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Toxin gene determination and evolution in scorpaenoid fish

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ABSTRACT

In this study, we determine the toxin genes from both cDNA and genomic DNA of four scorpaenoid fish and reconstruct their evolutionary relationship. The deduced protein sequences of the two toxin subunits in *Sebastapistes strongia*, *Scorpaenopsis oxycephala*, and *Sebastiscus marmoratus* are about 700 amino acid, similar to the sizes of the stonefish (*Synanceia horrida*, and *Synanceia verrucosa*) and lionfish (*Pterois antennata* and *Pterois volitans*) toxins previously published. The intron positions are highly conserved among these species, which indicate the applicability of gene finding by using genomic DNA template. The phylogenetic analysis shows that the two toxin subunits were duplicated prior to the speciation of Scorpaenoidei. The precedence of the gene duplication over speciation indicates that the toxin genes may be common to the whole family of Scorpaeniform. Furthermore, one additional toxin gene has been determined in the genomic DNA of *Dendrochirus zebra*. The phylogenetic analysis suggests that an additional gene duplication occurred before the speciation of the lionfish (Pteroinae) and a pseudogene may be generally present in the lineage of lionfish.

1. Introduction

Animal venoms have long been studied due to their potential applicability for pharmacological and physiological purposes and have provided a rich pharmacopoeia (Menez, 1998; Church and Hodgson, 2002; Tan et al., 2003; Baron et al., 2013). The development of antitoxins is another field that has been intensively studied (Theakston, 1983; Chippaux and Goyffon, 1998; Huston and George, 2001; Gazarian et al., 2005; Wesolowski et al., 2009). The large number of accidents caused by animal envenomation per year makes the development of effective antivenins essential and valuable. In addition to the pharmacological applications, the diversity of the targets and the specificity of animal toxins make them useful tools in investigating the physiological functions or cellular localization of various ion channels and cellular receptors (Terlau and Olivera, 2004; Dutertre and Lewis, 2010; Baron et al., 2013). Although numerous toxins extracted from terrestrial creatures have been studied and many of them have been in various stages of drug development (Newman and Cragg, 2012), marine toxins are largely ignored due to the difficulty in accessing them (Church and Hodgson, 2002; Jha and Zi-Rong, 2004; Smith and Wheeler, 2006). The majority of marine toxin studied have been from mollusca such as marine snails (Terlau and Olivera, 2004; Lu et al., 2014), leaving fish as a potent pool for new toxin discoveries.

Among the acanthomorpha, more than 1200 species are believed to be venomous (Smith and Wheeler, 2006). The phylogenetic tree constructed by Smith and Wheeler (2006) suggests that a high density of venomous species are present in the Scorpaenoidei, the suborder containing the most notorious venomous fishes: stonefish (genus *Synanceia*), lionfish (genera *Pterois*, *Parapterois* and *Dendrochirus*), and scorpionfish (genus *Scorpaena*). However, studies on the venom's properties are frequently impeded

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by the labiality of these proteins. To date, only several toxins from stonefish (Kreger, 1991; Garnier et al., 1997b; Khoo, 2002; Ueda et al., 2006), lionfish (Kiriake and Shiomi, 2011), scorpionfish (Schaeffer et al., 1971; Hahn and O'connor, 2000, Carrijo et al., 2005; Nagasaka et al., 2009; Andrich et al., 2010), devil stinger and waspfish (Shiomi et al., 1989) have been characterized for their bioactivities. Although these toxins have not been compared systematically, they all show hemolytic activity against various animal erythrocytes (Church and Hodgson, 2002). Since no phospholipase A_2 activity was found in any of these fish toxins (Shiomi et al., 1989; Poh et al., 1991; Hahn and O'connor, 2000, Andrich et al., 2010, Gomes et al., 2013), the hemolysis is believed to be from a non-enzymatic mechanism, as seen in some reptilian and amphibian toxins (Kini and Evans, 1989a). Studies using chemical modification methods further demonstrated that free thiol groups, tryptophans, and positively charged amino acid residues (such as Lys and Arg) play significant roles in the cytolytic activity of SNTX, the toxin extracted from *Synanceia horrida* (Chen et al., 1997; Khoo et al., 1998; Yew and Khoo, 2000). However, being that no mutagenesis studies have been conducted on this protein, the precise positions of the functional amino acid residues responsible for the hemolytic activity remain unknown.

In addition to the similarity in bioactivities, the cross-reactivity of the commercial stonefish antivenom (SFAV) against toxins from different scorpaenoid indicates the presence of structural similarity between these toxins (Shiomi et al., 1989; Andrich et al., 2010, Gomes et al., 2011). A thorough comparative study of the amino acid sequences might improve our understanding of the reacting sites and the operating mechanism of these molecules. Unfortunately, only four toxins from the stonefish (three from *Synanceia* and one from *Inimicus japonicus*), three from genus *Pterois*, and one from the waspfish (*Hypodytes rubripinnis*) have had their primary structures determined (Ghadessy et al., 1996; Garnier et al., 1997a; Ueda et al., 2006; Kiriake and Shiomi, 2011; Kiriake et al., 2013). The determined toxins belong to a novel protein family composed of two different subunits with no similar protein or peptide found in GenBank. Meanwhile, multiple alignments of these venom protein sequences show high similarities between (50%–80%) and within (>80%) the two subunits (Ueda et al., 2006; Kiriake and Shiomi, 2011; Kiriake et al., 2013). It was therefore hypothesized that these toxins evolved from a common ancestor peptide (Ghadessy et al., 1996; Ueda et al., 2006; Kiriake and Shiomi, 2011). However, testing this hypothesis has not been possible due to the scarcity of the sequence data and the narrow distribution of these sequences to only four genera (*Synanceia*, *Pterois*, *lnimicus* and *Hypodytes*).

In this study we cloned the toxin genes of several scorpaenoid fish collected from northern to northeastern Taiwan. By determining the gene sequences, we aim to: 1) reconstruct the evolutionary history of the toxin genes in Scorpaenoidei and clarify their evolutionary origin, and 2) identify the conserved regions of the venom peptides and infer the probable roles of these amino acid sites.

2. Materials and methods

2.1. Sample collection and species identification

Three species of live scorpaenoid fish (the barchin scorpionfish *Sebastapistes strongia*, the tassled scorpionfish *Scorpaenopsis oxycephala*, and the false kelpfish *Sebastiscus marmoratus*) and one species of dead fish (the zebra lionfish *Dendrochirus zebra*) were purchased from Nanfang-ao and Keelung and transferred to the laboratory for further experiments. The pictures of the collected samples are shown in Fig. 1 and the detailed information is listed in Table 1.

2.2. RNA and DNA extraction

Total RNA of *S. strongia*, *S. oxycephala*, and *S. marmoratus* was extracted from the dorsal spines following the Trizol RNA isolation protocol. For all study species, DNA was also extracted from muscle tissue using the DNeasy Blood & Tissue Kit (QIOGEN) following the manufacturer's instructions.

2.3. Primer design

Gene-specific primers for the four species were designed from the determined nucleotide sequences obtained from Section 2.4. For the two subunit genes of *S. strongia*, *S. oxycephala*, and *S. marmoratus*, two degenerate forward primers (Uni_A and Uni_B) were designed from the conserved regions to amplify the middle regions of each of the two subunits. Degenerate primers were also designed from the sequences around start and stop codon according to cDNA sequences obtained in this study and from GenBank. These primers were named by their annealing regions (beginning) and their target genes (ending) (Table 2): start for forward primers annealing to the start-codon regions, stop for the reverse primers annealing to the stop-codon regions; primer names ending with A or B correspond to the α and β subunits of the toxin genes; primer names ending with syn or pter were designed from the toxin genes in genus *Synanceia* or *Pterois*; and those ending with mar were species-specific primers for *S. marmoratus*.

For *D. zebra*, three distinct sequences were obtained by PCR using primers DegF1 and DegR. Three gene-specific primers (zebA, zebB, and zebC) were thus designed to amplify each individual gene. The sequences determined were then used to design the gene-specific primers to amplify the upstream and downstream sequences of the toxin genes of *D. zebra*. All the primers designed and used in this study are shown in Table 2.

2.4. RT-PCR

The first-strand cDNA was directly synthesized from 5 μg of total RNA using the 3′ RACE System for Rapid Amplification of cDNA Ends (invitrogen). The three degenerate primers (DegF1, DegF2, and DegR) designed by Kiriake and Shiomi (2011) were used and the reaction of RT-PCR was conducted following the protocol described

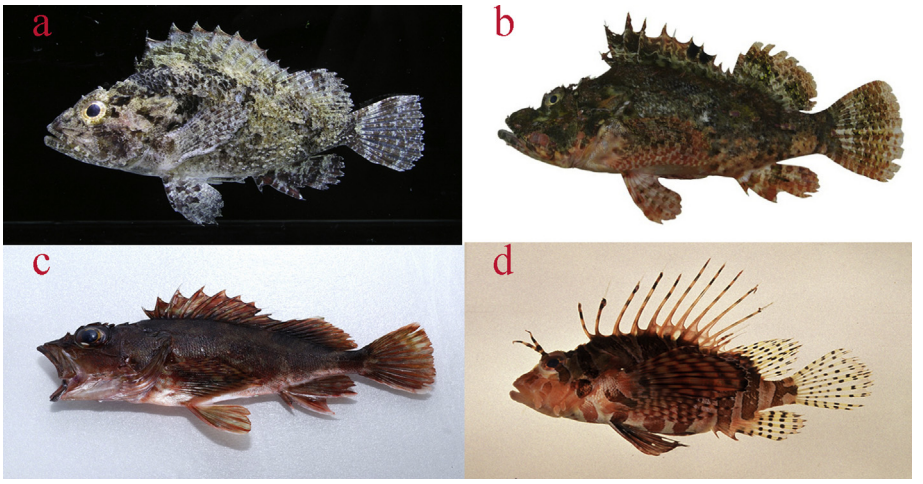

Fig. 1. Scorpaenoid fish of *Sebastapistes strongia* (a), *Scorpaenopsis oxycephala* (b), *Sebastiscus marmoratus* (c) and *Dendrochirus zebra* (d). Image source: the fish database of Taiwan, courtesy of Kwang-Tsao Shao, Wei-Chuan Chiang and Nian-Hong Jang-Liaw.

by Kiriake and Shiomi (2011), with a total volume of 25 μL containing 0.2 μL of *TaKaRa Ex Taq* (5 U/μL, TaKaRa), 2.5 μL of 2.5 mM dNTP Mixture (TaKaRa), 2.5 μL 10× *Ex Taq* Buffer (20 mM Tris–HCl, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidt P-40, 50% Glycerol, 20 mM $MgCl_2$, pH 8.0, TaKaRa), and 0.4 μM of each primers. The PCR condition was set with an initiation step at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and finished with a final extension step at 72 °C for 5 min. The PCR products were subcloned into the pGEM-T Easy Vector (Promega) and sequenced by the Center for Biotechnology, National Taiwan University.

2.5. RACE

For 3′ RACE, the cDNA synthesis procedure was the same as described in Section 2.4. Following cDNA synthesis, nested-PCR was carried out to amplify the 3′ UTR. The first reaction of nested-PCR was conducted with AUAP (5′-GGCCACGCGTCGACTAGTAC-3′) and the designed gene-specific primer (str3A1/str3B1 for the two subunits of *S. strongia*, oxy3A1/oxy3B1 for *S. oxycephala*, mar3A1/mar3B1 for *S. marmoratus*) and run for 20 cycles. Following that, the PCR product was 50× diluted and served as template for the second PCR using primer AUAP and another gene-specific primer (str3A2/str3B2 for *S. strongia*, oxy3A2/oxy3B2 for *S. oxycephala*, mar3A2/mar3B2 for *S. marmoratus*) and run for 35 cycles.

For 5′ RACE, the first-strand cDNA was synthesized from 5 μg of total RNA using the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (invitrogen) with the gene-specific primer (str5_RACE for *S. strongia*, oxy5_RACE for *S. oxycephala*, and mar5_RACE_A/mar5_-RACE_B for *S. marmoratus*). The first PCR was carried out using the gene-specific primer (str5A1/str5B1 for *S. strongia*, oxy5A1/oxy5B1 for *S. oxycephala*, mar5A1/mar5B1 for *S. marmoratus*) and AAP (5′-GGCCACGCGTCGACTAG-TACGGGGGGGGGGGGGGGG-3′) and was run for 20 cycles as in 3′ RACE. The PCR product was 50× diluted and the nested-PCR was run with the gene-specific primers (str5A2/str5B2 for *S. strongia*, oxy5A2/oxy5B2 for *S. oxycephala*, mar5A2/mar5B2 for *S. marmoratus*) and primer AUAP (5′-GGCCACGCGTCGACTAGTAC-3′) for 35 cycles. The PCR conditions for both 3′ and 5′ RACE were the same as described in Section 2.4 and the final PCR products were subjected to DNA sequence.

2.6. PCR from genomic DNA

For the genomic DNA of the three collected-live species (*S. strongia*, *S. oxycephala*, and *S. marmoratus*), Uni_A and Uni_B in combination with DegR were used to amplify each of the two subunits. The degenerate primers designed from start-codon and stop-codon sequences were used in combination with the corresponding gene-specific primers to obtain the whole toxin genes (StartA + str5A2/oxy5A2/mar5A2, StartB + str5B2/oxy5B2/mar5B2, StopA + str3A2/oxy3A2, StopB + str3B2/oxy3B2, StopA_mar + mar3A2, StopB_mar + mar3B2).

For *D. zebra*, PCR was conducted in the same manner as described above with the following primer pairs (Startsyn + zeb5A1, Startpter + zeb5B1/zeb5C1, StopA + zeb3A1, Stoppter + zeb3B1/zeb3C1).

2.7. Sequence analysis

The sequences obtained in this study and those downloaded from the NCBI database (AB262392, AB262393, AB623220–AB623223, AB775453–AB775458, U32516, U36237) were aligned and analyzed using Molecular Evolutionary Genetics Analysis (MEGA) 5.2.1 (Tamura et al., 2011). The nucleotide composition was estimated and the selection pressure was estimated by counting the d_N/d_S value (Muse and Gaut, 1994). The similarity of the deduced amino acid sequences was analyzed by an online tool called SIAS (Sequences Indentifies and Similarities) with PAM250 as the matrix for global similarity test.

Table 1
Biological information of the scorpaenoid fish used in this study.

Species	*N*	Sampling location	Length (cm)	Weight (g)
Sebastapistes strongia	2	Nanfang-ao	9.9–10.6	34–44
Scorpaenopsis oxycephala	3	Nanfang-ao and Keelung	13.2–22.4	93–411
Sebastiscus marmoratus	4	Keelung	15.1–22.3	57–330
Dendrochirus zebra	3	Nanfang-ao	13.3–15.6	86–137

2.8. Phylogenic analysis

The outgroup sequence was searched from the whole genome data by a BLAST search of the α subunit of *stonustoxin* (U36237) in the Ensembl database. Two open reading frame (ORF) sequences were found in the genome of the three-spined stickleback *Gasterosteus aculeatus*(-groupXXI: 7994877–7996973 and groupXXI: 7958278–7960098) and were used as the outgroup (no homology of the β subunit of *stonustoxin* (U32516) could be found in *G. aculeatus*). Molecular models for the deduced amino acid sequences were tested by the model testing program in MEGA 5 and the JTT model combined with the Gamma distributed mutation rates was chosen to construct the phylogenic trees using the Maximum Likelihood and Maximum Parsimony methods.

3. Results

3.1. Toxin genes determination by RT-PCR

RT-PCR using the primer pair DegF1 + DegR amplified a 1500 bp product in *S. strongia*, *S. oxycephala*, and *S. marmoratus*. Subcloning of the PCR products showed that two different gene fragments exist (named *XxTx_A* and *XxTx_B*, Xx represents the abbreviation of species names), as in the cases of the stonefish toxins *stonustoxin* (U32516 and U36237) and *neoverrucotoxin* (AB262392 and AB262393) and the lionfish toxins discovered by Kiriake and Shiomi (2011). For *S. marmoratus*, cloning of the 1500 bp product determined only one type of gene, which was similar (75–77%) to the α subunits of the stonefish toxins (thus was named *SmTx_A*). To get the β subunit gene of *S. marmoratus*, another primer pair DegF2 + DegR was used and a product about 1300 bp was amplified. Subcloning of the 1300 bp product resulted in two distinct gene fragments as found in the other two species, *S. strongia* and *S. oxycephala*.

3.2. Toxin genes determination by RACE

5′ RACE experiments succeeded in both toxin subunits in *S. strongia*, *S. oxycephala*, and *S. marmoratus*. The 5′ UTRs amplified by nested-PCR are about 95 bp (92–97 bp) for all the toxin genes. For 3′ RACE experiments, nested-PCR successfully amplified the 3′ UTRs in *S. oxycephala* and *S. marmoratus*, but did not result in any product in *S. strongia*. To get the whole coding region (CDR) sequences of *S. strongia*, the degenerate primers designed from the stop-codon regions were used to replace the universal primer AUAP. Using the same cDNA template as in the 3′ RACE, primer pairs str3A2 + StopA and str3B2 + StopB successfully amplified a single product in the two toxin subunit genes in *S. strongia*, respectively. The determined 3′ UTRs in *S. oxycephala* and *S. marmoratus* varied from 207 bp to 321 bp, while for *S. strongia*, the 3′ UTRs were undetermined. The whole lengths of determined cDNA sequences of the two subunits are 2209 and 2194 bp in *S. strongia*, 2410 and 2428 bp in *S. oxycephala*, and 2437 and 2519 bp in *S. marmoratus*. The deduced amino acid sequences are 703 a.a. for the α subunits of *S. strongia* (*SsTx_A*) and *S. oxycephala* (*SoTx_A*) and 702 a.a. for *S. marmoratus* (*SmTx_A*). The β subunits are 698 a.a. for *S. strongia* (*SsTx_B*) and *S. oxycephala* (*SoTx_B*) and 700 a.a. for *S. marmoratus* (*SmTx_B*). Table 3 summarizes the accession numbers, the lengths of UTRs, and the lengths of CDR of all the cDNA sequences determined in this study.

3.3. Toxin genes determination from genomic DNA templates

Degenerate primers Uni_A and Uni_B amplified the corresponding subunit genes in *S. strongia*, *S. oxycephala*, and *S. marmoratus* when using genomic DNA as the template. The PCR products were about 2200 bp for the α subunit genes and 2500 bp for the β subunits. The dissimilarity in the lengths of PCR products from cDNA and genomic DNA indicates the existence of introns in the toxin genes. The whole sequences of the toxin subunit genes in the genomic DNA of *S. strongia*, *S. oxycephala*, and *S. marmoratus* were determined using gene-specific primers and degenerate primers designed from start- and stop-codon sequences. The lengths (from start codon to stop codon) of these two toxin genes are 5011 bp (*SsTx_A*) and 3988 bp (*SsTx_B*) in *S. strongia*, 4838 bp (*SoTx_A*) and 3488 bp (*SoTx_B*) in *S. oxycephala* and 3158 bp (*SmTx_A*) and 3821 bp (*SmTx_B*) in *S. marmoratus*.

Since *D. zebra* data was collected from dead individuals, genomic DNA was used to amplify the toxin genes. Primer set DegF1 + DegR was used and the result showed three bands on the agarose gel, which ranged from 1500 to 2200 bp. Subcloning of the PCR products showed that three different products exist (named as *DzTx_A*, *DzTx_B*, and *DzTx_C*). GSPs were then designed for each of the three genes. Combined with DegR, the designed GSPs (zebA, zebB, zebC) successfully amplified a single product for each gene. A preliminary phylogenetic analysis using the neighbor-joining method demonstrated that *DzTx_A* is more similar to α subunit of *stonustoxin* (U36237), while *DzTx_B* and *DzTx_C* are grouped with the two toxin subunit genes in the genus *Pterois* (data not shown). The upstream sequence of *DzTx_A* was therefore amplified using a primer designed from the start-codon regions of the α subunits of *stonustoxin* and *neoverrucotoxin* (primer named Startsyn). For *DzTx_B* and *DzTx_C*, an additional primer was designed specifically for the start-codon region of lionfish (named Startpter). In an attempt to get the whole toxin sequences of *D. zebra*, primer sets Startsyn + zeb5A1, Startpter + zeb5B1 and Startpter + zeb5C1 successfully amplified the upstream sequences of these three toxin genes, respectively. However, we were unable to determine the downstream

Table 2
Primers used in this study.

Primer	Sequence	Position	Reference sequence	Source
Universal/degenerate primers for all species in this study				
FishF2	5′-CGACTAATCATAAAGATATCGGCAC-3′			Ward et al. (2005)
FishR2	5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′			
DegF1	5′-GATGGATATTGAAGCMTCTC-3′	260–279	*stonustoxin_α* (U36237)	Kiriake and Shiomi (2011)
DegF2	5′-GGGGCMAATGCYTTCTTTGT-3′	498–517	*stonustoxin_α* (U36237)	
DegR	5′-CCACTCYAMCTCCCAGTAAT-3′	1823–1804	*stonustoxin_α* (U36237)	
Uni_A	5′-GATATTGAAGCTTCTCTGGG-3′	305–324	cDNA of *SsTx_A* (KJ689803)	This study
Uni_B	5′-GAYATWGAAGCTTCTCTGAA-3′	305–324	cDNA of *SsTx_B* (KJ689804)	
StartA	5′-ATGYCTTCAGAYATCWTGRTAA-3′	98–119	cDNA of *SsTx_A* (KJ689803)	
StartB	5′-ATGYCTTCAGAHATCWTGGTKG-3′	98–119	cDNA of *SsTx_B* (KJ689804)	
Startsyn	5′-ATGTCTTCAGATTTGGTAATGC-3′	60–81	*stonustoxin_α* (U36237)	Ghadessy et al. (1996)
Startpter	5′-ATGTCYTCASAAATCTTGRTRG-3′	80–101	*PvTx-a* (AB623222)	Kiriake and Shiomi (2011)
StopA	5′-TYAAASTAATCTGASASTTCCRT-3′	3032–3054	*neoverrucotoxin_α* (AB262392)	Ueda et al. (2006)
StopB	5′-TTACAATAKTTTAATYYGACCATT-3′	2158–2181	*neoverrucotoxin_β* (AB262393)	
Stop_marA	5′-TCAAAGTAAATTGGCCTTTCCG-3′	2181–2202	cDNA of *SmTx_A* (KJ689807)	This study
Stop_marB	5′-TTAAAGTAATTTAATCTGGCCCTTGC-3′	2173–2198	cDNA of *SmTx_B* (KJ689808)	
Gene specific primers for *S. strongia*				
str3A1	5′-CGACCTACAAGACACTTCC-3′	771–789	cDNA of *SsTx_A* (KJ689803)	This study
str3A2	5′-CATGGCTCCTGCGAAGACTATC-3′	1615–1636		
str3B1	5′-CCTACCATCAACTTCCACAA-3′	774–793	cDNA of *SsTx_B* (KJ689804)	
str3B2	5′-GATCATCAATGACTTTAGCAAG-3′	1582–1603		
str5_RACE	5′-TTGTCACTGTCAAAGAC-3′	557–573	cDNA of *SsTx_A* (KJ689803)	
str5A1	5′-GTTGCCACGTTCTTGTCAGA-3′	513–494		
str5A2	5′-GGAGGAGCTTTGAATTGTTGAT-3′	462–441		
str5B1	5′-GTTGCCTCAATGTTCTGAAA-3′	513–494	cDNA of *SsTx_B* (KJ689804)	
str5B2	5′-AGATTAGTCATCAGCTGTTTGA-3′	462–441		
Gene specific primers for *S. oxycephala*				
oxy3A1	5′-GATTCCTACTGTTAGCATTGAG-3′	626–647	cDNA of *SoTx_A* (KJ689805)	This study
oxy3A2	5′-GAGAGGTTTGACAACTACCG-3′	1767–1786		
oxy3B1	5′-GATAGCAGCACTCCAATACT-3′	877–896	cDNA of *SoTx_B* (KJ689806)	
oxy3B2	5′-AACAAGAAGGCAATGTCTGG-3′	1714–1733		
oxy5_RACE	5′-CTTTATCACAGCTTCC-3′	623–608	cDNA of *SoTx_A* (KJ689805)	
oxy5A1	5′-TGTCCTGAAGGTTGCTGTCC-3′	594–575		
oxy5A2	5′-CCAGGAGGAGCTTTGAAT-3′	460–443		
oxy5B1	5′-CACTACATGAGTTGCCTCAATG-3′	519–498	cDNA of *SoTx_B* (KJ689806)	
oxy5B2	5′-GGTTTCAAGATTAGTCATCAGC-3′	465–444		
Gene specific primers for *S. marmoratus*				
mar3A1	5′-CCAAGTCACCATTCAGCAAT-3′	1139–1158	cDNA of *SmTx_A* (KJ689807)	This study
mar3A2	5′-GACCCAAACACAGTACACAA-3′	1690–1709		
mar3B1	5′-TGTGGATGGAATGTGAAGAA-3′	1168–1187	cDNA of *SmTx_B* (KJ689808)	
mar3B2	5′-GCCCTGACTCTGTCTGA-3′	1701–1717		
mar5_RACE_A	5′-AGCCACACCTTCATT-3′	836–822	cDNA of *SmTx_A* (KJ689807)	
mar5A1	5′-AGTCCTGGAAGATTCTGGTATG-3′	791–770		
mar5A2	5′-GTTGCTATCCTCTAACTTGTC-3′	585–565		
mar5_RACE_B	5′-AGGTGCTTCAGAGTAA-3′	866–851	cDNA of *SmTx_B* (KJ689808)	
mar5B1	5′-GTGGAAGTTGCTGGTAGG-3′	789–772		
mar5B2	5′-AAGAAGTCGCCGTGGAA-3′	730–714		
Gene specific primers for *D. zebra*				
zebA_F	5′-TCCAACTGACTGATGAAGAA-3′	814–833	gDNA of *DzTx_A* (KJ689800)	This study
zebB_F	5′-CTCTGGAAGACAACATAGTC-3′	2347–2366	gDNA of *DzTx_B* (KJ689801)	
zebC_F	5′-CTCTGGAAGACAACACAGTG-3′	1339–1358	gDNA of *DzTx_C* (KJ689802)	
zeb3A1	5′-TCAAGGAGCAAGCATCTAC-3′	1709–1727	gDNA of *DzTx_A* (KJ689800)	
zeb3A2	5′-TCTTTAGCACTTCCCTCCT-3′	1866–1884		
zeb3B1	5′-ACCTCTGTGGAAGACTACG-3′	2709–2727	gDNA of *DzTx_B* (KJ689801)	
zeb3B2	5′-AACTCCAACCACCACCAA-3′	2791–2808		
zeb3C1	5′-TGCTATGTCTTCACCTATGT-3′	1689–1708	gDNA of *DzTx_C* (KJ689802)	This study
zeb3C2	5′-ACTTCTTGGATTCACCTACA-3′	1744–1763		
zeb5A1	5′-CGGACATATCTTCTGTTCTTC-3′	1675–1655	gDNA of *DzTx_A* (KJ689800)	
zeb5B1	5′-TTCAGCACCTTTCTAATTTGA-3′	2398–2378	gDNA of *DzTx_B* (KJ689801)	
zeb5C1	5′-TGTAGGTGAATCCAAGAAGT-3′	1763–1744	gDNA of *DzTx_C* (KJ689802)	

regions. The determined lengths of the three partial genes in *D. zebra* are 2220 bp (*DzTx_A*), 3704 bp (*DzTx_B*), and 3091 bp (*DzTx_C*). The accession numbers of all the determined genomic DNA sequences and the lengths of introns and exons are listed in Table 3.

3.4. Intron–exon location in S. strongia, S. oxycephala, and S. marmoratus

Alignment of the toxin genes' cDNA with sequences determined from genomic DNA shows that both toxin

Table 3
Accession numbers and lengths of the toxin genes determined from cDNA and genomic DNA templates.

	Accession number	Total length	5′ UTR	CDR (DNA/amino acid)	3′ UTR
cDNA					
SsTx_A	KJ689803	>2209 bp[a]	97 bp	2112 bp/703 a.a.	Undetermined[a]
SsTx_B	KJ689804	>2194 bp[a]	97 bp	2097 bp/698 a.a.	Undetermined[a]
SoTx_A	KJ689805	2410 bp	92 bp	2112 bp/703 a.a.	206 bp
SoTx_B	KJ689806	2428 bp	93 bp	2097 bp/698 a.a.	238 bp
SmTx_A	KJ689807	2437 bp	93 bp	2109 bp/702 a.a.	235 bp
SmTx_B	KJ689808	2519 bp	95 bp	2103 bp/700 a.a.	321 bp

	Accession number	Total length	Exon_1	Intron_1	Exon_2	Intron_2	Exon_3
Genomic DNA							
SsTx_A	KJ689794	5011 bp	91 bp	2273 bp	1473 bp	626 bp	548 bp
SsTx_B	KJ689795	3988 bp	91 bp	542 bp	1467 bp	1349 bp	539 bp
SoTx_A	KJ689796	4838 bp	91 bp	2114 bp	1473 bp	612 bp	548 bp
SoTx_B	KJ689797	3488 bp	91 bp	542 bp	1467 bp	849 bp	539 bp
SmTx_A	KJ689798	3158 bp	91 bp	243 bp	1485 bp	806 bp	533 bp
SmTx_B	KJ689799	3821 bp	91 bp	542 bp	1473 bp	1176 bp	539 bp
DzTx_A	KJ689800	>2220 bp	88 bp	248 bp	1485 bp	214 bp	>185 bp[a]
DzTx_B	KJ689801	>3704 bp	91 bp	1484 bp	1479 bp	465 bp	>185 bp[a]
DzTx_C	KJ689802	>3091 bp	91 bp	476 bp	1473 bp	866 bp	>185 bp[a]

[a] The lengths of the 3′ UTRs of *SsTx_A* and *SsTx_B*, and exon_3 in *DzTx_A*, *DzTx_B*, and *DzTx_C* are undetermined since no PCR products were obtained.

subunits in all three species are constructed by 3 exons and 2 introns. The positions of intron_1 in both two toxin subunits of all three species are at the first base of Gly 31 (intron phase 1). For the α subunit genes of *S. strongia* (*SsTx_A*) and *S. oxycephala* (*SoTx_A*), the positions of intron_2 are at the first base of Tyr 522 (phase I), while for the α subunit gene of *S. marmoratus* (*SmTx_A*), intron_2 is located at the first base of Tyr 526 (phase I). For the β subunit genes of *S. strongia* (*SsTx_B*) and *S. oxycephala* (*SoTx_B*), the positions of intron_2 are at the first base of Tyr 520 (phase I) and for *S. marmoratus* (*SmTx_B*), the intron_2 is positioned at the first base of Tyr 522 (phase I). The distribution patterns of exons and introns in the two subunit genes of *S. strongia*, *S. oxycephala*, and *S. marmoratus* are depicted in Fig. 2.

3.5. Intron–exon prediction in D. zebra

Since the *stonustoxin* gene determined by Ghadessy et al. (1996) and the toxin genes of *S. strongia*, *S. oxycephala*, and *S. marmoratus* examined in this study are conserved in intron positions, we predicted that the ORFs of the three toxin genes in *D. zebra* are interrupted by introns in similar positions (intron_1: Gly 30 in *DzTx_A*, Gly 31 in *DzTx_B* and *DzTx_C*, intron_2: Tyr 522 in *DzTx_A*, Tyr 525 in *DzTx_B*, and Tyr 524 in *DzTz_C*). The partial ORFs of the three subunits in *D. zebra* were predicted to be 1758 bp (*DzTx_A*), 1755 bp (*DzTx_B*) and 1749 bp (*DzTx_C*). The GenBank accession numbers and the predicted lengths of the introns and exons of these three toxin genes in *D. zebra* are listed in Table 3 and the predicted exon–intron distributions are shown in Fig. 3.

3.6. Coding region and deduced amino acid sequences analysis

For the whole available scorpaenoid toxin sequences, the averaged d_N/d_S value is 0.719. The similarity test of the deduced amino acid sequences is shown in Table 4. The similarity of the α subunits of *S. strongia*, *S. oxycephala*, and *S. marmoratus* (*SsTx_A*, *SoTx_A* and *SmTx_A*) to the α subunits of *stonustoxin* and *neoverrucotoxin* are about 73% (69–77%), while similarity of the β subunits (*SsTx_B*, *SoTx_B* and *SmTx_B*) to the β subunits of *stonustoxin* and *neoverrucotoxin* are about 78% (76–80%). The two subunits in each species are about 55% similar. Alignment of the deduced amino acid sequences shows that 208 out of the 710 amino acid residues (including gaps) are conserved in all the sequences. Among them, 5 Cys (205, 375, 407, 528 and 575) and 6 Trp (247, 361, 525, 585, 589 and 620) are conserved in all sequences. For positively charged residues (Lys and Arg), 23 sites (13, 24, 65, 91, 97, 98, 104, 110, 159, 177, 178, 271, 272, 286, 305, 327, 332, 373, 385, 395, 474, 497, and 520) are charge-conserved among all sequences (Supplementary Table 1).

3.7. Phylogenetic analysis

When using JTT + G as the substitution model, both tree-constructing methods (ML and MP) depict similar topologies (Fig. 4). The two subunit genes from all species form distinct groups (named clade A and clade B) except for the two subunits of *Pterois*, which are grouped together and are branched from clade B (named subclade P). In both clade A and B, the sequences of Synanceiidae (*S. horrida*, *Synanceia verrucosa*, and *I. japonicus*) and Tetrarogidae (*H. rubripinnis*) are grouped together and form the basic branches. Within these two clades, *S. strongia* and *S. oxycephala* are grouped together and *S. marmoratus* forms a sister group. If we disregard the toxin genes of *Pterois* (subclade P), the sequences in both clades almost form a mirror image. For the three species of genus *Pterois*, these two subunit genes form distinct groups but the intra-group relationships are not perfectly mirrored. The main branches are all strongly supported by the ML and MP methods with bootstrap values higher than 90%.

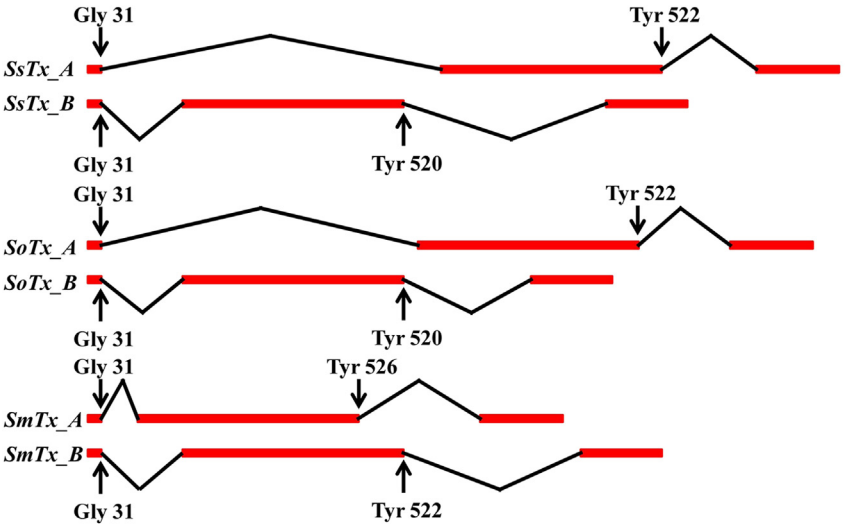

Fig. 2. The distribution of introns and exons of the two toxin subunit genes in *S. strongia*, *S. oxycephala*, and *S. marmoratus*. The exons are represented as red bars and introns as black lines connecting the exons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Combining the predicted ORFs of the three toxin genes of *D. zebra* into the phylogenetic analysis shows similar topologies as illustrated in Fig. 4. In both phylogenetic trees, *DzTx_A* is posited at clade A, while *DzTx_B* and *DzTx_C* are grouped with the sequences of *Pterois* in the subclade P (Fig. 5). For the main branches in these trees, bootstrap values are all higher than 90%, suggesting the robustness of the grouping results. However, the finer branches within subclade P are not strongly supported by the trees, with bootstrap values lower than 70% and the intra-group relationships of the toxin genes of the four species are not consistent in the trees.

4. Discussion

4.1. High similarity and evolutionally negative selection of the toxins

The toxin cDNA sequences obtained in this study are 2209 bp and 2194 bp for the α and β subunits in *S. strongia*, 2410 bp and 2428 bp in *S. oxycephala*, and 2437 bp and 2519 bp in *S. marmoratus*. Although we were not able to get the 3′ UTR sequences in *S. strongia*, the degenerate primers stopA and stopB succeeded in amplifying the toxin genes from the cDNA templates and thus have completed the coding region sequences. For the lengths of all these toxin genes obtained in this study, the coding regions (2094–2109 bp) are about the same size as in *stonustoxin* (U32516, U36237), *neoverrucotoxin* (AB262392, AB262393), and in lionfish and waspfish (Ghadessy et al., 1996; Ueda et al., 2006; Kiriake and Shiomi, 2011; Kiriake et al., 2013). The value of d_N/d_S (0.719), together with the high similarities between the toxin genes, suggests that these genes are under negative selection, which prevents deleterious mutations from accumulating (Fay et al., 2001). Since the venoms of these fish are mainly used as defensive weapons (Khoo, 2002; Lee et al., 2004; Gomes et al., 2011), the conservation (or convergence) of the toxin structures can enhance the memorization of predators. When being hurt by these toxins, it might keep the predator them from hunting morphologically similar fishes in the future.

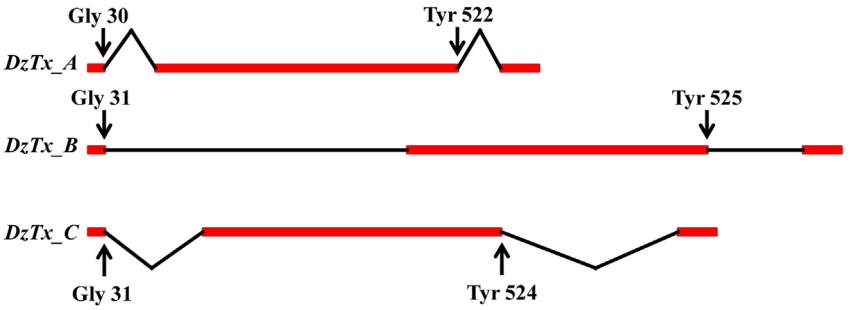

Fig. 3. The distribution of introns and exons of the three toxin genes in *D. zebra*. The exons are represented as red bars and introns as black lines connecting the exons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Global similarity of the deduced amino acid sequences of the toxin genes obtained in this study and those from the database.

	S. strongia		*S. oxycephala*		*S. marmoratus*		*S. horrida*		*S. verrucosa*		*H. rubripinnis*		*I. japonicus*		*P. antennata*		*P. volitans*		*P. lunulata*	
	SsTx A	SsTx B	SoTx A	SoTx B	SmTx A	SmTx B	SNTX A	SNTX B	neoVTX A	neoVTX B	HrTx A	HrTx B	IjTx A	IjTx B	PaTx A	PaTx B	PvTx A	PvTx B	PlTx A	PlTx B
SsTx_A	1	0.53	0.92	0.54	0.77	0.54	0.69	0.52	0.69	0.52	0.71	0.53	0.69	0.52	0.54	0.54	0.54	0.53	0.54	0.54
SsTx_B		1	0.54	0.95	0.55	0.84	0.53	0.78	0.53	0.78	0.54	0.78	0.53	0.77	0.8	0.84	0.8	0.83	0.8	0.83
SoTx_A			1	0.54	0.79	0.55	0.69	0.53	0.69	0.53	0.72	0.54	0.69	0.53	0.53	0.54	0.53	0.53	0.53	0.53
SoTx_B				1	0.56	0.84	0.54	0.78	0.54	0.78	0.55	0.78	0.54	0.77	0.79	0.83	0.8	0.82	0.8	0.83
SmTx_A					1	0.56	0.75	0.54	0.76	0.55	0.75	0.56	0.76	0.54	0.55	0.56	0.55	0.55	0.55	0.56
SmTx_B						1	0.54	0.8	0.54	0.8	0.56	0.82	0.54	0.8	0.79	0.84	0.8	0.84	0.8	0.84
SNTX_A							1	0.53	0.88	0.53	0.85	0.53	0.86	0.52	0.52	0.54	0.52	0.53	0.53	0.54
SNTX_B								1	0.52	0.96	0.53	0.87	0.52	0.92	0.74	0.78	0.75	0.77	0.74	0.78
neoVTX_A									1	0.53	0.85	0.53	0.91	0.53	0.53	0.55	0.53	0.54	0.53	0.55
neoVTX_B										1	0.53	0.87	0.52	0.92	0.74	0.78	0.74	0.77	0.74	0.77
HrTx_A											1	0.54	0.83	0.53	0.53	0.55	0.54	0.54	0.54	0.54
HrTx_B												1	0.53	0.87	0.74	0.79	0.74	0.79	0.74	0.79
IjTx_A													1	0.53	0.52	0.54	0.52	0.53	0.53	0.54
IjTx_B														1	0.72	0.76	0.73	0.76	0.73	0.76
PaTx_A															1	0.81	0.98	0.8	0.98	0.81
PaTx_B																1	0.83	0.98	0.83	0.98
PvTx_A																	1	0.82	0.98	0.82
PvTx_B																		1	0.83	0.97
PlTx_A																			1	0.82
PlTx_B																				1

4.2. Conservation of amino acid residues with important functions

The conserved regions seen in the deduced amino acid sequences provide useful information for inferring possible reaction sites or sites significant in structuring the venom proteins. By forming inter- or intrachain disulfide bonds, cysteine is considered as a critical amino acid in shaping and stabilizing the conformation of proteins (Bardwell et al., 1991; Doig and Williams, 1991; Sevier and Kaiser, 2002). Since the 5 Cys conserved in all the venom peptides are the same as published by Kiriake and Shiomi (2011) and these cysteine residues were inferred to be involved in intrachain S–S linkages (Ghadessy et al., 1996), it is plausible that the two subunits possess similar structures and functions.

In contrast to the conformational role of cysteines, tryptophan and cationic residues such as lysine and arginine have been shown to be essential in the cytolytic activities of various pore-forming toxins by constituting the hydrophobic surfaces of the proteins (Kini and Evans, 1989b; Yew and Khoo, 2000). In the stonefish toxin SNTX, oxidation of tryptophan residues by NBS (N-bromosuccinimide) can modify about 10 of the 20 total tryptophan residues in the toxin and cause the loss of the hemolytic activity (Yew and Khoo, 2000). Our study shows that 6 tryptophan residues are conserved among all the venom peptides (12 tryptophan residues per venom protein). It is possible that these tryptophan residues are the corresponding amino acid sites being oxidized as reported by Yew and Khoo (2000). As for the cationic residues, a previous study showed that modifying the positively charged side chains of lysine or arginine reduces the hemolytic activity of SNTX, but does not significantly change its secondary structure (Chen et al., 1997). Our result shows that 23 amino acid sites are positively charge-conserved in all the scorpaenoid toxin sequences. These residues are therefore assumed to play essential roles in the hemolytic and lethal activities of these toxins. Systematic comparative studies of the hemolytic activities of all these toxins and site-directed mutagenesis studies should provide further information about the roles of these residues.

4.3. Functional unit of the toxins

The venom proteins have long been acknowledged as heterodimers due to the observation of about 150–160 kDa products in the native condition and two different subunits in SDS–PAGE (Poh et al., 1991; Ueda et al., 2006). However, the study on *S. verrucosa* showed another venom protein (named VTX) that possesses two subunits but has a tetrameric structure (Garnier et al., 1995). The venom protein of *Scorpaena plumieri* was also reported to be capable of aggregating into even larger complexes (Gomes et al., 2013). By uniting into functional complexes, the toxins form transmembrane pores that lead to the lysis of the cells (Chen et al., 1997; Ueda et al., 2006; Gomes et al., 2013). The released cellular components may bind to the SP receptors on the endothelium and trigger the synthesis of nitric oxide (NO), which then causes the subsequent vasorelaxation and hypotension (Ghadessy et al., 1996; Sung et al., 2002; Liew et al., 2007). Although there is still some controversy about the actual mechanism of hemolysis, the functional diameters of the transmembrane pores have been determined by studies using osmotic protectants (polyethylene glycol polymers) in *S. horrida* and *S. plumieri* (Chen et al., 1997; Gomes et al., 2013). However, the impedance results shown in these studies occur in a gradual way rather than showing a threshold of osmotic protectant size (Fig. 1 in Chen et al., 1997 and Fig. 3 in Gomes et al., 2013). It is likely that the sizes of the pores formed by the toxins are not constants but are associated with the number of toxin molecules involved.

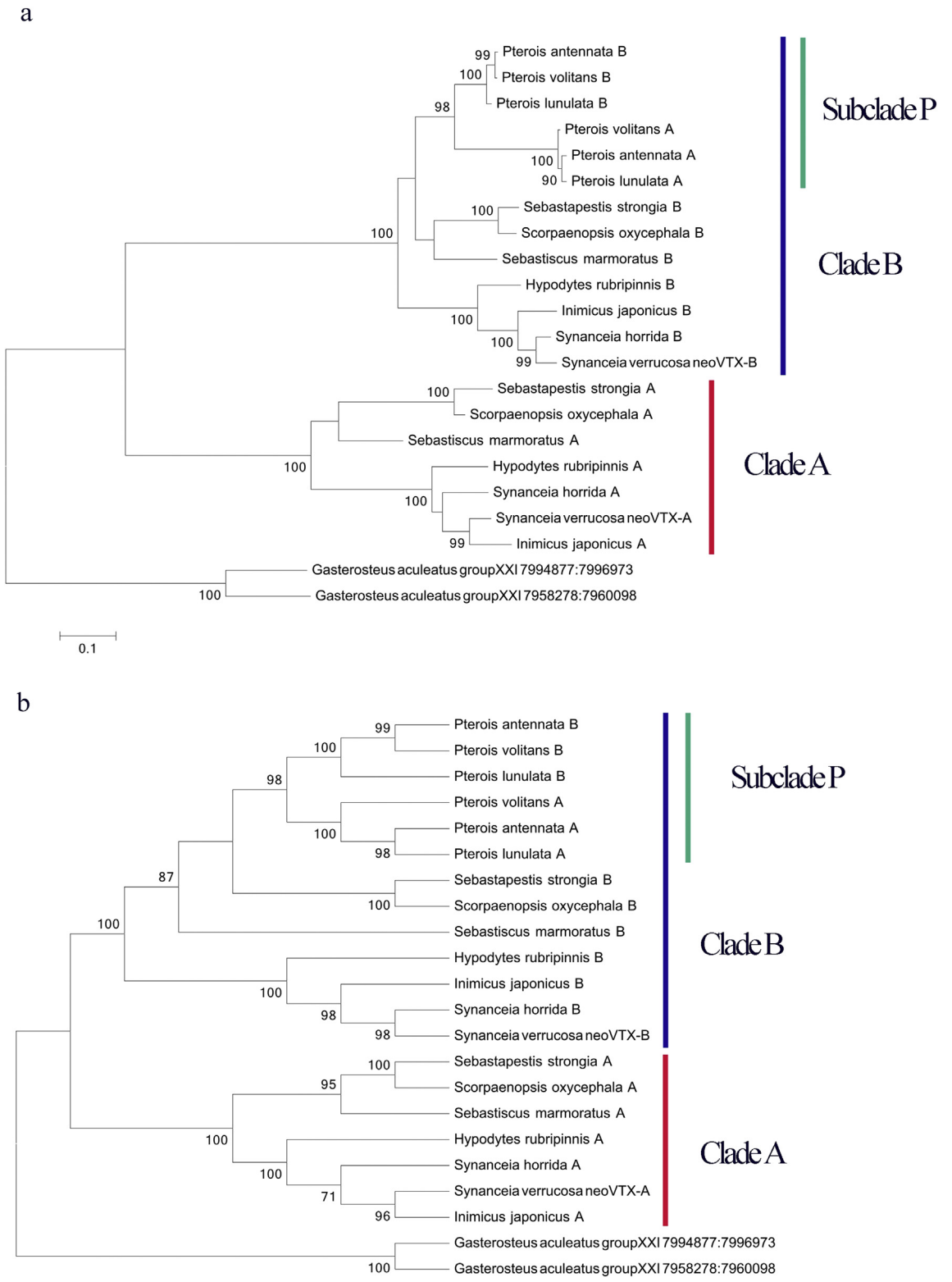

Fig. 4. Phylogenetic analyses of the toxin genes from *S. strongia*, *S. oxycephala*, and *S. marmoratus* using the maximum likelihood (panel a) and maximum parsimony (b) methods. Only bootstrap values >70% are shown in the figure.

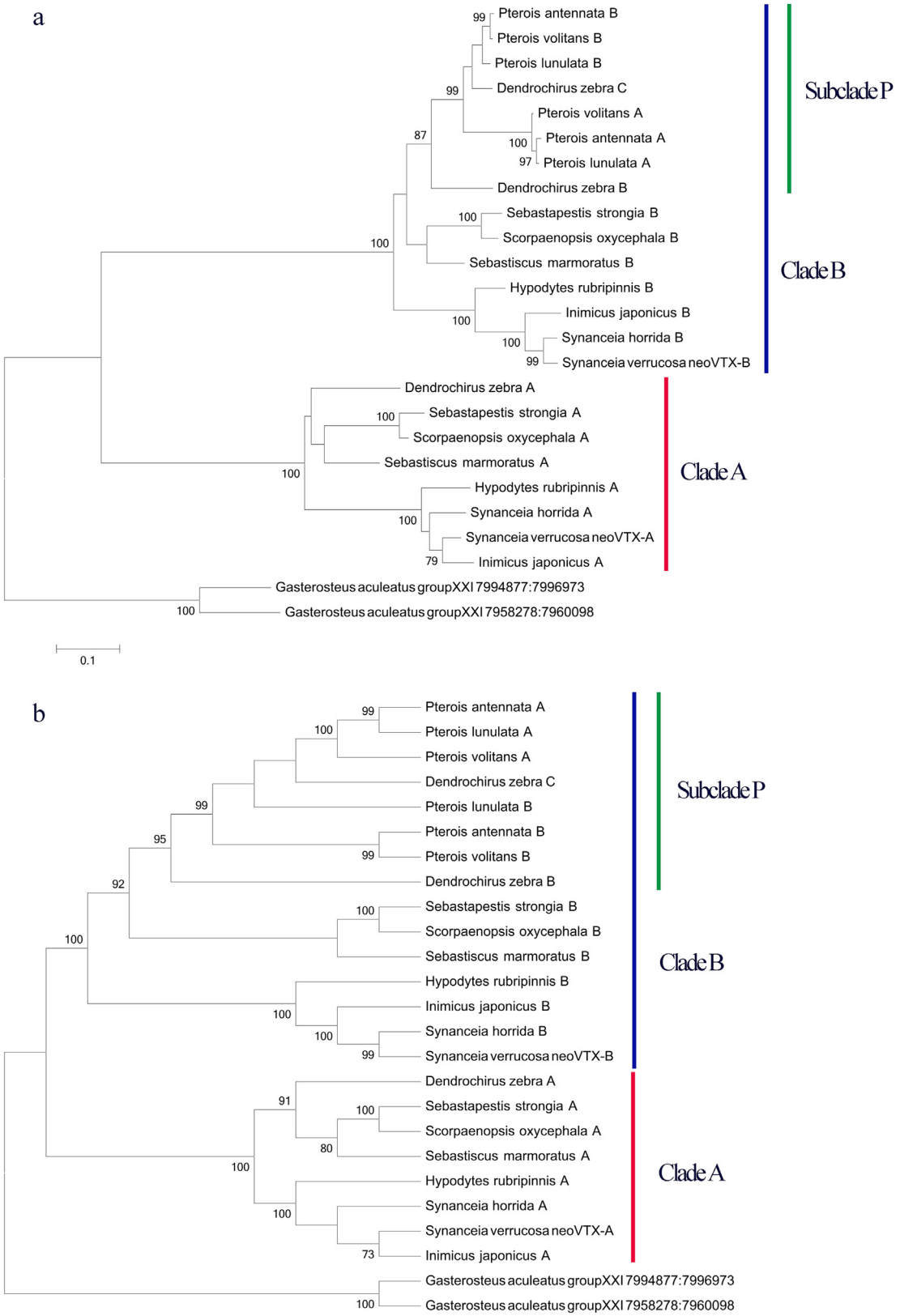

Fig. 5. Phylogenetic analyses of the toxin genes from *S. strongia*, *S. oxycephala*, *S. marmoratus*, and *D. zebra* using the maximum likelihood (panel a) and maximum parsimony (b) methods. Only bootstrap values >70% are shown in the figure.

Our study shows that these two toxin subunits are highly similar in primary (sequences similarity) and secondary structures (cysteine positions) and therefore may have redundant functions. In considering this redundancy and the gradualness of the impedance described above, the functional unit of the toxins may not be obligatorily heterodimeric as previously acknowledged. In our conjecture, the toxins are protein complexes that are formed by the two subunits with no fixed number or ratio. Since no studies have been conducted on the properties of the two subunits independently, the actual functional unit and the roles of the two individual subunits deserve further investigation.

4.4. Conservative nature of intron positions

Alignment of the CDRs (predicted CDRs in *D. zebra*) of the cDNA with the determined genomic toxin genes shows similar intron–exon distribution patterns, with 3 exons separated by 2 introns. The introns are all categorized as phase I and are flanked by GT and AG at the 5′ and 3′ ends, the most common consensus splicing sites discovered (Mount, 1982). For all the toxin sequences determined in this study, the positions of the two introns are largely conserved. Though there are some inconsistencies between the positions of introns_2 in the toxin genes, it can be attributed to the independent insertions/deletions in the two subunits. Disregarding the minor difference in intron_2 positions, the whole distribution of exons and introns are highly conserved among all sequences obtained in this study. The conservative nature of the positions and sequences on splicing sites indicates that the occurrences of the introns in these toxin genes are earlier than the duplication of the two subunits and the differentiation of these species. In addition, the conservative nature also implies that the open reading frames of the toxin genes in other scorpaenoid species can be predicted from their genomic DNA without obtaining the cDNA sequences.

4.5. Toxin gene duplication before scorpaenoid speciation

The deduced amino acid sequences of the toxin genes obtained in this study are highly similar to the toxin genes of stonefish and lionfish. The similarities from both toxin structures and amino acid sequences suggest the existence of a common ancestor for these genes in the Scorpaenoidei, or even at the higher level of Scorpaeniformes. Although this idea has been proposed in many studies (Ghadessy et al., 1996; Ueda et al., 2006; Kiriake and Shiomi, 2011), no one ever analyzed the toxin genes with an evolutionary perspective. The phylogenic trees constructed in this study show a general topology that the two subunits genes form distinct groups (clade A vs. clade B) and the finer branches are largely mirrored (disregarding those genes from the subfamily, Pteroinae). The clear separation of α and β subunits indicates that the duplication and differentiation of these two genes occurred prior to the speciation of the Scorpaenoid fish. Unfortunately, the lack of fossil evidence and reliable evolutionary data precludes the estimation of the timings for the speciation and the toxin gene duplication event.

4.6. Gene gain and loss in subfamily Pteroinae

Regarding the toxin genes in lionfish, the two subunits of genus *Pterois* as well as *DzTx_B* and *DzTx_C* (from *D. zebra*) are grouped together and form a distinct group branching from clade B. The position of this branch (subclade P) indicates that the toxin genes of *Pterois* (and *DzTx_B* and *DzTx_C*) originated from the β subunit genes of Scorpaenoidei and an additional duplication event occurred prior to the speciation of subfamily Pteroinae. However, the precise timings of this duplication and speciation are speculative due to the insufficiency of evidence.

Another point of interest is that the toxin genes obtained from the cDNA libraries of three *Pterois* species showed no homology to *DzTx_A* in these lionfish (Kiriake and Shiomi, 2011; Kiriake et al., 2013). Since *DzTx_A* was found more homologous to the α subunit genes of Scorpaenoidei (clade A), one possible explanation for the presence of this clade's A-homology was that the *DzTx_A* was a pseudogene and its function was replaced by the two genes duplicated from clade B (*DzTx_B* and *DzTx_C*). However, we cannot exclude the possibility that the existence of *DzTx_A* is a species-specific case and all these three toxin genes in *D. zebra* are functional, while the clade A gene in genus *Pterois* is completely lost. The genomic DNA sequencing of the toxin genes in *Pterois* species can provide useful evidence as to which hypothesis is correct.

Since the purified toxins from both lionfish (PvTx and PaTx) and stonefish (SNTX and neoVTX) show no significant difference in their properties, the duplication and pseudogenization seem to have occurred as random events, However, the possibility that this replacement processes confers some advantages for lionfish cannot be excluded.

4.7. Possible evolutionary pathway of the toxin genes in the Scorpaenoidei

From the perspective of gene duplication, a possible evolutionary pathway for the whole gene family is summarized and depicted in Fig. 6. Since the two outgroup sequences searched from GenBank are *stonustoxin_α*-homology and no homology of the β subunit of *stonustoxin* was found in the genome of the three-spined stickleback (*G. aculeatus*), the first origin of the toxin genes may be traced back to the lineage leading to the Gsterosteiformes and Scorpaeniformes. However, the phylogenetic position of Gasterosteidae is controversial (Cuvier and Latreille, 1829; Cushing, 1984, Chen et al., 2003; Miya et al., 2003; Smith and Wheeler, 2004) and some studies suggested that Gasterosteidae should be a member of the Socrpaeniformes (Cuvier and Latreille, 1829; Regan, 1913; Miya et al., 2003). It is therefore possible that the toxin genes originated in the lineage leading to Scorpaeniformes and evolved into the toxin function specifically in Scorpaenoidei. In the lineage of the Scorpaeniformes, the ancestor toxin gene was duplicated in the ancestral species of the Scorpaenoidei (or in the mother species of the Scorpaeniformes) and persisted through the speciation of the Scorpaenoidei, where it evolved to α and β subunit genes in the scorpaenoid analyzed in this study. Before the speciation of Pteroinae, the original β subunit gene duplicated once again and became two subunit genes in *Pterois* (*DzTx_B* and *DzTx_C*

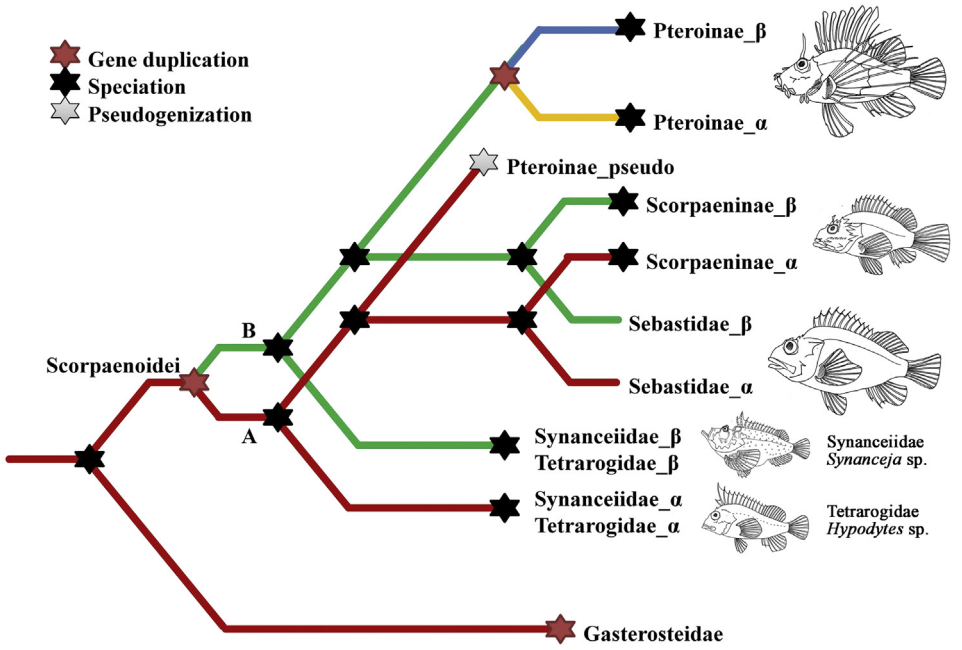

Fig. 6. Demonstration of the evolutionary history of the toxin genes in the Scorpaenoidei. Gray star indicates the pseudogenization of the toxin gene, brown stars indicate the duplication events of the toxin genes and black stars indicate the timing of speciation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in *D. zebra*). The speciation of the subfamily Pteroinae is likely an adaptive radiation and thus the intra-subfamily relationships could not be clearly resolved. In Pteroinae, the newly derived toxin subunit might have replaced the role of the original α subunit as a component of the toxin, while the original α subunit gene became a pseudogene. Since our evolutionary analysis is based on a relatively small number of species in the Scorpaenoidei, further study with more samples in the Scorpaeniformes should be conducted to test the origin of these toxin genes.

5. Conclusion

In this study we determined the toxin genes from four scorpaenoid species and reconstructed the possible evolutionary history of these genes. The results show that these toxins are under negative selection and suggest that they are commonly present in the whole Scorpaenoidei. Sequence alignment and phylogenetic results indicate that gene duplication explains the origin of all of these genes. Although we cannot determine the actual timing of toxin gene origination, we believe it to be prior to the speciation of the Scorpaeniformes. We did not test the bioactivity of the toxins of *S. strongia*, *S. oxycephala*, and *S. marmoratus*. However, it is likely they all share similar bioactivities and hemolytic activities as the toxins in stonefish and lionfish. The actual mechanism of the cytolytic activity of these toxins deserves further investigation.

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Conflicts of interests

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2014.06.013.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.06.013.

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