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Short Communication

P-like conotoxins detected in *Turris babylonia*

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Abstract: Toxin homologs are common across turrid species and reflect on similarities of their prey types. P-like conotoxin is one type isolated in various turrid snails, which exhibits conserved sequences in their precursor peptides with marked diversity and subtype variations in similar groups of turrid snails, a toxin repertoire patterning, termed as "P-coding" system, employed by turrid snails as a toxin diversification strategy to better target their prey. This study aimed to determine if P-like type 1 and type 3 conotoxin genes are present in *Turris babylonia* using gene-specific primers used in *Gemmula* species. Total RNA was extracted from venom duct and then used to prepare double-stranded (ds) cDNA. The ds cDNA was used as template for P-like type 1 and type 3 conotoxins amplification. Then, the amplicons generated were sent to Macrogen, Inc., Seoul, South Korea for sequencing and analyzed using DNAsis. Results showed that P-like conotoxin type 1 and type 3 were amplified from *T. babylonia*. The gene sequences showed similar framework IX scaffold. The P-like conotoxin type 1 has 85 amino acid residues with the characteristic six-cysteine residues and a conserve region of YEDGE similar to *T. babylonia*. The P-like conotoxin type 3 is the first of this type ever reported in this gastropod species. It has 62 amino acid residues with six-cysteine residues but with divergent amino acid sequence from the P-like type 1 conotoxin. P-like conotoxin type 1 and 3 were detected in *T. babylonia* using gene specific primers for *Gemmula* species. The detection of these P-like conotoxins provides support on the hypothesis of a possible "P-coding" system among the turrid snails. Similar approach can be done in other turrid species.

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Keywords: *Turris babylonia*, P-like conotoxins, venom peptides, conidae, turripeptide

1. Introduction

Venomous marine snails of the superfamily Conoidea are considered one of the most diverse invertebrate lineages attributing to more than 10,000 species (Olivera *et al*. 2014; Imperial *et al*. 2014). Among the Conoidea, turrids are the most primitive, appearing around 120 million years ago (Heller 2015).

Turrids are a paraphylectic group from the marine Gastropoda and were previously designated as the family Turridae, encompassing more than 3,600 named living species with several new species described every year (Kantor *et al*. 2017). However, there is no distinct turrid shell shape by which all members can easily be identified (Omaga *et al*. 2017). Due to numerous examples of homoplasy in their shell characteristics, there have been species delimitation and difficulty in morphological identification of turrids (Kantor *et al*. 2017).

Nevertheless, there are few iconic turrid species, such as *Turris babylonia*, the "tower of babel" (see Figure 1) which has a characteristic shell shape, sculpture and color pattern, thus allowing an unambiguous identification. For 200 years, *Turris babylonia* has been considered an easily identifiable and nominate species for its genus (Kantor *et al*. 2017). In order to capture their prey, these snails are equipped with feeding guilds and complex venoms known as turritoxins which diverge between species through hypermutation within gene families, each having a specific target prey. Although they are still characterized with highly conserved signal and pro sequences (Olivera *et al.* 2014; Omaga *et al*. 2017; Heller 2015).

Since the first characterization of turritoxins, numerous toxins are reported in the literature. Toxin homologs are common across turrid species and reflect on similarities of their prey-types. P-like conotoxin is one type isolated in various turrid snails. P-like conotoxins exhibit conserved sequences in their precursor peptides with marked diversity and subtype variation in similar groups of turrid snails, a toxin repertoire patterning, termed by Heralde (2007) as "P-coding" system, employed by turrid snails as a toxin diversification strategy to better target their prey. Hence, this study was conducted to determine if P-like type 1 and type 3 conotoxin genes are present in *Turris babylonia* using gene-specific primers used in *Gemmula* species, a genus belonging to same family as *Turris babylonia.* The use of genus-specific primers among different genera in a family of marine snails like the turrids may prove the presence of conserved genes among the genus within the family and a simpler approach in conotoxin discovery.

Figure 1. *Turris babylonia*. The shell with angular whorls. Taken from the coast of Cebu and Bohol, Philippines, May 2006, 20- meter depth, 6 cm. (Heralde, 2007) Reproduced with permission.

2. METHODOLOGY

2.1. Sample Collection, Authentication, and Total RNA Extraction

The sample was taken from the authenticated collection of Francisco M. Heralde III and collected from the coasts of Cebu and Bohol, Philippines 300 to 500 meters off the coastline at the depth of 20 meters using tangled nets. The venom ducts were isolated, collected, and placed in RNAlater™ (Ambion, TX, USA) and stored at -80^{0}C until further use. Total RNA were first extracted from the venom ducts of the snails (*Turris babylonia*) with RNEasy™ Mini Tissue Kit (Qiagen, Hilden, Germany) and kept at -80^{0}C until further use.

2.2. Double stranded cDNA Preparation

Extracted total RNA from the venom ducts of the snails was then used in the preparation of the double-stranded (ds) cDNA, following the SMART cDNA (Clontech Laboratories, Inc., CA, USA) protocol following the manufacturer's protocol.

2.3. PCR Amplification using primers for *Gemmula* Species

The putative conotoxin genes were amplified from two µL dsDNA product as template in 20 µL PCR reaction mix containing two µL PCR buffer (10X), dNTPs (200 µM), primers (0.5 µM each), Taq DNA polymerase (1U) and diethylpyrocarbonate (DEPC)-treated water. The primers used to amplify P-like conotoxin 1 was (5'-A(G/T)CGAAG(A/C)GCT(C/G)CATTCG-3') and P-like conotoxin 3 was (5'-ATC (G/C)A(T/G)(C/T)GAT (C/A)TGTT(T/G)TG-3') which were gene-specific primers designed for peptide conotoxins of *Gemmula speciosa.* The PCR profile used were as follows: an initial denaturation of one min at 95 °C, followed by 40 cycles of 20 sec at 95 °C, 20 sec at 54 °C and 30 sec at 72 °C, and a final extension of 5 min at 72 °C. All amplicons were analyzed by gel electrophoresis using agarose gel.

2.3 DNA Sequencing

The cDNA was then used as template for P-like type 1 and type 3 conotoxin amplification. The amplicons were analyzed and visualized using gel electrophoresis and the generated amplicons were sent to Macrogen, Inc., Korea for sequencing. The sequence was analyzed for open reading frame and protein sequence determined using DNAsis (Miraibio, Inc., CA, USA).

3. RESULTS AND DISCUSSION

Turrids produce venom that interferes with the neuromuscular ion channels by preventing the prey from closing the sodium gates and opening the potassium gates, thereby disrupting the electric signals that leave the nerve cell. This eventually results to the continuous paralysing twitch of all the body muscles, which then immobilizes their prey (Heller 2015; Gonzales & Saloma 2014). The turrid snails pose significant challenges in its taxonomy as being the largest family in the superfamily Conoidea and with largely unknown turrid peptides found in their venom (Olivera, Seronal, & Fedosov 2010; Kendel *et al.* 2013).

The enormous resource of natural peptide toxins from this venom has great pharmacological and research potential. Each species has its own distinct complement of highly structured peptide toxins, which then has a specific, physiologically relevant protein target. The molecular, physiological and pharmacological characterizations of these diverse and numerous peptide toxins have evolved in a new class of bioactive drugs. These highly bio-diversified groups of venomous marine gastropods still have so much to be discovered for potential new species and pharmacologic uses. The peptide toxin discovery from conoideans has revolutionized the way scientists try to harvest the natural resources available for pharmacological application (Olivera *et al*. 2014; Heller 2015; Gonzales & Saloma 2014).

As applied in this study, the determination of P-like conotoxins using gene-specific primers developed from various turrids such as *Gemmula* species was successful in detecting similar toxins in *Turris babylonia*. In the study by Heralde (2008), the Pg-gene superfamily of *Gemmula* venom peptides are identified and characterized. The similar primers from *Gemmula speciosa* and *Gemmula lisajoni* coding for P-like conotoxin type 1 and type 3 respectively were used in this study.

The PCR amplification of the synthesized double stranded cDNA using the gene-specific primers for P-like conotoxin types 1 and 3 of *Gemmula* species showed distinct bands as seen from the agarose gel (see Figure 2). These bands corresponded to the expected amplicon size of approximately 250 bases. The utility of primers for *Gemmula* species in amplification of P-like conotoxin types 1 and 3 in *Turris babylonia* as used in this study provided evidence that

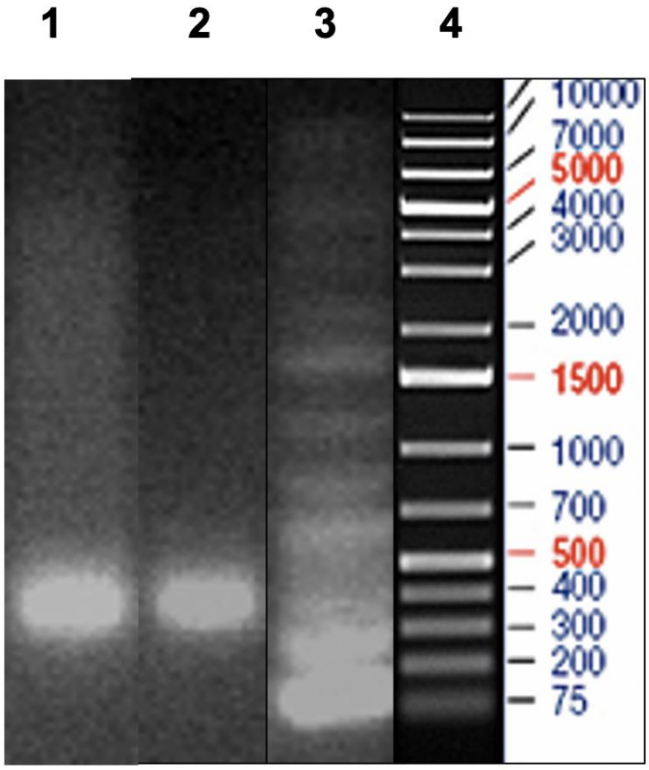

Figure 2. The agarose gel of PCR amplicons of P-like toxin type 1 and 3. Lane 1 is the P-like toxin type 1 PCR product. Lane 2 is the P-like toxin type 3. Lane 3 is the actual 1 kb GeneLadderTM run with the sample. Lane 4 is the 1 kb GeneLadderTM bands for comparison taken from the manufacturer's website (http://www.fementas.com/).

the conotoxins are conserved throughout evolution making this diverse group of marine gastropods as one of the most successful species of venomous marine organisms which use toxins for prey capture, avoidance of predation, and successful competition in the highly competitive marine ecosystem (Watkins, Hillyard, & Olivera 2006, Fu *et al*. 2018; Jin *et al*. 2019).

The gene sequences of P-like conotoxin types 1 and 3 showed similar framework IX scaffold (C-C-C-C-C-C) with differences in the mature peptide sequences. The toxin precursor of P-like conotoxin type 1 has 85 amino acid residues with the characteristic six-cysteine residues and a conserve region of YEDGE similar to the one reported by Heralde (2007), also in *T. babylonia* (see Table 1). The toxin precursor of P-like conotoxin type 3 is the first of this type ever reported in this gastropod species. It has 62 amino acid residues with the same six-cysteine residues but with divergent amino acid sequence from the P-like type 1 conotoxin. The toxin precursor peptide sequences of both toxins are shown in Table 1. Despite the presence of structural similarities of P-like conotoxin 1 and 3 with the general conotoxins with the distinctive cysteine framework, the biological function of these toxins may not necessarily be similar. The idea that the structural characteristics of these toxins follows the purported biological functions may not hold true to this group of marine gastropods. There is such a wide functional diversity among conotoxins due to post-translational modifications, sequence variation of the mature peptide toxins, and structural framework variations (Miles *et al.* 2002).

The conotoxins are complex polypeptides composed of three major sequences wherein the polypeptides are cleaved to release the functionally mature peptide sequence. These are short sequence polypeptides with a signal sequence in the "pre" region at the N-terminal, followed by a pro sequence, and the biologically active sequence at the C-terminal part. The several superfamilies of conotoxins derived from the cone snails (*Conus*) are well studied than its more abundant cousins, the auger snails (*Hastula*) and the turrid snails (*Turrid*). The diversity of conotoxins found in cone snails is also expected among auger and turrid snails. Hence, the body of knowledge about the conotoxins provides the foundation in our effort to elucidate the functional diversity of peptide toxins in auger and turrid snails (Watkins, Hillyard, & Olivera 2006; Becker & Terlau 2008).

The detection of P-like conotoxins type 1 and type 3 in *Turris babylonia* strengthens the hypothesis of the possible "P-coding" system among the generic members of the subfamily *Turrinae* (H. Adams and A. Adams1853 (1838)) and possibly among the *Conoidea*. Such P-like toxin gene homolog is present in most members of the subfamily *Turrinae*. In 2007, Heralde reported a toxin repertoire patterning, termed as "P-coding" system, which is used by these snails to effectively capture their prey. This coding system allows identification of potential toxins in Conoidean snails other than cone snails that have similar P-like pattern in its precursor peptide as used in categorizing conotoxins among cone snails (Heralde 2007; Heralde 2008; Robinson & Norton 2014). The approached used in this study to isolate potential peptide toxins in less studied and researched families of Conoidean snails can be

Table 1. Toxin precursor sequences of P-like conotoxin types 1 and 3 of *Turris babylonia* from amplicons using the designed P-like conotoxin type 1 and 3 primers for *Gemmula spp*. Peptide cleavage site is shown underlined while the conserved cysteine scaffold in bold letters. Conserved sequence relevant to clustering is shown italicized.

P-like toxin type 1 of *Turris babylonia*
MMAKLMITVMTVLLLSLQQGADGRSERWRKNQMAASRIMRNLITARLDPPRY**C**THKI**C***YEDGE***C**NQW**C**TAG**C**NLILGN**C**DTL **As reported in Heralde (2007) with 82 amino acids** MDVKGMITVMNVLGPSLQQGADGRSERWRKNQMAASRIMRNLIVARLDGPRY**C**THKI**C***YEGDE***C**NQW**C**TLG**C**NLILGS**C**DTAVVI **As reported in this study with 85 amino acids**
P-like toxin type 3 of *Turris babylonia* as reported in this study with 62 amino acids
MKVY**C**LLLVFVVFLSSQAPGRLDPR**C**SGV**C**FRPYSLL**C**VFSYPFTPKW**C**LIILFD**C**PVQFYN

used to further the research initiatives and our understanding of the diversity of the turrid peptides.

4. CONCLUSION

P-like conotoxin types 1 and 3 were detected in *T. babylonia* using gene specific primers for *Gemmula* species. The P-like type 3 toxin detected in this study was the first ever reported in this gastropod species. The detection of these P-like toxins provides support on the hypothesis of a possible "P-coding" system among the turrid snails. Similar approach can be done in other turrid species and the information utilized to correlate with their polychate-prey profiles.

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