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# Diversity of conotoxin types from *Conus californicus* reflects a diversity of prey types and a novel evolutionary history

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ABSTRACT

Most species within the genus *Conus* are considered to be specialists in their consumption of prey, typically feeding on molluscs, vermiform invertebrates or fish, and employ peptide toxins to immobilize prey. *Conus californicus* Hinds 1844 atypically utilizes a wide range of food sources from all three groups. Using DNA- and protein-based methods, we analyzed the molecular diversity of *C. californicus* toxins and detected a correspondingly large number of conotoxin types. We identified cDNAs corresponding to seven known cysteine-frameworks containing over 40 individual inferred peptides. Additionally, we found a new framework (22) with six predicted peptide examples, along with two forms of a new peptide type of unusual length. Analysis of leader sequences allowed assignment to known superfamilies in only half of the cases, and several of these showed a framework that was not in congruence with the identified superfamily. Mass spectrometric examination of chromatographic fractions from whole venom served to identify peptides corresponding to a number of cDNAs, in several cases differing in their degree of posttranslational modification. This suggests differential or incomplete biochemical processing of these peptides. In general, it is difficult to fit conotoxins from *C. californicus* into established toxin classification schemes. We hypothesize that the novel structural modifications of individual peptides and their encoding genes reflect evolutionary adaptation to prey species of an unusually wide range as well as the large phylogenetic distance between *C. californicus* and Indo-Pacific species.



## 1. Introduction

Cone snails are carnivorous molluscs that are justly acclaimed for their potent venoms used in prey capture. The genus *Conus* contains more than 700 species (Röckel et al., 1995; Daly and Craik, 2009), each of which may elaborate over 100 peptides within their venom. Over 3000 entries for these substances have been listed in the useful web-based database, ConoServer (http://research1t.imb.uq.edu.au/conoserver/), with additional accessions added on a virtually daily basis (Kaas et al., 2008). The term conopeptide refers to venom peptides and includes cysteine (Cys)-rich peptides as well as those that possess two Cys residues or none (Halai and Craik, 2009). The Cys-rich peptides, known as conotoxins, are typically 10 to 40 amino acids in length and contain up to 10 cysteines (Terlau and Olivera, 2004; Daly and Craik, 2009).

Conotoxins are categorized into frameworks according to the number and respective positions of their Cys residues, and they are further classified by the signal sequence

*Abbreviations:* Cys, cysteine; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; PTM, posttranslational modification.

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of the prepro-peptide (leader). Because this leader is cleaved during biosynthesis, its amino acid sequence must be determined from the corresponding cDNA. The signal sequences comprise the initial 18–22 amino acids of the leader and tend to be highly conserved among toxins having identical Cys-frameworks. These features has been used to define at least 15 superfamilies of conotoxins (Terlau and Olivera, 2004; Loughnan et al., 2009). Conopeptides also typically contain multiple posttranslational modifications (PTMs) that may be modulated by sequences within the propeptide region (Buczek et al., 2005).

At present, 22 Cys-frameworks have been identified, with most conotoxins falling into eight categories (Norton and Olivera, 2006). Within a given framework, the specificity of action may vary, depending on amino acid composition and inter-cysteine spacing (Halai and Craik, 2009). For example, certain μ-type toxins (M superfamily, Cys-framework 3) block different isoforms of voltage-gated Na channels (McIntosh and Jones, 2001; Norton and Olivera, 2006).

Most *Conus* species are restricted in their hunting to one prey type: worm-like invertebrates (annelids and enteropneusts), molluscs or fish (Röckel et al., 1995), and interspecific competition within this genus is thought to have led to feeding-niche specialization and toxin diversification (Duda et al., 2001; Duda and Palumbi, 2004; Remigio and Duda, 2008). Most studies supporting this conclusion have focused on *Conus* species from the tropical Indo-Pacific region where species diversity is by far the greatest. Relatively few conotoxins have been identified from Eastern Pacific (Hopkins et al., 1995) or Atlantic (Aguilar et al., 2009; Zamora-Bustillos et al., 2009) species, but these generally fit within the conceptual framework described above.

*Conus californicus* Hinds 1844 is an atypical member of the genus. Although probably descended from vermivorous ancestors (Duda et al., 2001), it has evolved into a generalist feeder that consumes at least 56 different species of all three prey types, in addition to scavenging (Kohn, 1966; Saunders and Wolfson, 1961; Stewart and Gilly, 2005). *C. californicus* is endemic to the temperate northeast Pacific Ocean, and over most of its range from the Farallon Islands of central California to Bahia Magdalena in Baja California Sur, Mexico, it is the only representative of the genus (Morris et al., 1980), a situation that may have existed since the late Miocene (Stanton, 1966). Sympatric congeneric species occur only in the southernmost portion of its range (Magdalena Bay to Cabo San Lucas).

Phylogenetic studies indicate a distant relationship between *C. californicus* and the rest of the genus, including other eastern Pacific members (Espiritu et al., 2001; Duda and Kohn, 2005). This outlying position and the potentially relaxed interspecies competition in *C. californicus* presumably underlie the extreme dietary diversity of this species. These features suggest that *C. californicus* may express novel peptide toxins, and yet this species has been largely overlooked, with initial studies involving semi-purified venom components (Cottrell and Twarog, 1972; Elliott and Raftery, 1979). The present study describes the identification of a large assortment of conotoxin types expressed by *C. californicus*, many of which are difficult to accommodate within the conventional conopeptide classification scheme.

## 2. Materials and methods

### 2.1. General

Specimens of *C. californicus* (shell length 1.5–3.5 cm) were collected from shallow subtidal areas in southern Monterey Bay, CA and La Jolla, CA. Intertidal specimens were collected in Bahia Asuncion, BCS, Mexico. Snails were dissected, and venom ducts weighing 2.5–5 mg were separated from the attached muscular bulb and radular sac. In some cases venom was manually extruded before proceeding with RNA isolation.

Total RNA was isolated from venom ducts using either RNaqueous (Applied Biosystems/Ambion, Austin, TX, USA) or Perfect RNA, Eukaryotic (Eppendorf, Westbury, NY, USA) kits and quantified by UV absorbance at 260 nm. For construction of a cDNA library, mRNA was prepared and fractionated using the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR was carried out using Pfu Turbo or Pfu Ultra DNA polymerases (Stratagene, La Jolla, CA, USA) on reverse-transcribed templates or with Taq DNA polymerase on bacterial clones according to the manufacturers' instructions.

Amplifications were performed using a PTC Thermal Cycler (MJ Research, Waltham, MA, USA) with 1.5–2 mM $MgCl_2$, 200 μM dNTP and 250 nM primers. After an initial denaturation of 7 min at 94 °C, reaction mixtures were subjected to 40 cycles of 94 °C (10 s) followed by 46 °C (30 s), to 72 °C at a rate of 0.6 °C/min and 1 min at 72 °C. After cycling was complete, an additional extension time of 7 min at 72 °C was allowed. Purification of PCR products utilized Wizard Prep (Promega, Madison, WI, USA) or Qiaquick (Qiagen, Valencia, CA, USA) kits.

Sequencing was accomplished with Big Dye terminator chemistry (Applied Biosystems, Foster City, CA, USA) utilizing either ABI Model 377 or 3100 automatic sequencers to give electropherograms that were examined visually and edited as required. Sequence data were imported into either Gene Works 2.4 (Intelligenetics, Inc., Mountain View, CA, USA) or Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and examined manually for peptide sequences typical of conopeptides.

### 2.2. Identification of conotoxin sequences using a cDNA library

RNA (12 μg) was extracted from pooled venom duct tissue (less venom) from 12 Monterey snails, size fractionated, and applied to an oligo-dT column to give 0.12 μg of mRNA. This was then used for cDNA synthesis with the SuperScript II kit and primers containing Sal I and Not I restriction sites. Products were ligated into the polylinker of the pSport1 vector between Sal I and Not I sites and introduced into Top10 Electrocompetent cells (Invitrogen) by electroporation. After growth on ampicillin medium (100 μg/ml), random colonies were inoculated into ampicillin L-Broth for overnight growth. Plasmids were purified with a conventional mini-prep procedure and assayed for an insert length of *ca.* 300–600 bp by digestion with Mlu I (one site of which is contained within the Sal I adapter of the cDNA insert) followed by gel