Journal of Biochemistry and Molecular Biology, Vol. 38, No. 4, July 2005, pp. 386-390

JOURNAL OF Biochemistry and Molecular Biology

Genomic Sequence Analysis and Organization of BmKαTx11 and BmKαTx15 from Buthus martensii Karsch: Molecular Evolution of α-toxin genes

XiuLing Xu, ZhiJian Cao, JiQun Sheng, WenLan Wu, Feng Luo, YongGang Sha, Xin Mao, Hui Liu, DaHe Jiang and WenXin Li*

Department of Biotechnology, College of Life Sciences, Wuhan University,Wuhan 430072, Hubei Province, PR China

Received 4 December 2004, Accepted 2 March 2005

Based on the reported cDNA sequences of BmKαTxs , the genes encoding toxin BmKαTx11 and BmKαTx15 were amplified by PCR from the Chinese scorpion Buthus martensii Karsch genomic DNA employing synthetic oligonucleotides. Sequences analysis of nucleotide showed that an intron about 500 bp length interrupts signal peptide coding regions of BmKαTx11 and BmKαTx15. Using cDNA sequence of BmKαTx11 as probe, southern hybridization of BmK genome total DNA was performed. The result indicates that BmKαTx11 is multicopy genes or belongs to multiple gene family with high homology genes. The similarity of BmKα-toxin gene sequences and southern hybridization revealed the evolution trace of BmKα-toxins: BmKα-toxin genes evolve from a common progenitor, and the genes diversity is associated with a process of locus duplication and gene divergence.

Keywords: Evolution, Intron, Organization structure, Scorpion toxin

Introduction

Scorpion venom is a mixture of various toxic proteins with different functions. Many of them can interfere with the activity of ion channels and modulate their functional properties. Four different families of toxins have been described, which are associated with the ion channels: Na^+, K^+, Cl^-, Ca^{2+}. The best-studied peptides are long chain toxins containing 60-70 amino acid residues cross-linked by four disulfide bridges. These peptides are mainly active on sodium channels (Possani, 1999; Goudet, 2002). Based on the different binding site to the sodium channel receptor, they are classified into two major classes (α-toxins and β-toxins). Scorpion α-toxins bind to receptor site 3 of voltage-gated sodium channels, slow or inhibit the Na^+ current inactivation and thus induce prolongation of action potentials. Moreover, α-toxins can be divided in four groups: (i) the classic α-toxins , which are highly specific to mammals; (ii) the insect α-toxins, highly active on insects; (iii) the α-like toxins active on both mammals and insects (Goudet, 2002), (iv)the intermediate α-toxins, active to both the mammals and insects, but more toxic to the mammals (Couraud, 1982; Kopeyan, 1985).

At present, the research work is mainly focused on the isolation and identification of BmKα-toxin proteins and genes (Xiong, 1997; Zhu, 2000), the 3D structure resolution (Housset, 1994; He, 1999), pharmacology (Yan-Feng, 2002; Yan-Feng, 2003), the application as the molecular probe, insecticides. But the study on BmKα-toxin gene evolution is still rare. Up to now, 23 α-toxins genes from different scorpion species have been identified, including 15 BmKα-toxins genes. Only 8α-toxin gene introns are cloned, including 4 introns from the BmK (from the website http://sdmc.i2r.a-star.edu.sg/scorpion/). To illuminate the genetic basis of diversification in BmKα-toxins, the genomic sequences of BmKαTxs were cloned and molecular evolution of BmKα-toxin genes was investigated. In this report, the sequence and gene organizations of two α-toxin genes (BmKαTx11 and BmKαTx15) from BmK are reported, and the evolution of BmKα-toxin genes is elucidated.

Material and Methods

Preparation and purification of genomic DNA The total

Abbreviations: BmK, Buthus martensii Karsch; PCR, polymerase chain reaction; UTR, untranslated region; bp, base pair

Data deposition: The sequences of two BmKα-toxin genes reported in this paper have been deposited in the GenBank database under accession number AY647170 (BmKaTx11) and AY647171 (BmKaTx15)

*To whom correspondence should be addressed.
Tel: 86-027-68752831; Fax: 86-027-68752146
E-mail: liwxlab@whu.edu.cn

genomic DNA was isolated from whole scorpion BmK as described (Corona, 1996).

Synthesis of oligonucleotide Based on the determined cDNA seqences of BmKαTx11 and BmKαTx15 (Zhu, 2000), the forward primer1 (5'CAAGAAATTTCCWTAAAACGR, corresponding to 5'UTR of BmKαTx11 and BmKαTx15) and the reverse primer2 (5'TTAACCGCCATTGCATCTTCC, corresponding to GRCNGG coding sequence and the TAG terminal codon of BmKαTx11 and BmKαTx15) were designed and synthesized.

Polymerase chain reaction (PCR) The PCR reaction was carried out with 25 μl reaction buffer containing 1 μg genomic DNA, 10 mM Tris-HCL (pH 8.3), 50 mM $MgCl_2$, 0.1 mMdNTP, 50 pM primers and 1 U Taq polymerase. A thermal cycle was used for 31 cycles of reaction under condition of denaturing at 94°C for 50 s, annealing at 55°C for 50 s, and extension at 72°C for 90 s, followed by 10 min at 72°C.

Cloning and DNA sequencing PCR product was electrophoresed in 1% agarose gel and purified by Gel extraction Kit (Omega, USA). Purified PCR product was ligased into the EcoR I site of pMD18-T vector (Takara, Kyoto, Japan). The ligased product was used to transform *E. coli* TG1 competent cells. Positive clones were selected and their plamids was sequenced on both strands by chain termination method (Sanger, 1977). Primers for sequencing were M13+ and M13- universal primers.

Southern hybridization of chromosomal DNA After overnight digest of chromosomal DNA from the BmK with the restriction enzyme EcoRI, HindIII, ClaI, PstI, the digested DNA were separated by electrophoresis in 0.8% agrose gels and transferred onto nylon membranes (Boehringer, Mannheim, Germany). BmKαTx11 cDNA was labeled with ^{32}P according to the protocol of the Exo-free Klenow Type Random Primer DNA Labeling Kit (Takara, Japan). Hybridization was carried out according to the methods Sambrook *et al.* (Sambrook, 2001) with ^{32}P-labeled BmKαTx11 cDNA.

Results and Discussions

α-toxin gene cloning and analysis Using the specific primers designed on the reported two α-toxin cDNA sequences, we obtained two genomic fragments of approximately 800 bp and 700 bp from directed PCR amplification of total DNA extracted from the BmK. The cloning and sequencing of these PCR products revealed that the two fragment of PCR products correspond to two different α-toxin gene: BmKαTx11 and BmKαTx15. Sequence comparison with the corresponding α-toxin cDNA showed that two genes share the same genomic organization: the gene is interrupted by a phase-1 intron at the 16th amino acid residue (G) of signal peptide coding region (Fig. 1). The genomic organization is consistent with the structure of other α-toxin genes (Delabre, 1995; Xiong, 1997). The two introns vary in the length: the intron of BmKαTx11 is 432 bp while the intron of BmKαTx15 is 509 bp.

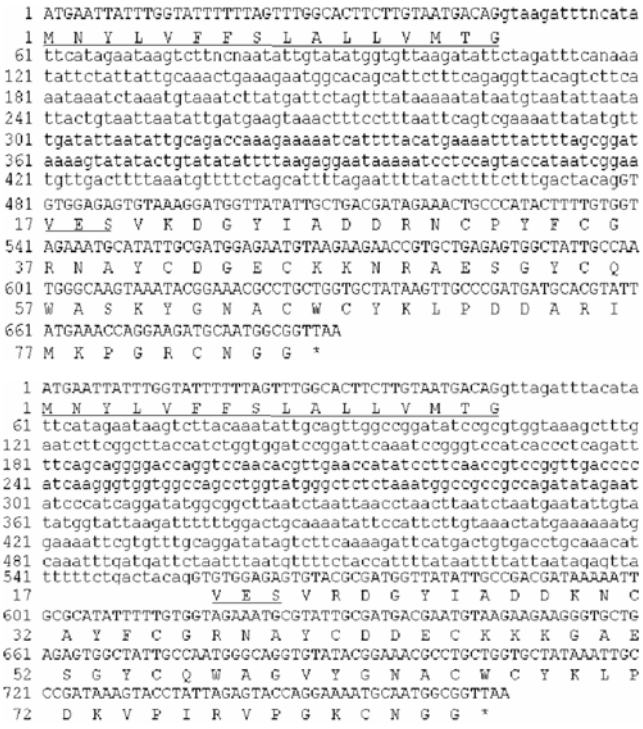

Fig. 1. Nucleotide sequence of genomic DNA encoding the α-Toxin precursor from the BmK. Exon I and exon II are written in capital letters. The introns are written in lowercase letters.The deduced amino acid sequences are given below the nucleotide sequence. Nucleotide and amino acids are numbered at the right side of corresponding sequences. The signal peptide is underlined.

BmKα-toxin intron analysis **Comparison** of the two α-toxins' (BmKαTx11, BmKαTx15) introns reveales that they have the same 5' splicing donor (5' G|gttagatt3') and 3' splicing receptor (5' gactacag|G3'). The introns have a mean A+T content of 76.6% (18.1% higher that the neighbouring exons). A-runs and T-runs are rich in the two introns, which could help splicing factors locate 3'splice sites, or may play a nonspecific role in limiting secondary structure or reducing the likelihood of AG dinucleotides occurring in this region (Csank,1990). Sequence alignment of four BmKα-Toxin (BmKαTx11, BmKαTx15, BmKM1, BmKM10) introns showed that common sequences (ACE) and copy-specific sequences (B in the BmKαTx15 and D in BmKαTx11, BmKM1, BmKM10) are present, as shown in Fig. 2. The common sequences share high homology, which indicate that they arose from common ancestor sequence. It is worth noting that palindrome sequence "AATATT" is present at the 5'end and 3'end of B region, implying two possible mechanism about the evolution of the sequence. 1) Sliding occur during the BmKαTx11 replicated, resulting in the loss of B region. 2) B region is integrated into the gene of BmKαTx15 as an original transposable element. It is still not clarified which explanation is more reasonable.

Southern hybridization When total DNA from the BmK

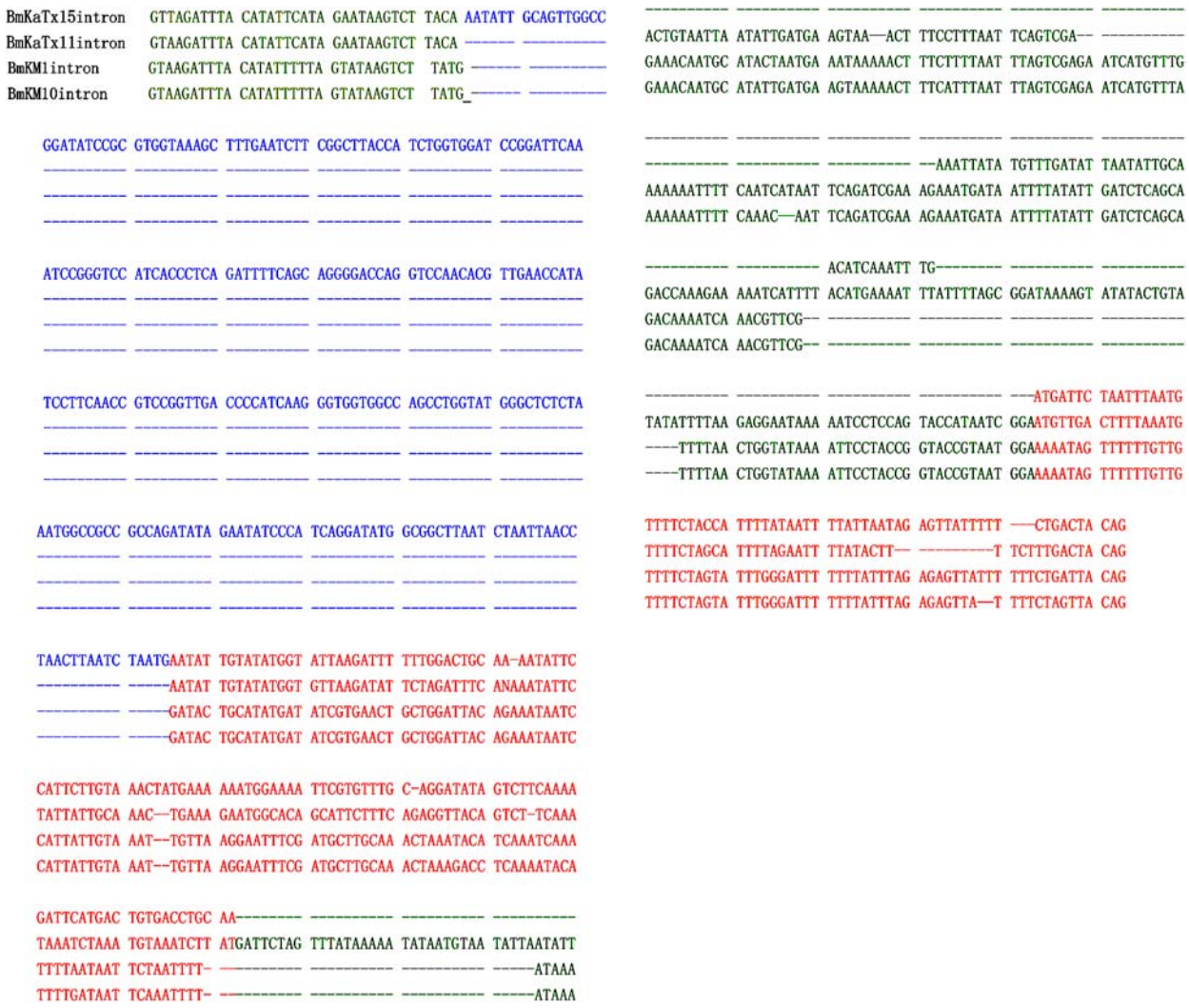

Fig. 2. Sequence Alignment of BmKα-Toxin Gene Introns. The nucleotide sequences of four BmK a-toxin intron are divided into five regions (A-E) marked with different color. Region A: yellow; Region B: blue; Region C: red; Region D: green; Region E: orange.

was digested overnight with either EcoRI, HindIII, ClaI, or PstI, and then probed with the BmKαTx11 cDNA, we observed multibands in each cases, suggesting the existence of multimembers in the α-toxin family (Fig. 3). This result coincides with the blast search of BmKαTx11 cDNA in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). A series of α-toxin genes with high sequence homology from BmK have been identified, for example: BmKaTx15, AGSP, BmKT, BmK unknown toxin, alpha toxin 1, BmKM1, BmKM10 (Fig. 4).

BmK α-toxin Family is from the common ancestor gene

A conclusion on the evolution of α-toxin family can be draw from the comparison of genomic DNA and the southern blotting analysis: the α-toxins are from the common progenitor. During the early stage of α-gene family evolution, the ancestral gene duplicated and integrated into different sites of genome, which could be proved from the results of

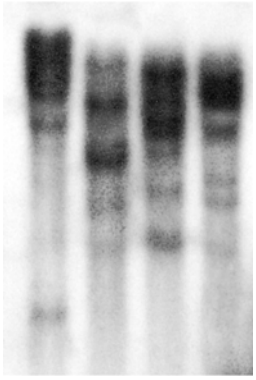

Fig. 3. Southern Blotting Result. Southern blot analysis of BmK genomic DNA digested by EcoRI, HindIII, ClaI or PstI with BmKαTx11 cDNA as a probe. Lane 1, EcoRI digests of genomic DNA. Lane2, HindIII digests of genomic DNA. Lane 3, ClaI digests of genomic DNA. Lane 4, PstI digests of genomic DNA.

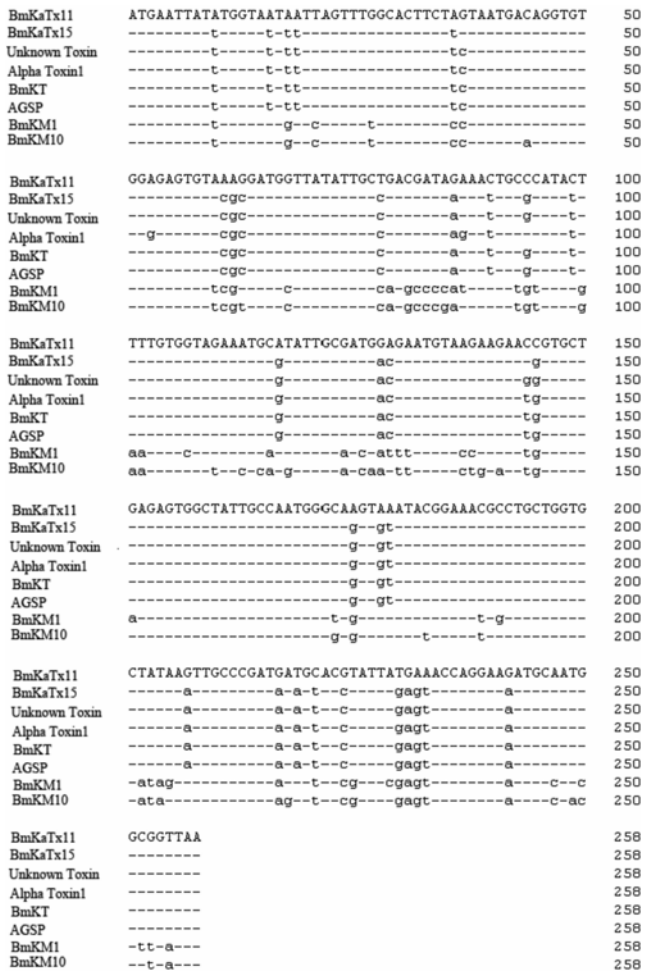

Fig. 4. cDNA sequence alignment of BmKαTx11 gene and its homologous genes from BmK. The genbank accession numbers of these α-toxin genes are as follows: BmKαTx11: AF155364, BmKαTx15: AF163016, AGSP: AF464898, BmKT: AF370023, BmK unknown toxin: AAG09657, alpha toxin 1: AF288607, BmKM1: AAC13693.1, BmKM10: AAC16697.1

southern blotting and blast search in GenBank. The ancestral α-toxin gene duplication provides molecular substance for function diversity, permitting the natural mutagensis and selection. In the late stage, the gene fragment deletion and mutation occurred. Accelerated mutation occurred in the mature peptide coding region (Zhu, 2002), resulting in the different neurotoxins for scorpion defense and prey. Moreover, introns of α-toxins changed much more than exons, which is associated with the different evolution pressure. The divergence pattern of α-toxins introns involves both changes in size (due to deletion and insertions) and base substitutions. The sequences alignment of four BmKα-toxin introns revealed that the sequences of theα-toxin intron was able to be divided into five regions (ABCDE). Among the five regions, the BmKαTx11, BmKM1, BmKM10 had four regions (ACDE), while the BmKαTx15 have A, B, C, E regions. Comparison of the four introns suggests that the ancestral α-toxin gene had five regions (A-E) before the divergence of functions. During the functional divergence by accelerated evolution, intron sequences varied through the loss of some regions. Discussion of the α-toxin evolution in this report are summarized and indicated as the hypothetical model in Fig. 5.

Acknowledgment This work was supported by a National Natural Science Foundation of China (No. 30370349), and the foundation of HuBei provincial Key laboratory of Biotechnology of traditional Chinese medicine, HuBei University.

References

Couraud, F., Jover, E., Dubios, J. M. and Rochat, H. (1982) Two types of scorpion toxin receptor siteds, one related to the activation, the other to the inactication of the action potential sodium channel. *Toxicon* **20**, 9-16.

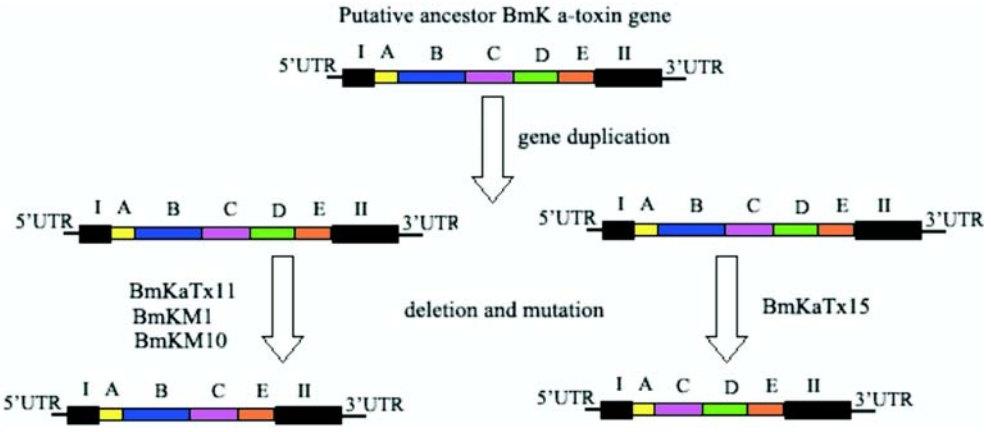

Fig. 5. Hypothetical Model on α-Toxin Evolution. Exon I and exon II are presented by black boxes. The coloured bars present different regions of intron, the colours are consistent with Fig. 2.

Corona, M., Zurita, M., Possani, L. D. and Becerril, B. (1996) Cloning and characterization of the genomic region encoding toxin IV-5 from the scorpion Tiyus serrulatus. *Toxicon* **34**, 251-256.

Csank, C., Taylor, F. M. and Martindale, D. W. (1990) Nuclear pre-mRNA introns: analysis and comparison of intron sequence from Tetrdhymena thernophila and other eukaryotes. *Nucleic Acids. Res.* **18**, 5133-5041.

Delabre, M. L., Pasero, P., Marilley, M. and Bougis, P. E. (1995) Promoter structure and intron-exon organization of a scorpion alpha-toxin gene. *Biochemistry* **34**, 6729-6736.

Goudet, C., Chi, C. and Tytgat, J. (2002) An overview of toxins and genes from the venom of the Asian scorpion Buthus martensii Karsch. *Toxicon* **40**, 1239-1258.

Goudet, C., Huys, I., Clynen, E., Schoofs, L., Wang, D., Waelkens, E. and Tytgat, J. (2001) Electrophysiological characterization of BmKM1, an alpha-like toxin from Buthus martensi Karsch venom. *FEBS Lett.* **495**, 61-65.

He, X. L., Li, H. M., Zeng, Z. H., Liu, X. Q., Wang, M. and Wang, D. C. (1999) Crystalstructures of two á-like scorpion toxins: Non-proline cis peptide bonds and implications fornew binding site selectivity on the sodium channel. *J. Mol. Biol.* **292**, 125-135.

Housset, D., Habersetzer-Rochat, C., Astier, J. P. and Fontecilla-Camps, J. C. (1994) Crystal structure of toxin II from the scorpion Androctonus australis Hector refined at 1.3 Åresolution. *J. Mol. Biol.* **238**, 88-103.

Kopeyan, C., Martinez, G. and Rochat, H. (1985) Primary structure of toxin IV of Leiurus quinquestriatus, characterization of a new group of scorpion toxins. *FEBS Lett.* **181**, 211-217.

Liu, Y., Ma, R. and Wang, S. (2003) Expression of an antitumor-analgesic peptide from the venom of Chinese scorpion Buthus martensii kirsch in *Escherichia coli*. *Protein Expr. Purif.* **27**, 253-258.

Liu, Y., Hu, J. and Zhang, J. (2002) Isolation, purification, and N-terminal partial sequence of an antitumorpeptide from the venom of the Chinese scorpion Buthus martensii kirsch. *Preparative Biochem. Biotech.* **32**, 317-327.

Possani, L. D., Becerril, B., Delepierre, M. and Tytgat, J. (1999) Scorpion toxins specific for Na^{+}-channels. *Eur. J. Biochem.* **264**, 287-300.

Sambrook, J. and Russell, D. W. (2001) Molecular Cloning: A laboratory manual. 3rd. Cold Spring Harbor Laboratory Press, New York, USA.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA seqencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Xiong, Y. M., Ling, M. H., Wang, D. C. and Chi, C. W. (1997) The cDNA and genomic DNA sequences of a mammalian neurotoxin from the scorpion Buthus martensii Karsch. *Toxicon* **35**, 1025-1031.

Zhu, S. Y., Li, W. X. and Zeng, X. C. (2000) Nine novel precursors of Buthus martensii scorpion a-toxin homologues. *Toxicon* **38**, 1653-1661.

Zhu, S. and Li, W. X. (2002) Molecular evolution of scorpion a-toxin: accelerated evolution and function divergence. *Progress Nature Scicence* **12**, 271-276.