Genetic and Ecological Correlates of Intraspecific Variation in Pitviper Venom Composition Detected Using Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) and Isoelectric Focusing

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Abstract. The ability to detect biochemical diversity in animal venoms has wide-ranging implications for a diverse array of scientific disciplines. Matrix-assisted laser desorption time-of-flight mass spectrometry (and, for comparative purposes, isoelectric focusing) were used to characterize venoms from a geographically diverse sample of *Trimeresurus stejnegeri* ($n < 229$) from Taiwan. Previously unrealised levels of heterogeneity were detected in venom phospholipase A_2 isoforms (PLA_2) and in whole venom profiles. Geographic variation in venom was primarily between Taiwan and two Pacific islets. Despite the common assumption that venom variation is a product of neutral molecular evolution, statistical testing failed to link venom variation with phylogenetic descent convincingly. Instead, pronounced differences in venom composition may be the product of natural selection for regional diets or of independent founder effects. More data are required on the functional differences between the isoforms to distinguish between these alternatives.

Key words: Venom — Phospholipase A_2 — MALDI-TOF mass spectrometry — Geographic variation — *Trimeresurus stejnegeri* — Taiwan

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Introduction

The ability to evaluate biochemical diversity is instrumental in aiding our understanding of the evolution of molecular complexity in biological systems. The complex venoms of pitvipers (Serpentes: Viperidae: Crotalinae) (Meier and Stocker 1995; Williams and White 1997) offer an ideal system on which to test emerging analytical tools and evolutionary hypotheses. Intraspecific geographic variation in crotaline venom composition is widespread (reviewed by Chippaux et al. 1991), and studies on the underlying causes and mechanisms of this variation are of considerable theoretical interest in the field of molecular evolution and in the development of new research tools, diagnostic agents, and medicinal drugs (Daltry et al. 1996a; Bowersox and Luther 1998; Stöcklin and Favreau 2002). They also have important implications for antivenom production strategies (Warrell 1986, 1997; Anderson et al. 1993; Chippaux and Goyffon 1998).

To date, electrophoresis has been the biochemical tool of choice for qualitatively analyzing large numbers of individual venoms from geographically disparate sources (Jones 1976; Gregory-Dwyer et al. 1986; Daltry et al. 1996a). Alternatively, cDNA cloning from venom gland extracts is routinely used for sequence analysis of target compounds (Danse

et al. 1997). While electrophoresis offers a rapid way of comparing large numbers of individual venoms, biological interpretations of banding patterns are compromised, as the precise identity of the proteins is unknown and, therefore, may have no adaptive value (Sasa 1999; Mebs 2001). Conversely, cDNA cloning offers precise characterization of venom components, but the labor-intensive nature of the technique means it cannot be applied for screening large numbers of samples. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) offers an accurate, fast, and efficient alternative to both methods, by directly measuring the molecular weight of proteins from complex mixtures (Mirgorodskaya et al. 2000). MALDI-TOF-MS has already been used to identify the masses of key components of arachnid venoms (Escoubas et al. 1999), but no contemporary studies have focused on geographic variation in pitviper venom.

The bamboo viper, *Trimeresurus stejnegeri* (Schmidt), is a nocturnal and arboreal snake predominantly occupying riparian niches (Mao 1993) in Southeast and East Asia (McDiarmid et al. 1999). *T. stejnegeri* (often incorrectly referred to as *Trimeresurus gramineus*) is one of the most thoroughly studied of all Asian pitvipers by toxinologists. Although generally limited to higher altitude forests (>800 m) on the continent, the species is widespread throughout the island of Taiwan from sea level to 1800 m (Creer et al. 2003). With a steep mountain range (reaching almost 4000 m) rising from the eastern coast and a shallower, descending western aspect, Taiwan is climatically and ecologically diverse. Furthermore, recent studies have revealed pronounced mitochondrial DNA (mtDNA) genetic differentiation between two major lineages of *T. stejnegeri* within Taiwan (Creer et al. 2001). These are likely to have resulted from colonisation events from the mainland during periods of landbridge formation (Huang 1984; Yu 1995) dating from the late Pliocene onward (Figs. 1A and B). Therefore, this study model offers the molecular and ecological heterogeneity that has the potential to generate diversity in venom composition.

A widespread assumption is that variation in venom composition is the product of phylogenetic descent (Mebs 2001) or biogeographical vicariance (Williams et al. 1988), but some studies point toward selection (Wilkinson et al. 1991; Daltry et al. 1996b; Sasa and Barrantes 1998). The present study therefore aims (a) to identify patterns of geographic venom variation of mature *T. stejnegeri* within Taiwan (and associated Pacific Coast insular populations), using MALDI-TOF-MS and isoelectric focusing (Righetti and Drysdale 1976), and (b) to test if venom variation is best correlated with phylogenetic, spatial, or ecological factors.

Materials and Methods

The samples for this study were collected throughout 38 localities on the main island of Taiwan and two offshore Pacific islands, Green and Orchid Islands (Fig. 1B, Appendix). Venom was usually extracted within 24 h of capture from single milkings of mature snakes (snout–vent length [SVL] >300 mm) and desiccated over silica gel.

Mass Spectrometry. MALDI-TOF-MS analysis was performed on 104 (male $n = 72$, female $n = 32$) individual crude *T. stejnegeri* venoms sampled from snakes from mainland Taiwan ($n = 88$) and Orchid ($n = 10$) and Green ($n = 6$) Islands. A limited number of samples were analyzed more than once, to check the accuracy and reproducibility of results. All MALDI-TOF-MS analyses were carried out on a PE Biosystems (Framingham, MS) PerSpetive Voyager-Elite II instrument equipped with a 2-m ion flight tube and delayed extraction system in the positive ionization mode. A fresh 10 mg/ml solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 30% CH_3CN containing 0.1% trifluoroacetic acid (TFA) was used as the matrix. The sample (2 mg/ml in 0.1% TFA) was diluted 1:10 with the matrix solution, and 1 μl of the mixture was loaded on a 100-well gold target plate and allowed to dry. Spectra were obtained in the linear mode using 337-nm radiation from a nitrogen laser, with 25-kV accelerating voltage, and 256 scans were averaged. Molecular species were detected within the mass-to-charge ratio (m/z) range 1000 to 60,000. External calibration of the mass scale was performed with bovine insulin. Mass spectra were analyzed using Data Explorer Version 3.4.0.0 (PE Biosystems), and dominant peak masses recorded (molecular weight [mw] + 1 dalton [Da]). This method involves recording quantified relative peak heights, serving as comparative internal standards across all samples (Rosche et al. 2000). Matrix adducts (mw + 205–206 Da), dimers (2mw + H^+), and double- and triple-charged species ([mw + $2H^+$]/2, [mw + $3H^+$]/3) as well as nonsignificant signals were eliminated from the analysis. A Student t test (Sokal and Rohlf 1995) was performed to establish whether there was any significant difference in dominant mass peaks between male and female venoms (Marsh and Whaler 1984; Daltry et al. 1996a, 1997). Mean dominant peak masses were contoured on a map of Taiwan using UNIRAS UNIMAP 2000 (European Software Contractors A/S).

To verify the accuracy of the MALDI-TOF-MS analysis, a single venom sample from Luku (site 18) was subjected to reversed-phase high-pressure liquid chromatography (HPLC) coupled online to electrospray mass spectrometry (ES-MS). ES-MS is another "soft" ionization (Fenn et al. 1989) mass spectrometric technique capable of an extremely high sensitivity and accuracy (±0.01%) in molecular weight determination (Stöcklin et al. 2000; Raftery 2000).

The crude venom was dissolved in 0.1% TFA (2 mg/ml) and 15 μl was injected for analysis. The chromatographic separation was performed using an ABI140B dual-syringe gradient pump (Applied Biosystems, Foster City, CA) operated at a flow rate of 40 μl/min. The reverse-phase column (150 × 1-mm diameter) was packed by ourselves with Nucleosil C_{18}, 5 μm, 300 Å (Macherey–Naggel, Germany). Solvent A was 0.1% TFA in water and solvent B was 90% CH_3CN in water containing 0.1% TFA. The system was equilibrated with 0% solvent B prior to injection. Initial conditions were maintained for 5 min after loading the sample, whereupon a linear gradient was initiated to 60% solvent B in 60 min and then to 100% solvent B over 5 min. The effluent was monitored at 214 nm by UV absorbance on an Applied Biosystems 757 detector equipped with a 35-nl UZ View capillary flow cell (LC Packings, The Netherlands). The effluent was then directly introduced into the mass spectrometer. Mass spectrometric analyses were carried out on a Micromass Platform II instrument (Micromass, UK). The mass spectrometer was equipped with an electrospray ion source (ES-MS) and operated in positive ionization mode under control of
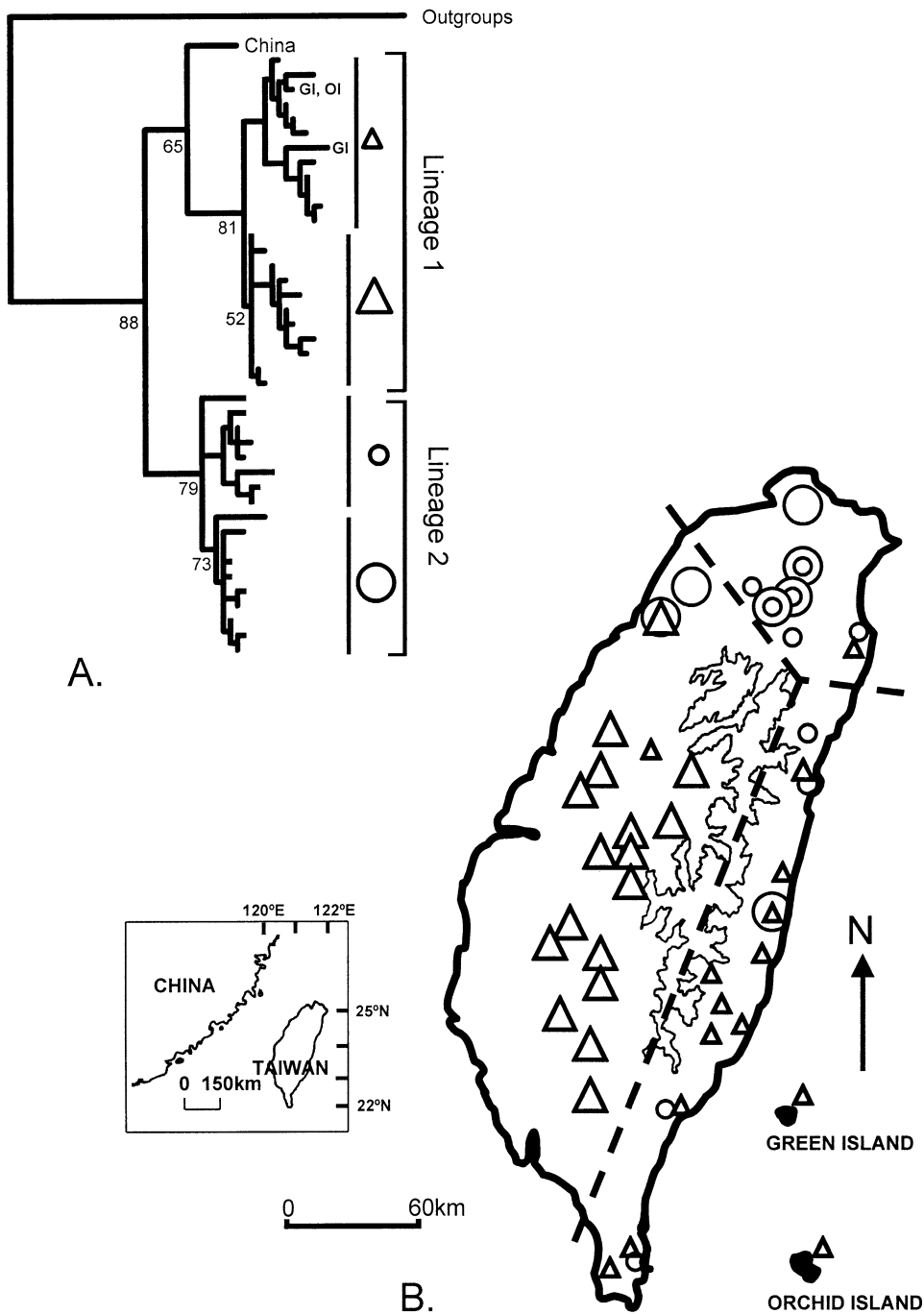
Fig. 1. A Fifty percent majority rule consensus mtDNA cytochrome *b* gene tree summarizing 688 maximum-parsimony trees. Bootstrap values are shown adjacent to nodes defining major lineages only. The two major lineages are subdivided into two further sublineages (*large* and *small triangles* and *circles*, representing Lineage 1 and Lineage 2, respectively). Green Island (GI) shares an identical haplotype with one site on the mainland (site 31). Orchid Island (OI) shares identical haplotypes with Green Island and two sites on the mainland (sites 12 and 34). **B** Geographic distribution of the lineages. The 2000-m contour line is shown. Locations where more than one lineage were present are represented by all-corresponding phylogenetic symbols. The *dashed lines* superimposed on the main island of Taiwan (North, west and east Coasts and southern peninsula), together with Green and Orchid islands, show the five regional groups used in the dietary comparisons.

the Mass Lynx data system. The quadrupole was scanned at 5 s/scan cycle from m/z 400 to m/z 1600 during the whole chromatographic process. The mass spectra corresponding to each LC peak present in the TIC chromatogram were averaged to improve the ion statistics, leading to a better molecular weight determination. External calibration of the mass scale was performed with horse heart myoglobin (Sigma, Switzerland) as a standard.

Isoelectric Focusing. As some previous venom comparative analyses have been based on isoelectric focusing (IEF), it is of interest whether this method will yield results comparable to those with MALDI-TOF-MS. IEF was performed on 216 (male $n = 149$, female $n = 67$) venoms from 39 localities from mainland Taiwan ($n = 172$) and Green ($n = 21$) and Orchid ($n = 23$) Islands. Wide-range pH gels, 3.5–9.5 (Precast Ampholine PAG),

were used on an LKB flat-bed electrofocusing apparatus Multiphor II 2117. The venoms were individually rehydrated with ultrapure water to a concentration of 10 mg/ml soluble protein immediately prior to focusing (for 80 min, at 1500 V). The gels were fixed and stained with Coomassie blue R-250 according to the manufacturer's instructions. Gels were scored by eye and bands recorded as present or absent. The isoelectric point (pI) was assigned according to a linear regression of the distance travelled of calibration marker proteins of known pI (Pharmacia, Herts., UK). The relationship between venom bands and independent variables was examined using a combination of matrix correspondence tests (MCT) and partial matrix correspondence tests (PMCT) (Manly 1991; Thorpe et al. 1994; Daltry et al. 1996b). These tests compare observed patterns of the dependent variable (venom data) to multiple independent variables in pairwise (MCT), or partial (PMCT), comparisons within a spatial framework (calculated from longitude and latitude coordinates). All venom distance matrices were calculated as taxonomic distance on nonstandardized data (equivalent to a matching coefficient where shared absences are equal to shared presences). All calculations were performed using R.S.T.'s adaptation of Manly's RT-MANT program (commercially available from www.west-inc.com) and employed 10,000 randomizations (Manly 1991) unless otherwise stated. Sequential Bonferroni tests (Rice 1989; Sokal and Rohlf 1995) were performed on the probabilities derived from the PMCTs according to the equations $1-(1-\sigma)^{1/k}$, $1-(1-\sigma)^{1/k-1}$, etc., where σ is the experimental error value, and k is the number of independent matrices.

Evaluating the Geographic Component of Venom Composition Variation. To determine whether individual venom protein bands and/or overall venom composition displayed a geographic pattern of occurrence, MCTs were used to correlate them against geographic proximity (independent variable). These MCTs employed 1000 random permutations only due to computational time limitations and were performed for males and females separately (for individual bands) or on combined male and female data (for the entire venom data set). All bands involved in a significant regression ($p < 0.05$ for any of the spatial tests) were included in further statistical comparisons and a principal-component analysis (PCA). The mean first and second principal-component scores were contoured onto a map of Taiwan using UNIRAS UNIMAP 2000 (European Software Contractors A/S) to visualize the pattern of geographic variation in venom composition.

Testing Patterns of Venom Variation Against Phylogenetic and Ecogenetic Variables. The venom data generated from the MALDI-TOF-MS (mean dominant peak mass) and isoelectric focusing (proportion of venom bands present within each population) analyses were summarized into population indices. Comparisons incorporating phylogeny could not be performed on genetically heterogeneous populations (Fig. 1B), hence where more than one major phylogenetic lineage was present in any single location, the population was split into two subpopulations. A combination of MCTs and PMCTs was used to test associations between patterns of venom composition variation against the following three independent variables (Daltry et al. 1996b, 1997), which have the potential to affect venom evolution. 1. Geographic distance/gene flow (based on longitude and latitude): This comparison allows one to test for a geographic pattern in a MCT or allows for spatial effects to be taken into account in a PMCT (Legendre and Fortin 1989; Manly 1991).
2. Phylogeny: This comparison tests whether differences in venom composition are associated with phylogenetic differentiation. Phylogenetic structure can be represented by a patristic distance matrix (i.e., the sum of the branch lengths between pairs of
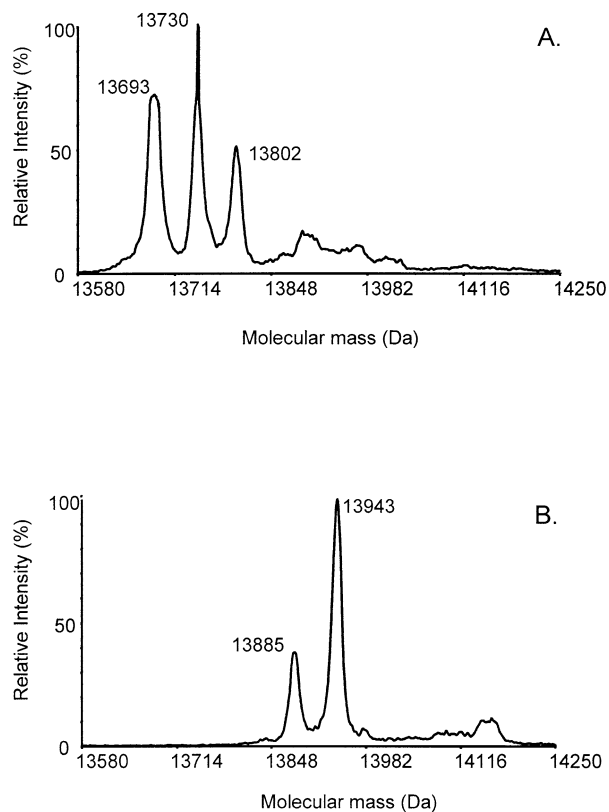

Fig. 2. Representative spectra from MALDI-TOF-MS analysis of *T. stejnegeri* venoms from different regions of Taiwan, showing the molecular masses measured for the different PLA$_2$'s present in each sample: (A) Luku, Nantou Co. (120.77E, 23.76N); (B) Green Island, Taitung Co. (121.48E, 22.67N).

haplotypes on a phylogenetic tree). The matrix was calculated from pairwise comparisons of 41 haplotypes (654 base pairs in length, mtDNA, cytochrome *b* gene; GenBank accession numbers AF277676–AF277716) between each of the major lineages shown by Creer et al. (2003) and illustrated in Fig. 1A.
3. Diet: This comparison tests whether patterns in venom variation are associated with geographic variation in diet composition. Geographical diet indices were calculated for the sampled populations from gut content analysis (Creer et al. 2002). Populations were assigned to one of five dietary/geographic regions (Fig. 1B), chosen to represent most accurately the trophic composition of areas with similar biogeographical history, geographical proximity, and microenvironmental characteristics while maximizing the sample size within each category.

To test further the association of venom with diet, independent contrasts were performed between mean haplotype diet data against mean haplotype values for the first principal-component scores from the IEF data ($n = 39$) and for the dominant mass peak data from the MALDI-TOF-MS analyses ($n = 31$). The contrasts were calculated using COMPARE 4.2 (http://darkwing.uoregon.edu/~compare4/v42/comparehigh.html). The program COMPARE 4.2 starts with a set of comparative data and a phylogeny and transforms these into a set of normally distributed, statistically independent, and standardized "contrasts," which conform to the requirements of most parametric statistical tests, outlined by Felsenstein (1985). The results of the independent contrasts were then subjected to a simple bivariate regression using least-squares estimation for continuous variables.
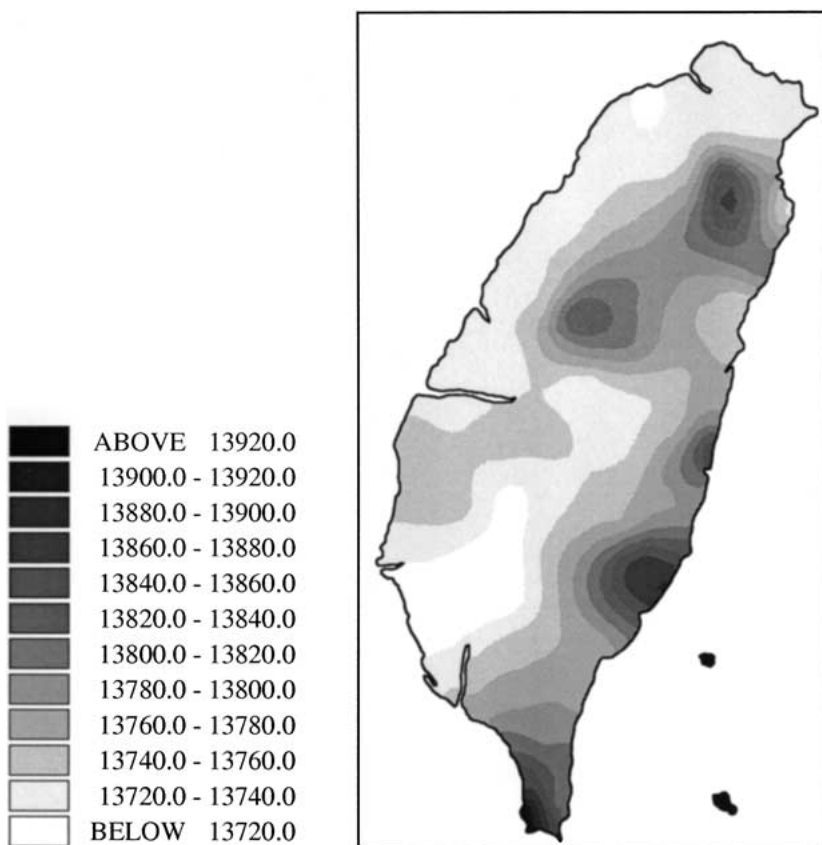
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Fig. 3. Mean dominant mass peaks (Da) from MALDI-TOF-MS analysis of individual venoms contoured onto a map of Taiwan.

Results

Mass Spectrometry. The MALDI-TOF-MS analysis revealed a number of characteristic mass peaks at approximately 1886, 7500, 12,500, 13,670–14,150, and 25,000 Da throughout the individual venoms. Mass peaks of replicated samples were within approximately 5 Da of each other, showing that separate data acquisition sets could be combined for analysis. For comparative purposes, all peaks in the mass range between 13,670 and 14,150 Da (over 10% maximum peak intensity) were recorded (Fig. 2). This revealed a suite of masses falling into 26 discrete weight classes (see the Appendix for peak masses). Analysis of dominant peaks showed no difference between male and female venoms ($t = -0.69$, 57 df, $p = 0.491$) but revealed pronounced geographic variation between the main island of Taiwan and the offshore Pacific islands (Fig. 3). Both Green and Orchid island venom types exhibit an increase in dominant peak masses in addition to the very southern tip and the southeast of Taiwan.

Online HPLC-ES-MS identified 63 compounds from the analysis of the replicated Luku venom sample (showing the greater sensitivity of this method). Two discrete compounds with molecular masses 13,801.92 and 13,801.97 Da (most likely to be the same molecular species) were within 6 Da of the mass determined for one of the dominant mass peaks from the MALDI-TOF-MS analysis (13,806 Da). Another compound with an estimated mass of 13,732.84 Da was within 10 Da of an additional mass peak derived from the MALDI-TOF-MS analysis (13,742 Da), illustrating a minimum of 99.93% similarity between the two analyses and, thus, confirming the accuracy of the MALDI-TOF-MS mass determination.

Isoelectric Focusing. Between 18 and 55 bands were observed in any single venom profile. Thirty-four bands were consistently scorable, and of these, 14 were correlated with geographic proximity and used for PCA. Separate PCAs for male and female venoms separately (not shown) gave highly concordant patterns to each other and the combined analysis.

The first two principal components account for 29.9% of the variation evident in the venom banding profiles. The mean first principal-component scores (16.2%) reveal a weak east–west pattern of venom types on the main island of Taiwan (Fig. 4A). The principal difference is shown in venom types from Green Island, which remain separate from both Orchid Island and mainland Taiwan. The mean second principal-component scores (13.7%) reveal differentiation in venom types from southerly populations (Fig. 4B). To a lesser degree, venoms from a number
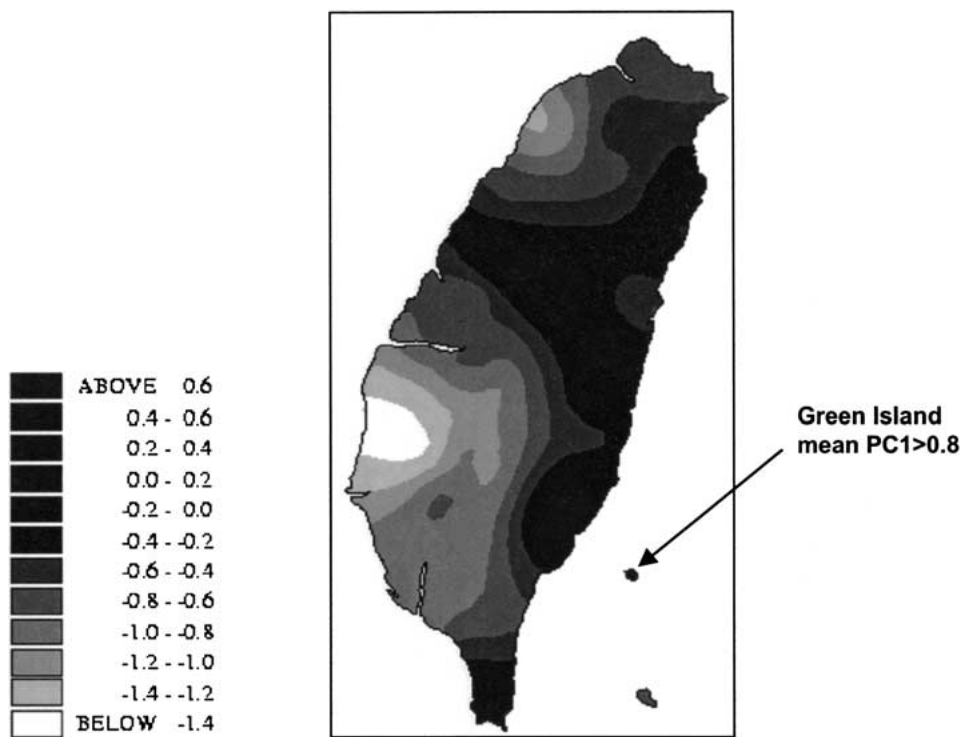
A.
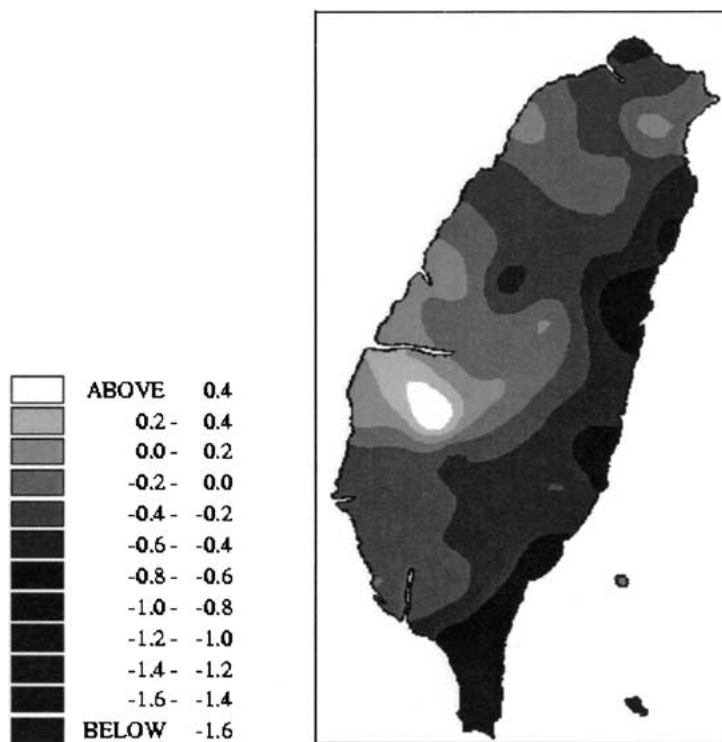
B.

Fig. 4. A Mean first principal-component (PC1) scores of IEF venom analysis contoured onto a map of Taiwan. Green Island highlighted with mean PC1 scores over 0.8, with the rest of the main island of Taiwan and Orchid Island not exceeding 0.4. B Mean second principal-component scores showing NE–SW clinal variation in venom types on the main island of Taiwan.

of populations throughout the east coast and Orchid Island form the lower limit of a north–east, south–west clinal variation in venom types extending to the west coast of Taiwan.

Pairwise correlation of the MALDI-TOF-MS dominant mass peaks revealed that all three independent variables were significantly correlated with the pattern of geographic variation in venom com-

ponents, but only diet remained significant when subjected to simultaneous testing. Whole IEF venom profiles showed no significant association with any of the three alternative variables. However, 5 of the 14 individual IEF venom bands that showed significant geographic structure showed a significant association with at least one of the three tested alternative variables (Table 1). Of these, two showed a significant association with phylogeny (8.72, 9.33), three with diet (3.6, 4.17, 8.72), and three with geographic proximity (8.72, 4.17, and 8.25) in a MCT. When bands with multiple significant associations were further tested in a PMCT, this declined to two significant associations with diet (8.72, 3.6), two with phylogeny (8.72, 9.33), and one with geographic proximity (8.25). Of the two bands still associated with phylogeny, further scrutiny reveals that band 8.72 is only poorly represented in one sublineage of Lineage 1 (denoted by small triangles in Figs. 1A and B) and in one sublineage of Lineage 2 (denoted by small circles in Figs. 1A and B). These sublineages are phylogenetically distinct from each other but are sympatric throughout the east coast and the islands. Band 9.33 was significantly associated with phylogeny in all geographical comparisons. This appears to be the result of the reduced occurrence of band 9.33 from venoms of populations from one sublineage of Lineage 1 (denoted by the large triangle in Fig. 1A). Analyses of the independent contrasts generated by COMPARE 4.2 resulted in a highly significant relationship between mean IEF PC1 venom data and diet ($r = 0.473$, $p = 0.003$) but no relationship between MALDI-TOF-MS dominant mass peak data and diet ($r = 0.141$, $p = 0.802$).

Discussion

The suite of masses (13,670–14,150 Da) detected by MALDI-TOF-MS analysis of *T. stejnegeri* venoms correspond to the range of published masses of phospholipase A_2 (PLA$_2$) isoforms (Table 2) of "*T. gramineus*" obtained from the SWISS-PROT protein sequences database (Bairoch and Apweiler 2000). These PLA$_2$'s are calcium-dependent (Harris and MacDonell 1981; Harris 1985), low-molecular weight, group II PLA$_2$ enzymes characteristic of viperid and crotaline venoms (Kini 1997). Functionally, they catalyze the breakdown of phospholipids, which are a common constituent of all biological membranes, and have a potent inhibitory effect on platelet aggregation and, thus, blood clotting function (Feng et al. 1996). In *T. stejnegeri* venoms, PLA$_2$'s would most likely perform a hemorrhagic/digestive function (Kuo and Wu 1972; Warrell 1995). However, functional differences between PLA$_2$ isoforms (e.g., variation in myotoxicity) of *T. stejne-*

Table 1. Results of the MCTs and PMCTs showing significance levels of individual venom bands (IEP) and MALDI-TOF-MS (PLA$_2$) analyses compared with geographic distance, phylogeny, and dietary variables outlined in the text

Venom component (IEP)	G distance	Phylogeny	Diet
3.6	0.1461	0.3067	0.0037**
4.17	0.0248*	0.0797	0.0456*
8.25	< 0.0001**	0.1607	0.5393
8.72	0.0047**	0.0003**	0.0003**
(PMCT)	0.2634	< 0.0001**	< 0.0001**
9.33	0.3969	< 0.0001**	0.6474
PLA$_2$	0.0005**	0.0217**	0.0003**
(PMCT)	0.0543	0.0416	0.0067*

Note. (PMCT) denotes the results of PMCTs following more than one significant MCT between venom and the independent variables.
*Significant at $p < 0.05$ level.
**Significant at $p < 0.01$ level. Significance levels for simultaneous comparisons have been adjusted for sequential Bonferroni correction (see text).

geri have been noted (Fukagawa 1992; Nakai et al. 1995).

Although the occurrence of multiple isoforms of snake venom components has been described in other snake species (Trikha et al. 1994; Harris 1997; Kini 1997; Monteiro et al. 1997; Tyler et al. 1997), the levels of intraspecific protein diversity detected in this study (approximately 22 PLA$_2$ isoforms) is considerably higher than has been described previously. For example, only up to 10 species-specific enzyme isoforms have been described from the venoms of the Indian cobra, *Naja naja*, Russell's viper, *Daboia russeli*, and king brown, *Pseudechis australis* (Kini 1997). The detection of increased levels of enzyme diversity in the present example is most likely a result of the combination of a large and diverse sampling regime and the sensitivity of the mass spectrometric analysis. Nevertheless, the results clearly show the existence of previously unrecognized biochemical heterogeneity within a single crotaline species from a relatively limited geographic range.

Although biochemical phenotypes may be interpreted as either a form of phenotypic plasticity or the result of posttranslational modification, a range of studies suggests that venom components are genetically inherited. Venoms of captive-bred Malayan pitvipers, *Calloselasma rhodostoma*, closely matched those of wild specimens sampled from the original location of the captive reptiles, although the housed animals were fed on an artificial all mouse diet (Daltry et al. 1997). Additionally, intraspecific variation is evident in captive specimens maintained on homogenous diets (Bonilla et al. 1973; Gregory-Dwyer et al. 1986) and even among the same litter (Chippaux et al. 1982). These data, alongside research

Table 2. Estimated molecular masses of PLA$_2$ isoforms calculated from a search of known proteins isolated from *T. gramineus* (i.e., *T. stejnegeri*) venoms in the SWISS-PROT protein database (expasy.ch), given here with their primary accession numbers (AC)

Phospholipase A$_2$	Mass (Da)	Reference(s)[a]	AC
Isozyme I	13,734.34	1,2,4	P20476
Isozyme II	13,779.32	3	P81478
Isozyme III	13,711.3	3	P81480
Isozyme IV	13,778.35	3	P81479
Isozyme V	13,892.16	4	P70090
Isozyme VI	13,938.51	4	P70088
Isozyme VII	13,917.36	4	P70089

Note. Average molecular masses of the enzymes with oxidized disulfide bridges were calculated with the MassLynx NT Biolynx software (Micromass Ltd., Altrincham, UK). Nakashima et al. (1995) refer to the specimens as *T. gramineus*. However, since no other green pitviper species inhabit Taiwan, where the specimens were obtained, these are attributable to *T. stejnegeri*.
[a](1) Oda et al. (1987) (from venom). (2) Oda et al. (1991) (from venom). (3) Fukugawa et al. (1992) (from venom). (4) Nakashima et al. (1995) (from venom gland).

investigating the interspecific (Aird et al. 1989; Glenn and Straight 1989; Rael et al. 1992) and intraspecific (Schenberg 1958; Wilkinson et al. 1991) hybridization of venom types, strongly suggest that venom type is predominantly heritable (Daltry et al. 1996a). Furthermore, significant correlations between electrophoretic analyses and variation in enzymatic and biological activities have been demonstrated for *C. rhodostoma* (Daltry et al. 1996a) and *D. russeli* (Chijiwa et al. 2000).

Genomic analyses suggest that a form of accelerated evolution in crotaline venom coding genes contributes to PLA$_2$ phenotypic diversity. Comparisons between the noncoding regions including introns and coding regions of genes (exons) encoding *Trimeresurus "gramineus"* (i.e., *stejnegeri*) and *Protobothrops flavoviridis* PLA$_2$ isozymes revealed a three- to eightfold increase in nucleotide substitutions for the protein coding regions of exons (Nakashima et al. 1994, 1995; Ogawa et al. 1996). Similar results from comparisons of the molecular regions encoding serine proteases in *Trimeresurus* (Deshimaru et al. 1996), PLA$_2$ genes in elapid *Naja* species (Chuman et al. 2000), and even conotoxins from *Conus* spp. (Duda and Palumbi 1999) additionally suggest that higher rates of substitution are not uncommon in venom coding genes. Whether hypervariability in venom isoforms is attributed to Darwinian accelerated evolution (Nakashima et al. 1995), positive Darwinian selection (Gubenšek and Kordiš 1997), or diversifying selection (Duda and Palumbi 1999; Conticello et al. 2001), evidence suggests that the mode of molecular evolution of certain protein coding regions may be adaptive for organisms confronted with variable environments. This has been exemplified in the somatic hypermutation in the immune system, the SOS mutagenesis response of *E. coli*, the enrichment of mouse MHC genes (Conticello et al. 2001), and the surface antigen-switching mechanisms of trypanosomes (Metzgar and Wills 2000). Thus, the increased

diversity in venom isoforms is likely to confer a selective advantage to pitvipers subjected to dynamic predator–prey interactions and/or environmental change.

Following the detection of intraspecific variation in venom composition, elucidating the evolutionary causal mechanisms behind observed patterns remains a complex evolutionary problem. Due to the complexity of crotaline venoms, venom variation cannot be unequivocally attributed to a single factor in the present model. Significant associations were observed between some venom bands/PLA$_2$ isoforms and all tested variables. However, only IEF venom band 9.33 was found to be significantly associated with phylogeny. Although a single IEF band can comprise more than one venom component, a common phylogenetic origin of similar venom types is not well substantiated in the current analyses.

The relationship between geographic distance and venom composition implicates spatial scale and localised ecogenetic factors in determining geographic variation in patterns of venom composition. A degree of clinal east–west variation in venom composition can be seen from the contouring of the second principal-component scores of the IEF venom analysis (Fig. 4B). According to these data, venom types are most divergent from scattered populations throughout the east coast and the southern tip of the main island of Taiwan. Given the impressive topography of Taiwan, it is likely that the vicariant effect of the Central Mountain Range has played a role in defining these patterns. Similar climatic factors interacting with localized topography and geology are likely to have created locally uniform habitat types with homogeneous fauna and flora. Thus, localized selection may have played a role in generating diversity in venom variation (Wilkinson et al. 1991; Daltry et al. 1997), or the patterns may simply reflect ongoing gene flow at nuclear loci.

Many aspects of the venom analyses showed a significant association with diet composition. This is predominantly the result of the relationship between Pacific insular variation in venom composition (Figs. 3 and 4) and a biogeographical- or microenvironmental (Schwaner 1985; Campbell and Solorzano 1992; King 1993)-driven shift in diet composition (Creer et al. 2002). The predominate diet of *T. stejnegeri* sampled on the main island of Taiwan is amphibians; however, reptiles appear to be regularly preyed upon on Green Island. All identifiable reptilian matter found in the gastrointestinal tract of *T. stejnegeri* was remains of *Japalura* species. These arboreal agamid lizards pose a greater threat than amphibians to the snakes by possessing sharp teeth and claws. Therefore, selection on either the pharmacological effect or the digestive properties (or both) of venom composition might be expected if a switch was made between a diet comprising mainly of amphibian prey items to a diet including more robust prey. A similar scenario has recently been described for the venom of *Protobothrops flavoviridis* inhabiting Okinawa Island, which lacks the main hemorrhagic (Sadahiro and Omori-Satoh 1980) and myotoxic (Chijiwa et al. 2000) PLA$_2$ principles present in conspecifics inhabiting Amami Island in Japan. This has been attributed to a dietary shift, as *P. flavoviridis* feeds mainly on rats (86%) on Amami Oshima Island but primarily on Holst's frogs, *Rana holsti* (approximately 90%), on Okinawa Island (Chijiwa et al. 2000).

However, the step from demonstrating a correlation to assuming causation is large. Although different PLA$_2$ isoforms have been shown to have significantly different physiological/pharmacological effects (Fukugawa et al. 1993; Nakai et al. 1995; Kini 1997; Harris 1997), the only way of unequivocally testing the potency or digestive actions of venoms is on the prey (Kini 1997). Animal testing is out of the scope of the current study; however, testing of various activities (phospholipase activity, hemorrhagic activity, neurotoxicity, and myotoxicity) of the different isoforms detected in this study are in progress. An alternative explanation, which is also consistent with this pattern, is that the disparate venom composition of the two Pacific island populations of *T. stejnegeri* is due to a founder event. Venoms sampled from individuals from the southernmost tip of Taiwan exhibit PLA$_2$ isoforms of similar mass to those of the Pacific island snakes (Fig. 3 and the Appendix). In this case, the PLA$_2$ coding genes of the initial founding population may have simply spread throughout the island populations, resulting in homogeneous venom types. Distinguishing between these two alternatives is not possible with the current data.

Conclusions

MALDI-TOF-MS proved to be a very efficient and accurate analytical method for assaying large numbers of individual venoms and establishing the identity of component compounds. Given these advantages, the potential for MALDI-TOF-MS as a baseline tool in venom research is particularly promising. Although significant associations were observed between some venom components and all tested variables, phylogeny does not appear to be the principal causal factor of venom variation. Crotaline venoms are multicharacter phenotypes, which may be subject to a combination of spatial, ecological, and genetic factors. Consequently, there may be no single answer regarding causation of variation in venom composition. A fuller understanding of venom genomics, and how venom coding genes are expressed, is required. It is still not clear whether the isoforms detected in animal venoms at finer evolutionary levels are the result of *de novo* mutation or are the products of differential gene expression. A multidisciplinary approach to venom evolution is still developing, and by combining toxinology and molecular biology in an explicitly tested evolutionary and ecological framework, further studies will no doubt advance the understanding of the molecular basis of venom evolution.

Appendix

Table A1. Mass peaks (>10% signal intensity) for individual venoms and site means recorded from the MALDI-TOFMS analysis

Site	Mass peaks in order of Site mean intensity (Da)		
1. Yangminshan Park, Taipei City Taipei Co., 121.55E, 25.15N	13,890, 13,779		
	13,725		
	13,769, 13,720		
	13,738, 13,914		13,781
2. Wulei, Taipei Co., 121.55E, 24.85N	13,752, 13,905		
	13,750, 13,941, 13,693		
	13,739, 13,898		13,747
3. Fushan, Ilan Co., 121.51E, 24.80N	13,897, 13,750		
	13,893, 13,745		13,895

Table A1. (*Continued*)

Site	Mass peaks in order of Site mean intensity (Da)	
4. Paling, Taoyaun Co., 121.38E, 24.68N	13,735, 13,891, 13,802	
	13,755, 13,910	13,745
5. Pa-shen-san, Taichung Co., 120.74E, 24.22N	13,740, 13,812, 13,960	13740
6. Takeng, Taichung Co., 120.65E, 24.14N	13,751	
	13,729, 13,947, 13,798	
	13,751	13,744
7. Chichi, Nantou Co., 120.80E, 23.84N	13,737	13,737
8. Chushan, Nantou Co., 120.68E, 23.76N	13,803	13,803
9. Hsitou, Nantou Co., 120.81E, 23.68N	13,752	13,752
	13,721	
10. Shanping, Kaohsiung Co., 120.56E, 23.00N	13,693	13,693
11. Liangshan, Pingtung Co., 120.66E, 22.70N	13,891, 13,730, 13,810	
	13,725, 13,891	
	13,756, 13,912, 14,090	13,791
12. Chufengshan, Pingtung Co.,120.84E, 22.14N	13,940, 13,777	
	13,904, 13,795	
	13,792, 13,910	13,879
13. Chihpen, Taitung Co., 121.06E, 22.72N	13,733, 13,897	
	13,755, 13,909, 14,058	13,744
14. Green Island, Taitung Co., 121.48E, 22.67N	13,939	
	13,941	
	13,943, 13,885	
	13,938	
	13,940, 13,883	
	13,917	13,936
15. Tungyanshan, Taipei Co., 121.39E, 24.80N	13,737, 13,897	13,737
16. Chingshan, Taichung Co., 120.87E, 24.18N	13,894	13,894
17. Chutyukeng, Taichung Co.,120.64E, 24.04N	13,907, 13,775	
	13,776	
	13,739	
	13,726	13,787
18. Luku, Nantou Co.,120.77E, 23.76N	13,713	
	13,753, 13,702	
	13,806, 13,742	
	13,730, 13,693, 13,802	
	13,936, 13,762, 13,724	
	13,941, 13,753	
	13,721	
	13,716	
	13,718	
	13,711	
	13,748	
	13,735	13,769
19. Chuyunshan, Kaohsiung Co., 120.82E, 23.13N	13,740, 13,807, 13,696	
	13,746	13,748
20. Dong Ha Farm, Taitung Co., 121.31E, 22.96N	13,892, 13,735	
	13,742, 13,905	
	13,889, 13,756	13,841
21. Chimei, Coastal Range HighWay, Hualien Co., 121.45E, 23.49N	13,741, 13,806	
	13,902, 13,765	13,822
22. Shuiyuan, Hualien Co., 121.56E, 24.02N	13,739, 13,893	
	13,747, 13,909	13,743
23. Hsincheng, Hsinchu Co., 120.95E, 24.74N	13,729, 13,954, 13,799	13,729
24. Chaochiao, Miaoli Co., 120.87E, 24.64N	13,738, 13,893	
	13,730	13,734
25. Sanmei, Chiai Co., 120.68E, 23.37N	13,700	13,700
26. Quantzelin, Tainan Co., 120.51E, 23.35N	13,743, 13,691, 13,801	13,743
27. Sanpin, Kaohsiung Co., 120.67E, 22.92N	13,709, 13,886, 13,796	
	13,789, 13,903	
	13,715	13,738
28. Paoli, Pintung Co., 120.77E, 22.10N	13,939	13,939
29. Kuanshan, Taitung Co., 120.16E, 23.06N	13,891, 13,742, 14,074	13,891
30. Wushei, Nantou Co., 120.12E, 24.02N	13,737	
	13,800, 13,979, 14,140	13,769

Table A1. (Continued)

Site	Mass peaks in order of Site mean intensity (Da)	
31. Suao, Ilan Co., 121.85E, 24.59N	13,734, 13,892	
	13,894, 13,736	
	13,729, 13,891	13,786
32. Taroko National Park, Hualien Co., 121.50E, 24.17N	13,734, 13,903	
	13,731, 13,809	
	13,736, 13,899	13,734
33. Fuyuan, Hualien Co., 121.38E, 23.57N	13,730, 13,882, 13,952	
	13,732	
	13,903, 13,745	
	13,745	
	13,731, 13,898	
	13,895, 13,749	13,768
34. Litau, Taitung Co., 121.05E, 23.18N	13,727, 13,869	
	13,909, 13,757	
	13,735, 13,886	
	13,743, 13,885	13,779
35. Chan-na, Taitung Co., 121.13E, 22.96N	13,882	13,882
36. Antung Hot Springs, Hualien Co., 121.35E, 23.30N	13,887, 13,757	
	13,760, 13,901	13,824
37. Ming-chi, Ilan Co., 121.52E, 24.60N	13,890	13,890
38. Lienhwachih, Nantou Co., 120.90E, 23.85N	13,686	13,686
40. Orchid Island, Taitung Co.,121.60E, 22.13N	13,937	
	13,936, 13,879	
	13,941, 13,884	
	13,930	
	13,887, 13,941	
	13,937, 13,876	
	13,936, 13,880	
	13,938, 13,882	
	13,940, 13,886	
	13,945, 13,890	13,933

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