

Diet and snake venom evolution

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VENOM composition within snake species can show considerable geographical variation¹, an important consideration because bites by conspecific populations may differ in symptomatology and require different treatments²⁻⁵. The underlying causes of this phenomenon have never been explained. Here we present evidence that the variation in the venom of the pitviper *Calloselasma rhodostoma* (Serpentes: Viperidae) is closely associated with its diet. We also evaluated other possible causes of geographic variation in venom using partial Mantel tests⁶⁻¹⁰ and independent contrasts¹¹, but rejected both contemporary gene flow (estimated from geographical proximity) and the phylogenetic relationships (assessed by analysis of mitochondrial DNA) among populations as important influences upon venom evolution. As the primary function of viperid venom is to immobilize and digest prey¹²⁻¹⁴ and prey animals vary in their susceptibility to venom^{15,16}, we suggest that geographical variation in venom composition reflects natural selection for feeding on local prey.

The Malayan pitviper *C. rhodostoma* is the leading cause of venomous snakebite across much of southeast Asia³. Its venom contains many potent digestive enzymes¹⁷ which can cause human victims to suffer severe tissue damage, often leading to permanent deformities among survivors. The precise symptomatology shows some geographical variation³, suggestive of variation in venom composition.

For our investigation of the intraspecific variation in venom composition, we collected venom from 67 wild adult *C. rhodostoma* from 36 localities in Vietnam, Thailand, Malaysia and Java (Fig. 1a). Each sample was isoelectrically focused (IEF) across polyacrylamide gels containing carrier ampholytes. Ordination analysis of the electrophoretograms revealed strong geographical variation in the venom composition of this species (Fig. 1b).

Three hypotheses to account for the geographical variation in *C. rhodostoma* venom were considered. Firstly, variation in venom could be a function of the geographical distance between groups. The opportunity for exchange of venom-coding genes is expected to be higher between spatially close populations and might cause them to produce more similar venom than remote conspecifics. Furthermore, neighbouring populations are more likely to share a similar biotic and abiotic environment, potentially resulting in similar unspecified selection pressures. Secondly, variation in venom may be associated with the patristic phylogenetic relationships among groups. This hypothesis predicts that populations of recent common ancestry produce more similar venoms than populations separated by greater patristic distances¹⁰. The intraspecific phylogeny of *C. rhodostoma* was reconstructed, as free as possible from perturbation by ecogenetic selection, using restriction-fragment length polymorphism (RFLP) analysis of a

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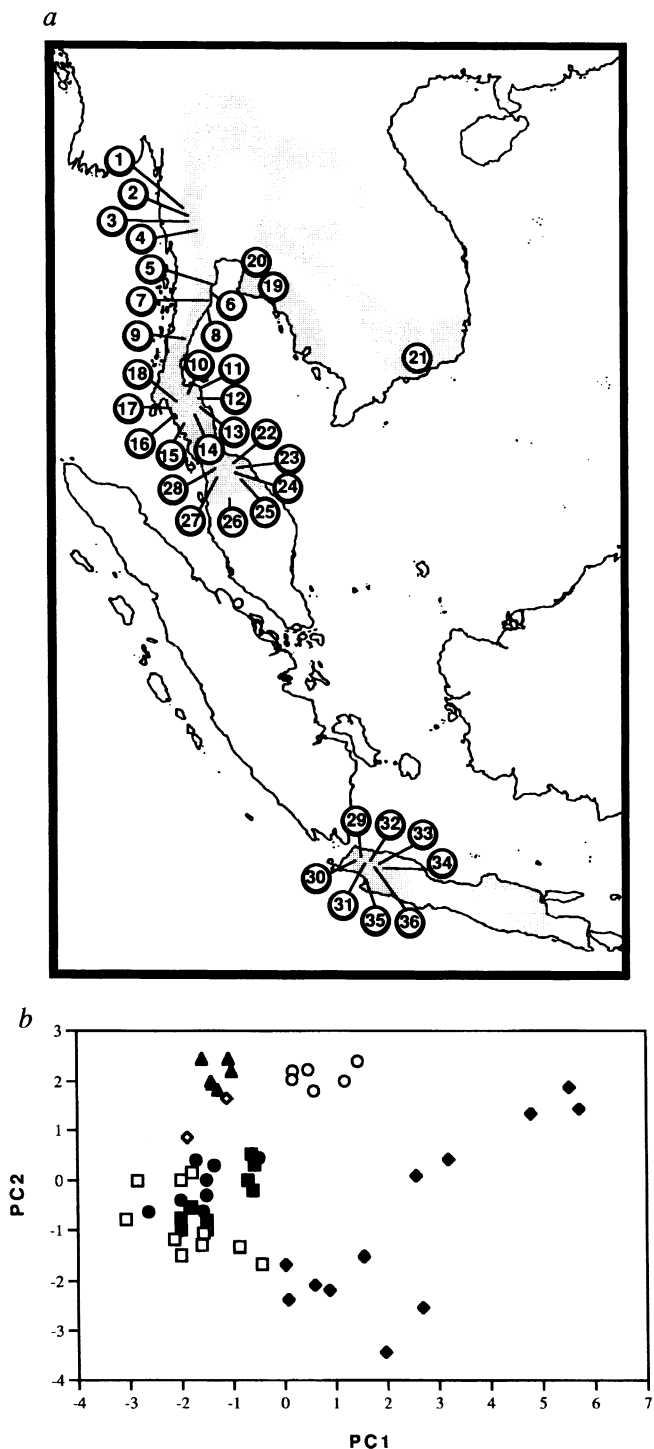


FIG. 1 a, The distribution of 36 sites in Southeast Asia from which venom samples of *Calloselasma rhodostoma* were collected. 67 adult specimens of >400 mm snout-vent length were captured in the Thai provinces of Kanchanaburi (sites 1–4); Prachuap Khiri Khan (5–8); Chumphon (9); Nakhon Si Thammarat (10–14); Krabi (15–18); Rayong (19, 20); also Vung Tau province, Vietnam (21); Kedah state, Malaysia (22–28); West Java, Indonesia (29–36). Shading represents the known distribution range of *C. rhodostoma*. b, Principal components analysis of isoelectrically focused electrophoretograms of venom samples collected in Kanchanaburi (○), Prachuap Khiri Khan (▲), Chumphon (△), Nakhon Si Thammarat and Krabi (●), Rayong (◇), Vietnam (◆), Malaysia (■) and Java (□). Venom was extracted and desiccated within 12 h of capture. The composition of individual samples was compared using isoelectric focusing across precast Ampholine PAGplate gels (Pharmacia), pH range 3.5–9.5. 15 μ l of each rehydrated venom (10 mg soluble protein per ml) was loaded and run for 80 min at 1,500 V before staining with Coomassie blue. The influence of gender was removed by excluding bands produced by females only (p/ 5.90

767-base-pair (bp) fragment of mitochondrial DNA amplified using polymerase chain reaction (PCR). Thirdly, variation in venom might be associated with geographical variation in diet. Faeces were palpated from each snake upon capture and prey items were identified to taxonomic class. The dietary analysis was supplemented by analysis of the stomach contents and faeces of preserved specimens from the same localities. Adult *C. rhodostoma* eat amphibians, reptiles and endotherms, but the relative contribution of each prey type shows highly significant geographical variation (Fig. 2).

Venom samples were grouped according to geographical origin (Fig. 1a), and thirteen IEF bands varied in distribution among the

TABLE 1 Intercorrelations between venom composition and matrices representing potential causal hypotheses

	Venom	Diet	Geography
Diet	0.5957*		
Geography	0.4021*	0.5535*	
Fitch–Margoliash with molecular clock	0.2119*	0.1563	0.1342
Fitch–Margoliash without molecular clock	0.0670	–0.0354	–0.0649
Maximum likelihood	0.0649	–0.0796	0.1332
Neighbour joining	0.2346*	0.3091*	0.1091
Dollo parsimony	0.0479	–0.0902	0.0946

Intercorrelations between distance matrices representing differences in overall venom composition, diet composition, geographical distance, and patristic distances based on phylogenetic trees generated by five algorithms. With the 67 venom samples grouped by geographical origin (Fig. 1a), thirteen distinct bands corresponding to different isoelectric points (pI); Table 2b) varied between the 36 groups. Overall mean venom composition of each group was recorded as a series of numbers denoting the occurrence of each band (so for example, the number 1 indicates that it is present in all group members; 0.5, that it is present in 50%; and 0, that it is absent from all). A 36 \times 36 distance matrix representing the variation in overall mean venom composition was compared to similarity matrices derived from three causal hypotheses (see text). Diet was recorded as the percentage of amphibians, reptiles and endotherms (birds and mammals) constituting the diet in each region (Fig. 2). Geographical proximity was computed from the mean latitude and the mean longitude of each group. The phylogenetic relationships among the snakes was assessed using PCR–RFLP analysis of mtDNA. A few millimetres of caudal tissue were removed from all 67 specimens under anaesthesia and stored in 70% ethanol. A 767-base-pair (bp) fragment of mtDNA, chiefly comprising the cytochrome *b* gene (709 bp), was amplified from these biopsies using the PCR oligonucleotide primers (5') L14841 (ref. 25) and (3') MVZ16 (ref. 26). Seven restriction endonucleases (*DdeI*, *EcoRI*, *HinfI*, *NciI*, *NlaIII*, *ScrFI*, *TaqI*) identified 15 different haplotypes among these fragments. Phylogenetic trees were generated based on the modal haplotype of each of the 36 regional groups (maximum likelihood and Dollo parsimony algorithms²⁷) or on the genetic distances between them, obtained using program DA, REAP²⁸ (neighbour-joining and Fitch–Margoliash with and without a molecular-clock assumption²⁷). The distance-based methods have the advantage that they consider within-population variation. Three main lineages are resolved by all algorithms: Western Thailand (Kanchanaburi and Prachuap Khiri Khan provinces); Vietnam with the rest of Thailand; Malaysia with Java (plus one group from Rayong province in Thailand). Patristic distance matrices were computed between each pair of groups for each of the 5 phylogenetic trees.

*Indicates significance beyond $P < 0.05$ after sequential Bonferroni correction to all tests²⁹.

and 6.90). Among the 67 venom samples, 53 different types were recognized; snakes that produced identical venoms invariably came from the same locality. Each variant was coded to denote the presence (1) or absence (0) of each variable pI band and a principal components (PC) analysis was conducted upon these data. The first two component scores (PC1, PC2) account for 27.97 and 14.67% of the total variation respectively. Mantel tests were run on matrices representing the totality of the between-population variation in venom composition.

36 groups. Partial Mantel tests were used to evaluate the association between the observed patterns of venom variation among groups (expressed as a taxonomic distance matrix) with patterns predicted by the three hypotheses, while simultaneously partialling out the effects of intercorrelation between the hypotheses (Table 1)⁶⁻¹⁰. Regressing out the patristic distances from the largely selectively neutral molecular phylogeny allows one to test for ecogenetic adaptation free of patristic phylogenetic relationships¹⁰. The partial Mantel test with overall venom composition (combining all variable bands) as the dependent variable, and geographical proximity, patristic distance and diet as independent variables, found diet alone to be significantly partially correlated (Table 2). When the pattern of variation in each band was tested in turn, at least 5 of the 7-9 bands showing significant association with any of the causal hypotheses were significantly associated with average diet at each locality. Only one band was significantly associated with population phylogeny, casting serious doubt upon the hitherto unchallenged reliability of venom as a taxonomic tool¹⁸⁻²⁰. These conclusions are supported by independent contrast methods¹¹ which show diet and venom to be correlated, free of phylogenetic effects (Table 2).

The variable components have not yet been identified, but preliminary data indicates that the variation in IEF electropho-

retograms is congruent with geographical variation in the venoms' enzymatic activities (unpublished data). Pitvipers typically ingest relatively large prey items²¹ and their venoms seem to have two main functions, namely prey immobilization and digestion¹²⁻¹⁴. As prey taxa vary markedly in their susceptibility to snake venom^{15,16}, it would seem that natural selection has caused different *C. rhodostoma* populations to produce venoms appropriate for subduing and digesting the local diet. This conclusion is further supported at the within-population level by the simultaneous ontogenetic changes in diet and venom composition.

It should be noted that venoms of captive-bred *C. rhodostoma* produced electrophoretic profiles identical to those of wild specimens from the same locality as their parents, in spite of their unnatural diet in captivity. This indicates that the venom/prey association is inherited rather than environmentally induced; studies of other snakes support the conclusion that venom composition is under strict (non-plastic) genetic control^{22,23}.

The findings of this study have important implications for snakebite therapy. The compositional variation elucidated using IEF may well reflect variation in the venoms' immunological properties²⁴, and it should be feasible to produce a more widely effective antivenom against *C. rhodostoma* by taking the geographical variation into account¹. Unfortunately, our results also

TABLE 2 Significance of partial regressions between venom composition and causal hypotheses

	Geographic proximity	Patristic distance	Diet
(a) Overall venom composition			
Venom versus geographical distance and diet	0.2525		0.0001*
Fitch-Margoliash with molecular clock	0.5358	0.0225	0.0001*
		0.0217	0.0002*
Fitch-Margoliash without molecular clock	0.2255	0.1329	0.0001*
		0.1435	0.0001*
Maximum likelihood	0.3792	0.1338	0.0001*
		0.0905	0.0001*
Neighbour joining	0.2272	0.2490	0.0001*
		0.3075	0.0001*
Dollo parsimony	0.3578	0.0976	0.0001*
		0.0916	0.0001*
(b) Individual venom bands†			
Band 1, p/ 9.40	0.0576	0.7012	0.0046‡
Band 2, p/ 9.15§	0.0009*	0.0018	0.0506
Band 3, p/ 8.55	0.0041	0.1680	0.1023
Band 4, p/ 8.45	0.6542	0.2217	0.3542
Band 5, p/ 8.25	0.0083	0.6280	0.0011*
Band 6, p/ 7.85	0.6182	0.0862	0.4000
Band 7, p/ 7.45	0.8052	0.0015*	0.7296
Band 8, p/ 6.55	0.0321	0.6700	0.0012*
Band 9, p/ 6.10	0.1113	0.6429	0.7233
Band 10, p/ 6.00	0.0009*	0.0160	0.0624
Band 11, p/ 5.80	0.0542	0.4829	0.0012*
Band 12, p/ 5.70	0.0247	0.4727	0.0002*
Band 13, p/ 5.45	0.0044	0.4944	0.0006*

a, Partial Mantel matrix association tests: null hypothesis probabilities for the partial regression between overall venom variation and causal hypotheses (diet, geographical distance and each of the 5 phylogenetic trees). Other potential ecogenetic factors, such as rainfall, temperature and vegetation, were tested but showed no association with any venom band. Partial Mantel tests⁶⁻¹⁰ show that the pattern of geographical variation in overall venom composition is strongly associated with diet, but not with geographical distance, or phylogeny, irrespective of the phylogenetic algorithm used. To control for possible confounding effects from the significant intercorrelation between the causal hypotheses, further Mantel tests were run that excluded either geographical distance or patristic distance from the analyses. In all cases, venom composition retains its significant partial regression with diet, but not with the other causal hypotheses. Each partial Mantel test entailed 10,000 randomizations. The comparative method of independent contrasts under the gradual change model¹¹ (as well as other models) supports these conclusions as it gives a significant correlation ($r = 0.669$; $P < 0.01$) between diet and venom, where the continuous variables representing venom and diet variation are the largest principal coordinate of the venom and diet matrices respectively. However, this procedure is less appropriate than the partial Mantel test because it cannot control for possible gene flow between populations (or other multidimensional hypotheses), and does not, on its own, consider multiple competing hypotheses free of distribution. b, Null hypothesis probabilities for the partial correlation between the 13 individual venom components and causal hypotheses. At least 5 of the 7-9 bands showing significant association with any causal hypothesis were significantly associated with diet, two with geographical proximity (probably reflecting the greater opportunity for gene flow between spatially close demes) and only one with patristic distance (phylogeny).

*Significance beyond $P < 0.05$ after sequential Bonferroni correction to all tests within the table²⁹.

†Probability values for individual bands are based on the Fitch-Margoliash tree with molecular clock assumption. The use of the other phylogenetic trees resulted in identical patterns of significant association except where noted below.

‡Significantly associated with diet when phylogeny is represented by the Dollo parsimony tree.

§Not significantly associated with any causal hypothesis if phylogeny is represented by the maximum likelihood tree.

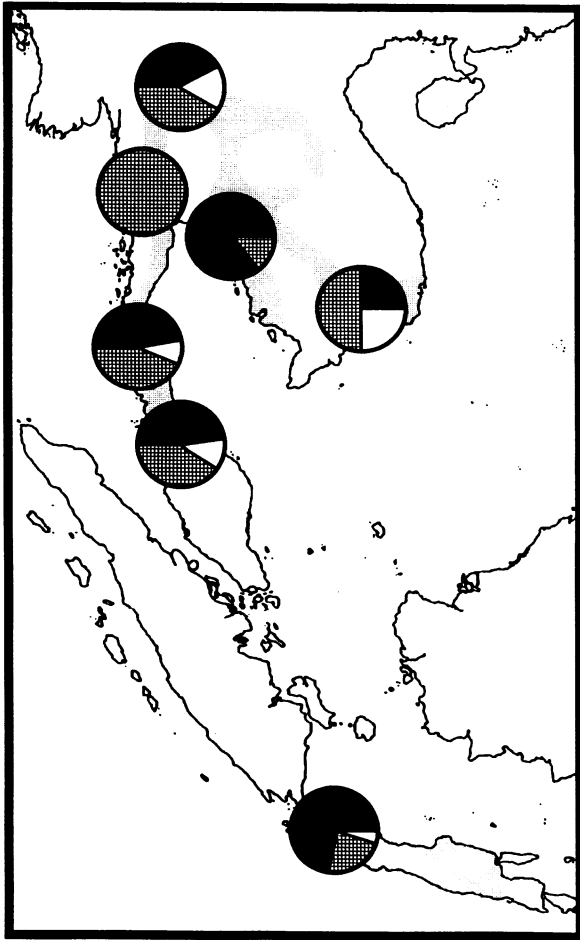


FIG. 2 Geographical variation in the diet of adult *C. rhodostoma*. 216 prey items were identified from faeces and stomach contents. The pie charts represent the contribution of amphibians (white), reptiles (cross-hatched) and endotherms (black) as a percentage of the number of items identified from specimens of >400 mm snout-vent length. Background shading represents the known distribution range of this snake.

indicate that it may not be easy to predict how the venom of other medically important snakes will vary; the population phylogeny may not provide a reliable guide and few species have been subjected to sufficiently rigorous ecological studies to enable patterns of venom variation to be inferred from their diet. □

Received 25 April; accepted 22 November 1995.

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ACKNOWLEDGEMENTS. We thank N.-H. Tan and G. Ponnudurai for enzyme assays, J. Norman for advice on molecular methods, B.J.F. Manly and T. Garland for programs, and the museums that loaned specimens to us. This research was primarily funded by a SERC studentship (J.C.D.) and the Leverhulme Trust (R.S.T. and A. Malhotra), with support from NERC (W.W.) and the Royal Society (R.S.T.).