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Evolution of *Conus* peptide toxins: Analysis of *Conus californicus* Reeve, 1844

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ABSTRACT

Conus species are characterized by their hyperdiverse toxins, encoded by a few gene superfamilies. Our phylogenies of the genus, based on mitochondrial genes, confirm previous results that *C. californicus* is highly divergent from all other species. Genetic and biochemical analysis of their venom peptides comprise the fifteen most abundant conopeptides and over 50 mature cDNA transcripts from the venom duct. Although *C. californicus* venom retains many of the general properties of other *Conus* species, they share only half of the toxin gene superfamilies found in other *Conus* species. Thus, in these two lineages, approximately half of the rapidly diversifying gene superfamilies originated after an early Tertiary split. Such results demonstrate that, unlike endogenously acting gene families, these genes are likely to be significantly more restricted in their phylogenetic distribution. In concordance with the evolutionary distance of *C. californicus* from other species, there are aspects of prey-capture behavior and prey preferences of this species that diverges significantly from all other *Conus*.

1. Introduction

The study of model organisms has revealed that many polypeptide gene families with endogenous functions are evolutionarily conserved (*Drosophila* 12 Genomes Consortium, 2007); it is their expression patterns that characteristically differ between taxa. A well known example among eukaryotes is the set of enzymes that are encoded by highly conserved "housekeeping genes" (Tatusov et al., 1997). However, although phenotypic differences among species are in part a consequence of different expression patterns, this alone would seem inadequate to explain the great exuberance of life, the millions of different living species.

It was recently suggested that specific classes of genes diverge very rapidly and the functional gene products they encode differ significantly, even among closely related species (Olivera, 2006). One class of these rapidly diversifying genes include those whose gene products do not act endogenously within the organism, but instead play a role in interactions with other organisms. Even closely related species may exhibit distinct biotic interactions if they differ in their prey, predators and competitors. Thus, those gene products that either directly or indirectly mediate interactions specific to an organism's particular ecological niche would be expected to diverge rapidly within a biodiverse lineage. The persistence of these gene families, however, has not been systematically evaluated.

Venomous animals seem particularly suitable for studying such genes, since a large proportion of the major gene products expressed in venom clearly target other organisms. It is becoming clear that acquiring venom is often correlated with major adaptive radiation events. At least three major taxa (reptiles, arachnids and prosobranchs) have undergone such events (Espiritu et al., 2001; Vidal, 2002; Vidal and Hedges, 2002; Fry et al., 2003a,b; King, 2004). One of the characters underlying these radiation events must be the acquisition of an effective venom delivery system; i.e., a hollow tooth (Vonk et al., 2008). Yet fundamental questions regarding the molecular evolutionary processes that occur still remain: does the appearance of novel toxin gene families promote speciation events? Are the venom components a result of common selective pressures?

The assessment of the linkage between venom components and phylogeny began with snakes: work on phylogenetic distribution and recruitment of venom toxins of snakes was initiated over fifty years ago (Kochva, 1963, 1965, 1987). In the 90s, some began to cast their doubts upon the utility of venom as a taxonomic tool (Chippaux et al., 1991; Daltry et al., 1996). Yet in 2006, Fry and colleagues

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demonstrated that venom evolved in lizards prior to the appearance of snakes and as a result, recruitment of venom is now recognized as a synapomorphy of Toxicofera, a new reptilian clade consisting of suborders Iguania and Serpentes (Fry et al., 2003a). Regardless of this major breakthrough in snake venom evolution, the utility of venom peptide sequences as characters for phylogenetic work is still being debated (reviewed extensively in Chipman, 2009 and Fry et al., 2009). For venomous molluscs, the relationship of venom components to phylogeny is only beginning to be assessed.

Given the extensive molecular data sets collected for many of the living representatives, the superfamily Conoidea (Puillandre et al., 2008; Taylor et al., 1993), is especially useful for this purpose (Olivera et al., 2002). Species encoding peptide toxins produced by the Conoidea include about 10,000 species of venomous marine snails (Olivera, 1997, 2006; Olivera et al., 2002; Terlau and Olivera, 2004). These venoms are extremely complex, with each species producing a repertoire of up to 200 different products within the venom duct alone. In the best-studied group, the cone snails, it has been shown that these powerful cocktails are encoded by a relatively small number of gene superfamilies that exhibit an unprecedented rate of accelerated evolution (Duda and Palumbi, 1999, 2000; Duda, 2008; Duda and Remigio, 2008; Remigio and Duda, 2008). These provide a basis for evaluating the presence or absence of venom gene families in different lineages of this large and diverse molluscan group, including among others the Conidae, the Turridae and the Terebridae (Bouchet and Rocroi, 2005; Taylor et al., 1993).

Approximately 10 different superfamilies have been characterized from cone snails; three of these are a major presence in the venom ducts of most cone snail species: the A, M, and O gene superfamilies that encode *Conus* peptides containing 2–3 disulfide bonds (Jones and Bulaj, 2000; Terlau and Olivera, 2004). Are these gene superfamilies prominently expressed across the entire range of venomous molluscs? A tentative answer was provided by the analysis of genes expressed in the venom duct of *Lophiotoma olangoensis*, a species belonging to the family Turridae, generally acknowledged to be distant from *Conus* both by morphological and molecular criteria (Puillandre et al., 2008; Taylor et al., 1993). All three major gene superfamilies of *Conus* peptides were missing, and a different spectrum of gene superfamilies were found—only one minor superfamily was present in both Conidae and Turridae (Watkins et al., 2006). A similar recent analysis of *Impages hectica* (Conoidea, Terebridae), revealed a lack of overlap with gene superfamilies expressed in *Conus* venom ducts (Imperial et al., 2007).

In order to further assess the distribution of *Conus* gene superfamilies, we have undertaken a comprehensive investigation of a species traditionally included within the genus, *Conus californicus.* Previous molecular work indicated (and our work using three mitochondrial gene fragments support) that this species is distant from the majority of other *Conus*. The phylogenetic divergence from other *Conus* revealed by the molecular data make this species ideal for assessing which of the standard conopeptide-gene superfamilies are conserved throughout the genus *Conus*.

We also address a complementary issue: gene products expressed in the venoms of cone snails are highly post-translationally modified. In at least some branches of the superfamily Conoidea (such as in the family Terebridae), venom peptides are not highly post-translationally modified (Imperial et al., 2007). We determined whether the post-translational modifications found in cone snail peptides persist in this outlier. As will be demonstrated, this comprehensive study of superfamilies and their gene products from *C. californicus* venom ducts has provided definitive answers for these issues. The results of this study provide a base-line data set for the distribution of superfamilies across diverging phylogenetic lineages.

2. Materials and methods

2.1. Rapid population assessments and collection of specimens

Individual specimens (15–22 mm) of *Conus californicus* were collected during an afternoon ebb tide on the western coast of Baja California, 40 km north of Ensenada, Mexico. Collection was planned so that it would coincide with a slack tide event, which occurred on the first three days of December, 2005. On this intertidal reef flat of mostly solid, exposed limestone benches, haphazardly-spaced sand-filled tidal pools were completely engulfed by seagrass. Population densities within the pools were rapidly assessed by first estimating an area equivalent one of the plastic, five (5) gallon buckets used for field collections ($\sim$0.073 m^2), and then counting every individual within that area. Mean density values were calculated from five such rapid assessments. A total of 150 specimens were collected; as a mater of policy, all individuals <15 mm were not taken.

2.2. Laboratory observations of foraging behavior

On December 5, 2005, the *Conus californicus* were transported to the University of Utah and transferred to a preconditioned artificial seawater aquarium. A second precoditioned aquarium ($70 \times 35 \times 40$ cm^3) containing sand 5 cm deep and live rock was divided in three by positioning two perforated, acrylic glass dividers 0.5 cm thick at 23 cm intervals along the length of the tank. Of the 150 specimens collected, 30 were divided into groups of 10 and placed into each aquarium partition; the remaining specimens were either dissected or maintained in separate aquaria.

Observations of foraging behavior began the following day and continued for 12 months. During these observations, *C. californicus* were presented a variety of fresh and salt water fish purchased from local aquarium stores and/or Canadian night crawlers (*Lumbricus terrestris*) and closely monitored for 4–6 h. Multiple still photographs were taken using a digital camera (Canon Powershot S3 IS) during attack sequences, which usually lasted for $\sim$30 min, and at intervals of 6, 12, and 24 h, thereafter. Prey not attacked after 6 h were removed from the tank.

Behavioral observations of predation on shrimp were conducted by the Centro de Investigacion Cientifica Y de Educacion (CICESE; Ensenada, Mexico). CICESE's close proximity to the collection site afforded repeated opportunities for collecting additional specimens and field observations. *Conus californicus* used in these experiments were collected two different times per year (i.e., from both rainy and dry seasons) over the course of three different collect trips. Snails were housed (65 snails per tank) in 40-L polycarbonate tanks ($40 \times 30 \times 30$ cm^3) supplied with filtered, continuous-flow seawater at an exchange rate of 20% per day. The temperature of this natural water supply is maintained at 20 °C. These snails were fed with two live shrimp every two weeks and were observed for a period of 9 months.

2.3. Isolation of peptides from venom ducts

Venom ducts were dissected from 21 live snails and immediately flash frozen. The ducts remained frozen until resuspension on ice in 1.5 mL of B45 (45% AcN:55% H_2O w/0.2% TFA). The suspension was quickly homogenized using a hand-held Teflon pestle that fits into an Eppendorf tube. The resulting homogenate was further mixed via sonication for 15 s, and spun in a Jouan CR412 refrigerated tabletop centrifuge at 5000 rpm for 15 min. The supernatant was diluted 5$\times$ with 0.1% TFA, and applied in seven separate runs onto a Vydac C_{18} analytical column. HPLC elution was performed using a gradient of 5–50% B90 in 0.1% TFA. All major elution peaks were collected as separate fractions.