

The utility of AFLPs for supporting mitochondrial DNA phylogeographical analyses in the Taiwanese bamboo viper, *Trimeresurus stejnegeri*

S. CREER,* R. S. THORPE,* A. MALHOTRA,* W.-H. CHOU† & A. G. STENSON*

*School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, UK

†National Museum of Natural Science, 1, Kuan Chien Road, Taichung, Taiwan, 404, China

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Abstract

An amplified fragment length polymorphism (AFLP) assay was performed on individuals representing discrete haplotypes from two genetically distinct mtDNA lineages of the bamboo viper, *Trimeresurus stejnegeri* (Schmidt), within Taiwan. AFLP (525 polymorphic markers from five primer pairs) and mtDNA genetic distances were highly correlated and an analysis of molecular variance, and a Bayesian approach similarly partitioned estimates of genetic similarity according to the mtDNA phylogeographical pattern. These results are discussed in relation to biogeographical hypotheses, comparative rates of mtDNA molecular evolution, and in the identification of evolutionary significant units of Taiwanese *T. stejnegeri*. In spite of the high degree of congruence between the genetic datasets, the AFLP phylogenetic analysis did not support the mtDNA tree, suggesting that no contemporary barriers to gene flow exist between individuals from the two mtDNA lineages.

Introduction

The comparative study of mtDNA gene sequences revolutionized the field of intraspecific phylogeography during the late twentieth century (Avise *et al.*, 1987; Avise, 1998). As a result, a large number of biogeographical hypotheses have been formulated based on mtDNA phylogeographical data. Whilst a large proportion of these studies will accurately represent population processes, the nonrecombining mode of transmission of mtDNA leaves the inferences of many studies vulnerable to the confounding phenomenon of stochastic lineage sorting (Avise, 1989, 1994). Lineage sorting can occur when a combination of ancestral polymorphism coupled with the differential survival of alleles throughout evolutionary time results in a gene phylogeny not matching the organismal phylogeny (Avise, 1994; Page & Holmes, 1998). Thus, the need for independent, genomic molecular markers to support mtDNA analysis is clear. To date however, no single technique (e.g. allozymes, restriction fragment length

polymorphisms, random amplified polymorphic DNA, microsatellites, single copy nuclear DNA variation, single-stranded conformation polymorphisms) has proven to be optimal for resolving genetic relatedness at a range of taxonomic levels (Mueller & Wolfenbarger, 1999; Parsons & Shaw, 2001). Each technique has strengths and weaknesses generally based upon the equilibrium between repeatability, cost and development time and the detection of genetic polymorphism. The answer to these problems may lie in the analysis of amplified fragment length polymorphism (AFLP) fragments. AFLP analysis (Vos *et al.*, 1995; Mueller & Wolfenbarger, 1999) is claimed to generate a large number of repeatable genomic polymorphic markers without the necessity for any prior research and development. Thus, the simultaneous analysis of larger numbers of genomic loci aims to reduce the confounding effects of historical lineage sorting that may prevail over single gene analyses. Although the dominant nature of inheritance of AFLP markers currently limits their utility for applied population genetics (Gaudeul *et al.*, 2000; Ogden & Thorpe, 2002), the method appears to be ideal for obtaining genomic support for intraspecific mtDNA analyses. Not only would this offer verification for hypothesized biogeographical reconstructions, or historical population processes (e.g. bottlenecks and expansions), but also offer an added degree of confidence

Correspondence: Simon Creer, Brambell Building, School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, UK
Tel.: +44 1248 351151; fax: +44 1248 371644;
e-mail: s.creer@bangor.ac.uk

in obtaining robust organismal phylogenies for any genetic comparative analyses. An extensive number of studies have used AFLPs to assess the intraspecific genetic diversity of plant (Sharbel *et al.*, 2000; Larson *et al.*, 2001; Zawko *et al.*, 2001) and fungal (Redecker *et al.*, 2001) populations. However, AFLP assays of animals (García *et al.*, 2002; Salvato *et al.*, 2002), and in particular, vertebrates (Albertson *et al.*, 1999; Mock *et al.*, 2002) are still limited.

A recent phylogeographical study of mtDNA cytochrome *b* (*cyt b*) gene sequences of the bamboo viper, *Trimeresurus stejnegeri* (Schmidt), has revealed the presence of two genetically distinct mtDNA lineages (separated by up to 5.9% corrected pairwise sequence divergence) within Taiwan (Creer *et al.*, 2001). Preliminary analyses suggest that the two Taiwanese populations are the result of separate colonization events from mainland Asia, but the pattern may have also arisen simply by historical lineage sorting via founding populations within Taiwan. Therefore, here it was assessed whether levels of genetic differentiation measured by polymorphic AFLP markers can be used in the identification of delimited gene pools which correspond to mtDNA analyses in Taiwanese *T. stejnegeri* (as a model system). Furthermore, if mtDNA pattern is correlated with AFLP genetic differentiation, a phylogenetic analysis may address whether gene exchange occurs between discrete mtDNA lineages.

Materials and methods

Population sampling and DNA extraction

In accordance with the primary aim of the AFLP assay, only a subset of individuals were selected to represent all of the discrete mtDNA haplotypes identified in Creer *et al.* (2001) corresponding to geographically disparate localities throughout Taiwan (Fig. 1a, b). Whole genomic DNA was extracted according to the protocol outlined in Creer *et al.* (2001). Template DNA was visualized by ethidium bromide staining (Dowling *et al.*, 1996) and quantified according to known concentration standards on 1% agarose electrophoresis gels. Subsequently, 250 ng of template DNA was pelleted via standard sodium acetate/ethanol precipitation (Sambrook *et al.*, 1989) and used for the AFLP assay.

AFLP assay

The AFLP assay (Vos *et al.*, 1995) was carried out according to the manufacturer's protocol (AFLP™ Plant Mapping, PE Applied Biosystems, Foster City, CA, USA) with the following modifications. All PCR reactions were carried out on a PE Applied Biosystems GeneAmp® PCR System 9700. Half, and not full volume reactions were used for restriction–ligation reactions. Preselective amplification products were diluted with 140 µL of 0.1xTE, rather than 190 µL. AFLP *EcoRI* 5'GACTGCGTACCAA-

TTC+A3' and *MseI* 5'GATGAGTCTGAGTAA+C3' preselective primers were used in a combined premix form (PE, Applied Biosystems). AFLP bands were generated using five combinations of *EcoRI* (fluorescent 5' end-labelled) and *MseI* selective reaction primers, i.e. *EcoRI* and *MseI* preselective primers plus two additional bases shown in Table 1. The systematic utility of these selective primer pairs has been previously demonstrated in *T. albolabris* (Giannasi *et al.*, 2001). Selective amplification PCR products (1 µL FAM, 2 µL JOE and 3 µL TAMRA fluorescent end-labelled, to optimize detection of blue, green and yellow colours respectively) were dry-pelleted (70 °C) and resuspended in 2.5 µL loading buffer (60% formamide, 20% dextran Blue and 20% PE Applied Biosystems, Warrington, UK, GS500 ROX size marker). Following denaturing at 95 °C for 2 min and snap chilling (0 °C), 1 µL of each product was electrophoresed through a 5% Long Ranger® (BMA) polyacrylamide gel (Amresco, Solon, OH, USA) on an ABI377 DNA sequencer, using 36 cm plates. Chromatograms were exported using Genescan® and the presence (coded 1) or absence (coded 0) of bands between 100–500 bp (>100 rescaled peak height) were identified using Genotyper®. Only polymorphic bands were scored, and so common labels were cleared (tolerance of 1.0 bp) and categories (i.e. bands) were made from labels with 2.0 bp tolerance. A consequence of loading more than one selective amplification product on the same automated sequencer lane may be that high intensity bands in one colour have the potential of producing the same sized low intensity band in another colour. In order to test this, the exact sizes of all polymorphic bands from the *EcoRI* plus ACC (FAM, blue), *MseI* plus CAG, and the *EcoRI* plus AGG (TAMRA, yellow), *MseI* plus CTA selective amplifications loaded on the same gel were compared to check for coincidental peak sizes. The above modifications were made to optimize detection, and accurate size calling of AFLP bands in the present study using Genescan® and Genotyper® with the ABI377 DNA sequencer.

Data analysis

A combination of molecular variance, distance, and parsimony analyses (Parsons & Shaw, 2001) in addition to a Bayesian hierarchical model (Holsinger *et al.*, 2002) were used to assess genetic relatedness and phylogenetic structure of operational taxonomic units (OTUs) representing haplotypes from the genetically distinct mtDNA lineages identified in Creer *et al.* (2001). For all analyses, data from all five primer pair combinations were combined in a single binary data matrix.

Partitioning genetic diversity

Following the assumption that the same mating pattern prevails in all sampled populations, an estimate of genomic population structure was determined via an analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) using

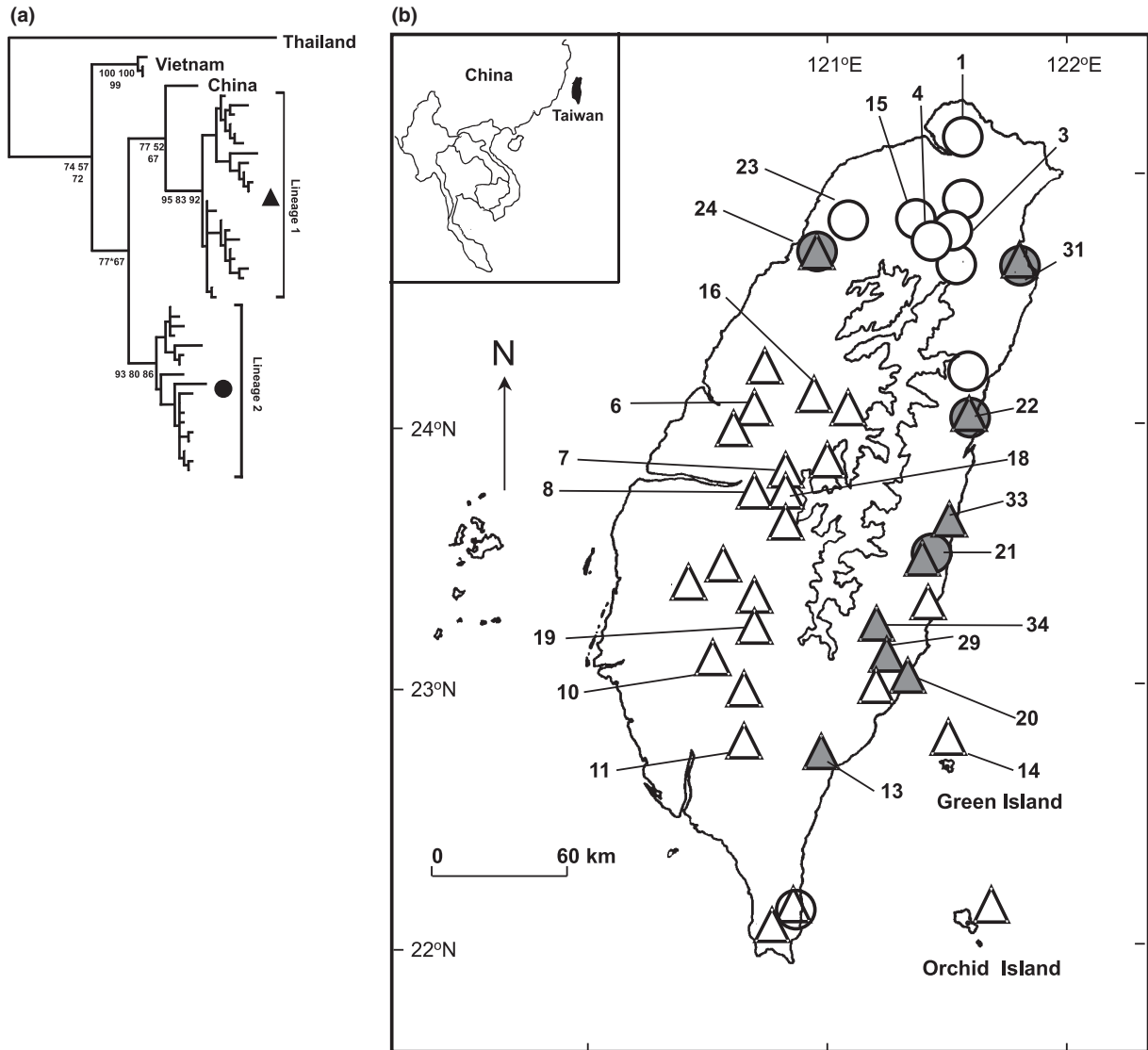


Fig. 1 (a) MtDNA *cyt b* maximum likelihood tree with bootstrap values (neighbour-joining, maximum parsimony, and maximum likelihood) reading left to right, top to bottom respectively shown adjacent to corresponding nodes. Bootstrap values support major phylogenetic lineages within Taiwan depicted by the triangle (Lineage 1), and circle (Lineage 2). (b) Map of Taiwan and SE Asia (insert) showing all locations of the two major Taiwanese mtDNA phylogenetic lineages detected in (Creer *et al.*, 2001) used in the AFLP analysis. The contour represents 2000 m a.s.l. Within Taiwan, triangles and circles correspond to Lineage 1 and 2 respectively, and sites are only numbered where individuals were used for the AFLP analysis. The shaded sites correspond to the region where Lineage 1 and 2 overlap (sympatric) used for the ΔMOVA and Bayesian analyses.

Arlequin version 1.1 (Schneider *et al.*, 1997). In order to compare the level of genomic genetic differentiation with mtDNA *cyt b* sequence phylogeography, variation was partitioned among three pseudopopulations according to OTU's representing the two nonoverlapping individual mtDNA lineages, and the central region where the two lineages overlap (sympatric, Fig. 1b). This population subdivision aimed to investigate the levels of genetic dissimilarity between OTUs representing the two 'pure' mtDNA lineages and OTUs (sympatric) which may exhibit

intermediate levels of genomic differentiation. The same population structure was further analysed using the Bayesian method implemented by Hickory version 0.6 (Holsinger & Lewis, 2002) that facilitates direct estimation of F_{ST} (eqn 1) from dominant markers.

$$F_{ST} = Q_s - Q_t / 1 - Q_t, \quad (1)$$

Where Q_s is the probability that two alleles chosen at random from within a population are identical and Q_t is the probability that two alleles chosen at random from

Table 1 The five combinations of *EcoRI* and *MseI* selective reaction primers used to generate AFLP bands. The fluorescent, end-labelling dye plus colour is given according to PE Applied Biosystems virtual filter set A (in parentheses).

Primer pair	<i>EcoRI</i> plus	<i>MseI</i> plus	Number of polymorphic markers
1	CC (FAM, blue)	AG	114
2	GC (JOE, green)	TG	98
3	GG (TAMRA, yellow)	TC	80
4	GG (TAMRA, yellow)	TA	123
5	CC (FAM, blue)	AC	110

the entire set of populations are identical. The Bayesian statistic that estimates F_{ST} is referred to as θ^B . For full explanation of the etymology of θ^B , see Holsinger *et al.* (2002).

Phylogenetic reconstruction

Neighbour-joining (NJ) was conducted on total pairwise distances with ties broken randomly. Maximum parsimony analysis was performed with AFLP banding profiles added randomly. Bootstrapping (1000 replicates) was performed to obtain a relative measure of node support for the resulting trees (Felsenstein, 1985). All bands were weighted equally and trees rooted using a *T. stejnegeri* from Loei province, north east Thailand.

Mantel tests

Finally, in order to test for a correlation between the AFLP data, geography, and mtDNA haplotypes, pairwise Mantel tests (Gaudeul *et al.*, 2000) were performed on distance matrices based upon total number of pairwise differences in shared AFLP bands against geographic distance (measured by longitude and latitude co-ordinates), and pairwise genetic distances based upon the substitutional model of molecular evolution assigned to the mtDNA dataset using MODELTEST (Posada & Crandall, 1998).

Results

Total sets of AFLP markers were obtained for 34 *T. stejnegeri* from 24 localities in Taiwan and the Thai

sample (Appendix and Fig. 1b). Each of the five primer pair combinations produced between 80 and 123 polymorphic bands resulting in a total of 525 AFLP markers (Table 1). No coincidental peaks were observed between the *EcoRI* plus ACC, *MseI* plus CAG, and the *EcoRI* plus AGG, *MseI* plus CTA selective amplifications, suggesting that polymorphic loci were not recorded more than once across the different primer pair combinations.

The Mantel tests showed that AFLP genetic distance was highly correlated (standardized correlation coefficient $r = 0.235$; $P < 0.001$) with mtDNA genetic distance (654 bp, HKY + gamma 0.8490), but not with geographic distance ($r = -0.066$; n.s.). The results of the AMOVA and Bayesian analyses gave concordant results showing that the highest measures of genetic dissimilarity were obtained between the comparison of Lineage 1 and Lineage 2 only, and the comparison including all three pseudopopulations (Table 2). Conversely, the comparisons between Lineage 1 and the overlapping region and Lineage 2 and the overlapping region shared the highest proportion of genetic similarity. The NJ (Fig. 2) and MP (not shown) phylogenetic trees were poorly supported, highly pectinate, trees showing no concordance with the genetically differentiated, two lineage structure of the mtDNA tree in Fig. 1a.

Discussion

AFLPs and mtDNA phylogeography

In the present example, the utility of AFLP markers for the identification of delimited gene pools that correspond to well defined, intraspecific mtDNA phylogeographical patterns has been confirmed. The AFLP and mtDNA measures of genetic distance are highly correlated and the AMOVA and Bayesian analyses proportionally partition estimates of genetic dissimilarity according to the mtDNA lineages. The AMOVA estimates of F_{ST} were lower than the Bayesian estimates of θ^B . This has been observed in other empirical studies (Holsinger *et al.*, 2002), and may be due to different statistical methodologies, the nonidentical estimates of F_{ST} involved (Cockerham, 1969; Schneider *et al.*, 1997), or an artefact of smaller sample sizes (Holsinger *et al.*, 2002).

Table 2 Results of the AMOVA and Bayesian analyses. All comparisons were performed on variation partitioned between hypothetical populations corresponding to (i) the region in which only Lineage 1 occurs, (ii) the region in which only Lineage 2 occurs and (iii) the region in which both overlap (sympatric, Fig. 1b). For the Bayesian analysis, θ^B (mean values) corresponds to F_{ST} (Holsinger *et al.*, 2002). Credible intervals (CI) play a role in Bayesian statistics similar to confidence intervals (Sokal and Rohlf, 1995) in classical statistics.

Source of variation	AMOVA			Bayesian		
	d.f.	SS	Variance component	F_{ST}	θ^B	95% CI
Lineages 1, 2, and sympatric	2	161.5	2.54	0.045**	0.101	0.079–0.126
Lineage 1 vs. 2	1	85.65	3.06	0.053**	0.123	0.093–0.159
Lineage 1 vs. sympatric	1	70.42	2.02	0.040*	0.087	0.056–0.121
Lineage 2 vs. sympatric	1	86.58	2.63	0.043*	0.084	0.055–0.119

** $P < 0.01$, * $P < 0.05$

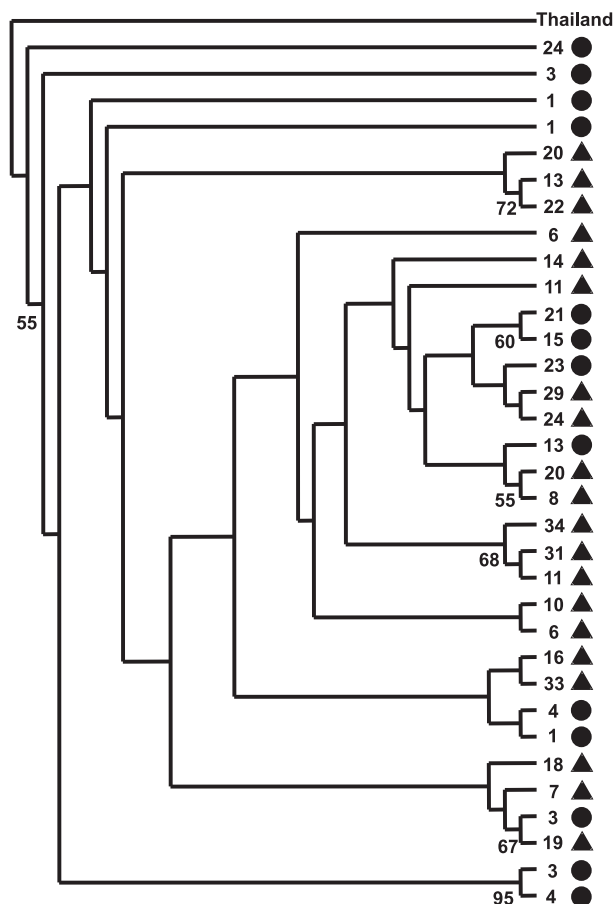


Fig. 2 AFLP NJ tree with bootstrap values shown adjacent to corresponding nodes. Site numbers (Fig. 1b, Appendix) and open triangles (Lineage 1) and circles (Lineage 2) identify taxa assigned to the two mtDNA lineages.

Whilst the AFLP molecular variance analyses are congruent with the mtDNA phylogeographical pattern, the phylogenetic analyses suggest that genetic exchange does occur between individuals from the two mtDNA lineages. Although the use of cluster analyses on AFLP data do not give strict measures of gene flow, they are commonly used to define interspecific boundaries, and detect hybridization between *a priori* defined genetic entities (O'Hanlon *et al.*, 1999; Congiu *et al.*, 2001; Teo *et al.*, 2002; Nijman *et al.*, 2003). Thus, the lack of any clear separation between individuals representing the two mtDNA lineages in the phylogenetic analyses indicate that there are no significant barriers to gene flow between the mitochondrial lineages of *T. stejnegeri* within Taiwan. Conversely, the deep ocean trench (between 2–500 m) (Couper, 1983) between the offshore Pacific islands and the main island of Taiwan offers a significant barrier to gene flow. However, the *T. stejnegeri* from Green Island was not phylogenetically distinct from the populations on the main island of Taiwan (Fig. 2)

suggesting that the island colonization event has occurred relatively recently.

Taiwanese *T. stejnegeri* biogeography and comparative rates of mtDNA evolution

The tectonic processes that resulted in the present island of Taiwan began approximately 4 million years ago (Ma) during the Pliocene (Hsu, 1990). A simultaneous rise in sea level is hypothesized to have resulted in the initial isolation of the island from mainland China for approximately 2 million years (Liu & Ding, 1984), which preceded an unspecified number of landbridge connections throughout the Pleistocene (Huang, 1984). The present genetic analyses offer support for the biogeographical hypothesis of colonization of Taiwan by *T. stejnegeri* via at least one Pleistocene landbridge connection (Huang, 1984; Liu & Ding, 1984) to the mainland since the initial isolation of Taiwan in the late Pliocene (Creer *et al.*, 2001). Following the assumption that the mtDNA and AFLP phylogeographical interpretations are correct, the two lineages of *T. stejnegeri* are hypothesized to have been initially separated approximately 4 Ma. Given that mean corrected mtDNA *cyt b* sequence divergence between the lineages is 4.4% (range 3.2–5.9%, SD = 0.6%), this would imply that *T. stejnegeri* would have an estimated maximum corrected rate of *cyt b* molecular evolution of 1.1% per million years (pmy) (range 0.8–1.5%). This rate is in accordance with an estimated 'ball park' mtDNA range of 0.47–1.32% pmy previously suggested for small to medium sized ectotherms (Zamudio & Greene, 1997), and overlaps considerably with the range 1.09–1.77% pmy specifically calculated from a concatenated *cyt b*-NADH subunit 4 (mtDNA) partition in neotropical piperids (Wüster *et al.*, 2002). Thus, despite the number of methodological and natural factors which are known to affect the calculation of rates of molecular evolution (Martin & Palumbi, 1993; Avise, 1994; Rand, 1994; Page & Holmes, 1998), congruent estimates of mtDNA molecular clocks are emerging for squamates from disparate data sources.

Identification of evolutionary significant units within Taiwan

Modern conservation strategies utilize knowledge of spatial patterns of species diversity, and historical population processes (Moritz, 1995) in the identification of evolutionary significant units (Brooks *et al.*, 1992; Moritz & Faith, 1998). The mechanisms which are likely to have had a major affect on Taiwanese biogeographical assemblages are mainland colonization events, and the vicariant effect of the Central Mountain Range (Fig. 1b). It is interesting therefore, to note that the phylogeographical patterns confirmed for *T. stejnegeri* have been broadly recognized in previous herpetological studies

using alternative genetic markers. The marked east–west genetic heterogeneity in addition to the paraphyly of Taiwanese populations have been shown using allozymes in *Rana limnocharis* (Indian Rice Frog) (Toda *et al.*, 1997, 1998), and an independent study of small mammals (Yu, 1995). An RFLP study of *Japalura* lizards (Ota, 1997) throughout Taiwan additionally emphasized the presence of east–west genetic differences amongst populations studied. Consequently, these data may outline recurrent phylogeographical patterns of faunal communities sharing similar life history characteristics and biogeographical origins (Avisé, 1998; Da Silva & Patton, 1998; Moritz & Faith, 1998). Although specific case studies would have to genetically appraised, this suggests that the regions east and west of the Central Mountain Range may deserve appropriate recognition in future conservation programs. Moreover, further molecular data gathered from a range of fauna from diverse biogeographical origins (Ota, 1997) and life histories, including endemic species (Yang *et al.*, 1994) may reveal additional evolutionary significant areas for special consideration in conservation strategies.

The level of intraspecific genetic discrimination achieved by AFLPs in the current example is in agreement with a number of studies performed on *Aedes aegypti* (yellow fever mosquito: Yan *et al.*, 1999), *Nasutitermes takasagoensis* (winged termites: García *et al.*, 2002), the *Thaumetopoea pityocampa* – *wilkinsoni* complex (winter pine processionary moths: Salvato *et al.*, 2002), *Anolis* lizards (Ogden & Thorpe, 2002) and *Meleagris gallopavo* (wild turkey: Mock *et al.*, 2002), but in sharp contrast to the lack of any congruence found between AFLP and mtDNA analyses within the *Larus argentatus* species ring complex (Herring Gull: de Knijff *et al.*, 2001; Liebers *et al.*, 2001). Undoubtedly, the use of AFLP's in comparisons of animal taxa will continue to grow and more examples using diverse combinations of primers will lead to a greater understanding of the potential possibilities of this emerging molecular systematic tool.

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Appendix Table showing mtDNA *cyt b* gene GENBANK accession code, location (samples are preceded with the haplotype and site numbers) and National Museum of Natural Science (NMNS), Taiwan field number (*represents missing code).

GENBANK accession code	Location	Field number
1. AF277700	4. Paling	12336
2. AF277701	1. Yangminshan	*
3. AF277708	1. Yangminshan	12322
4. AF277712	1. Yangminshan	12324
7. AF277696	3. Fushan	12260
9. AF277703	3. Fushan	12263
10. AF277704	3. Fushan	12264
11. AF277706	4. Paling	12337
12. AF277689	6. Takeng	4110
13. AF277707	6. Takeng	4117
14. AF277688	7. Chichi	3759
15. AF277694	8. Chushan	*
16. AF277678	11. Liangshan	*
17. AF277709	11. Liangshan	12250
18. AF277680	14. Green Island	12338
19. AF277697	13. Chihpen	4259
21. AF277676	13. Chihpen	4160
22. AF277685	31. Suao	12256
23. AF277693	15. Tungyanshan	12317
24. AF277698	16. Chingshan	*
26. AF277716	18. Luku	12197
27. AF277715	19. Chyunshan	12307
28. AF277691	10. Shanping	3932
29. AF277687	20. Dong Ha Farm	4266
30. AF277710	20. Dong Ha Farm	12281
31. AF277681	21. Chimei	4330
32. AF277711	22. Hsuei-yuan	4348
33. AF277682	23. Hsinscheng	12308
34. AF277692	24. Chaochiao	12315
35. AF277713	24. Chaochiao	12316
36. AF277683	29. Kuanshan	12328
39. AF277714	33. Fuyuan	12227
40. AF277702	34. Litau	12255
41. AF171898	North East Thailand	*