Venom phospholipases A_2 of bamboo viper (*Trimeresurus stejnegeri*): molecular characterization, geographic variations and evidence of multiple ancestries

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Phospholipases A_2 (PLA_2s) were purified from the *Trimeresurus stejnegeri* venom obtained from various localities in Taiwan and three provinces in China, by gel filtration followed by reversed-phase HPLC. The precise molecular mass and N-terminal sequence of each PLA_2 were determined. In addition to the six previously documented PLA_2 isoforms of this species, we identified ten novel isoforms. The venom gland cDNAs of individual specimens of the viper from four localities were used for PCR and subsequent cloning of the PLA_2s. The molecular masses and partial sequences of most of the purified PLA_2s matched with those deduced from a total of 13 distinct cDNA sequences of these clones. Besides the commonly known Asp49 or Lys-49 PLA_2s of crotalid venoms, a novel type of PLA_2 with Asn-49 substitution at the Ca^{2+}-binding site was discovered. This type of PLA_2 is non-catalytic, but may cause local oedema and appears to be a venom marker of many tree vipers. In particular, we showed that *T. stejnegeri* displayed high geographic variations of the PLA_2s within and between their Taiwanese and Chinese populations, which can be explained by geological isolation and prey ecology. A phylogenetic tree of the acidic venom PLA_2s of this species and other related Asian vipers reveals that *T. stejnegeri* contains venom genes related to those from several sympatric pit vipers, including the genera *Tropedolaemus* and *Gloydius* besides the *Trimeresurus* itself. Taken together, these findings may explain the exceptionally high variations in the venom as well as the evolutionary advantage of this species.

Key words: Asn-49 variant, bamboo viper (*Trimeresurus stejnegeri*), geographic variation, phospholipase A_2, phylogeny tree, venom.

INTRODUCTION

Bamboo viper *Trimeresurus stejnegeri* is widely distributed in south China, Taiwan and northern areas of southeastern Asia. It is the most common cause of snakebites in Taiwan and North Vietnam. Although studies on its venom components have been continued for many years, recent studies revealed significant geographic variations in the morphologies [1], diets [2], mitochondrial DNA [3,4] and the venom components of this species [5,6]. According to nested clade analyses, two or three lineages of Taiwanese *T. stejnegeri* (formerly named *Trimeresurus gramineus*) are existent [5,6], but phylogeny does not appear to be the principal causal factor of its venom variations [6]. It was not clear whether or not the variations detected in the venom were the result of differential gene expression.

Combining protein chemistry and functional genomics in a previously tested ecological framework [6], the present study aims to elucidate the venom evolution of *T. stejnegeri* using phospholipases A_2 (PLA_2s; EC 3.1.1.4) as a reference. The 14 kDa PLA_2s are common components in pit-viper venoms. They usually display multiple isoforms with various functional specificities, including neurotoxic, myotoxic, oedema-inducing, anti-platelet or anti-coagulating activities [7]. Five PLA_2s were previously purified from the pooled venom of Taiwanese *T. stejnegeri* and their full amino acid sequences were determined [8–10]. They are apparently different from the five PLA_2s purified from a pooled venom sample of Chinese *T. stejnegeri* [11].

A recent survey by mass analyses of the PLA_2s in 104 geographic venom samples of Taiwanese *T. stejnegeri* revealed that although an individual sample contains only 1–4 PLA_2s, a total of 22 distinct PLA_2 isoforms were detected for all the localities [6]. The structures and functions of these isoforms were not studied. The high diversity was rather exceptional, since other pooled viper venoms usually express less than six PLA_2 isoforms [12,13]. In the present study, we purified the PLA_2s from individual juvenile and adult *T. stejnegeri* venoms from various localities in Taiwan, and pooled venoms from three provinces in China. The enzymic and pharmacological activities of the PLA_2s were characterized. The cDNAs encoding 13 novel PLA_2s of this venom species were also cloned and fully sequenced. The results help to understand better the structure–activity relationships and specificities of the PLA_2s. Moreover, phylogeny relationships between the acidic PLA_2s in the venoms of *T. stejnegeri* and several Asian pit vipers were analysed in an effort to trace the origin of the venom proteins.

EXPERIMENTAL

Snakes and venoms

Individual *T. stejnegeri* venoms were collected from more than 12 regions in Taiwan. Pooled venoms of *T. stejnegeri* were also obtained from southern Fu-Jian, Zeh-Jiang and southern An-Huei in China. Venom powders of *Trimeresurus albolabris* and

Abbreviations used: CTs, Chinese *T. stejnegeri*; PLA_2, phospholipase A_2; PRP, platelet-rich plasma; Ts, Taiwanese *T. stejnegeri*; for brevity we have used single-letter codes for amino acids, e.g. D49 stands for Asp-49.

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The nucleotide sequence data for *T. stejnegeri* venom PLA_2s will appear in EMBL Nucleotide Sequence Database under the accession numbers AY211932–AY211944.

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Table 1 Primers used and the *T. stejnegeri* PLA_2 clones obtained in PCR

UTR, untranslated region.

Primer no.	Nucleotide sequence	Design rationale	PLA_2 clone obtained when used with primer 1 in PCR
1	GCCTGCAGRACTTAGGCA	Stop ~ 3′-UTR, antisense	None
2	TCTGGATTSAGGAGGATGAGG	5′-UTR, sense	Ts-K49a, Ts-R6, Ts-A3, Ts-A4 & Ts-A6, CTs-K49a, CTs-R6, CTs-A6
3	AGYCTNATNCARTTYGARAC	Residues 1–7, sense	Ts-A3, CTs-A3
4	AGYGTNATHGARTTNGGNAA	Residues 1–7, sense	CTs-K49b
5	ATGAAAGTGGCGGGGAGA	Residues 10–15, sense	Ts-A1 and Ts-A5
6	GGGAGGATGATHAARGARGA	Residues 6–12, sense	Ts-G6D49

Trimeresurus popeorum were purchased from Sigma (St. Louis, MO, U.S.A.). To obtain the venom glands, five adult *T. stejnegeri* were collected from four localities (Ilan, Sandemen and Manzhou in Taiwan and southern Fu-Jian in China), and a specimen of *Tropedolaemus wagleri* was purchased from Bali, Indonesia.

Cloning and sequencing of PLA_2

Venom of each specimen of *T. stejnegeri* was extracted 3 days before killing, and the fresh venom glands were dissected for RNA extraction. The cDNAs for the mRNA were subsequently synthesized [13]. Specific primers of 18–21 oligonucleotides were designed based on the highly conserved 5′- and 3′-untranslated regions [13,14] or specific N-terminal sequences of the purified PLA_2 (Table 1). PCR was conducted using Super Taq DNA polymerase to amplify the cDNAs encoding PLA_2s [15]. A 0.4 kb fragment was specifically amplified, as shown by 1 % agarose gel electrophoresis. After being treated with polynucleotide kinase, the amplified DNA fragment was first inserted into the pGEM-T easy (Promega, Madison, WI, U.S.A.) and then transformed into the *Escherichia coli* strain JM 109. White transformants were screened and cDNA clones were selected. Both cDNA strands were sequenced by the dideoxynucleotide method [16].

Purification and characterization of venom PLA_2s

Venoms of *T. stejnegeri* (5–15 mg) were dissolved in up to 0.2 ml of reagent-grade water. After repeated centrifugations at 20000 ***g*** for 5 min, aliquots of 100 μl were injected into a gel-filtration column (Superdex75, HR10/30) on an FPLC system (Pharmacia). The column was pre-equilibrated and eluted at 1.0 ml/min with 0.1 M ammonium acetate (pH 6.2) at room temperature. Fractions containing PLA_2 activities were separately collected and freeze-dried. They were further purified by reversed-phase HPLC with a Vydac C_{18} column (4.5 mm × 250 mm) [13].

Purified PLA_2s were dried in a vacuum-centrifuge device (Labconco, U.S.A.). Their molecular masses were determined by electrospray ionization–MS on a mass spectrometer (API100, PerkinElmer). Protein sequences were determined by a gas-phase amino acid sequencer coupled with a phenylthiohydantoin amino acid analyser (model 120A; Applied Biosystems, Foster City, CA, U.S.A.).

Enzymic activities and other functional assays

Concentration of PLA_2 was determined by reading the absorbance at 280 nm and assuming a molar absorption coefficient of 1.5 at 1.0 mg/ml of the protein. The hydrolytic activities of PLA_2s towards mixed micelles of L-dipalmitoyl phosphatidylcholine (99 %, Avanti polar lipid, U.S.A.) and deoxycholate or Triton X-100 (Sigma) were assayed at pH 7.4 and 37 °C on a pH-stat apparatus (Radiometer, Denmark) [13].

For oedema test, a Wistar rat (approx. 200 g body mass) was anaesthetized by injection of sodium pentobarbital. The rat was then injected with a sterile solution of PLA_2 in saline buffer on the hind-paw. Local swelling of the paw was monitored by a plethysmometer (type 7150, Ugo Basile) [14]. For platelet aggregation experiments, the anti-platelet activity of PLA_2s was assayed with PRP (platelet-rich plasma) prepared from a healthy human donor. The aggregation was initiated by the addition of 10 μM ADP and measured by an aggregometer (Payton, module 600B, Canada) at 37 °C [13].

The effect of venom PLA_2s on blood coagulation was studied by APTT (activated partial thromboplastin time) with a Hemostasis Analyzer (model KC1Δ, Sigma Diagnostics, U.S.A.). The myotoxicities on mouse thigh and the direct haemolytic activities towards human erythrocytes of basic PLA_2s were studied as described previously [17].

Phylogenetic analysis of the acidic PLA_2s of *T. stejnegeri* venom

The sequences closely related to each of the acidic *T. stejnegeri* PLA_2 isoforms were selected by BlastP search (NCBI). Our unpublished sequences of the acidic PLA_2s from the venoms of several Asian pit vipers (*T. wagleri*, *T. albolabris* and *T. popeorum*) were also included in the dataset. Amino acid-sequence alignment was made using PILEUP program. Cladograms were constructed based on these sequences by neighbour-joining algorithm using PHYLIP program [18]; degree of confidence for the internal linage was determined by bootstrap methods [19].

RESULTS AND DISCUSSION

Purification and characterization of PLA_2s

The monomeric and dimeric PLA_2s in *T. stejnegeri* venom were separated by gel-filtration column (Figure 1, fractions 1 and 2 respectively). From the *T. stejnegeri* samples of western Taiwan, an abnormally late peak (Figure 1, fraction 3) containing G6D49-PLA_2 was eluted. All PLA_2s were further purified by HPLC, and some examples are shown in Figure 2. The PLA_2s were eluted during HPLC in the following order: basic R6-PLA_2, K49-PLA_2, weakly basic G6D49-PLA_2 and the acidic PLA_2s. Content of each PLA_2 in the crude venom (%, w/w) was estimated from the UV absorbance peak both during gel filtration and HPLC. Total content of PLA_2s in each sample was found to vary between 1 and 24 % (w/w), depending on the age of snake and locality (see Table 4).

After determining the N-terminal sequence and the molecular mass, each of the venom PLA_2s was annotated based on the abbreviated species name (i.e. Ts for Taiwanese *T. stejnegeri* and CTs for Chinese *T. stejnegeri*) and its apparent structural subtype (e.g. A for acidic PLA_2) [7]. We successfully identified a total of ten Ts-PLA_2s and seven CTs-PLA_2s (Tables 2 and 3), including Ts-K49a, A1, A2 and A5, which have been previously isolated from

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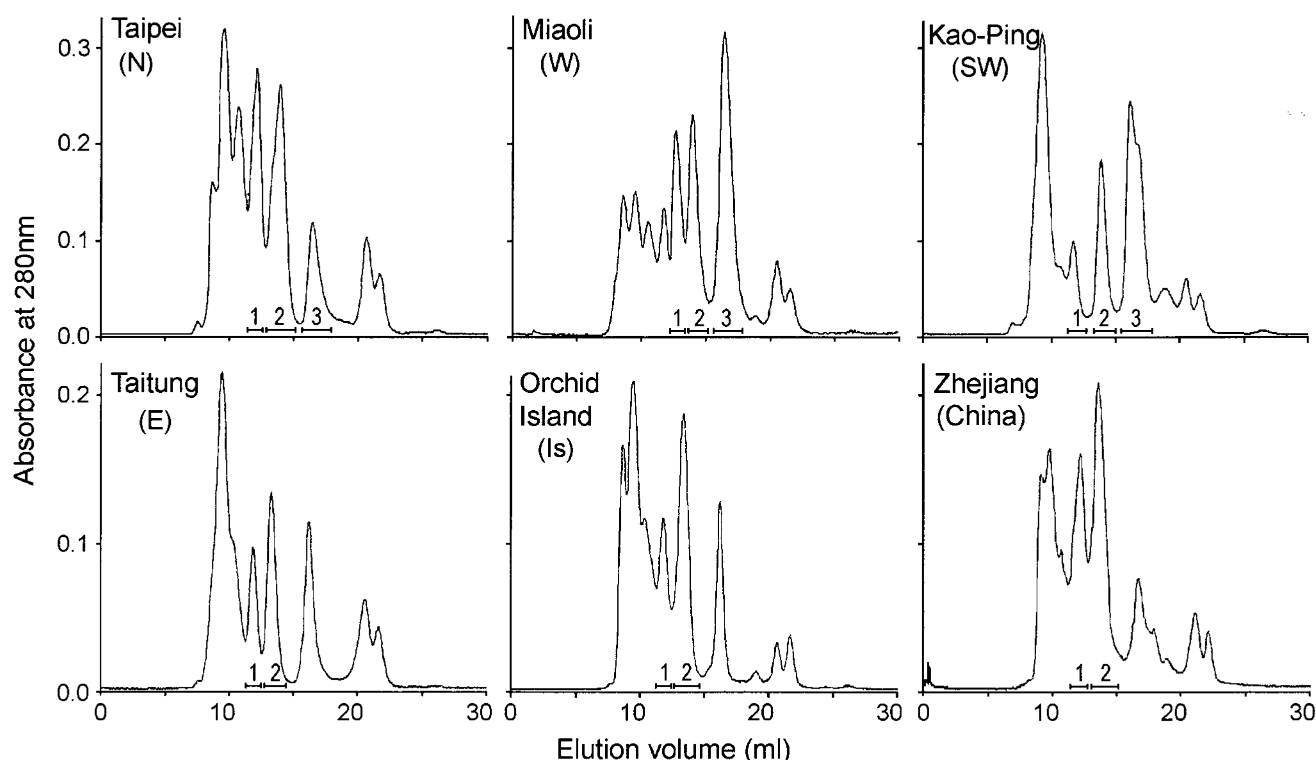

Figure 1 Gel filtration of the venom samples of *T. stejnegeri*

Elution profiles for the *T. stejnegeri* venoms from six representative localities are shown, and the diet subset (in parentheses) is indicated below each locality. The dissolved venom was eluted at a flow rate of 1.0 ml/min at room temperature (25 °C) on an FPLC system with a Superdex G75 (HR 10/30) column in equilibration with 0.1 M ammonium acetate (pH 6.2). PLA_2-containing fractions (indicated by bars) were pooled for further purification.

the pooled venom [8–10]. From a south Taiwanese *T. stejnegeri*, we also purified Tgr-PL-VI, which was previously cloned, but not isolated. However, we have not found Tgr-PL-IV (theoretical mass 13.778 kDa) [8] that differs from Ts-A1 by only one amino acid residue.

A recent report has identified a total of 22 distinct molecular masses of venom PLA_2s in 104 Taiwanese *T. stejnegeri* samples from 38 localities [6]. This is more than twice the number that we identified (Table 2). Overestimation of the number of venom PLA_2s resulted, probably, from the limitation of MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry) used in the previous survey (mass precision of approx. 0.06 %) [6], and also from possible post-translational modifications of the proteins, such as oxidation of methionine and de-amidation of glutamine and asparagine residues.

Cloning and sequence determination

About 160 PLA_2 clones were obtained from the cDNAs prepared individually from the venom glands of four *T. stejnegeri* specimens and sequenced. After comparing the cDNA sequences, we identified nine distinct clones encoding Ts-PLA_2 variants and six distinct clones encoding CTs-PLA_2 variants (Table 1). We failed to clone CTs-K49c, CTs-G6 and CTs-A1 from the venom glands of Fu-Jian specimen, although the three PLA_2s were purified from the venoms of Zhe-Jiang or An-Hui. Each specimen apparently expressed several, but not all, of the venom PLA_2s. Notably, the catalytic activity and amino acid sequence 1–32 of CTs-A1 are very similar to those of CTs-A3. A few additional PLA_2 cDNA sequences (not shown) obtained probably encode pseudogenes [13].

The complete amino acid sequences of the acidic and basic PLA_2 deduced were aligned respectively in Figures 3(A) and 3(B) according to a commonly used numbering system [20]. Assuming all the conserved cysteine residues in the PLA_2s form disulphide bonds, the mass and pI value of each PLA_2 were calculated from the cDNA predicted protein sequence, and then matched one-to-one with the sequences of the purified PLA_2s (Tables 2 and 3), whose masses were successfully confirmed with electrospray ionization–MS.

Catalytic activities and functions of the PLA_2s

The *in vitro* enzymic activities of these PLA_2s towards zwitterionic and cationic micellar substrates were determined (Table 5). As expected, the catalytic activities of all the R6- and K49-PLA_2s of this venom species were hardly detectable (not shown), owing to the lack of D49 at their catalytic sites essential for Ca^{2+} binding [21]. Ts-A5 differs from Ts-A1 by a substitution N1H, which causes a significant reduction of its enzymic activity especially when assayed with the mixed micellar substrate, containing Triton X-100. Notably, Ts-G6D49 is a potent enzyme and its specificity towards the micelles containing Triton X-100 is relatively high.

Previously, the acidic venom PLA_2 of *T. stejnegeri* (Jian-Xi, China) was reported to be a platelet aggregation inhibitor [22]. On the other hand, Ts-A2, but not Ts-A1 or A5, was found to induce contracture of guinea-pig ileum [8,9]. Figure 4 shows the anti-platelet activities of the purified acidic PLA_2s using human PRP; most of the PLA_2s show moderate anti-platelet activities. Although differing in a single substitution (A40P), Ts-A3 and CTs-A3 exhibit similar enzymic and anti-platelet activities. The enzymic activities of venom acidic PLA_2s have been shown to be

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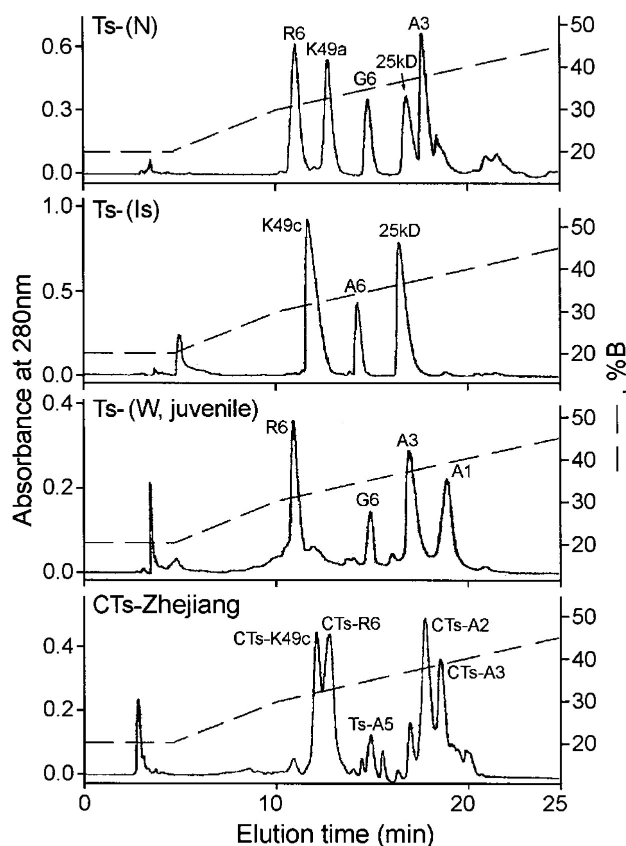

Figure 2 Purification of the venom PLA_2s by reversed-phase HPLC

After gel filtration and freeze-drying, the redissolved PLA_2-containing fractions were purified on a Vydac C_{18} HPLC column with a gradient of B solvent (acetonitrile). Four representative examples of the elution profiles are shown. The PLA_2 peaks were analysed by ESI–MS and N-terminal sequencing and annotated accordingly.

Table 2 Molecular data and N-terminal sequences of all the PLA_2s found in Taiwanese *T. stejnegeri* venoms

Residues at site 6 are shown in bold.

PLA_2	Previous name*	Mass	pI	N-terminal sequences
Ts-R6		13 689	9.4	HLLQL**R**KMIKKMTNKEPILSYGK
Ts-K49a	Tgr-PLA-V	13 892	9.5	SVIEL**G**KMIFQETGKNPATSYGL
	Tgr-PLA-VI	13 917	9.6	GVIEL**T**KMIVQEMGKNALTSYSL
Ts-K49b		13 931	9.4	GVIEL**T**KMFVQEMGKNALTSYSL
Ts-K49c		13 876	9.5	SVIEL**G**KMIFQETGKNPATSYGL
Ts-G6D49		13 805	7.8	SLLEF**G**RMIKEETGKNPLSSYFS
Ts-A1	Tgr-PLA-I	13 734	4.8	HLMQF**E**TLIMKVAGRSGVWYYGS
Ts-A2	Tgr-PLA-II	13 779	5.0	NLLQF**E**NMIRNVAGRSGIWWYSD
Ts-A3		13 750	5.3	SLIQF**E**TLIMKVAKKSGMFSYSA
Ts-A4		13 905	5.5	HLLQF**E**TMIIKMTKQTGLFSYSF
Ts-A5	Tgr-PLA-III	13 711	4.7	NLMQF**E**TLIMKVAGRSGVWYYGS
Ts-A6	Tgr-PLA-VII	13 939	4.7	HLMQF**E**NMIKKVTGRSGIWWYGS

* See [8–10].

essential, but not simply proportional to their anti-platelet effects [23]. Each acidic PLA_2 may have evolved with distinct specificity towards platelets of different species [12]. To understand the specificities of each acidic PLA_2, the platelets of various potential preys of the species should be included in the study.

The hydrolytic activities of all the K49- or N49-PLA_2s of *T. stejnegeri* venoms were too low to be determined (results not shown). However, local oedema was prominent within few hours after injection of 5–10 μg of these basic PLA_2 homologues/paw (Figure 5). The oedematous potency of Ts-K49c was higher than that of Ts-K49a, whereas the potencies of Ts-R6 and CTs-R6 ranked between both the Ts-K49 isoforms. We also tested other functions of these basic PLA_2 homologues. The anti-coagulating and myotoxic effects of the K49-PLA_2s were approx. 4–8-fold lower than those of trimucrotoxin (a myo-/neuro-toxin from *Protobothrops mucrosquamatus* [24]) and the anti-coagulating R6-PLA_2 from *Gloydius* [25] (results not shown). Inhibition of platelet aggregation and direct haemolysis of washed human erythrocytes by Ts-R6 and CTs-R6 were found to be very weak.

The active enzyme Ts-G6D49-PLA_2 induced fast and sustaining local oedema (Figure 5), and prolonged the coagulation time of human plasma (results not shown). A similar type of D49-PLA_2 with myotoxicity has been found in *Bothrops* venom, and its three-dimensional structure has recently been solved [26].

Table 3 Molecular data and *N*-terminal sequences of all the PLA_2s found in Chinese *T. stejnegeri* venoms

Ts-R6, Ts-A5 and Ts-A6 in this list were identical with those found in the Taiwanese venoms. The pI values were predicted from cDNA deduced protein sequences. Residues at site 6 are shown in bold.

PLA_2	Mass	pI	N-terminal sequences
Ts-R6	13 689	9.4	HLLQL**R**KMIKKMTNKEPILSYGK
CTs-R6	13 576	9.4	SLLQL**R**KMIKKMTNKEPILSYSK
CTs-K49a	13 817	9.5	SLVQL**G**KMIFQETGKNPATSYGL
CTs-K49b	13 771	9.4	SVIEL**G**KMIFQETGKNPATSYGL
CTs-K49c	13 512	–	SVIEL**G**KMILQETGKNPVTHYGA
CTs-G6	13 758	–	NLVQL**G**KMIFQETGKNPATSYGL
CTs-A1	13 774	–	SLIQF**E**TLIMKVAGQSGMFSYSA
CTs-A2	13 675	4.9	NLMQF**E**LLIMKVAGRSGIVWYSD
CTs-A3	13 776	5.3	SLIQF**E**TLIMKVAKKSGMFSYSA
Ts-A5	13 711	4.7	NLMQF**E**TLIMKVAGRSGVWYYGS
Ts-A6	13 939	4.7	HLMQF**E**NMIKKVTGRSGIWWYGS

Structure–function relationships of the PLA_2s

It is known that the venom acidic PLA_2s from pitvipers *Gloydius* [27] and *Calloselasma* [12] are potent platelet inhibitors. It has been shown that the residues E6, D114 or D115, W20, W21, Y113 and W119 of the PLA_2s form a surface site to which platelets may possibly bind [27]. The low or moderate anti-platelet effects of Ts-A1, Ts-A5 and CTs-A2 (Figure 4) may be owing to the lack of W20, W21 or D114 (Figure 3A). The relationships between membrane-disturbing effect and the basic residues of K49-PLA_2s have been studied previously [28]. The lysine residues at positions 116–119, 123, 127, 128, and the RRKPK sequences at positions 34–38 are, probably, responsible for the heparin-binding capability, oedematous effect (Figure 4) and local myonecrosis of these basic and non-catalytic PLA_2 homologues [14,28]. Among the six isoforms of *T. stejnegeri* K49-PLA_2s identified, the amino acid sequences are at least 89 % identical (Figure 3B), but only Ts-K49a and Ts-K49c contain all the 14 cysteine residues of group II PLA_2s and are expressed abundantly in the venom.

Notably, unusual substitutions of C91H in CTs-K49a, CTs-K49b and Ts-K49b, as well as C105R in Tgr-PL-VI, apparently result in the low expression level or instability of these mutants (Table 4). Ts-K49b (cloned in the present study) differed from Tgr-PL-VI (cloned previously [29]) by only eight amino acid substitutions. It is known that seven disulphide bonds are conserved in all the viperid venom PLA_2s except the disulphide C91-C62 being absent in the venom PLA_2s from *Bitis* (a Viperinae

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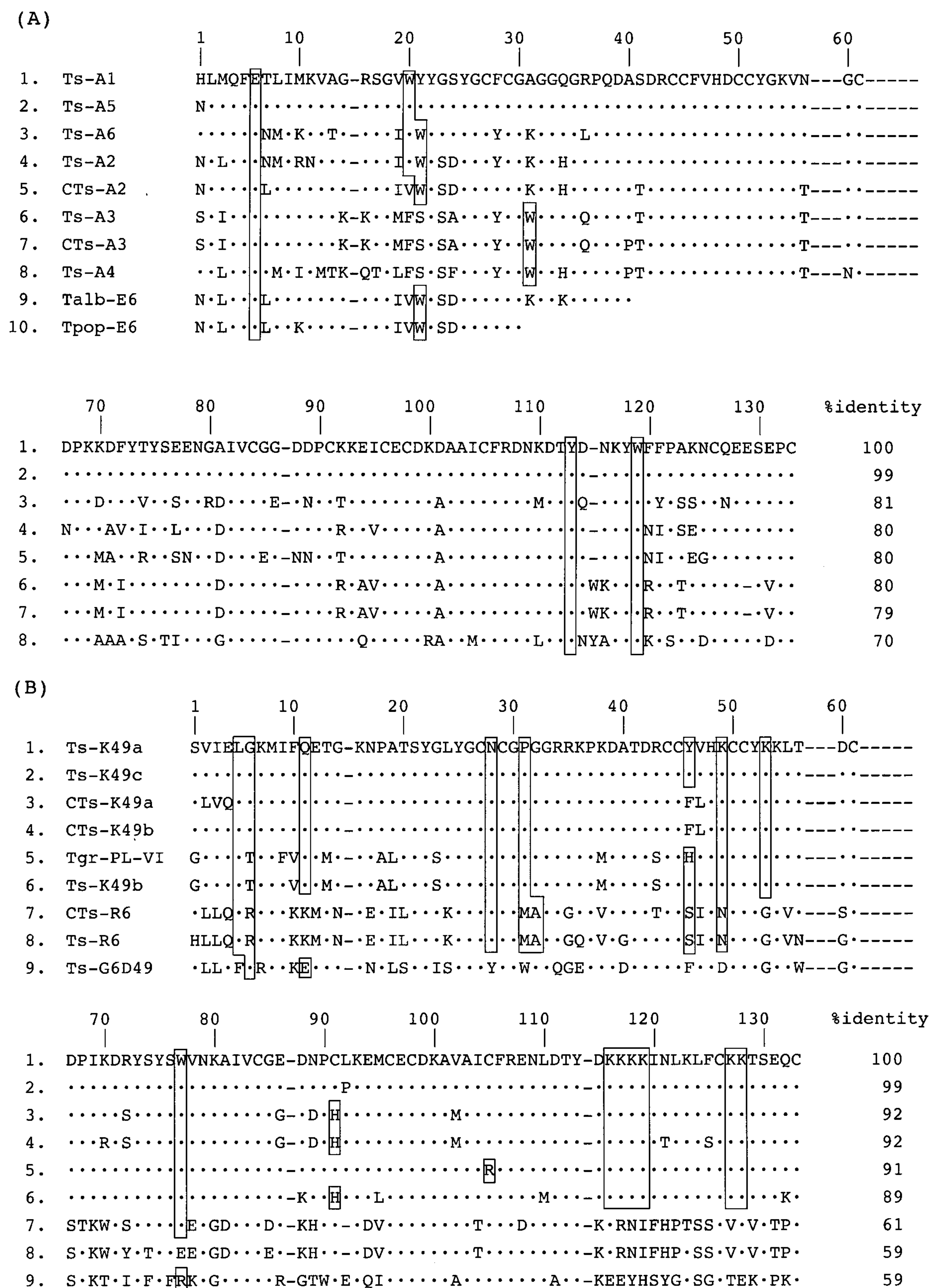

Figure 3 Multiple sequence alignments of the acidic (A) and basic (B) PLA_2s of *T. stejnegeri* venom

Single-letter codes for amino acids are used, and the numbering system follows that of Renetseder et al. [20]. The residues identical with those in the top line are denoted with dots, and the gaps are marked with hyphens. Special or function-related substitutions are boxed. The partial sequences of the acidic PLA_2s from *T. albolabris* (Talb-E6) and *T. popeorum* (Tpop-E6) venoms are also listed in (**A**).

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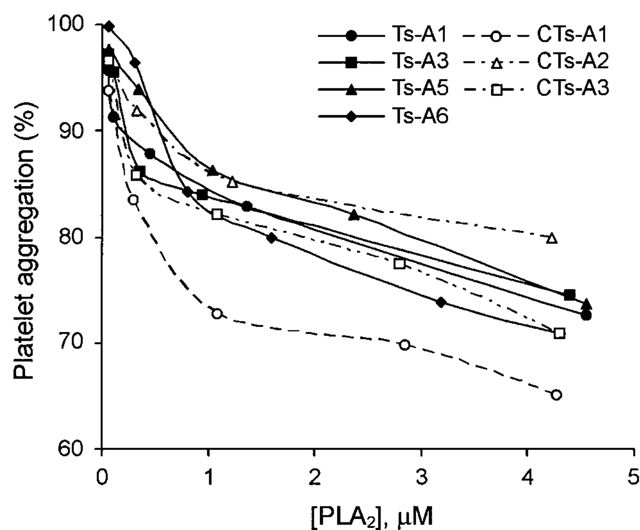

Figure 4 Inhibition of platelet aggregation by the acidic PLA_2s

ADP-induced aggregation of human platelets was studied after incubation of the acidic PLA_2 with the freshly prepared PRP for 5 min at 37 °C. Dose-dependent inhibition of the aggregation was calculated by a comparison with the control (without adding PLA_2). Data points are the averages of 2–3 independent experiments.

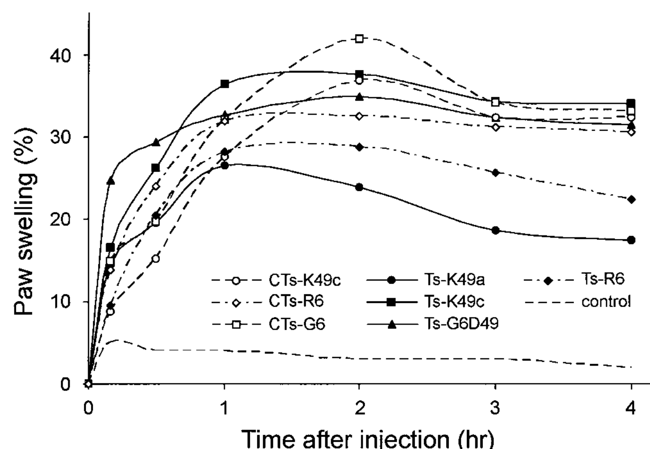

Figure 5 Oedema-inducing activities of the basic PLA_2s on rat paw

Time course of the swelling on the hind-paw was followed after injection of 10 μg of the venom protein dissolved in PBS. Volume of the paw was measured, and % swelling relative to original volume was the average from at least two independent experiments.

genus) [30]. The absence of C91-C62 may be a character of primitive type of the venom group II PLA_2s. It remains to be investigated whether the missing C91-C62 is common in the venom K49-PLA_2s of other arboreal vipers of the genus *Trimeresurus sensu stricto* [3,4]. The disulphide bonds 50–131, 11–77 of the PLA_2 contribute more to stability and enzyme activity than disulphide bonds 61–91 and 44–105; the last two contribute to the stability by 2.3 and 3.4 kcal/mol (1 cal ≈ 4.184 J), respectively [31,32].

Ts-R6 and CTs-R6 are apparently novel types of group II PLA_2 lacking a Ca^{2+}-binding site. Their relatively low molecular masses could be attributed to a distinct gap at position 92 and the presence of many glycine, serine and threonine substitutions. As compared with the K49-PLA_2s, both R6-PLA_2s contain less basic residues at positions 115–128 but more basic residues at the N-terminal regions (e.g. positions 6, 7, 10, 11 and 24), and have several acidic substitutions at positions 71–86 that may confer specific pharmacological properties [21]. Since a similar type of R6-PLA_2 was found in the venom of other arboreal *Trimeresurus* (e.g. *T. albolabris* and *T. popeorum*; results not shown), the pharmacological effects of the PLA_2s towards the major prey, frogs, are currently being investigated.

Table 4 Geographic variations of the *T. stejnegeri* venom PLA_2s

Protein content of each PLA_2 in crude venom (%, w/w) was estimated based on relative A_{280} areas of the protein peaks in both Figures 1 and 2.

(A) Individual venoms of adult Taiwanese *T. stejnegeri*

Locality (diet subset)	Ts-PLA_2 isoform	Content (%, w/w)
Taipei (N)	R6	5–6
	K49a	4–7
	G6D49	3–4
	A3	5–8
Miaoli (W)	R6	10–12
	G6D49	5–6
	A3	6–8
Kao-Ping (SW)	R6	5–8
	K49a	2–10
	G6D49	5–9
	A3	2–4
Ilan (NE)	R6	2
	K49a	9
	A3	3
Taitung (SE)	K49a	10–15
Orchid Island (Is)	K49c	12
	A6	3

(B) Venoms of juvenile Taiwanese *T. stejnegeri*

Locality (diet subset)	Ts-PLA_2 isoform	Content (%, w/w)
Miaoli (W)	R6	8
	G6D49	3
	A3	4
	A1	4
Kao-Ping (SW)	R6	5
	G6D49	1
	A3	2
	A1	2
Orchid Island (Is)	K49c	1

(C) Pooled venoms of adult Chinese *T. stejnegeri*

Provinces	PLA_2 isoforms	Content (%, w/w)
Zhe-Jiang	CTs-K49c	7
	CTs-R6	11
	Ts-A5	1
	CTs-A2	1
	CTs-A3	4
An-Hui	Ts-R6	2
	CTs-R6	7
	CTs-G6	1
	CTs-A1	6
	CTs-A2	3
Fu-Jian	CTs-R6	0.5
	CTs-A1	0.5
	CTs-A2	4

Geographic variations of the venom PLA_2s

Subtropical Taiwan is climatically and ecologically diverse, owing to a central mountain range approx. 4000 m above sea level extending across the country from north to south. According to a

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previous survey [2], the content of frogs in the diet of adult Taiwanese *T. stejnegeri* could be separated into five diet subsets: (N), northern subset containing 100 % frogs; (W), western subset containing 92 % frogs; (SW), southwestern subset containing 85 % frogs; (E), eastern subset containing 78–80 % frogs; and (Is) offshore Island subset containing 47–52 % frogs. Our results of the PLA_2s variations of the Taiwanese *T. stejnegeri* were empirically grouped according to these five subsets (Tables 4A and 4B). At least 3–5 individual samples were analysed for each locality or diet subset to ensure that the results were representative. The results reveal that most of the venoms from western Taiwan contain Ts-R6, Ts-G6D49 and Ts-A3, whereas the venoms from eastern Taiwan contain Ts-K49a. Thus, the differences in the venom between the eastern and the western groups are obvious [6].

Our results in Table 4(A) are not contradicted by the findings of previous surveys of the geographic variations of *T. stejnegeri* venom. For example, of the ten samples from Luku (subset W) previously analysed [6], PLA_2s with molecular masses similar to those of Ts-R6, Ts-G6D49 and Ts-A3 were frequently found, and the molecular masses matching those of Ts-A6 and Ts-K49c were reported for the venom from two offshore populations [6]. Differing in a single substitution of L92P, the oedema-inducing activity of Ts-K49c for the rats is more prolonged and 2-fold higher than that of Ts-K49a (Figure 5). The abundant Ts-K49c in the venoms from two offshore islands would probably have evolved from Ts-K49a in the eastern populations by a founder effect [6]. The isoforms Ts-A2 and A5 were found only in a few samples collected in southwestern Taiwan in the vicinity of where the pooled *T. stejnegeri* venoms were obtained for previous studies [8–10] (C. C. Chang, personal communication).

Taiwan and China are separated by Taiwan Strait. Land bridges have connected Taiwan to the Asian continent possibly 2–3 times during the Pliocene and Pleistocene, and therefore made the exchanges of *T. stejnegeri* between Taiwan and China possible [6]. Among the eight distinct PLA_2s purified from the samples from three Chinese provinces (Table 2), two (Ts-R6 and Ts-A5) were identical with those from Taiwanese venom, but of very low venom content (Table 4C). The present-day *T. stejnegeri* on both sides of Taiwan Strait differ significantly in their venom genes, due to geological separation and a rapid venom evolution. Notably, the K49- and G6D49-PLA_2s are weakly expressed in the Chinese samples. Its ecological meaning is not clear, since the diets of Chinese *T. stejnegeri* have not been investigated.

Ontological variations of the venom PLA_2s

The venoms were collected from juvenile *T. stejnegeri* below 5 months from three locations in Taiwan. By purification and identification of the venom PLA_2s, the ontological variations of the PLA_2s are shown in Table 4(B). The venom from juvenile vipers was particularly abundant in Ts-R6 and Ts-A1, whereas Ts-A1 was rarely found in the venom of adult viper. Notably, K49-PLA_2s were rarely expressed in the venom of juvenile vipers, possibly correlated with a diet lacking rodents.

Possible ecological correlations of the venom variations

To interpret the venom variations, both the functional specificities of the venom proteins and the ecological factors for the viper need to be examined. It appears that the presence of oedematous and myotoxic K49-PLA_2s correlates with the rodent-rich diet of pit vipers. The venoms from eastern Taiwan and the offshore islands are abundant in K49-PLA_2s, the *T. stejnegeri* in these localities have been reported to consume relatively more mammals than in other localities [2]. Moreover, the higher specificity of Ts-K49c than Ts-K49a for rodents (Figure 5) is also in accordance with the diet of offshore island populations [2,6]. Abundance of K49-PLA_2s in many South American *Bothrops* venoms [14,27] is also consistent with the mammal-rich diets of the snakes [34]. On the other hand, the frog-dominant diet [2] is often associated with the lack of venom K49-PLA_2 in the juveniles of the northern and western populations of Taiwanese *T. stejnegeri* (Table 4A). Similarly, the unusual absence of K49-basic protein in the venom has been reported for the *T. flavoviridis* on Okinawa Island with a frog diet [33].

Table 5 Enzymic activities of purified Asp-49 PLA_2s of *T. stejnegeri* venom towards micellar substrates

Initial rates were measured by pH-stat method at 37 °C and pH 7.4. dPPC, dipalmitoyl L-phosphatidylcholine.

PLA_2	Specific activity (μmol · mg^{-1} · min^{-1})	
	dPPC + deoxycholate	dPPC + Triton X-100
Ts-G6D49	308 ± 8	372 ± 2
Ts-A1	406 ± 32	246 ± 3
Ts-A2	254 ± 4	81 ± 7
Ts-A3	573 ± 53	215 ± 15
Ts-A5	376 ± 28	44 ± 6
Ts-A6	178 ± 20	101 ± 5
CTs-A1	444 ± 9	147 ± 10
CTs-A2	457 ± 46	352 ± 9
CTs-A3	681 ± 17	133 ± 3

The acidic PLA_2s in the viper venoms have, in general, evolved more isoforms than the basic PLA_2s [12,13]. Whether the hydrolytic activities of acidic venom PLA_2s contribute to the digestion of preys [35] is not clear, and have been shown to be inhibitors of platelet aggregation. The presence of multiple isoforms of *T. stejnegeri* acidic PLA_2s is probably an adaptation to diverse preys with different platelet properties [13].

PLA_2 cladogram and possible ancestry of *T. stejnegeri*

Apparently, the venom PLA_2 variations may be attributed to the differential expression of a set of about nine PLA_2 isoforms in either the Taiwanese or Chinese *T. stejnegeri* (Table 5). We look deeper into this unusually high polymorphism by the phylogenetic analysis based on the sequences of venom acidic PLA_2s, which are the common venom components of most pit vipers [6,7,31,36]. The resultant tree displays a polyphyletic character for the PLA_2s from *T. stejnegeri*, and the bootstrap values are high when the PLA_2 of *Protobothrops* venom is assigned as outgroup (Figure 6). Interestingly, Ts-A1, -A5 and -A6 are linked with the acidic venom PLA_2s of *Tropedolaemus*, Ts-A2 and CTs-A2 are linked with those from other tree vipers of the same genus (*T. sensu stricto*), and Ts-A3 and CTs-A3 are linked with those of the Chinese *Gloydius* (formerly named *Agkistrodon*). All these related genera or species are viviparous, like *T. stejnegeri* [37], and are probably sympatric to *T. stejnegeri* [38].

Since *T. stejnegeri* is not at a basal position in the mitochondrial DNA phylogeny tree of pit vipers [3], and the other venom species studied so far contained only a few highly similar acidic PLA_2 isoforms [7,12,30], it seems less probable that most of the venom genes of the other pit vipers were lost during evolution, whereas those in *T. stejnegeri* were conserved. This can be best explained by the theory that *T. stejnegeri* was derived probably from interbreeding between several pit-viper species in the same or different genera, e.g. *Gloydius* (Figure 6). Merger of the

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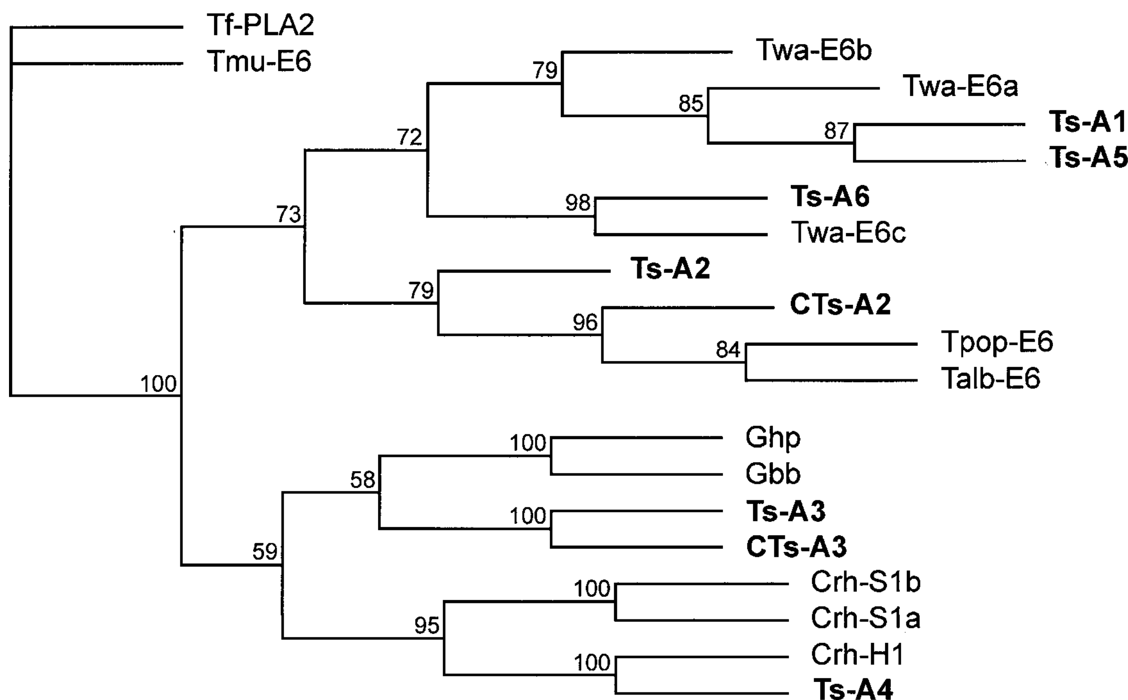

Figure 6 Phylogenetic relationships of the acidic PLA_2s from venoms of *T. stejnegeri* and selected Asian pit vipers

Data used for phylogenetic analysis include all the amino acid sequences listed in Figure 3(A) (indicated in boldface) and the complete sequences of the E6-PLA_2s of *T. wagleri* (Twg-E6a–c, results not shown), *Calloselasma rhodostoma* [12] and *Gloydius halys* [31]. Venom sources and GenBank® accession numbers are: Ghb-E6 of *Gloydius b. bravicaudus* (P14418); GhP-E6 of *G. halys Pallas* (AF015246); Crh-E6 isoforms of *C. rhodostoma* (AF104067-70); Tf-PLA2 (AB072175) of *Trimeresurus flavoviridis*; and Tmu-PL-I (X77 088) of *Trimeresurus mucrosquamatus*, which was assigned as the outgroup for making the phylogeny tree using the computer program. Values are calculated bootstrap values, indicating the confidence level of the branching.

venom genes from the ancestral species and subsequent selective expression of some of the genes through natural selection may have increased the diversity of the present-day *T. stejnegeri* venom.

The rich isoforms were found not only in the acidic PLA_2s but also in the K49-PLA_2s (Tables 2 and 3) and other venom protein families (e.g. the serine proteases; I.-H. Tsai and Y.-M. Wang, unpublished work). The interbreeding between present-day vipers of the same genus have been documented [39,40] and those between different genera have been rare [41] but may have happened by some means when the genetic gaps were not as wide as the present. The interbreeding must have occurred before the geographic separation of Taiwan from China, since the orthologous *T. stejnegeri* PLA_2s in each branch of the phylogeny tree (Figure 6) are existent in the venoms from both areas. Namely, Ts-A5 is found in the venoms of both Chinese and Taiwanese *T. stejnegeri*, CTs-A3 differs from Ts-A3 by only a substitution of A40P, and CTs-A2 is 84 % identical with Ts-A2 (Figure 3).

Conclusion

The venom proteins are heritable [6] and have been known to adapt a positive Darwinian evolution and accelerated mutations [29]. The intra-species variations of the venom proteins in *T. stejnegeri* reflect a dynamic adaptation and evolution of the venom. The present study draws five important conclusions: (1) *T. stejnegeri* is especially rich in venom genes, and the venom variations within the population of *T. stejnegeri* in Taiwan or China could be explained by differential expression of a set of genes in response to ecological and ontological conditions; (2) different sets of venom genes are present in Chinese and Taiwanese *T. stejnegeri*, suggesting a long history of separation of both populations; (3) a new type of oedematous R6N49-PLA_2s has been discovered, and it appears to be a marker protein in the venoms of Asian arboreal pit vipers; (4) some of the *T. stejnegeri* venom proteins are related to those from the same genus, but some are related more to those from other genera, suggesting possibly multiple ancestors for this species; and (5) increased venom diversity is likely to confer an adaptation advantage for the snake, as testified by the high density and wide distribution of *T. stejnegeri*.

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