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The use of amplified fragment length polymorphism in determining species trees at fine taxonomic levels: analysis of a medically important snake, *Trimeresurus albolabris*

NICHOLAS GIANNASI, ROGER S. THORPE and ANITA MALHOTRA
School of Biological Sciences, University College of North Wales, Bangor, Gwynedd, LL57 2UW, UK

Abstract

There are a huge number of phylogenetic studies based on mitochondrial DNA (mtDNA); however, these may represent gene trees that may not be congruent with the species tree. A solution to this problem is to include additional, independent, loci from the nuclear genome. At fine taxonomic levels, i.e. between populations and closely related species, previously suggested nuclear markers such as intron sequence data may not be appropriate. In this study we investigate the use of amplified fragment length polymorphisms (AFLP) to aid determination of the species tree for 24 specimens of a medically important snake, *Trimeresurus albolabris*. This is of particular importance for many venomous snakes as venom often varies intraspecifically. Five different primer combinations produced 434 bands and were analysed by constructing a phylogenetic tree using neighbour joining and principal component analysis. Results were similar across all methods and found distinct groupings. The results were compared with mtDNA data and a reconciled tree was constructed in order to determine the species tree for *T. albolabris*. We found that *T. albolabris* (*sensu lato*) is not monophyletic. Specimens from the Indonesian islands (except West Java) form a distinct clade and we propose elevation to species level. A specimen from Nepal is also distinct and suggests that this population also deserves specific status. We suggest that AFLPs may prove a valuable aid in determining species trees as opposed to gene trees at fine taxonomic levels and this should facilitate the incorporation of molecular data into such activities as antivenom production and conservation management.

Keywords: AFLPs, mtDNA, phylogeny, pit viper, reconciled trees, species tree

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Introduction

The last decade has seen a huge number of vertebrate phylogenetic studies based on mitochondrial DNA (mtDNA) (Pagel 1999), many of which have proven extremely valuable. However, because of the lack of recombination, the 37 different genes of the animal mitochondrial genome are inherited as a single unit, and hence phylogenies derived from one or many mtDNA genes are not independent estimates of organismal phylogeny (Moore 1995; Page 2000). Furthermore, a phylogeny produced from mtDNA represents a gene tree that may not be congruent with the species tree because of lineage sorting (Moore 1995). If one is attempting to infer the evolutionary history of species and populations, rather than genes, this poses a significant problem (Palumbi & Baker 1994). The only apparent solution is to include additional, independent, loci from the nuclear genome in phylogenetic studies (Wu 1991).

At the intergeneric and distinct species level, exon primed intron crossing (EPIC) conserved primers appear to offer a solution to the gene tree/species tree problem (Palumbi & Baker 1994; Friesen *et al*. 1997; Prychitko & Moore 1997). However, because of the slow rate of intron sequence evolution (Moore 1995) these markers have very limited use at finer taxonomic levels, i.e. for population and closely related species. Because the gene tree/species tree problem still exists at these finer levels an alternative source of loci, independent of mtDNA, is required. This is particularly relevant for venomous snakes because intraspecific variation in venom composition has been detected in numerous species (Chippaux *et al*. 1991).

Correspondence: R. S. Thorpe. Fax: +44 1248 371644; E-mail: r.s.thorpe@bangor.ac.uk

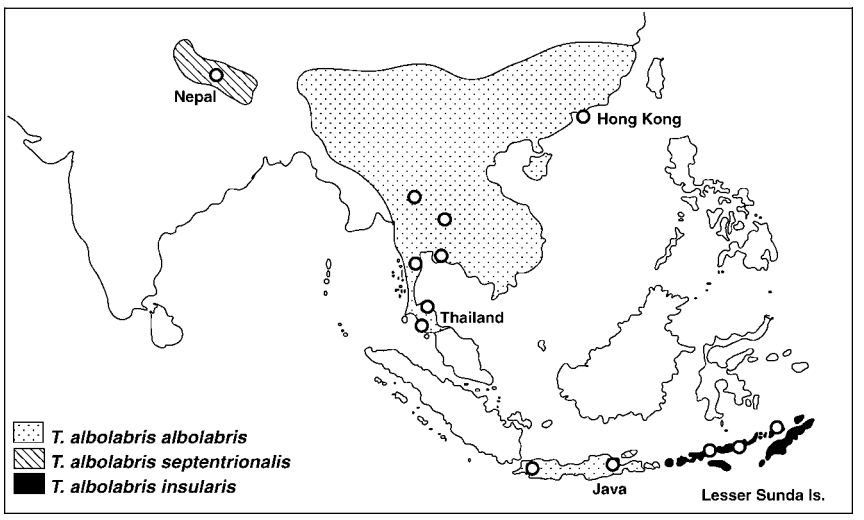

Fig. 1 Distribution of the three currently recognized subspecies of *Trimeresurus albolabris*, according to Regenass & Kramer (1981). Circles indicate populations sampled.

Furthermore, because snakebite is very common in tropical, developing countries and treatment with antivenom remains the only specific treatment for envenomation by snakes (Theakston 1997), attempting to determine the species tree is of more than esoteric interest in these animals.

Although a variety of mtDNA-independent loci exist that do not have the drawbacks of EPIC at fine taxonomic levels, e.g. microsatellites (Petren *et al*. 1999), the costs of developing a suitable set of microsatellites for a given organism may be prohibitive. A recently developed polymerase chain reaction (PCR)-based assay, amplified fragment length polymorphism (AFLP; Vos *et al*. 1995) has proven to be a rich and relatively inexpensive source of such independent loci for fine taxonomic levels for invertebrates (McMichael & Prowell (1999), plants (Beismann *et al*. 1997) and microbes (Janssen *et al*. 1996). In this study we investigate the value of AFLPs as loci that are independent of mtDNA and apply them to the venomous snake, *Trimeresurus albolabris*. We chose this snake because it is frequently the most common cause of snakebite in many parts of its range and is responsible for significant levels of mortality and morbidity (Romer 1963; Hutton *et al*. 1990; Viravan *et al*. 1992), and it also demonstrates significant mtDNA structure (Malhotra & Thorpe 2000).

Materials and methods

Population sampling and DNA extraction

Twenty-four *Trimeresurus albolabris* (*sensu lato*) individuals were sampled across the range shown in Fig. 1. In addition, samples of the closely related species *T. purpureomaculatus*, *T. macrops* and *T. erythrurus* were also included to evaluate the monophyly of *T. albolabris* (*sensu lato*). Samples were in the form of tail-tip biopsies preserved in 80% ethanol, liver tissue in 80% ethanol, or 100–200 μL of blood taken from the caudal vein, placed in 1 mL 5% EDTA, and stored in 2 mL SDS–Tris buffer (100 mM Tris, 3% SDS). Whole genomic DNA was extracted using the protocol of Maniatis *et al*. (1989).

Table 1 The number of marker bands obtained for each of five primer combinations

Primer pair	*Eco*RI	*Mse*I	Number of markers
1	ACC	CAG	87
2	AGC	CTG	96
3	AGG	CTC	104
4	AGG	CTA	95
5	ACC	CAC	52

AFLP procedure

The AFLP technique (Vos *et al*. 1995) consists of two consecutive PCR (preselective and selective amplifications), which amplify the DNA fragments generated by double-restriction digestion and subsequent ligation of specific nucleotide adapters. A selected group of fragments is amplified as selective nucleotides are added to the primers used. AFLP reactions were performed using the GIBCO BRL AFLP Analysis System 1 following the manufacturer's instructions. AFLP reactions for each primer pair combination were then run on separate 6% polyacrylamide gels with a tomato DNA reference which were then dried and exposed to X-ray film (Kodak XLS-1) for 48 h. Because of the number of specimens used in this study each primer pair combination was run on a separate gel, thus avoiding the potential problems of scoring bands across gels. Five selective primer pair combinations, with three selective nucleotides, were used and are shown in Table 1.

Data analysis

The presence or absence of AFLP markers was scored by hand from the autoradiographs and coded as a binary character. For all analyses, data from the five primer combinations were then combined to form a raw data matrix consisting of all bands. Two approaches were then used to ascertain the relationships of the 27 specimens. First, the raw data matrix was tested for adequacy of phylogenetic signal using PAUP* Version 4.0b2a (Swofford 1998) by plotting the distribution of 100 000 random trees, with calculation of g_1 (Hillis & Huelsenbeck 1992). A distance based on the mean number of pairwise character differences (adjusted for missing data) was then employed to estimate divergence between specimens. A phylogenetic tree was constructed from this distance matrix using the neighbour-joining procedure (Saitou & Nei 1987). The reliability of this, and all other trees, was tested by bootstrap analysis (Felsenstein 1985) with 1000 replications. In addition, a permutation test probability analysis (PTP; Faith & Cranston 1991) was performed as an overall statistical assessment of the resulting tree, because bootstrap analysis sometimes lacks statistical power at the intraspecific level (Templeton *et al*. 1992).

The second approach consisted of using MVSP Version 3.1 (Kovach 1999) to perform a R-mode principal component analysis (PCA) on the correlation matrix. Data were standardized and the resulting principal component scores plotted.

In order to produce a single phylogeny for *T. albolabris* (*sensu lato*) it is necessary to combine the information from both the AFLP and mtDNA data sets. We used reconciled trees (Goodman *et al*. 1979; Page 1994) to infer the species tree. This approach uses an optimality criterion to choose a species tree or trees; more precisely, given a gene tree *G*, it is possible to estimate the actual species phylogeny *T** which when reconciled with *G* has the lowest cost (Page & Charleston 1997). This concept is readily extended to multiple gene trees as the cost of reconciling all gene trees is simply the sum of the costs of reconciling the individual gene trees (Page & Charleston 1997). Because the number of evolutionary trees increases rapidly with increasing numbers of species (Felsenstein 1978) we used the program GENETREE (Page 1998) to search tree space using a heuristic search. Specifically, we used the total number of evolutionary events ('duplications' and 'losses') as the optimality criterion and alternated between nearest neighbour interchanges and subtree pruning and regrafting as tree perturbations from a random starting tree with 100 searches, retention of equal cost trees and with the steepest ascent option implemented.

Results

After pooling the data from the five AFLP primer combinations used for analysis (Table 1), 434 fragments were scored, with an average of 86.8 bands per primer combination.

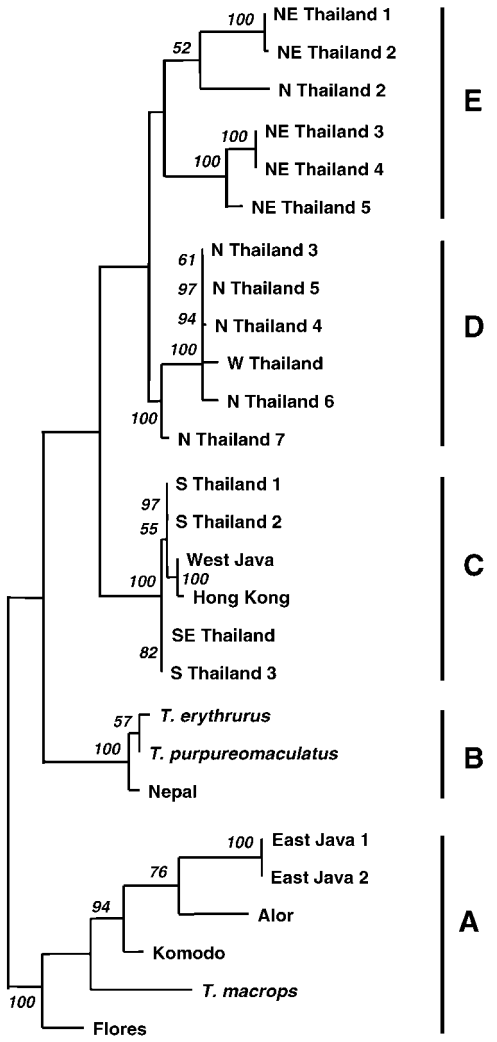

Fig. 2 Neighbour-joining tree derived from the mean number of pairwise character differences distance matrix. Bootstrap support values (> 50%), derived from 100 replications, are shown. This is an unrooted tree, which has been arbitrarily rooted to be consistent with the mitochondrial tree (see Fig. 4). The short branch lengths in clades C and D make the internal structure difficult to discern, so these are given here. Clade C: (((ST1, ST2)97, (WJ, HK)100)55, (SET, ST3)82); Clade D: ((((NT3, NT5)61, NT4)97, (WT)94, NT6)100, NT7).

Across all specimens, 21 bands were fixed and 413 were polymorphic. The presence of a significant phylogenetic signal in the data set was indicated by the skewness parameter $g_1 = -0.66$, corresponding to $P < 0.001$ for the number of characters and taxa involved (Hillis & Huelsenbeck 1992). Five clades could be recognized from the neighbour-joining analysis (Fig. 2) and bootstrap support throughout the tree was generally high (i.e. > 75%). Furthermore, the PTP test was significant ($P < 0.01$), indicating a low probability that this tree arose by chance. Clade A contains individuals from East Java, the Indonesian islands of Alor, Komodo and Flores, and *Trimeresurus macrops*.

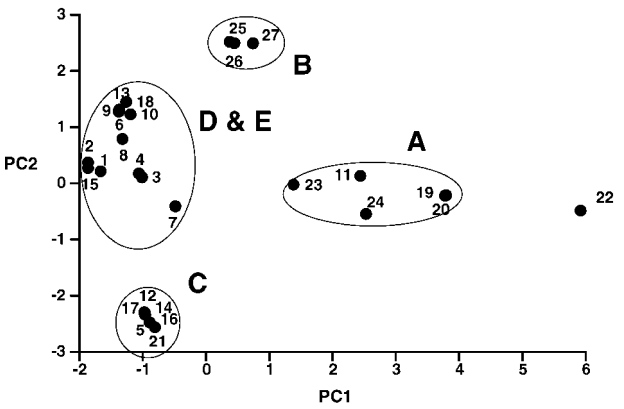

Fig. 3 Plot of first and second principal components of principal components analysis on amplified fragment length polymorphism characters, together showing 42% of the total variation. The numbers refer to the following specimens: 1–4, NE Thailand; 5, 12, 17, S Thailand; 6–10, 13, 15, N Thailand; 11, *Trimeresurus macrops*; 14, SE Thailand; 16, W Java; 18, W Thailand; 19, 20, East Java; 21, Hong Kong; 22, Alor; 23, Flores; 24, Komodo; 25, *T. erythrurus*; 26, *T. purpureomaculatus*; 27, Nepal.

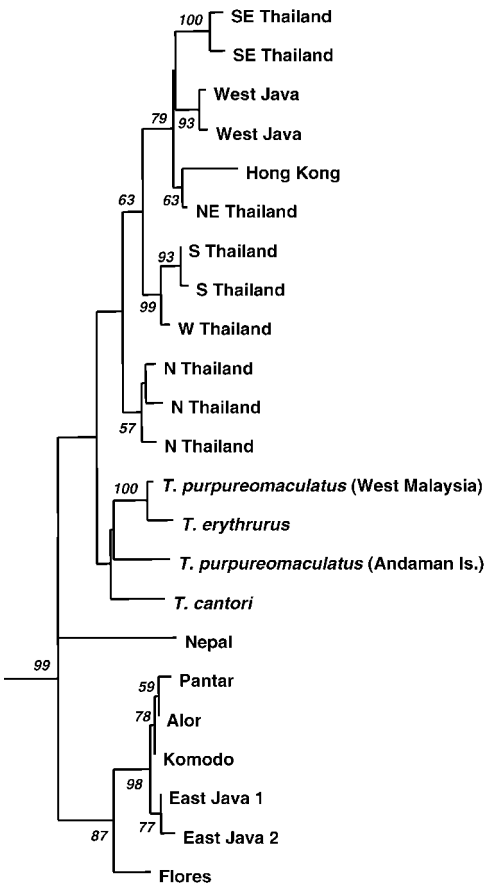

Fig. 4 Relationships for 19 specimens of *Trimeresurus albolabris* (*sensu lato*) and three other *Trimeresurus* species (*T. purpureomaculatus*, *T. erythrurus* and *T. cantori*) based on a maximum likelihood analysis of a 660-bp fragment of cytochrome *b* mitochondrial DNA sequence data (Malhotra & Thorpe, 2000). Bootstrap support values (> 50%) from 100 bootstrap replications are shown.

Clade B contains *T. purpureomaculatus*, *T. erythrurus* and an individual *T. albolabris* (*sensu lato*) from Nepal. Clade C contains individuals from southern and southeast Thailand, West Java and Hong Kong. The fourth clade (D) features specimens from northern and central Thailand, whereas the fifth (E) contains specimens from northeastern Thailand, plus a single specimen from northern Thailand.

PCA also revealed five main clusters (Fig. 3). The first two components accounted for 26 and 16% of the total variation, respectively. The plot of the first two principal components indicates the presence of four main clusters, which correspond to the clades in the phylogenetic analysis, with the exception that clades D and E (northern and northeastern Thailand) are not distinguished from each other on two axes alone. The remaining distinct specimen is from the Indonesian island of Alor (22), which groups with clade A in the phylogenetic analysis.

Clearly, both analyses produce similar results, namely that *T. albolabris* (*sensu lato*) is not monophyletic and clusters of individuals are consistently recognizable. Furthermore, when the AFLP data are compared with the phylogenetic relationships from mitochondrial sequence data (Fig. 4) from Malhotra & Thorpe (2000) there are a number of clear parallels. First, *T. albolabris* (*sensu lato*) is clearly not monophyletic, with other species of *Trimeresurus* consistently nested within *T. albolabris* (*sensu lato*). Second, specimens from East and West Java do not group together.

The reconciled tree analysis found a single parsimonious species tree (Fig. 5a) with a cost of 55 events (7 duplications and 48 losses). Six groups can clearly be recognized and there are a number of points of particular interest. First, group 6 is very distinct and is comprised of specimens from the Indonesian islands of East Java, Alor, Komodo and Flores. The Nepalese specimen (group 5) is also very distinct and appears separate from all other *T. albolabris* populations. Group 4 contains two clearly distinct species, namely *T. purpureomaculatus* and *T. erythrurus*. This is of particular note as these render *T. albolabris* (*sensu lato*) paraphyletic (Malhotra & Thorpe 2000). Group 3 forms a geographically coherent grouping of specimens from north Thailand, whereas Group 2 consists of southern and western Thai specimens. Group 1 contains specimens from West Java, southeast Thailand, Hong Kong and northeast Thailand.

Discussion

The results demonstrate the usefulness of the AFLP technique for obtaining molecular information in venomous snakes at a fine taxonomic level, i.e. within a species and genus. Indeed, g_1 analysis demonstrated the presence of a significant phylogenetic signal in the AFLP data set and

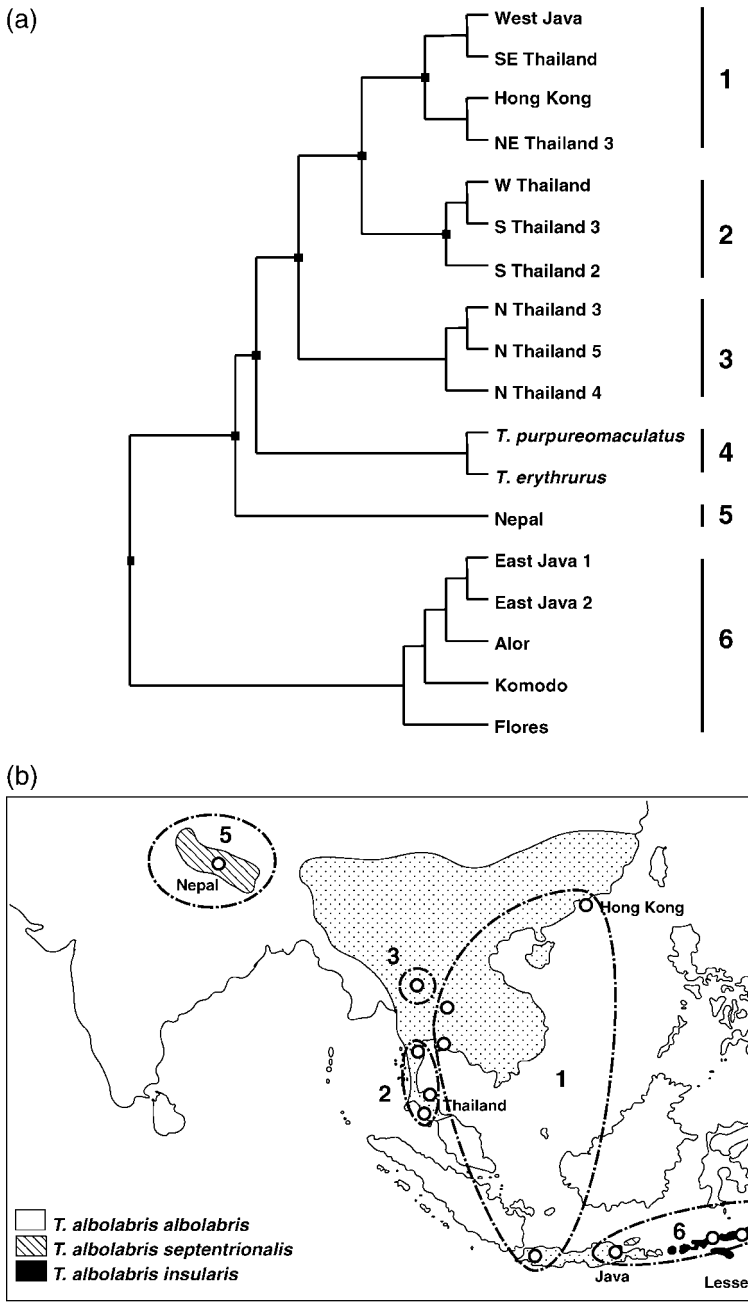

Fig. 5 (a) Reconciled tree for the mitochondrial DNA gene tree shown in Fig. 4 with the amplified fragment length polymorphism-based topology shown in Fig. 2. There are seven duplication events represented by black squares. These events may be gene duplications resulting in paralogous copies of genes, or may describe cladogenesis of genes independent of speciation. Branch lengths are arbitrary. (b) Map of south-east Asia showing the geographical distribution of the groups from the reconciled tree (numbers correspond to groups shown in (a)). There is a clear lack of correspondence between genetic and traditional subspecific categories.

phylogenetic analysis demonstrates that a well-supported and well-resolved tree can be reconstructed from this data set. When the data are subjected to a neighbour-joining or ordination analysis (PCA), similar results are obtained, providing a further demonstration of the robustness of the AFLP data set. Indeed, when the AFLP results are compared with those obtained using mtDNA sequence data they are largely similar.

This finding has significant implications for researchers interested in systematics. First, because the AFLP technique requires no a priori knowledge of sequence information it is easily applied to any species from which it is possible to extract DNA. Second, AFLP markers are representative of the whole genome and offer a diverse source of genetic data that appears to have substantial phylogenetic information and thus aid in the construction of the evolutionary history of species and populations rather than genes. For venomous snakes, attempting to determine the species tree is of more than esoteric interest because snakebite is very common in tropical, developing countries and treatment with antivenom remains the only specific treatment for envenomation by snakes (Theakston 1997).

If venom composition differs between snake taxa, then a monovalent antivenom against the venom of one taxa is unlikely to be effective in neutralizing the venom of another taxa. Hence, knowing which species are present in which geographical locations enables a sound basis for the production and clinical application of antivenoms.

A previously proposed solution to this gene tree vs. species tree problem has been to sequence additional, independent loci from the nuclear genome (Wu 1991). There have recently been several investigations of the utility of a range of intron sequences as a source of such loci, in particular, exon primed intron crossing (EPIC) conserved primers have been used in studies of actin in cetaceans (Palumbi & Baker 1994), fibrinogen (Prychitko & Moore 1997) and aldolase, glyceraldehyde-3 phosphate dehydrogenase (G3PD), alpha enolase and lamin in birds (Friesen *et al*. 1997). While this approach has been successful, the slow rate of intron sequence evolution (Moore 1995) has limited the usefulness of these markers to deep taxonomic splits (i.e. between relatively divergent species and genera) and they are not suitable for population- and species-level studies.

Our results clearly demonstrate the value of AFLP markers as an aid to gene tree–species tree problems at finer taxonomic levels, in which intron sequence data are not informative. Specifically, our AFLP analysis finds that *Trimeresurus albolabris* is clearly not monophyletic as three other species, *T. purpureomaculatus*, *T. erythrurus* and *T. macrops*, are nested within all trees. Furthermore, distinct groups or clades that do not correspond to the distribution of the conventionally recognized subspecies are recognized from all analyses (Fig. 1).

Although the value of different molecular markers for systematic purposes may continue to be debated, in order to propose taxonomic amendments a final tree must be constructed from often conflicting data sets. There is a number of different approaches available to construct phylogenetic trees when more than one data source exists. These approaches can be split into two methods, those that combine the data and those that keep the data separate and then combine the resulting trees.

The simplest data combination is to unconditionally combine all data sets in a 'total evidence' analysis as advocated by Kluge (1989). The main attraction of combining all data into a single analysis is that it makes use of all the available data. However, it has been suggested that some data sets can be 'positively misleading' (Bull *et al*. 1993) and incorporation into a total evidence analysis can produce incorrect estimates of phylogeny (e.g. Flynn & Nedbal 1998). A further weakness of the total evidence approach is that it assumes that all the data reflect the same evolutionary history. This may not be the case because, in many species, one may expect mtDNA and nuclear genes to have different, conflicting, histories that may represent gender-biased migration (e.g. humpback whales Palumbi & Baker 1994).

A second data combination approach is that of conditional data combination (Bull *et al*. 1993; de Queiroz *et al*. 1995), which advocates combining data sets unless there is evidence for significant conflict among them. However, this approach raises another problem of how to test statistically for the degree of heterogeneity between data partitions. A variety of statistical tests has been suggested as appropriate. These tests include the partition homogeneity test (Farris *et al*. 1994), the Wilcoxon signed ranks test (Templeton 1983), the Kishino/Hasegawa test (Kishino & Hasegawa 1989), level of nodal support (Flynn & Nedbal 1998) and the likelihood heterogeneity test (Huelsenbeck & Bull 1996). However, Sullivan (1996) suggested that while tests of homogeneity provide evidence to keep data partitions separate in phylogenetic analyses, combining certain heterogeneous partitions can lead to a more robust phylogeny in some details.

An alternative approach is to keep data sets separate, construct trees from each data set and then use a consensus approach to provide a final tree. A drawback of using consensus approaches is that they do not use all the available data; rather, they only use data that are consistent between trees and nearly always result in a less than fully resolved tree. It has been suggested that although a lack of resolution exists, this is countered by the certainty that all the groups in the final consensus tree are supported by all the data. However, as Barrett *et al*. (1991) demonstrated, this is not necessarily true.

Another method that keeps the data sets separate is to apply reconciled tree concepts (Goodman *et al*. 1979; Page 2000). The optimal species tree or final tree is simply the tree in which all the trees constructed from the separate data can be embedded with the least cost. Although this approach also has limitations, in that weakly supported and highly supported nodes are given equal weighting (Page 2000), it appears to provide a practical way to infer species trees from one or more gene trees from a variety of data sets. The results of this analysis clearly show that the specimens from East Java, Alor, Komodo and Flores form a distinct clade and should be elevated to species status, for which the name *T. insularis* (Kramer 1977) is available. The presence of *T. macrops* nested within this clade in the AFLP tree is problematic and requires further study. Unfortunately, no mitochondrial data (cytochrome *b*) are currently available for this species as it fails to amplify with a number of different primer sets (Malhotra & Thorpe 2000). The Nepalese specimen also appears distinct from all other *T. albolabris* (*sensu lato*) specimens and thus also warrants specific status under the name *T. septentrionalis* (Kramer 1977). The remaining three groups (1–3) may represent geographical variation of *T. albolabris*, or may represent distinct species, and it is not immediately obvious which is the case. Their parapatric distribution and the presence of some overlap between geographical areas in the AFLP

tree (Fig. 2) suggests that it may be best to retain them as geographical variants of a single species (*T. albolabris sensu stricto*) for the time being. More detailed sampling to address this question is in progress.

The affinity of the west Javan specimens with group 1, which is otherwise restricted to the mainland, is of note. On the basis of current geography, this makes little biogeographic sense. However, during the Pleistocene, sea levels were lower and these islands formed part of the Sunda shelf (Heaney 1991). This biogeographic pattern is supported by evidence from another venomous snake species, *Calloselasma rhodostoma*, that also shows boundaries between southern Thailand and northern west Malaysia (Daltry *et al*. 1997). However, the distinctness of the east and west Javan populations is less easy to explain; it is possible that the east Javan populations represent a westward colonization of Java from the Lesser Sunda islands (Malhotra & Thorpe 1997).

Although AFLP markers are being used more widely in vertebrate studies (e.g. Ajmone-Marsan *et al*. 1997; Liu *et al*. 1998) we believe this is the first study to report the use of AFLP markers in a biogeographic context. We suggest that AFLP markers may prove an extremely valuable source of loci, independent of mtDNA, for defining population and species boundaries.

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References

Ajmone-Marsan P, Valentini A, Cassandro M, Vecchiotti Antaldi G, Bertoni G, Kuiper M (1997) AFLP markers for DNA fingerprinting in cattle. *Animal Genetics*, **28**, 418–426.

Barrett M, Donoghue MJ, Sober E (1991) Against consensus. *Systematic Zoology*, **40**, 486–493.

Beismann H, Barker JHA, Karp A, Speck T (1997) AFLP analysis sheds light on distribution of two *Salix* species and their hybrid along a natural gradient. *Molecular Ecology*, **6**, 989–993.

Bull JJ, Huelsenbeck JP, Cunninghma CW, Swofford DL, Waddell PJ (1993) Partitioning and combining data in phylogenetic analysis. *Systematic Biology*, **42**, 384–397.

Chippaux J-P, Williams V, White J (1991) Snake venom variability: methods of study, results and interpretation. *Toxiconology*, **29**, 1279–1303.

Daltry JC, Wuster W, Thorpe RS (1997) The role of ecology in determining venom variation in the Malayan pitviper, *Calloselasma rhodostoma*. In: *Venomous Snakes: Ecology, Evolution and Snakebites* (eds Thorpe RS, Wuster W, Malhotra A), pp. 155–171. Clarendon Press, Oxford.

Faith DP, Cranston PS (1991) Could a cladogram this short have arisen by chance alone? On permutation tests for cladistic structure. *Cladistics*, **7**, 1–28.

Farris JS, Kallersjo M, Kluge AG, Bult C (1994) Testing significance of incongruence. *Cladistics*, **10**, 315–319.

Felsenstein J (1978) The number of evolutionary trees. *Sytematic Biology*, **27**, 27–33.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Flynn JJ, Nedbal MA (1998) Phylogeny of the Carnivora (Mammalia): congruence vs incompatibility among multiple data sets. *Molecular Phylogenetics and Evolution*, **9**, 414–426.

Friesen VL, Congdon BC, Walsh HE, Birt TP (1997) Intron variation in marbled murrelets detected using analyses of single-stranded conformational polymorphisms. *Molecular Ecology*, **6**, 1047–1058.

Goodman M, Czelusniak J, Moore GW, Romero-Herrera AE, Matsuda G (1979) Fitting the gene lineage into its species lineage: a parsimony strategy illustrated by cladograms constructed from globin sequences. *Systematic Zoology*, **28**, 132–168.

Heaney LR (1991) A synopsis of climatic and vegetational change in south-east Asia. *Climatic Change*, **19**, 53–61.

Hillis DM, Huelsenbeck JP (1992) Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity*, **83**, 189–195.

Huelsenbeck JP, Bull JJ (1996) A likelihood ratio test to detect conflicting phylogenetic signal. *Systematic Biology*, **45**, 92–98.

Hutton RA, Looareesuwan S, Ho M (1990) Arboreal green pit vipers (genus *Trimeresurus*) of south-east Asia—Bites by *T. albolabris* and *T. macrops* in Thailand and a review of the literature. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84**, 866–874.

Janssen P, Coopma R, Huys G *et al*. (1996) Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology*, **142**, 1881–1883.

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order of the Hominoidea. *Journal of Molecular Evolution*, **29**, 170–179.

Kluge AG (1989) A concern for evidence and a phylogenetic hypothesis of relationships among *Epicrates* (Boidae, Serpentes). *Systematic Zoology*, **38**, 7–25.

Kovach WL (1999) *A Multivariate Statistical Package for Windows, ver 3.1*. Kovach Computing Services, Pentraeth, UK.

Kramer U (1977) Zur schlangenfauna Nepals. *Review Suisse Zoologique*, **84**, 721–761.

Liu Z, Nichols A, Li P, Dunham RA (1998) Inheritance and usefulness of AFLP markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their F1, F2 and backcross hybrids. *Molecular and General Genetics*, **258**, 260–268.

Malhotra A, Thorpe RS (1997) New perspectives on the evolution of south-east Asian pit vipers (genus *Trimeresurus*) from molecular studies. In: *Venomous Snakes: Ecology, Evolution and Snakebites* (eds Thorpe RS, Wuster W, Malhotra A), pp. 115–127. Clarendon Press, Oxford.

Malhotra A, Thorpe RS (2000) A phylogeny of the *Trimeresurus* group of pit-vipers: new evidence from a mitochondrial gene tree. *Molecular Phylogenetics and Evolution*, **16**, 199–211.

Maniatis TE, Frisch EF, Sambrook J (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbour Laboratory, Cold Spring Harbor, NY.

McMichael M, Prowell DP (1999) Differences in amplified fragment-length polymorphisms in fall armyworm (Lepidoptera: Noctuidae) host strains. *Annals of the Entomological Society of America*, **92**, 175–181.

Moore WS (1995) Inferring phylogenies from mtDNA variation: mitochondrial gene trees versus nuclear gene trees. *Evolution*, **49**, 718–726.

Page RDM (1994) Maps between trees and cladistic analysis of historical associations among genes, organisms, and areas. *Systematic Biology*, **43**, 58–77.

Page RDM (1998) GENETREE: comparing gene and species phylogenies using reconciled trees. *Bioinformatics*, **14**, 819–820.

Page RDM (2000) Extracting species trees from complex gene trees: reconciled trees and vertebrate phylogeny. *Molecular Phylogenetics and Evolution*, **14**, 89–106.

Page RDM, Charleston MA (1997) From gene to organismal phylogeny: reconciled trees and the gene tree/species tree problem. *Molecular Phylogenetics and Evolution*, **7**, 231–240.

Pagel M (1999) Inferring the historical patterns of biological evolution. *Nature*, **401**, 877–884.

Palumbi SR, Baker CS (1994) Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. *Molecular Biology and Evolution*, **11**, 426–435.

Petren K, GrantBR, GrantPR (1999) A phylogeny of Darwin's finches based on microsatellite DNA length variation. *Proceedings of the Royal Society of London, Series B*, **266**, 321–329.

Prychitko TM, Moore WS (1997) The utility of DNA sequences of an intron from the β-fibrinogen gene in phylogenetic analysis of woodpeckers (Aves: Picidae). *Molecular Phylogenetics and Evolution*, **8**, 193–204.

de Queiroz A, Donaghue MJ, Kim J (1995) Separate versus combined analysis of phylogenetic evidence. *Annual Review of Ecology and Systematics*, **26**, 657–681.

Regenass U, Kramer E (1981) Zur Systematik der grunen Grubenottern der Gattung *Trimeresurus* (Serpentes, Crotalidae). *Review Suisse Zoologique*, **88**, 163–205.

Romer JD (1963) Notes on the white-lipped pit viper, *Trimeresurus albolabris* Gray, in Hong Kong. In: *Venomous and Poisonous Animals and Noxious Plants of the Pacific Region* (eds Keegan HL, MacFarlane WV), pp. 379–384. Pergamon Press, Oxford.

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425.

Sullivan J (1996) Combining data with different distributions of among-site variation. *Systematic Biology*, **45**, 375–380.

Swofford DL (1998) PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sinauer Associates, Sunderland, MA.

Templeton AR (1983) Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution*, **37**, 221–244.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III Cladogram estimation. *Genetics*, **132**, 619–633.

Theakston RDG (1997) The kinetics of snakebite envenoming and therapy. In: *Venomous Snakes Ecology, Evolution and Snakebite* (eds Thorpe RS, Wuster W, Malhotra A), pp. 251–257. Clarendon Press, Oxford.

Viravan C, Looareesuwan S, Koskarn W *et al*. (1992) A national hospital-based survey of snakes responsible for bites in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86**, 100–106.

Vos P, Hogers R, Bleeker M *et al*. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.

Wu C-I (1991) Inferences of species phylogeny in relation to segregation of ancient polymorphisms. *Genetics*, **127**, 429–435.

This study forms part of a long-term molecular phylogenetic study of Asian vipers (particularly *Trimeresurus*) led by Anita Malhotra and Roger S Thorpe. Nick Giannasi's interests lie in determining species trees from molecular data and a multidisciplinary approach to speciation. This study reflects Roger Thorpe's interests in using nuclear markers to support mtDNA phylogenies. Other aspects of the group's work include evolutionary studies of island lizards.
