

# Unusual venom phospholipases A<sub>2</sub> of two primitive tree vipers *Trimeresurus puniceus* and *Trimeresurus borneensis*

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## Keywords

phospholipase A<sub>2</sub>; phylogenetic analysis; snake venom; *Trimeresurus borneensis*; *Trimeresurus puniceus*

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## Note

Novel cDNA sequences encoding PLA<sub>2</sub>s have been submitted to EMBL Databank and are available under accession numbers: AY355171 to AY355175 for Tpu-K49a, Tpu-K49b, Tpu-G6D49, Tpu-E6a and Tpu-E6b; AY355177 to AY355179 for Tbo-K49, Tbo-E6 and Tbo-G6D49, respectively.

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For a long time, the genus *Trimeresurus* (*sensu lateral*) has been known to consist of over 40 species of Asian pit vipers. It is now divided into four recognized genera: *Trimeresurus*, *Ovophis*, *Protobothrops* and *Tropidolaemus* [1]. The arboreal *Trimeresurus* (*sensu stricto*) are indigenous to south and south-eastern Asia. It should be noted that data from morphological and mtDNA analyses suggest that *Trimeresurus* (*sensu stricto*) is possibly derived from more than one ancestral species and should be considered as a polyphyletic group [1–3]. However, their venom components have not been well studied except for those of the green

To explore the venom diversity of Asian pit vipers, we investigated the structure and function of venom phospholipase A<sub>2</sub> (PLA<sub>2</sub>) derived from two primitive tree vipers *Trimeresurus puniceus* and *Trimeresurus borneensis*. We purified six novel PLA<sub>2</sub>s from *T. puniceus* venom and another three from *T. borneensis* venom. All cDNAs encoding these PLA<sub>2</sub>s except one were cloned, and the molecular masses and N-terminal sequences of the purified enzymes closely matched those predicted from the cDNA. Three contain K49 and lack a disulfide bond at C61–C91, in contrast with the D49-containing PLA<sub>2</sub>s in both venom species. They are less thermally stable than other K49-PLA<sub>2</sub>s which contain seven disulfide bonds, as indicated by a decrease of 8.8 °C in the melting temperature measured by CD spectroscopy. The M110D mutation in one of the K49-PLA<sub>2</sub>s apparently reduced its edematous potency. A phylogenetic tree based on the amino-acid sequences of 17 K49-PLA<sub>2</sub>s from Asian pit viper venoms illustrates close relationships among the *Trimeresurus* species and intergeneric segregations. Basic D49-PLA<sub>2</sub>s with a unique Gly6 substitution were also purified from both venoms. They showed edema-inducing and anticoagulating activities. It is notable that acidic PLA<sub>2</sub>s from both venoms inhibited blood coagulation rather than platelet aggregation, and this inhibition was only partially dependent on enzyme activity. These results contribute to our understanding of the evolution of *Trimeresurus* pit vipers and the structure–function relationships between various subtypes of crotalid venom PLA<sub>2</sub>.

bamboo vipers *Trimeresurus stejnegeri* [4]. Distinguished by a yellow–brown skin color, *Trimeresurus puniceus* and *Trimeresurus borneensis* only inhabit Sumantra, Java and adjacent areas [5]. Previous phylogenetic studies suggest that both species are primitive and closely related to several *Trimeresurus* species in the Indian subcontinent [1].

Snake venoms are characteristic, with specific pharmacological activities and bioavailability, and thus have potential for medical applications. In addition, investigating the diversity of venom proteins may help us to understand snake systematics and their venom

## Abbreviations

PLA<sub>2</sub>, phospholipase A<sub>2</sub>; Tbo, *Trimeresurus borneensis*; Tpu, *Trimeresurus puniceus*; Tst, *Trimeresurus stejnegeri*; APTT, activated partial thromboplastin time.

protein evolution. Phospholipase A<sub>2</sub> (PLA<sub>2</sub>; EC 3.1.1.4) is one of the most widely studied snake venom proteins because of its abundance, small size, and structural stability. The amino-acid sequences of ≈ 290 snake venom PLA<sub>2</sub>s have been determined, and many of their 3D structures resolved [6–8]. PLA<sub>2</sub>s of pit viper venoms have evolved into several subtypes, each playing distinct functional roles such as platelet aggregation inhibitor [9], neurotoxin [10], anticoagulant [11], and myotoxin [12].

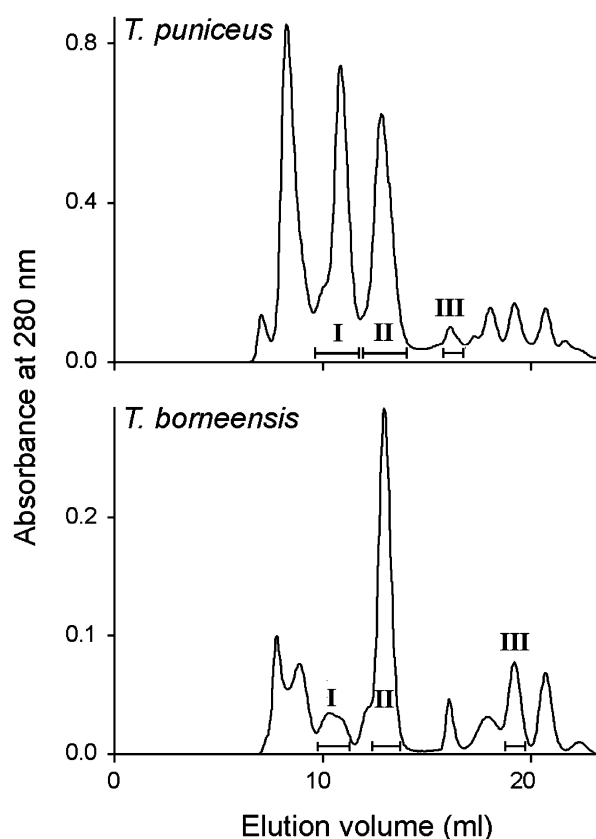
In this study, we purified the PLA<sub>2</sub>s from *T. puniceus* and *T. borneensis* venoms, cloned the cDNAs, and solved their full amino-acid sequences. The functions of these purified PLA<sub>2</sub>s and the effects of a missing disulfide bond at C61–C91 were investigated. On the basis of amino-acid sequences of orthologous venom PLA<sub>2</sub>s, we built a phylogenetic tree to study evolutionary relationships among the Asian pit vipers.

## Results

### Purification and characterization of venom PLA<sub>2</sub>

The results of gel filtration indicated that *T. puniceus* (Tpu) venom contained abundant high molecular mass components, whereas *T. borneensis* (Tbo) venom was relatively rich in smaller proteins (< 20 kDa) (Fig. 1). By RP-HPLC of fraction II (Fig. 1, corresponding to  $14 \pm 2$  kDa), we purified six PLA<sub>2</sub> isoforms from Tpu venom (Fig. 2A) and two from Tbo venom (Fig. 2B). They were designated K49-PLA<sub>2</sub>, G6D49-PLA<sub>2</sub> and E6-PLA<sub>2</sub> according to substitutions at residue 6 or 49 as previously suggested [4,13]. The PLA<sub>2</sub>s were eluted in the order K49-PLA<sub>2</sub>s, weakly basic G6D49-PLA<sub>2</sub>, and finally acidic E6D49-PLA<sub>2</sub>s. Unexpectedly, G6D49-PLA<sub>2</sub>s of both Tpu and Tbo venom were eluted in fraction III (Figs 1 and 2C), suggesting their affinity for the column. Unlike previous experience with pit viper venoms [4,14], we did not find dimeric PLA<sub>2</sub>s in this two venom species.

The protein content of each PLA<sub>2</sub> in the crude venom (% w/w) was estimated from the relative UV absorbance during the two chromatographic steps. Molecular mass and N-terminal sequence of the purified PLA<sub>2</sub>s were determined. The results are summarized along with the predicted pI values in Table 1. The total protein content of Tpu and Tbo venom PLA<sub>2</sub>s was ≈ 19% and 27%, respectively. The activities of the purified enzymes with micellar substrates are shown in Table 2. Consistent with previous reports [4,12], the catalytic activity of K49-PLA<sub>2</sub>s was hardly detectable.

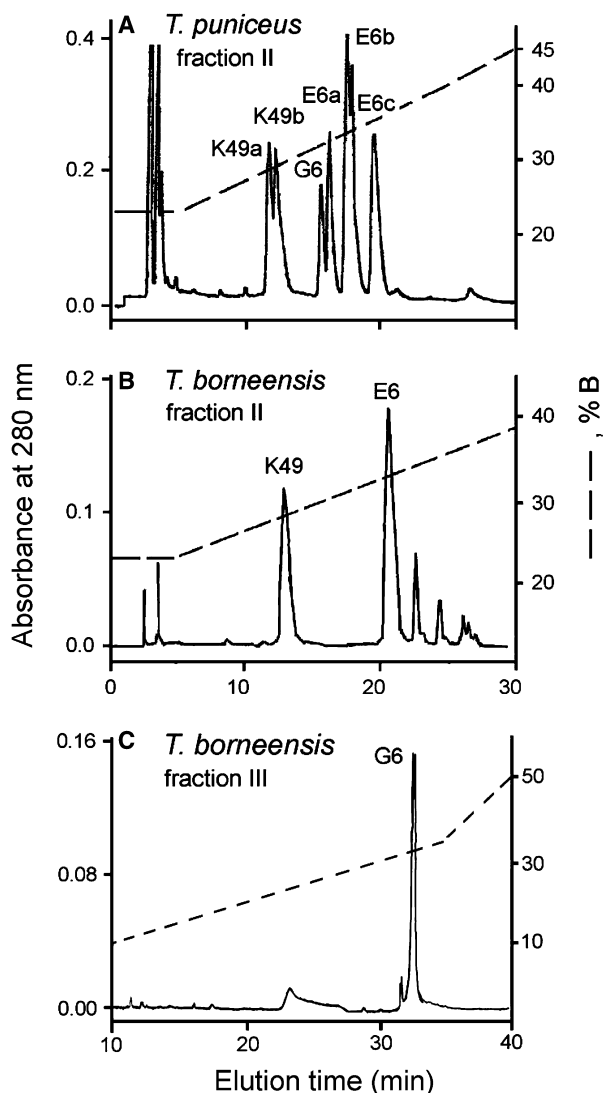


**Fig. 1.** Gel filtration of the crude venom. Dissolved venom of *T. puniceus* or *T. borneensis* was loaded on to a Superdex G75 (HR10/30) column on a FPLC system. The elution was carried out with equilibration buffer, 0.1 M ammonium acetate (pH 6.4), at a flow rate of 1.0 mL·min<sup>-1</sup>. Fractions I–III (shown by bars) were pooled separately.

### Cloning and full sequencing of the PLA<sub>2</sub>s

Cloning of venom PLA<sub>2</sub>s has been facilitated by PCR using properly designed primers and conditions [4,15]. Five and three distinct cDNAs encoding the venom PLA<sub>2</sub>s from Tpu and Tbo, respectively, were identified after more than 60 cDNA clones for each species were sequenced. All the deduced PLA<sub>2</sub> sequences consist of a signal peptide of 16 amino-acid residues followed by an enzyme domain of 122 residues. Assuming that all the conserved cysteine residues in PLA<sub>2</sub> form disulfide bonds, the mass and pI value of each cloned PLA<sub>2</sub> were calculated. Exact matches were found for eight PLA<sub>2</sub>s purified from both venoms (Table 1). In addition, complete amino-acid sequences of the basic (Fig. 3A,B) and acidic (Fig. 3C) PLA<sub>2</sub> were aligned with closely related or similar sequences, respectively.

However, we failed to clone Tpu-E6c in spite of a great number of clones selected for sequencing or



**Fig. 2.** Purification of PLA<sub>2</sub>s by RP-HPLC. Lyophilized pooled fractions II and III from gel filtration were redissolved and fractionated on a C<sub>8</sub>-Vydac HPLC column with a gradient of B solvent (dashed lines). The PLA<sub>2</sub> peaks were assessed by ESI-MS and enzyme assay. Annotations of the PLA<sub>2</sub>s are the same as those shown in Table 1.

using alternative primers based on its N-terminal residues 1–9 (AA YCTNCTNCARTTYGARATGATGAT) or residues 5–11 (TTYGARATGATGATHYTNA A). We therefore used peptide mass spectra fingerprinting to analyze the peptides derived from trypsin digestion of reduced and alkylated Tpu-E6c. By the rationale that more acidic PLA<sub>2</sub> was eluted later in the RP-HPLC, a hypothetical sequence for Tpu-E6c was deduced from the peptide mass spectra fingerprint data by assuming the presence of D70–E71, which hampers the cleavage at the K69–D70 bond in Tpu-E6c

(Table 3). The calculated mass (13 794.39 Da) of the hypothetical sequence for Tpu-E6c in Fig. 3C matched that obtained from ESI-MS of the purified protein (13 792.8 ± 4.1 Da, Table 1).

### CD and stability of K49-PLA<sub>2</sub>

The CD spectra of Tpu-K49a and Tst-K49a [4] at 27 °C were very similar (Fig. 4A). Based on computer analyses of the two spectra, the calculated contents of α-helices, β-sheets and β-turns were 34%, 18% and 22%, respectively. The molar ellipticities at 222 nm, which reflect the helical contents of the proteins, were also measured at various temperatures between 20 °C and 80 °C to evaluate the thermal stabilities. One melting temperature was observed for each protein, i.e. 54.3 °C for Tpu-K49a and 63.1 °C for Tst-K49 (Fig. 4B).

### Functional studies

Local edema was obvious on the foot a few hours after injection of the basic venom PLA<sub>2</sub>s (Fig. 5). The edematous potencies of Tpu-K49a and Tbo-K49 were similar to that of the CTs-K49c isoform [4], whereas Tpu-K49b was ≈ 50% less potent. Tpu-G6D49 was also capable of inducing fast and sustained local edema. The inhibition of ADP-induced platelet aggregation by acidic E6-PLA<sub>2</sub>s or the weak basic G6-PLA<sub>2</sub> from both venoms was also studied using platelet rich plasma prepared from human and rabbit blood. Inhibition was not large: 15–25% at ≈ 5–10 μg PLA<sub>2</sub> per ml platelet-rich plasma (data not shown).

Significantly, some of the E6-PLA<sub>2</sub>s and G6D49-PLA<sub>2</sub>s prolonged the blood coagulation time in a dose-dependent manner (Table 4). A strongly anticoagulating R6-PLA<sub>2</sub> purified from *Protobothrops tokarensis* venom [13] served as a positive control. During the measurement of activated partial thromboplastin time (APTT), the anticoagulating effect of Tpu-E6a was not affected by increasing the preincubation time from 1 min to 10 min. We then used His48-methylated and inactivated Tpu-E6a to study the dependence of the anticoagulation effect on enzyme activity. After 1 h and 4 h of treatment with the affinity label, the enzymatic activity remaining was 14% and 5%, respectively. After 6 h of treatment and with < 4% of the original hydrolytic activity, the methylated PLA<sub>2</sub> retained 35% of the original anticoagulation activity. Native Tpu-G6D49 and Tbo-G6D49 also prolonged the blood coagulation time (Table 4). The former was twice as potent as the latter although their enzymatic activities were about the same.

**Table 1.** Inventory of PLA<sub>2</sub> purified from *T. puniceus* and *T. borneensis* venom. Values of pI were predicted from protein sequences deduced from the cDNA sequences. Molecular masses were determined by ESI-MS.

| PLA <sub>2</sub> | Protein (%) | pI  | Molecular mass (Da) | N-Terminal sequences 1–23 |
|------------------|-------------|-----|---------------------|---------------------------|
| Tpu-K49a         | 3.5         | 9.3 | 14221.5 ± 1.7       | SVIQLGKMILQETGKNPVKYYGA   |
| Tpu-K49b         | 3           | 9.1 | 14112.8 ± 2.7       | SVIQLGKMILQETGKNPVKYYGA   |
| Tpu-G6D49        | 1.5         | 8.4 | 13912.8 ± 2.2       | SLLEFGRMIIKEETGKNPLFSYIS  |
| Tpu-E6a          | 3.5         | 5.3 | 13723.2 ± 5.7       | NLLQFELMIKKMSGRSGIRWYSD   |
| Tpu-E6b          | 1.5         | 4.5 | 13978.8 ± 2.2       | HLMQFETMIMKVAGRSGVWVWYGS  |
| Tpu-E6c          | 4           | 4.7 | 13792.8 ± 4.1       | NLLQFEMMILKMAGRSGIRWYSD   |
| Tbo-K49          | 10          | 9.0 | 14034.0 ± 5.3       | SVIELGKMILQETGKNPVTTYSA   |
| Tbo-G6D49        | 3           | 8.4 | 13959.6 ± 0.9       | SLLEFGRMIIKEETGKNPLFSYIS  |
| Tbo-E6           | 14          | 5.3 | 13723.0 ± 3.6       | NLLQFEMMINKMAGRSGIRWYSD   |

**Table 2.** Enzymatic activities of venom D49-PLA<sub>2</sub>s with micellar substrates. Hydrolysis of L-dipalmitoyl phosphatidylcholine was measured at pH 7.4, 37 °C in the presence of 3 mM deoxycholate or 6 mM Triton X-100, 10 mM CaCl<sub>2</sub> and 0.1 M NaCl.

| Purified PLA <sub>2</sub> | Specific activity (μmol·mg <sup>-1</sup> ·min <sup>-1</sup> ) |               |
|---------------------------|---|---------------|
|                           | +Deoxycholate   | +Triton X-100 |
| Tpu-G6D49                 | 279 ± 35  | 353 ± 30      |
| Tpu-E6a                   | 661 ± 11  | 393 ± 14      |
| Tpu-E6b                   | 188 ± 11  | 69 ± 1        |
| Tpu-E6c                   | 504 ± 11  | 446 ± 40      |
| Tbo-G6D49                 | 272 ± 16  | 509 ± 7       |
| Tbo-E6                    | 701 ± 17  | 471 ± 45      |

### Molecular phylogeny of venom K49-PLA<sub>2</sub>s from Asian pit vipers

A phylogenetic tree was built to study the structural relationships among venom K49-PLA<sub>2</sub>s from 10 Asian pit viper venom species (Fig. 6). The outgroup in this tree was a K49-PLA<sub>2</sub> (Bothropstoxin-I) from the venom of a New World species *Bothrops jararacussu* [16].

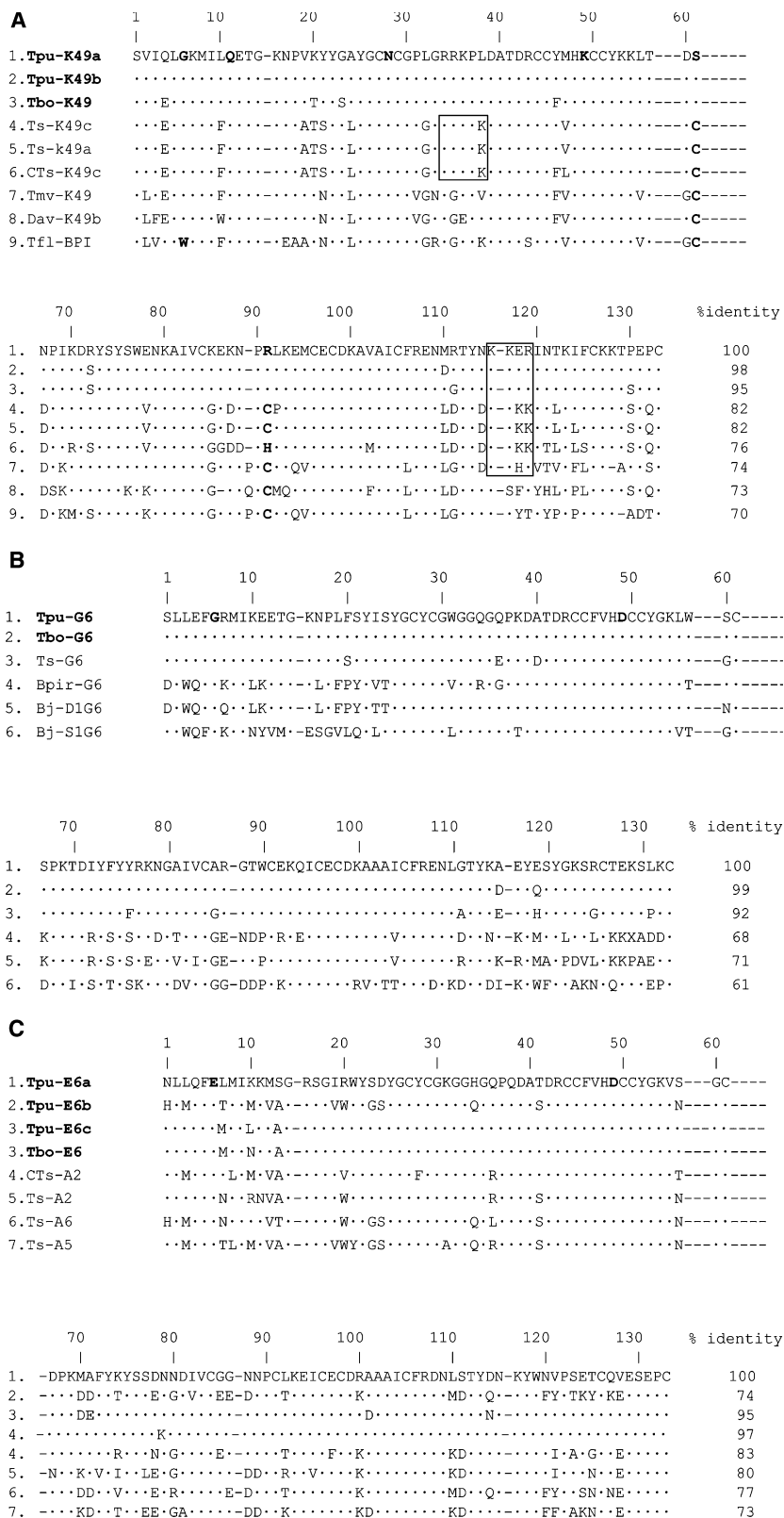
### Discussion

In contrast with all PLA<sub>2</sub>s previously purified from Crotalinae venom [6–8], PLA<sub>2</sub>s containing six disulfide bonds have been isolated from the venom of two African Viperinae, *Bitis gabonica* [17] and *Bitis nasicornis* [18]. Unlike the D49-PLA<sub>2</sub>s from Tpu and Tbo venoms, Tpu-K49a, Tpu-K49b and Tbo-K49 contain only six disulfide bonds, although K49-PLA<sub>2</sub>s missing residue C91 but retaining C61 were cloned from the venom glands of *T. stejnegeri*, but the proteins could not be found in the venom. In fact, all the K49-PLA<sub>2</sub>s purified from different geographic venom samples of *T. stejnegeri* contain seven disulfide bonds [4]. This is

the first report on venom K49-PLA<sub>2</sub>s with six disulfide bonds. As the numbers of disulfide bonds in many secreted protein families are increasing through evolution (e.g. the serine protease family [19]), Tpu and Tbo may be considered as relatively primitive among the *Trimeresurus* species, as also suggested by the phylogenetic analysis of their mtDNA [1,2].

It is widely accepted that disulfide bonds play an important role in maintaining conformational stability and tolerance to environmental factors such as heat, proteolytic enzymes and detergent [20]. Disulfide bonds at 50–131 and 11–77 of the secreted PLA<sub>2</sub> contribute significantly to conformational stability, whereas the disulfide bond at 61–91 contributes much less [21]. Mutagenesis of C61–C91 resulted in a decrease of 2.3 kcal·mol<sup>-1</sup> (9.63 kJ·mol<sup>-1</sup>) of unfolding free energy and lowered hydrolytic activity in the case of bovine pancreatic PLA<sub>2</sub>, or 11 kJ·mol<sup>-1</sup> decrease of unfolding free energy in the case of porcine pancreatic PLA<sub>2</sub> [22]. It was found that the melting temperature of Tpu-K49 was 8.8 °C lower than that of Tst-K49 (Fig. 5). This temperature reduction is consistent with that observed in a mutagenesis study of T4 lysozyme [23].

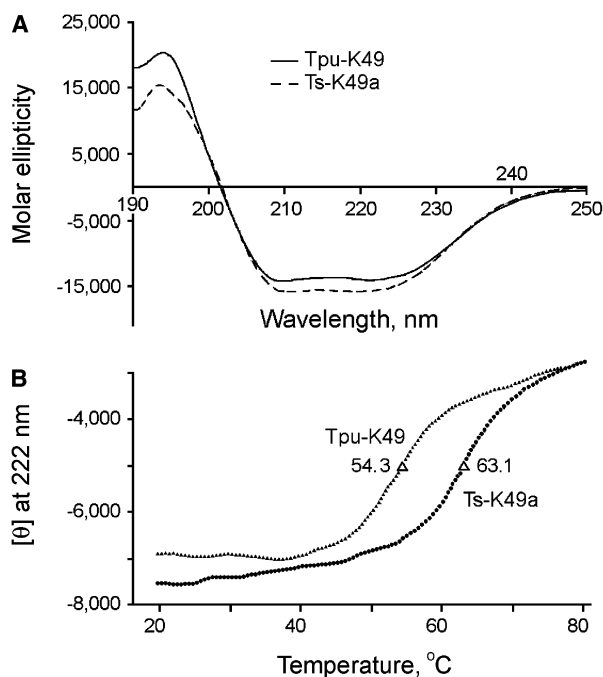
The K49-PLA<sub>2</sub>s display several Ca<sup>2+</sup>-independent activities, including myotoxicity, bactericidal and edema-inducing effects [12]. These activities are possibly related to certain conserved residues which are unique to the K49-PLA<sub>2</sub> family, but absent from the D49-PLA<sub>2</sub>s, including L5, Q11, E12, N28, R34, K49, K53, W77, K80, V102, K115, K117, K123, K127 and K128 (Fig. 3A). The presence of the bulky amino acids V, M or F at position 102 possibly prevents the access of phospholipids to the active site [24]. A common heparin-binding motif is present near the C-termini of all the K49-PLA<sub>2</sub>s (Fig. 3A and [25,26]). Notably, sequences of Tpu-K49a and Tpu-K49b differed by only two substitutions, i.e. R72 and M110 in



**Fig. 3.** Alignment of the amino-acid sequences of three subtypes of PLA<sub>2</sub>s. (A) K49-PLA<sub>2</sub>s. (B) G6D49-PLA<sub>2</sub>s. (C) E6-PLA<sub>2</sub>s. Single-letter codes of amino acids and the numbering system of Renetseder *et al.* [43] were used. Residues identical with those in the top line were denoted with dots, and gaps were marked with hyphens. New sequences and special substitutions are shown in bold. Heparin-binding motifs are boxed. GenBank (SwissProt) accession numbers for the PLA<sub>2</sub>s and the species are: Ts-A2 (P81478), Ts-A6 (P70088), Ts-A5 (P81480), Ts-K49c (AY211936), Ts-K49a (AY211935), CTs-K49c (AY211938), Ts-G6D49 (AY211944) and CTs-A2 [4] from *T. stejnegeri*; Tmv-K49 (X77647) from *Protobothrops mucrosquamatus*; Dav-K49b (AF269132) from *Deinagkistrodon acutus*; Tfl-BPI (P20381) from *Protobothrops flavoviridis*; Bpir-G6D49 (1GMZ\_A) from *Bothrops pirajai*; Bj-D1G6 (AY185201), Bj-S1G6 (AY145836) from *Bothrops jararacussu*.

**Table 3.** Comparison of molecular mass (Da) of the tryptic peptides of Tpu-E6c (MM<sub>T</sub>) determined by peptide mass spectra fingerprinting with the calculated molecular mass (MM<sub>C</sub>). Sequences that differed from those of Tpu-E6a are shown in bold. Segment numbering follows that in Fig. 3C.

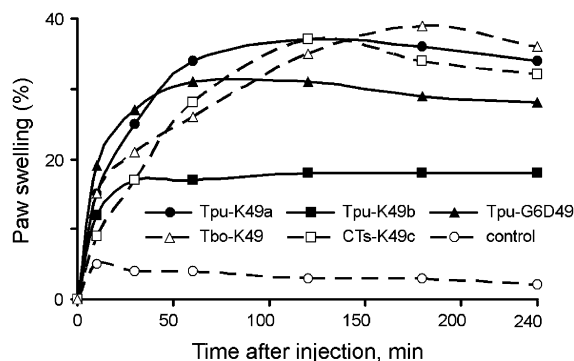
| Peptide sequence  | Position | MM <sub>T</sub> | MM <sub>C</sub> |
|-------------------|----------|-----------------|-----------------|
| NLLQFEMMILK       | 1–11     | 1380.66         | 1379.82         |
| WYSDYGCYCGK       | 21–31    | 1458.80         | 1460.51         |
| GGHGQPQDATDR      | 32–43    | 1239.56         | 1238.55         |
| CCFVHDCCYGYK      | 44–54    | 1509.78         | 1509.48         |
| VSGCDPKDEFYK      | 55–74    | 1466.76         | 1464.65         |
| YSSDNDIVCGGNNPCLK | 75–93    | 2028.96         | 2028.83         |
| EICECDR           | 94–100   | 982.45          | 983.34          |
| <b>DAAICFR</b>    | 101–107  | 853.93          | 853.40          |
| <b>DNLSTYNNK</b>  | 108–117  | 1067.9          | 1068.49         |
| YWNVPSETCQVESEPC  | 118–133  | 1987.11         | 1986.77         |



**Fig. 4.** CD spectra and conformational stability. (A) CD spectra of the K49-PLA<sub>2</sub>s with six and seven disulfide bonds. (B) Changes in helical content of the PLA<sub>2</sub>s during thermal denaturation as followed by molar ellipticity [θ] at 222 nm. Melting temperatures were calculated from the reflection points.

Tpu-K49a and S72 and D110 in Tpu-K49b. The higher basicity and hydrophobicity at these two positions explain the twofold higher edema-inducing activity of Tpu-K49a than Tpu-K49b (Fig. 5).

So far, up to 10 3D structures of the K49-PLA<sub>2</sub> family have been solved by X-ray crystallography [24,27,28]. In common with other structures of



**Fig. 5.** Time course of rat foot edema induced by the PLA<sub>2</sub>s. A rat foot was injected with 10 μg purified venom PLA<sub>2</sub> in 100 μL sterile NaCl/P<sub>i</sub>. The control group received only NaCl/P<sub>i</sub>. Swelling or size of the foot was measured with a plethysmometer. Experiments were performed in duplicate, and data points were averaged results.

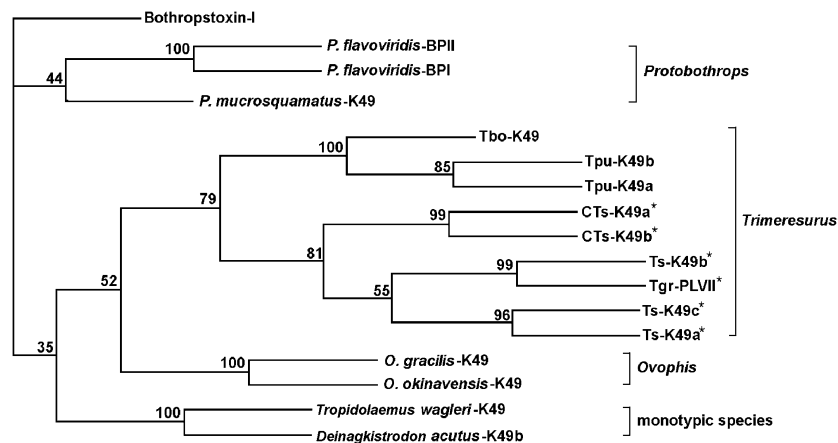
**Table 4.** Anticoagulant activities of purified venom D49-PLA<sub>2</sub>s. APTT was measured twice (final volume 150 μL). Results shown are mean ± SEM.

| PLA <sub>2</sub>            | Dose (μg) | Coagulation time (s) |
|-----------------------------|-----------|----------------------|
| Control                     | 0         | 29.0 ± 1.0           |
| Tpu-E6a                     | 3.0       | 78.7 ± 2.5           |
|                             | 1.0       | 55.7 ± 1.1           |
|                             | 0.3       | 48.0 ± 0.5           |
| Methyl-Tpu-E6a <sup>a</sup> | 3.0       | 55.2 ± 0.7           |
| Tpu-E6c                     | 3.0       | 44.3 ± 0.2           |
| Tpu-G6D49                   | 3.0       | 48.3 ± 1.1           |
|                             | 1.0       | 42.2 ± 0.3           |
|                             | 0.3       | 35.1 ± 0.5           |
| Tbo-E6                      | 3.0       | 43.4 ± 0.3           |
| Tbo-G6D49                   | 3.0       | 45.3 ± 0.3           |
|                             | 2.0       | 42.3 ± 0.1           |
|                             | 1.0       | 38.6 ± 0.4           |
| Pto-R6-PLA <sub>2</sub>     | 0.42      | 73.5 ± 2.0           |
|                             | 0.10      | 47.0 ± 1.0           |

<sup>a</sup> The enzyme was inactivated by methylation at imidazole of His48.

D49-PLA<sub>2</sub>s, the K49-PLA<sub>2</sub>s consist of three α-helices, two antiparallel β-strands, and a few connecting loops. Two long α-helices are interlocked by disulfide bonds at C44–C105 and C51–C98 which form a rigid platform stabilizing the overall structure. Despite the difference in one disulfide bond, the amino-acid sequence of Tpu-K49a was ≈ 80% identical with that of Ts-K49a from *T. stejnegeri* venom [4]. Potential heparin-binding motifs at positions 115–119 and 35–39 or 69–72 of most of the K49-PLA<sub>2</sub>s [12,26] were also conserved (Fig. 3A). Therefore, the pharmacological activities of these six-disulfide-bonded K49-PLA<sub>2</sub>s were

**Fig. 6.** Phylogenetic analysis of K49 PLA<sub>2</sub>s from Asian pit viper venoms. Dataset used were 17 complete amino-acid sequences of K49 PLA<sub>2</sub>s, including those from the venom of *Tropidolaemus wagleri* and *Ovophis gracilis* (I.-H. Tsai, Y.-M. Wang & C.M. Tu, unpublished data). The isoforms from *T. stejnegeri* venom are denoted with asterisks. In addition to those shown in the legend of Fig. 3, accession numbers of the K49-PLA<sub>2</sub>s are: Ts-K49b (AY211937), CTs-K49a (AY211934), Tgr-PLVII (P70089), Ook-K49 (Q92152), and Bothropstoxin-I (Q90249).



nearly the same as other K49-PLA<sub>2</sub>s at ambient temperature.

Both K49-PLA<sub>2</sub> and E6-PLA<sub>2</sub>s are marker proteins of pit viper venoms [13,14]. A previous phylogenetic tree of the K49-PLA<sub>2</sub>s showed separate clusters for the venom proteins from the Old World pit vipers and the New World pit vipers [15]. Herein we focus on the evolutionary relationships among K49-PLA<sub>2</sub>s of Old World pit vipers (Fig. 6). The robustness of this cladogram is supported by high bootstrap values at most nodes. It is notable that most *Trimeresurus* species, including *T. borneensis*, *T. puniceus* and *T. stejnegeri*, are linked. Clustering of these *Trimeresurus* species in the cladogram may be attributed to a unique deletion at residue 89, specific conservation of V2, R35, I69, F106 and N121, and a charged residue 118 in their K49-PLA<sub>2</sub>s, in contrast with those from other Asian pit vipers (Fig. 3A). Whether and how these structural diversities affect K49-PLA<sub>2</sub> function are not clear.

The G6D49-PLA<sub>2</sub>s have so far been found only in the venom of a few venomous genera, including *T. stejnegeri* [4] and South American *Bothrops* (Fig. 3B). These PLA<sub>2</sub> isoforms were potent, with specificity for micelles containing Triton X-100 (Table 2). Under weakly acidic conditions, Tpu-G6D49 and Tbo-G6D49 were eluted from the Superdex gel-filtration column later than expected, as has also been reported for the purification of a few other basic venom PLA<sub>2</sub>s [28]. The enzymes are capable of inducing local edema (Fig. 6) and are more potent anticoagulants than K49-PLA<sub>2</sub>s (Table 4). A previous study showed that a G6D49-PLA<sub>2</sub> (i.e. myotoxin MT-III) from *Bothrops asper* venom increased mouse vascular permeability and induced edema and inflammation *in vivo* [29].

The mechanism behind the anticoagulation effect of PLA<sub>2</sub> is probably its binding via basic residues to coagulation factors in the prothrombinase complex, thus inhibiting thrombin activation [30,31]. But which

residues are crucial for hindering the prothrombinase is puzzling. Despite differing by only two amino-acid substitutions at 115–119 (Fig. 3B), Tbo-G6D49 was 50% less potent than Tpu-G6D49, suggesting that this interface-recognition region affects the anticoagulating activity. Moreover, by careful sequence comparison (Fig. 3C), we noticed that basic residues K10, R16, R20, and K69 in Tpu-E6a, Tbo-E6 and Pto-R6 PLA<sub>2</sub>s [13] possibly contribute to the anticoagulation activity (Table 4). Some of these residues have been suggested to be important for the anticoagulation effect of crotalid venom PLA<sub>2</sub>s [13,30,31]. However, venom PLA<sub>2</sub>s from elapid snakes or true vipers may have different anticoagulating sites [32].

Multiple acidic E6-PLA<sub>2</sub>s are present in the venom of many pit vipers, and each enzyme may play different roles [9,33]. Many of them have been found to affect platelet function [9,34]. However, we found that the acidic PLA<sub>2</sub>s of Tpu and Tbo inhibit platelet aggregation only relatively weakly. We also found that Tpu-E6a at a concentration of 0.1–1 μM significantly prolonged the blood coagulation time. After methylation at His48 and inactivation, Tpu-E6a retained considerable anticoagulation activity (Table 4). Moreover, the APTT was hardly affected by the duration of the Tpu-E6a preincubation time. In fact, many strongly anticoagulating venom PLA<sub>2</sub>s show low hydrolytic activity [30,35]. It has also been shown that an acidic PLA<sub>2</sub>, Cvv-E6f, from *Crotalus v. viridis* venom induced severe edema [33]. Therefore, acidic E6-PLA<sub>2</sub>s probably have evolved with more diversity than previously recognized. Their target proteins remain the challenge for future investigations.

The morphologies of *T. puniceus* and *T. borneensis* are remarkably similar. Previous phylogenetic analyses suggested a close relationship between *T. puniceus*, *T. borneensis* and the cogeneric species in southern Asia (e.g. *Trimeresurus trigonocephalus* and *Trimeresurus*

*malabaricus*) [1,2]. All three subtypes of venom PLA<sub>2</sub>, K49, E6 and G6D49 (Fig. 3A–C), are present in *T. puniceus*, *T. borneensis* and *T. stejnegeri*. The amino-acid sequence of Tbo-K49 differs from that of Tpu-K49 by only four substitutions (Fig. 3A), and Tbo-E6 is structurally very similar to Tpu-E6a and Tpu-E6c, while the sequence of Tpu-E6b is 92% identical with that of Ts-A6 of *T. stejnegeri* (Fig. 3C), and their specific hydrolytic activities were very similar and relatively low (Table 2 and [4]). The cladogram in Fig. 6 also supports the previous conclusion that cogenetic species contain similar venom PLA<sub>2</sub>s [13]. However, basic R6-PLA<sub>2</sub>s, which are present in venoms of *T. stejnegeri* [4] and *Trimeresurus popeorum* (our unpublished data), are absent in venoms of *T. puniceus* and *T. borneensis*. Thus, present day arboreal *Trimeresurus* are probably derived from more than one ancestral species, or it is not a monophyletic genus [1–3].

The venom of *T. borneensis* used in this study was collected from a single specimen whereas that of *T. puniceus* was pooled venom. As intraspecies variations of acidic E6-PLA<sub>2</sub>s of pit viper venom may be common [4,33], the three isoforms of E6-PLA<sub>2</sub>s purified from the *T. puniceus* venom may be combined contributions from different snakes. Tpu-E6c is probably an ortholog of Tpu-E6a (Fig. 3C) and is possibly absent or hardly expressed at all in the snake we killed. Why these E6-PLA<sub>2</sub>s do not form homodimers is not certain, but it may be related to the lack of Pro113 [34]. It appears that the presence of K69 in a PLA<sub>2</sub> is not a sufficient condition for forming dimers [36].

In conclusion, full sequencing and phylogenetic analyses of the venom PLA<sub>2</sub>s of two primitive species *T. puniceus* and *T. borneensis* confirms their close relationship to the cogenetic *T. stejnegeri* (Figs 3 and 6). However, the venom diversities of *T. puniceus* and *T. borneensis* PLA<sub>2</sub>s are not as great as those observed with *T. stejnegeri* [4]. We also show the presence of unusual K49-PLA<sub>2</sub>s with six pairs of disulfide bonds and rare basic G6D49-PLA<sub>2</sub>s in these venoms. Their acidic PLA<sub>2</sub>s showed significant anticoagulating effects. This study on the diversity of venom PLA<sub>2</sub>s also helps us to understand the structure–function relationships of the venom protein isoforms and the evolution of pit vipers.

## Experimental procedures

### Venoms and other materials

A live specimen and pooled venom powder of *T. puniceus* were purchased from Ramba Reptile Park, Bali, Indonesia. A live specimen of *T. borneensis* was purchased from Glades Herp Inc. (Fort Myers, FL, USA). Venom was col-

lected from the snakes 2 days before the venom glands were removed and the snake killed. All measures were taken to minimise pain. NIH guidelines for animal experiments were followed. The glands were immediately preserved in RNA-later solution (Ambion, Austin, TX, USA) until ready for RNA extraction. The mRNA extraction and the cDNA synthesis kits were purchased from Stratagene (La Jolla, CA, USA). Modification and restriction enzymes were from Promega. Synthetic L-dipalmitoyl glycerophosphocholine was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Other chemicals were from Merck and Sigma.

### Purification and enzymatic activities of venom PLA<sub>2</sub>

Crude venom (15 mg) was dissolved in 100 µL buffer and centrifuged at 15 000 *g* for 5 min to remove insoluble material. The supernatant was injected into a gel-filtration column (Superdex G75, HR10/30) on an FPLC system. Venom proteins were eluted in 0.1 M ammonium acetate at room temperature. Fractions containing PLA<sub>2</sub> activities were pooled and freeze-dried. They were further purified by RP-HPLC using a column of silica gel (Vydac C<sub>8</sub>, 4.5 mm × 250 mm; Hesperia, CA, USA) equilibrated with 0.07% aqueous trifluoroacetic acid (solvent A), and eluted with a 25–45% linear gradient of acetonitrile containing 0.07% trifluoroacetic acid (solvent B). Purified PLA<sub>2</sub>s were dried in a vacuum-centrifuge device (Labconco, Kansas City, MO, USA).

The concentration of PLA<sub>2</sub> was determined from the *A*<sub>280</sub>, assuming an absorption coefficient of 1.5 at 1.0 mg·mL<sup>-1</sup>. The hydrolytic activities of PLA<sub>2</sub> towards mixed micelles of L-dipalmitoyl phosphatidylcholine and deoxycholate or Triton X-100 were assayed in 2.5 mL solution at pH 7.4 and 37 °C, on a pH-stat apparatus (RTS 822; Radiometer, Copenhagen, Denmark). The reaction rate was corrected for the nonenzymatic spontaneous rate.

### Amino-acid sequence and molecular mass of PLA<sub>2</sub>

The N-terminal sequences of purified PLA<sub>2</sub>s were determined using a gas-phase amino-acid sequencer coupled with a phenylthiohydantoin amino-acid analyzer (model 477A; Perkin-Elmer, Foster City, CA, USA). The molecular masses of the PLA<sub>2</sub>s [dissolved in 0.1% (v/v) acetic acid with 50% (v/v) CH<sub>3</sub>CN] were analyzed by ESI-MS on a mass spectrometer (model API100; Perkin-Elmer) equipped with the computer software BIOMULTIVIEW 1.2.

For peptide mass spectra fingerprinting, PLA<sub>2</sub> was reduced with dithioerythritol and alkylated with iodoacetamide in the dark. Alkylated PLA<sub>2</sub> was digested overnight with sequencing grade, modified trypsin (Promega, Madison, WI, USA). Enzyme digestion was stopped with acid before injection into the nanoLC-MS/MS system, which



comprising a four-pumping Ultra-Plus™ II system (Micro-Tech Scientific, Vista, CA, USA) connected to the Q-ToF Ultima™ API mass spectrometer in place of the Micro-mass CapLC™ system. The masses of peptides obtained were sorted and matched to the calculated molecular masses of the most possible fits predicted from a known orthologous PLA<sub>2</sub> sequence.

### Cloning and sequence determination

RNA was isolated from venom glands, and the cDNA to mRNA was prepared using a kit [10,15]. To amplify and clone venom PLA<sub>2</sub>s, PCR [37] was conducted using SuperTaq DNA polymerase with a pair of mixed-base oligonucleotide primers (primer 1: 5'-TCTGGATTSAGG AGGATGAGG-3'; primer 2: 5'-GCCTGCAGAGACT TAGCA-3'), which were designed according to the highly conserved cDNA regions of the group-II venom PLA<sub>2</sub>s [38]. In addition, another primer (5'-CAYCTNATGC ARTTYGARAC-3') was designed to replace primer 1 based on the amino-acid sequences 1–7 of Tpu-E6b, to make the amplification successful. Fragments of 0.4 kb were specifically amplified by PCR as shown by electrophoresis of the products on a 1% agarose gel.

After treatment with polynucleotide kinase, the amplified DNA was inserted into the pGEM-T easy vector (Promega). It was then transformed into *Escherichia coli* strain JM109. White transformants were picked up to select the cDNA clones. The DNA Sequencing System (model 373A) and the Taq-Dye-Deoxy terminator-cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) were used to determine the sequences [39]. All the cDNA sequences reported were cloned at least twice, and both nucleotide strands were sequenced.

### CD and thermal stability

The concentration of venom protein in phosphate-buffered saline (NaCl/P<sub>i</sub>)/NaF-saturated buffer (pH 7.4) was determined by the UV absorbance at 280 nm and adjusted to 0.3 mg·mL<sup>-1</sup>. CD measurements were carried out on a J720 spectropolarimeter (Jasco, Tokyo, Japan) under constant flushing of nitrogen at 27 °C. All results were the average of five scanning measurements. Thermal stability of the protein in the NaCl/P<sub>i</sub>/NaF buffer was investigated by measuring the ellipticity at 222 nm with stepwise temperature increments of 0.5 °C from 20 °C to 80 °C using a thermostatically controlled sample holder.

### Edema induction and effects on platelets and blood coagulation

For testing of the edematous effect of venom PLA<sub>2</sub>, Wistar rats (male, ≈200 g body weight) were anaesthetized with

sodium pentobarbital. One of the hind feet was injected with 10 µg purified PLA<sub>2</sub> in 100 µL sterile NaCl/P<sub>i</sub>, and the other received NaCl/P<sub>i</sub> only. The size of the foot was measured at several intervals with a plethysmometer (type 7150; Ugo Basile, Comerio, Italy), and the time course of the swelling was recorded [4,15].

Blood was collected from rabbit and healthy human donors. Dose-dependent inhibition of ADP-induced aggregation of platelet-rich plasma by purified PLA<sub>2</sub> was measured with an aggregometer (model 600B; Payton, Scarborough, Ont, Canada) at 37 °C after the addition of 10 µM ADP [4]. The effects of PLA<sub>2</sub>s on blood coagulation time (i.e. APTT) were studied using a Hemostasis Analyzer (model KC1; Sigma Diagnostics). To inactivate PLA<sub>2</sub>, methylation of His48 at the active site was performed by incubating purified 0.14 mM PLA<sub>2</sub> in 0.1 M sodium phosphate buffer (pH 7.9) with 2.86 mM methyl *p*-nitrobenzenesulfonate and 9% (v/v) acetonitrile at 25 °C [40]. The remaining catalytic and anticoagulating activities were measured.

### Phylogenetic analysis of K49-PLA<sub>2</sub>s

Phylogenetic analysis was based on the 17 available amino-acid sequences of venom K49-PLA<sub>2</sub>s from Old World pit vipers. Our unpublished amino-acid sequences of K49-PLA<sub>2</sub> from venom glands of *Tropidolaemus wagneri* [14] and *Ovophis gracilis* were also included in the dataset. Multiple alignments of the sequences were made using the PILEUP program and neighbor-joining methodology. Then the tree was built by the program PHYLIP (<http://www.evolution.genetics.washington.edu/phylip.html>) [41]. The degree of confidence of the lineage at each node was determined by bootstrap analyses of 1000 replicates [42].

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