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<th>Molecular characterization on the genome structure of hemolysin toxin isoforms isolated from sea anemone Actineria villosa and Phyllodiscus semoni.</th>
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Molecular characterization on the genome structure of hemolysin toxin isoforms isolated from sea anemone *Actineria villosa* and *Phyllodiscus semoni*

Gen-ichiro Uechi¹, Hiromu Toma², Takeshi Arakawa³, and Yoshiya Sato²*

¹Department of Virology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto Nagasaki City, Nagasaki 852-8523, Japan; ²Department of Parasitology and International Health, Graduate School of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan; ³Molecular Microbiology Group, COMB, Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

*Corresponding author: Yoshiya Sato, Ph.D.

Department of Parasitology and International Health, Graduate School of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0215, Japan.

Tel: +81-98-895-1129

Fax: +81-98-895-1409

E-mail: ysato@med.u-ryukyu.ac.jp
Abstract

We recently identified the existence of new isoforms of Avt-I (from sea anemone *Actineria villosa*) and Pstx20 (from sea anemone *Phyllodiscus semoni*) hemolytic toxins, and named them Avt-II and Pst-I. Avt-II and Pst-I differ in length by 14 and 7 bp, respectively, as compared to their corresponding isoform genes. Both newly found isoform genes have the coding regions with the identical length of 1,033 bp. The restriction fragment length polymorphism analysis with endonuclease *Hph*I was able to clearly distinguish between the two Avt isoforms, but not Pstx isoforms, and based on the densitometric analysis of DNA bands, it indicated that relative expression levels of Avt-I and Avt-II genes were 18.3% and 81.7%, respectively. PCR amplification of the two Avt isoform genes using the genomic DNA as template indicated the existence of two introns within each toxin isoform gene. The first intron with the identical 242 bp in length for both Avt isoform was found within the 5′-untranslated region, and the second intron with lengths of 654 bp and 661 bp in Avt-I and Avt-II isoforms, respectively, was found within the signal sequence coding region. This is for the first time to identify the existence of introns within hemolysin genes of sea anemone. Having several unique characteristics that have identified only for a new member of actinoporin family of *A. villosa* and *P. semoni*, e.g., strong toxicity and genes with introns, it is plausible to speculate that these toxins have a unique genetic evolutionary lineage differed from that for other sea anemone hemolytic toxins.

*Keywords*: Sea anemone; *Actineria villosa*; *Phyllodiscus semoni*; actinoporin; hemolysin; intron; isoform; multigene
1. Introduction

*Actineria villosa* (Japanese name “Fusa-Unbach Isoginchaku”), whose distribution in the coastal sea of the Okinawa islands was recently reported (Oshiro 2001), is a sea anemone that is morphologically quite similar to coral, but its venomous proteins are known to be extremely toxic to skin and muscle, as in the case of the closely related sea anemone *Phyllodiscus semoni* (Japanese name “Unbach Isoginchaku”) (Nagai, et al. 2002). Both *A. villosa* and *P. semoni* belong to Aliciidae family. Globular vesicles surrounding the surface of the body readily discharge huge nematocysts by chemical and physical stimulus.

In the present study, we isolated a novel hemolytic toxin from the globular vesicles of the tentacles and found that it has biochemical and physiological properties similar to those of hemolysins from other sea anemone species. We recently cloned the hemolytic toxin gene Avt-I from *A. villosa*, which was characterized as a typical member of the hemolysin family and exhibited 99% homology with Pstx20 from *P. semoni* (Uechi, et al. 2005).

Hemolysin is a eukaryotic pore-forming toxin exclusively found in sea anemones. This is a family of basic cysteine-less proteins with molecular weights ranging from 18,000 to 20,000 that bind to sphingomyelin (SM) as their target (Anderluh 2002). They form cation-selective pores with a diameter of 2 nm on erythrocyte membranes, particularly in sheep erythrocytes (Varanda, et al. 1980, Belmonte, et al. 1993, Tejuca, et al. 2001). In addition, cardiostimulatory anti-tumor and anti-parasite activity has also been reported for some toxins from sea anemones (Thomson, et al. 1987, Norton, et al. 1990, Simpson, et al. 1990, Tejuca, et al. 1999, Monastyrnaya 2002).


However, the ion-channel-blocking toxin HmK from *Heteractis magnifica* is interrupted by two introns (Gendeh, et al. 1997), while the homologous toxin ShK from *Stichodactyla helianthus* has no introns (Castaneda, et al. 1995).

Here, we provide the first report on the existence and novel genome structure of hemolysin isoforms in *A. villosa* and *P. semoni*.
2. Materials and methods

2.1 Animals and sample collection

Samples of the sea anemones *A. villosa* and *P. semoni* were collected along the Odo coast near Itoman City, Okinawa, Japan.

2.2 Preparation of genomic DNA from *A. villosa* and *P. semoni*

Tentacle fragments were homogenized and placed in 1.5-ml microcentrifuge tubes containing 500 µl of Y-PER reagent (Pierce) in which proteinase K had been dissolved at a concentration of 1 mg/ml. The suspension was incubated at 55°C for 3 h with gentle stirring. Twenty microliters of RNase A (5 U/µl) was then added to each tube, and a second incubation was carried out at 37°C for 1 h. The resulting DNA solution was extracted once with phenol saturated with Tris-HCl (pH 8.0) and twice with chloroform:isoamyl alcohol (24:1). After the addition of 1/10 volume of 3M sodium acetate solution and 2.5 volumes of absolute ethanol, samples were incubated at –20°C for 1 h. Samples were centrifuged at –20°C for 30 min, and the pellets were rinsed with 70% ethanol and dissolved in 100 µl of TE solution.

2.3 RNA purification and reverse transcription

Total RNA was isolated from sea anemones *A. villosa* and *P. semoni* using ISOGEN Reagent (Nippongene) and was further treated with DNase I. The cDNA was
synthesized using SuperScript III (Invitrogen) and oligo dT primer.

2.4 Amplification of Avt-I homologous gene from cDNA and genomic DNA

The following primers were used for DNA amplification: 5’UTR, 5’-GAAATGATCAGTTCATTTACTTTC-3’, and 3’UTR, 5’-TAGGGTTCATAAGTTTTTATTGGG-3’. The first-strand cDNA and genomic DNA were used as templates for polymerase chain reaction (PCR) amplification under the following conditions: 1 µM of primers 5’UTR and 3’UTR, 1.5 mM MgCl₂, 0.2 mM of each dNTP, and 2.5 U of ExTaq DNA polymerase (Takara) in a volume of 50 µL. PCR amplification of Avt-I was carried out in an MJ Research PTC-100 programmed with the following protocol: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min. Finally, samples were held at 72°C for 5 min. PCR products were separated on a 1.5% agarose gel (Nacalai Tesque), and excised DNA fragments were purified using a QIAquick PCR purification kit (Qiagen). The purified PCR product was sequenced directly and then cloned into pGEM-T Easy Vector (Promega). Insert DNA sequences of several clones were subsequently sequenced by T7 and SP6 primers with the ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit on a ABI PRISM 310 DNA sequencer (Applied Biosystems), according to the manufacturer’s instructions.

2.5 Restriction fragment analysis

The PCR products from A. villosa and P. semoni were digested using endonuclease
*HphI* (New England Biolab) at 37°C for 6 h. The digested samples were separated on a 1.25% agarose gel. The gel was stained with ethidium bromide, and the band intensities were quantified by a Gel-Doc 2000 (Bio-Rad) and Scion-image (Scion Corp.). Sequence alignments were performed using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; Hall 1999). Phylogenetic analyses were performed using the neighbor-joining and parsimony-based algorithms in the Clustal W and PHYLIP software packages, respectively. Finally, unrooted phylogenetic trees were drawn and visualized using TreeView (Win32; version 1.5.2).
3. Results

3.1 The homologous gene structure of Avt-I

When the amplified PCR product from cDNA was sequenced directly, some polymorphism was observed as a double chromatogram peak. Further analysis using several clones obtained by TA cloning indicated that the actinoporins of *A. villosa* and *P. semoni* consist of at least two genes. Avt-I and Pstx20 were previously cloned from *A. villosa* and *P. semoni*, respectively, and later other novel actinoporins—Avt-II and Pst-I—were cloned and their DNA sequences accurately determined. The cDNA (Acc. No. AB175824) and genomic sequences (Acc. No. AB512460-AB512463) are available in the DDBJ.

The Avt-II and Pst-I cDNAs were both determined to be 1046 bp long, consisting of an open reading frame (ORF) of 630 bp, a 5′ untranslated region (UTR) of 179 bp, and a 3′ UTR of 186 bp (Fig. 1a). Avt-I differed by 8, 7, and 8 bp from Avt-II, Pst-I, and Pstx20, respectively. Avt-I showed a high degree of sequence identity at the amino acid level: one amino acid difference for Avt-II, one for Pst-I, and three for Pstx20 (Fig.1b). The calculated masses from the predicted sequences were 19673.54 for Avt-I, 19699.58 for Avt-II, 19699.58 for Pstx20, and 19751.66 for Pst-I. The base at position 66 is C in *A. villosa*, but T in *P. semoni*. This sequence of AGTACT was susceptible to digestion by *RsaI* or *Scal*, while AGCACT could not be digested; thus, this sequence can be used to discriminate between *A. villosa* and *P. semoni*.

Restriction endonuclease analysis using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/) indicated that *HphI* is suitable for isoform
determination. *HphI* endonuclease digests full-length Avt-I (at positions 702 and 834), Avt-II (at positions 834 and 900), Pst-I (at positions 702, 834, and 900), and Pstx20 (at positions 697 and 829) (Fig. 2). Densitometric analysis of the fragmented bands suggested that the expression level of Avt-I was 18.3%, while that of Avt-II was 81.7%. Pst-I and Pstx20 genes could be discriminated by *BstXI* enzyme at the position of 755 of Pstx20. Avt-I and Avt-II were expressed in *E. coli*, but there were no significant differences between their hemolytic activities (data not shown). The base A is found at the –3 position upstream of the ATG codon. This has also been found in the mRNA of eukaryotes, where it is known as the Kozak sequence. The 3′ end of the Avt-I, Avt-II, and Pst-I genes contains a polyadenylation signal AATAAA. The Avt-I, Avt-II, and Pst-I genes have a long 5′UTR region, which may increase the efficiency of translation and mRNA stability.

### 3.2 Gene structure of the Avt-I toxin

PCR amplification of Avt-I was carried out using genomic DNA as the template. The length of the Avt-I cDNA is 1033 bp, but a fragment of approximately 1.8 kbp was amplified from genomic DNA (Fig. 3). This result indicates that the Avt-I gene contains an intron. The genomic DNA sequences of the hemolysin toxins from *A. villosa* and *P. semoni* indicated that the genes were interrupted by two introns. Avt-II and Pst-I were also interrupted at same position. The first intron disrupted the 5′-UTR region, and the second intron was located almost at the end of the signal peptide sequence. No intron was observed in the mature region. The first intron was found to be 248 bp long; the
second intron was 650 bp long in Avt-1, but 658 bp in Avt-II (Fig. 4). The intron-exon junctions that are typical donor and acceptor splice sites have followed the GT/AG rule, in which the introns begin with GT and end with AG.

The A+T content of introns 1 and 2 was 69.42% and 64.37%, respectively, and that of exons 1, 2, and 3 was 65.48, 57.41, and 58.25%, respectively. The A+T content of the intron was significantly higher than that of any exon except exon 1 (Table 1).

3.2 Phylogenetic analysis

DNA alignment was analyzed with both the parsimony and distance matrix methods. Figure 5 shows an unrooted neighbor-joining tree, where the divisions of the Avt-I and Avt-II genes into defined groups are evident. In this tree, the Avt-I, Avt-II, Pst-I, and Pstx20 genes from *A. villosa* and *P. semoni* appear to form a clade that is separate from Actiniidae, Sagartiidae, and Stichodactylidae.
4. Discussion

The Okinawa islands are isolated from the main island of Japan and have evolved characteristicly. Many endemic species have been observed in Okinawa, and its unique natural environment is known as “the eastern Galapagos.” Okinawa is particularly famous for coral and is a base for the breeding of marine species.

The distribution of *A. villosa* was first reported in 1833 (Quoy and Gaimard, 1833), and its biotope was determined in Okinawa in 2000. Following a severe typhoon, the habitat of *A. villosa* was damaged, and the number of *A. villosa* had decreased drastically by 2003. We could not find any new populations of *A. villosa* after 2003.

The similarity in amino acid sequence between the Avt-I coding region and that of the other actinoporins (e.g., EqtII, HmT, and StII) suggests that these toxins probably evolved from the same ancestral gene. *Actinia equina* is a European sea anemone, *Stichodactyla helianthus* is found in the Caribbean, and *A. villosa* and *P. semoni* are from Japan. The distances separating these species and their unique environments might lead to characteristic evolution of genome structure. The actinoporin sequence and genomic structure of *A. villosa* and *P. semoni* are nearly the same, but morphological similarity was not observed, with the exception of the nematocyst vesicle. Therefore, both species might have evolved to conserve the nematocyst vesicle for defense against predators.

Two sea anemones have been studied at the genomic level. The actinoporins HMgIII from *H. magnifica* and equinatoxin II from *A. equina* do not have introns within the coding region. The relative low position of cnidarians in the evolutionary tree tends to lead to an intron-less gene. It might be supposed that quick expression of mRNA and
reloading of the protein toxin into the nematocyst is an advantageous system for survival.

The A+T content of the intron was significantly higher than that of any exon except exon 1. It has been proposed that the high A+T content of the intron may limit strong secondary structure formation or be recognized by specific factors (Csank, et al. 1990), but the mechanism underlying this is still unknown.

Comparison of Avt-I intron-exon organization with other known toxin gene structures

We present here the first data on an intron-containing hemolysin gene from a sea anemone, although two other groups of toxins from other species have been studied at the genomic level. Genomic structures similar to those of Avt-I have been found in neurotoxic peptides calitoxins (clx-1 and clx-2) from Calliactis parasitica (Spagnuolo, et al. 1994, Moran, et al. 2009) and the K⁺ channel toxin (HmK) from H. magnific (Gendeh, et al. 1997). These genes are interrupted by two introns and their exon-intron organization is quite similar with Avt-I genome structure.

The intron-exon junctions that are typical donor and acceptor splice sites have followed the GT/AG rule, in which the introns begin with GT and end with AG (Breathnach, et al. 1981, Iida, et al. 1983).

_A. villosa_ and _P. semoni_ have nematocytes on the surface of globular cells that detach very easily from the body. These nematocytes are used against foes and must be replaced immediately. The production of toxin may be accelerated by multiple copies of the toxin gene in the genomic DNA. _A. villosa_ and _P. semoni_ have acquired
advantageous toxin production through evolutionary processes. The color, texture, and morphology of *A. villosa* have been found to be different from those of *P. semoni*, but interestingly, they have almost the same multiple genes and gene structure. However, very little is known about the sea anemone *A. villosa* and its toxin evolution; therefore, more genomic data and comparison with another species are required.

**Acknowledgements**

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Figure legends

(A)

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<th>Avt-II</th>
<th>Pst-I</th>
<th>Pstx20</th>
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<tr>
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<td>CACTTTTTTGGC</td>
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<td>GATATTTTAACT</td>
<td>GATATTTTAACT</td>
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<tr>
<td>4</td>
<td>CACTTTTTTGGC</td>
<td>CACTTTTTTGGC</td>
<td>CACTTTTTTGGC</td>
<td>CACTTTTTTGGC</td>
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</table>

(B) All of the hemolysins are 226 amino acids long, consisting of 47 amino acids of prepropeptide and 179 amino acids of mature protein without cysteine residue.

Figure 1. Multiple alignments of nucleic acid sequences and deduced amino acid sequences of hemolytic toxin isoform genes.

(A). The lengths of Avt-I, Avt-II, and Pst-I are 1046, 1033, and 1033 bp, respectively. Avt-I and II were isolated from *Actineria villosa*; Pst-I and Pstx20 (Oshiro, et al) were isolated from *Phyllodiscus semoni*. The polyadenylation signal is indicated by the boxed sequence. The cDNA and genomic sequences are available in the DDBJ under accession numbers AB175824 and AB512460-AB512463.
PCR fragments generated with 5’ and 3’ UTR-specific primers using *A. villosa* and *P. semoni* cDNA as templates were digested with the restriction endonuclease *HphI*. The isoform was observed in *A. villosa* (Lane 1; upper arrow). All of the isoforms were digested in the *P. semoni* sample (Lane 2; lower arrow).
Figure 3. Amplification of the Avt-I gene from cDNA and genomic DNA.

Lane 1: 2-Log DNA ladder markers. Lane 2: Amplification of Avt-I from cDNA. Lane3: Amplification from genomic DNA. Both PCR samples were amplified by 5’ and 3’ UTR primer (see materials and methods).
Figure 4. Exon-intron structure of Avt-I and II.

The exon-intron boundaries were determined by comparison with the cDNA sequences. ORFs are represented by boxes, and UTR regions are represented by solid lines. Intron 1 (242 bp) is located in the 5′ UTR region, and intron 2 (654 bp in Avt-I, 661 bp in Avt-II) is located almost at the end of the signal peptide.
Avt-I, Avt-II, Pst-I, and Pstx20 genes from *A. villosa* and *P. semoni* appear to form an Aliciidae clade, and this clade is distant from the Actiniidae (equinatoxin I and II from *Actinia equina*), Stichodactylidae (HMgIII and HmT from *Heteractis magnifica* and Sticholysin I and II from *Stichodactyla helianthus*), and Sagartiidae (Src-I from *Sagartia rosea*) families.
Table 1
AT content of exon and intron.

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<tr>
<td>Exon 2</td>
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<tr>
<td>Exon 3</td>
<td>58.25</td>
</tr>
<tr>
<td>Intron 1</td>
<td>69.42</td>
</tr>
<tr>
<td>Intron 2</td>
<td>64.37</td>
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</table>

Table 1. AT content of exon and intron.
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