal regions. Most residues of signal regions are hydrophobic residues (M, F, L, V, and A). They have a common sequence, MGMRMMFX$_1$X$_2$FX$_3$LVVLATVX$_5$X$_6$, in which conserved residues are shaded, and where X$_1$X$_2$ represents TV, IM, or VV; X$_3$ represents L or M; X$_4$ represents T, I, or S; and X$_5$X$_6$ represents VS, DT, or VT residues. Prominent propeptide regions are composed of a high percent of hydrophilic residues (52–75%) and many residues are basic amino acids (R,K,H) (15–30%), which might facilitate the precursor to process into mature peptides by proteolytic function (Figure 3). The last one or two residues before the mature regions are usually basic K or R, requiring proteolytic cleavage. There are 8–10 conservative residues among the proregions of 21–28 amino acids in all the 18 precursors. However, there are

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Figure 3 Comparison of the three conotoxin propeptide sequences that are newly found in this paper with other homologue α4/7 conotoxins. The conserved residues are shaded, and the mature toxins are underlined. Symbol * before G (Gly) indicates COOH-terminal amidation. The GenBank accession numbers of the previously identified α4/7 conotoxins are as follows: lp1.2 AY580322, MII P56636, Mr1.1 AY580325, Mi1.1 AY588975, LiC22P DQ141133, lt1b DQ345365, lp1.1 AY580321, LeD2P DQ141134, QC1.3 AY588972, qc1.1 AY580319, PIA P69658, PeIA DQ008450, lp1.3 AY580323, Vc1.1 P69747, Tx2 AF146353, Tx1 AF146352, TeA21P DQ141135, GIC AF526267.

only four Cys residues that are completely conservative among the mature toxin regions with a α4/7 conotoxins common motif (-CCX4CX7C-). In view of the specific residues in the mature region, it is especially striking that most α4/7 conotoxins are *C*-terminal amidation with standard processing at the *C*-terminus from the sequence $-XG_{1-3}(K)R_{3-1}$- to –X-NH2 (Figure 3). The relationship between the high ratio of some specific residues in different regions of α4/7 conotoxin precursors and their post-translation modifications and their toxins bioactivities remains intriguing. However, it is noteworthy that conotoxin structure analysis would be beneficial for function research of the corresponding gene families in spite of the fact that their potential biological activity is not clear.

In order to understand the evolutionary trend among the α4/7 conotoxins, each phylogenetic tree and percent identities of the signal, propeptide, and mature regions for this scaffold subfamily, both in DNA and peptide levels, were constructed by comparing the 18 isoforms from 11 cone snail species (Figure 3, Tables 2–3, Figures 4–6). We also analyzed the cysteine codon conservation within the hypervariable toxin regions (Figure 4(A)), substitutions of different apparent nucleotide (Figure 4(B)), and amino acid residue (Figure 4(C)), respectively, in the different domains of current alignment.

In the present study, we observe a significant homology in the prepro regions of the α4/7 subfamily, while the predicted toxin regions are hypervariable within a conserved cysteine framework (Tables 2–3, Figure 4). It was striking to note that cysteine codons within the hypervariable mature domain are conspicuously conserved (Figure 4(A)). A conserved arrangement of cysteine residues generally implies a conserved disulfide configuration. The 1st, 2nd, and 3rd cysteines in these peptides exhibit a highly position-specific codon conservation: TGC for Cys1, TGT for Cys2, and Cys4. The codon triplets for cysteine 3 preferred TGT (89%) to TGC (11%). Cysteine codon conservancy in this study differs a bit from that shown in the literature [23]. Research by Santos *et al.* showed that the first two Cys residues of three α3/5 conotoxins have an apparent reversal in codon usage from all the other α-conotoxins (TGT TGC in GI, GIB, and SI *versus* TGC TGT in all others). DNA sequence identity (%) between the signal peptides of the 11 α-conotoxins range from 90.5% to 100% (Table 2). Peptide sequence identity (%) between them are larger, with 76.2–100% (Table 3). Percent identity (%) of DNA and peptide sequences between the propeptides of the 18 α-conotoxins have a range of 56.1–100% and 25–100%, respectively, which is similar to 57.9–100% and 25–100% for the DNA and peptide of mature regions, respectively (data

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Table 2 DNA Sequence percent identity (%) between the signal peptides of the 11α-conotoxins

	Vc1.1s	lp1.1s	lp1.2s	lp1.3s	lt1bs	Mi1.1s	Mr1.1s	qc1.1s	QC1.3s	Tx1s	Tx2s
Vc1.1s	***	93.7	98.4	93.7	92.1	100	100	93.7	93.7	93.7	96.8
lp1.1s		***	92.1	96.8	98.4	93.7	93.7	90.5	90.5	90.5	90.5
lp1.2s	—	—	***	92.1	90.5	98.4	98.4	92.1	92.1	92.1	96.8
lp1.3s	—	—	—	***	95.2	93.7	93.7	90.5	90.5	90.5	90.5
lt1bs	—	—	—	—	***	92.1	92.1	88.9	88.9	88.9	88.9
Mi1.1s	—	—	—	—	—	***	100	93.7	93.7	93.7	96.8
Mr1.1s	—	—	—	—	—	—	***	93.7	93.7	93.7	96.8
qc1.1s	—	—	—	—	—	—	—	***	100	88.9	92.1
QC1.3s	—	—	—	—	—	—	—	—	***	88.9	92.1
Tx1s	—	—	—	—	—	—	—	—	—	***	90.5
Tx2s	—	—	—	—	—	—	—	—	—	—	***

Table 3 Peptide sequence percent identity (%) between the signal peptides of the 11α-conotoxins

	lt1bs	lp1.2s	lp1.1s	Mr1.1s	Mi1.1s	QC1.3s	qc1.1s	lp1.3s	Tx1s	Tx2s	Vc1.1s
lt1bs	***	76.2	90.5	76.2	76.2	76.2	76.2	90.5	71.4	76.2	76.2
lp1.2s	—	***	81	100	100	90.5	90.5	85.7	90.5	100	100
lp1.1s	—	—	***	81	81	81	81	95.2	76.2	81	81
Mr1.1s	—	—	—	***	100	90.5	90.5	85.7	90.5	100	100
Mi1.1s	—	—	—	—	***	90.5	90.5	85.7	90.5	100	100
QC1.3s	—	—	—	—	—	***	100	85.7	85.7	90.5	90.5
qc1.1s	—	—	—	—	—	—	***	85.7	85.7	90.5	90.5
lp1.3s	—	—	—	—	—	—	—	***	81	85.7	85.7
Tx1s	—	—	—	—	—	—	—	—	***	90.5	90.5
Tx2s	—	—	—	—	—	—	—	—	—	***	100
Vc1.1s	—	—	—	—	—	—	—	—	—	—	***

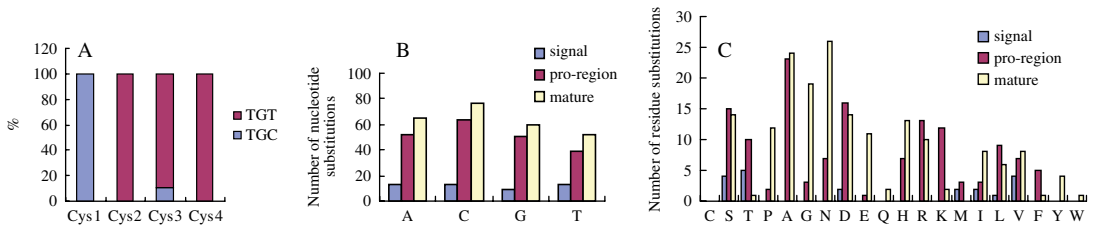

Figure 4 Cysteine codon conservation within the hypervariable toxin regions (A); Different apparent nucleotide (B) and amino acid residue (C) substitutions, respectively, in the different domains of the current alignment α4/7 conotoxins precursor.

not shown). Examination of cDNA sequences of the *C*-terminal regions next to the stop codon of the current alignments revealed that they had 2–3 preferred stop codons TGA, the sequences were hyperconservative and become identical once again except for a few nucleotide substitutions (Figure 5). This sequence conservation summary in signal and *C*-terminal untranslational regions would be significant to design probes and PCR primers for more novel cloning of conotoxin genes.

Notably, signals of five precursors (Vc1.1, Tx2, lp1.2, Mi1.1, and Mr1.1) and that of two peptides (QC1.3 and qc1.1) are completely same, with 100% identity (Table 3). However, one synonymous substitution occurred at the 33rd locus of lp1.2 and Tx2 nucleotide sequence, where both CTA of lp1.2 and CTC of Tx2 encoding the 11th amino acid Leu, which results in the DNA sequences of lp1.2 and Tx2, are not the same as Vc1.1, Tx2, and lp1.2 (CTG also encoding Leu). Owing to the fact that no mutation occurred at the DNA sequences of QC1.3 and qc1.1 signals, they have completely same DNA and amino acid sequences, with 100% identity (Table 2). Nucleotide substitution number in the signals ranges from 9 to 13, which is the lowest as compared with that of the propeptides

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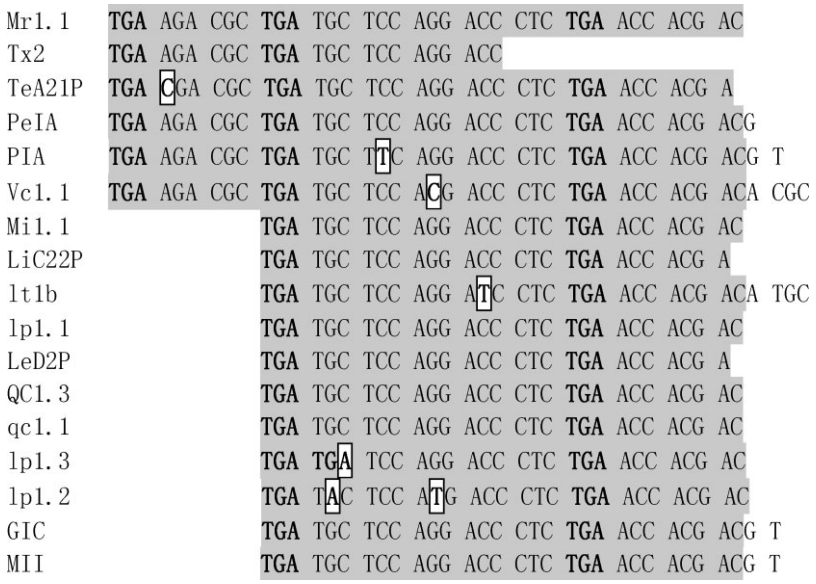

Figure 5 Comparison of cDNA sequences of the *C*-terminal regions next to the stop codon of the current alignments. The conserved nucleotide sequences are shaded, the variable nucleotides are framed.

(39–52) and the mature regions (52–77) (Figure 4(B)). It is surprising that the nucleotide substitution number in the propeptide is a little less than in the mature regions, both of which appear in highly frequent nucleotide substitution. Residue substitution number in different regions in these conotoxin precursors is also reflected in their degree of divergence, as demonstrated in Figure 4(C).

Residue substitutions number in the mature and propeptide region is similar, both of which are significantly higher than in the signal regions (Figure 4(C)). Examination of mature peptide domain showed that residue substitutions between Asn(N) and four other residues, including Ser (S), Gly (G), Asp (D), and His (H), are typically very common, which also happened between G (Gly) and Arg (R), Ala (A), N, and Glu (E), between A and Val (V), R, E, G, and S, as well as between L and I. Whereas substitutions between C (Cys), M (Met), and the other residues are rare, no residue substitutions occurred. As regards the propeptide region, residue substitutions between R and K, between D and A and H, as well as between A and S, T, D, and L, between S and A, L, and F are the most common, whereas no substitutions happened between C, Q, Y(Tyr), W (Trp) and the other residues; substitutions between P, G, E, M, I and the rest of the residues are very rare too. As regards the signal domain, the lowest residue substitution number (less than 5) appeared in the signal regions, but no substitutions occurred at most amino acid sites. Many residue substitutions are very rare, except for a low number occurring between S, T, D, M, I, L, and V. Marked deviations from typical patterns like C, S, P, 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 appears in the propeptide domain that forms part of the propeptide cleavage site. This may be the reason that the highest residue substitutions happened between K and R, whereas it was 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(C).

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 (%) (Figure 6). It indicates that the family of α4/7 conotoxins has a prominent characteristic of diversity both in mature and propeptide regions. Nucleotide and residue substitution analysis in different regions of the conopeptides in the present research confirms that conotoxins are undergoing accelerated evolution, and their mature domain is undergoing accelerated mutation in α4/7 conotoxins, which is consistent with the suggestions of previous authors. [14,15,19]

There are only two-residue differences in propeptide of LiC22 and QC1.3. For LiC22, the 6th Asp(D) is against the 16–18th GAC of DNA, and the 17th Val (V) is against the 49–51th GTT of DNA. For QC1.3, the 6th Thr(T) is against the ACC of DNA, and the 17th Ala (A) is against the 49–51th GCT of DNA. However, predicted LiC22 and QC1.3 mature toxins are completely similar with 100% identity both in DNA and peptide levels. Percent identities between LiC22 and QC1.3 proregions are 94.7% (DNA) and 89.5% (peptide), which were induced by three nucleotide substitutions. LeD2 and lp1.1 have completely same mature peptides

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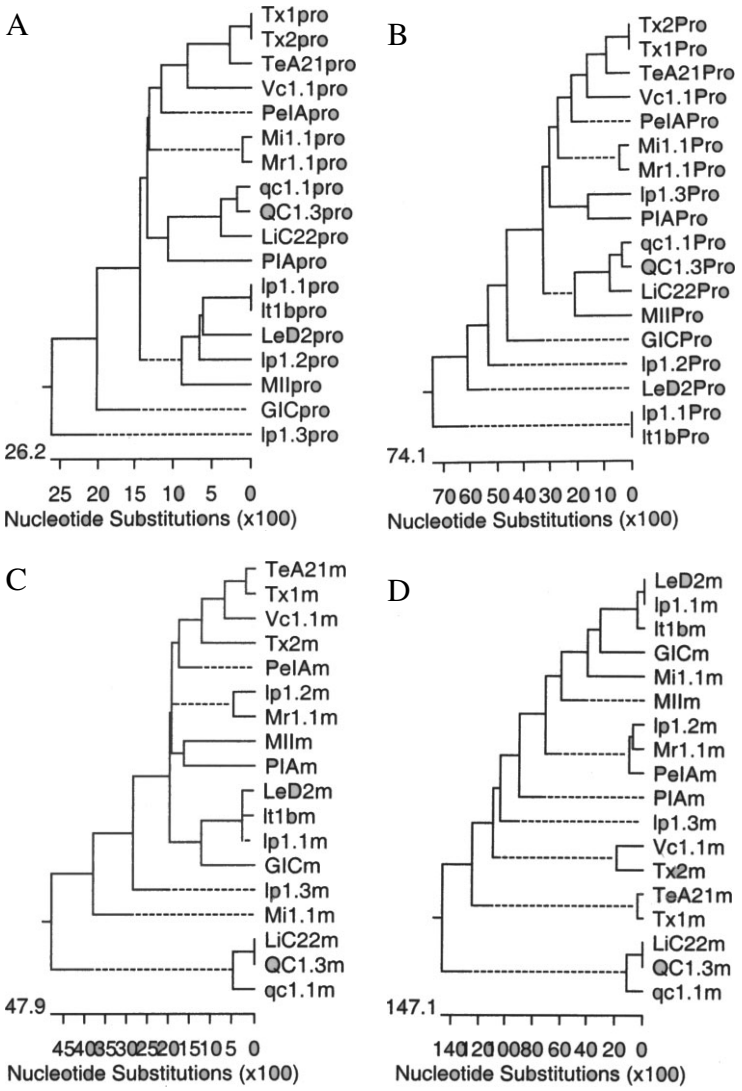

Figure 6 Phylogenetic trees to view evolutionary relationships of current alignment α4/7 conotoxin precursors. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. (A) for DNA sequences of proregion; (B) for amino acid residue sequences of proregion; (C) for DNA sequences of mature peptides; (D) for amino acid residue sequences of mature peptides.

and very different propeptides (76.2% identity), seven nucleotide substitutions occurred in DNA proregion. Vermivorous lp1.1 and lt1b have completely same DNA and peptide sequences in their proregions, which have only one residue difference both in signal and mature region owing to one nonsynonymous substitution in signal region, and one nonsynonymous and two synonymous nucleotide substitutions in mature region. It is surprising that predicted posttranslational LeD2, lp1.1, and lt1b mature toxins by COOH-terminal amidation are completely the same. TeA21 and Tx1 from the same species *C. textile* have three residue differences both in propeptide and mature region because of three nonsynonymous nucleotide substitutions. It is also strange that predicted posttranslational TeA21 and Tx1 toxins are completely same. It means that there are two–three different genes (alleles) encoding correspondingly different toxin precursors, finally obtaining a completely same mature toxin by posttranslational process in α4/7 conotoxins. Substitutions involving smaller distances are chosen over alternative residue substitutions that involve greater distances.

The α4/7 conotoxins were clearly divisible into four distinct clades on the basis of the 8th and 9th variable residues and the 11 signal sequence identities (Table 2–3, Figure 3). Five sequences (Vc1.1, Tx2, lp1.2, Mi1.1, and Mr1.1) from the worm-hunting and snail-hunting species studied in this research are found to cluster together in a big clade (M-TV). While another three sequences (lp1.1, lt1, and lp1.3) from the worm-hunting cone snail are found to cluster together to form the second clade (M-IM). QC1.3 and qc1.1 from the vermivorous *C. quercinus* cluster together to form

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the third clade (M-TM). Tx1 from the molluscivorous *C. textile* forms the fourth clade independently (M-VV). Designed primers of LiC22, LeD2, and TeA21 from the present research, as well as GIC, MII, PeIA, and PIA from earlier researches, were found to correspond to the conserved intron in the front proregions and 3′-untranslated region sequences of α-conotoxin prepropeptides. Therefore, their signals were excluded in the resulting PCR products and were not available in the current alignments.

Proregion sequences are clearly divisible into five distinct clades on the basis of conserved sequences in DNA proregions (Figure 6(A)). Seven sequences (Tx1, Tx2, TeA21, Vc1.1, PeIA, Mi1.1, and Mr1.1) cluster together in clade I, which is the biggest. Five sequences (lp1.1, lt1b, LeD2, lp1.2, and MII) cluster together in clade II, which is the second biggest. Four propeptides (qc1.1, QC1.3, LiC22, and PIA) cluster together in clade III. GIC and lp1.3 form clade IV and clade V independently. Evolutionary relationships in DNA proregions appeared consistent with peptide level, except for lp1.3 and PIA, within the same peptide clade and within different DNA clades (Figure 6(A), (B)). Most evolutionary relationships of the proregion in the present alignment are consistent with signal peptides, besides, GIC and lp1.3 separated from signal clade II and formed two clades independently. The mean pairwise distances among peptide sequences from these five clades ranged from 59 to 79.9. The α4/7 conotoxins in the present alignment share a somewhat high conservancy in the propeptides, with an average residue identity of 30–76%.

Mature-region sequences are clearly divisible into six distinct clades (Figure 4(c)). Most evolutionary relationships of mature region in the present alignment are very different with signal and propeptides, most toxins separated from signal and propeptide clades to form independent clades, besides, LiC22, QC1.3, and qc1.1, with almost the same sequence (91–100% identity), and LeD2, lt1b, and lp1.1 with 95.2–100% identity fall into their own two separate clades, respectively. Evolutionary relationships of DNA mature region are different from peptide levels too (Figure 6(C), (D)). The mean pairwise distances among sequences from these six clades ranged from 73.1 to 195. For the toxin region, the identity mainly originates from the conserved disulfide pattern with an average residue identity of 25–60%. The divergence (D) relationships between sequence pairs of the three regions are Dmature peptide region > Dpro-region ≫ Dsignal. So the sequence of the mature peptides, particularly of their intercysteine regions, showed high intra- and interspecies variability (Figure 6(C), (D)). The divergence of the propeptide and the mature toxin region confirmed the allelic selection inducing a prominent characteristic of diversity [15] (Table 2–3, Figure 6).

In this study, we have identified three novel α4/7 conotoxins by gene cloning. Properties so far known about the α4/7 subfamily α-conotoxins were discussed in detail. Phylogenetic analysis of the α4/7 subfamily, which were systematically performed here, suggests how this subfamily branches may have originated; this has been very rarely reported in previous researches. The data presented here are new and are of importance, and it should attract the interest of researchers in the field. The elucidated cDNAs of these toxins will facilitate a better understanding of the relationship of their structure and function, as well as the process of their evolutionary relationships.

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