

Molecular characterization on the genome structure of hemolysin toxin isoforms isolated from sea anemone *Actinaria villosa* and *Phyllodiscus semoni*

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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 *HphI* 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-Unbachi 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 “Unbachi 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).

Avt-I and other hemolysins contain four tryptophan residues (Trp-45, 116, 117, and 149) that play a crucial role in binding to erythrocyte membranes (Turk, et al. 1992, Malovrh, et al. 2000). Although most hemolysins [e.g., EqII (Anderluh, et al. 1996), HmT (Samejima, et al. 2000), and StI (Lanio, et al. 2001)] contain an Arg-Gly-Asp (RGD) motif that promotes attachment to integrin receptors on the surface of cells (Leininger, et al. 1991, Ruoslahti 1996, Anderluh 2002, Monastyrnaya, et al. 2010) and some synthetic RGD peptide induce apoptosis (Buckley, et al. 1999), but Avt-I and Pstx20 lack such a motif.

In general, hemolysins (e.g., EqII and StII) constitute several isoforms that are members of a multigene family and have high homology with each other (Kem, et al. 1988, Anderluh, et al. 1999, Monastyrnaya 2002). Previous studies have revealed that the actinoporin gene lacks an intron within its coding region (Anderluh, et al. 1995, Anderluh, et al. 1996, Pungercar, et al. 1997, Wang, et al. 2000, Klyshko, et al. 2004).

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 μ l 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/ μ l) 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 μ l 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 μ M 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 μ L. 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

*Hph*I (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 *ScaI*, 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 characteristically. 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.

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Figure legends

(A)

Avt-I	1	GAATGATCAGTTCACTTACTTTCTGAGTAAGATAAGGTCAAAGTCTGAACAGATCCTTTAGCCTCATTGGTATAAAAAGGTTGAGAAGCCATT	100
Avt-II	1	GAATGATCAGTTCACTTACTTTCTGAGTAAGATAAGGTCAAAGTCTGAACAGATCCTTTAGCCTCATTGGTATAAAAAGGTTGAGAAGCTATT	100
Pst-I	1	GAATGATCAGTTCACTTACTTTCTGAGTAAGATAAGGTCAAAGTCTGAACAGATCCTTTAGTACTCATTGGTATAAAAAGGTTGAGAAGCCATT	100
Pstx20	1	-----GATCAGTTCACTTACTTTCTGAGTAAGATAAGGTCAAAGTCTGAACAGATCCTTTAGTACTCATTGGTATAAAAAGGTTGAGAAGCCATT	95
Avt-I	101	AGCAAGAGCCAGAATTAGTCGCTTTCAAGGTCCTTTTAGCTTCCATAACTCGTCAAGCAAGCAGACACTAAGAAGAAAGATGAGACATTCGTGGTCTTC	200
Avt-II	101	AGCAAGAGCCAGAATTAGTCGCTTTCAAGGTCCTTTTAGCTTCCATAACTCGTCAAGCAAGCAGACACTAAGAAGAAAGATGAGACATTCGTGGTCTTC	200
Pst-I	101	AGCAAGAGCCAGAATTAGTCGCTTTCAAGGTCCTTTTAGCTTCCATAACTCGTCAAGCAAGCAGACACTAAGAAGAAAGATGAGACATTCGTGGTCTTC	200
Pstx20	96	AGCAAGAGCCAGAATTAGTCGCTTTCAAGGTCCTTTTAGCTTCCATAACTCGTCAAGCAAGCAGACACTAAGAAGAAAGATGAGACATTCGTGGTCTTC	195
Avt-I	201	CTGTACATGTTCCCTAGCCCTTTTCGATACCCACGGCTTTTGCTAAGAAACACATCGTTACAAAGAAAGGAAACCATCAAGACATCACAACGACAAATGAAG	300
Avt-II	201	CTGTACATGTTCCCTAGCCCTTTTCGATACCCACGGCTTTTGCTAAGAAACACATCGTTACAAAGAAAGGAAACCATCAAGACATCACAACGACAAATGAAG	300
Pst-I	201	CTGTACATGTTCCCTAGCCCTTTTCGATACCCACGGCTTTTGCTAAGAAACACATCGTTACAAAGAAAGGAAACCATCAAGACATCACAACGACAAATGAAG	300
Pstx20	196	CTGTACATGTTCCCTAGCCCTTTTCGATACCCACGGCTTTTGCTAAGAAACACATCGTTACAAAGAAAGGAAACCATCAAGACATCACAACGACAAATGAAG	295
Avt-I	301	GGGAGAATGCCGAAAAGAAATCTGCTGCCGTTGCCGGAGCCGTCATCGCTGGAGGCGAGCTTGCCTTAAAAATCCTTACTAAAATCTCGATGAATAGG	400
Avt-II	301	GGGAGAATGCCGAAAAGAAATCTGCTGCCGTTGCCGGAGCCGTCATCGCTGGAGGCGAGCTTGCCTTAAAAATCCTTACTAAAATCTCGATGAATAGG	400
Pst-I	301	GGGAGAATGCCGAAAAGAAATCTGCTGCCGTTGCCGGAGCCGTCATCGCTGGAGGCGAGCTTGCCTTAAAAATCCTTACTAAAATCTCGATGAATAGG	400
Pstx20	296	GGGAGAATGCCGAAAAGAAATCTGCTGCCGTTGCCGGAGCCGTCATCGCTGGAGGCGAGCTTGCCTTAAAAATCCTTACTAAAATCTCGATGAATAGG	395
Avt-I	401	TAAAATCGATCGCAAGATTGCAATTGGCGTTGATAACGAATCTGGCCCTTAAATGGACAGCTTTGAATACTTACTATAAATCTGGAGCCTCGGATGTCAC	500
Avt-II	401	TAAAATCGATCGCAAGATTGCAATTGGCGTTGATAACGAATCTGGCCCTTAAATGGACAGCTTTGAATACTTACTATAAATCTGGAGCCTCGGATGTCAC	500
Pst-I	401	TAAAATCGATCGCAAGATTGCAATTGGCGTTGATAACGAATCTGGCCCTTAAATGGACAGCTTTGAATACTTACTATAAATCTGGAGCCTCGGATGTCAC	500
Pstx20	396	TAAAATCGATCGCAAGATTGCAATTGGCGTTGATAACGAATCTGGCCCTTAAATGGACAGCTTTGAATACTTACTATAAATCTGGAGCCTCGGATGTCAC	495
Avt-I	501	TTGCCATATGAAGTAGAAAATCCAAAGCTCTCTTTGACACCGCAAGGAAGAGCAAAAGACCTGTAGCGCGGGGCGCTGTGGCGCTCCTTGCCTACAAAA	600
Avt-II	501	TTGCCATATGAAGTAGAAAATCCAAAGCTCTCTTTGACACCGCAAGGAAGAGCAAAAGACCTGTAGCGCGGGGCGCTGTGGCGCTCCTTGCCTACAAAA	600
Pst-I	501	TTGCCATATGAAGTAGAAAATCCAAAGCTCTCTTTGACACCGCAAGGAAGAGCAAAAGACCTGTAGCGCGGGGCGCTGTGGCGCTCCTTGCCTACAAAA	600
Pstx20	496	TTGCCATATGAAGTAGAAAATCCAAAGCTCTCTTTGACACCGCAAGGAAGAGCAAAAGACCTGTAGCGCGGGGCGCTGTGGCGCTCCTTGCCTACAAAA	595
Avt-I	601	TGAGCAGCGGTAATACTCTGGCAGTAATGTTCCAGCGTCCCTTTGACTACAACCTTATACCCAATTGGTGAATGTTAAGATTTACGATGGTGAGAAAAA	700
Avt-II	601	TGAGCAGCGGTAATACTCTGGCAGTAATGTTCCAGCGTCCCTTTGACTACAACCTTATACCCAATTGGTGAATGTTAAGATTTACGATGGTGAGAAAAA	700
Pst-I	601	TGAGCAGCGGTAATACTCTGGCAGTAATGTTCCAGCGTCCCTTTGACTACAACCTTATACCCAATTGGTGAATGTTAAGATTTACGATGGTGAGAAAAA	700
Pstx20	596	TGAGCAGCGGTAATACTCTGGCAGTAATGTTCCAGCGTCCCTTTGACTACAACCTTATACCCAATTGGTGAATGTTAAGATTTACGATGGTGAGAAAAA	695
Avt-I	701	AGCGGATGAAAAGATGTATAATGAGCTGTATAATAACAACAACCCAATTAACCCAGTATATGGGAGAAACGAGATCTAGGACAGGATGGACTCAAGCTT	800
Avt-II	701	AGCGGATGAAAAGATGTATAATGAGCTGTATAATAACAACAACCCAATTAACCCAGTATATGGGAGAAACGAGATCTAGGACAGGATGGACTCAAGCTT	800
Pst-I	701	AGCGGATGAAAAGATGTATAATGAGCTGTATAATAACAACAACCCAATTAACCCAGTATATGGGAGAAACGAGATCTAGGACAGGATGGACTCAAGCTT	800
Pstx20	696	AGCGGATGAAAAGATGTATAATGAGCTGTATAATAACAACAACCCAATTAACCCAGTATATGGGAGAAACGAGATCTAGGACAGGATGGACTCAAGCTT	795
Avt-I	801	AGAGGATTCATGACAAGCAATGGTGATGCGAACTTGTGATCCACATTGAGAAGAGCTAAAACCTGATAAATTTACCCGGCGCCATTGAGTGTATAATTTAA	900
Avt-II	801	AGAGGATTCATGACAAGCAATGGTGATGCGAACTTGTGATCCACATTGAGAAGAGCTAAAACCTGATAAATTTACCCGGCGCCATTGAGTGTATAATTTAA	900
Pst-I	801	AGAGGATTCATGACAAGCAATGGTGATGCGAACTTGTGATCCACATTGAGAAGAGCTAAAACCTGATAAATTTACCCGGCGCCATTGAGTGTATAATTTAA	900
Pstx20	796	AGAGGATTCATGACAAGCAATGGTGATGCGAACTTGTGATCCACATTGAGAAGAGCTAAAACCTGATAAATTTACCCGGCGCCATTGAGTGTATAATTTAA	895
Avt-I	901	ATCCACGCTAAAGGTTTTTGACAGTTAGTAGTTTCATTGAAGTCTCTGTGTGAAACCTCCATATTCCTAATGACCTGACGCAAACTATTGTATAGA	1000
Avt-II	901	ATCCACGCTAAAGGTTTTTGACAGTTAGTAGTTTCATTGAAGTCTCTGTGTGAAACCTCCATATTCCTAATGACCTGACGCAAACTATTGTATAGA	1000
Pst-I	901	ATCCACGCTAAAGGTTTTTGACAGTTAGTAGTTTCATTGAAGTCTCTGTGTGAAACCTCCATATTCCTAATGACCTGACGCAAACTATTGTATAGA	1000
Pstx20	896	ATCCACGCTAAAGGTTTTTGACAGTTAGTAGTTTCATTGAAGTCTCTGTGTGAAACCTCCATATTCCTAATGACCTGACGCAAACTATTGTATAGA	995
Avt-I	1001	ATTATGAATCCCAATAAAAACTTATGAACCCATAAAAAAAAAAAAAA	1046
Avt-II	1001	ATTATGAATCCCAATAAAAACTTATGAACCCATA	1033
Pst-I	1001	ATTATGAATCCCAATAAAAACTTATGAACCCATA	1033
Pstx20	996	ATTATGAATCCCAATAAAAACTTATGAACCCATAAAAAAAAAAAAAA	1041

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.

(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.

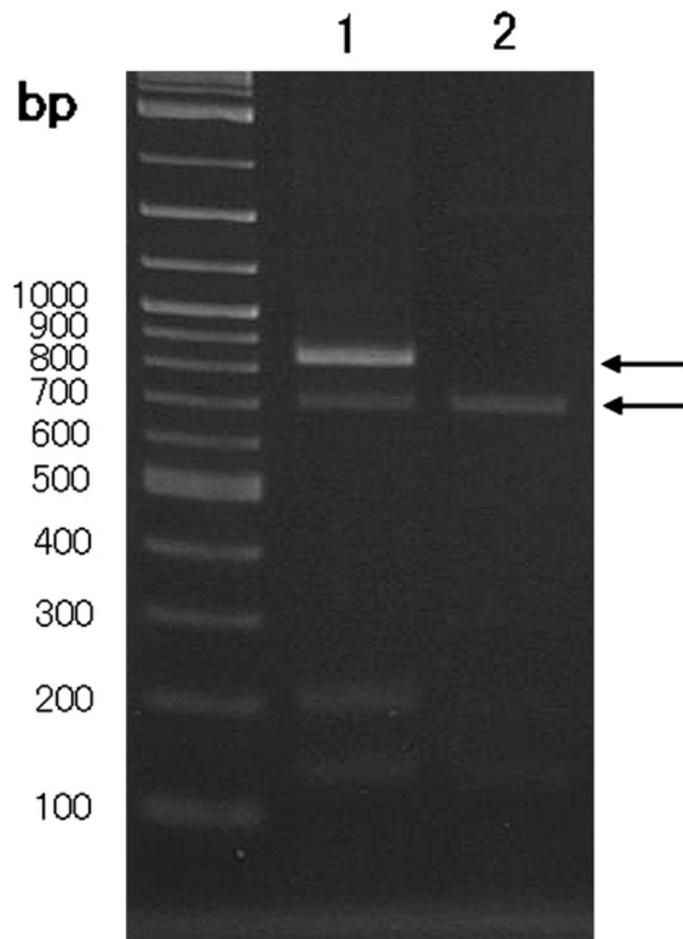


Figure 2. Digestion of hemolysin by *Hph*I restriction endonuclease.

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 *Hph*I. 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).

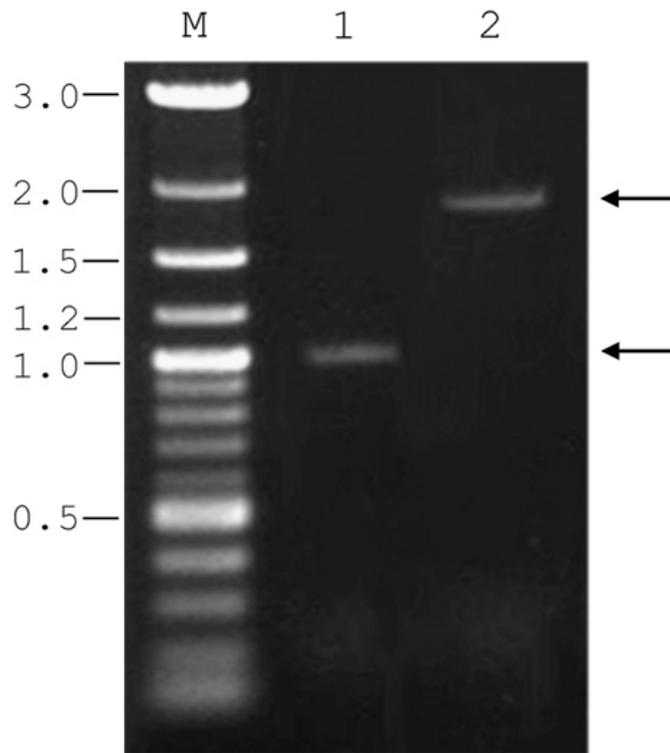


Figure 3. Amplification of the Avt-I gene from cDNA and genomic DNA.

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).

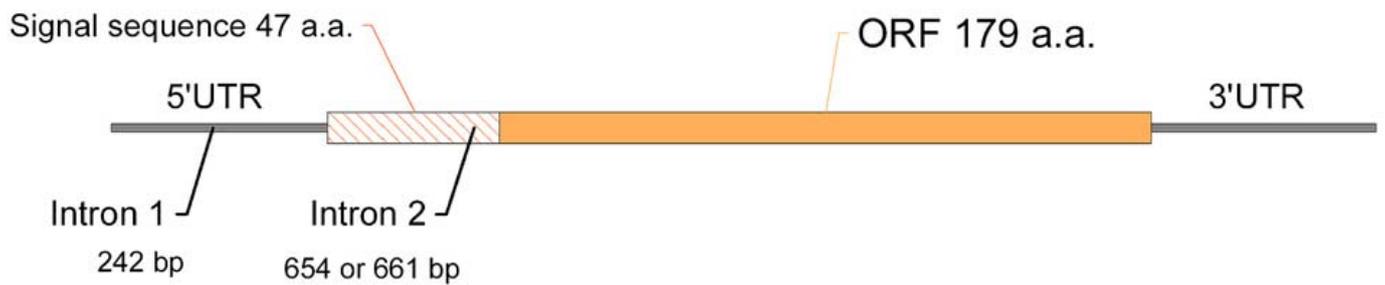


Figure 4. Exon-intron structure of Avt-I and II.

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, 661bp in Avt-II) is located almost at the end of the signal peptide.

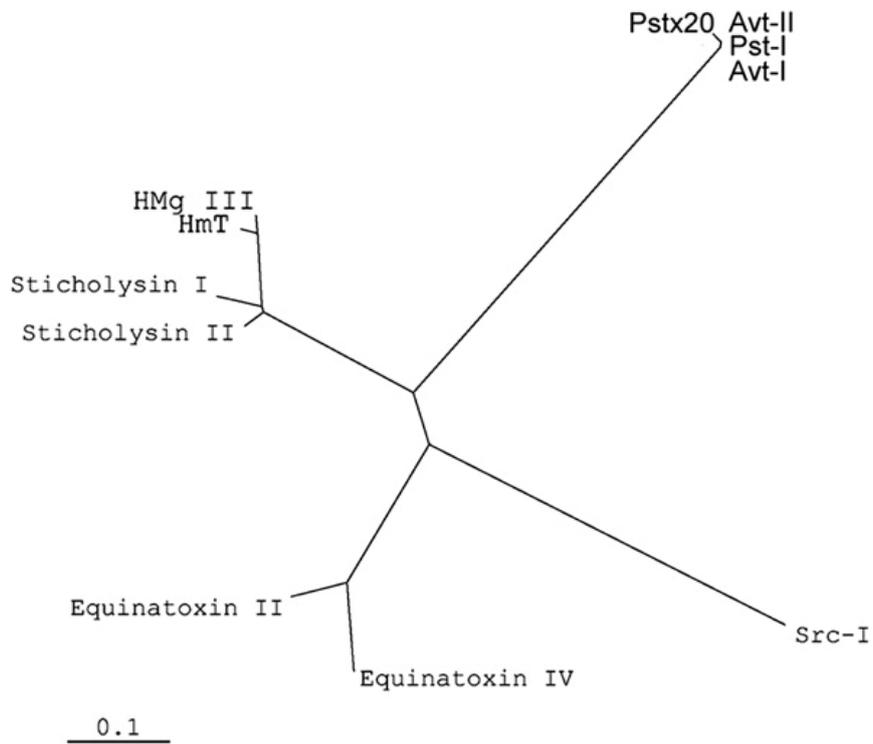


Figure 5. Phylogenetic analysis of hemolysin genes

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.

	AT content (%)
Exon 1	65.48
Exon 2	57.41
Exon 3	58.25
Intron 1	69.42
Intron 2	64.37

Table 1. AT content of exon and intron.

References:

Anderluh, G., Pungercar, J., Štrukelj, B., Maček, P., Gubenšek, F., 1995. The Coding Region of the Equinatoxin II Gene Lacks Introns. . *Croatica Chemica Acta* 68, 533-542.

Anderluh, G., Pungercar, J., Strukelj, B., Macek, P., Gubensek, F., 1996. Cloning, sequencing, and expression of equinatoxin II. *Biochem Biophys Res Commun.* 220, 437-442.

Anderluh, G., Krizaj, I., Strukelj, B., Gubensek, F., Macek, P., Pungercar, J., 1999. Equinatoxins, pore-forming proteins from the sea anemone *Actinia equina*, belong to a multigene family. *Toxicon.* 37, 1391-1401.

Anderluh, G., Macek, P., 2002. Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria). *Toxicon.* 40, 111-124.

Belmonte, G., Pederzoli, C., Macek, P., Menestrina, G., 1993. Pore formation by the sea anemone cytolysin equinatoxin II in red blood cells and model lipid membranes. *J Membr Biol.* 131, 11-22.

Breathnach, R., Chambon, P., 1981. Organization and expression of eucaryotic split genes coding for proteins. *Annu Rev Biochem.* 50, 349-383.

Buckley, C. D., Pilling, D., Henriquez, N. V., Parsonage, G., Threlfall, K.,

Scheel-Toellner, D., Simmons, D. L., Akbar, A. N., Lord, J. M., Salmon, M., 1999. RGD peptides induce apoptosis by direct caspase-3 activation. *Nature*. 397, 534-539.

Castaneda, O., Sotolongo, V., Amor, A. M., Stocklin, R., Anderson, A. J., Harvey, A. L., Engstrom, A., Wernstedt, C., Karlsson, E., 1995. Characterization of a potassium channel toxin from the Caribbean Sea anemone *Stichodactyla helianthus*. *Toxicon*. 33, 603-613.

Csank, C., Taylor, F. M., Martindale, D. W., 1990. Nuclear pre-mRNA introns: analysis and comparison of intron sequences from *Tetrahymena thermophila* and other eukaryotes. *Nucleic Acids Res.* 18, 5133-5141.

Gendeh, G. S., Chung, M. C., Jeyaseelan, K., 1997. Genomic structure of a potassium channel toxin from *Heteractis magnifica*. *FEBS Lett.* 418, 183-188.

Iida, Y., Sasaki, F., 1983. Recognition patterns for exon-intron junctions in higher organisms as revealed by a computer search. *J Biochem.* 94, 1731-1738.

Kem, W. R., Dunn, B. M., 1988. Separation and characterization of four different amino acid sequence variants of a sea anemone (*Stichodactyla helianthus*) protein cytolysin. *Toxicon*. 26, 997-1008.

Klyshko, E. V., Issaeva, M. P., Monastyrnaya, M. M., Il'yna, A. P., Guzev, K. V., Vakorina, T. I., Dmitrenok, P. S., Zykova, T. A., Kozlovskaya, E. P., 2004. Isolation, properties and partial amino acid sequence of a new actinoporin from the sea anemone

Radianthus macrodactylus. *Toxicon*. 44, 315-324.

Lanio, M. E., Morera, V., Alvarez, C., Tejuca, M., Gomez, T., Pazos, F., Besada, V., Martinez, D., Huerta, V., Padron, G.de los Angeles Chavez, M., 2001. Purification and characterization of two hemolysins from *Stichodactyla helianthus*. *Toxicon*. 39, 187-194.

Leininger, E., Roberts, M., Kenimer, J. G., Charles, I. G., Fairweather, N., Novotny, P.Brennan, M. J., 1991. Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proc Natl Acad Sci U S A*. 88, 345-349.

Malovrh, P., Barlic, A., Podlesek, Z., MaCek, P., Menestrina, G.Anderluh, G., 2000. Structure-function studies of tryptophan mutants of equinatoxin II, a sea anemone pore-forming protein. *Biochem J*. 346 Pt 1, 223-232.

Monastyrnaya, M., Leychenko, E., Isaeva, M., Likhatskaya, G., Zelepuga, E., Kostina, E., Trifonov, E., Nurminski, E.Kozlovskaya, E., 2010. Actinoporins from the sea anemones, tropical *Radianthus macrodactylus* and northern *Oulactis orientalis*: Comparative analysis of structure-function relationships. *Toxicon*. in press.

Monastyrnaya, M. M., Zykova, T. A., Apalikova, O. V., Shwets, T. V., Kozlovskaya, E. P., 2002. Biologically active polypeptides from the tropical sea anemone *Radianthus macrodactylus*. *Toxicon*. 40, 1197-1217.

Moran, Y., Gordon, D., Gurevitz, M., 2009. Sea anemone toxins affecting voltage-gated sodium channels--molecular and evolutionary features. *Toxicon*. 54, 1089-1101.

Nagai, H., Oshiro, N., Takuwa-Kuroda, K., Iwanaga, S., Nozaki, M., Nakajima, T., 2002. A new polypeptide toxin from the nematocyst venom of an Okinawan sea anemone *Phyllo-discus semoni* (Japanese name "unbachi-isoginchaku"). *Biosci Biotechnol Biochem*. 66, 2621-2625.

Norton, R. S., Bobek, G., Ivanov, J. O., Thomson, M., Fiala-Beer, E., Moritz, R. L., Simpson, R. J., 1990. Purification and characterisation of proteins with cardiac stimulatory and haemolytic activity from the anemone *Actinia tenebrosa*. *Toxicon*. 28, 29-41.

Oshiro, N., Iwanaga, S., Nozaki, M., Nakanose, T., Uchida, H., 2001. New distributional record and venom toxicity of the sea anemone *Actinaria villosa* (Quoy et Gaimard, 1833). *Annual Report of Okinawa Prefectural Institute of Health and Environment*. 35, 133-136.

Pungercar, J., Anderluh, G., Macek, P., Franc, G., Strukelj, B., 1997. Sequence analysis of the cDNA encoding the precursor of equinatoxin V, a newly discovered hemolysin from the sea anemone *Actinia equina*. *Biochim Biophys Acta*. 1341, 105-107.

Ruoslahti, E., 1996. RGD and other recognition sequences for integrins. *Annu Rev Cell*

Dev Biol. 12, 697-715.

Samejima, Y., Yanagisawa, M., Aoki-Tomomatsu, Y., Iwasaki, E., Ando, J., Mebs, D., 2000. Amino acid sequence studies on cytolytic toxins from sea anemone *Heteractis magnifica*, *Entacmaea quadricolor* and *Stichodactyla mertensii* (Anthozoa). *Toxicon*. 38, 259-264.

Simpson, R. J., Reid, G. E., Moritz, R. L., Morton, C., Norton, R. S., 1990. Complete amino acid sequence of tenebrosin-C, a cardiac stimulatory and haemolytic protein from the sea anemone *Actinia tenebrosa*. *Eur J Biochem*. 190, 319-328.

Spagnuolo, A., Zanetti, L., Cariello, L., Piccoli, R., 1994. Isolation and characterization of two genes encoding calitoxins, neurotoxic peptides from *Calliactis parasitica* (Cnidaria). *Gene*. 138, 187-191.

Tejuca, M., Anderlueh, G., Macek, P., Marcet, R., Torres, D., Sarracent, J., Alvarez, C., Lanio, M. E., Dalla Serra, M., Menestrina, G., 1999. Antiparasite activity of sea-anemone cytolytins on *Giardia duodenalis* and specific targeting with anti-Giardia antibodies. *Int J Parasitol*. 29, 489-498.

Tejuca, M., Dalla Serra, M., Potrich, C., Alvarez, C., Menestrina, G., 2001. Sizing the radius of the pore formed in erythrocytes and lipid vesicles by the toxin sticholysin I from the sea anemone *Stichodactyla helianthus*. *J Membr Biol*. 183, 125-135.

Thomson, M., Moritz, R. L., Simpson, R. J., Norton, R. S., 1987. Tenebrosin-A, a new cardiostimulant protein from the Australian sea anemone *Actinia tenebrosa*. *Biochem Int.* 15, 711-718.

Turk, T., Macek, P., Gubensek, F., 1992. The role of tryptophan in structural and functional properties of equinatoxin II. *Biochim Biophys Acta.* 1119, 1-4.

Uechi, G., Toma, H., Arakawa, T., Sato, Y., 2005. Biochemical and physiological analyses of a hemolytic toxin isolated from a sea anemone *Actinaria villosa*. *Toxicon.* 45, 761-766.

Uechi, G., Toma, H., Arakawa, T., Sato, Y., 2005. Molecular cloning and functional expression of hemolysin from the sea anemone *Actinaria villosa*. *Protein Expr Purif.* 40, 379-384.

Varanda, W., Finkelstein, A., 1980. Ion and nonelectrolyte permeability properties of channels formed in planar lipid bilayer membranes by the cytolytic toxin from the sea anemone, *Stoichactis helianthus*. *J Membr Biol.* 55, 203-211.

Wang, Y., Chua, K. L., Khoo, H. E., 2000. A new cytolytic toxin from the sea anemone, *Heteractis magnifica*: isolation, cDNA cloning and functional expression. *Biochim Biophys Acta.* 1478, 9-18.