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Multiple causation of phylogeographical pattern as revealed by nested clade analysis of the bamboo viper (*Trimeresurus stejnegeri*) within Taiwan

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Abstract

In order to assess the utility of nested clade analysis, both standard phylogenetic algorithms and nested clade analysis were performed on a geographically widespread survey of mitochondrial DNA haplotypes of the bamboo viper, *Trimeresurus stejnegeri*, within Taiwan. Gross tree topologies were congruent for all analyses and indicated the presence of two geographically overlapping clades within Taiwan. The smaller lineage was restricted to the north and east coasts, whereas the larger lineage occupied all but the northern range of the species within Taiwan including the Pacific offshore populations of Green and Orchid Islands. The phylogeographical pattern supports the existence of at least one colonization event from the continent since the initial isolation of Taiwan from the mainland in the Pliocene. However, determining the exact number of colonization events was not possible due to the simultaneous vicariant forces of hypothesized continental landbridge connections and the occurrence of dramatic *in situ* orogenesis throughout the Pleistocene. Nested clade analysis provided multiple temporal and spatial population historical inferences that are not possible with standard analyses and therefore should become widely applied to future phylogeographical studies.

Keywords: mitochondrial DNA, nested clade analysis, phylogeography, Taiwan, *Trimeresurus stejnegeri*

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Introduction

Since the inception of the field of intraspecific phylogeography (Avise *et al*. 1987), advances in, and increased availability of molecular ecological techniques and associated computer-based algorithms have seen studies within the field diversify and increase exponentionally. Contemporary studies often present an array of slightly conflicting trees (or a consensus summary tree) produced by a combination of distance and character based approaches, supported by several alternative and often computationally time-consuming measures. The resulting phylogenetic affinities of haplotypes are subsequently overlain on geography and demographical, ethological and historical details of the species involved incorporated into its interpretation (Avise 1998). One of the primary disadvantages of standard phylogeographical methodology is that biological inferences are often presented with no statistical analysis or hypothesis testing of the spatial distribution of haplotypes. This has been addressed by using random permutation (Mantel) tests to correlate alternative historical events against phylogeographic pattern (Thorpe & Malhotra 1996; Thorpe *et al*. 1996) and nested clade analysis (Templeton *et al*. 1995; Crandall & Templeton 1996; Hammer *et al*. 1998; Durand *et al*. 1999; Gómez-Zurita *et al*. 2000).

Nested clade analysis uses a statistically supported haplotype tree ($P > 0.95$) to define a nested series of clades, thereby allowing an evolutionary nested analysis of the spatial distribution of genetic variation. More importantly, such nested analysis has the power to discriminate between phylogeographical patterns due to restricted gene flow vs. historical events operating at the population level (Templeton 1998). In this paper, therefore, we aim to use both standard tree building algorithms (e.g. neighbour-joining, maximum parsimony, and maximum-likelihood) and nested clade analysis for inferring underlying causes of phylogeographical pattern, using mitochondrial cytochrome *b* (cyt*b*) gene sequences of *Trimeresurus stejnegeri* (Schmidt), within Taiwan.

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The subtropical island of Taiwan (395 km long and 144 km wide) provides an excellent opportunity for comparing contemporary phylogeographical patterns with biogeographical hypotheses. Taiwan is topographically, climatically and ecologically diverse (Lin 1990; Su 1992), with a mountain range approaching an elevation of 4000 m rising steeply from the eastern coast and giving way to a broad western plain. *T. stejnegeri* is a nocturnal, arboreal snake predominately favouring riparian habitats (Mao 1993). Subject to the presence of suitable microhabitat requirements, the snake occurs almost ubiquitously in Taiwan from sea level to 1800 m. The distribution and abundance of *T. stejnegeri* within a relatively small yet diverse island such as Taiwan provides an excellent model for phylogeographical studies. Geographical variation in morphology has been described in the species (Castellano *et al*. 1994) and partial Mantel tests attributed environmental heterogenity as the possible underlying cause, but the geological history of Taiwan may have contributed significantly to the phylogeographical composition of fauna.

Presently located 160 km across the shallow Strait of Taiwan from mainland China, Taiwan was first isolated by rising sea levels 4 million years ago (Ma; Hsu 1990). However, both faunistic analysis (Ota 1991, 1997) and geological evidence indicate landbridges have connected the island to the Asian continent possibly 2–3 times, initially during the Pliocene (Yu 1995) and potentially twice during the Pleistocene (Gascoyne *et al*. 1979; Fairbanks 1989). The geological history therefore suggests conspecific populations within Taiwan may comprise of more than one evolutionary lineage resulting from faunal exchanges with the continent. The phylogeographic structure of the hypothesized colonizing populations may additionally have been subject to restricted gene flow by *in situ* orogeny (Chai 1972; Page & Suppe 1981). The tectonic formation of the large-scale Central Mountain Range and, to a lesser extent, the tectonics and volcanism forming the East Coast Mountain Range are further vicariant forces potential of causing allopatric barriers to gene flow. Existing Taiwanese biogeographical studies based on faunal similarity (Hikida & Ota 1997), morphological (Chou & Lin 1997), restriction fragment length polymorphism (RFLP; Ota 1997) and allozyme studies of herpetofauna (Toda *et al*. 1997, 1998) and small mammals (Yu 1995) emphasize the importance of both vicariance due to uplifting of the Central Mountain Range and continental incursion events.

Materials and methods

Population sampling and outgroup choice

Trimeresurus stejnegeri (n = 201) were collected from 40 geographically widespread populations throughout Taiwan (Appendix I) including the offshore Pacific, Orchid (Lanyu) and Green (Ludau) Islands. This sampling regime aimed to comprehensively represent the inter- and intraspecific population variation of *T. stejnegeri* within Taiwan. In order to examine the phylogenetic relationships between the mainland and Taiwanese populations of *T. stejnegeri* a mainland Chinese haplotype (Fujian province), and three haplotypes from Vietnam (Tam Dao, Quang Thanh provinces) were included in the analyses (GenBank accession numbers given in Appendix II). Although *T. stejnegeri* is widely distributed throughout Asia [from India and Nepal through Myanmar and SE Asia to China (McDiarmid *et al*. 1999; unpublished data)], no alternative overseas colonization events were considered as *T. stejnegeri* has not been recorded from the adjacent island chains of the Phillippines to the southeast of Taiwan or from the Ryukyu islands extending to the northeast to Japan.

A *Trimeresurus popeorum* (Chang Rai province, Thailand) and a haplotype from a geographically and morphologically distant population currently assigned to *T. stejnegeri* but likely to belong to a closely related species (Loei province, Thailand, unpublished data) were chosen as outgroups according to preliminary molecular systematic data for the genus *Trimeresurus* as a whole (Malhotra & Thorpe 2000; unpublished data). Since the biogeographical analysis focused on Taiwan, and nested clade analysis is designed for intraspecific data sets (Crandall & Templeton 1996; Templeton 1998) the two outgroups and the Vietnamese sequences were excluded from the nesting algorithm.

DNA extraction, amplification and sequencing

Samples were either in the form of blood [approximately 100 μL, homegenized in 1 mL 5% EDTA and preserved in 2 mL SDS-Tris buffer (100 mM Tris, 3% SDS)], or liver tissue (in 80% ethanol) (see Appendix I for museum voucher numbers). Whole genomic DNA was extracted from 200 to 600 μL of blood/buffer, or 10–20 mg of ethanol-preserved liver using standard protocols (Sambrook *et al*. 1989).

A fragment of approximately 650 bp of the mitochondrial DNA (mtDNA) cytochrome *b* (cyt*b*) gene was amplified via polymerase chain reaction (PCR) (Saiki *et al*. 1988) with modified versions of the primers Mt-A: CTCCCAGCCCCATCCAACATCTCAGCATGATGAAACTTCG (Lenk & Wink 1997) and Mt-F: AGGGTGGAGTCTTCTGTTTTTGGTTTACAAGACCAATG (Wink 1995). Thermal cycling parameters were denaturation at 94 °C for 3 min followed by 35 cycles of: denaturation at 93 °C for 1 min; annealing at 47 °C for 2 min and extension at 72 °C for 2 min. Final extension at 72 °C for 3 min terminated each reaction. A negative (upH_2O) control was always included to eliminate the possibility of contamination. Double stranded PCR products were electrophoresesed on 1% agarose gels and visualized by ethidium bromide staining (Dowling *et al*. 1996). Unincorporated nucelotides and primers were removed using a number

of commercially available kits e.g., Wizard minicolumns (Promega), QIA quick columns (QIAGEN) and rapid gel extraction kits (CONCERT). Single stranded sequencing was either carried out using a modification of the Sequenase version 2.0 protocol (Perkin-Elmer), or sequenced using dye-labelled terminators (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit), and subsequently run on an ABI Prism 377 DNA sequencer. Two additional primers H15149: CCCTCAGAATGATATTT-GTCCTCA (Kocher *et al*. 1989) and MVZ16: GGCAAAT-AGGAAGTATCATTCTG (Moritz *et al*. 1992), were used for sequencing, often from several different PCR products.

Preliminary sequence analyses

Sequences were aligned by eye, and translated into amino acid sequences to check for the presence of stop codons which might indicate the amplification of pseudogenes (Sorenson & Fleischer 1996; Zhang & Hewitt 1996). For distance analyses, the substitutional model was assigned according to distance analyses performed using the log likelihood function of MODELTEST 3.0 (Posada & Crandall 1998). MODELTEST compares 56 different nested models of DNA substitution in a hierarchical hypothesis-testing framework and uses log likelihood scores to establish the model of DNA evolution that best fits the data. This model was then used for subsequent distance-based analyses. To test for saturation, the probability model selected from the above procedure was plotted (data not shown) against the number of pairwise differences due to transitions at the first, second and third codon positions (Wakeley 1996).

Phylogenetic analyses

All analyses were performed on unique haplotypes using the beta test version (b2A) of PAUP* 4.0 (Swofford 1998), with explicit assumptions replacing default parameters where highlighted below. Replicated haplotypes were excluded from the analysis in order to reduce computational time. Neighbour-joining (NJ) was conducted on the probability model identified above, with ties broken randomly. Maximum parsimony (MP) and maximum likelihood (ML) analyses were conducted using a random addition heuristic search with tree–bisection–reconnection (TBR) and subtree pruning–regrafting (SPR) branch swapping, respectively. Bootstrapping [1000 replicates for NJ (NJ option) and MP (FASTSTEP option), 500 replicates for ML (nearest-neighbour interchange, NNI, branch swapping) due to computational time limitations] was performed to obtain a relative measure of node support for the resulting trees (Felsenstein 1985). All sites were equally weighted.

The G and C composition of all sequences were subjected to a G-test of heterogeneity (Sokal & Rohlf 1981). McDonald & Kreitman's (1991) test was used to evaluate the possibility of non-neutral evolution of the cyt*b* gene (Ballard & Kreitman 1995). All ingroup operational taxonomic units (OTUs) were tested against five outgroup taxa from *Trimeresurus sensu lato* (*T. popeorum*, *T. albolabris*, *T. gramineus*, *Ovophis monticola*, and *Protobothrops mucrosquamatus*) (GenBank accession numbers listed in Appendix II). The overall rate constancy of the data was investigated by performing the Kishino & Hasegawa (1989) test, which compares the likelihood of trees both with, and without the incorporation of a molecular clock. Relative rates between the various lineages were tested using the two-cluster test of Takezaki *et al*. (1995) as incorporated in PHYLTEST. Each monophyletic lineage was tested against sister lineages, using the closest interior clade as an outgroup.

Testing the paraphyly/monophyly of the Taiwanese populations

Two-tailed Wilcoxon signed-ranks tests (Templeton 1983) and the Kishino and Hasegawa test were used to test whether alternative tree topologies were significantly different from the most parsimonious tree and the maximum likelihood tree. For the two-tailed Wicoxon signed-ranks tests, a strict consensus analysis was performed on the total maximum parsimony data set (Tree Paraphyly) and edited to represent the alternative hypothesis. The resulting tree was then used as a constraint tree in a replicate parsimony analysis, constraining the analysis to retain only the most parsimonious trees compatible with the alternative tree topology to be tested. Another strict consensus analysis on these trees subsequently resulted in the alternative strict consensus topology (Tree Monophyly). Differences in tree length between Tree 1 and Tree 2 were tested for significance using the Wilcoxon signed ranks test, as implemented by PAUP* 4.0 (Templeton 1983). The Kishino and Hasegawa test was performed on the maximum likelihood tree and an edited tree to represent the alternative hypothesis.

Nested clade analysis

Nested clade analysis as presented by Templeton *et al*. (1995), involves a number of steps which can be summarized as follows. A preliminary calculation evaluates the probability that homoplasious sites or multiple hits will reject strict parsimony. This estimate is based on the number of individuals studied and the number of polymorphic nucleotides relative to the total number of nucleotides examined (Hudson 1989; Templeton *et al*. 1992). If overall parsimony is rejected (as will often be the case in intraspecific data sets) the probability (P_j) of a parsimonious linkage between two haplotypes that differ at j polymorphic sites and share m sites can be calculated according to Templeton *et al*. (1992), thus evaluating the limits of parsimony (parsimony accepted when $P_j > 0.95$).

Given the limits of parsimony, a minimum spanning network is constructed showing a 95% set of plausible networks including the maximum parsimony subset plus nonparsimonious alternatives consistent with quantifiable limits to the deviation from parsimony (Templeton *et al*. 1992). Subsequently, the nesting algorithm as outlined in Templeton *et al*. (1987, 1992), Templeton & Sing (1993) and updated specifically for DNA sequences rather than restriction site data in Crandall (1996) assigns a series of nested clades to the set of plausible cladograms. In brief, the nesting rules start at the tips of the cladogram and move one mutational step into the interior, uniting all haplotypes connected by this procedure into a '1-step clade.' Following pruning off the initial 1-step clades from the tips, the procedure is repeated on the more interior portions of the haplotype tree until all haplotypes have been placed into 1-step clades. The next level of nesting uses the 1-step clades as units, rather than individual haplotypes. The nesting rules are the same; however, '2-step clades' are now formed. The nesting procedure is repeated until a nesting level is reached such that the next higher nesting level would result in only a single category spanning the entire original haplotype network. The resulting nested clades are designated by 'C-N' where 'C' is the nesting level of the clade and 'N' is the number of a particular clade at a given nesting level (Hammer *et al*. 1998; Templeton 1998).

Once the cladogram has been converted into a nested series of linked haplotype networks, the geographical data are quantified either directly from longitude and latitude records or via a distance matrix in kilometres into two distance statistics: (i) the clade distance, D_c, which measures the geographical range of a particular clade; and (ii) the nested clade distance, D_n, which measures how a particular clade is geographically distributed relative to its closest evolutionary sister clades. The GEODIS program (Posada *et al*. 2000) designed to perform these calculations is available at http://bioag.byu.edu/zoology/crandall_lab/programs.htm. Specifically, the clade distance measures the average distance that an individual bearing a haplotype from the clade of interest lies from the geographical centre of all individuals bearing haplotypes from the same clade. The nested clade distance measures the mean distance that an individual bearing a haplotype from the clade of interest lies from the geographical centre of all individuals bearing haplotypes from the next higher-level nesting clade that contains the clade of interest (Templeton 1998). Contrasts in distance measures between tip clades and the clades immediately interior to them in the cladogram are important in determining geographical structuring of genetic variation (Templeton *et al*. 1995). The statistical significance of the different distance measures and the old–young (interior-tip) contrasts are determined by random permutation testing as in Mantel tests (Manly 1991). This procedure simulates the null hypothesis of a random geographical distribution for all clades within a nesting category given the marginal clade frequencies and sample sizes per locality. The two major reasons for the failure to reject the null hypothesis are: (i) the samples are inadequate to detect geographical structuring even though it exists; and (ii) the population is panmictic over the sample area. As there is no way of discriminating between the two alternatives, biological inference is confined to clades in which the null hypothesis is rejected (Hammer *et al*. 1998).

If statistically significant patterns are detected, they can be interpreted using the inference key presented in Templeton *et al*. (1995) and duplicated in Templeton (1998). This summarizes three major biological factors that can cause a significant spatial/temporal association of haplotype variation (restricted gene flow, past fragmentation, and range expansion) throughout the hierarchical clade levels.

The minimum spanning tree was constructed using all Taiwanese haplotypes and the Chinese haplotype. The haplotypes from Orchid and Green Islands were, however, omitted from the geographical distance based analysis. Orchid and Green Islands are volcanic in origin (Bowin *et al*. 1978) separated from mainland Taiwan by deep ocean trenches (Couper 1983), and are hypothesized never to have been connected to Taiwan. The current geographical analysis focused primarily on historical terrestrial migrations (including landbridge formation) and, therefore, these haplotypes were removed to avoid misleading terrestrial associations that are unlikely to have existed in the past.

Results

Preliminary sequence analysis

A total of 654 bp from 46 haplotypes (including outgroups, Vietnamese, and Chinese haplotypes) were included in the final phylogenetic analyses (GenBank accession numbers are given in Appendix II). Identical sequences were obtained when using products from single DNA extracts amplified using different primer sets, as well as from multiple PCR products amplified with identical primer sets. No insertions, deletions or stop codons were found in the data. Mean pairwise ingroup taxa comparisons showed a relatively high transition, transversion ratio (11.2 : 1, respectively) and a marked skewed transition bias at the third codon typical of cyt *b* (Irwin *et al*. 1991), additionally refuting the possibility that a nuclear pseudogene had inadvertently been amplified (Arctander 1995). The substitutional model of molecular evolution assigned by MODELTEST was HKY (Hasegawa–Kishino–Yano; Hasegawa *et al*. 1985) with a gamma distance shape parameter of 0.8490, and this was used in subsequent phylogenetic distance-based analyses. No saturation was observed for transitions at the first, second or third codon position. There were no significant differences between the base

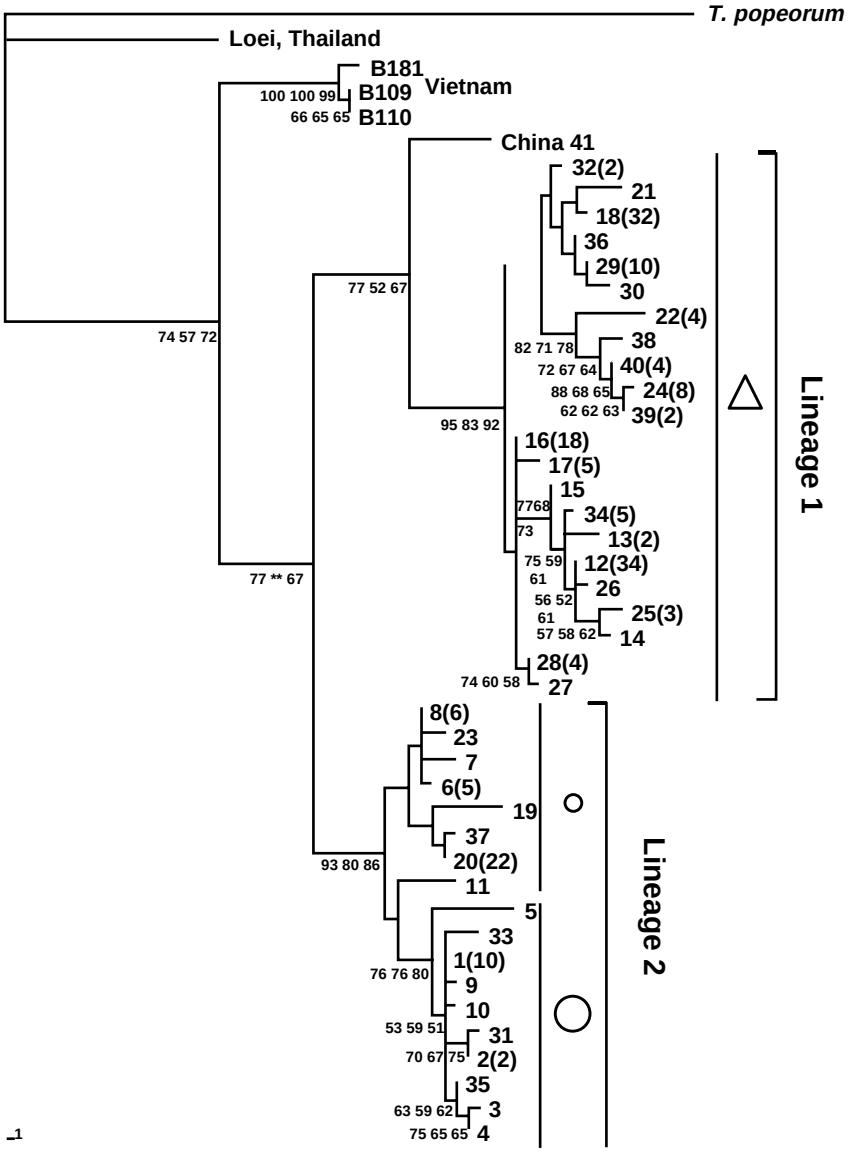

Fig. 1 Maximum-likelihood tree with all bootstrap values [neighbour-joining (NJ), maximum parsimony (MP) and maximum-likelihood (ML), respectively, reading left to right, top to bottom] shown adjacent to corresponding nodes. Lineage 1 is represented by large triangles and Lineage 2 is divided into two further sublineages (large and small circles). No MP bootstrap value was assigned to the basal node of the monophyletic group comprising Lineage 1 and 2 and the Chinese haplotype, as this node was collapsed in the strict consensus of the parsimony analyses. Haplotype numbers are shown followed by number of replicates in parentheses.

frequency distributions of all sequences tested (G_HG = 3.75, G_HC = 2.16, $P > 0.995$). The McDonald and Kreitman test failed to reject the null hypothesis of neutral evolution for any comparison. Levels of corrected pairwise sequence divergence between the 201 Taiwanese *Trimeresurus stejnegeri* and the Chinese *T. stejnegeri* ranged from 2.2 to 4.9%, and from 0 to 5.9% between Taiwanese populations. Thus, pairwise sequence divergence among Taiwanese samples can be higher than between some Taiwanese and Chinese haplotypes.

Phylogenetic analyses

Among the 46 haplotypes there were 152 variable sites, 71 of which were parsimony informative. MP analysis revealed 11161 equally parsimonious trees (243 steps) each with a consistency index (CI) of 0.695, retention index (RI) of 0.876 and rescaled consistency index (RC) of 0.610. Bootstrapped NJ, MP (strict consensus 252 steps) and ML analyses gave largely congruent tree topologies with proportionally similar bootstrap values supporting major lineage divergences (summarized in the fully resolved maximum likelihood tree presented with all bootstrap values in Fig. 1). The tree areas susceptible to multifurcations can be seen in Fig. 1 as minor lineages lacking bootstrap support. The Kishino and Hasegawa test failed to reject the null hypothesis of no variation in overall rate constancy between trees both with and without the incorporation of a molecular clock. The relative rate tests failed to detect significant variation between relative rates of any combination of lineages tested.

The analyses support the presence of two distinct, lineages of *T. stejnegeri* within Taiwan and tree topologies consistently reject the monophyly of the Taiwanese populations

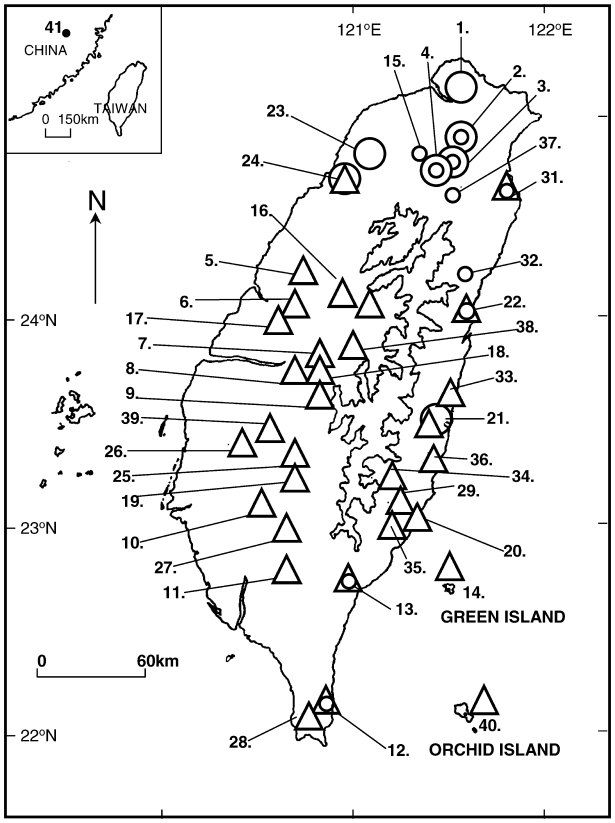

Fig. 2 Map of Taiwan illustrating the geographical location of the two main lineages of *Trimeresurus stejnegeri* resulting from the phylogenetic analyses. Large triangles, and large and small circles correspond to Lineage 1 and 2, respectively, outlined in Fig. 1. Locations where more than one lineage were present are represented by all corresponding phylogenetic symbols. Sample numbers are highlighted by locality in the Appendix I.

(with bootstrap values NJ 77, MP 52, ML 67). The paraphyly of the Taiwanese populations are additionally supported via the rejection of the null hypothesis ($P = 0.045$) of no difference between the unconstrained Tree Paraphyly {252 steps, (outgroups, (Vietnam, (lineage1, (lineage2, China))))} and the constrained Tree Monophyly {264 steps, (outgroups, (Vietnam, China, (lineage1, lineage2)))} using the two-tailed Wilcoxon signed-ranks test. The Kishino and Hasegawa test however, failed to reject the null hypothesis of no difference between the presented maximum likelihood tree and the edited tree representing the Monophyly topology above. The geographical distribution of lineages can be seen in Fig. 2. Lineage 1 is distributed over most of the island excluding the extreme north. The eastern limits of lineage 1 includes all haplotypes from Orchid (site 40) and Green (site 14) Islands. Orchid Island shares identical haplotypes with Green Island, and two sites on the mainland (12, 34). Green Island shares an identical haplotype with one site on the mainland (31). Lineage 2 is distributed throughout the northern region of Taiwan and also extends south along the east coast to Chufengshan (12).

Table 1 Nested contingency analysis of geographical associations

Clade	Permutational chi-square statistic	Probability
1-1	20.50	0.000
1-2	48.97	0.035
1-3	16.00	0.004
1-4	18.10	0.000
2-1	36.00	0.000
2-3	38.00	0.000
3-1	466.67	0.000
3-3	226.49	0.001
Whole cladogram	155.71	0.000

Nested clade analysis

The Hudson estimator failed to reject the null hypothesis of nonparsimony for the entire data set ($P = 0.056$) and

Table 2 Full results from the inference key of geographical distance analyses

Clade	Chain of Inference	Inference
Haplotypes nested in 1-1	1-2-3-4-9-10-NO	Unable to distinguish between fragmentation and restricted gene flow with isolation by distance.
Haplotypes nested in 1-2	1-2-11-17-4-NO	Restricted gene flow with isolation by distance.
Haplotypes nested in 1-3	1-2-3-5-15-NO	Past fragmentation.
Haplotypes nested in 1-4	1-2-3-4-NO	Restricted gene flow with isolation by distance.
1-step clades nested in 2-1	1-2-3-5-15-16-YES	Allopatric fragmentation.
1 step clades nested in 2-3	1-2-3-4-9-10-YES	Allopatric fragmentation.
2-step clades nested in 3-1	1-2-3-5-15-16-YES	Allopatric fragmentation. Additionally confirmed as clades mutationally connected by larger than average mutational steps.
2-step clades nested in 3-3	1-2-3-4-9-NO	Past fragmentation. Additionally confirmed as clades mutationally connected by larger than average mutational steps.
Whole cladogram	1-2-3-4-9-10-YES	Allopatric fragmentation. Additionally confirmed as clades mutationally connected by larger than average mutational steps.

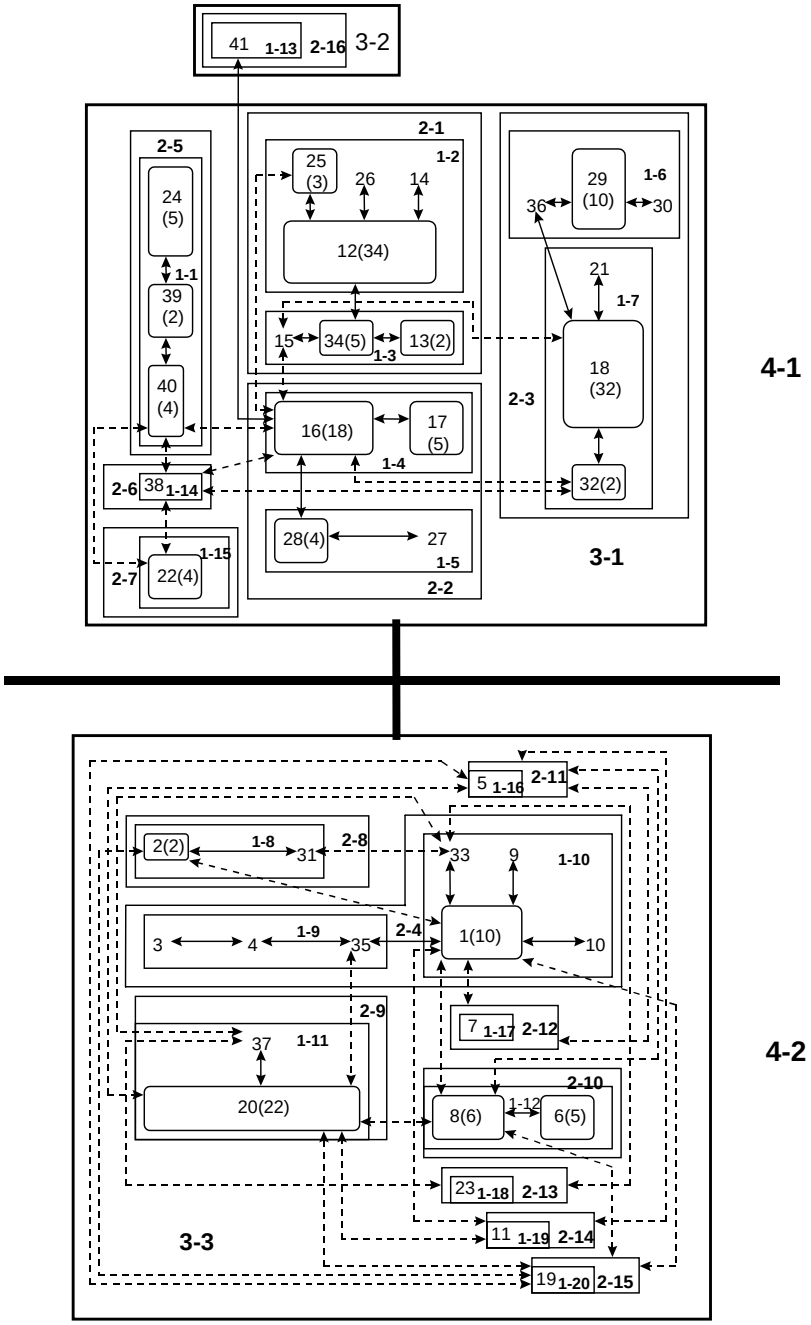

Fig. 3 The nested 95% set of plausible cladograms for the *Trimeresurus stejnegeri* mtDNA data set. Unique haplotypes are unboxed and only nesting levels are indicated. Solid arrows indicate unambiguous single mutations whereas dashed arrows indicate equally likely alternative connections. The solid, bold line connecting clades 4-1 and 4-2 represents a multistep nonparsimonious linkage (16 mutational steps between haplotypes 23 and 18). Nested clades are enclosed in square-edged rectangles and all nesting levels are indicated in bold type, including degenerate clades.

subsequently parsimony was accepted for up to 11 mutational steps (P_{11} = 0.95). Given these constraints, the minimum spanning network (Fig. 3) was constructed. The large number of homoplasious loops describe a set of 829 440 plausible networks (P > 0.95) (Templeton & Sing 1993).

Focusing just on the unambiguous areas of the cladogram, 12 1-step networks were constructed, leaving a further eight degenerate clades (comprising single or identical haplotypes) at the 1-step level. Four unambiguous 2-step clades were constructed leaving a further 12 degenerate clades at the 2-step level. The 3-step networks simply joined all 2-step networks connected by ambiguous loops (up to nine mutational steps within clades 3-1 and 3-3) (Templeton & Sing 1993). In creating 4-step clades, the Chinese haplotype (clade 3-2) was joined to clade 3-1 (11 mutational steps) forming clade 4-1 leaving clade 4-2 degenerate at this level. The nesting algorithm was terminated with the nonparsimonious union of clades 4-1 and 4-2 (16 mutational steps between haplotypes 23 and 18) shown in Fig. 3.

Nested contingency analysis (Templeton *et al.* 1995) detected significant geographical associations within eight individual

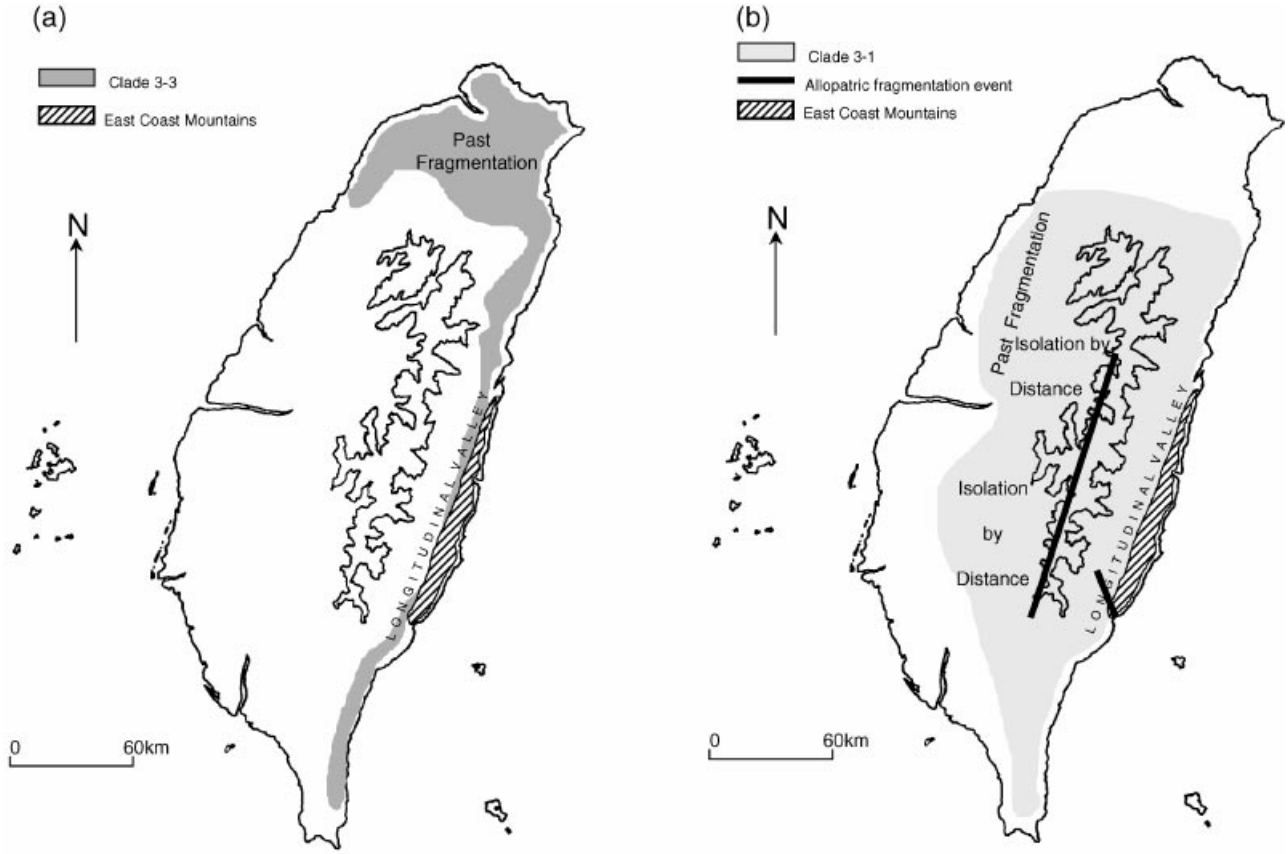

Fig. 4 Maps of Taiwan summarizing the major biological inferences generated by the use of the biological inference key of geographical distance analyses below the whole cladogram level. (a) The distribution of 2-step clades nested within clade 3-3 which are inferred to have been subjected to past fragmentation along the north and east coasts of Taiwan. (b) The hypothesized location of allopatric fragmentation events spanning the middle of the Central Mountain Range and the southern tip of the East Coast Mountains, and the approximate locations of all the lesser clades nested within clade 2-2 across central-western Taiwan. (Position of corresponding biological inferences represent geographical location of clades. See text for further explanation).

clades and for the whole cladogram (Table 1) and, therefore, only these were included in the overall spatial distance analysis. The inference key results are listed in full in Table 2. Briefly, the distance analyses describe a combination of population historical events throughout the hierarchical nested clade structure of haplotypes (Fig. 4). Allopatric fragmentation was hypothesized between the 3-step clades within the whole cladogram. The three major 3-step clades translate exactly to the Chinese haplotype, lineage 1 and lineage 2 of the phylogenetic analysis. Three additional allopatric events were hypothesized. These were between the north and south 1-step clades nested within clade 2-3 restricted to the southeast coast and between the east and west 2-step clades of clade 3-1 (incorporating the east and west components of clade 2-1) either side of the Central Mountain Range. Past fragmentation was inferred for the 2-step clades nested in clade 3-3 that are restricted to the north and east coasts. The remaining resolved hypothesized population histories comprise past fragmentation events and restricted gene flow with isolation by distance amongst all lesser clades of clade 2-2 situated throughout the mid-western plain of Taiwan.

Discussion

The mtDNA cyt*b* data set for *Trimeresurus stejnegeri* within Taiwan exhibited a degree of homoplasy. This is indicated in low CI, RI, and RC values (Farris 1989) alongside the common occurrence of polytomies in bootstrapped phylogenetic analyses and the large number of plausible cladogram alternatives in the minimum spanning network. Homoplasy confounds the finer phylogenetic resolution in traditional analyses (Smouse 1998) but is only included in the nested clade analysis once unambiguous mutational connections between haplotypes have been established (Templeton & Sing 1993). This removes a degree of uncertainty from the interpretation of intraspecific haplotype trees and avoids the assignation of spurious relationships between haplotypes at shallow tree levels which may not exist.

Phylogenetic analyses

Gross tree topologies for all phylogenetic analyses were identical and congruent with the nested minimum spanning network. All analyses suggest the presence of two, genetically well-differentiated, lineages (the term lineage from here on refers to the phylogenetic analysis) or clades (the term clade from here on refers to the nested clade analysis) of *T. stejnegeri* within Taiwan. In all analyses, the larger lineage 1 or clade 4-1 forms a monophyletic group which includes the Chinese haplotype. The paraphyly of the Taiwanese populations is further supported by the parsimony based two-tailed Wilcoxon signed-ranks tests and the 95% confidence interval assigned to the minimum spanning network, but not by the maximum likelihood Kishino and Hasegawa test. It appears, therefore, that two biogeographical scenarios could be suggested for the observed tree topologies. The first indicates that present day Taiwanese *T. stejnegeri* populations have resulted from at least one incursion event from mainland China since their initial isolation. This biogeographical interpretation is supported via the inference key hypothesis of allopatric fragmentation for the whole cladogram distance analysis. Alternatively, if the Taiwanese populations are monophyletic with respect to the mainland, the observed topologies and phylogeographical patterns may be the result of historical lineage sorting (Avise 1986) of the original *T. stejnegeri* populations isolated when Taiwan first became isolated from the mainland. Only comprehensive sampling of a geographically wide range of mainland Chinese *T. stejnegeri* (data not available) would be able to unequivocally support or refute either hypothesis. Although the lineage sorting hypothesis remains a possibility, the incursion scenario correlates well with current concepts regarding the tectonic evolution of Taiwan and subsequent changes in sea level.

The present elevation of Taiwan began approximately 4 Ma during the Pliocene (Hsu 1990) and resulted from the collision of the Phillippine Sea and continental Asian tectonic plates (Page & Suppe 1981). This was accompanied by a sea level rise connecting the South Yellow and East China Seas with the South China Sea and the subsequent isolation of Taiwan from mainland China for approximately two million years (Liu & Ding 1984). The connection with the mainland is believed to have been restored periodically throughout the Pleistocene and the island status of Taiwan was only restored very recently at the end of the late Dali glaciation (15 000 years before present) (Huang 1984). If the continental colonization hypothesis were correct, the genetic discontinuity between the two main lineages/clades of *T. stejnegeri* within Taiwan may therefore have resulted from the long-term initial isolation of Taiwan from mainland China from the mid-Pliocene to the early Pleistocene.

Nested clade analyses

Below the level of the whole cladogram, the past fragmentation (Fig. 4a) inference of the 2-step clades nested within clade 3-3 suggest the existence of remnant populations of the evolutionary oldest components of *T. stejnegeri* throughout the north and east coasts of Taiwan. mtDNA haplotypes have a finite lifespan and so will inevitably be succeeded throughout space and time. Three allopatric fragmentation events were highlighted from the nested clade analysis of clade 3-1 (and subsequently clade 2-1) and clade 2-3 (summarized geographically in Fig. 4b). The central and southeastern allopatric events could therefore be a result of *in situ* genetic isolation of populations caused by the prevention of gene flow across mountain ranges, or by continental incursion events. Toward the end of the Pliocene and the beginning of the Pleistocene a geological compression break resulted in the beginning of the upthrust of the Central Mountain Range (with its highest peak, Yü Shan, at 3997 m) and the East Coast Mountain Range (mean altitude 1000 m, lower towards the north) separated by the establishment of a transform fault, the Longitudinal Valley shown in Fig. 4 (Chai 1972; Bowin *et al*. 1978). The restriction of gene flow in the latter case may have been exacerbated as the East Coast Mountains represent a totally different geological province (Phillippine sea plate) to the rest of Taiwan (comprised of continental Asian plate) separated by a transform fault, the Longitudinal Valley (see Fig. 4). It is feasible, therefore, that the East Coast Mountains have been separated from the rest of Taiwan by a shallow water barrier (Chai 1972). The historical potential barrier to gene flow in addition to the contemporary lack of suitable habitat for *T. stejnegeri* throughout the Longitudinal Valley would expected to have exerted a notable vicariant effect on localized gene flow. However, the roles of continental incursion and large and small scale oregenies and plate tectonics in restricting gene flow cannot be separated.

The remaining key inferences highlight the spatial-temporal movements of haplotypes situated within clades predominately located in the central western region of Taiwan (Fig. 4b). It is inferred that clade 2-2 [spanning from Luku (site 18) in the north to Liangshan (site 11) in the south] has experienced restricted gene flow with isolation by distance. Further lateral range expansions would be unexpected due to the lack of suitable habitat along the southwest coast and the marked decrease in temperature as altitude increases to the east into the central mountain range. To the north, populations of *T. stejnegeri* occupying the area bordered by Chaochiao (site 24), Takeng (site 6), Hsitou (site 9) and Wushei (site 30) (clade 1-2) are hypothesized to have experienced past fragmentation in addition to restricted gene flow with isolation by distance. As above, restricted gene flow with isolation by distance would be

expected for any species colonizing a landscape and the past fragmentation events could be attributed to microvariant events (Templeton *et al*. 1995) either prior to colonization or *in situ*.

T. stejnegeri may been introduced by man to the offshore Pacific islands. Recent mtDNA analyses have revealed an unusually rapid colonization sequence of the lizard *Lipinia noctua*, throughout Polynesia. This has been interpreted as being facilitated by human-mediated dispersal, presumably as stowaways on early Polynesian canoes (Austin 1999). Alternatively, the natural occurrence of rafting is an equally plausible explanation for the colonization of Orchid and Green Islands (for example, see Censky *et al*. 1998). Part of the Luzon volcanic arc, Orchid and Green Islands date from the mid-Miocene to the Pliocene and are surrounded by water ranging from 500 to 2000 m in depth (Bowin *et al*. 1978; Page & Suppe 1981). Therefore, even at the height of the late Pleistocene glaciations, in which sea level was between 110 and 155 m lower than present levels (Huang 1984; Liu & Ding 1984; Zhang 1984) it is highly unlikely that a landbridge would have been created. Therefore, some form of over water dispersal (natural or assisted) remains the only plausible mechanism of colonization.

The combination of standard phylogenetic analyses and nested clade analysis, although highly informative, was unable to clarify the particular roles of palaeoclimate and tectonics within aspects of the population historical events of *T. stejnegeri* within Taiwan. The marked east–west genetic heterogeneity of herpetofauna within Taiwan has been previously illustrated via allozyme data of the Indian Rice Frog, *Rana limnocharis* (Toda *et al*. 1997, 1998) and RFLP data of *Japalura* species of lizards (Ota 1997). Likewise, the paraphyly of Taiwanese populations with continental stock has been highlighted by allozyme studies of small mammals (Yu 1995) and *R. limnocharis* (Toda *et al*. 1997, 1998). However, although the current data strongly suggest current clades of *T. stegnegeri* within Taiwan have resulted from at least one incursion event since the initial isolation of Taiwan in the Pliocene, the exact biogeographic scenario remains unknown. The nested clade analysis highlighted a number of allopatric events below the whole cladogram level, yet distinguishing between the two major vicariant mechanisms with the current data was not possible. It should be noted however, that the two vicariant forces are not mutually exclusive and present day phylogeographical patterns may have resulted from a dynamic interaction of both tectonic and palaeoclimatic forces.

Implications for future studies on T. stejnegeri *within Taiwan*

The current analyses summarize phylogeographic relationships of *T. stejnegeri* throughout Taiwan based on data derived from a single mitochondrial gene. It would, therefore, be highly advantageous to investigate the level of nuclear introgression within and between the major lineages or clades revealed by the present analyses (Avise 1998). Moreover, once the phylogeographic structure has been satisfactorily resolved further studies can be conducted investigating the historical influences phylogenetic relationships have had regarding geographical variation in morphology (Castellano *et al*. 1994) and ongoing geographical variation in venom composition studies.

Summary of phylogeographical analytical methods

Both standard phylogenetic analyses and nested clade analysis gave identical gross tree topologies. However, the geographical distance analyses from nested clade analysis were considerably more informative in inferring statistically tested historical events throughout the nested clade hierarchy. The central goal of both nested clade analysis and matrix correspondence tests (as used in Thorpe *et al*. 1996) are the same, i.e. separating contemporary phylogeographic pattern from population historical processes. However, the individual hypothesis testing frameworks are different. The matrix correspondence tests aim to discriminate between alternative putative historical scenarios that have been formulated to address a specific phylogeographical pattern (Thorpe *et al*. 1996). Nested clade analysis however, assigns one of three key historical population processes (or variations within) according to a genetic-spatial analysis of haplotype distribution and geography. Thus, nested clade analysis does not test the plausibility of alternative historical processes pertaining to a specific phylogeographical pattern. Given the conflicting biogeographical mechanisms of vicariance by both palaeoclimate and tectonic orogeny however, it was not feasible to generate a hypothesis testing framework similar to that used by Thorpe *et al*. (1996) for the current scenario. Both nested clade analysis and matrix correspondence tests require specialist knowledge and still remain relatively unused in elucidating population historical processes. However, both aim to test phylogeographic pattern, rather than simply present it and, therefore, the use of both techniques should be encouraged in developing the field of spatial-genetic analysis of genealogical distributions. More specifically, nested clade analysis needs a wider understanding and further worked examples. The method possesses the advantage of operating from a single, statistically supported haplotype tree representing all possible parsimonious and nonparsimonious alternatives (Templeton *et al*. 1992). This removes the need to discriminate between separate conflicting phylogenetic analyses and avoids the presentation of weakly supported clades that only confound extrapolations from the data rather than support

them. While standard phylogenetic reconstructions remain widely accepted and understood, the inherent simplicity and increased inferential power gained by incorporating hypothesis testing through nested clade analysis should not be overlooked in the future of phylogeographical studies.

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This work forms part of a larger study of the systematics of *Trimeresurus* pitvipers begun by Anita Malhotra and Roger S. Thorpe in 1992. Simon Creer's PhD in particular focused on the causes of geographical variation in the venom composition of *Trimeresurus stejnegeri* in Taiwan. Wen-hao Chou's research interests focus on the biodiversity and biogeography of Taiwanese and Asian herpetofauna.

Appendix I

List of sampling localities and approximate geographical location of *Trimeresurus stejnegeri* within Taiwan and mainland China (numbers plotted in Fig. 2). Figures in parentheses refer to the number of individuals with that haplotype where more than one copy present

Sample site number and name	Haplotype numbers	National Museum of Natural Science, Taiwan Field No. (in order, * represents missing code)
1. Yangminshan	1(2), 2(2), 3, 4	12325, 12323, 12321, *, 12322, 123241
2. Wulei	1(5), 5, 6	*, 3782, 3781, 3783, 12304, *, 3786
3. Fushan	1, 6(4), 7, 8, 9, 10	12266, 12261, 12265, 12267, 12268, 12260, 12262, 12263, 12264
4. Paling	1, 11	12336, 12337
5. Pa-shen-san	12(2)	3780, 3779
6. Takeng	12(10), 13(2)	4108, 4109, 4110, 4111, 4113, 4114, 4115, 4116, 4118, *, 4112, 4117
7. Chichi	12, 14	3758, 3759
8. Chishan	15	*
9. Hsitou	12(2)	3755, 3754
10. Shanping	16, 28	3931, 3932
11. Liangshan	16, 17(5)	*, 12244, 12245, 12248, 12249, 12250
12. Chufengshan	8, 18(9)	4170, 4168, 4169, 4171, 4172, 4173, 4174, 4175, 4176, 4177
13. Chihpen	18(9), 19, 20, 21	4125, 4126, 4123, 4127, 4122, 4128, 4129, 4124, 4130, 4259, 4258, 4160
14. Green Island	18, 22(3)	12338, 12339, 12274, 6231
15. Tungyanshan	23	12317
16. Chingshan	24	*
17. Chutyukeng	12(5), 25	12287, 12288, 12289, 12290, 12291, 12292
18. Luku	12(14), 16(5), 26	12176, 12177, 12178, 12179, 12180, 12181, 12182, 12199, 12200, 12201, 12202, 12203, 12196, 12198, 12192, *, 12193, 12194, 12195, 12197
19. Chuyunshan	16(7), 27, 28	12185, 12183, 12186, 12187, 12188, 12189, 12190, 12307, 12191
20. Dong Ha Farm	29(7), 30	4266, 12280, 12282, 12283, 12284, 12285, 12286, 12281
21. Chimei	24, 31	12231, 4330
22. Hsuei-yuan	20(6), 32(2)	12205, 12206, 12207, 12208, 12209, 12210, 4348, 4347
23. Hsincheng	33	12308
24. Chaochiao	1, 34(5), 35	99, 12314, 12311, 12315, 12312, 12313, 12316
25. Sanmei	28	12306
26. Quantzelin	16	12332
27. Sanpin	16(3)	12294, 12295, 12296
28. Paoli	18	12319
29. Kuanshan	29, 36	12329, 12328
30. Wushei	25(2)	6235, 12331
31. Suao	8(3), 20(3), 22	12257, 12258, 12259, 12271, 12272, 12273, 12256
32. Taroko	20(12), 37	12218, 12219, 12220, 12221, 12222, 12212, 12213, 12214, 12215, 12216, 12217, 12223, 12211
33. Fuyuan	24(6), 38, 39	12225, 12226, 12228, 12229, 12230–1, 12230–2, 12224, 12227
34. Litau	18(4), 40(4)	12254, 12340, 12341, 12343, 12253, 12255, 12342, 12344
35. Chan-na	18	12276
36. Antung	29(2), 39	12276, 12278, 12277
37. Ming-chi	8	12279
38. Lienhwachih	12	12270
39. Chukou	28	*
40. Orchid Island	18(7)	12232, 12233, 12234, 12235, 12236, *, *
41. Wu-yi shan	41	*

Appendix II

GenBank Accession numbers for sequences used in study

Organism	Location	Accession nos
Trimeresurus popeorum	North Thailand	AF171902
T. albolabris	Alor Island Indonesia	AF171882
Ovophis monticola	Taiwan	AF171907
Protobothrops mucrosquamatus	Taiwan	AF171897
T. gramineus	South India	AF171905
T. stejnegeri	North East Thailand	AF171898
T. stejnegeri	Vietnam, Tam Dao	AF278709–10
T. stejnegeri	Vietnam, Quang Thanh	AF278711
T. stejnegeri	China	AF277677
T. stejnegeri	Taiwan	AF277676–716