INTRODUCTION

Studying the geographic variation within a species by combining molecular phylogeography (Avise, 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, vipers: 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 (Loosareesuwan 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 vipers are 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
subcontinent, and another including all the populations
to the east of the Bay of Bengal) that they named as sub-
species, 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 dif-
ferent 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 corrobo-
ration, 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 mito-
chondrial 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 ND2 primers
were from Arévalo et al. (1994) and the ND4 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 prod-
ucts were purified using the Wizard® PCR purification
system (Promega), then sequenced by cycle sequencing
using dye-labelled terminators (ABI Prism BigDye Termi-
nator 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

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**Table 1.** Sample localities and GenBank accession numbers.

<table>
<thead>
<tr>
<th>Locality</th>
<th><em>Cyt</em> b Accession</th>
<th>ND2 Accession</th>
<th>ND4 Accession</th>
</tr>
</thead>
<tbody>
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<td>AY165089</td>
<td>AY165077</td>
<td>AY165064</td>
</tr>
<tr>
<td>Tuban, W of Gresik, East Java, Indonesia</td>
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<td>AY165070-1</td>
<td>AY165057-8</td>
</tr>
<tr>
<td>Tonggerambang, Mbay, Flores, Indonesia</td>
<td>AY165085-6</td>
<td>AY165072-3</td>
<td>AY165059-60</td>
</tr>
<tr>
<td>Hauk Kyunt, Myanmar</td>
<td>AY165080</td>
<td>AY165067</td>
<td>AY165054</td>
</tr>
<tr>
<td>Thailand (southern central)</td>
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<td>AY165078-9</td>
<td>AY165065-6</td>
</tr>
<tr>
<td>Thayur, Tamil Nadu, India</td>
<td>AY165087</td>
<td>AY165075</td>
<td>AY165062</td>
</tr>
<tr>
<td>Gampola, Sri Lanka</td>
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<td>AY165076</td>
<td>AY165063</td>
</tr>
<tr>
<td>Poipet, Sisophon, Cambodia</td>
<td>AY165081</td>
<td>AY165068</td>
<td>AY165055</td>
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<td>Guangdong, China</td>
<td>AY165082</td>
<td>AY165069</td>
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<td>Pakistan*</td>
<td>AJ275723*</td>
<td>AY165074</td>
<td>AY165061</td>
</tr>
</tbody>
</table>

*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 g2 statistics calculated from a search of 10^4 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 specimens studied by Wüster et al., 1998, 1999) were then searched on 12 OTUs (12 is the maximum number of OTUs possible for this test). The 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 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 ratio = 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 lower 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 g1=–1.389; search constrained to specific strongly supported nodes: g1=–0.909 and –0.700 respectively). Base frequencies were found to be homogeneous among OTUs (x2=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,


χ²=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 (–lnL = 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 adjacent 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 fulfills 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*,

### Table 2. Uncorrected genetic distances (× 100) between localities (lower diagonal) and their standard errors (upper diagonal).

<table>
<thead>
<tr>
<th></th>
<th>Cambodia</th>
<th>China</th>
<th>East Java</th>
<th>Flores</th>
<th>Myanmar</th>
<th>Pakistan</th>
<th>India</th>
<th>Sri Lanka</th>
<th>Taiwan</th>
<th>Thailand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cambodia</td>
<td>0.4</td>
<td>0.3</td>
<td>0.3</td>
<td>2.6</td>
<td>0.8</td>
<td>0.7</td>
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<tr>
<td>China</td>
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<td>0.3</td>
<td>2.9</td>
<td>0.8</td>
<td>0.8</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>East Java</td>
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<td>0.3</td>
<td>2.7</td>
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<td>0.3</td>
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<tr>
<td>Flores</td>
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<td>1.1</td>
<td>2.2</td>
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<td>0.7</td>
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<tr>
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<td>10.4</td>
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<tr>
<td>Taiwan</td>
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<tr>
<td>Thailand</td>
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<td>10.1</td>
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</tr>
</tbody>
</table>

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*et al.*
Phylogeography of Russell’s viper

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, stenocoeous.

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,
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 division 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.

Phylogenetically equivocal effect may be that the cycles of expansion and bottlenecking (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).

ACKNOWLEDGEMENTS

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