JOURNAL OF EXPERIMENTAL ZOOLOGY (MOL DEV EVOL) 308B:744–756 (2007)

Venomous Auger Snail *Hastula* (*Impages*) *hectica* (Linnaeus, 1758): Molecular Phylogeny, Foregut Anatomy and Comparative Toxinology

JULITA S. IMPERIAL[1], YURI KANTOR[2], MAREN WATKINS[3], FRANCISCO M. HERALDE III[4], BRADFORD STEVENSON[1], PING CHEN[1], KARIN HANSSON[5], JOHAN STENFLO[5], JOHN-PAUL OWNBY[1], PHILIPPE BOUCHET[6], AND BALDOMERO M. OLIVERA[1]*
[1]*Department of Biology, University of Utah, Salt Lake City, Utah*
[2]*Laboratory of Ecology and Morphology of Marine Invertebrates, A. N. Severtzov Institute of Problems of Evolution, Russian Academy of Sciences, Moscow, Russia*
[3]*Department of Pathology, University of Utah, Salt Lake City, Utah*
[4]*National Institute of Molecular Biology and Biotechnology, University of the Philippines, Diliman, Quezon City, Philippines*
[5]*Department of Clinical Chemistry, University of Lund, University Hospital, Malmö, Sweden*
[6]*Muséum National d'Histoire Naturelle, Paris, France*

ABSTRACT The >10,000 living venomous marine snail species [superfamily Conoidea Fleming, 1822] include cone snails (*Conus*), the overwhelming focus of research. *Hastula hectica* (Linnaeus, 1758), a venomous snail in the family Terebridae Mörch, 1852 was comprehensively investigated. The Terebridae comprise a major monophyletic group within Conoidea. *H. hectica* has a striking radular tooth to inject venom that looks like a perforated spear; in *Conus*, the tooth looks like a hypodermic needle. *H. hectica* venom contains a large complement of small disulfide-rich peptides, but with no apparent overlap with *Conus* in gene superfamilies expressed. Although *Conus* peptide toxins are densely post-translationally modified, no post-translationally modified amino acids were found in any *Hastula* venom peptide. The results suggest that different major lineages of venomous molluscs have strikingly divergent toxinological and venom-delivery strategies. *J. Exp. Zool. (Mol. Dev. Evol.) 308B:744–756, 2007.*

How to cite this article: Imperial JS, Kantor Y, Watkins M, Heralde FM III, Stevenson B, Chen P, Hansson K, Stenflo J, Ownby J-P, Bouchet P, Olivera BM. 2007. Venomous auger snail *Hastula* (*Impages*) *hectica* (Linnaeus, 1758): molecular phylogeny, foregut anatomy and comparative toxinology. J. Exp. Zool. (Mol. Dev. Evol.) 308B:744–756.

The toxoglossate gastropods (suborder Toxoglossa or superfamily Conoidea Fleming, 1822) are molluscs that generally envenomate their prey. They are traditionally divided into three major groups: the cone snails (*Conus* Linnaeus, 1758), the auger snails (Terebridae Mörch, 1852) and the turrids (Turridae Swainson, 1840; *sensu lato*); the latter group is definitely paraphyletic (Taylor et al., '93). The toxoglossate venoms that have been investigated extensively are almost exclusively those from cone snails. Venom characterization of this large and complex genus, with 500–700 species, has proven to be important for neuropharmacology (Olivera et al., '90; Terlau and Olivera, 2004); some cone snail peptide toxins are even being developed as therapeutic drugs (Miljanich, '97, 2004; Olivera, 2006). Furthermore,

Grant sponsor: National Institute of General Medical Sciences; Grant number: GM 48677; Grant sponsor: Research funds from the University of Utah.

Julita S. Imperial, Yuri Kantor and Maren Watkins have contributed equally to this work.

*Correspondence to: Baldomero M. Olivera, Department of Biology, University of Utah, Salt Lake City, UT 84112.
E-mail: Olivera@biology.utah.edu

Received 11 June 2007; Revised 11 June 2007; Accepted 25 July 2007
Published online 21 September 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21195

the analysis of *Conus* venom peptides and the genes that encode them provide insights into both phylogeny and pathways of evolution (Olivera, 2002; Imperial et al., 2007).

Compared to the significant progress made with cone snails, other groups of venomous mollusks have been almost completely neglected. Only one study carried out so far has characterized toxins from the venoms of the auger snails (Imperial et al., 2003); three toxins from the venom of *Terebra subulata* (Linnaeus, 1767) were identified. In this study, *Hastula* (*Impages) hectica* (Fig. 1) was targeted for comprehensive analysis. This species is in a different major division of toxoglossate gastropods (the Terebridae) from *Conus*, and likely represents a divergent branch of the Terebridae from *T. subulata*; as a group, terebrids seem to have diverged from cone snails early in conoidean evolution.

The Terebridae are a family with ~300 known species (Bratcher and Cernohorsky, '87; Terryn, 2007a) and many additional unnamed deep-water forms. A number of studies have been carried out on the anatomy of the family (Marcus and Marcus, '60; Miller, '71, '75, '79; Taylor, '90). On the basis of a small number of species directly examined, great variability was found in the anatomy of the anterior foregut. Taylor ('90) recognized two major divisions of the family based on foregut anatomy and radular morphology: the first includes species with non-hollow teeth and a well-developed radular membrane. These species, found in both the genus *Duplicaria* Dall, 1908 as well as in *Terebra* Bruguière, 1789, lack a proboscis and venom apparatus, but have salivary glands. The second group of species have hypodermic radular teeth, a venom apparatus, proboscis, salivary glands and occasionally, accessory salivary gland(s). Finally, there is a group lacking both the radula and venom apparatus, as well as a proboscis and sometimes, salivary glands; these species could be derived from either one of the radulate groups.

Actual feeding has been observed only for a small number of species, but the anatomy of radulate species with a venom apparatus suggests that they use a strategy similar to the Conidae for envenomating prey: a radular tooth is transferred to the proboscis tip and venom is injected through the hollow tooth into the prey (usually different polychaete worms). There is little congruence between shell morphology and digestive system anatomy (and therefore, feeding mechanism). For example, *T. subulata* and *Terebra areolata* (Adams et Reeve, 1850) are strikingly similar in shell pattern (Terryn, 2007b) (one is often confused for the other; see Fig. 1), but they differ considerably in internal anatomy; a venom duct is present in *T. subulata*, but absent in *T. areolata*.

Fig. 1. Shells of some species of Terebridae discussed in this report. Left panel, *Hastula hectica* shells demonstrate the wide variation in shell pattern found in this species; specimens analyzed in this study were most similar to the bottom specimen. Right panel, bottom row: Two species of venomous *Terebra* believed to feed on tube-dwelling polychaetes. Left, *Terebra subulata*, the species from which three polypeptide toxins were characterized previously; right, *Terebra guttata*. Right panel, middle row: Two *Terebra* species that have no venom ducts. Left, *Terebra crenulata*; right, *Terebra areolata*. Note the striking similarity between *T. subulata* (bottom row, left) and *T. areolata* (middle row, right); these species, which are shown to be phylogenetically quite unrelated in this work, are often mistaken for each other because of their similar shell patterns. Right panel, top row: left, *H. hectica*; right, *Hastula lanceata*. The bar represents 1 cm; all specimens (right panel) to scale. Photographs by Kerry Matz.

A group of auger snails are found living where wave action apparently allows them to devour worms that become exposed (Miller, '70). Many of these species are in the genus *Hastula* (H. and A. Adams, 1853) including the species analyzed below, *H. hectica*. This species is assigned to the genus *Impages* E. A. Smith, 1873 by some taxonomists, but *Impages* is regarded as a subgenus in most systematic treatments (Bratcher and Cernohorsky, '87).

H. hectica specimens collected on the island of Panglao in the Philippines were dissected for anatomical and molecular studies. Venom ducts were accumulated from specimens from several locations in the Pacific and stored. The anatomy of

the foregut and the radula of *H. hectica* was investigated. Venom ducts were used for both toxin purification and characterization as well as for an in-depth analysis of messenger RNA. The combination of biochemical purification and molecular cloning has provided a broad characterization of the toxins expressed in the venom duct of this species. These studies have provided the first comprehensive interdisciplinary comparison between a non-*Conus* conoidean and the cone snails; the similarities and differences provide insight into phylogenetically divergent conoidean lineages.

METHODS

Isolation and identification of peptidic components from venoms ducts of Hastula hectica

H. hectica venom ducts were resuspended in 1 mL of 40% acetonitrile—0.2% trifluoroacetic acid (TFA), and homogenized using a hand-held Teflon pestle that fits into an Eppendorf tube. The homogenate was spun in a Beckman F0650 (Fullerton, CA) rotor at 20 k rpm for 15 min. The supernatant was diluted five fold with 0.1% TFA, and applied to a Vydac C18 analytical column (Hesperia, CA). High-performance liquid chromatography (HPLC) elution was done with a gradient of 1.8–54.0% CH3CN in 0.1% TFA. Most of the major peaks were collected as separate fractions.

Fractions containing major peaks were scanned by matrix-assisted laser desorption ionization mass spectrometry, and those that had components within the range of 1–5 kDa were reduced in the presence of 10 mM dithiothreitol and alkylated using 4-vinylpyridine reagent (Imperial et al., 2003). The alkylated components were sequenced by Edman degradation using the Applied Biosystems Model 492 Sequenator (courtesy of Dr. Robert Schackmann of the DNA/Peptide Facility, University of Utah).

Western blot analysis

Crude venom extract of *H. hectica* was prepared, freeze-dried, dissolved in water, and centrifuged for 15 min at 10,000 rpm. Both the collected supernatant and pellet fraction, respectively, were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis employing the γ-carboxyglutamate (Gla)-specific mouse monoclonal antibody M3B. The M3B antibody had been purified and prepared as described previously (Brown et al., 2000). A venom extract from *Conus figulinus* was used as a positive control for the detection of Gla-containing venom components.

Protein samples were reduced, alkylated and separated in 15% (w/v) sodium dodecyl sulfate polyacrylamide gels and stained with Gelcode Blue stain reagent (Pierce, Chemical Co., Rockford, IL). For Western blot analysis proteins were electrotransferred to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) using wet electrophoretic transfer. The membrane was blocked for 1 hr in quench buffer (5% skim milk powder in 10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) and subsequently incubated for 1 hr in a solution containing 10 μg/mL of the Gla-specific monoclonal antibody M3B in quench buffer. After washing with quench buffer the membrane was incubated for 1 hr with alkaline phosphatase-conjugated rabbit anti-mouse IgG (DAKO A/S, Glostrup, Denmark) and then washed with quench buffer and developed with a substrate solution containing 50 mg/mL BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate, Sigma, St. Louis, MO) in 100% dimethyl sulfoxide and 50 mg/mL NBT (Nitro Blue Tetrazolium, Sigma) in 80% dimethyl sulfoxide. All incubations were performed at room temperature.

Bioassays using Caenorhabditis elegans

The peptides that were present in relatively large quantities were purified by subfractionation in the Vydac C18 analytical HPLC column using gradients at 0.18% CH3CN in 0.1 TFA/min. The purified peptides were lyophilized and assayed by injection into the pseudocoelomic space of the nematode *C. elegans* using standard injection techniques (Mello et al., '91).

Cloning and sequencing of 12S rRNA

Venom ducts were dissected out of live snails collected in the vicinity of Panglao Island, Philippines; these were quick-frozen and transported to the laboratory in Salt Lake City. Genomic DNA was prepared from 20 mg of tissue using the Gentra PUREGENE DNA Isolation Kit (Gentra System, Minneapolis, MN) according to the manufacturer's standard protocol. The resulting genomic DNAs were used as templates for polymerase chain reaction (PCR) using oligonucleotides corresponding to the 12S rRNA gene segment of mtDNA (Oliverio and Mariottini, 2001), and PCR products were purified using the High Pure PCR

Product Purification Kit (Roche Diagnostics, Indianapolis, IN).

The eluted DNA fragments were annealed to pAMP1 vector. The resulting products were transformed into competent DH5α cells, using the CloneAmp pAMP System for Rapid Cloning of Amplification Products (Life Technologies/Gibco BRL, Grand Island, NY), and the nucleic acid sequences of the 12S rRNA-encoding clones were determined. 12S phylogenetic trees were created using the Megalign program (LaserGene sequence analysis software, DNASTAR, Inc., Madison, WI).

Preparation of cDNA

One *H. hectica* venom duct (0.012 g) was homogenized in 1 mL of TRIzol reagent. The RNA was isolated by phase separation and precipitation according to the manufacturer's standard protocol (TRIzol Total RNA Isolation; Life Technologies/Gibco BRL, Grand Island, NY). First-strand cDNA was prepared from 1 μg *H. hectica* RNA using the Clontech SMART PCR cDNA Synthesis Kit (Clontech Laboratories, Palo Atlo, CA) according to the manufacturer's standard protocol. Second-strand synthesis and amplification were performed using LD PCR (Clontech SMART PCR cDNA Synthesis, Clontech Laboratories, Palo Alto, CA) according to the manufacturer's standard protocol, using an MJ Research PTC-200 Peltier Thermal Cycler (Waltham, MA).

Cloning

The resulting PCR product was purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's suggested protocol. A second amplification, using tailed PCR primers, was performed on the cDNA. The resulting PCR product was gel-purified and recovered from agarose using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's protocol for recovery of DNA from agarose. The eluted DNA was annealed to pAMP1 vector, and the resulting product transformed into competent DH5α cells using the CloneAmp pAMP System for Rapid Cloning of Amplification Products (Life Technologies/Gibco BRL, Grand Island, NY). The nucleic acid sequences of the resulting clones were determined using standard ABI (Applied Biosystems, Foster City, CA) automated sequencing protocols.

DNA sequence analysis

Sequence data were analyzed for translation products using the EditSeq program (LaserGene sequence analysis software, DNASTAR, Inc. 1228 Park St., Madison, WI). Signal peptide cleavage sites were predicted by both Analyze Signalase 2.03, a freeware program for applying the algorithm of von Heijne (von Heijne, '86) to the prediction and analysis of mammalian signal sequences (©1988–1992, Ned Mantei, Department of Biochemistry, Swiss Federal Institute of Technology, ETH-Zentrum CH-8092 Zurich, Switzerland) and by SignalP 3.0, a website-based computational method from the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/SignalP/).

Molecular phylogeny

Nucleic acid sequences (12S mtDNA) were aligned manually using MEGA version 3.0 (Kumar et al., 2004). MrBayes (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) was used to construct the phylogenetic tree. The final tree is a 50-majority rule consensus tree created from two independent runs; 1,000,000 were made in each run, 100,000 of which were saved. For the final consensus tree, the first 150 of each of those 100,000 were discarded as part of the ''burn-in'' phase. The standard deviation after 1,000,000 generations was 3.624×10^{-3}. We used a GTR (general time reversible) model, with the rate variation of some sites being invariable and the remaining rates drawn from a γ distribution.

RESULTS

Overview of molecular phylogeny

Although several proposals for the phylogeny of the Terebridae have been made, molecular markers have not been used widely. We carried out a molecular analysis of six species representing three major ecological groups. The first group, including *T. subulata* and *Terebra guttata* (Röding, 1798), consists of relatively large, predatory snails of the tropics that live in sandy bottoms in calm waters below the tide line; they are believed to use venom to prey on tube dwelling polychaete worms. The second group, including *T. areolata* and *T. crenulata* (Linnaeus, 1758), lacks a venom apparatus and therefore unable to envenomate prey. Finally, the third group, including *Hastula lanceata* Linnaeus, 1767 and *H. hectica* possess a venom duct, but have a different feeding strategy

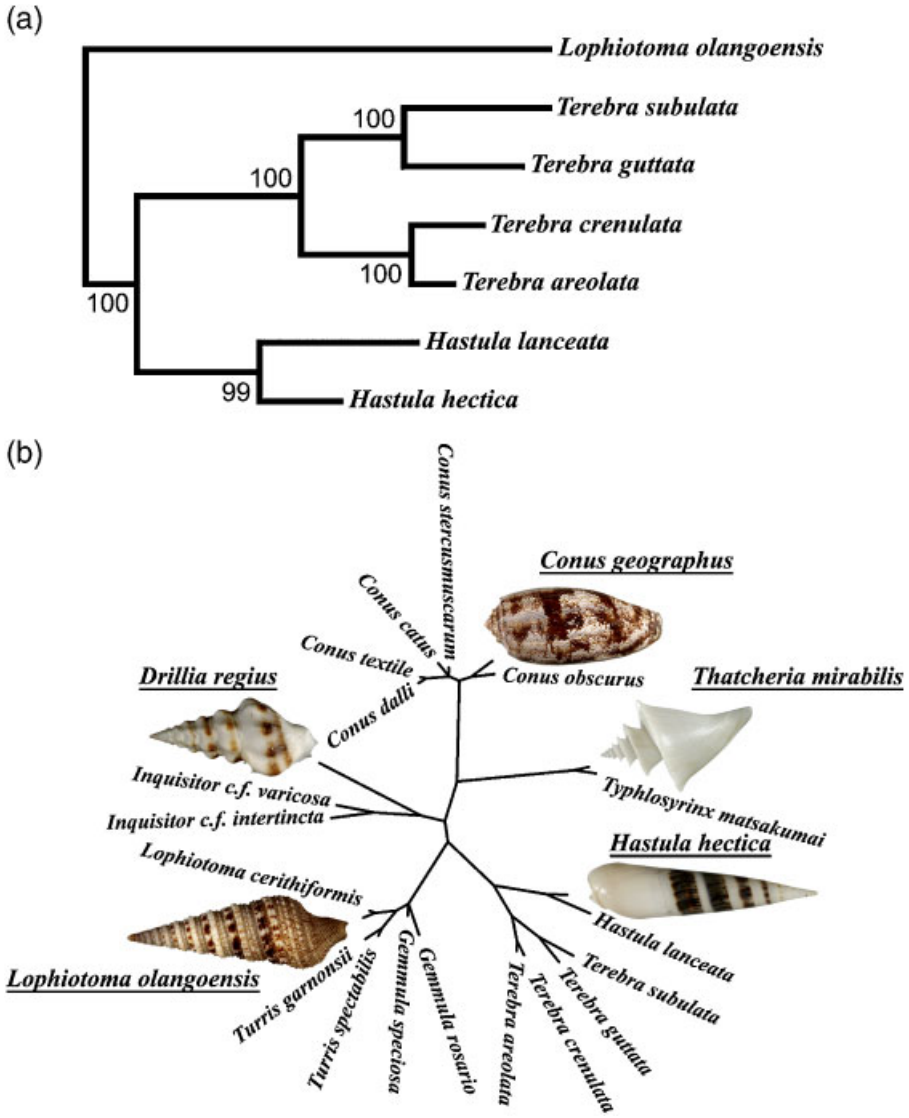

Fig. 2. (**a**) Phylogenetic relationship of *Hastula hectica* to other Terebridae. Sequences of 12S mitochondrial DNA sequences were obtained as described in Methods, and a phylogenetic tree was constructed. In addition to the six species of Terebridae (*Terebra subulata*, *T. guttata*, *T. areolata*, *T. crenulata*, *Hastula lanceata* and *H. hectica*), one turrid species (*Lophiotoma olangoensis*) was analyzed as the outgroup taxon. The phylogenetic tree shown was constructed using the MrBayes software program and a time-reversible model. The results are from a run of 10^6 generations, sampling every hundred with a burn-in of 250 trees. The results were verified by multiple tree-building software programs; these all yielded similar results. (**b**) Phylogeny of *H. hectica* relative to other conoideans. 12S DNA sequences of various conoidean taxa were obtained as described in Methods, and a phylogenetic tree was constructed as described above.

from *T. subulata* (see Introduction). A turrid species, *Lophiotoma olangoensis* Olivera, 2002 was included to serve as the outgroup taxon for the analysis.

DNA was prepared and sequenced as described in Methods. The phylogenetic tree obtained from the sequence analysis is shown in Figure 2a; the results support division of these species of Terebridae into three distinct groups. Both conventional taxonomic criteria and the molecular data are consistent with the conclusion that *H. hectica* is not closely related within the Terebridae to *T. subulata* (Fig. 2a). The three major branches of the phylogenetic tree are very well supported. A similar tree with identical major branches was independently constructed using 16S mtDNA sequences (not shown).

The general phylogeny of species in Terebridae in the larger context of the superfamily Conoidea was assessed. Additional Conoidean taxa were

included in the 12S mtDNA analysis; the results are shown in Figure 2b. The six species of Terebridae analysed form one of five major branches and comprise a well-defined monophyletic group within the superfamily. Other major branches include the turrid species traditionally assigned to the subfamily Turrinae, and the cone snails. A feature of the tree worth noting is that the six *Conus* species analysed have relatively short branches, compared to the relatively deeper branching observed for the six terebrid species. The results shown in Figure 2b support the general perception that cone snails and auger snails diverged from each other early in conoidean evolution.

The separation of *H. hectica* and *H. lanceata* into a different genus from *Terebra* is supported by the results of the molecular analysis (Fig. 2a). It should be noted that on the basis of shell morphology, *T. areolata* and *T. subulata* seem very similar and are often confused with each other; a recent comprehensive treatment of *Terebra* species has separated these two species together into a distinct genus different from other *Terebra* species. The molecular analysis appears to show that species with venom ducts (i.e. *T. subulata* and *T. guttata*) and the *Terebra* species without venom ducts (*T. crenulata* and *T. areolata*) are in different clades, and the presence or absence of a venom duct is more reliable as an indicator of phylogenetic affinity than the shell morphological criteria that have been used traditionally. Before a reliable key for correlating true phylogenetic affinities with shell morphological similarities can be obtained, a much more comprehensive molecular analysis is required.

Morphology of the digestive system of Hastula hectica

H. hectica is anatomically typical of terebrids with venom glands. Like other Terebridae, it possesses a long extensible labial tube (introvert formed by the extension of the walls of the rhynchodaeum), which usually is inverted within the rhynchocoel cavity (Fig. 3a—ri). This labial tube supposedly facilitates prey capture when everted. We were not able to observe the evertion of the introvert in our specimens. The proboscis is relatively long; in a contracted form, it is at least as long as the rhynchocoel itself, and therefore is folded to provide space for the inverted labial tube. The thin-walled buccal tube (Fig. 3b—bt) leads from the mouth of the proboscis tip to the muscular oval buccal mass, situated at the proboscis base (Fig. 3b—bm), where it is clearly seen through the walls of the buccal tube. The foregut glands are typical for terebrids: the long venom gland (Fig. 3a and b—vg) with a large muscular bulb (Fig. 3a—mb) occupies a significant portion of the body sinus and opens into the buccal cavity near the proboscis base (Fig. 3b). Immediately anterior to the venom duct, the buccal sac of the radula opens into the buccal cavity (for terminology see Taylor et al., '93; Fig. 3b). The radular diverticulum is of typical morphology and consists of a medium-sized curved radular sac (where the radular teeth are formed) (Fig. 3b—rad), a rather short and narrow radular cecum (Fig. 3b—rc) (where the completely formed teeth are stored before usage on the proboscis tip) and a very short buccal sac, which leads from the radular cecum to the buccal cavity.

The salivary gland is single (fused from initially paired glands), narrow and semicircular, embracing the esophagus from the dorsal side (Fig. 3b—sg). The paired salivary ducts pass from both sides of the esophagus to open nearly symmetrically into the buccal sac of the radular diverticulum. *H. hectica* possesses paired tubular, comparatively large accessory salivary glands, situated ventrally on both sides of the esophagus (Fig. 3b—asg); the narrow ducts of the glands enter the proboscis base. We were not able to trace them on dissection, but in most neogastropods they open into anterior part of the buccal tube, leading from the mouth opening to the buccal cavity.

The radular morphology is unique. The radular sac contains about 25 rows of teeth (around 50 in overall), five of which not completely formed (Fig. 4A), whereas the radular cecum has only about 12 teeth. The radular teeth are around 770 μm long (6.2% of aperture length). The teeth are of the typical hypodermic type, enrolled, with a wide opening at the base (Fig. 4D) and barbed at the tip, where the large oval opening is situated (Fig. 4F). A very unusual character is that the tooth is punctured along most of its length with numerous round to oval holes, which are the largest in the middle and anterior third of the tooth (Fig. 4B, 4C) and gradually diminish in diameter to become obsolete approaching the tooth base. Thus, in contrast to the hypodermic needle-like teeth of other conoideans that inject venom, the *H. hectica* radular tooth looks like a perforated spear.

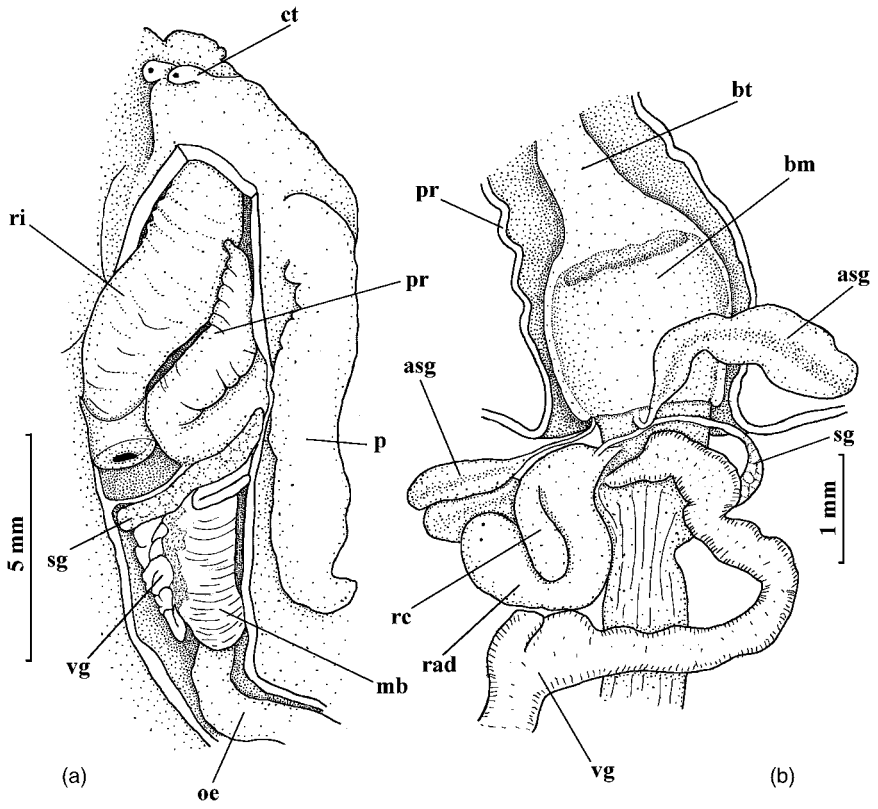

Fig. 3. Anatomy of the foregut of *Impages hectica*. (**a**) Rhynchocoel and body sinus opened by dorsal incision. (**b**) Posterior part of proboscis and anterior esophagus from the ventral side. Buccal mass is seen through the walls of the transparent buccal tube. All cuts left white. asg, accessory salivary gland; bm, buccal mass; bt, buccal tube; ct, cephalic tentacles; mb, muscular bulb of the venom gland (duct); oe, esophagus; p, penis; pr, proboscis; rad, radular sac; rc, radular cecum; ri, labial tube or rhynchostomal introvert; sg, salivary gland; vg, venom gland (duct).

Purification and biochemical characterization of toxins from Hastula hectica *venom*

Specimens of *H. hectica* were dissected, and venom extracts prepared as described in Methods. Venom peptides were fractionated using HPLC, and major peaks that could be purified to homogeneity were analyzed using standard Edman sequencing protocols. The HPLC profile obtained from crude venom is shown in Figure 5 (Imperial, 2007); eight of the toxins were further purified by HPLC to homogeneity in sufficient amounts for amino acid sequencing. The sequences obtained are shown in Table 1 (Imperial, 2007). Two of the peptides have four Cys residues (two disulfides), two have six Cys residues (three disulfides) and four have eight Cys residues. The peptides characterized vary in size from 11 to 37 amino acids; what is noteworthy is that except for two of the peptides with eight Cys residues (hheTx4 and hheTx5), which clearly exhibit strong sequence similarity, the rest differ in their Cys patterns; thus there are seven different Cys patterns, including a very unusual one with three vicinal Cys residues (C … C … C … C … C … CCC) for hheTx2.

The biological activity of four of the partially purified fractions was assessed by injecting the samples into the nematode *C. elegans* (see Methods). Of the four tested, only one fraction, which contained the peptide, hhe7a, was active. Upon injection of the purified peptide, the worms had an abnormally twisted body and became immobile. When a fivefold dilution of the toxin was tested, the worms exhibited uncoordinated twisting for 10–20 min. Thus, the hhe7a peptide clearly affects locomotion of *C. elegans*.

A striking contrast between the peptides directly isolated from *H. hectica* venom ducts and conopeptides is the complete absence of any posttranslational modification. It is conceivable that eight peptides from this complex venom that were not posttranslationally modified happened to be purified and other venom components might be

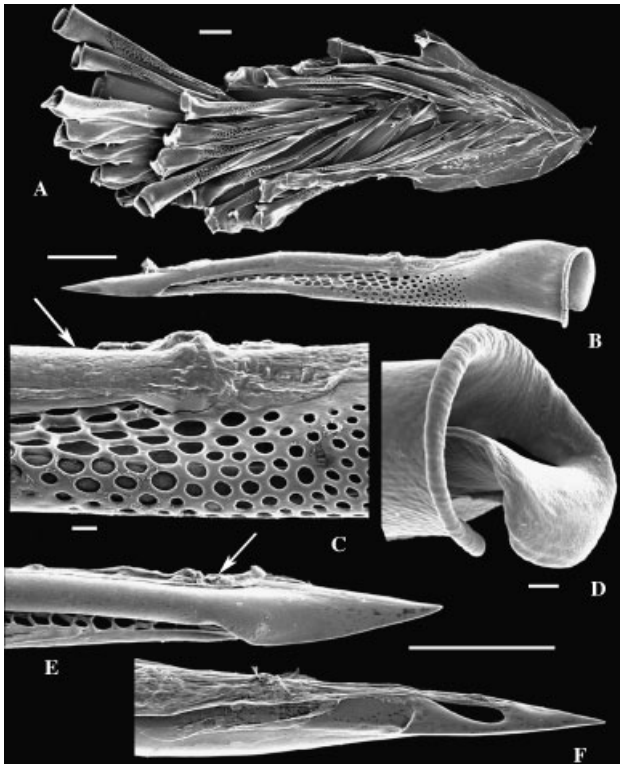

Fig. 4. Radula of *Impages hectica*. (**A**) Part of radular membrane from the radular sac, position of teeth undisturbed. (**B**) Separate radular tooth. (**C**) Central portion of the radular tooth. (**D**) Base of the tooth. (**E–F**) Tip of the tooth in different views. Scale bars: 100 μm for A, B, E and F, 10 μm for C, D. Arrows indicate the remains of the radular membrane.

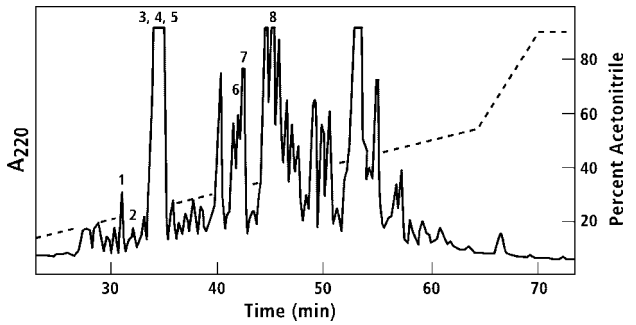

Fig. 5. Fractionation of peptide toxins from the venom of *Hastula hectica*. Crude *H. hectica* venom was collected as described in Methods. The venom extracts from nine specimens were pooled, and a separation using reversed-phase high-performance liquid chromatography was carried out on the crude venom extract; the peaks marked are those that were purified further, and for which homogeneous components were obtained in sufficient quantities to complete amino acid sequencing (Peak 1 = hhe1a; Peaks 2,3,4 = hhe7a, hheTx1 and hhe9a; Peak 5 = hheTx2; Peak 6 = hheTx3). The sequences of the peptides obtained are shown in Table 1.

highly posttranslationally modified. One characteristic posttranslational modification of *Conus* peptides is the γ-carboxylation of glutamate to Gla, which requires the vitamin K-dependent carboxylation of glutamate. The availability of a specific antibody for this posttranslational modification made it possible to assay sensitively whether any peptide in *H. hectica* venom has Gla; in a typical *Conus* venom, ~10% of peptides has this modification.

A Western blot analysis for the presence of Gla of a crude venom extract of *H. hectica* was carried out as described in Methods. The M3B Gla-specific monoclonal antibody was used to detect this posttranslational modification; as a control, a venom extract from a worm-hunting *Conus* species, *C. figulinus*, was analyzed in parallel. The results of this analysis are shown in Figure 6: although comparable amounts of *C. figulinus* and *H. hectica* venoms were analyzed, intensely labeled bands were detected only in the *C. figulinus* venom, but there was little or no staining of *H. hectica* venom components. Thus, in comparison to *Conus* venoms, it would appear that the venom of *H. hectica* has either no, or exceedingly low levels of γ-carboxylation. Thus, both a random sample of major peptides in the venom as well as a comprehensive analysis of the entire venom suggest that compared with *Conus* venoms, this posttranslational modification is either completely absent or occurs only at very low levels in *H. hectica* venom.

Identification of toxin-encoding cDNA clones from Hastula hectica *venom ducts*

cDNA was prepared from *H. hectica* venom ducts as described in Methods. The sequence analysis of 118 randomly chosen cDNA clones was carried out. A description of the classes of clones found is summarized in Table 2; a number of clones (5) had identities to known polypeptides, including some ribosomal proteins; an even larger number (24) encoded polypeptides without any sequence similarity to known proteins, and which do not seem to be toxins. However, a significant number of clones (26) encoded secreted disulfide-rich polypeptides; these are the genes that encode the putative peptide toxins.

In total, eight distinct mature toxins are predicted from the clones sequenced. If the rules established for *Conus* toxins are followed with regard to proteolytic processing of the toxin precursors, mature peptide sequences can be deduced; these are shown in Table 3. The

TABLE 1. Summary of peptides purified from Hastula hectica venom

	Sequence	Peak no. (see Fig. 5)
Two disulfides		
hhe1a	GE**C**C**C**TD**C**AQTAAANY**C**	1
hheTx1	D**C**LP**C**GHDV**C**C	3
Three disulfides		
hhe7a	AR**C**EQ**C**PSY**C**C**C**QSDSPPE**C**DG**C**E	4
hhe9a	YEEN**C**GTEY**C**TSKIG**C**PGR**C**V**C**KEYNYNGEITRR**C**RA	5
Four disulfides		
hheTx2	S**C**SSG**C**SD**C**NSDS**C**Q**C**TLNQFTNSDS**C**C**C**	2
hheTx3	KQ**C**TSNM**C**SAD**C**SPG**C**C**C**IIDKLEW**C**T**C**D**C**	6
hheTx4	NEV**C**PPGE**C**QQY**C**C**C**DLRK**C**K**C**INLSFYGLT**C**N**C**DS	7
hheTx5	NEV**C**PPGR**C**EPY**C**C**C**DPRK**C**K**C**LSIDFYGLV**C**N**C**DS	8

The sequences were obtained from the high-performance liquid chromatography peaks shown in Fig. 5; the indicated high-performance liquid chromatography fractions were purified further and sequenced as described in Methods.

predicted toxins varied in size from 19 to 41 amino acids. The sequences shown may be further processed at the C-terminal end to yield even smaller gene products. Only one clone, Hhe9.1, encoded a gene product (hhe9a) isolated from venom; Hhe9.1 was represented by over 10% of all of the sequences analyzed (13/118). The assumption that conotoxin processing rules are followed can be tested for this one toxin that was both encoded by a cDNA clone, and directly isolated from venom. The entire precursor sequence derived from the open reading frame of clone Hhe9.1 is shown in Table 4; the predicted processing does indeed yield the mature toxin actually isolated from venom, hhe9a. Thus, the precursor for hhe9a has the canonical prepropeptide organization of conotoxins—in this case, a precursor of 88 AA with a standard signal sequence (21 AA), an intervening ''pro: region (30 AA), and the mature toxin (37 AA) in single copy, at the C-terminal end of the precursor.

A high fraction of the predicted mature toxins was peptides with six cysteine residues (presumably crosslinked into three disulfide bonds), whereas two of the predicted mature toxins had four cysteine residues. Of the six different cDNA clones encoding predicted mature peptides with six cysteine residues, two have an arrangement similar to the P-conotoxin superfamily (we refer to these as P-type *Hastula* peptides), and four have Cys patterns similar to the O-conotoxin family (these are referred to as the O-type peptides). We have followed the nomenclature adopted for conopeptides both with regard to the naming of the peptides and of the corresponding clones. Thus, the P-type *Hastula* peptide hhe9a is encoded by clone Hhe9.1, and has the class IX

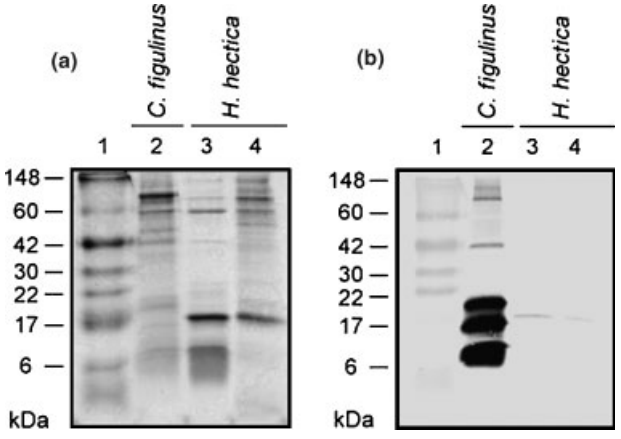

Fig. 6. Gel electrophoresis and Western blot analysis of venom extract from *H. hectica*. Crude venom extracts (25 μg) from *Hastula hectica* and *Conus figulinus* were reduced, alkylated, and electrophoresed in a sodium dodecyl sulfate/15%/-polyacrylamide gel. (**a**) Gelcode Blue stained gel. (**b**) Western blot employing the Gla-specific mouse monoclonal antibody M3B. Lane 1, molecular weight marker; lane 2, crude extract from *C. figulinus*; lane 3, crude extract from *H. hectica*; lane 4, pellet fraction after centrifugation of crude extract from *H. hectica*.

conotoxin Cys pattern (… C … C … C … C … - C … C …). In contrast, four of the clones are of the ''O-type'', with a class VI/VII Cys pattern (… C … C … CC … C … C …). An overview of conotoxin Cys patterns and nomenclature has been discussed in several review articles (Terlau and Olivera, 2004; Olivera, 2006).

DISCUSSION

The combined phylogenetic, anatomical and toxinological analysis accomplished above on the auger snail *H. hectica* is the most extensive carried

out so far on any species of Terebridae. The molecular phylogeny of *H. hectica* was assessed both within the family Terebridae and across the superfamily Conoidea. Then, the anatomy of *H. hectica* was described and finally the similarities and differences in toxinology between *Conus* peptides and *H. hectica* were discussed.

The molecular analysis was carried out to more precisely define the phylogenetic placement of *H. hectica* in relation to other Terebridae, and to other conoideans. *H. hectica* and *T. subulata* belong to divergent branches within Terebridae (see Fig. 2A). As expected, the family Terebridae seems to be a monophyletic group within the broader conoidean phylogenetic tree. From the sample of taxa analyzed, there are at least five major groups within the superfamily suggested by the tree in Figure 2B. From the species set shown, the terebrids comprise one of these major branches. The phylogeny obtained suggests that a comparison between *H. hectica* and *T. subulata*, and between *H. hectica* and *Conus* are between highly divergent taxa within Teribridae, and within the superfamily Conoidea, respectively.

The anatomy of the foregut and radula of *T. subulata* was described previously by Taylor ('90); the entire arrangement of the organs of the foregut is generally similar in *H. hecti*ca. A major difference is the presence of a septum in *T. subulata*, and its absence in *H. hecti*ca. The septum is the muscle membrane with an orifice, which separates the rhynchocoel (the cavity where the retracted proboscis and labial tube are situated) into two compartments. In a retracted position, the labial tube is situated in front of the septum, whereas the proboscis is posterior to it; the proboscis can be protruded, when everted, through the orifice. The septum is sporadically found in different Terebridae, in species of the subfamily Raphitominae and in the genus *Conus*; its function remains unknown. In this respect, *T. subulata* is similar to *Conus*, whereas *H. hectica* differs from both.

The radular teeth of *H. hectica* are unique and differ markedly from that *T. subulata* by the presence of the numerous holes that penetrate the walls of the tooth (see Fig. 4F). The function of the unusual morphology of the *H. hectica* radular tooth is a matter of speculation. This peculiar radular morphology was noted previously by Taylor ('90).

Although there are no direct observations of *H. hectica* feeding, a mechanism is suggested by the radular and foregut anatomy of the species. The presence of the subradular membrane in Terebridae has not previously been noted; from our studies, it is clear that the subradular

TABLE 2. Summary of cDNA clones analyzed from Hastula hectica venom ducts

Name	No. clones
Clones encoding toxins	26
Hhe9.1	13
Hhe9.2	3
Hhe6.1	3
Hhe6.2	3
Other toxins	4
Clones encoding polypeptides that are not toxins	29
Blast identity assigned	5
Unknown identity[a]	24
No ORFs or very small ORF	44
Small inserts	19
Total	118

ORF, open reading frame.
[a]One of the unknown polypeptides in this class was represented by ten different clones.

TABLE 3. Mature toxin sequences derived from cDNA clones from Hastula hectica

Three disulfides	
P-type pattern	
Hhe9.1	YEEN**C**GTEY**C**TSKIG**C**PGR**C**V**C**KEYNYNGEITRR**C**RA
Hhe9.2	DEEVG**C**FPNV**C**KNDGN**C**SIETSTGMTR**CQC**LEGYTGHV**C**ENPL
O-type pattern	
Hhe6.1	GMGIGINLPP**C**IKNGEY**C**NPWTGSIILGGA**CC**GT**C**TDYE**C**H
Hhe6.2	ALP**C**PYG**C**PLR**CC**HMTDGV**C**LRNKQG**C**
Hhe6.3	VLFTPPELLG**C**GNR**C**SDD**CC**KWGR**C**QPG**C**TD
Hhe6.4	SSLH**C**GDDPW**C**PTG**CC**ENED**C**DIG**C**KRDWEKSRSQP
Two disulfides	
Clone Hhe9	ALSVLLQUS**C**TM**C**LF**CC**YL
Clone Hhe53	GLSQSG**C**QAFTGRW**C**VG**C**ERLRSRVVWE**C**SPKRVVNSI

The cDNA clones were sequenced, and open reading frames identified. Sequences of the mature toxins that are predicted after posttranslational processing of the precursors are shown.

TABLE 4. Comparison of Hhe9.1 precursor sequence from Hastula hectica to a Lophiotoma olangoensis clone

```
                1         10        20        30        40        50
Hhe9.1          MMTKTGLVLLFAFLLVFPVSSLPMDAEAGHARLEMDKR-DAGNEAWTRLL
Lol142          MATTMTVVLITSVLLVLAEIF-PRDTDGQAAHKSTDQLSQLVDHLKLRKL

                ↓         60        70        80
                KRYEENCGTEYCTSKIGCPGRCVCKEYNYNGEITRRCRA
                SR-RQYCPSNTCNERDECPGNCNDCEYVPGTGPNKRCVKK
```

Hhe, *Hastula hectica*; Lol, *Lophiotoma olangoensis*. Arrow shows where proteolytic cleavage occurs to generate the mature peptide toxin.

membrane is relatively strong in *H. hectica*. The integrity of the radula persists after dissolving the radular sac in bleach and even in the radular cecum the teeth are attached to the membrane. In contrast, in *Conus* spp. which also have some vestigial subradular membrane (Kantor and Taylor, 2000), the teeth lying in the radular cecum are entirely separate from the membrane. This difference suggests significant divergence between the feeding mechanisms of *Conus* and *H. hectica*.

In *Conus*, as well as in some other conoidean groups that have ''hypodermic radula'', numerous ready-to-use teeth are available in the radular cecum for immediate use. Multiple teeth can be used on a single prey, as is observed in mollusc-feeding *Conus* spp. Thus, delivery of the tooth from the radular cecum to the proboscis tip occurs rapidly, probably by contraction of the proboscis wall (Schulz et al., 2004). However, in *H. hectica*, because the teeth in the radular cecum are still attached to the membrane, these cannot be immediately used for stabbing prey when required.

In the process of radular growth the oldest part of the membrane (situated in the radular cecum) is permanently destroyed and the teeth become dislodged. When the tooth is separated from the membrane, it is transferred to the proboscis tip, where it is presumably held until it is used. This is also assumed for members of the Turridae sensu stricto that have a strong subradular membrane. In most specimens examined, there was a tooth at the proboscis tip held by the sphincter(s) (Kantor and Taylor, '91). Because the tooth in *Hastula* is held at the proboscis tip for a significant period of time before an attack and is concealed completely within the proboscis with its base resting on the large sphincter, this suggests that during prey envenomation, only a small portion of the radular tooth protrudes from the proboscis. Therefore, the radula would not penetrate the body of the prey as deeply as would occur in a fish-hunting *Conus* envenomation, for example.

The radular morphology in *H. hectica* seems to be unique within the family; there are no other known Terebridae species with the similar radular teeth. Several species of *Mangelia* (Mangeliinae) (Rolán and Otero-Schmidtt, '99) apparently have holes penetrating the walls of the hollow hypodermic tooth; unfortunately the publication had line drawings without a detailed discussion of the radular morphology. This report suggests that holes penetrating the walls of the hypodermic tooth may have originated independently at least twice in Conoidea.

We have no data on the prey preference of *H. hectica* in Philippines, but according to Miller ('79), in Hawaii it feeds on sand-dwelling polychaete worms *Nerinides* sp. (= *Scolelepis*) (Spionidae). There is one more species of Terebridae in Hawaii, *Hastula strigillata* that has the usual radular morphology without any holes but feeding on the same polychaete worms (J. Taylor, personal communication). Thus, any connection between diet and the radular morphology for *Hastula* remains tenuous.

Previous work on *T. subulata* (Taylor, '90; Imperial et al., 2003) characterized the anatomy and three toxins purified from venom; in this study, a more comprehensive characterization of *H. hectica* venom has been carried out; eight toxins were elucidated by venom purification and eight from cDNA clones derived from venom duct mRNA. The spectrum of peptides characterized is biased clearly toward those that are highly expressed because major toxin peaks were analyzed (see Fig. 5) and the more abundant cDNA clones (such as Hhe9.1) were more likely to be sequenced by the molecular cloning approach used. Surprisingly, only one toxin purified from venom was identified in the cDNA analysis. This toxin, hhe9a, was the most abundant cDNA clone found in the *H. hectica* cDNA library, and was a major component of the venom. The minimal degree of overlap observed between the cDNA

clones and the toxins purified from venom suggests that the total number of toxins in the venom of *H. hectica* is likely to be very much larger than the 15 different sequences identified by the combined biochemical/molecular analysis. This assertion is also supported by the HPLC profile shown in Figure 5. Thus, the different *Hastula* toxins identified in this study comprise only a small minority of the total toxin diversity in the venom of this species, suggesting a venom complexity comparable with that of the cone snails.

The *H. hectica* toxins purified from venom were generally smaller (11–37 AA) than the three toxins purified previously from *T. subulata* venom (~40 AA). Because the latter only involved three putative toxins, a more extensive characterization of toxins from *T. subulata* and *H. hectica* venom ducts may reveal toxins with sequence similarities. Unexpectedly, the only discernible homology observed for any *Hastula* toxins in this study was with a toxin identified previously by molecular cloning from the turrid *L. olangensis* (see Table 4; Watkins et al., 2006). Thus, the genes expressed in turrid and terebrid venoms may overlap more than might have been expected from the presumed long divergence between the two groups.

In both *H. hectica* and *Conus* venoms, a diverse spectrum of unusually small peptidic toxins are present; four of the toxins identified from *H. hectica* venom are under 25 amino acids in length, a size comparable with the smaller conopeptides. Furthermore, like most *Conus* peptides, *Hastula* toxins have two to four disulfide linkages. The precursors of *Hastula* toxins have the prepropeptide organization characteristic of conopeptide precursors, and proteolytic processing of precursors is required in both instances to generate the mature peptide toxin. Thus, the general biochemical characteristics of venom components of *H. hectica* and *Conus* are quite similar.

However, there are also striking differences. First is the apparent lack of posttranslationally modified amino acids in the *Hastula* peptides. No toxins identified in the Terebridae so far show evidence of C-terminal amidation, and there is a surprising absence in *Hastula* toxins of even the more common posttranslationally modified amino acids found in *Conus* peptides (such as hydroxyproline). Even with this small sample, this is a rather striking result; a random sample of the same number of *Conus* peptides in the same size range would have revealed a number of diverse posttranslational modifications. Analysis of the whole venom extract suggested that one of the characteristic modifications found in ca. 10% of *Conus* peptides, Gla, is absent in *H. hectica* venom. The second difference is the complete lack of overlap between the gene families expressed in the two types of venom. These results suggest a fundamental divergence in pathways of toxin evolution in *Hastula* and in *Conus*, although there is a convergence in the complexity of the venoms, and in the use of small disulfide-rich peptides as the biologically active venom components.

ACKNOWLEDGMENTS

This research was supported in part by Program Project GM 48677 from the National Institute of General Medical Sciences, and from research funds from the University of Utah.

LITERATURE CITED

Bratcher T, Cernohorsky WO. 1987. Living Terebras of the world. New York, NY: American Malacologists, Inc.

Brown MA, Stenberg LM, Persson U, Stenflo J. 2000. Identification and purification of Vitamin K-dependent proteins and peptides with monoclonal antibodies specific for gamma-carboxyglutamyl (gla) residues. J Biol Chem 275:19795–19802.

Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294:2310–2314.

Imperial JS. 2007. Novel peptides from *Conus planorbis*, *Terebra subulata*, and *Hastula hectica* (Ph.D. dissertation). Brisbane: The University of Queensland.

Imperial JS, Watkins M, Chen P, Hillyard DR, Cruz LJ, Olivera BM. 2003. The augertoxins: biochemical characterization of venom components from the toxoglossate gastropod *Terebra subulata*. Toxicon 42:391–398.

Imperial JS, Silverton N, Olivera BM, Bandyopadhyay PK, Sporning A, Ferber M, Terlau H. 2007. Using chemistry to reconstruct evolution: on the origins of fish-hunting in venomous cone snails. Proc of the American Philosophical Society 151:185–200.

Kantor YI, Taylor JD. 1991. Evolution of the toxoglossan feeding mechanism: new information of the use of radula. J Molluscan Stud 57:129–134.

Kantor YI, Taylor JD. 2000. Formation of marginal radular teeth in Conoidea (Neogastropoda) and the evolution of the hypodermic envenomation mechanism. J Zool Lond 252:251–262.

Kumar S, Tamura K, Nei M. 2004. MEGA 3: Integrated software for molecular evolutionary genetic analysis and sequence alignment. Brief Bioinform 5:150–163.

Marcus E, Marcus E. 1960. On *Hastula cinerea*. Boletins da Faculdade de Filosofia, Ciencias e Letras. Universidade de São Paulo, Zoologica 23:25–66.

Mello CC, Kramer JM, Stirchcomb D, Ambros V. 1991. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J 10:2959–3970.

Miljanich GP. 1997. Venom peptides as human pharmaceuticals. Sci Med 4:6–15.

Miljanich GP. 2004. Ziconotide: neuronal calcium channel blocker for treating severe chronic pain. Curr Med Chem 11:3029–3040.

Miller BA. 1970. Studies on the biology of Indo-Pacific Terebra (Ph.D. dissertation). Durham, NH: University of New Hampshire.

Miller BA. 1971. Feeding mechanisms of the family Terebridae. Rep Am Malacological Union AMU Pacific Division 1970:72–74.

Miller BA. 1975. The biology of *Terebra gouldi* Deschayes, 1859, and a discussion of life history similarities among other hastulas of similar proboscis type. Pacific Sci 29:227–241.

Miller BA. 1979. The biology of *Hastula inconstans* (Hinds, 1844), and a discussion of life history similarities among other hastulas of similar proboscis type. Pacific Sci 33:289–306.

Olivera BM. 2002. *Conus* venom peptides: reflections from the biology of clades and species. Annu Rev Ecol Syst 33:25–42.

Olivera BM. 2006. *Conus* peptides: biodiversity-based discovery and exogenomics. J Biol Chem 281:31173–31177.

Olivera BM, Rivier J, Clark C, Ramilo CA, Corpuz GP, Abogadie FC, Mena EE, Woodward SR, Hillyard DR, Cruz LJ. 1990. Diversity of *Conus* neuropeptides. Science 249:257–263.

Oliverio M, Mariottini P. 2001. A molecular framework for the phylogeny of *Coralliophila* and related muricoids. J Molluscan Stud 67:215–224.

Rolán E, Otero-Schmidtt J. 1999. The family Turridae s.l. (Mollusca, Neogastropoda) in Angola, 2. subfamily Mangeliinae Fischer, 1883. Argonauta 13:5–26.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics (Oxford, England) 19:1572–1574.

Schulz JR, Norton AG, Gilly WF. 2004. The projectile tooth of a fish-hunting cone snail: *Conus catus* injects venom into fish prey using a high-speed ballistic mechanism. Biol Bull 207:77–79.

Taylor JD. 1990. The anatomy of the foregut and relationships in Terebridae. Malacologia 32:19–34.

Taylor JD, Kantor YI, Sysoev AV. 1993. Foregut anatomy, feeding mechanisms, relationships and classification of the Conoidea (= Toxoglossa) (Gastropoda). Bull Nat Hist Mus Lond (Zool) 59:125–170.

Terlau H, Olivera BM. 2004. *Conus* venoms: a rich source of novel ion channel-targeted peptides. Physiol Rev 84:41–68.

Terryn Y. 2007a. A Collectors Guide to Recent Terebridae (Mollusca: Neogastropoda). Hackenheim: ConchBooks & NaturalArt.

Terryn Y. 2007b. Genus Terebra Bruguière, 1789. In: ConchBooks, editor. A Collectors Guide to Recent Terebridae (Mollusca: Neogastropoda). Hackenheim: ConchBooks & Natural Art. p Plate 6.

von Heijne G. 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 14: 4683–4690.

Watkins M, Hillyard DR, Olivera BM. 2006. Genes expressed in a turrid venom duct: divergence and similarity to conotoxins. J Mol Evol 62:247–256.