Toxin Reviews, 24:63–78, 2005

ISSN: 0731-3837 print / 1525-6057 online
DOI: 10.1081/TXR-200046396

Taylor & Francis
Taylor & Francis Group

REVISED SYSTEMATICS OF TAIWANESE VIPERID SNAKES AND THE CORRELATION TO VENOM DIVERSITY AND EVOLUTION

INN-HO TSAI

Institute of Biological Chemistry, Academia Sinica; Institute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan

The major viperid snakes present in Taiwan today are: Daboia russelli siamensis, Deinagkistrodon acutus, Protobothrops mucrosquamatus, Trimeresurus stejnegeri, Ovophis monticola and Ovophis gracilis. As suggested by the species trees deduced from mtDNA sequences of the family Viperidae, these species apparently belong to five different genera. Molecular cloning, N-terminal sequencing, and mass spectrometry have facilitated the sequence-determination of venom proteins. Previous examples of using venom proteins as characteristics for the phylogenetic and evolutionary studies are reviewed herein. Two new phylogeny trees using protein sequence datasets for the crotalid venom acidic phospholipases and the disintegrins are shown. Both trees grossly reveal phylogeographic relationships between the species, although some inconsistencies exist. Moreover, distinct protein markers in each of the Taiwanese viperid venoms and their relations to the snakebite symptoms are discussed in terms of the known snake systematics.

Abbreviations used:

HPLC	high performance liquid chromatography
PCR	polymerase chain reaction
PLA	phospholipase A_2

I. Introduction

The present-day venomous snakes are about 450 species and under four families: Colunbridae, Atractaspididae, Elapidae, and Viperidae. Many species under the last two families are medically important and their venoms have been investigated extensively. The two sub-families of Viperidae, Viperinae, and Crotalinae, are comprised of true vipers and pit vipers, respectively. Recent

Address correspondence to Dr. Inn-Ho Tsai, P. O. Box 23-106, Taipei, Taiwan. E-mail: bc201@gate.sinica.edu.tw

progress of the molecular approach has revolutionized our understanding of venomous snakes and venom genes. Aided by computer technology and phylogenetic theory, molecular genetics has also been applied to uncover evolutionary histories of venomous snakes. Correct taxonomy of the vipers would be important for the purpose of antivenin production, snakebite treatment, and conservation management.

Previous cladogram analyses using mtDNA sequences have separated the two viperid subfamilies (Heise et al., 1995), and also revised the systematics of *Trimeresurus* (sensu lateral) (Creer et al., 2003; Malhotra and Thorpe, 2000) and *Agkistrodon* complex (Klaus et al., 1996; Parkinson, 1999; Parkinson et al., 2000) among Asian pit vipers. Based on these and other related studies on venomous snake systematics, it is apparent that taxonomy of the six poisonous viperid species in Taiwan should be updated and their taxonomies properly revised.

Findings of the structural diversities of venom proteins suggested that inter-family differences are too obvious to be an issue in snake systematics, e.g., it is easy to distinguish between the phospholipase A_2 (PLA, EC3.1.1.4) in elapid venoms (group I PLA) and those in viperid venoms (group II PLA) by disulfide bond patterns of the proteins (Davidson and Dennis, 1990). It is the intra-family or intra-subfamily relationships of the vipers that warrant investigation. Previous results of phylogenetic analyses of the group-I PLA (Tsai, 1997) and three-fingered neurotoxins (Slowinsky, 1997) were consistent with the phylogeography of species under Elapidae and Hydrophiidae. For Viperidae, the venom protein families include: acidic and basic Group II PLAs (Danse et al., 1997), Zn^{2+}-metalloproteases, disintegrins (Huang et al., 1989), serine proteases, C-type lectinlike proteins, Kunitz-type protease inhibitors (Siddiqi et al., 1991), vascular endothelium growth factors (VEGF) (Junqueira de Azevedo et al., 2001), *L*-aminoacid oxidases (Du and Clemetson, 2002), and cystein-rich secretory proteins (CRISP) (Yamazaki et al., 2003). Sequences of these protein families could be useful datasets for phylogenetic analyses of the species, as long as sufficient homologous sequences from representative venom species are available. In the present review, taxonomy of Taiwanese viperid species will be discussed. Using their venom proteins as examples, the following questions will be addressed: whether the venom

may serve as useful characteristics for high-order systematics of the viperid snakes? are there genera-specific venom-markers and associated snakebite symptoms?

II. Materials and Methods

Venoms were extracted from each snake 2 days before it was sacrificed, at which point the venom glands were removed immediately for RNA extraction. The mRNA preparation and the cDNA synthesis kits were purchased from Stratagene. Restriction enzymes and other enzymes were purchased from Promega. Other chemicals were of reagent grade.

Crude venom dissolved in buffer was fractionated on a FPLC system with a Superdex 75 column (HR10/30, Pharmacia) in 0.1 M ammonium acetate (pH 6.5) at room temperature. Freeze-dried fractions were further purified by reversed-phase HPLC using a C18 column (4.5 $\times$ 250 mm, 10 μ, Vydac) equilibrated with 0.07% aqueous trifluoroacetic acid (solvent A), and eluted with a gradient of CH_3CN containing 0.07% trifluoroacetic acid (solvent B). The disintegrins were eluted before 20 % solvent B and PLAs were eluted before 38% solvent B.

The N-terminal sequences of purified venom proteins were determined by an automated amino acid sequencer (Model 477A, PE-Applied Biosystems). Their molecular weights were determined by ESI-MS spectrometer (Sciex mass analyzer, API100, Perkin-Elmer). The full amino acid sequences of venom proteins were deduced from the cDNA cloning. The amino acid sequences of related venom proteins were searched and selected by BlastP (NCBI). The amino acid sequence alignment was made by PILEUP program. Cladograms were constructed based on these sequences by neighbor-joining algorithm using program PHYLIP (Felsenstein, 1992), and the degree of confidence for the internal linage was determined by bootstrap methods (Felsenstein, 1985).

III. Results and Discussion

A. Systematics and Taxonomy of Taiwanese Viperid Snakes

The only Taiwanese true-viper (Viperinae), *Daboia r. siamensis* (formerly *V. r. formosensis*), resides in a limited range in the south

and east regions. It has been found to be similar to the subspecies of Russell's vipers in southeastern Asia (e.g. Thailand) (Belt et al., 1997; Tsai et al., 1996). The major pit vipers inhabiting Taiwan are: *Protobothrops mucrosquamatus* (formerly *Trimeresurus mucrosquamatus*), *Deinagkistrodon acutus* (formerly *Agkistrodon acutus*), *Trimeresurus stejnegeri* (formerly *Trimeresurus gramineus*), *Ovophis gracilis,* and *Ovophis monticola* (formerly *T. gracilis.* and *T. monticola*). These species are apparently under five different genera. Their revised species names are in accordance with the systematics inferred from recently published species trees based mainly on mitochondrial and ribosomal DNA sequences. Although the sister relationship between *Ovophis okinavensis* and *Ovophis gracilis* was supported by several studies (Malhotra and Thorpe, 2000; Parkinson, 1999), the systematics of *Ovophis monticola* remain controversial (Tu et al., 2000).

B. Venom Protein Evolution

An ancestral gene may go through speciation, duplication, or exon-shuffling events. Speciation and gene duplication lead to orthologous genes and paralogous genes, respectively. In most of the cases, only the paralogous genes and the products of exon shuffling will be observed in the genome. Since venoms are gene products and snakes of different genera can not cross, the cladogram of venom proteins or their genes should be related to or agree with the species tree. Functions of venom proteins are dependent on their molecular structures; thus it may be expected that different functional subtypes or paralogous venom proteins might be segregated in the tree for a venom protein family, while the tree of orthologous venom proteins (of a same functional subtype) would show the phylogeographic or evolutionary relationship of the species. Moreover, it seems reasonable to expect that snakes of the same genus contain similar subtypes or paralogs of the venom proteins.

Previous findings on various venom proteins have revealed that gene duplication followed by accelerated evolution of their functional domains have made a significant contribution to the functional diversities of viperid venom proteins (Deshimaru et al., 1996; Nakashima et al., 1995; Tani et al., 2002). For adaptation and endurance of the snakes, less frequent mutations in the

venom molecules may have occurred for functional conservation after specific subtypes of the venom protein were fixed by positive Darwinian selection (Chijiwa et al., 2003). It also has been shown that the functional differences between members of a venom protein family usually are related to substitutions at the surface sites while the protein folds are conserved (Kini and Chan, 1999).

However, the genetic relationships may also be complicated by intra-species variations, possibly resulting from prey-ecology (Daltry et al., 1997), geographic separation, ontology (Warrell, 1997), or other factors. Indeed, we found that the three Taiwanese viperid species, *D. r. siamensis* (Tsai et al., 1996), *P. mucrosquamatus* venoms (Tsai et al., 2001), and *T. stejnegeri* (Tsai et al., 2004), show considerable intra-species variations in their venom PLA isoforms, mainly due to differential expression of each isoforms and geographic separation of the snakes. Usually, more PLA sequences can be deduced from cloning the venom gland cDNAs than are predicted from venom purification because not all of the mRNA are translated into venom proteins (Chijiwa et al., 2000). mRNA of recombinant hybrids or pseudogenes of PLAs have also been found in venom glands (Tsai et al., 2000b).

In addition, the 14 kDa PLAs in Viperinae venoms have been found to be structurally distinct from those in Crotalinae venoms in terms of the numbers of introns and 5'-untranslated regions (Gubensek, 1997). Previous studies also revealed that subtypes of PLA in the venoms of the two subfamilies are different, e.g., basic Lys49-PLA and Glu6-PLA are present exclusively in venoms of pit vipers, while Ser49-PLAs are present only in venoms of true vipers (Bharati et al., 2003; Danse et al., 1997).

C. Review on the Phylogeny Trees Based on Venom Protein Sequences

Although immunological cross-reactivity between related viperid venoms is common, it is difficult to use the data quantitatively or to infer snake systematics (Harrison et al., 2003). Molecular evolution or structural relationships of the venoms are better understood by cladogram analyses based on protein or cDNA sequences. The sequences of other homologous venom proteins may be searched from databanks on the Internet. Finally, multiple sequence alignment and cladogram analysis are performed to

trace the phylogenetic relations of these proteins. Out-group is properly chosen and the bootstrap values are references or guidance during optimization of the tree.

Moreover, the tree may serve as a basis for structure-function classification and annotation of newly identified venom protein. Since functions of venom proteins are associated with their sequences or certain domain structures, the function of a newly identified venom protein may be predicted by examining its position in the phylogenetic tree or its relationship to other molecules with known pharmacological properties. Some insight into their structural and functional relationships may also be obtained by sequence alignment and comparison. Similar functions would be observed for orthologous proteins and distinct functions would be observed for paralogous proteins.

Previously, molecular phylogeny has been used to investigate the structural relationships or subtypes of protein families from viperid venom, including Zn^{+2}-metalloproteases (Tsai et al., 2000), disintegrins (Calvet et al., 2003), serine proteases (Wang et al., 2001), Asp49-PLA (Chijiwa et al., 2003), and Lys49-PLA (Angulo, et al., 2002; Tsai et al., 2001), as well as those from elapid venom, e.g., three-fingered toxins (Jeyaseelan et al., 2003; Slowinski et al., 1997). For example, the phylogenetic tree of venom serine proteases revealed three tentatively classified functional subtypes: clotting enzymes, kininogenases, and plasminogen activators (Wang et al., 2001). The phylogenetic tree of the small metalloproteases from pit viper venom also revealed three major functional subtypes, i.e., acidic strong hemorrhagins (possibly collagenases), basic weak hemorrhagins, and fibrinogenases (Tsai et al., 2000). Since most of the pit viper venoms express more than two or all the protease-subtypes, these subtypes may have evolved before the speciation of these vipers.

D. New Examples of Venom Disintegrin Tree

Common and low molecular weight venom proteins, e.g., disintegrins and PLAs, are relatively convenient references for studying snake biosystematics or comparing intergeneric differences. Probably all the Taiwanese viperid venoms except that of *Daboia russelli* contain disintegrins (Huang et al., 1989; Tsai et al., 1994; Tsai

et al., 2000a). This family of venom proteins evolved from a common ADAM scaffold with high diversity in disulfide bonding pattern and specific amino acids in their inter-cysteine loop containing RGD or KGD (or other motif). Disintegrins may be divided into four or five groups according to their polypeptide length and state of oligomerization (Calvete et al., 2003). Herein, a cladogram was constructed based on the protein sequences of the short and medium viperid venom disintegrins (Fig. 1).

Notably, the *D. acutus* disintegrin with 49 amino acid residues (Tsai et al., 2000a) is smaller than those from other crotalid venoms, and was thus assigned as an out-group for the cladogram analysis. The disintegrins from Viperinae are smaller than most of those from Crotalinae (approx. 70–84 amino acid residues). The phylogenetic tree (Fig. 1) reveals that all disintegrins of the Viperinae venoms are well separated from those of the Crotalinae venoms, and the crotalid disintegrins are related roughly by genera, with a few exceptions. Unfortunately, none of disintegrin from *Ovophis* venoms is available. Notably, disintegrins of *T. stejnegeri* showed high diversification and more than four isoforms could be identified. This is in accord with the unusually high diversity of its venom PLAs (Tsai et al., 2004). The disintegrins of *T. stejnegeri* and *Gloydius halys* are at the basal positions relative to those from other crotalid genera, and trigramin β is identical to albolabrin of *T. albolabris*. However, the disintegrins of some Old World crotalid species (*Protobothrops*, *Calloselasma*, and *Gloydius* in northeastern Asia) could not be completely separated from those of the New World species.

To obtain a better understanding of the systematics of crotalid snakes, the results (Fig. 1) should be examined carefully together with the data of other species trees (Murphy et al., 2003; Parkinson, 1999) and the natural history of their migration to the New World (Wuster et al., 2002). Assuming the venom relationships reflect the species systematics, it appears that at least two ancestral lines of the Old World pit vipers migrated to the New World. The American *Sistrurus*, C*rotalus atrox,* and *Crotalus durissus* are possible descendents of *Gloydius* of northeastern Asia, while many other *Crotalus* (e.g., *C. v. viridis* and *C. h. horridus*) and the nonrattlers (*Agkistrodon*, *Lachesis*, and *Bothrops*) are possible descendents of *Protobothrops* and *Calloselasma*.

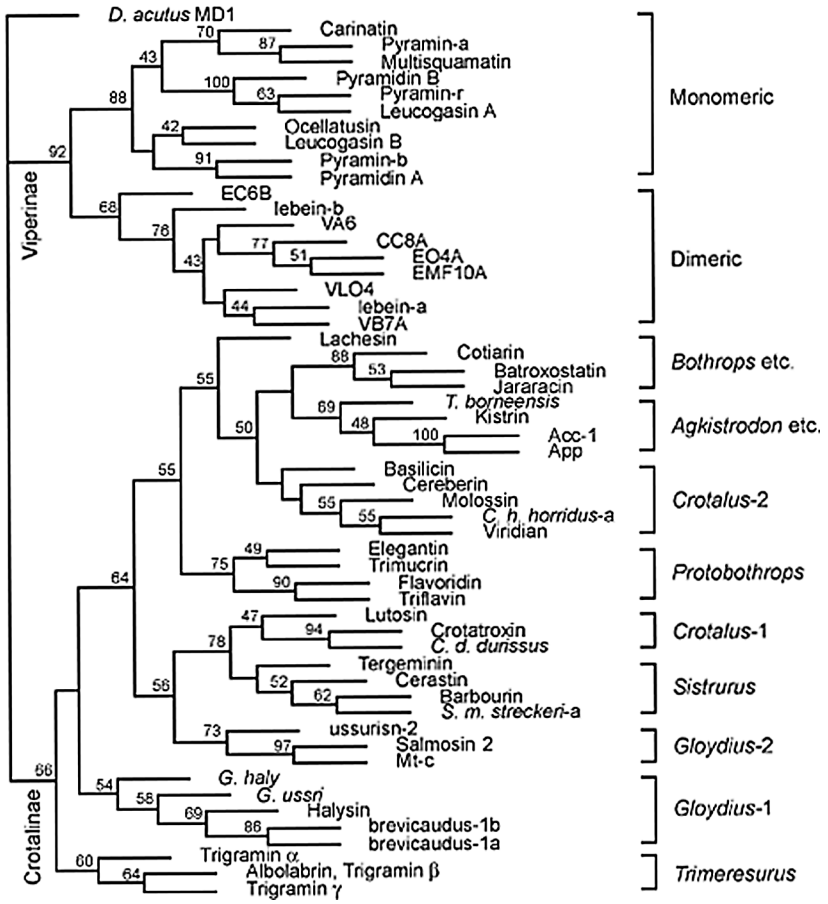

FIGURE 1 Phylogenetic tree showing the relationships of disintegrins from viperid venoms. Amino acid sequences used includes those listed in Calvete et al. (2003) and our unpublished sequences of Sms-a (*Sistrurus m. streckeri*), Tbo (*Trimeresurus borneensis*), and Chh-a (*Crotalus h. horridus*). Other disintegrin sequences (and accession numbers) include: Carinatin (P17347), Pyramin-a (A32029), Pyramin-r (AAB33186-1), Pyramin-b (S53431), *C. d. durissus* (D43019), ussuris-2 (A59412), Acc-1 (BAC55944), *A. p. piscivorus* (CAD29068), Trimucrin (X77089), *G. haly* (1IQ2-A), *G. ussri* (AAP20644), brevicaudus-1b (A59410), and brevicaudus-1a (A59411). The sequence of *D. acutus* disintegrin is assigned as out-group but long disintegrins were not included in the dataset. Values are calculated bootstrap values, indicating the confidence level of the branching.

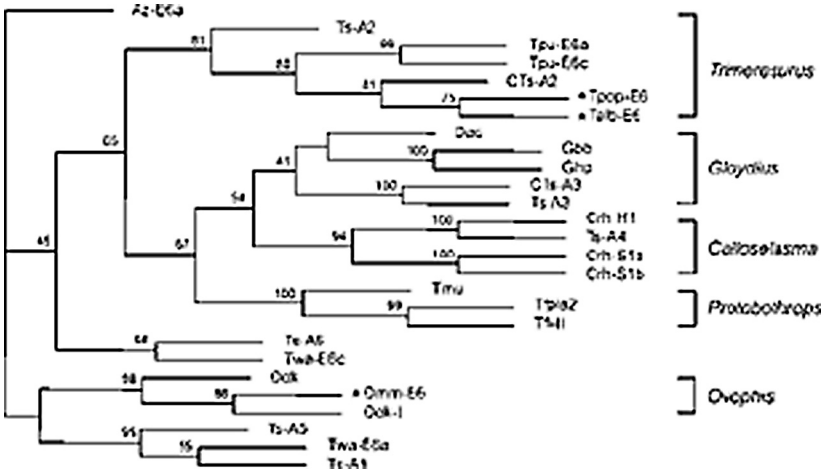

FIGURE 2 Phylogenetic tree of acidic E6-PLA from Asiatic pit viper venoms. Az-E6a (the out-group) is an unpublished full sequence for the acidic PLA from *Azemiops feae* venom. Other E6-PLA sequences used are published amino acid sequences (Tsai et al., 2004; Wang et al., 1996b). Three partial sequences (with *N*-terminal 32 residues) were denoted with asterisks; Omm-E6-PLA is an E6-PLA from *O. m. monticola* venom. Values on branching points are calculated bootstrap values.

E. New Tree of Acidic Glu6-PLAs

We have previously purified, cloned, and characterized PLAs from the venom of Taiwan vipers, including *D. r. siamensis* (Tsai et al., 1996; Wang et al., 1992), *P. mucrosquamatus* (Liu et al., 1991; Tsai et al., 1995; Tsai et al., 2001; Wang and Tsai, 1994), *D. acutus* (Wang et al., 1996a; Wang et al., 1996b), and *T. stejnegeri* (Tsai et al., 2004). One of the most common components found in the pit viper venoms are the acidic Glu6-PLAs (Wang et al., 1999; Tsai et al., 2004), which may inhibit the aggregation of platelets or play other roles (Tsai et al., 2003). The phylogeny tree of venom acidic E6-PLA from Asiatic pit vipers is shown in Fig. 2. *Ovophis* (O. *monticolar* and *O. okinavensis*) appears to be rather distinct relative to the other genera of Asian pit vipers in this cladogram. While all species under *Protobothrops* appear to be monophyletic, *Trimeresurus* (sensu stricto) (Fig. 2) and *Gloydius* (Fig. 1) show more diversifications.

Notably, *T. stejnegeri* appear to have more venom E6-PLA variants that are similar to those from several genera (e.g. *Gloydius* and *Tropidolaemus*) other than *Trimeresurus* (sensu stricto) (Tsai et al., 2004). In the PLA tree (Fig. 2), *T. stejnegeri* is associated with the primitive semi-arboreal *T. puniceus* and *T. borneensis*, and the more recently evolved arboreal *T. albolabris* and *T. popeorum* (Creer et al., 2003), but in the disintegrin tree (Fig. 1) *T. stejnegeri* is associated with *T. albolabris* but separated from *T. borneensis*. Thus, the current taxonomy does not adequately represent the true diversity present in *Trimeresurus* (sensu stricto) (Malhotra and Thorpe, 2000), and the possibility of multiple ancestries of *T. stejnegeri* seem to exist.

It has been known that *Azemiops, Tropedolaemus*, and *Deinakistrodon* are monotypic genera and are relatively primitive (Parkinson, 1999). The topology of both venom protein trees (Fig. 1 and 2) are in accord with known snake systematics, showing clusters according to snake genera.

F. Venom Protein Markers and Snakebite Symptoms of Taiwanese Vipers

Epidemiology and symptoms of the snakebites in Taiwan have been reported (Kuo and Wu, 1972; Sawai, 1969). In general, envenoming by viperid snakes causes hypotension, hemorrhage, thrombocytopenia, edema, and inflammation. Symptoms of the snakebites by each viperid species in Taiwan are characteristic if not all distinct. Results obtained from animal experiments in some cases cannot be extrapolated to the human snakebite situations, possibly due to differences in the body size and target specificities. Notably, envenoming by *Daboia r. siamensis* (formerly *Vipera r. formosensis*) causes hypotension and neurotoxic symptoms on experimental animals due to high content of a dimeric PLA toxin (Wang et al., 1992); but it causes acute renal failure, hemolysis and bleeding diathesis, and incoagulable blood in human victims (Hung et al., 2002a and 2002b). The synergistic effects of hemorrhagic metalloprotease and Coagulation Factor V and X activating enzymes in the venom (Takeya et al., 1992; Tokunaga et al., 1988) may cause consumptive coagulopathy, bleeding, and thrombocytopenia (Hung et al., 2002a).

Envenoming by *P. mucrosquamatus* causes severe thrombocytopenia, due to the presence of collegen-like (Teng et al.,

1993) and C-lectin like integrin-agonist on the platelet (Huang et al., 2004); it also causes myonecrosis due to the effects of high content of N6-PLAs (Chen et al. 1994; Tsai et al., 1995) and K49-PLAs (Liu et al., 1991). Envenoming by *D. acutus* causes severe hemorrhage and coagulopathy due to the synergism of both the Zn^{+2}-metalloproteases (Tsai et al., 2000) and the thrombin like fibrinogenase (Wang et al., 2001), and rich contents of C-lectin like antiplatelet proteins (Chen et al., 2000; Tani et al., 2002). The envenoming by *T. stejnegeri* and *O. gracilis* causes local edema, apparently due to some basic PLAs (Tsai et al., 2004) and kininogenases. Thus, snakebite symptoms may well be explained by specific synergistic actions of purified toxins in each venom species.

The biodiversity of subtropical Taiwan, the home of many interesting venomous snakes, has been a valuable resource for snake venom research. Elucidation of both the snake phylogeny and molecular phylogeny of the venom allows us to understand better the systematics of various venomous species and the structural relationships of venom proteins. Furthermore, basic research on the Taiwanese venom components has revealed intergeneric difference of venom markers and snakebite symptoms. These findings should render valuable references for snakebite diagnosis and treatments on the island.

Acknowledgments

The author sincerely thanks Ms.Ying-Ming Wang for help with phylogenetic analyses of the venom proteins, and many other assistants and students whose contributions have made this review possible. The research has been supported by grants from National Science Council, Taiwan and Academia Sinica, Taiwan. This paper is dedicated to the late Prof. C. Y. Lee (1915-2001) on his memorial day.

References

Angulo, Y., Olamendi-Portugal, T., Alape-Giron, A., Possani, L. D., Lomonte, B. (2002). Structural characterization and phylogenetic relationships of myotoxin II from *Atropoides (Bothrops) nummifer* snake venom, a Lys49 phospholipase A_2 homologue. *Int. J. Biochem. Cell Biol.* 34:1268–1278.

Belt, P. J., Malhotra, A., Thorpe, R. S., Warrell, D. A., Wuster, W. (1997). Russell's viper in Indonesia: snakebite and systematics. In: Thorpe R. S., Wuster W., and Malhotra A. eds. *Venomous Snakes*, Clarendon Press, Oxford 219–234.

Bharati, K., Hasson, S. S., Oliver, J., Laing, G. D., Theakston, R. D., Harrison, R. A. (2003). Molecular cloning of phospholipases A_2 from venom glands of *Echis* carpet vipers. *Toxicon* 41:941–947.

Calvete, J. J., Moreno-Murciano, M. P., Theakston, R. D. G., Kisiel, D. G., Marcinkiewicz, C. (2003). Snake venom disintegrins: novel dimeric disintegrins and structural diversification by disulphide bond engineering. *Biochem. J.* 372:725–734.

Chen, L. N., Liu, C. S., Chang, C. C. (1994). Isolation and characterization of a toxic phospholipase A_2 from the venom of the Taiwan habu (*Trimeresurus mucrosquamatus*). *Biotech. Appl. Biochem.* 19:61–73.

Chen, Y. L., Tsai, K. W., Chang, T., Hong, T. M., Tsai, I. H. (2000). Glycoprotein Ib-binding protein from the venom of *Deinagkistrodon acutus*: cDNA sequence, functional characterization, and three-dimensional modeling. *Thromb. Haemost.* 83:119–126.

Chijiwa, T., Deshimaru, M., Nobuhisa, I., Nakai, M., Ogawa, T., Oda, N., Nakashima, K., Fukumaki, Y., Shimohigashi, Y., Hattori, S., Ohno, M. (2000). Regional evolution of venom-gland phospholipase A_2 isoenzymes of *Trimeresurus flavoviridis* snakes in the southwestern islands of Japan. *Biochem. J.* 347:491–499.

Chijiwa, T., Hamai, S., Tsubouchi, S., Ogawa, T., Deshimaru, M., Oda-Ueda, N., Hattori, S., Kihara, H., Tsunasawa, S., Ohno, M. (2003). Interisland mutation of a novel phospholipase A_2 from *Trimeresurus flavoviridis* venom and evolution of Crotalinae group II phospholipases A_2. *J. Mol. Evol.* 57:546–554.

Creer, S., Malhotra, A., Thorpe, R. S. (2003). Assessing the phylogenetic utility of four mitochondrial genes and a nuclear intron in the Asian pit viper genus, *Trimeresurus*: separate, simultaneous and conditional data combination analyses. *Mol. Biol. Evol.* 20:1240–51.

Daltry, J. C., Wuster, W., Thorpe, R. S. (1997). The role of ecology in determining venom variation in Malayan pitviper, *C. rhosdostoma*. In: Thorpe R. S., Wuster W., and Malhotra A. eds. *Venomous Snakes*, Clarendon Press, Oxford. 155–172.

Danse, J. M., Gasparini, S., Menez, A. (1997). Molecular biology of snake venom phospholipases A_2 In: Kini R. M. ed. *Venom Phospholipase A_2 Enzyme: Structure, Function and Mechanism*, J. Wiley & Sons, 29–71.

Davidson, F. F., Dennis, E. A. (1990). Evolutionary relationships and implications for the regulation of phospholipase A_2 from snake venom to human secreted forms. *J. Mol. Evol.* 31:228–238.

Deshimaru, M., Ogawa, T., Nakashima, K., Nobuhisa, I., Chijiwa, T., Shimohigashi, Y., Fukumaki, Y., Niwa, M., Yamashina, I., Hattori, S., Ohno, M. (1996). Accelerated evolution of crotalinae snake venom gland serine proteases. *FEBS Lett.* 397:83–88.

Du, X. Y., Clemetson, K. J. (2002). Snake venom *L*-amino acid oxidases. *Toxicon* Erratum in: *Toxicon* 40:1381 40:659–665.

Felsenstein, J. (1992). *PHYLIP: the PHYLogeny Inference Package*, version 3.573. Computer program distributed by the U. of Washington, Dept. of Genetics, Seattle, U.S.A.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791.

Gubensek, F., Kordis, D. (1997). Venom phospholipases A_2 genes and their molecular evolution In: Kini R. M. ed. *Venom Phospholipase A_2 Enzyme: Structure, Function and Mechanism*, J. Wiley & Sons, London &, New York 73–95.

Harrison, R. A., Wuster, W, Theakston, R. D. G. (2003). The conserved structure of snake venom toxins confers extensive immunological cross-reactivity to toxin-specific antibody. *Toxicon* 41:441–449.

Heise, P. J., Maxson, L. R., Dowling, H. G., Hedges, S. B. (1995). Higher-level snake phylogeny inferred from mitochondrial DNA sequences of 12S rRNA and 16S rRNA genes. *Mol. Biol. Evol.* 12:259–265.

Huang, K.-F., Ko, T.-P., Hung, C.-C., Chu, J., Wang, A. H.-J., Chiou, S.-H. (2004). Crystal structure of a platelet agglutinating factor isolated from the venom of Taiwan habu (*Trimeresurus mucrosquamatus*). *Biochem. J.* 378:1–9.

Huang, T. F., Holt, J. C., Kirby, E. P., Niewiarowski, S. (1989). Trigramin: primary structure and its inhibition of von Willebrand factor binding to glycoprotein IIb/IIa complex on human platelets. *Biochemistry* 28:661–669.

Hung, D. Z., Wu, M. L., Deng, J. F., Lin-Shiau, S. Y. (2002a). Russell's viper snakebite in Taiwan: differences from other Asian countries. *Toxicon* 40:1291–1298.

Hung, D. Z., Wu, M. L., Deng, J. F., Yang, D. Y., Lin-Shiau, S. Y. (2002b). Multiple thrombotic occlusions of vessels after Russell's viper envenoming. *Pharmacol. Toxicol.* 91:106–110.

Jeyaseelan, K., Poh, S. L., Nair, R., Armugam, A. (2003). Structurally conserved alpha-neurotoxin genes encode functionally diverse proteins in the venom of *Naja sputatrix*. *FEBS Lett.* 553:333–341.

Junqueira de Azevedo, I. L. M., Farsky, S. H. P., Oliveira, M. L. S., Ho, P. L. (2001). Molecular cloning and expression of a functional snake venom vascular endothelium growth factor (VEGF) from the *Bothrops insularis* pit viper. *J. Biol. Chem.* 276:39836–39842.

Kini, R. M., Chan, Y. M. (1999). Accelerated evolution and molecular surface of venom phospholipase A_2 enzymes. *J. Mol. Evol.* 48:125–132.

Klaus, F., Mink, D. G., Brown, W. M. (1996). Crotalin intergeneric relationships based on mitochondrial DNA sequence data. *Copeia* 1996:763–773.

Kuo, T. P., Wu, C. S. (1972). Clinico-pathological studies on snakebites in Taiwan. *J. Formos. Med. Assoc.* 47:65–98.

Liu, C. S., Chen, J. M., Chang, C. H., Chen, S. W., Teng, C. M., Tsai, I. H. (1991). The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*. *Biochim. Biophys. Acta* 1077:362–370.

Malhotra, A., Thorpe, R. S. (2000). A phylogeny of the *Trimeresurus* group of pit vipers: new evidence from a mitochondrial gene tree. *Mol. Phylog. Evol.* 16:199–211. Erratum in: *Mol. Phylogenet. Evol.* (2001), 19:164.

Murphy, R. W., Fu, J., Lathrop, A., Feltham, J. V., Kovac, V. (2002). Phylogeny of the rattlesnakes (*Crotalus* and *Sistrurus*) inferred from sequences of five mitochondrial DNA genes. In: Schuett G. W., & Höggren M. eds. *Biology of the Vipers*, Biological Sciences Press (Cooper Publishing Grp.), Indiana 69–92.

Nakashima, K., Ogawa, T., Oda, N., Hattori, M., Sakaki, Y., Kihara, H., Ohno, M. (1995). Accelerated evolution of *Trimeresurus flavoviridis* venom gland phospholipase A_2 isozymes. *Proc. Natl. Acad. Sci. USA* 90:5964–5968.

Parkinson, C. L. (1999). Molecular systematics and biogeographic history of pitviper as determined by mitochondrial and ribosomal DNA sequences. *Copeia* 1999:576–586.

Parkinson, C. L., Zamudio, K. R., Greene, H. W. (2000). Phylogeography of the pitviper clade *Agkistrodon*: historical ecology, species status, and conservation of cantils. *Mol Ecol.* 9:411–420.

Sawai, Y. (1969). Snakebites on Taiwan. *The Snake* 1:9–18.

Siddiqi, A. R., Zaidi, Z. H., Jornvall, H. (1991). Purification and characterization of a Kunitz-type trypsin inhibitor from leaf-nosed viper venom. *FEBS Lett.* 294:141–143.

Slowinski, J. B., Knight, A., Rooney, A. P. (1997). Inferring species trees from gene trees: a phylogenetic analysis of the Elapidae (Serpentes) based on the amino acid sequences of venom proteins. *Mol. Phylogenet. Evol.* 8:349–362.

Takeya, H., Nishida, S., Miyata, T., Kawada, S., Saisaka, Y., Morita, T., Iwanaga, S. (1992). Coagulation factor X activating enzyme from Russell's viper venom (RVV-X). A novel metalloproteinase with disintegrin (platelet aggregation inhibitor)-like and C-type lectin-like domains. *J. Biol. Chem.* 267:14109–14117.

Tani, A., Ogawa, T., Nose, T., Nikandrov, N. N., Deshimaru, M., Chijiwa, T., Chang, C. C., Fukumaki, Y., Ohno, M. (2002). Characterization, primary structure and molecular evolution of anticoagulant protein from *Agkistrodon actus* venom. *Toxicon* 40:803–813.

Teng, C. M., Ko, F. N., Tsai, I. H., Hung, M. L., Huang, T. F. (1993). Trimucytin: A collagen-like aggregaton inducer isolated from *Trimeresurus mucrosquamatus* snake venom. *Thromb. Haemost.* 69:286–292.

Tokunaga, F., Nagasawa, K., Tamura, S., Miyata, T., Iwanaga, S., Kisiel, W. (1988). The factor V-activating enzyme (RVV-V) from Russell's viper venom, identification of isoproteins and their complete amino acid sequences. *J. Biol. Chem.* 263:17471–17481.

Tsai, I. H., Wang, Y. M., Lee, Y. H. (1994). Characterization of a cDNA encoding the precursor protein of platelet aggregation inhibitor and metalloprotease of *T. mucrosquamatus* venom. *Biochim. Biophys. Acta* 1200:337–340.

Tsai, I. H., Lu, P. J., Wang, Y. M., Ho, C. L., Liaw, L. L. (1995). Molecular cloning and characterization of a neurotoxic phospholipase A_2 from the venom of Taiwan habu (*T. mucrosquamatus*). *Biochem. J.* 311:895–900.

Tsai, I. H., Lu, P. J., Su, Y. C. (1996). Two types of Russell's viper revealed by variation in phospholipases A2 from venom of the sub-species. *Toxicon* 34:99–109.

Tsai, I. H. (1997). Phospholipases A2 from Asian snake venom. *J. Toxicol. -Toxin Rev.* 16:79–113.

Tsai, I. H., Wang, Y. M., Chiang, T. Y., Chen, Y. L., Huang, R. J. (2000a). Purification, cloning and sequence analyses for pro-metalloprotease-disintegrin varients from *Deinagistrodon acutus* venom and subclassification of the small venom metalloproteases. *Eur. J. Biochem.* 267:1359–1367.

Tsai, I. H., Wang, Y. M., Au, L. C., Ko, T. P., Chen, Y. H., Chu, Y. F. (2000b). Phospholipases A_2 from *Callosellasma rhodostoma* venom gland: cloning and sequencing of ten of the cDNAs, three-dimensional-modelling and chemical modification of the major isozyme. *Eur. J. Biochem.* 267:6684–6691.

Tsai, I. H., Chen, Y. H., Wang, Y. M., Tu, M. C., Tu, A. T. (2001a). Purification, sequencing and phylogenetic analyses of novel Lys-49 phospholipases A_2 from the venoms of rattlesnakes and other pit vipers. *Arch. Biochem. Biophys.* 394:236–244.

Tsai, I. H., Chen, Y. H., Wang, Y. M., Liau, M. Y., Lu, P. J. (2001b). Differential expression and geographic variation of the venom phospholipases A_2 of *Calloselasma rhodostoma* and *Trimeresurus mucrosquamatus*. *Arch. Biochem. Biophys.* 387:257–264.

Tsai, I. H., Wang, Y. M., Chen, Y. H., Tu, A. T. (2003). Geographic variations, cloning and functional analyses of the venom acidic phospholipases A_2 of *Crotalus viridis viridis*. *Arch. Biochem. Biophys.* 411:289–296.

Tsai, I. H., Wang, Y. M., Chen, Y. H., Tsai, T. S., Tu, M. C. (2004). Venom phospholipases A_2 of bamboo viper (*Trimeresurus stejnegeri*): molecular characterization, geographic variations and evidence of multiple ancestries. *Biochem. J.* 377:215–223.

Tu, M. C., Wang, H. Y., Tsai, M. P., Toda, M., Lee, W. J., Zhang, F. J., Ota, H. (2000). Phylogeny, taxonomy, and biogeography of the oriental pitvipers of the genus *Trimeresurus* (Reptilia: Viperidae: Crotalinae): a molecular perspective. *Zool. Sci.* 17:1147–1157.

Wang, Y. M., Lu, P. J., Ho, C. L., Tsai, I. H. (1992). Characterization and molecular cloning of neurotoxic phospholipases A_2 from Taiwan viper *(Vipera russelli formosensis). Eur. J. Biochem.* 209:635–641.

Wang, Y. M., Tsai, I. H. (1994). Cloning and sequencing of an acidic phospholipase A_2 from Taiwan habu *(T. mucrosquamatus)* venom. *J. of Chinese Biochem Soc. (R.O.C.)* 23:53–58.

Wang, Y. M., Wang, J. H., Pan, F. M., Tsai, I. H. (1996a). Lys-49 phospholipase A_2 homologs from venoms of *Deinagkistrodon acutus* and *T. mucrosquamatus* have identical protein sequence. *Toxicon* 34:485–489.

Wang, Y. M., Wang, J. H., Tsai, I. H. (1996b). Molecular cloning and deduced primary structures of the acidic and basic phospholipases A_2 from the venom of *Deinagkistrodon acutus*. *Toxicon* 34:1191–1196.

Wang, Y. M., Liew, Y. F., Chang, K. Y., Tsai, I. H. (1999). Purification and characterization of the venom phospholipases A_2 from four monotypic Crotalinae snakes. *J. Nat. Toxin* 8:331–340.

Wang, Y. M., Wang, S. R., Tsai, I. H. (2001). Serine protease isoforms of *Deinagkistrodon acutus* venom: cloning, sequencing and phylogenetic analysis. *Biochem. J.* 354:161–168.

Warrell, D. A. (1997). Geographic and intraspecies variation in the clinical manifestations of envenoming by snakes. In: Thorpe R. S., Wuster W., and Malhotra A. eds. *Venomous Snakes*, Clarendon Press, Oxford. 189–204.

Wüster, W., Salomão, M. G., Quijada-Mascareas, J. A., Thorpe, R. S. (2002). Origin and evolution of the South American pitviper fauna: evidence from mitochondrial DNA sequence analysis. In: Schuett G. W. ed. *Biology of the Vipers*, Biological Sciences Press (Cooper Publishing), Indiana 111–128.

Yamazaki, Y., Hyodo, F., Morita, T. (2003). Wide distribution of cysteine-rich secretory proteins in snake venoms: isolation and cloning of novel snake venom cysteine-rich secretory proteins. *Arch. Biochem. Biophys.* 412:133–141.