Chapter

Venomics Study of *Protobothrops flavoviridis* Snake: How Venom Proteins Have Evolved and Diversified?

Tomohisa Ogawa and Hiroki Shibata

Abstract

Venomics projects have been conducted to disclose the divergent profiles and evolution of various venomous animals. Here, we describe the venomics project including genome and transcriptome of habu snake, leading to drug discovery. Venomics project including the decoding of their whole genomes revealed partly a producing mechanism of various venom proteins including accelerated evolution and alternative splicing and how the toxic organisms have evolved from the nontoxic ones. In addition, the venomics analysis of transcriptomes and proteomes beyond species reveals the relationship between the geographical distribution and evolution of toxic organisms. The abundance of different gene products within a gene family caused by accelerated evolution and alternative splicing may contribute to expand the repertoire of effective weapons to prey capture accompanied with neofunctionalization.

Keywords: alternative splicing, evolution, snake, venomics, whole genome analysis

1. Introduction

A wide variety of creatures, bio-diversified life, have been evolved over 3.8 billion years on earth. Some organisms can produce the biological weapons called "toxins." How have these poisonous organisms evolved to become poisonous? Recent progress on genome sequencing technology has made it possible to analyze the whole genomes of non-model organisms other than model ones and become an important tool for understanding their evolutionary history [1, 2]. "Venomics project" has been undertaken, in which the genomes of a venomous animals are deciphered, and their entire contents of venom are revealed by proteomics and transcriptomics in addition to genome analysis.

2. Toxin-producing organisms

There are various creatures with "toxins" in nature. Toxin derived from living organisms are originally toxic organic compounds such as alkaloids and polyethers produced by plants and phytoplankton and then accumulated through the food chain. On the other hand, the toxins produced by venomous animals are called

"venom" and mainly consist of "cocktails" of various bioactive peptides and proteins. In nature, various poisonous animals such as spider (~38,000 species), scorpions (~1400 species), cone snail (~3200 species), and snakes (~800 species) exist. These peptide/protein toxins act specifically on various target sites as a mixture, disrupting the biological function of prey species.

Since each toxic component have been shown to be very selective, specific, and potent, they represent the lead compounds for the development of new drugs, pharmaceutical lead with few side effects, and useful tool which reveals complex mechanisms of life such as central and peripheral nervous, cardiovascular, blood coagulation, complement, and immune systems. In fact, some successful examples of venom-derived peptides and proteins have been already developed and in practical use such as Prialt and ziconotide. Ziconotide is a 25-amino acid peptide ω-conotoxin M isolated from *Conus magus* and blocks neuronal N-type voltage-gated calcium channels, causing a potent analgesic effect for chronic severe pain with 1000 times more effective than morphine. Captopril is also a notable example of pharmaceutical drug derived from toxins as a bradykinin-potentiating peptide isolated from the venom of *Bothrops jararaca* snake and widely used as an anti-hypotensive by inhibition of angiotensin-converting enzyme (ACE). Thus, a variety of toxins are useful as pharmaceutical leads.

3. Venomics projects

The "venomics projects," international joint research projects on various venomous animal genome, transcriptome, and proteome analyses, were proposed at the 14th International Society on Toxinology (IST) held in Adelaide, Australia, in 2003 [3]. These projects aim to obtain the common information to toxic organisms such as snakes, scorpions, spiders, bees, poisonous frogs, cone snails, sea anemones, jellyfishes, etc. They also provided new knowledge that leads to understanding the venom production and transport systems, molecular mechanisms of diversity of venomous proteins, search for new toxic components related to the drug discovery and pharmacological agents that directly relate to unmet medical needs for diseases, and new therapeutic treatments for venom animal bites. For example, the European Venomics Project (completed in October 2015) was based on the several omics analyses (mainly proteome and transcriptome analyses) of 203 venomous animal species ranging from scorpions, corn snails, poisonous spiders, snakes, and lizards, resulting in the identification of 25,000 toxic protein/peptide sequences, of which 4000 were functionally analyzed [4].

In Japan, to elucidate the novel toxins and the diversification mechanism of venom proteins by accelerated evolution, we deciphered the whole genome sequence of habu snake (*Protobothrops flavoviridis*).

To date, genome analysis of venomous animals including sea anemones [5], mites [6], scorpions [7], poisonous frogs [8], and bees [9] has been performed. Furthermore, whole genome sequence analyses of several venomous snakes that include king cobra [10], pygmy rattlesnake [11], saw-scaled viper [12], five-pacer viper [13], Taiwan habu [14], habu [15], hot-spring snake [16], and some sea snakes [17] have been reported in addition to nontoxic snakes such as python [18], boa [11], corn snake [19], and common garter snake [20] (**Table 1**). More recently, near chromosome-level assembly has been also achieved for Indian cobra [21] and garter snake (**Table 1**).

Here, we describe what we have learned from the venomics analyses on the genome and transcriptome decoding of habu snake (*P. flavoviridis*).

	Spiecies	Common name	Accession	ID	Total length (Mb)	GC%	Assembly	Number of genes	Registration date	Ref.	Remarks
1	*Ophiophagus hannah*	King cobra	PRJNA73575	73575	1594.07	40.6	GCA_000516915.1; OphHan1.0; scaffolds: 296,399; contigs: 816,633; N50: 5,201; L50: 71,224	18,445	26-Sep-11	Vonk FJ et al. (2013) [10]	
2	*Python bivittatus*	Burmese python	PRJNA61243	61243	1435.04	39.8	GCA_000186305.2; Python_molurus_bivittatus-5.0.2; scaffolds: 39,113; contigs: 274,244; N50: 10,658; L50: 38,694	25,385	9-Sep-13	Castoe TA et al. (2013) [18]	Non-venomous
3	*Boa constrictor*	Boa constrictor	PRJNA210004	210004	1415.23		scaffolds: 144,256; N50: 16,487	10,793	28-Jun-13	Vicoso B et al., (2013) [11]	Non-venomous
4	*Sistrurus miliarius*	Pygmy rattlesnake	PRJNA210004	210004	1309.02		scaffolds: 187,303; N50: 12,501	11,939	28-Jun-13	Vicoso B et al., (2013) [11]	
5	*Echis coloratus*	Saw-scaled viper	PRJNA252690	252690	1717.11		scaffolds: 4,790,800; N50: 5,576; contig: 4,973,413; N50: 3,857		13-Jun-14	Hargreaves AD et al., (2014) [12]	
6	*Pantherophis guttatus*	Corn snake	PRJNA268069	268069	1404.22	38.3	GCA_001185365.1; PanGut1.0; scaffolds: 883,920; contigs: 1,320,171; N50: 2,394; L50: 138,295	24,258	21-Jul-15	Ullate-Agote A et al. (2014) [19]	Non-venomous
7	*Thamnophis sirtalis*	Common garter snake	PRJNA290790	290790	1424.9	41.8	GCA_001077635.2; Thamnophis_sirtalis-6.0; scaffolds: 7,930; contigs: 175,977; N50: 10,447; L50: 28,683		23-Jul-15	Castoe TA et al. (2011) [20]	Non-venomous TTX-resistance
8	*Deinagkistrodon acutus*	Five-pacer viper / Chinese moccasin	PRJNA314443	314443	1526.36/1473.4		Female: scaffolds: 183,158; N50: 2,018,329; contigs: 297,390; N50: 26,709; Male: scaffolds:160,256; N50: 2,122,253; contigs: 287,757; N50: 22,424	21,194	7-Mar-16	Yin W et al. (2016) [13]	
9	*Protobothrops mucrosquamatus*	Taiwan habu	PRJNA313429	313429	1673.88	40.6	GCA_001527695.3; P.Mucros_1.0; scaffolds: 52,280; contigs: 167,851; N50: 21,948; L50: 19,485	20,122	15-Jan-16	Aird SD et al (2017) [14]	
10	*Protobothrops flavoviridis*	Amami habu	PRJDB5507	484141	1413.2	38.2	GCA_003402635.1; HabAm_1.0; scaffolds: 84,502; contigs: 218,011; N50: 18,879; L50: 20,311	25,134	2-Aug-18	Shibata H et al.(2018) [15]	
11	*Thermophis baileyi*	Bailey's snake / hot-spring snake	PRJNA473624	473624	1747.68		GCA_003457575.1; DSBC_Tbai_1.0; scaffolds: 20,729; contigs: 179,554; N50: 18,227; L50: 25,622	20,995	5-Sep-18	Li JT et al.(2018) [16]	
12	*Laticauda colubrina*	Yellow-lipped sea krait	PRJDB7284	513505	2024.69	35.6	GCA_004320045.1; latCor_1.0; scaffolds: 62,906; contigs: 164,306; N50: 26,721; L50: 18,756		7-Jan-19	Kishida T et al.(2019) [17]	
13	*Hydrophis melanocephalus*	Slender-necked sea snake	PRJDB7271	513504	1402.64	34.8	GCA_004320005.1; hydMel_1.0; scaffolds: 122,022; contigs: 306,746; N50: 7,411; L50: 46,598		7-Jan-19	Kishida T et al.(2019) [17]	
14	*Laticauda laticaudata*	Blue-ringed sea krait	PRJDB7226	513503	1558.71	40.1	GCA_004320025.1; latLat_1.0; scaffolds: 83,587; contigs: 89,677; N50: 35,581; L50: 12,655		7-Jan-19	Kishida T et al.(2019) [17]	
15	*Emydocephalus ijimae*	Ijima's turtleheaded sea snake	PRJDB7221	513502	1625.2	40.3	GCA_004319985.1; emyIji_1.0; scaffolds: 157,858; contigs: 171,534; N50: 18,545; L50: 24,83		7-Jan-19	Kishida T et al.(2019) [17]	
16	*Naja naja*	Indian cobra	PRJNA527614	527614	1768.54	40.39	GCA_009733165.1; Nana_v5; scaffolds: 1,897; contigs: 13,805; N50: 302,474; L50: 1,397	23,248	5-Dec-19	Suryamohan K et al (2020) [21]	Chromosome-level assembly
17	*Thamnophis elegans*	Western terrestrial garter snake	PRJNA561997	561997	1672.19		GCA_009769535.1; rThaEle1; Scaffolds: 365; Contigs:1,883; N50: 4,620,601; L50: 83		19-Dec-19		Non-venomous/ Chromosome-level assembly
18	*Notechis scutatus*	Mainland tiger snake	PRJEB27871	483163	1665.53	40.2	GCA_900518725.1; TS10Xv2-PRI; scaffolds: 52,414; contigs: 131,885; N50: 31,763; L50: 13,462		27-Jul-18		
19	*Pseudonaja textilis*	Eastern brown snake	PRJEB27869	483162	1590.04	40.1	GCA_900518735.1; EBS10Xv2-PRI; scaffolds: 28,550; contigs: 88,019; N50: 50,443; L50: 8,240		27-Jul-18		
20	*Hydrophis cyanocinctus*	Asian annulated sea snake	PRJNA506024	506024	1389.86	37.6	GCA_004023725.1; ASM402372v1; scaffolds: 546,690; contigs: 1,037,439; N50: 3,626; L50: 96,206		19-Nov-18		
21	*Hydrophis hardwickii*	Hardwick's sea snake	PRJNA506024	506024	1296.39	37.2	GCA_004023765.1; ASM402376v1; scaffolds: 616,046; contigs: 1,057,848; N50: 2,852; L50: 115,477		19-Nov-18		

Table 1.
*Whole genome decoding project for habu snake (*P. flavoviridis*) is highlighted in orange color.*

4. What is habu snake, *P. flavoviridis*?

Habu snakes inhabiting in Nansei Islands (Southwest Islands) of Okinawa and Kagoshima prefectures are the most dangerous domestic snakes in Japan (**Figure 1**). Due to their relatively large body size, long attacking range, and a large amount of delivered venom, still many snakebites and envenoming occur especially during farming (about 80 to 100 cases per year). While habu snakes are specific animals designated by Japanese laws, they are subject to extermination as sanitary animals in many habitats, and some are also consumed commercially such as habu liquor and leather products including the Okinawan musical instrument, Sanshin.

Among the 14 species of *Protobothrops* (The Reptile Database: http://www.reptile-database.org/), three species, *P. flavoviridis* from the Amami and the Okinawa islands, *P. tokarensis* from the Tokara Islands, *P. elegans* from the Yaeyama Islands, are endemic to Japan (**Figure 2A**).

In addition to *Protobothrops*, *Ovophis okinavensis* (hime-habu) are distributed from the Amami and Okinawa islands. From the view of geographical history of the Nansei Islands of Japan and Taiwan, it was expected that these *Protobothrops* snakes including the Taiwan habu (*P. mucrosquamatus*), which are distributed in Taiwan, have been diversified from the beginning of the Quaternary Pleistocene to 2.0 million years ago when the islands began to be separated from the continent. Isolated environment on each island resulted in the differentiation and the

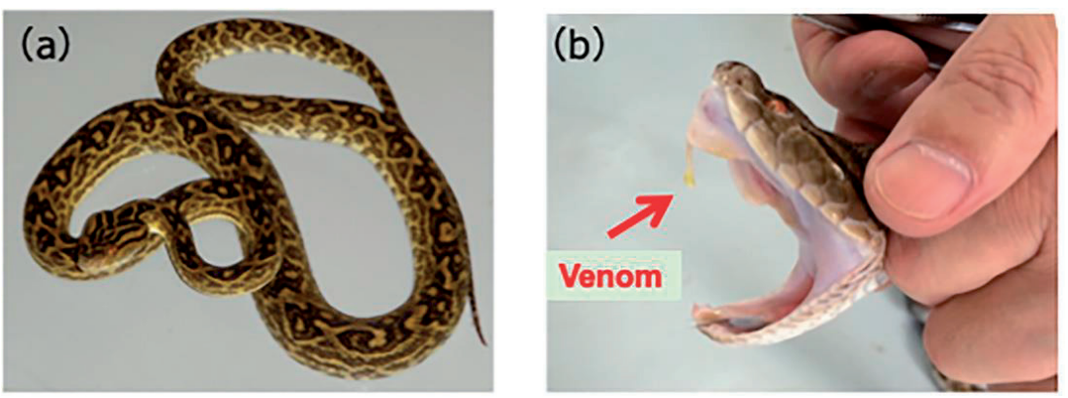

Figure 1.
Protobothrops flavoviridis *snake (a) and its venom (b).*

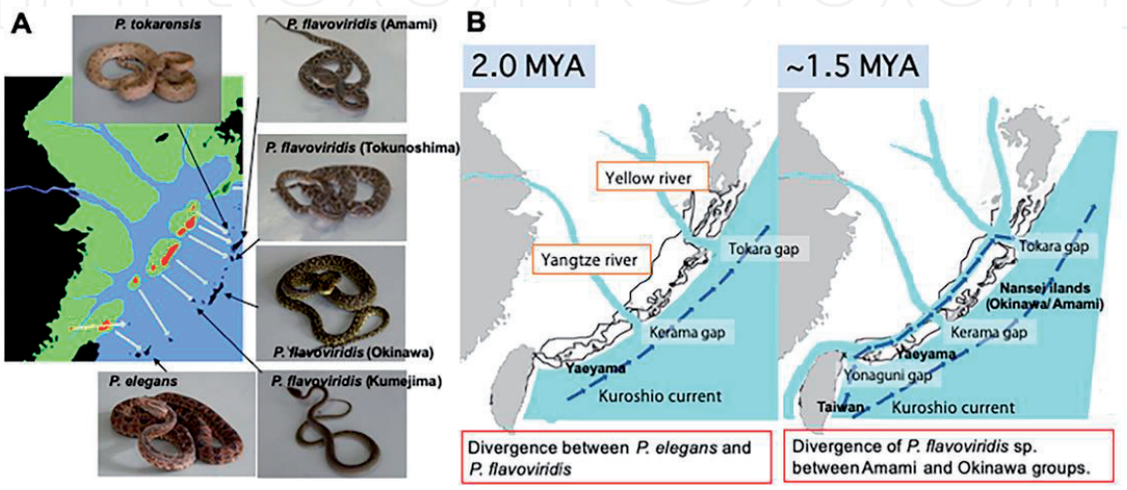

Figure 2.
Distribution of Protobothrops *snakes (a) and geological history of the Nansei Islands in Japan (B).*

speciation of *Protobothrops* species. Molecular phylogenetic analyses using the full-length mtDNA genome sequences of *Protobothrops* snakes showed that the habu snake (*P. flavoviridis*) is close to the Tokara habu (*P. tokarensis*) and Sakishima habu (*P. elegans*) is close to the Taiwan habu (*P. mucrosquamatus*), respectively [22]. These observations are consistent with the geographical history of the Nansei Islands, that is, Okinawa-Amami islands and Taiwan-Yaeyama islands were first separated by Yangtze River (corresponding to the Kerama Gap) and diverged into two groups, the habu (*P. flavoviridis*) and Tokara habu (*P. tokarensis*) species groups and the Sakishima habu (*P. elegans*) and Taiwan habu (*P. mucrosquamatus*) species groups. Further, a remarkable genetic gap between the Amami and Okinawa clades within *P. flavoviridis* was observed. Interestingly, the Tokara habu (*P. tokarensis*) was found to be genetically very close to the Amami clade of *P. flavoviridis* than the Okinawa clade. This indicates that some populations of the Amami clade of *P. flavoviridis* have distributed on the Tokara Islands (Takara and Kodakara islands) and become differentiated to the Tokara habu (*P. tokarensis*) after the divergence of Amami and Okinawa clades. In addition, the Sakishima habu and Taiwan habu diverged as the Yaeyama Islands are separated from Taiwan due to the Yonaguni Gap (**Figure 2B**). Due to the gap of the mouth of the old Yellow River (equivalent to the Tokara Gap), there is no *Protobothrops* snake in the mainland of Japan beyond the Tokara Gap. In summary, the evolutionary history of the speciation of *Protobothrops* in the Nansei Islands is closely associated with the geographical history of the islands.

Snake venoms are potentially lethal complex mixtures composed of proteins and peptides encoded by multigene families that function specific but synergistically to incapacitate the prey or opponent. Venom components can be classified based on their effects as neurotoxic, cardiotoxic, cytotoxic, and hemorrhagic. The viper venoms are known as hemorrhagic toxins that include a wide variety of physiological activities such as metalloproteases (MPs) that destroy blood vessels, phospholipase A_2 (PLA2) that causes inflammation and necrosis, C-type lectin-like proteins (CTLP) and serine proteases (SP) that effect on blood clotting, and so on. Since each of these peptide/protein toxins has very high specificity, it is expected to be a useful tool for clarifying the complex mechanism of life and as a pharmaceutical lead compound. To fully characterize snake venom repertoires and to understand the molecular mechanisms involved in evolution and physiological functions of snake venoms, "venomics studies" including whole genome decoding has been much anticipated.

5. Habu venomics: decoding of the habu genome reveals the evolutionary mechanism of venom-related genes that create a wide variety of venoms

The genome of habu (*P. flavoviridis*) consists of 8 pairs of macro-chromosomes including ZW sex chromosomes and 10 pairs of micro-chromosomes (total 2n = 36). The genome size was estimated to be approximately 1.8 Gb or 1.41 Gb in size by FACS and *k*-mer analysis, respectively [15]. Recently, we decoded the whole genome sequence of habu (*P. flavoviridis*) snake, that is, a total of 136 Gb of shotgun sequences were analyzed and successfully decoded with a sequencing depth of about 96-fold, resulting in the draft genome of habu snake, HabAm1, that include 25,134 protein-coding genes (**Table 1**) [15]. Among 20,540 annotated gene models of HabAm1, we validated 284 genes as venom-related genes, 60 toxic protein genes (SV), and 224 of their non-venom paralog genes (NV). Finally, 18 gene families can be identified as venom-related genes, that is, metalloprotease, serine protease, C-type lectin-like protein, phospholipase A_2, three-finger toxin (3FTX),

aminopeptidase (APase), Cys-rich secreted protein (CRISP), 5 'nuclease (5Nase), hyaluronidase (Hyal), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), L-amino acid oxidase (LAAO), bradykinin-enhancing peptide and C-type diuretic peptide (BPP and CNP), and so on (**Figure 3**).

Furthermore, the venom-related genes can be classified into three categories according to the degree of gene duplication (**Figure 3**). Category III consists of four gene families of MP, SP, CTLP, and PLA2, which are major components of venom and highly multiplexed in both SV gene copies and NV paralog. Category II includes 3FTX, APase, and CRISP, which showed moderate multiplexing in both SV gene copies and NV paralogs. Finally, category I, which consists of only 1 SV copy and 2 to 10 copies of NV paralogs, contained other venom-related genes such as LAAO, NGF, VEGF, Hyal, 5Nase, etc. Phylogenetic analyses of these venom-related genes revealed the unique evolutionary aspects of venomous proteins, that is, only one gene out of four copies have gained venom functions during two-round whole genome duplications (2R-WGD) that occurred in the early evolution of vertebrates.

The accelerated evolution phenomenon in venom proteins was first found in the habu snake PLA2 genes [23, 24] and was later found in other animal venom proteins and peptides such as conotoxin [25, 26], scorpion toxins [27, 28], and spider toxins [29]. Although accelerated evolution has been demonstrated in the genes involved in the biodefence molecule and reproduction in addition to the toxin genes [30], their mechanisms are unknown. Using the complete set of SV and NV gene families in the habu genome, molecular evolution rates analysis by computing numbers of synonymous (K_S) and non-synonymous (K_A) nucleotide substitutions per site for each pair suggested that accelerated evolution was observed only in category III and category II, such as SP, PLA2, and CTLP (K_A/K_S ratios: mean +/−SE =1.047 +/−0.438 for svMPs, 1.253 +/− 0.090 for svSPs,

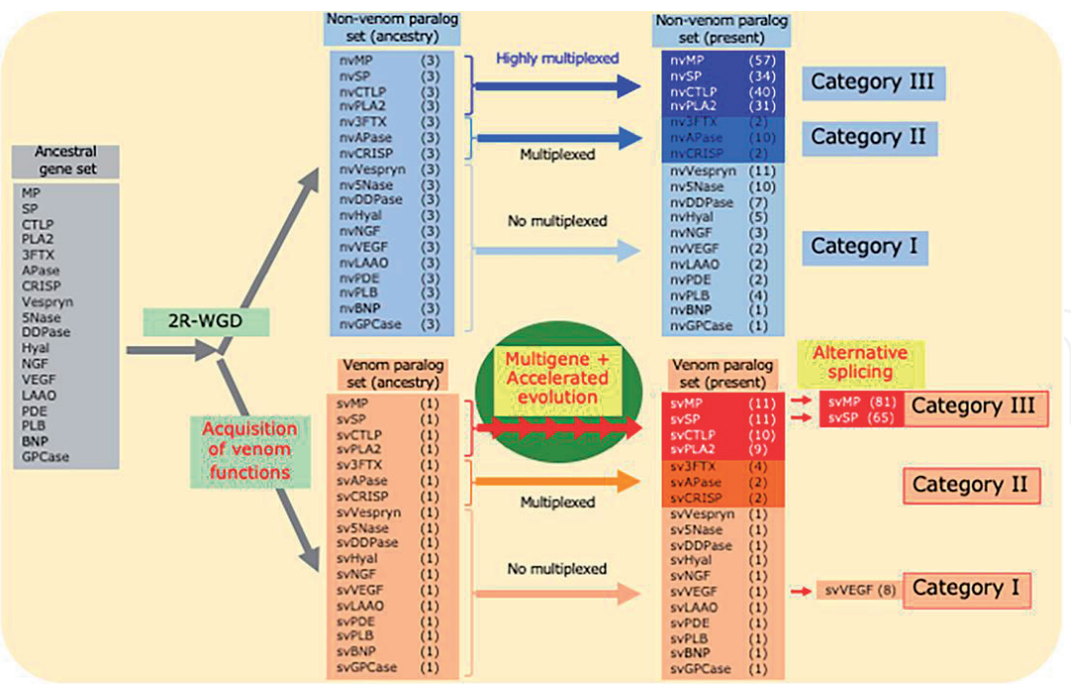

Figure 3.
Deduced evolutionary and diversification history of Protobothrops flavoviridis *snake venom proteins. Through two rounds of whole genome duplication, original set of 18 venom-related gene families (shown in a gray box) became 72 genes (four copies each). Then, a single copy of each family had been a co-option to gain toxic functions, resulting in one toxic gene (shown in an orange box) and three non-venom paralogs (shown in a blue box). Four families of major protein components in the venom (category III: MP, SP, CTLP, and PLA2) have experienced repeated duplication, resulting in complex configurations with 9–11 SV genes and 31–57 NV genes. SV genes in category III showed accelerated evolution. svMP, svSP, and svVEGF were also diversified by alternative splicing.*

0.871 +/− 0.071 for svCTLPs, and 1.093 +/− 0.062 for svPLA2s) [15]. On the other hand, the venom-related genes in category I and NV paralogs in all categories I–III showed no accelerated evolution (K_A/K_S ratios: 0.512 +/− 0.018).

Furthermore, RNA-seq (total of 1.7 billion read pairs, 348 Gb sequence, 1.11 million transcripts identified) from 18 tissues of habu snake and the comprehensive transcript analysis in the venom gland by using PacBio sequencing (~97,000 transcripts) were conducted [31]. Extensive alternative splicing was observed in three venom protein gene families, metalloproteinase (MP), serine protease, and vascular endothelial growth factors (VEGF) with a total of 81, 65, and 8 transcript variants, respectively (**Figure 3**). Especially, svMP showed that over 80 splice variants were transcribed from 11 genes diversified by gene duplication. MPs are key toxins that cause venom-induced pathogenesis such as hemorrhage, fibrinolysis, and apoptosis. According to their domain architecture, svMPs are classified into four groups (P-I to P-IV) (**Figure 4A**). P-I type MPs possess only the metalloproteinase domains and are largely non-hemorrhagic. P-II type MPs contain MP domains and disintegrin domains. P-III type MPs contain Cys-rich domains as well as MP and disintegrin domains. P-IV type MPs harbor lectin-like domains linked by disulfide bonds to the P-III-like structures. These different types of MP proteins can be produced from single MP genes not only by proteolytic processing but also alternatively splicing, resulting in a wider variety of svMPs and disintegrin peptides (**Figure 4B**).

Thus, the alternative splicing is involved in a mechanism for generating diversity of venom proteins in addition to the accelerated evolution [15, 31]. The abundance of different gene products within a gene family caused by accelerated evolution and alternative splicing may contribute to expand the repertoire of effective weapons to prey capture accompanied with neofunctionalization.

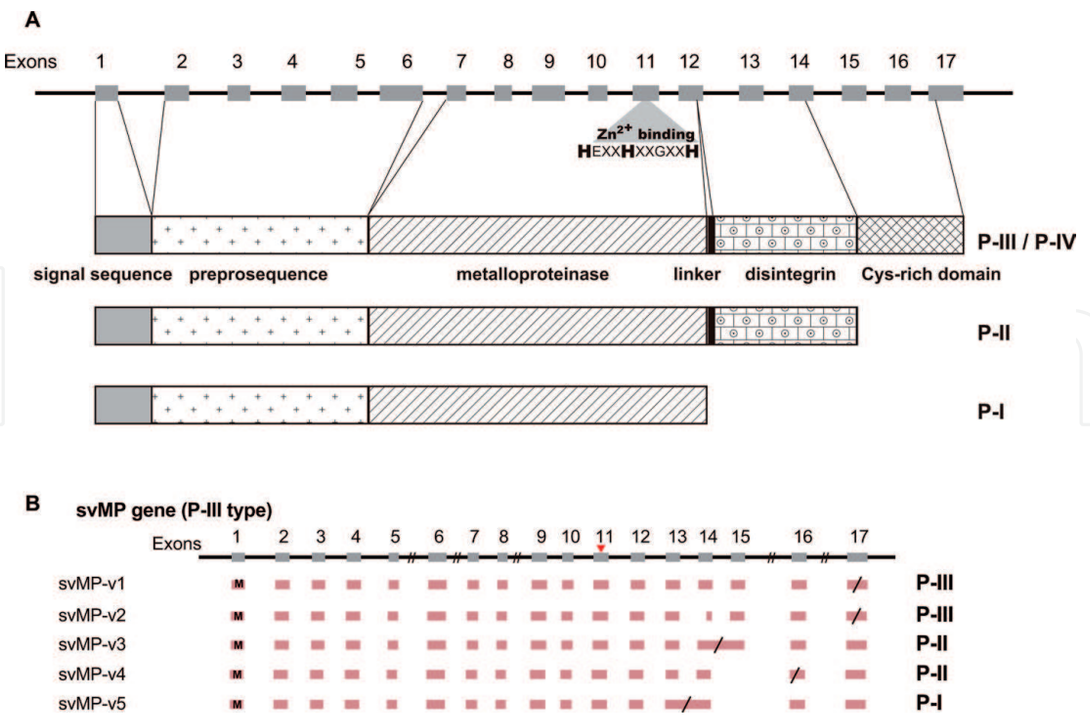

Figure 4.
Schematic structures (A) and alternative splicing (B) of metalloproteinase genes expressed in habu venom glands. (A) the gene structure is shown with 17 exons. A Zn-binding site is also shown in exon 11. Different domains in the three types of MP protein products (P-I, P-II, and P-III/P-IV) are shown as boxes. The active site is also shown by asterisk as "Zn-binding" motif, HEXGHNLGXXHD. P-IV contains lectin-like domain linked by disulfide bonds to the P-III structures. (B) Typical example of P-III type MP gene was shown with validated transcript variants verified in the venom gland, which encode P-I~III type MPs. Initiation and stop codons are marked with "M" and slashes, respectively. Red-colored arrowhead indicates Zn-binding site in exon 11.

6. What comes from venomics project

What did we learn from the "venomics" researches including the decoding of their whole genomes? It revealed partly a producing mechanism of various venom proteins including accelerated evolution and alternative splicing and how the toxic organisms have evolved from the nontoxic ones. In addition, the "venomics" analysis of transcriptomes and proteomes beyond species reveals the relationship between the geographical distribution and evolution of toxic organisms. Recent transcriptomic and proteomic analyses of several snake venoms have reconfirmed in detail that snake venom variation often occurs between individuals of not only interspecifically but also intra-specifically, of which distributions are different geographic locations, diverse environment, and eating habits [32]. For example, a proteomic analysis of 18 species of the genus *Micrurus* snakes in the American continent revealed that the toxic compositions of the major neurotoxins, PLA2, and 3FTX dramatically vary from species to species [32]. Terciopelo (*Bothrops asper*) inhabiting Costa Rica has been also shown to have different toxic compositions between populations from the Pacific coast and from the Caribbean coast. In addition another specie from the same genus, kaisaka (*Bothrops atrox*) inhabiting the same Latin America, also has been shown to have different venom components between Colombia and Brazil [33, 34]. These studies indicate that the composition and structure of the venom varies from region to region even within the same species and that the treatment with anti-venom for snakebites may not work in some areas due to the venom diversity. The envenoming by snakebites is estimated to be about 5 million people annually worldwide, of which about 125,000 die and 400,000 suffer from sequelae such as the loss of extremities [35]. Currently, although anti-venom is currently the only effective treatment for snakebites, there are some cases where the anti-venom production is discontinued due to the economical or political reasons. This serious situation was pointed out by the World Health Organization (WHO) as "neglected tropical disease" [36]. Venomics research is important to develop the anti-venom by using protein engineering techniques against unknown venom proteins, which are obtained by genome decoding, and to understand the mechanism of action of the venom. Venomics research will also lead to the discovery of new useful tools for clarifying the complex mechanisms of life and new functional molecules useful as pharmaceutical leads. For example, three-finger toxins, which have been known as major components of Elapidae and Hydrophiidae neurotoxins, were found in habu snake genome [15].

Whole genome analysis is a powerful tool to understand molecular mechanisms involved in snake venom evolution. We expect that the whole genome analyses of wider variety of venomous species will accelerate the acquisition of useful comprehensive information about different mixtures of venom proteins encoded by different sets of genes and the understanding of the evolutionary histories of venom systems and the common features of venomous animals.

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Conflict of interest

The authors declare no conflict of interest.

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