

Phylogeography of the Russell's viper (*Daboia russelii*) complex in relation to variation in the colour pattern and symptoms of envenoming

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The Russell's viper complex has a patchy (relict) distribution over large areas of Asia from Pakistan to Taiwan and the Lesser Sunda islands. In many areas it is the primary cause of snakebite mortality, and hence a serious medical problem. A multigene mitochondrial gene tree, supported by multivariate morphometry and basic colour pattern, suggests a primary split in the organismal phylogeny giving distinct, diagnosable, eastern and western forms that we recognize as full species: *Daboia russelii* (west of the Bay of Bengal) and *Daboia siamensis* (east of the Bay of Bengal). The clinical symptoms of human envenoming show marked geographic variations that are broadly unrelated to the phylogeny. The molecular phylogeny, together with current distribution and fossil record, suggests cycles of extreme expansion and contraction for this complex. Further studies on venom variation, diet and local population phylogeny are required, but the local and regional variation in symptoms may be the result of fixation of venom genes during cyclical bottlenecks which could explain the haphazard, non-phylogenetic pattern of symptoms of envenomation in this species complex.

Key words: multigene phylogeography, relict distribution, medically important venomous snake

INTRODUCTION

Studying the geographic variation within a species by combining molecular phylogeography (Avice, 2000) with an analysis of other systems, such as multivariate morphology (Brown et al., 2000; Malhotra & Thorpe, 2000a), size (Giannasi et al., 2000), colour (Thorpe, 2002), reproductive strategy (Surget-Groba et al., 2001), biochemistry (Daltry et al., 1996) or current gene flow (Thorpe & Richard, 2001; Ogden & Thorpe, 2002) can allow us to evaluate the roles of natural selection and population history (e.g. vicariance) in the processes of speciation and character evolution. Moreover, earlier work on the geographic variation of a species may leave a legacy of a suite of subspecies, and in these cases the molecular phylogeography combined with other approaches may enable us to evaluate their validity. In many cases the subspecies may reflect adaptation by natural selection of one particular character set (Thorpe & Stenson, 2003) rather than be taxonomically useful and predictive of other features. However, in other cases the subspecies actually warrant species status (Zamudio & Greene, 1997; Parkinson et al., 2000; Keogh et al., 2001). With medically important organisms, such nomenclatural changes may take on added importance (Wüster, 1998) and, in addition, a robust organismal phylogeny may enable other clinical/medical features to be properly evaluated. Examples of how a phylogenetic perspective facilitates an understanding of venom variation are afforded by Daltry et al.'s (1996) study of the Malayan pitviper and Creer et al.'s (2003) study of the Taiwanese bamboo viper, which elucidate the role of ecological factors, such as diet, in venom evolution.

The Asian snake *Daboia russelii* (Shaw & Nodder, 1797) is a medium to large, very dangerously venomous, viper: see David & Ineich (1999) and McDiarmid et al. (1999) for nomenclature. In many parts of the range it is a major cause of snakebite mortality and morbidity and an important health risk (Looareesuwan et al., 1988; Warrell, 1989). There is an apparent lack of agreement between many aspects of variation in this species complex, namely the conventional subspecies, the multivariate morphology, the colour pattern, the clinical symptoms of envenoming and anti-venom efficacy.

Russell's viper is distributed with varying densities and occurrence through the Indian subcontinent (Daniel, 1983) and then, with marked discontinuity, east to China and Taiwan and south to the Lesser Sunda islands (Fig. 1). The widespread patchy mainland distribution of allopatric populations across southern and eastern Asia presumably reflects a relict of a more complete distribution in the past. Generally, each allopatric patch has a "conventional" subspecies (i.e. based on superficial morphological criteria) with five to seven being recognized – see Wüster et al. (1992) and Wüster (1998) for reviews. These subspecies do not relate to the considerable geographic variation in the clinical manifestations of envenoming of humans reviewed in Warrell (1989, 1997) and Belt et al. (1997). While the clinical symptoms may be functionally related to the differences in venom composition and enzyme activity (Jayanthi & Gowda, 1988; Woodhams et al., 1990), the pattern of variation does not relate either to the conventional subspecies, or very obviously to the efficacy of anti-venoms (Wüster, 1998). A multivariate morphometric analysis by Wüster et al. (1992) showed two distinct forms (a western form on the Indian

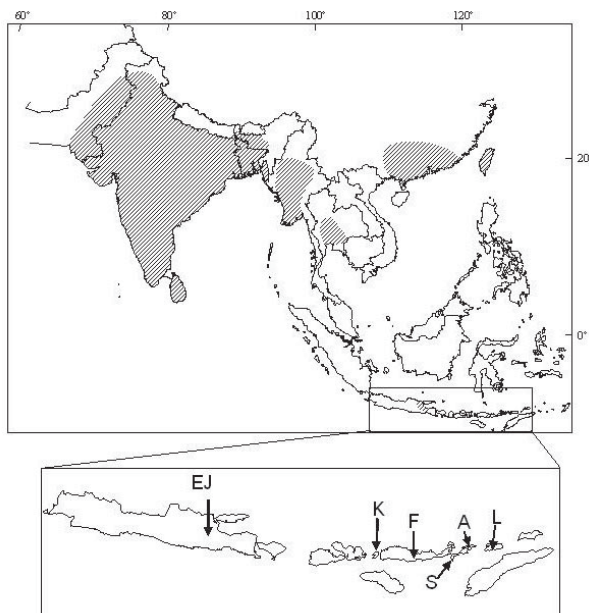


Fig. 1. The distribution of *Daboia russelii*. The Indian subcontinent distribution is not as continuous as the stylized diagram suggests (particularly in the Ganges delta). In the inset of the Lesser Sundas, there are records from East Java (EJ), Komodo (K), Flores (F), Solor (S), Adonara (A) and Lembata (L).

subcontinent, and another including all the populations to the east of the Bay of Bengal) that they named as subspecies, replacing the numerous conventional subspecies. Hence *Daboia russelii russelii* in the west incorporates the previous subspecies *russelii*, *pulchella* (Gray, 1842) and *nordicus* (Deraniyagala, 1945), while *D. russelii siamensis* (Smith, 1917) in the east incorporates the previous subspecies *siamensis*, *limitis* (Mertens, 1927), *sublimitis* (Kopstein, 1936) and *formosensis* (Maki, 1931). These two forms can be clearly diagnosed by different colour patterns (Wüster, 1998), but do not readily relate to the variation in envenoming symptoms or anti-venom efficacy (Belt et al., 1997) any more than did the conventional subspecies. Although the multivariate analysis suggested two very distinct forms, due to the lack of molecular phylogenetic studies or other corroboration,

Wüster et al. (1992) refrained from giving the two forms full species status. A fuller understanding of the taxonomic status of these forms, the role of ecology and other factors in the evolution of the venom, and variation in envenoming symptoms may be facilitated by a phylogeny of the complex. Here we present the first mitochondrial DNA phylogeny for the complex, and compare this multigene phylogeny to the morphological pattern and the pattern of clinical effects of the venom.

MATERIALS AND METHODS

Sampling and DNA template preparation

Samples (Table 1) were in the form of tail-tip or liver tissue preserved in 80% ethanol, or blood taken from the caudal vein and stored in 0.1M EDTA pH 8.0; 100mM Tris pH 8.0; 3% SDS. Whole genomic DNA was extracted and purified following the protocol of Sambrook et al. (1989).

Polymerase Chain Reaction (PCR) amplification and mtDNA sequencing

Fragments of three mitochondrial genes were amplified, cytochrome b (*cyt b*; 758 bp), NADH dehydrogenase subunit 4 (ND4; 900 bp), and NADH dehydrogenase subunit 2 (ND2; 363 bp). The *cyt b* primers were 5'-TCA AAC ATC TCA ACC TGA TGA AA-3' (703Bot L-strand – modified from Kocher et al., 1989) and 5'-GGC AAA TAG GAA GTA TCA TTC TG-3' (H-strand, modified version of primer MVZ 16 of Moritz et al., 1992). The ND4 primers were from Arévalo et al. (1994) and the ND2 primers from Hackett (1996 and references therein).

PCR cycling parameters were identical for all three genes: after an initial 5-minute period of denaturation at 94 °C, there were 30 cycles with 45 secs denaturation at 94 °C, 30 secs annealing at 50 °C and 60 secs extension at 72 °C, followed by a final 3-min extension at 72 °C. PCR products were purified using the Wizard® PCR purification system (Promega), then sequenced by cycle sequencing using dye-labelled terminators (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit) followed by electrophoresis on an ABI Prism 377 DNA sequencer.

Phylogenetic analyses

Sequences were aligned by eye and translated to amino acid sequences in MEGA version 2.1 (Kumar et al., 2001) to check that stop codons (indicating pseudogenes) were

Table 1. Sample localities and GenBank accession numbers.

Locality	Cyt <i>b</i>	ND2	ND4
Fong Shan, Pingtung, Taiwan	AY165089	AY165077	AY165064
Tuban, W of Gresik, East Java, Indonesia	AY165083-4	AY165070-1	AY165057-8
Tonggurambang, Mbay, Flores, Indonesia	AY165085-6	AY165072-3	AY165059-60
Hauk Kyunt, Myanmar	AY165080	AY165067	AY165054
Thailand (southern central)	AY165090-1	AY165078-9	AY165065-6
Thayur, Tamil Nadu, India	AY165087	AY165075	AY165062
Gampola, Sri Lanka	AY165088	AY165076	AY165063
Poipet, Sisophon, Cambodia	AY165081	AY165068	AY165055
Guangdong, China	AY165082	AY165069	AY165056
Pakistan*	AJ275723*	AY165074	AY165061

*From Lenk et al. (2001)

absent. All variable sites were double checked against the original ABI 377 chromatograms viewed in Chromas 1.51 (Technelysium Pty Ltd, 1998; www.technelysium.com.au/chromas.html). All further analyses were conducted in PAUP* 4.0 8a (Swofford, 2001) unless stated otherwise (e.g. the Bayesian analysis). Regression (with cytochrome *b* distances arbitrarily as the independent variable) was used to compare uncorrected pairwise distances of all genes to check that they reflected similar rates of base substitution. The mtDNA genes were then concatenated taking care not to disrupt the reading frames. Each mitochondrial fragment was designated as a separate partition. To ensure that there was phylogenetic congruence among the three adjoined fragments, we used a partition homogeneity test (Farris et al., 1994) specifying 100 heuristic search replicates, random addition of sequences and tree bisection–reconnection (TBR) branch swapping.

A range of preliminary tests was conducted prior to phylogenetic reconstruction. The presence of phylogenetic signal (Hillis, 1991; Hillis & Huelsenbeck, 1992) in the data was established from g_i statistics calculated from a search of 10^6 random trees. In order to determine if signal was spread throughout the tree, or concentrated at one particular node, all possible trees were then searched on 12 OTUs (12 is the maximum number of OTUs possible for this test, hence the Pakistan haplotype and outgroups were excluded) with iterative deletion of taxa from the two most basal and well-supported nodes in the tree. The homogeneity of base frequencies among haplotypes was compared using a chi-square test. Nucleotide saturation was assessed for transitions and transversions respectively at each codon position by plotting uncorrected *p*-distances against HKY85+ Γ corrected distances (the model HKY85+ Γ was found to be the one that best describes the mode of evolution among the ingroup haplotypes: see section on Bayesian and maximum likelihood analysis below). Neutrality (Kimura, 1983) was tested using a McDonald and Kreitman test (McDonald & Kreitman, 1991; Ballard & Kreitman, 1994) implemented in DnaSP Version 3 (Rozas & Rozas, 1999). This test is based on a comparison of synonymous and nonsynonymous (replacement) variation within and between two clades. Clade 1 included all OTUs from the eastern clade, and clade 2 included India, Pakistan and Sri Lanka. Using a chi-square test, the rate constancy of sequence evolution across OTUs was checked by comparing the likelihood score of a tree in which a molecular clock had been enforced with that of the unconstrained likelihood tree.

Using the combined data set, and including *Echis ocellatus* and *Vipera berus* as outgroups, phylogenies were reconstructed using Bayesian analysis (Huelsenbeck & Ronquist, 2001), maximum parsimony (MP; Swofford & Olsen, 1990) and maximum likelihood (ML; Felsenstein, 1981). Bayesian analysis was performed with MrBayes 3.0b4 using an HKY85+ Γ (Hasegawa et al., 1985) model of evolution selected for the ingroup by MODELTEST 3.0 (Posada & Crandall, 1998). The model parameters were: base frequencies = A: 0.3243, C: 0.2953, G: 0.0969, T: 0.2835; transition/transversion ra-

tio = 7.3743; rates = Γ , shape = 0.3440; proportion of invariable sites = 0. The Bayesian Markov chain Monte Carlo analysis was started with four simultaneous runs of four chains (three heated, one cold) for 5,000,000 generations, with trees being sampled every 200 generations giving a total of 25,002 trees. The first 6250 trees were discarded as “burn-in” (the number of trees before the likelihood scores converged), and the remaining trees were compared to determine the posterior probability of the nodes within the phylogeny. Bayesian analysis has computational advantages over ML (Leaché & Reader, 2002). The MP analysis involved a heuristic search of 5000 replicates, with TBR branch swapping and random addition of sequences. Heuristic searching (1000 replicates) was used in the ML analysis, imposing the model selected by MODELTEST (see above). Node support for ML and MP trees was estimated using the bootstrap (Felsenstein, 1985). Bootstrapping was carried out by heuristic searching (100 random replications, TBR swapping), with 1000 bootstrap replicates in the case of MP and 100 replicates for the ML analysis, while imposing the same model used for the tree construction. For the MP trees, branch support was also determined from Bremer support (Bremer, 1994), calculated in the program SEPAL 1.4 (Salisbury, 2000).

The clinical symptoms of envenoming of humans are derived from previous studies (Ariaratnam et al., 2001; Warrell, 1989, 1995; Belt et al., 1997 and references therein; Mukherjee et al., 2000; Hung et al., 2002), and the basic colour pattern is derived primarily from the 225 museum specimens studied by Wüster et al. (1992). These characteristics were then mapped on the Bayesian tree using MACCLADE v3.0 (Maddison & Maddison, 1992) with the unsupported nodes collapsed.

RESULTS

For the ingroup the fragment size, number of variable sites and number of parsimony informative sites for *cyt b* are 576, 87 and 69 bp respectively, for ND2 they are 306, 40 and 33 bp respectively and for ND4 they are 606, 94 and 65 bp respectively. Regression of the genetic distances was found to suggest similar rates of base substitution in the three mitochondrial gene fragments, with ND4 approximately the same rate as *cyt b* (slope = 1.00), and ND2 slightly slower than *cyt b* (slope = 0.85). The null hypothesis of congruence between character partitions could not be rejected ($P=0.39$). Strong phylogenetic signal was detected throughout the data set ($P<0.01$: unconstrained search $g_i=-1.389$; search constrained to specific strongly supported nodes: $g_i=-0.909$ and -0.700 respectively). Base frequencies were found to be homogeneous among OTUs ($\chi^2=19.15$; $P=0.99$). There was no saturation at the first and second codon positions and only negligible saturation of transitions at the third (plots not shown). The McDonald and Kreitman test failed to reject the null hypothesis of neutral evolution (Fisher's exact test, two tailed: $P>0.05$), and the interior branch test showed that all interior branch lengths were greater than zero ($P>0.05$). A non-significant difference in likelihood scores between the unconstrained and clock-enforced trees ($df=13$,

Table 2. Uncorrected genetic distances ($\times 100$) between localities (lower diagonal) and their standard errors (upper diagonal).

	Cambodia	China	East Java	Flores	Myanmar	Pakistan	India	Sri Lanka	Taiwan	Thailand
Cambodia		0.4	0.3	0.3	2.6	0.8	0.7	0.7	0.3	0.3
China	1.9		0.3	0.3	2.9	0.8	0.8	0.7	0.2	0.3
East Java	1.6	1.1		0.3	2.7	0.8	0.8	0.8	0.3	0.3
Flores	1.4	1.3	1.1		2.2	0.8	0.8	0.7	0.3	0.2
Myanmar	0.4	0.5	0.4	0.4		0.8	0.7	0.7	0.4	0.4
Pakistan	10.3	10.3	10.2	10.0	10.3		0.5	0.4	0.8	0.8
India	10.4	10.5	10.6	10.2	10.4	3.1		0.2	0.8	0.8
Sri Lanka	10.2	10.3	10.4	10.0	10.2	2.9	0.3		0.7	0.7
Taiwan	1.6	0.5	1.0	1.0	2.6	10.0	10.3	10.1		0.3
Thailand	1.4	1.7	1.4	0.8	2.3	10.0	10.1	10.0	1.4	

$\chi^2=14.9152$, $P>0.05$) suggests a consistent rate of evolution between branches.

The Bayesian analysis of combined sequence (1488 bp) from the mtDNA genes inferred a tree (Fig. 2) with the same topology as the MP (length 703 steps) and a very similar topology to the ML tree ($-\ln L = 4995.4270$). For all trees the primary split is between east and west clades and these have very strong node support (Fig. 2). The divergence between these two clades is substantial, and the ratio of between-clade to within-clade pairwise divergence is striking (Table 2). Between-locality divergence (uncorrected) within each respective primary clade only has a maximum of just over 3%, but between different primary clades it ranges from 10.0 to 10.6%. Within the western clade the Pakistan sample is basal, with the Sri Lankan and Indian southern samples as the sister group. Within the eastern clade, there is less divergence and little structure in this combined analysis of approximately 1.5 Kbp. There is effectively a “star” phylogeny as the five eastern lineages are separated with only very short branch lengths (generally with poorly supported nodes). They are: 1) Cambodia, 2) Myanmar, 3) Thailand, 4) Flores and 5) China/Taiwan/Java. The Myanmar sample is basal in the Bayesian and MP trees, but the Cambodian sample is basal in the ML tree. Subsequently, the Javan branch is sister to the geographically distant Taiwan/China branch, rather than the geographically adjacent Flores branch, and this generally has good support.

When colour pattern (Fig. 3) and the clinical symptoms of envenoming (Fig. 4) are plotted on the phylogeny it is evident that the colour pattern is unambiguously related to the east–west clades, but the envenoming symptoms bear little relation to the phylogeny (note that considerable caution is required in interpreting Fig. 4; see legend). The set of symptoms involving renal failure, bleeding and coagulopathy is widespread across the complex and occurs in all areas that have been studied (Fig. 4A). Apart from this, all other symptoms occur rather haphazardly, with some members of both eastern and western clades invoking symptoms of neuro-myotoxicity (Fig. 4B), intravascular haemolysis (Fig. 4C), primary shock and hypotension (Fig. 4E), pituitary infarction (Fig. 4F) and perhaps generalized capillary permeability (see legend for Fig. 4D). Moreover, within clades, geographically adja-

cent populations may invoke different symptoms. In the west the Sri Lankan and Indian populations differ in whether or not they cause pituitary infarction (Fig. 4F). In the east, for example, the Myanmar and Thai populations differ in whether or not they cause intravascular haemolysis (Fig. 4C), generalized capillary permeability (Fig. 4D), primary shock and hypotension (Fig. 4E) and pituitary infarction (Fig. 4F).

DISCUSSION

The mitochondrial gene tree clearly shows separate, well-supported east–west clades with relatively little divergence within each of these primary clades compared to the substantial divergence between them. This is supported by the multivariate morphology (Wüster et al., 1992) and the colour pattern. The multivariate morphology also shows relatively high between-group divergence for these east–west groups and the basic colour pattern is entirely diagnostic. West of the Bay of Bengal the snakes have a lateral row of circular/sub-circular blotches on either side of a vertebral row, giving three rows. East of the Bay of Bengal there is an additional double row of irregular triangular blotches “between” the vertebral and lateral rows giving five or seven rows depending on whether the double row is recognized as one or two rows per side (see Fig. 3 for a fuller description). Although a mtDNA gene tree may not necessarily reflect the species tree (Page & Holmes, 1998), in this case the primary division in the gene tree is likely to represent the primary division in the species tree, because of the corroboration of the basic colour pattern and multivariate morphometry. There is no introgression or contact zone between these forms and therefore no intermediates. Based solely on multivariate morphology, Wüster et al. (1992) named these forms as subspecies, recognizing that more study was required before raising them to species status. This molecular phylogeographic investigation fulfils this requirement and we name these two forms as full species. Hence, in the west, *D. r. russelii*, *sensu* Wüster et al. (1992), is raised to full species status, *D. russelii*, and incorporates *pulchella* and *nordicus*, while in the east, *D. r. siamensis*, *sensu* Wüster et al. (1992), is raised to full species status, *D. siamensis*, and incorporates *limitis*,

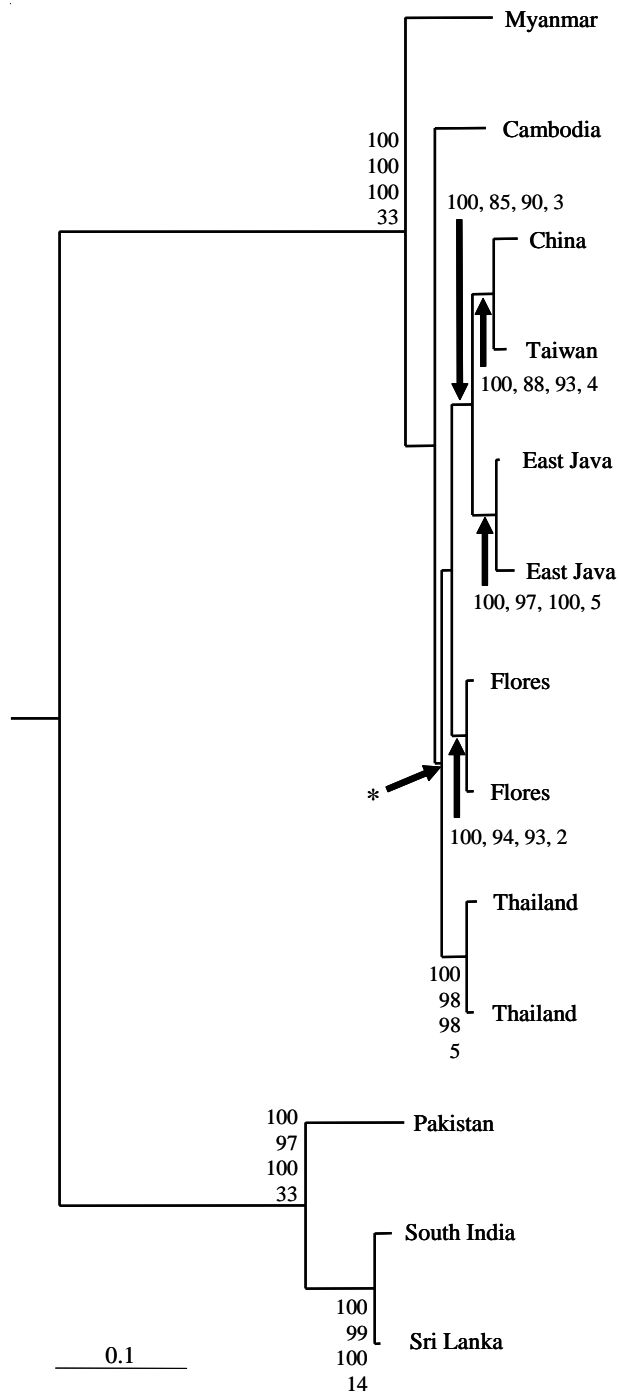


Fig. 2. Combined mtDNA gene tree. Bayesian tree with branch lengths and nodes support where it is greater than 95% (*this node has 88% support). Below (or after) the node support for the Bayesian tree is the bootstrap support for the node in the ML tree, followed by the bootstrap, then Bremer, support for the node in the MP tree.

sublimitis and *formosensis*. The full synonymy is given in Wüster (1998) and McDiarmid et al. (1999) and the basic colour pattern is diagnostic (Fig. 3).

The patchy distribution and relative lack of deep divergence within *D. siamensis* is notable. In fact there are long undivided branches for both eastern and western clades after the primary split. This may reflect extreme bottlenecking towards the end of this period. Timing

these periods exactly with a molecular clock is problematic (Zamudio & Greene, 1997; Wüster et al., 2002) and unnecessary as only an approximation is required together with an appreciation of the relative, rather than absolute, times. If we assume an approximate rate of change in Cyt *b* of 1–1.5% (uncorrected) per my for this fairly large-bodied ectotherm (Bromham, 2002; Wüster et al., 2002; Thorpe & Stenson, 2003 and references therein), this would suggest divergence of the primary east–west clades approximately 7–11 mybp, with a bottleneck for both clades occurring before their subsequent radiation at about 2–3 mybp. The phylogeny suggests that at approximately 2–3 mybp there was an almost simultaneous divergence of the eastern lineages (Cambodia; Myanmar; Thailand; Lesser Sundas; Java/China). This lineage divergence was likely to be at a time of mainland range expansion. In the east, the Sunda shelf appears to have been exposed at times of lower sea level (Heaney, 1991; Karns et al., 2000) and would have allowed the overland colonization of Java from ancestors held in common with those that colonized mainland China and Taiwan. The overland colonization of Java is reminiscent of the situation in the Malayan pit viper (Daltry et al., 1996) and the white-lipped pit viper (Giannasi et al., 2001). Taiwan has been joined to mainland China repeatedly over the Pleistocene (Huang, 1984). The lack of distinct divergence of the Taiwanese Russell's viper, as with the bamboo viper *Trimeresurus stejnegeri* (Creer et al., 2001), suggests fairly recent overland colonization of Taiwan.

On the other hand the Lesser Sunda islands occupied by Russell's viper (Komodo/Flores and islands eastwards) would not have been recently connected to the mainland and are distinct from the adjacent East Javan populations in both their multivariate morphometry (Wüster et al., 1992) and molecular phylogeny. This is in contrast to *Cryptelytrops (Trimeresurus) insularis* (*C. albolabris* group) (Malhotra & Thorpe, 2000b) where the East Javan populations are similar to populations in Flores (*C. insularis*), but distinct from the west Javan form (referred to as *C. albolabris sensu stricto*).

In the Russell's viper complex, with the exception of the over-water colonization of the Lesser Sundas, the eastern range was presumably colonized overland as part of a more continuous distribution, before more recent range contraction. Consequently, this species complex appears to have undergone cycles of substantial range expansion and contraction to produce this phylogenetic and distributional pattern. This is supported by a fossil similar to Russell's viper belonging to the broad Russell's viper group (*sensu* Szyndlar, 1988), in the mid Pliocene of Spain. These cycles, together with the current pattern of extreme abundance in some localities and rarity or absence in adjacent localities (Brongersma, 1958; Daniel, 1983) suggests that it is, in some critical aspect, stenoecious.

The incongruence between the evenomation symptoms and the primary phylogenetic division (supported by basic colour pattern and multivariate morphometry) stems from the evolutionary levels at which they vary. Although there is some multivariate morphometric variation within the eastern species, it is relatively slight and,

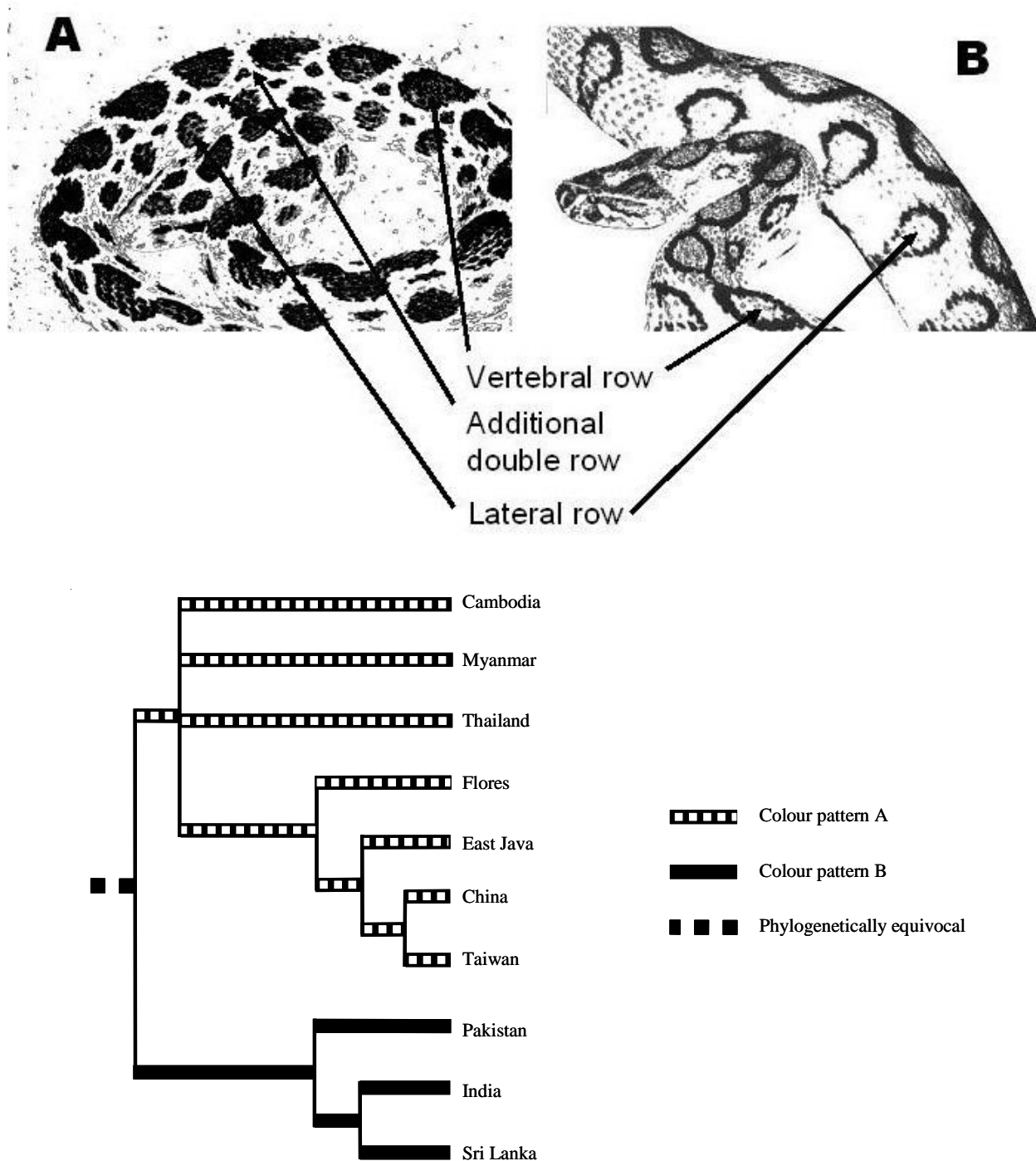


Fig. 3. Phylogenetic distribution of basic colour pattern types. Type A is found consistently in the east and type B consistently in the west. Note that pattern type A has an additional double row of dark blotches. The genetic basis of the diagnostic colour pattern is unknown, but in type A the additional double row of dark blotches appears to be due to the basic background colour being dark with the vertebral and lateral blotches being circled by a broad ring of lighter colour that isolates a double row of dark irregular triangles or blotches. The upper triangles are at the base of the vertebral row and point up, and the lower triangles are at the top of the lateral row and point down. In addition this darker background in the east tends to give the appearance of the vertebral and lateral blotches being more solid compared to the west.

as stated above, the molecular data shows relatively little variation within either the eastern or western lineages. However, the venom symptomatology varies at a much lower evolutionary level than the east–west split. Given the difference in scale between basic phylogenetic divi-

sion and the regional symptoms, no clear relationship is to be expected between venom evolution and phylogeny in this complex and the phylogenetic relationships do not allow prediction of the symptoms in areas where they have not been studied directly. Although one may very

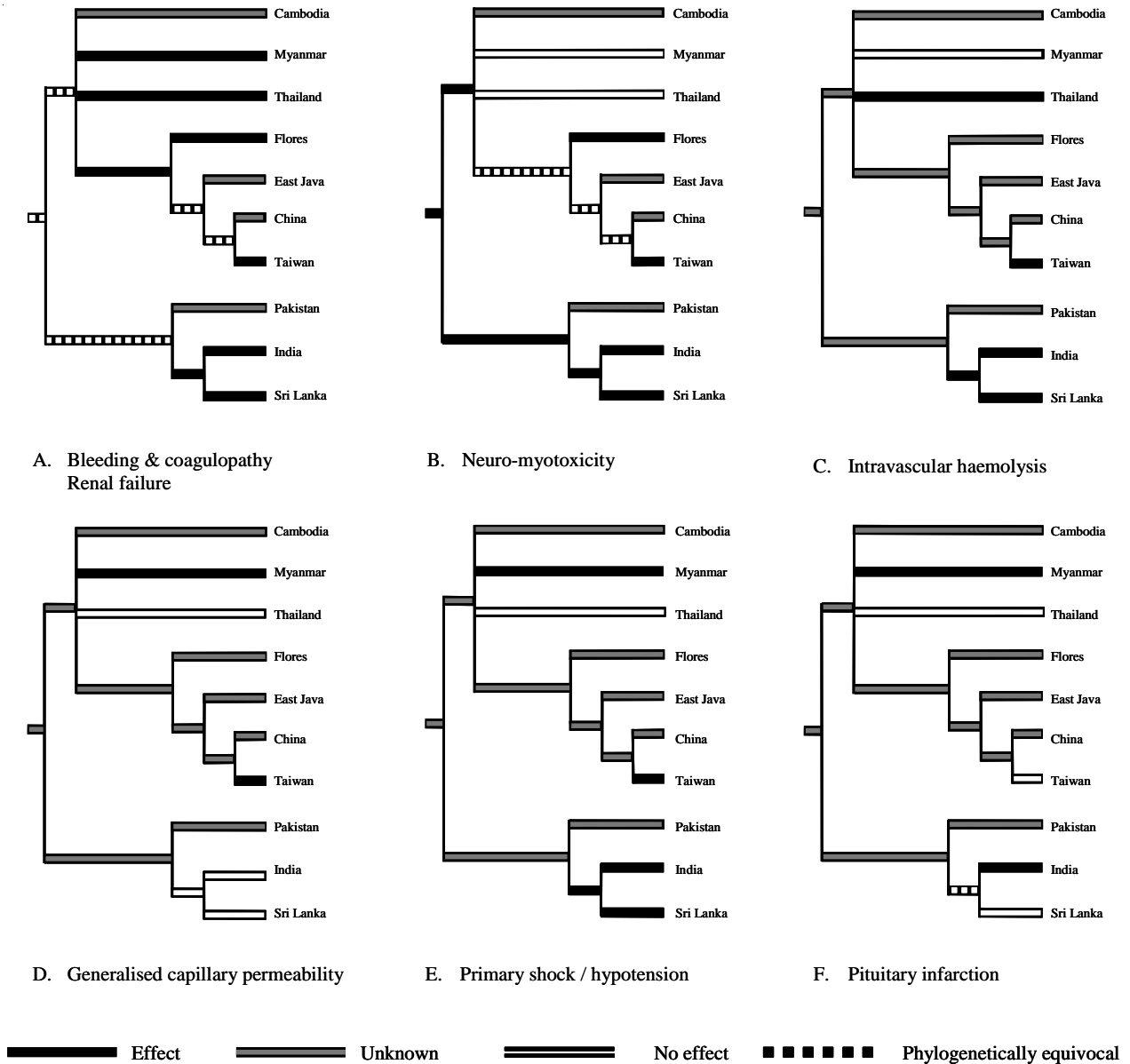


Fig. 4. Geographic variation in the clinical effects of Russell's viper venom in relation to phylogeny. There are no data available for Cambodia, East Java, China and Pakistan and this is indicated on the trees. In addition, considerable caution needs to be exercised in interpreting this figure. While more detailed data is available for areas such as Sri Lanka (Ariaratnam et al., 2001) and Myanmar (Warrell, 1989, 1995), in many areas occupied by this complex there is a shortage of rigorous clinical studies with large samples and proven identification of the envenoming snake. Data may be compromised because it is anecdotal rather than clinical, the identity of the envenoming species may be questionable, inconsistent reports exist, and clinical symptoms may have occurred for extraneous reasons. In particular, for Fig. 4B, the evidence for neurotoxicity in Flores (Belt et al., 1997) is admittedly weak, and there are inconsistencies in the reports from Taiwan where there may be causes other than envenomation for the neuromuscular symptoms. For Fig. 4C, the vulnerability to intravascular haemolysis may depend on G6PD status which was generally not determined, raising questions as to the reliability of the data. For Fig. 4D generalized capillary permeability is reliably recorded only for Myanmar as the evidence for this in Taiwan is questionable. There are anecdotal reports of generalized capillary permeability from India but in the absence of published evidence we have scored it as absent.

tentatively suggest that the symptoms of envenoming in China may be similar to those in Taiwan, it is more difficult to predict those in eastern Java, and impossible to predict the symptoms in Pakistan. It may be that, as with the Malayan pit viper (Daltry et al., 1996) and the bamboo viper

(Creer et al., 2003), there is ecological adaptation to diet. However, this is not necessarily the case and it may simply be that the cycles of expansion and bottlenecking have produced a somewhat "random" pattern of fixation of the venom genes (ultimately determining the symptoms

of envenoming) that now relates to neither phylogeny nor ecological factors such as diet. This is the interpretation that we support.

An investigation of venom evolution at this finer evolutionary scale may be desirable given the medical importance of this complex. This is likely to need a phylogenetic framework (Thorpe et al., 1995; Daltry et al., 1996; Creer et al., 2003), with resolution at the appropriate level, in order to critically test hypotheses. The lack of resolution at the finer levels of the current phylogeny is associated with extremely short branch lengths between the nodes separating the main eastern lineages (Cambodia; Myanmar; Thailand; Lesser Sundas; Java/China). Hence, it appears that the lack of resolution at this level is due to an almost simultaneous divergence of multiple lineages in one of the complex's range-expanding phases, rather than any inadequacy in the amount of sequence data (1550 bp). Consequently, it is likely that one will not readily be able to produce a resolved phylogeny for the main eastern lineages (Baker et al., 2001).

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REFERENCES

- Arévalo, E., Davis, S.K. & Sites, J.W. Jr. (1994). Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. *Systematic Biology* 43, 387–418.
- Ariaratnam, C.A., Sjöström, L., Raziak, Z., Abeyasinghe, S., Kularatne, M., Arachchi, R.W.K.K., Sheriff, M.H.R.R., David, G., Theakston, D.G. & Warrell, D.A. (2001). An open, randomized comparative trial of two antivenoms for the treatment of envenoming by Sri Lankan Russell's viper (*Daboia russelii russelii*). *Transactions of the Royal Society of Tropical Medicine and Hygiene* 95, 74–80.
- Avise, J.C. (2000). *Phylogeography*. Cambridge, Massachusetts: Harvard University Press.
- Baker, R.H., Wilkinson, G.S. & DeSalle, R. (2001). Phylogenetic utility of different types of molecular data used to infer evolutionary relationships among stalk-eyed flies (Diopsidae). *Systematic Biology* 50, 87–105.
- Ballard, J.W.O. & Kreitman, M. (1994). Unraveling selection in the mitochondrial genome of *Drosophila*. *Genetics* 138, 757–772.
- Belt, P., Malhotra, A., Thorpe, R.S., Warrell, D.A. & Wüster, W. (1997). Russell's viper in Indonesia: snakebite and systematics. In *Venomous Snakes. Ecology, Evolution and Snakebite*, 207–217. Thorpe R.S., Wüster, W. & Malhotra, A. (eds). Oxford: Clarendon Press.
- Bremer, K. (1994). Branch support and tree stability. *Cladistics* 10, 295–304.
- Bromham, L. (2002). Molecular clocks in reptiles: life history influences rate of molecular evolution. *Molecular Biology and Evolution* 19, 302–309.
- Brongersma, L.D. (1958). *Vipera russelii* in de Indische Archipel. *Natuurkunde* 67, 88–90.
- Brown, R.P., Campos-Delgado, R. & Pestano, J. (2000). Mitochondrial DNA evolution and population history of the Tenerife skink *Chalcides viridanus*. *Molecular Ecology* 9, 1061–1069.
- Creer, S., Malhotra, A., Thorpe, R.S. & Chou W-H. (2001). Multiple causation of phylogeographical pattern as revealed by nested clade analysis of the bamboo viper (*Trimeresurus stejnegeri*) within Taiwan. *Molecular Ecology* 10, 1967–1981.
- Creer, S., Malhotra, A., Thorpe, R.S., Stöcklin, R., Favreau, P. & Chou, W.-H. (2003). Genetic and ecological correlates of intraspecific variation in pitviper venom composition using matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) and isoelectric focusing. *Journal of Molecular Evolution* 56, 317–329.
- Daltry, J., Wüster, W. & Thorpe, R.S. (1996). Diet and snake venom evolution. *Nature* 379, 537–540.
- Daniel, J.C. (1983). *The Book of Indian Reptiles*. Bombay: Bombay Natural History Museum.
- David, P. & Ineich, I. (1999). Les serpent venimeux du monde: systematique et repartition. *Dumerilia* 3, 3–499.
- Farris, J.S., Källersjö, M., Kluge, A.G. & Bult, C. (1994). Testing significance of incongruence. *Cladistics* 10, 315–319.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* 17, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Giannasi, N., Thorpe, R.S. & Malhotra, A. (2000). A phylogenetic analysis of body size evolution in the *Anolis roquet* group. *Molecular Ecology* 9, 193–202.
- Giannasi, N., Thorpe, R.S. & Malhotra, A. (2001). The use of amplified fragment length polymorphism in determining species trees at fine taxonomic levels: analysis of a medically important snake, *Trimeresurus albolabris*. *Molecular Ecology* 10, 419–426.
- Hackett, S. (1996). Molecular phylogenetics and biogeography of tanagers in the genus *Ramphocelus* (Aves). *Molecular Phylogenetics and Evolution* 5, 368–382.
- Hasegawa, M., Kishino, H. & Yano, T. (1985). Dating the human–ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22, 160–174.
- Heaney, L.R. (1991). A synopsis of climatic and vegetational change in southeast Asia. *Climatic Change* 19, 53–61.
- Hillis, D.M. (1991). Discriminating between phylogenetic

- signal and random noise in DNA sequences. In *Phylogenetic Analysis of DNA Sequences*, 278–294. Miyamoto M. & Cracraft, J. (eds). New York: Oxford University Press.
- Hillis, D.M. & Huelsenbeck, J.P. (1992). Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* 83, 189–195.
- Huang, J. (1984). Change of sea-level since the late Pleistocene in China. In *The Evolution of the East Asian Environment*, 309–319. Whyte, R.O. (ed.). Hong Kong: Center for Asian Studies, University of Hong Kong.
- Huelsenbeck, J.P. & Ronquist, F.R. (2001). MRBAYES: Bayesian inference in phylogeny. *Bioinformatics* 17, 754–755.
- Hung, D-Z., Wu, M-L., Deng, J-F. & Lin-Shiau, S-Y. (2002). Russell's viper snakebite in Taiwan: differences from other Asian countries. *Toxicon* 40, 1291–1298.
- Jayanthi, G.P. & Gowda, T.V. (1988). Geographical variation in India in the composition and lethal potency of Russell's viper (*Vipera russelli*) venom. *Toxicon* 26, 257–264.
- Karns, D.R., O'Bannon, A., Voris, H.K. & Weigt, L.A. (2000). Biogeographical implications of mitochondrial DNA variation in the bockadam snake (*Cerberus rynchops*, Serpentes: Homalopsinae) in southeast Asia. *Journal of Biogeography* 27, 391–402.
- Keogh, J.S., Barker, D.G. & Shine, R. (2001). Heavily exploited but poorly known: systematics and biogeography of commercially harvested pythons (*Python curtus* group) in southeast Asia. *Biological Journal of the Linnean Society* 73, 113–129.
- Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.
- Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X. & Wilson, A.C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA* 86, 6196–6200.
- Kumar, S., Tamura, K., Jakobsen, I.B. & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* 17, 1244–1245.
- Leaché, A.D. & Reeder, T.W. (2002). Molecular systematics of the eastern fence lizard (*Sceloporus undulatus*): A comparison of parsimony, likelihood, and Bayesian approaches. *Systematic Biology* 51, 44–68.
- Lenk, P., Kalyabina, S., Wink, M. & Joger, U. (2001). Evolutionary relationships among the true vipers (Reptilia: Viperidae) inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* 19, 94–104.
- Looareesuwan, S.C., Viravan, C. & Warrell, D.A. (1988). Factors contributing to fatal snake bite in the tropics: analysis of 46 cases in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 82, 930–934.
- Maddison, W.P. & Maddison, D.R. (1992). *MACCLADE: Analysis of Phylogeny and Character Evolution. Version 3.0*. Sunderland, Mass.: Sinauer Associates.
- Malhotra, A. & Thorpe, R.S. (2000a). The dynamics of natural selection and vicariance in the Dominican anole: patterns of within-island molecular and morphological divergence. *Evolution* 54, 245–258.
- Malhotra, A. & Thorpe, R.S. (2000b). A phylogeny of the *Trimeresurus* group of pit-vipers: new evidence from a mitochondrial gene tree. *Molecular Phylogenetics and Evolution* 16, 199–211.
- McDiarmid, R.W., Campbell, J.A. & Toure, T.A. (1999). *Snake Species of the World. A Taxonomic and Geographic Reference. Volume 1*. Washington D.C.: The Herpetologist's League.
- McDonald, J.H. & Kreitman, M. (1991). Adaptive protein evolution at the Adh locus in *Drosophila*. *Nature* 351, 652–654.
- Moritz, C., Schneider, C.J. & Wake, D.B. (1992). Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Systematic Biology* 41, 273–292.
- Mukherjee, A.K., Ghosal, S.K. & Maity, C.R. (2000). Some biochemical properties of Russell's viper (*Daboia russelli*) venom from eastern India: correlation with clinico-pathological manifestations in Russell's viper bite. *Toxicon* 38, 163–175.
- Ogden, R. & Thorpe, R.S. (2002). Molecular evidence for ecological speciation in tropical habitats. *Proceedings of the National Academy of Sciences of the USA* 99, 13612–13615.
- Page, R.D.M. & Holmes, E.C. (1998). *Molecular Evolution: A Phylogenetic Approach*. Oxford: Blackwell Science.
- Parkinson, C.L., Zamudio, K.R. & Greene, H.W. (2000). Phylogeography of the pitviper clade *Agkistrodon*: historical ecology, species status, and conservation of cantils. *Molecular Ecology* 9, 411–420.
- Posada, D. & Crandall, K.A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Rozas, J. & Rozas, R. (1999). DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* 15, 174–175.
- Salisbury, B.A. (2000). *SEPAL: Strongest Evidence and Parsimony Analyzer*. New Haven: Department of Ecology and Evolutionary Biology, Yale University.
- Sambrook, J., Fritsch, E.F. & Maniatis, T.E. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbour Laboratory Press.
- Surget-Groba, Y., Heulin, B., Guillaume, C.P., Thorpe, R.S., Kupriyanova, L., Vogrin, N., Maslak, R., Mazzotti, S., Venczel, M., Ghira, I., Odierna, G., Leontyeva, O., Monney, J.C. & Smith, N. (2001). Intraspecific phylogeography of *Lacerta vivapara* and the evolution of viviparity. *Molecular Phylogenetics and Evolution* 18, 449–459.
- Swofford, D.L. (2001). *PAUP*: Phylogenetic Analysis using Parsimony (and Other Methods), Version 4.0b8*. Sunderland, Mass.: Sinauer.
- Swofford, D. & Olsen, G.J. (1990). Phylogeny reconstruction. In *Molecular Systematics*, 411–501. Hillis, D.M. & Moritz, C. (eds). Sunderland, Mass.: Sinauer.
- Szyndlar, Z. (1988). Two new extinct species of the genera *Malpolon* and *Vipera* (Reptilia, Serpentes) from the

- Pliocene of Layna (Spain). *Acta Zoologica Cracoviensia* 31, 687–706.
- Thorpe, R.S. (2002). Analysis of color spectra in comparative evolutionary studies: molecular phylogeny and habitat adaptation in the St. Vincent anole, *Anolis trinitatis*. *Systematic Biology* 51, 554–569.
- Thorpe, R.S., Malhotra, A., Black, H., Daltry, J.C. & Wüster, W. (1995). Relating geographic patterns to phylogenetic processes. *Philosophical Transactions of the Royal Society B* 349, 61–68.
- Thorpe, R.S. & Richard, M. (2001). Evidence that UV markings are associated with patterns of molecular gene flow. *Proceedings of the National Academy of Sciences of the USA* 98, 3929–3934.
- Thorpe, R.S. & Stenson, A.G. (2003). Phylogeny, paraphyly and ecological adaptation of the colour and pattern in the *Anolis roquet* complex on Martinique. *Molecular Ecology* 12, 117–132.
- Warrell, D.A. (1989). Snake venoms in science and clinical medicine 1. Russell's viper: biology, venom and treatment of bites. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 83, 732–740.
- Warrell, D.A. (1995). Clinical toxicology of snakebite in Asia. In *Handbook of Clinical Toxicology of Animal Venoms and Poisons*, 493–594 Meier, J. & White, J. (eds). Boca Raton, Florida: CRC Press.
- Warrell, D.A. (1997). Geographical and intraspecies variation in the clinical manifestations of envenoming by snakes. In *Venomous Snakes. Ecology, Evolution and Snakebite*, 189–203. Thorpe R.S., Wüster, W. & Malhotra, A. (eds). Oxford: Clarendon Press.
- Woodhams, B.J., Wilson, S.E., Xin, B.C. & Hutton, R.A. (1990). Differences between the venoms of two subspecies of Russell's viper: *Vipera russelli pulchella* and *Vipera russelli siamensis*. *Toxicon* 28, 427–433.
- Wüster, W. (1998). The genus *Daboia* (Serpentes: Viperidae): Russell's viper. *Hamadryad* 23, 33–40.
- Wüster, W., Otsuka, S., Malhotra, A. & Thorpe, R.S. (1992). Population systematics of Russell's viper: a multivariate study. *Biological Journal of the Linnean Society* 47, 97–113.
- Wüster, W., Salomão, M.G., Quijada-Mascareñas, J.A. & Thorpe, R.S. (2002). Origin and evolution of the South American pitviper fauna: evidence from mitochondrial DNA sequence analysis. In *Biology of the Vipers*, 111–128. Schuett, G.W., Höggren, M., Douglas, M.E. & Greene, H.W. (eds). Eagle Mountain: Eagle Mountain Publishing.
- Zamudio, K.R. & Greene, H.W. (1997). Phylogeography of the bushmaster (*Lachesis muta*: Viperidae): implications for neotropical biogeography, systematics, and conservation. *Biological Journal of the Linnean Society* 62, 421–442.

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