DOCTOR OF PHILOSOPHY

Cryptic speciation and biogeography in Indomalayan pitvipers (Trimeresurus)

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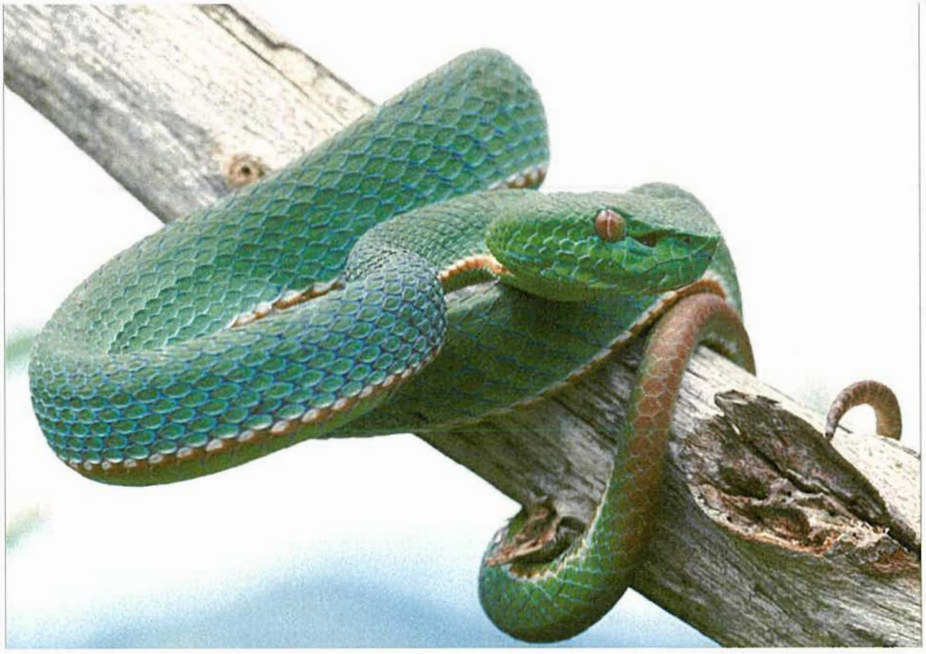

A thesis submitted to the University of Wales, Bangor

by

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Summary

Trimeresurus pitvipers are a conspicuous component of most Asian reptilian faunas and in recent years have become a subject of intense interest to systematists, ecologists and herpetoculturalists. The following study reviews cryptic speciation and biogeography in three *Trimeresurus* species groups from the Indomalayan region.

The systematics of two frequently misidentified closely related species, *Trimeresurus hageni* and *T. sumatranus,* are first investigated using multivariate morphometrics. Two morphological forms are found to correspond to *T. hageni* in West Malaysia, Thailand, Singapore and Sumatra, and *T. sumatranus* in Borneo and west Sumatra. Geographic variation and diagnostic characters are discussed.

Following the collection of new material for the *T. sumatranus* complex, the molecular and phenotypic evolution of the five species in this group is analysed using mitochondrial DNA sequencing and multivariate morphometrics. A well-resolved phylogeny shows each species to represent a distinct lineage. Phenotypic differentiation within the group is congruent between the sexes but does not reflect phylogenetic history. An adaptive explanation for the observed pattern of differentiation is supported by independent contrasts analysis, and hypotheses concerning the functional significance of traits are proposed. Ecological convergence in traits used for classification is found to have important implications for species identification where taxa are distributed over varying environments.

Molecular, morphological and ecological species criteria are used to delimit species boundaries in the widespread *T. popeiorum* complex. A mitochondrial DNA phylogeny for the complex indicates two well-differentiated northern and southern clades. Multivariate analysis of morphological characters reveals a generally conserved pattern of geographical variation, with strongest differentiation between island populations. The current subspecific taxonomy of *T. popeiorum* is found to be

inconsistent with the molecular, morphological and ecological divisions uncovered, and a conservative reorganisation of the group into three species is recommended. The limitations of several pattern-based species criteria are discussed, and the combined utility of molecular, morphological and ecological data is evaluated in relation to delimiting species boundaries in organisms that are difficult to sample in large numbers.

Finally, mitochondrial gene sequences are used in combination with 4-nucleotide extension AFLPs to infer systematic relationships and biogeographic history in the *T. albolabris* complex. A mitochondrial gene tree reveals four distinct clades, whose present distribution is closely linked to original patterns of vegetation cover. Parallels between ordination and phylogenetic analysis of AFLP markers and the mtDNA phylogeny support previous taxonomic revisions and provide further resolution of systematic relationships within the complex. Congruence between the divisions recovered by AFLP and mtDNA analysis demonstrates the utility of 4-nucleotide extension AFLPs for generating systematic information within a complex of closely related pitviper species.

Contents

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CHAPTER 1

General Introduction

1.1 Investigating biological diversity: patterns and processes

1.1.1 Species and speciation

Species are the fundamental units of biological diversity, yet despite more than a century of investigation, their nature and origins remain to be fully understood. The Biological Species Concept (BSC) defines species as groups of reproductively isolated interbreeding populations (Mayr, 1942), and since its conception during the Evolutionary Synthesis has formed the focus of speciation research (Coyne, 1994). Reproductive isolation can be premating, postmating or a combination of both, and can arise indirectly as a result of natural selection, or by direct selection for premating barriers due to hybrid inferiority (Dobzhansky, 1946). However, between species hybridisation has been reported in numerous sexually reproducing taxa (e.g. Grant and Grant, 1992; Rieseberg and Wendel, 1993; Mallet, 1995; Wu, 2001), and many workers now adhere to a relaxed version of the BSC that requires only a sufficient reduction in gene flow to maintain genetically distinct populations (Schluter, 1998; Mallet, 2001; Wu, 2001).

The once general view that the origin of reproductive isolation requires an allopatric distribution of populations is no longer valid (Mallet, 2001), and sympatric and parapatric models of speciation are now widely accepted (Gavrilets *et al.*, 2000; Turelli *et al.*, 2001; Via, 2001; Salewski, 2003). Ecological speciation has emerged as an important mode of speciation, and occurs when divergent selection on populations in contrasting environments leads to reproductive isolation due to

reduced hybrid fitness (Howard *et al.*, 1998; Mallet, 2001; Schluter, 2001). Adaptive divergence occurs most quickly in allopatry (Coyne, 1992) and is thought to be the primary mode of speciation in island radiations (Whittaker, 1998). Diversification during a period of isolation may also precede ecological speciation of sympatric and parapatric populations (Coyne, 1992).

At a practical level, determining whether two populations have the capacity to fuse or have diverged sufficiently to remain differentiated requires detailed knowledge of their genetics, ecology and reproductive biology (Mallet, 2001). This involves an impractical level of investigation for many taxa and is unfeasible for allopatric populations (Harrison, 1998). The operational shortcomings of the isolation criterion have prompted numerous alternative definitions of species (species concepts), each emphasising a different combination of species criteria (de Queiroz 1998). Mechanistic species concepts are based on the underlying processes that give rise to and maintain species (Luckow 1995; de Queiroz 1998). They include the recognition criterion, which requires reproductive unification due to common fertilisation and mate recognition systems (Patterson, 1985); the niche criterion, which recognizes species as lineages that occupy adaptive zones minimally different from those of other lineages (Van Valen, 1976); and the cohesion criterion, by which species are diagnosed as the most inclusive populations of genetically and ecologically exchangeable individuals (Templeton, 1989).

Alternatively, historical species concepts infer species status from observed patterns of variation (Luckow 1995; de Queiroz 1998). Examples are the phenetic criterion, which distinguishes species as separate clusters in multivariate morphometric analysis (Sokal and Sneath 1963; Sokal and Crovello 1970); phylogenetic and genealogical species criteria, which equate species with evolutionary lineages, or segments of phylogenetic trees (Wiley, 1978; Cracraft 1983; Baum and Shaw, 1995); the diagnosability criterion, according to which species are diagnosed on the basis of unique combinations of character states (Cracraft, 1989; Nixon and Wheeler 1990; Davies and Nixon 1992); and the apomorphy criterion, which diagnoses

species on the basis of unique derived character states (Baum, 1992). For a detailed review of the alternative species concepts see de Queiroz (1998) or Mallet (2001).

The distinction between mechanistic and historical species concepts breaks down in their implementation, which in either case depends on inferences from patterns of variation in morphological or molecular markers, or both (de Queiroz 1998). de Queiroz (1998) further emphasised the disparity between concepts of the nature of species and the criteria used to diagnose them, and that different criteria are based on predictions from different phenomena associated with the speciation process. For example, fixed differences or categorical disjunctions in morphological and molecular characters are most likely to arise in the context of reproductive isolation (Nixon and Wheeler 1990; Davies and Nixon 1992), and an evolutionary lineage is the inevitable product of a cohesive reproducing population (Templeton, 1998). However, the fact that different species concepts use different criteria for species is clearly incompatible with the need for a clear and consistent hypothesis testing procedure for recognizing species boundaries (Sites and Crandall, 1997; Puorto *et al.,* 2001). Consequently, instead of basing taxonomic decisions on a single species concept or criterion, a more reliable approach is to test the predictions of all of the various species criteria against independent sources of data (Mishler and Donoghue, 1982; Baum and Shaw, 1995; Mallet, 2001; Puorto *et al.*, 2001).

A realistic estimate of species boundaries is important on several levels. Conservation workers use the species category to identify areas of endemism, catalogue and quantify biodiversity, and select, design and manage wildlife reserves (Sites and Crandall, 1997). The taxonomic resolution of venomous snake taxa has further implications for the clinical management of envenomings (Theakston, 1997; Theakston *et al.,* 2003). Geographic variation in venom has been detected in numerous snake species (Chippaux *et al.*, 1991; Daltry *et al.*, 1996; Creer *et al.*, 2003) and can render antivenom prepared against one regional variant ineffective against a bite by another (Tan, 1991; Anderson *et al*, 1993; Theakston, 1997; Theakston *et al.,* 2003). Moreover, the molecular phylogenies derived from systematic studies can provide a framework for investigations of historical biogeography, character evolution and the

dynamics of adaptation and divergence (Miyamoto and Cracraft, 1991). This is the subject of the following section.

1.1.2 Phylogenetic applications

By incorporating the effects of shared evolutionary history, molecular phylogenies can provide a rigorous basis for generating and testing causal hypotheses of differentiation. At the intraspecific level, Mantel and Partial Mantel tests (Manly, 1991; Mantel and Valand, 1970; Thorpe *et al.,* 1994) can be used to disentangle the relative roles of ecogenetic geographic variation (resulting from natural selection for current ecological conditions) and phylogenetic geographic variation (due to historical events, such as shared ancestry or genetic isolation following vicariance) (Thorpe 1975, 1979, 1987; Malhotra and Thorpe 1991a). At the interspecific level, comparative methods, such as Felsenstein's independent contrasts, can be used to remove correlations due to common ancestry before alternative factors involved in differentiation are assessed by the distribution of traits in relation to current ecological factors (Felsenstein, 1985; Harvey and Purvis, 1991; Garland, 1992; Bauwens *et al.*, 1995; Martins and Hansen, 1997; Kohlsdorf *et al.,* 2001). Such incorporation of molecular data into a hypothesis-testing framework has provided key insights into the evolution and adaptive radiation of a diverse range of taxonomic groups (Losos, 1990; Moreno and Carrascal, 1993; Thorpe *et al.*, 1994; Garland *et al.*, 1999; Harris and Steudel, 1997; Castellano and Giacoma, 1998; Baldwin, 2000; Reinthal and Meyer, 2000).

In reptiles, most investigations of the causal factors involved in geographic variation have been carried out at the intraspecific level on island lizard populations (Thorpe, 1989; Brown *et al.,* 1991; Thorpe, 1991; Losos, 1994; Malhotra and Thorpe, 1997; Malhotra and Thorpe, 2000b). Such studies have elucidated the relative roles of ecogenetic and phylogenetic forces in the variation of island populations, and have revealed correlations between morphological characters and various facets of the environment, including temperature, rainfall, altitude and vegetational type.

Translocation studies of Neotropical *Anolis* lizards have revealed significant correlations between the magnitude of ecological change and the strength of selection (Malhotra and Thorpe 1991b) and in a longer-term study, between the magnitude of ecological variation and morphological differentiation (Losos *et al.*, 1997). Comparatively few attempts have been made to infer causal patterns of variation in pitvipers. Intraspecific variation in venom composition was found to be correlated with diet differences in the Malayan pitviper, *Calloselasma rhodostoma* (Daltry, 1996) and the Chinese Bamboo pitviper, *Trimeresurus stejnegeri* (Creer *et al.*, 2003). In the South American *Bothrops atrox* complex, significant associations were found between morphological characters and the current environment, this was attributed primarily to selection pressures involving camouflage (Wüster *et al.*, 1997).

Molecular phylogenies can also be used to infer the biogeographic history of a species or species complex from the geographic distribution of haplotype clades (e.g. Zamudio and Greene, 1997; Avise *et al.*, 1988; Puorto et al., 2001). This combination of geography and genealogy is termed phylogeography (Avise *et al.*, 1987; Avise 1989). Concordant patterns of variation in the distribution of haplotype lineages among different species provide evidence for biogeographic boundaries and extrinsic dispersal events (Avise, 1992, 1994). Molecular clocks can be applied (given a calibrated rate of sequence evolution) to date the approximate timing of divergence, allowing correlation with known geological events such as mountain formation, habitat fragmentation and sea level changes (Joseph *et al.*, 1995; Zamudio and Greene, 1997). Dispersal events are considered to be less frequent than vicariance, and are rarely associated with parallelism (Cox and Moore, 2000). Island colonisation dispersal sequences can be inferred from molecular phylogenies by assigning geographic localities to ancestral populations corresponding to the present distribution of their closest terminal taxa (Thorpe *et al.*, 1994).

Phylogeographic patterns can be further used to tentatively infer geographical modes of speciation (Harrison, 1991). Sympatric speciation is assumed when sister species show inclusive ranges (Lynch, 1989); whereas parapatric speciation is inferred when

sister taxa are distributed on opposite sides of an environmental gradient (Patton *et al.*, 1998). Allopatric divergence is indicated if reciprocally monophyletic groups are separated by large phylogeographic gaps (Avise, 1994). Models of allopatric divergence can be further divided into the isolation of a peripheral population, the colonisation of a new habitat by members of a single population, or vicariance (Harrison, 1991). Vicariance events are seen to be congruent with prior phylogeographic structure if genealogical and geographic patterns are concordant (Patton *et al.*, 1998).

1.2 The Indomalayan Region

This thesis focuses on three species groups of venomous snakes that are extensively distributed in the Indomalayan biogeographic realm. This contains the Indian subcontinent (Pakistan, India, Sri Lanka, Nepal, Bhutan and Bangladesh), Indochina (Myanmar, Vietnam, Laos, Cambodia and Thailand) and Malesia (Malaysia, Indonesia, Brunei, Singapore and the Philippines) (Figure 1.1) (Udvardy, 1975; MacKinnon and MacKinnon, 1986).

1.2.1 Climate and vegetation

The primary factors determining climate are altitude, latitude and rainfall. Equatorial areas receive more rainfall than sub-tropical areas at higher latitudes (2000-3000mm/yr versus 3000-4000mm/yr) (Whitmore, 1975; Anon, 1995). Near the equator rainfall is evenly distributed throughout the year, supporting evergreen forests. In structure and species composition these habitats can be divided into lowland forest below 1000m and montane forest above 1000m (MacKinnon, 1997). At higher latitudes, rainfall is seasonal with marked dry seasons, and deciduous or monsoon (moist deciduous) forests predominate (Whitmore, 1975). The division between lowland and montane forest occurs at progressively lower altitudes further north (MacKinnon, 1997). Subtropical lowland forests and tropical lower montane forests show similar structure and species composition, and are classed together by some authors (e.g. Whitmore, 1975). Eastern Java and the Lesser Sunda islands lie in the Australian rain shadow and, despite their proximity to the equator, have dry, seasonal climates and support predominantly deciduous vegetation (Whitmore, 1975). Figure 1.1 illustrates the original inter-glacial distribution of vegetation types in the Indomalayan region.

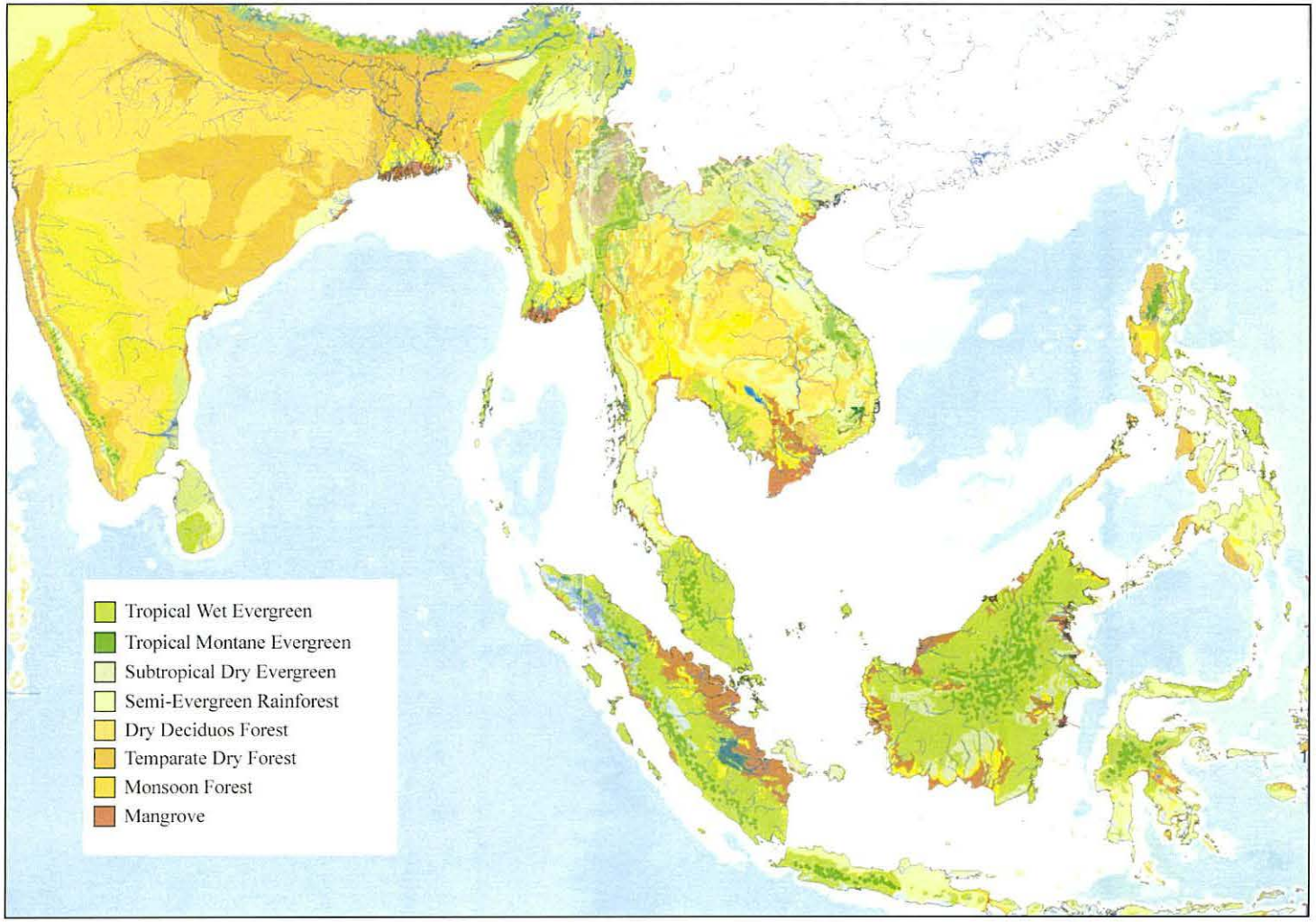

Figure 1.1: Original habitat distribution of the Indomalayan region (modified from MacKinnon, 1997).

1.2.2 Geological history

The geological history of the Indomalayan region involves two main tectonic events. The first was the collision between India and Eurasia 55-65 million years ago (Courtillot *et al.*, 1986; Beck *et al.*, 1995), which led to the formation of faults in Myanmar, Thailand, Malaysia and Sumatra (Hutchison, 1989; Polachan *et al.*, 1991). The second was the transverse collision of the Southeast Asian and Australian continental plates around fifteen million years ago (Whitmore, 1975), which led to the formation of the Lesser Sunda islands and parts of Sulawesi and the Philippines (Tapponnier *et al.*, 1982; Hutchison, 1989). The biogeographic history of the region has also been dramatically influenced by periodic fluctuations in climate. Warm inter-glacial periods have been intermittently separated by cool glacial periods (ice ages) every 100 000 years. The generally accepted model for this climatic cycle is based on astronomical forces originating from small changes in the ellipticity of Earth's orbit every 100 000 years. At times of glacial maxima global changes are thought to be amplified by two positive feedback mechanisms: the reduction of insulation due to lowered CO_2 levels, and the increase of total bond albedo due to the expansion of polar ice caps (Whitmore, 1975). This results in lowered sea levels, cooler climate and increased aridity (Whitmore, 1975; Morely, 1998).

The most recent ice age occurred during the late Pleistocene, between 15,000 and 22,000 years ago (Whitmore, 1975). During this time sea level dropped to 120m below the present level, resulting in the exposure of shallow inter-island platforms that connected the Greater Sunda islands (Borneo, Sumatra and Java) to mainland Asia and joined the Philippine islands into four mega-island groups (Whitmore, 1975; Heaney, 1985a) (Figure 2.1). The previous ice age occurred during the middle Pleistocene, 160,000 years ago, causing sea levels to drop 160m below their current level, joining Palawan (Philippine islands) and Borneo and connecting the Mentawai islands (west Sumatra) to the Sunda shelf (Whitmore, 1975; Heaney, 1985a). The presence of inter-island platforms facilitated an interchange of island fauna in the archipelago that is

reflected in the present distribution of numerous Indomalayan taxa (Leviton, 1963; Heaney, 1986).

The contiguous Indomalayan landmass experienced a more continental climate during the Pleistocene ice ages (Whitmore, 1975; Morely, 1998). The pollen record indicates that increased seasonality, reduced rainfall and lower temperatures resulted in the fragmentation of wet forest habitats; these retreated to higher altitudes and at low elevations were replaced by deciduous forest, scrub and savanna (Whitmore, 1975; Hall and Holloway 1998; Morely, 1998). The isolation of populations in glacial forest 'refugia' is a popular model of ecological vicariance (Haffer, 1969; Vanzolini, 1970; Whitmore, 1998), and has been implicated in the diversification of numerous rainforest species in tropical South America (e.g. Froehlich *et al.*, 1991), Africa (e.g. Mayr and O'Hara, 1986) and Indomalaya (e.g. Han and Sheldon, 2000). Pollen data also indicate lowered boundaries between upper and lower zones of montane forest (Whitmore, 1998), which is thought to have facilitated the migration of montane taxa (Whitmore, 1975; Heaney, 1985a).

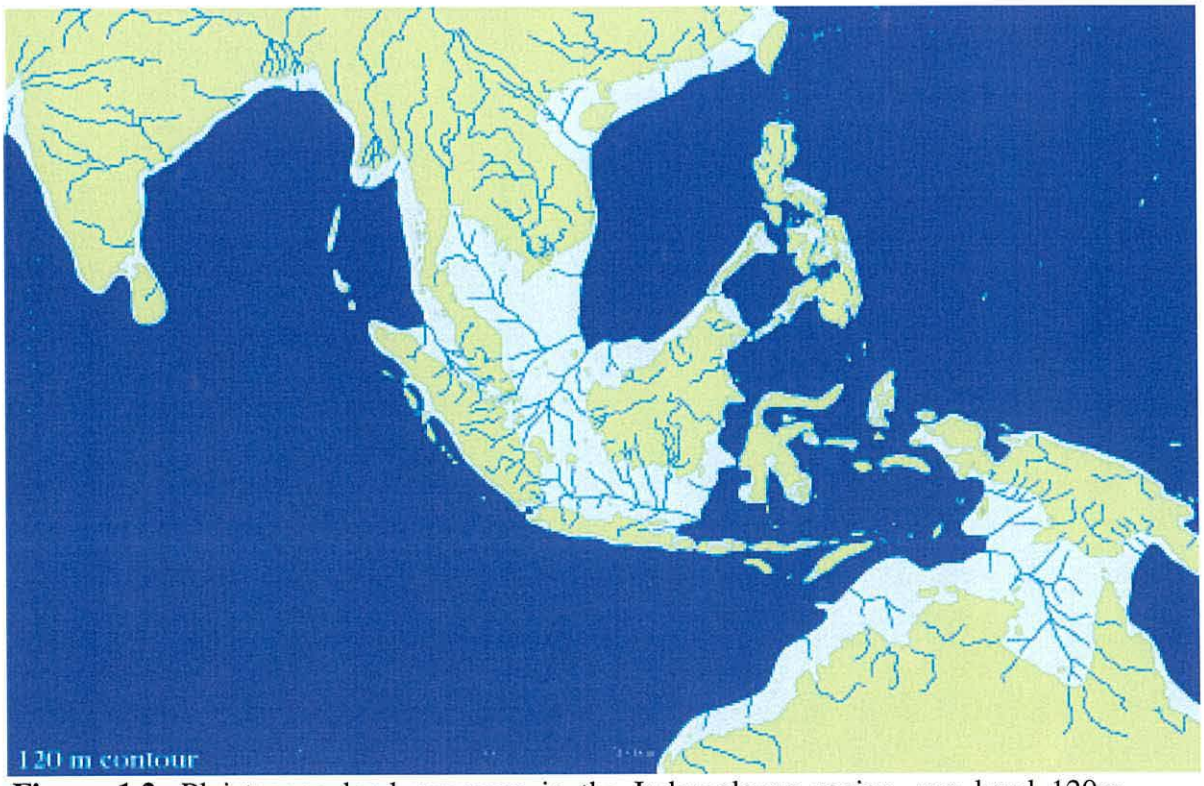

Figure 1.2: Pleistocene land exposure in the Indomalayan region, sea level 120m below present (Simpson, 2000).

1.2.3 Biodiversity

A history of mostly stable equatorial conditions combined with repeated geological changes of land connection and isolation have led to high levels of species richness and local endemism in the Indomalayan region (MacKinnon, 1997). The Indonesian, Malaysian and Philippine archipelagos constitute the largest aggregation of islands in the world (Heaney, 1986), and support especially high levels of biodiversity (MacKinnon, 1997; Whittaker, 1998). However, high human density and dependence on natural resources have led to higher rates of loss of biodiversity in the Indomalayan region than in any other biogeographic realm (MacKinnon and MacKinnon, 1986; MacKinnon, 1997). An estimated 60% of the original ecosystems illustrated in Figure 1.1 have now been cleared and much of what remains has been degraded due to uncontrolled logging, wildlife trade, land conversion, fire, climate change, pollution and the spread of exotic species (MacKinnon, 1997). Adequate knowledge of species richness and distribution is consequently essential for defining effective conservation strategies in the region.

1.3 Study Species

Venomous pitvipers (Reptilia: Serpentes: Viperidae: Crotalinae) of the *Trimeresurus* complex Lacepede, 1804 are a conspicuous component of most Asian reptilian faunas (McDiarmid *et al.*, 1999). The c. 34 species in the group occupy a wide range of arboreal habitats and show considerable diversity in aspects of morphology, diet and life history. However, striking phenotypic convergence, as well as substantial geographic variation and sexual dimorphism has long confounded taxonomic resolution of the group (Stejneger, 1927; Pope and Pope 1933; Maslin, 1942; Brattstrom, 1964; Regenass and Kramer 1981) and continues to impede field identification. In recent years, the application of molecular methods has resolved much of the confusion surrounding the systematics of the *Trimeresurus* complex (Kraus *et al.*, 1996; Parkinson, 1999; Malhotra and Thorpe, 2000; Parkinson *et al.*, 2002; Malhotra and Thorpe, in press a). Several smaller groups of related species have been defined within the complex (Malhotra and Thorpe, 2000; Malhotra and Thorpe, in press a) and numerous cryptic species have been identified (Malhotra and Thorpe, 2000; Giannasi *et al.*, 2001; Malhotra and Thorpe, in press b; Malhotra and Thorpe, submitted).

This thesis focuses on the systematic and evolutionary biology of three *Trimeresurus* species groups from the Indomalayan region. These are the Indomalayan (*T. sumatranus*), *T. popeiorum* and *T. albolabris* groups (*sensu* Malhotra and Thorpe, 2000; Malhotra and Thorpe, in press a). These groups were selected because each is the subject of considerable taxonomic confusion in the field, in museum collections and in much of the published literature (Pope and Pope 1933; Maslin, 1942; Brattstrom, 1964; Regenass and Kramer 1981; Tweedie, 1983; Lim, 1991; Jintakune, 1995; David and Vogel, 1996). In addition, each group has a wide geographic distribution, comprising transcontinental and island populations that display considerable morphological and ecological diversity, making them suitable for comparative analysis.

1.3.1 The T. sumatranus group

Five nominal species are currently recognised in this group (Figure 1.3) and are diagnosed by a long papillose hemipenis type and a separated first supralabial and nasal scale (Malhotra and Thorpe, in press a). They are: *T. sumatranus* (Raffles, 1822), *T. hageni* (Lidth de Jeude, 1886), *T. malcolmi* (Loveridge, 1938), *T. schultzei* (Griffin, 1909) and *T. flavomaculatus* (Gray, 1842). *T. sumatranus* and *T. hageni* are mostly found within the Malaysian and Indonesian archipelagos. *T. malcolmi* is endemic to Mt. Kinabalu and the Crocker range in Sabah, Borneo. *T. schultzei* is endemic to Palawan in the Philippine Islands. *T. flavomaculatus* occurs over much of the Philippines and is divided into three subspecies, each of which shows considerable colour pattern polymorphism. The *T. sumatranus* group species vary considerably in prey utilisation, altitudinal range and habitat preference, although all are restricted to undisturbed forests. They are thick-bodied egg-layers, and are unusual within the *Trimeresurus* complex in that, although dimorphic, both sexes display conspicuous pattern elements, including spots, bands, stripes and reticulated markings.

1.3.2 The T. popeiorum group

This group includes a single viviparous species, *T. popeiorum* Smith, 1937 (Figure 1.4), which is diagnosed by a long calyculate hemipenis and a separated first supralabial and nasal scale (Malhotra and Thorpe, in press a). *T. popeiorum* occurs from northeast India to the Sunda Shelf, inhabiting undisturbed rainforest habitats mostly at moderate and high altitudes. It shows the typical 'green pitviper' colouration of a uniform green background and a red tail, and is often confused with sympatric relatives *T. albolabris, T. gumprechti* and *T. macrops*. Regenass and Kramer (1981) split *T. popeiorum* into three subspecies on the basis of scalation characters. *T. p. popeiorum* was recognised on the mainland; *T. p. sabahi* refered to the Borneo population; and *T. p. barati* was restricted to the Barisan range in west Sumatra (Regenass and Kramer, 1981).

1.3.3 The T. albolabris group

This is the most widespread species group within the *Trimeresurus* complex, ranging from Nepal and China south to Java and the Lesser Sunda islands. It currently includes twelve viviparous species that are characterized by full or partial fusion of the first supralabial and nasal scale (Malhotra and Thorpe, in press a). They are *T. albolabris, T. erythrurus, T. purpureomaculatus, T. cantori, T. andersoni, T. septentrionalis, T. insularis, T. fasciatus, T. labialis, T. venustus, T. macrops* and *T. kanburiensis* (Figure 1.5). These species occupy a diverse range of habitat types, including rainforests, mangroves, degraded forests and plantations, at mostly low and moderate altitudes. *T. albolabris sensu stricto* displays a particularly generalist use of habitats and can often be found near human habitations. Consequently, this species is frequently the leading cause of venomous snakebite in many parts of its range (Romer, 1963; Hutton *et al.*, 1990; Viravan *et al.*, 1992).

Figure 1.3.1
T. sumatranus (female)
Bengkulu, Sumatra
Photo: K. Sanders

Figure 1.3.2
T. sumatranus (male)
Poring, Sabah, Borneo
Photo: K. Sanders

Figure 1.3.3
T. malcolmi (juvenile)
Mt. Kinabalu, Sabah,
Borneo
Photo: K. Sanders

Figure 1.3.4
T. hageni (female)
Satun, south Thailand
Photo: A. Malhotra

Figure 1.3.5
T. hageni (male)
Bengkulu, Sumatra
Photo: K. Sanders

Figure 1.3.6
T. schultzei (female)
Palawan, Philippines
Photo: A. Malhotra

Figure 1.3.7
T. flavomaculatus
Luzon, Philippines
Photo: A. Gumprecht

Figure 1.3.8
T. flavomaculatus
(juvenile)
Luzon, Philippines
Photo: A. Gumprecht

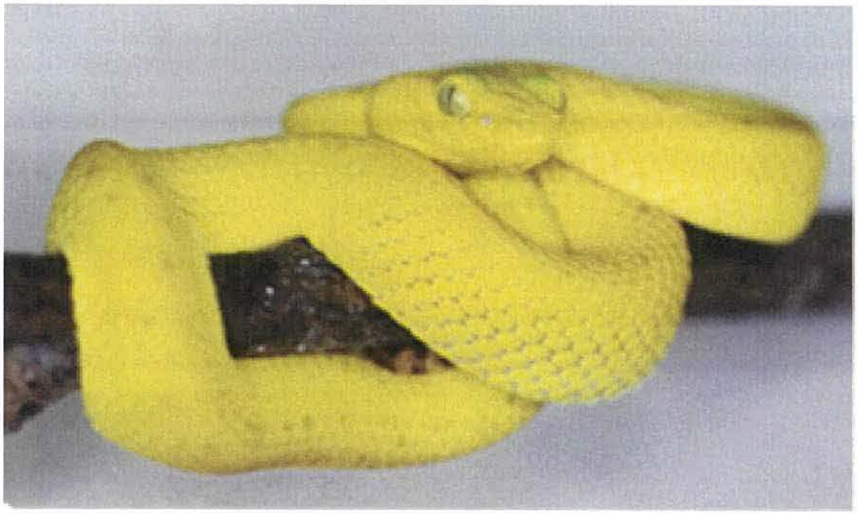

Figure 1.3.9
T. f. mcgregori
Batanes, Philippines
Photo: K. Sanders

Figure 1.3.10
T. f. mcgregori
Batanes, Philippines
Photo: A. Gumprecht

Figure 1.4.1
T. popeiorum (male)
Kaeng Krachan,
west Thailand
Photo: A. Malhotra

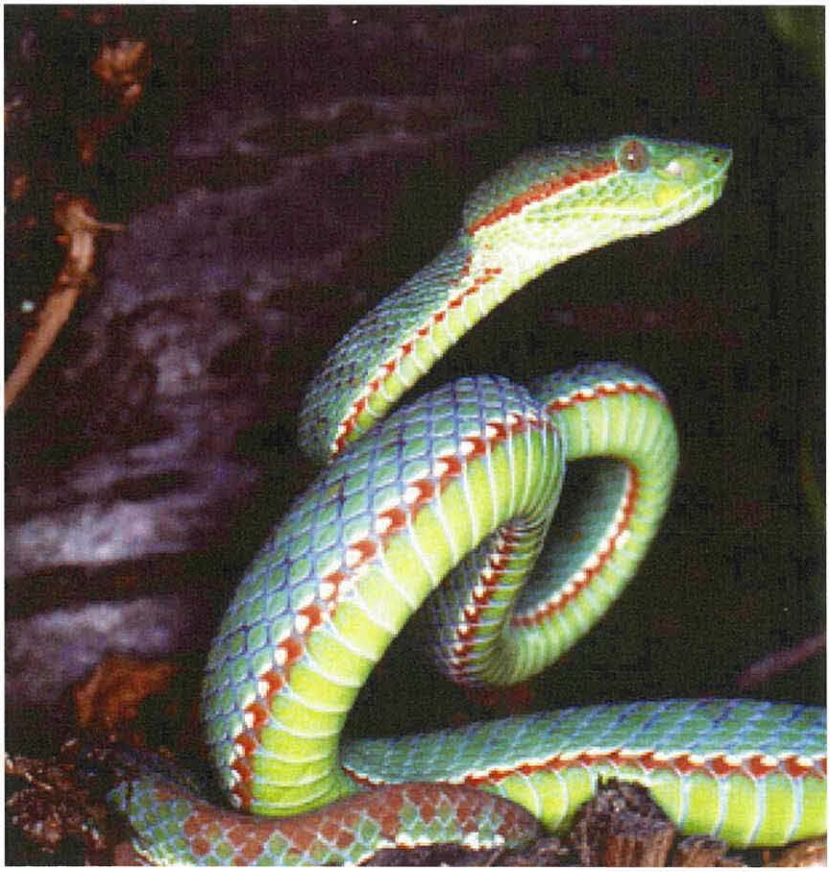

Figure 1.4.2
T. popeiorum (male)
Khao Lak,
south Thailand
Photo: A. Gumprecht

Figure 1.4.3
T. popeiorum
(juvenile)
south Thailand
Photo: D. Shoub

Figure 1.4.4
T. popeiorum (male)
south Thailand
Photo: D. Shoub

Figure 1.4.5
T. popeiorum (female)
Cameron Highlands,
West Malaysia
Photo: A. Malhotra

Figure 1.4.6
T. popeiorum (male)
Bukit Fraser,
West Malaysia
Photo: A. Gumprecht

Figure 1.4.7
T. popeiorum (male)
Mt. Kinabalu,
Sabah, Borneo
Photo: K. Sanders

Figure 1.4.8
T. popeiorum (female)
Mt. Kinabalu,
Sabah, Borneo
Photo: K. Sanders

Figure 1.4.9
T. popeiorum (female)
Bengkulu, Sumatra
Photo: K. Sanders

Figure 1.5.1
T. albolabris
Mandalay division
Myanmar
Photo: Cal. Acad. Sci.

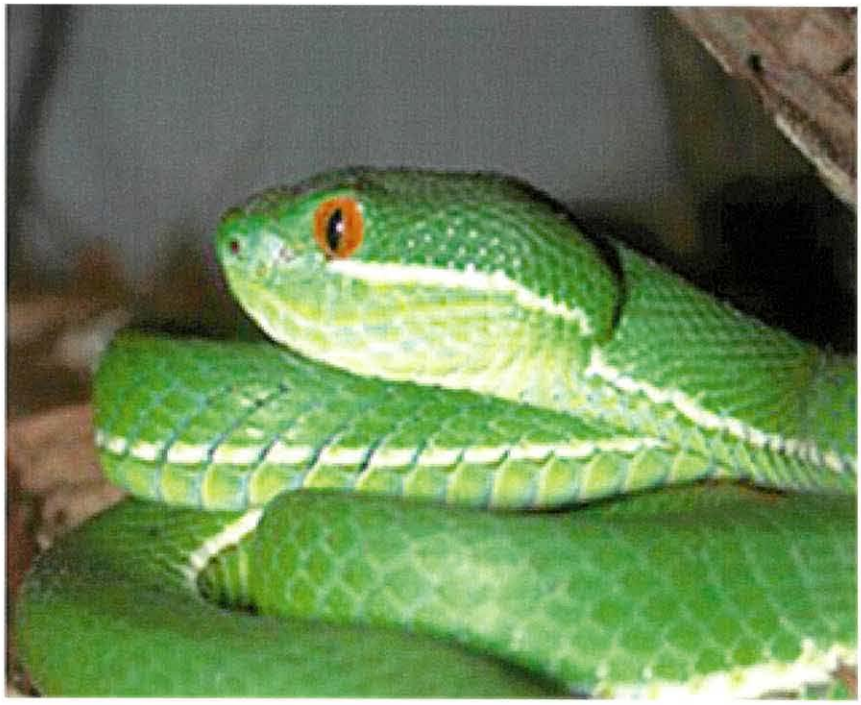

Figure 1.5.2
T. albolabris
south Thailand
Photo: M. Snipes

Figure 1.5.3
T. fasciatus
Tanadjampea Id.
Sulawesi
Photo: K. Sanders

Figure 1.5.4
T. insularis
Timor, Indonesia
Photo: W. Wüster

Figure 1.5.5
T. purpureomaculatus
Mandalay division
Myanmar
Photo: Cal. Acad. Sci.

Figure 1.5.6
T. purpureomaculatus
Perak, West Malaysia
Photo: A. Gumprecht

Figure 1.5.7
T. erythrurus
Rakhine state
Myanmar
Photo: Cal. Acad. Sci.

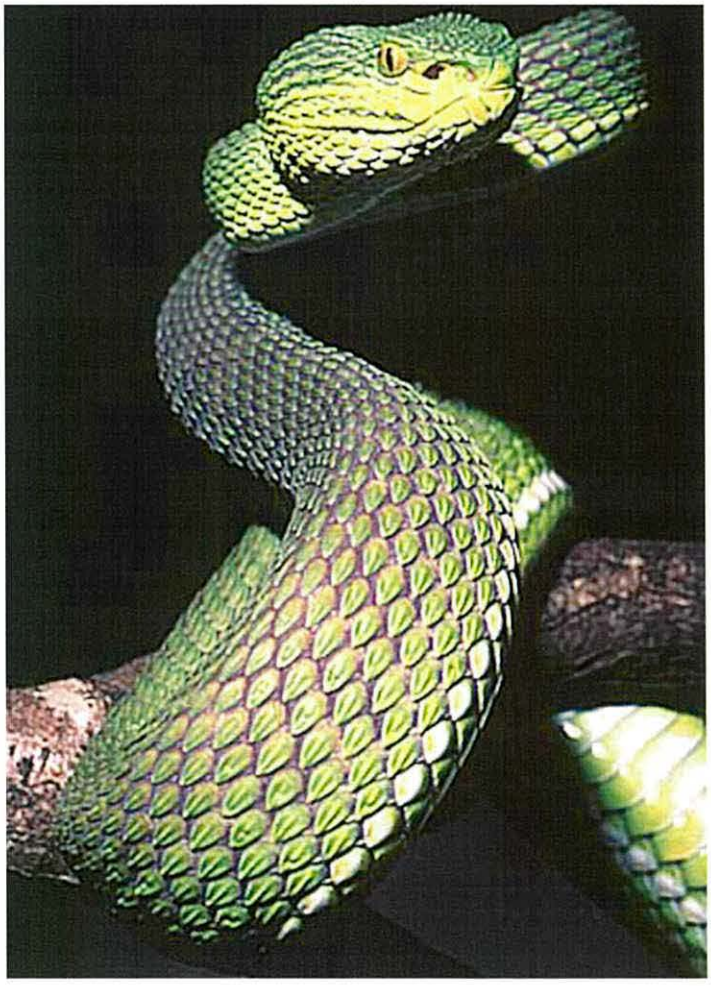

Figure 1.5.8
T. erythrurus
Yangon division
Myanmar
Photo: W. Wüster

1.4 Sytematic Methods

1.4.1 Molecular markers

1.4.1a Mitochondrial DNA

Direct sequencing of PCR amplified homologous DNA regions can yield very large data sets for phylogenetic reconstruction (Hillis *et al.*, 1996). The mitochondrial genome possesses a number of attributes that make it a particularly valuable source of sequence data in animal systematics (Avise et al., 1987; Hillis *et al.*, 1996). A high number of homoplasmic mitochondria occur per cell, allowing easy isolation and assay (Avise, 1994; Zevering *et al.*, 1991). Neutral mutations evolve at different rates in individual mitochondrial genes (Palumbi, 1996), allowing evolutionary relationships to be inferred at a wide range of taxonomic levels, free from the confounding effects of natural selection and plasticity (Kimura, 1986). Neutral substitutions also accumulate rapidly in mtDNA relative to nuclear DNA (Brown *et al.*, 1979; Vawter and Brown, 1986). This is explained by the lack of a repair mechanism in mitochondrial genome (Wilson *et al.*, 1985), and makes mtDNA particularly useful in resolving relationships at fine taxonomic levels (Goebel *et al.*, 1999).

Also important is the predominantly maternal mode of mtDNA inheritance (Avise and Vrijenhoek, 1987; Avise, 1994); 'paternal leakage' has been identified only in a minority of studies (e.g. Gyllensten *et al.*, 1991; Hoeh et al., 1991; Zouros *et al.*, 1992). Thus, mtDNA haplotypes represent primarily non-recombining characters. This results in a smaller effective population size, rapid lineage coalescence and a reduced risk of lineage sorting of ancestral polymorphisms, relative to nuclear DNA (Avise, 1994). Consequently, a mtDNA tree is more likely to accurately represent an organismal phylogeny than a tree based on nuclear sequence data (Avise, 1994; Johnson and Clayton, 2000).

In the absence of gene flow, mitochondrial sequence differences can be used to date approximately the divergence of two taxa from their most recent shared ancestor (Zuckerkandl and Pauling, 1965). However, the application of a 'molecular clock' requires a calibrated rate of sequence evolution in a comparable taxon. This can be derived from the fossil record, or less reliably, from the timing of known geological vicariance events (Hillis *et al.,* 1996). Numerous studies have shown between-taxon rate heterogeneity associated with generation time, body size and metabolic rate (e.g. Martin and Palumbi, 1993; Mindell and Thacker, 1996), indicating that estimates derived from molecular clocks are likely to be more reliable between closely related taxa (Caccone, *et al.* 1997). Limitations of mtDNA include the lack of independence of mitochondrial genes due to their inheritance as a single unit (Moore, 1995; Page, 2000). In addition, the maternal mode of mtDNA inheritance can confound estimates of species boundaries if gene flow is male mediated and females are philopatric (Palumbi and Baker, 1994).

1.4.1b Fragment analysis

Sequence information can also be investigated indirectly by electrophoretically comparing DNA fragments. Although fragment analysis provides lower resolution than direct sequencing, it is a powerful and cost effective alternative that allows screening of large numbers of loci from throughout the genome (Dowling *et al.*, 1996). Microsatellite and minisatellite methods are used to detect variation in fragment size of tandemly repeated sequences via PCR amplification (Tautz, 1989; Dowling *et al.*, 1996). Restriction enzymes are used to identify gain and loss of recognition sequences by digestion and electrophoretic separation of restriction fragment length polymorphisms (Dowling *et al.*, 1996). Alternatively, random amplified polymorphic DNAs are used to detect gain and loss of PCR priming sites by comparison of fragments randomly amplified by a combination of primers (Williams *et al.*, 1990).

A powerful new technique that combines the strengths of restriction fragment methods and PCR amplification is Amplified Fragment Length Polymorphism (AFLP) (Zabeau and Vos, 1993; Vos, 1995). Large numbers of reproducible markers are generated in three principal stages (Vos *et al.,* 1995). Whole genomic DNA is first digested using two restriction endonucleases and oligonucleotide adaptors are ligated to fragment restriction sites. A subset of the restricted fragments is then amplified by a two stage PCR, first using single nucleotide extension primers and then using more selective three (or more) nucleotide extension primers. One primer is end-labelled using a fluorescent dye or radio-label, allowing detection of fragments following electrophoretic separation on an acrylamide gel. Polymorphisms are identified by the presence or absence of a fragment next to a known size marker. Between 50 and 100 restriction fragments, of up to 500 base-pairs in length, are typically amplified and detected, providing a powerful estimate of genetic similarity without prior knowledge of the genome (Vos *et al.,* 1995).

AFLPs have been used effectively in a diverse range of taxa, including bacteria (e.g. Keim *et al.*, 1997), fungi (e.g. Rosendahl and Taylor, 1997), invertebrates (e.g. Semblat, *et al.*, 1998), vertebrates (e.g. Nijman *et al.,* 2003), and plants (e.g. Gaiotto *et al.,* 1997). AFLP markers are most often applied at congeneric and conspecific levels (Mueller and Wolfenbarger, 1999). They can be used to resolve systematic relationships using distance methods of phylogenetic reconstruction, and to detect hybridisation and estimate species boundaries using multivariate ordination methods (Beismann, *et al.,* 1997; Giannasi *et al.,* 2001b; Teo *et al.,* 2002; Semerikov *et al.,* 2003). The main disadvantage of AFLPs is that they generate dominant markers that do not provide information on whether the fragment occurs in the homozygous or heterozygous condition. This limits their use in population genetic studies, which often require precise assignment of allelic states (Mueller and Wolfenbarger, 1999).

1.4.2 Phylogenetic inference

The aim of phylogenetic inference is to convert molecular information into an evolutionary tree for the organisms from which those data are derived (Swofford *et al.*, 1996). A large range of methods has been developed for this purpose. These can be divided into distance and character-based approaches and may be based on clustering algorithms and optimality criteria, respectively (Quicke, 1993). Distance methods generally use a pairwise genetic distance versus taxon matrix to calculate the number of nucleotide differences between aligned sequences (Page and Holmes, 1998). Clustering methods provide an estimate of genetic similarity between taxa by progressively joining the most similar operational taxonomic units (OTUs) (Quicke, 1993). Sequence distance does not increase linearly with time due to saturation of substitutions at the same site, and distance correction models are available to account for unequal frequencies of nucleotides (e.g. F81 (Felsenstein, 1981)), unequal proportions of transitions and transversions (e.g. K2P (Kimura, 1980)) or both (e.g. HKY85 (Hasegawa *et al.,* 1985)). Distance methods are easy to implement and require relatively little computational time. However, their disadvantages include the loss of character state information that results from the conversion of sequence data into a distance matrix, and reduced ability to test the fit between alternative trees and the data (Swofford *et al.*, 1996). Character based approaches retain sequence information, allowing reconstruction of evolutionary relationships from inferences of the sequential evolution of character states. Furthermore, optimality criteria can be used to test between alternative evolutionary hypotheses using tree-searching algorithms (Swofford *et al.*, 1996). For these reasons, character based methods are used in preference to distance methods, despite the increased computational time required for their implementation. Maximum Parsimony, Maximum Likelihood and Bayesian methods are widely used character based methods of phylogenetic inference.

1.4.2a Maximum parsimony

Maximum Parsimony (MP) analysis searches for the phylogeny that minimizes the number of evolutionary steps required to explain the original data (Swofford *et al.*, 1996). This is based on the assumption that evolutionary change is rare and sequence evolution occurs by the fewest (most parsimonious) number of changes between two states (Swofford *et al.*, 1996). Generalised parsimony (Swofford and Olsen, 1990) compensates for saturation in quickly evolving base pair positions by weighting different kinds of substitutions. However, recent studies have shown that weighted parsimony is unlikely to return a more accurate phylogeny and can compromise the information content of the data (Milinkovitch and Lyons-Weiler, 1998; Vidal and Lecointre, 1998). Minimum length trees are selected using search algorithms. Exhaustive searches of up to 20-25 taxa can be carried out using branch-and-bound algorithms, which build all possible trees in a step wise manner, calculate tree length at an intermediate stage, and discard trees that contain a previous arrangement of taxa or exceed minimum length (Hendy and Penny, 1982; Platnick, 1987). For larger data sets, heuristic methods can be used to search for the optimum tree using a series of rearrangements of a subset of possible trees, but cannot guarantee finding the shortest tree (Swofford *et al.*, 1996; Page and Holmes, 1998). Parsimony methods may recover an inaccurate tree if long branches have acquired shared homoplasious changes due to variable rates of evolution or high levels of divergence, leading to incorrect grouping of long branches ('long branch attraction') (Felsenstein, 1988).

1.4.2b Maximum likelihood

In contrast to parsimony methods, maximum likelihood methods evaluate phylogenetic hypotheses in terms of the probability that the observed data would have resulted in a given tree (Swofford *et al.,* 1996). The log likelihoods of alternative trees are computed and compared using an explicit model of molecular character transformation. The model of evolution that best fits the data can be

determined using MODELTEST 3.0 (Posada and Crandall 2001). MODELTEST compares 56 different nested substitutional models and uses log likelihood scores to determine the model of molecular evolution that best fits the data. Maximum likelihood is considered generally robust and less subject to sampling error than parsimony methods (Page and Holmes, 1998; Nei and Kumar, 2000). However, it is the most computationally expensive method of phylogenetic inference, and is currently of limited value in analysing large data sets (Sanderson and Kim, 2000).

1.4.2c Bayesian inference

Bayesian methods of phylogenetic inference also use complex models of nucleotide substitution (Larget and Simon, 1999), but differ from maximum likelihood approaches in that they provide probabilities of hypotheses, not probabilities of data given a hypothesis (Lewis, 2001). Markov Chain Monte Carlo (MCMC) methods are used to generate an approximation of the posterior probability distribution of tree topology, branch lengths and substitution model parameters (Rannala and Yang, 1996; Mau *et al.*, 1999). All sample points prior to stationarity of the posterior probability distribution are essentially random and discarded as 'burn-in' samples (Huelsenbeck and Ronquist 2001). The remaining samples, each containing a tree topology with branch lengths and substitution model parameter values, are combined in a consensus tree (Huelsenbeck and Ronquist 2001). The percentage of samples that recover each clade are used to represent posterior clade probabilities (Huelsenbeck and Ronquist 2001). A recent analysis found high levels of congruence between tree topologies reconstructed using parsimony, likelihood and Bayesian methods (Leache and Reeder, 2002). Bayesian methods were also found to require 80% less computational time than likelihood methods (Leache and Reeder, 2002), enabling efficient analysis of large data sets.

1.4.2d Testing the reliability of trees

Several indices are available to estimate the closeness of fit between a data set and maximum parsimony tree. The consistency index (C.I.) of a character is the minimum number of possible transitions divided by the number of observed transitions; a C.I. can be calculated for the overall phylogeny as a measure of the extent of homoplasy (Kludge and Farris, 1969). The retention index (R.I.) also provides an indication of the proportion of homoplasious characters, but accounts for variation in the number of parsimoniously uninformative characters in different data sets (Archie, 1989; Farris, 1989). The rescaled consistency index (R.C.) is the product of the consistency index and the retention index, and accounts for differences in the number of character state transitions for characters that show equivalent levels of homoplasy (Farris, 1989).

Bootstrapping is used to assess support for individual nodes in neighbour joining, maximum parsimony and maximum likelihood trees (Felsenstein, 1985). This involves data resampling and replacement, resulting in a set of replicate matrices in which some characters are multiply represented and others are missed altogether (Nei and Kumar, 2000). Phylogenetic reconstruction is repeated for each new matrix; the proportion of bootstrap replicates that show a particular clade is used as a measure of statistical support for that clade (Nei and Kumar, 2000). Assuming equal rates of change, bootstrap proportions over 70% are thought to correspond to a probability of 95% or more that the clade relationship is genuine (Felsenstein, 1988).

Bayesian phylogenetic inference uses the percentage of post burn-in trees that recovered each clade as posterior probabilities of clade support (Huelsenbeck and Ronquist, 2001). The support derived from Bayesian posterior probabilities can be interpreted more clearly than bootstrap values in that they represent the probability that a clade is true given the model, priors and data (Rannala and Yang, 1996; Huelsenbeck *et al.,* 2002). However, posterior probabilities are often observed to be considerably higher than corresponding nonparametric bootstrap values and recent studies based on simulation methods indicate that there is a systematic difference

between the two methods (Erixon *et al.*, 2003). In particular, Bayesian posterior probabilities were found to be more likely to lead to erroneous conclusions (i.e. have a higher type I error rate), especially when the models used for analyses are underparameterised (Erixon *et al.*, 2003). Bayesian posterior probabilities were also found to be less conservative than bootstrap values when the model was correctly specified (Erixon *et al.*, 2003).

1.4.3 Multivariate morphometrics

Multivariate ordination of morphological characters is a powerful systematic method that is used to detect subtle patterns of variation within species and distinguish between cryptic species. Two commonly used methods are principal component analysis (PCA) and canonical variate analysis (CVA). Characters showing significant between-locality differences can be identified prior to ordination using two-way analysis of variance (ANOVA) or co-variance (ANCOVA). Prior to analysis, size-correlated characters should be adjusted to minimise the effects of ontogenetic variation. This can be achieved by log-transformation of the covariate (e.g. snout-to-vent length or head length) and size dependent characters, and covariance analysis on the residuals of the regression of each size dependent character against the covariate.

1.4.3a Principal component analysis

PCA does not require prior grouping of OTUs. Principal components (vectors or axes) are extracted from covariance or correlation matrices (Gower, 1966). The first principal component axis explains the maximum variation. Subsequent principal components are orthogonal (and therefore independent) to previous principal components and explain the maximum amount of the remaining variance in the data (Gower, 1966). PCA can be used in conjunction with CVA in case there is heteroscedasticity in the data (inequality of within-group covariation matrices) and

multiple taxa within a group. Principal coordinates analysis (PCO) can be dual to PCA, but differs in that the coordinates are extracted from a between-OTU distance matrix, and is more often applied to genetic distance data, such as restriction fragment analysis (e.g. Ogden and Thorpe, 2002).

1.4.3b Canonical variate analysis

CVA is a discriminant technique that ordinates *a priori* grouped individuals. This allows within group correlation between characters to be taken into account, reducing information redundancy in the data set (Thorpe, 1976). A between taxon similarity coefficient, Mahalanobis D^2, is implied (Sneath and Sokal, 1973). The first canonical variate axis maximizes the separation of the groups with subsequent axes representing proportionally less between-group variation (Gower, 1966). It is a more powerful means of discrimination than PCA, but requires homoscedastic data and hence the exclusion of characters that show zero variance within a group.

1.4.4 Comparative analysis: Independent contrasts

Due to the hierarchical nature of phylogenetic relationships, closely related species tend to share more characteristics through descent from common ancestors than distantly related species (Garland, 1992). Felsenstein's (1985) method of independent contrasts attempts to account for this lack of independence in cross-species data sets, allowing comparative analysis of relationships between different phenotypes or between phenotypes and environmental variables. Correlations are estimated using information on the amount of character change between pairs of closely related species since their most recent shared ancestor.

The values of N species are transformed into N - 1 standardised independent contrasts that are statistically independent and identically distributed and can be applied in regression or correlation analysis (Garland, 1992). Pairs of species at the

tips of the phylogeny are contrasted (the phenotype of one is subtracted from the phenotype of the other), the phenotype of their ancestral node is estimated and they are pruned from the tree. Further contrasts are computed moving down the tree. Contrasts involving phenotypes of internal nodes are down weighted (as they are estimated values) when each contrast is divided by the square root of sum of its branch lengths (Garland *et al.*, 1999).

Computer simulations have shown independent contrasts to be robust with respect to violation of assumptions, including deviation from a Brownian model of character evolution and errors in branch lengths (Purvis *et al.*, 1994; Martins, 1996). Furthermore, given accurate phylogenetic topology and branch lengths, the statistical power of independent contrasts was found to be equivalent to that of conventional correlation applied to non-phylogenetic data (Garland and Adolph, 1994).

1.4.5 Sample acquisition

In this study, an attempt was made to obtain as many specimens directly from the field as possible. This increased confidence in the data and provided correspondence between genetic, morphological and ecological data sets. Fieldwork was carried out in and around forest habitats at night, both on foot and by driving at low speed on quiet roads. At each locality, additional field personnel were sought to maximise the number of samples obtained. This was necessary given time constraints and the challenges of finding cryptic animals that occur at low densities in undisturbed habitats. Aboriginal people were the most productive snake catchers, but forest workers and national park staff also located and occasionally collected specimens. Additional blood and tissues were obtained from museum and private collections when locality information was available and species identity could be verified. Blood samples were taken from the caudal vein with a hypodermic syringe, placed in 1 ml 0.1M EDTA, and stored in SDS-Tris buffer (2% SDS, 100mM Tris); tail tips, liver and muscle tissue was preserved in 80% ethanol.

1.4.6 Division of labour

The data analysed in this thesis was collected jointly by Anita Malhotra (AM) and the author. AM provided approximately one third and three quarters of the morphometric data used in *T. sumatranus* and *T. popeiorum* group analyses, respectively. Care was taken to ensure that data was collected consistently between the workers, and was confirmed by ordination analysis. AM also provided outgroup sequences and approximately two thirds of the *T. abolabris* group sequences used. All data analysis was carried out by the author.

1.5 Thesis Aims

The following study attempts to review the systematic and evolutionary biology of three species groups of Indomalayan *Trimeresurus* pitvipers. The second chapter uses multivariate morphometric methods to identify diagnostic characters, uncover patterns of geographic variation and determine the distributional range of two commonly confused pitvipers from the Malesian archipelago, *Trimeresurus hageni* and *T. sumatranus*. The third chapter focuses on the five species in the *Trimeresurus sumatranus* complex. Systematic relationships are examined at interspecific and intraspecific levels using a combination of multivariate analysis of scalation and colour pattern characters and phylogenetic analysis of four combined mitochondrial gene regions. Ecological diversification is investigated using independent contrasts analysis of morphological traits and various facets of the current environment. In the fourth chapter, an attempt is made to delimit species boundaries in the widely distributed *T. popeiorum* complex using molecular, morphological and ecological pattern-based species criteria. Mitochondrial DNA sequences from four genes are used to reconstruct phylogenies, estimate divergence times and compare between-OTU frequencies of fixed nucleotide differences; geographic morphological variation is investigated using multivariate analysis of morphological characters. This data is used to assess the limitations of several pattern-based species criteria and their combined utility in delimiting species boundaries in organisms that are difficult to sample in large numbers. The fifth chapter is part of an ongoing investigation of the systematics and biogeography of the *T. albolabris* complex. Evolutionary history is reviewed using new and existing sequence data for two combined mitochondrial genes and, independently, using ordination and phylogenetic analysis of AFLP markers generated with four-nucleotide extension primers. The utility of four-nucleotide extension AFLP primers is evaluated in relation to systematic studies of closely related pitviper species.

CHAPTER 2

A contribution to the systematics of two commonly confused pitvipers from the Sunda Region: *Trimeresurus hageni and T. sumatranus*

2.1 Abstract

The systematics of two Southeast Asian green pitviper species, *Trimeresurus hageni* and *T. sumatranus,* are investigated by canonical variate analysis. Preliminary results reveal two morphological forms corresponding to mainly *T. hageni* in West Malaysia, Thailand and Singapore and *T. sumatranus* in Borneo. Allopatric populations of both taxa are examined from Sumatra. Geographic variation is present in both species, which are distinguished mainly by head scalation, but also by colour and pattern.

2.2 Introduction

Trimeresurus sumatranus (Raffles, 1822) and *T. hageni* (Lidth de Jeude, 1886) are closely related species, occupying low elevations in undisturbed forests and having largely overlapping ranges. The systematics of these species and their precise distribution is an area of long-standing confusion. Many workers assign both species to *T. sumatranus* by default (Tweedie, 1983; Lim, 1991; Jintakune, 1995; David and Vogel, 1996) and the status of *T. hageni* has been in dispute since its initial description (Lidth de Jeude, 1886; Lidth de Jeude, 1890; Boulenger, 1896; Brongersma, 1933).

T. hageni was described as a separate species from *T. sumatranus* on the basis that only one or two supralabial scales are in contact with the subocular (compared with three in *T. sumatranus*), and the dark edges on head and body scales and dorsal cross-bands that are characteristic of *T. sumatranus* are not present (Lidth de Jeude, 1886). The species' distribution is widely debated, but specimens from south Thailand, West Malaysia and Singapore are normally assigned to *T. hageni*, and specimens from Borneo are normally assigned to *T. sumatranus* (David and Vogel, 1996; Cox *et al.*, 1998; Stuebing and Inger, 1999). Both species are thought to occur on Sumatra and surrounding islands (Brongersma, 1933; Dring, 1989; Cox *et al.*, 1998).

There have been few attempts to resolve the systematics of *T. hageni* and *T. sumatranus* since their initial description; these have been based on small sample sizes and a traditional character-by-character approach (Boulenger, 1896; Brongersma, 1933). Given the levels of geographic, ontogenetic and sexual variation usually present in viper species (Wüster *et al.*, 1992; Malhotra and Thorpe, 1997), the systematics of these taxa is best approached using modern statistical methods based on a broad range of morphological characters. In this paper, we present preliminary results from an ongoing investigation of the systematics and interrelationships of *T. hageni* and *T. sumatranus*.

2.3 Materials and Methods

We examined 78 specimens from museum collections in the United States, Europe and Malaysia (Figure 2.1). A total of 93 characters relating to scalation, colour and pattern were recorded for each specimen. Ventral scales were counted from head to vent, with the first ventral identified according to the method of Dowling (1951). The positions of scale reductions along the body (recorded as the number of the ventral or subcaudal scale opposite which it was situated) were transformed to percentage ventral scale (%VS) or caudal scale (%CS) position, in order to compensate for variation in ventral and subcaudal scale number. Male and female specimens were treated separately in all analyses to avoid bias caused by sexual dimorphism.

Specimens were grouped by locality into operational taxonomic units (OTUs). Two groups dominated the analysis, one was comprised of specimens from Thailand, West Malaysia and Singapore, and another was comprised of specimens from Borneo (Sabah and Sarawak). These groups were shown to be monophyletic by molecular analysis (unpublished data), which revealed a clear distinction between western specimens that lacked dorsal cross-bands and had at most two supralabials connected to the subocular scale, and eastern specimens that had dorsal cross-bands and had three supralabials in contact with the subocular scale. Molecular data was not available for specimens from Sumatra, and these were grouped individually to avoid combining sympatric species in one OTU.

Each OTU was checked prior to further analysis using Principal Component Analysis, which does not require that individuals be assigned groups prior to the analysis. The integrity of the OTUs was confirmed with the exception of one specimen from Betong (south Thailand), which had dark banding and in the PCA ordination was closest to the Borneo OTU. In subsequent analysis this specimen was

grouped separately from the other western specimens. The OTUs used and their sample size for each sex is listed in Table 2.1.

Variation between OTUs was tested for individual characters by means of one-way analysis of variance (ANOVA). Only characters showing significant between-OTU variation were used in subsequent analyses. These are presented in Appendix 2.3.

Canonical variate analysis (CVA) was used to investigate patterns of geographic variation between OTUs. This method maximises the separation between groups relative to variation within groups. It is a standard multivariate method and has been applied successfully to numerous models of geographic variation in reptiles (Wüster *et al.,* 1992; Thorpe *et al.*, 1994; Daltry *et al.,* 1996).

OTU	Sample Size	
	Males	Females
Thailand, West Malaysia, Singapore	16	15
North Sumatra 1 (Medan)	1	1
North Sumatra 2 (Medan)	0	1
Central Sumatra 1 (Padang)	1	1
Central Sumatra 2 (Padang)	0	1
South Sumatra 1 (Palembang)	0	1
Nias	1	10
Siberut	3	3
East Malaysia	4	18
Betong (south Thailand)	1	0
Total	27	51

Table 2.1: OTUs and sample size for each sex.

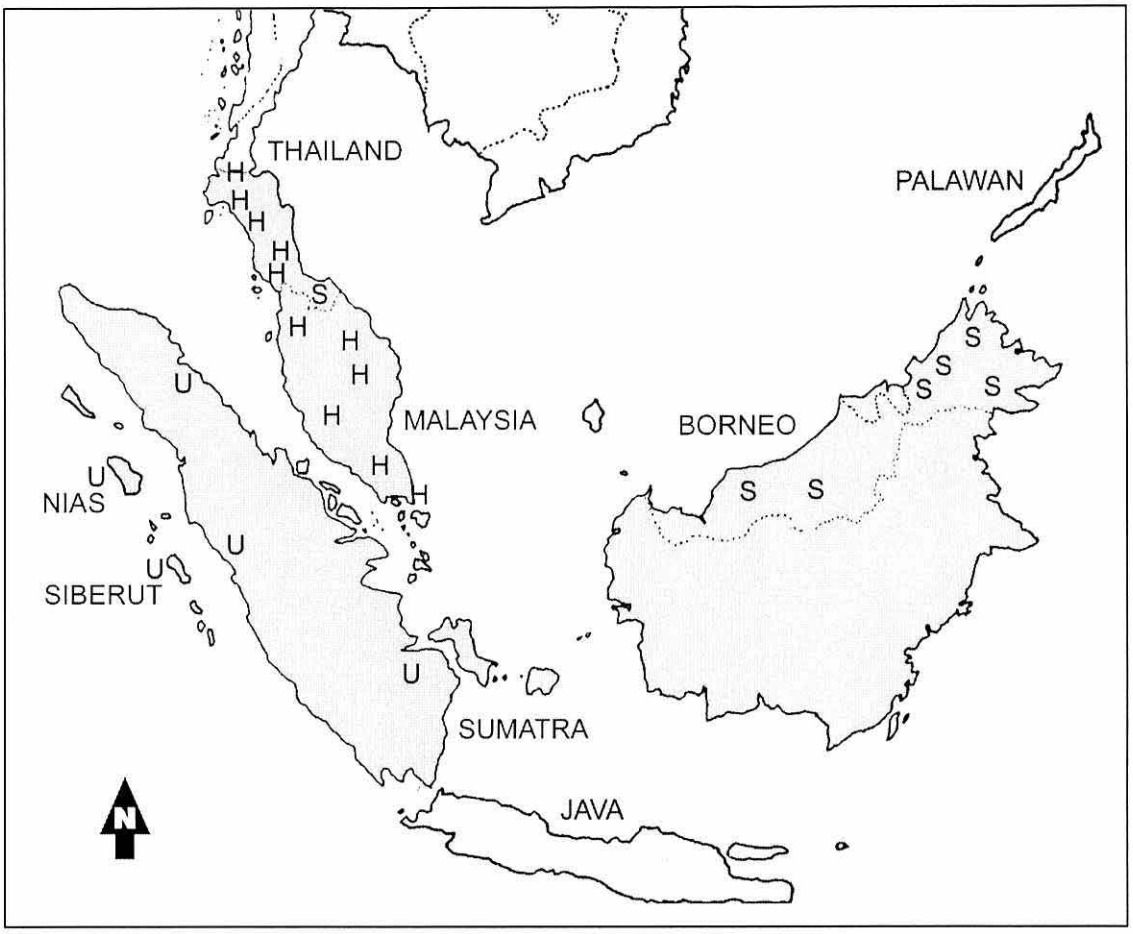

Figure 2.1: Geographic origin of specimens used in multivariate analysis.
S = *Trimeresurus sumatranus*; H = *Trimeresurus hageni*; U = unidentified specimens.
Shading represents the known distribution of *T. hageni* and/or *T. sumatranus.*

2.4 Results

The CVA of males shows clear separation along the first canonical variate of specimens normally assigned to *T. hageni* from Thailand, West Malaysia and Singapore and those normally assigned to *T. sumatranus* from East Malaysia. The Siberut OTU and the single specimens from Nias and northern Sumatra are closest to the mainland *T. hageni* population. The specimens from Betong, Thailand and central Sumatra are closest to the Borneo OTU, but are well differentiated on CV2.

Analysis of females also shows strong differentiation between the Thailand, West Malaysia and Singapore OTU and the Borneo OTU. The Siberut and Nias specimens are phenotypically close to *T. hageni* from Thailand, West Malaysia and Singapore. Specimens from north and south Sumatra are also closely affiliated to this mainland population. The specimens from central Sumatra are closest to the Borneo population along CV1, although are clearly differentiated on CV2.

CVA analysis can be used to identify the characters that account for most variation between groups. In both sexes scalation characters were more important in distinguishing between the taxa than were characters relating to colour and pattern. The most important character is the fifth supralabial scale, which meets the subocular scale in *T. sumatranus* and in *T. hageni* is separated from the subocular by one scale. Also important is the frequent presence of an internasal scale in *T. sumatranus*, which is usually lacking in *T. hageni*. In addition, *T. sumatranus* has fewer supralabial scales and fewer scales between supraoculars than *T. hageni*. Our work verifies two of the original diagnostic characters used by Lidth de Jeude (1886) who described *T. hageni* as a distinct species that lacks dorsal cross-bands and has fewer supralabial scales in contact with the subocular scale. However, we did not find dark edging on head and body scales to be a valid diagnostic character on the basis that *T. hageni* specimens from Nias have very strong dark edges on their head and body scales.

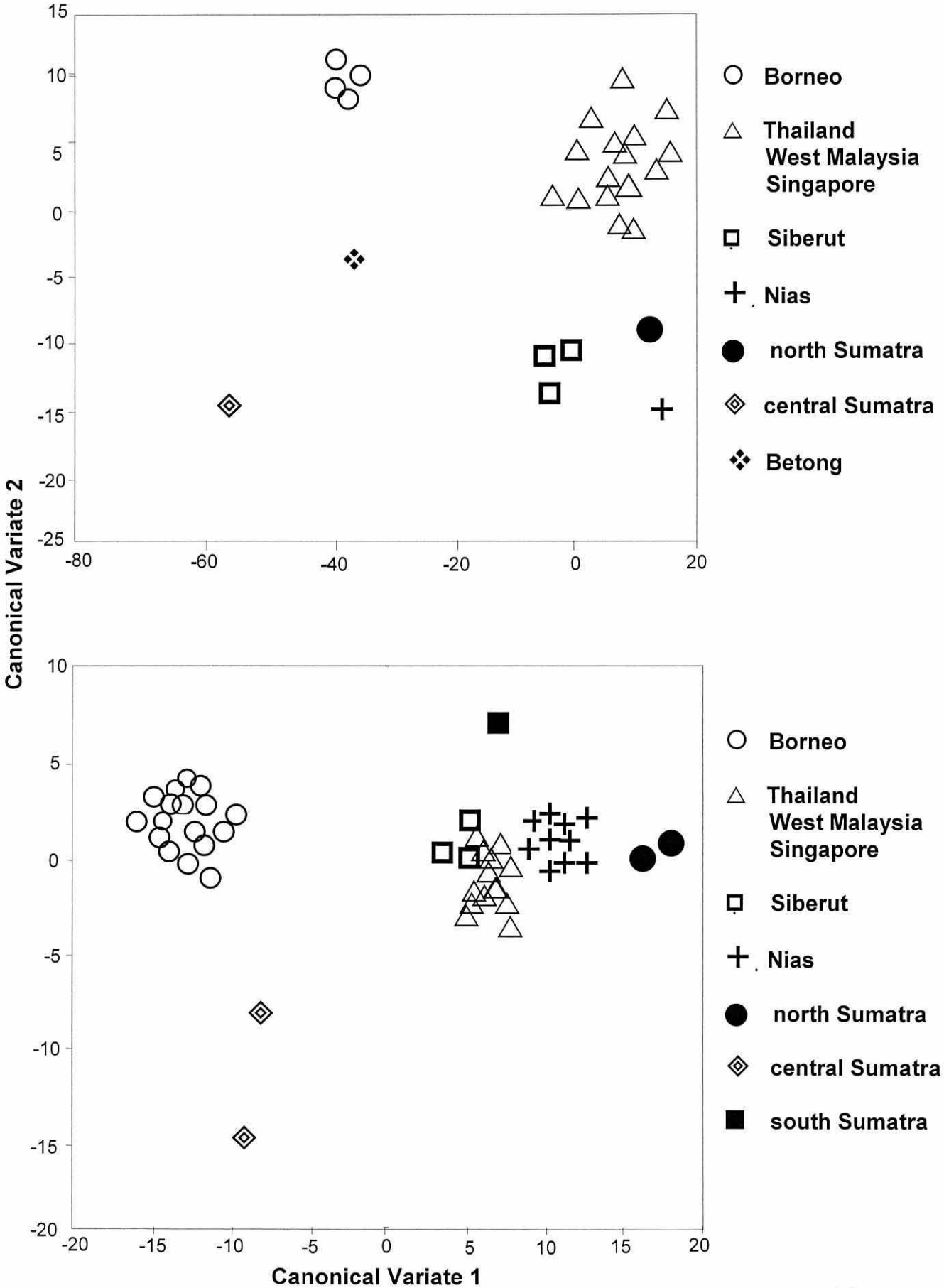

Figure 2.2: Canonical Variate Analysis of *T. hageni* and *T. sumatranus* populations (top = males; bottom = females).

2.5 Discussion

The results of this preliminary analysis reveal a major phenotypic division in both sexes. This corresponds to *T. sumatranus* in Borneo, central Sumatra and southern Thailand and *T. hageni* in southern Thailand, West Malaysia, Singapore, north Sumatra, south Sumatra, Nias and Siberut. The species are best distinguished by head scalation, but can also be identified by colour and pattern.

Geographic variation is also present at the intra-specific level. The Siberut and Nias specimens show stronger differentiation in males than in females. Their phenotypic similarity to mainland *T. hageni* is based mainly on scalation characters. Moreover, on the basis of colour and pattern, the Nias population is quite distinct with head and body scales strongly edged in black. Nias was last connected to Sumatra in the geologically recent past (c. 18,000 years ago), whereas Siberut has been isolated for around one million years (Dring, 1989). The extent to which these populations have diverged from the mainland population will be investigated using molecular methods and may lead to taxonomic revisions.

Sumatran populations are represented by few specimens, but these exhibit the same general pattern in males and females: *T. sumatranus* from central Sumatra appear to be strongly differentiated from the Borneo OTU, whereas *T. hageni* from north and south Sumatra are only weakly differentiated from the mainland OTU. This pattern will be tested when additional data becomes available. An analysis of the phylogenetic relationships of these populations, using mitochondrial sequence data, is also underway and should help to clarify their status.

CHAPTER 3

Ecological diversification in the *Trimeresurus sumatranus* complex: convergence in taxonomically important traits has implications for species identification

3.1 Abstract

We analyse molecular and phenotypic evolution in a group of taxonomically problematic Indomalayan pitvipers, the *Trimeresurus sumatranus* group. Mitochondrial DNA sequencing provides a well-resolved phylogeny, with each species representing a distinct lineage. Multivariate morphological analysis reveals a high level of phenotypic differentiation, which is congruent between the sexes but does not reflect phylogenetic history. An adaptive explanation for the observed pattern of differentiation is supported by independent contrasts analysis, which shows significant correlations between current ecology and many of the scalation and colour pattern characters that most account for the variation between taxa, including those that are presently used to identify the species. Reduced precipitation and altitude, and increased temperature, are correlated with higher numbers of scales on the head, body and tail. It is hypothesised that scale number plays an important role in heat and water exchange by influencing the area of exposed interstitial skin, and that colour pattern variation reflects selection pressures involving camouflage and thermoregulation. Ecological convergence in traits used for classification is found to have important implications for species identification where taxa are distributed over varying environments.

3.2 Introduction

Despite the increased availability of molecular systematic techniques that allow phylogeny to be inferred independent of phenotype, most species are still described on the basis of morphological traits of unknown phylogenetic content. When shared ancestry is taken into account in comparative studies of character variation, the evolutionary forces involved in phenotypic differentiation can be assessed by the distribution of traits within a group of taxa in relation to current ecological factors (Felsenstein, 1985; Harvey & Purvis, 1991; Garland, 1992; Bauwens *et al.*, 1995; Martins & Hansen, 1997; Kohlsdorf *et al.,* 2001). Ecological convergence in traits used for classification can confuse species identification in groups that are widely distributed over varying environments. Comparative studies can provide an indication of the utility of morphological characters used in classification, and can lead to new hypotheses concerning the adaptation of organisms to their environments (Brooks & McLennan, 1991).

Numerous studies of geographic variation in reptilian scalation and colour pattern have been reported in the literature. However, only a minority have addressed the role of natural selection in the observed pattern of variation, and most of these have been carried out at the intraspecific level on island lizard populations (e.g. Thorpe, 1989; Thorpe, 1991; Losos, 1994; Malhotra & Thorpe, 1997). Such investigations have elucidated the relative roles of ecogenetic and phylogenetic factors in the variation of island populations, and have revealed correlations between morphological characters and various facets of the environment, including temperature, rainfall, altitude and vegetational type. Additional studies have shown that adaptive differences can arise very quickly at the population level. This has been observed in *Anolis* lizards, in which scalation and colour pattern characters were found to be prone to rapid adaptive change in response to change of ecotype (Malhotra & Thorpe, 1993).

The *Trimeresurus sumatranus* group of Indomalayan pitvipers provides an excellent opportunity to investigate the role of ecological divergence in differentiation at the

interspecific level. The taxa are closely related, yet have isolated gene pools due to their allopatric distribution across an environmentally varying archipelago (Malhotra and Thorpe, submitted). In this study, we use mtDNA sequences to infer phylogenetic relationships in the *Trimeresurus sumatranus* group, and elucidate the pattern of phenotypic differentiation between taxa using multivariate analysis on scalation and colour pattern characters. The characters that account for most of the variation in the morphological analysis, and those that are currently used to identify the species, are then subjected to the method of phylogenetically independent contrasts (Felsenstein, 1985). Our aims are to investigate the role of environmental factors in the differentiation of taxa, and to assess the implications of ecological adaptation in taxonomically important traits for species identification. Felsenstein's (1985) independent contrasts analysis is a standard phylogenetic method and has proved useful in detecting correlations in numerous comparative studies at the interspecific level (Garland *et al.*, 1993; Butler & Losos, 1997; Barraclough *et al.* 1998; Martins *et al.* 2001).

The *T. sumatranus* group comprises five nominal species, which are widely distributed over the Indomalayan archipelago (Figure 3.1) and display considerable morphological and ecological diversity. They are: *T. sumatranus, T. hageni, T. malcolmi, T. schultzei* and *T. flavomaculatus.* These species are arboreal and oviparous, occurring almost exclusively in undisturbed rainforests. There is substantial variation in the forest types they occupy, reflecting the latitudinal range of the complex; *T. schultzei, T. f. flavomaculatus* and *T. f. mcgregori* occupy seasonal forests in the Philippine Islands, which receive consistently lower rainfall than those in equatorial Indonesia (2000-3000mm/yr versus 3000-4000mm/yr) (Whitmore, 1975; Anon, 1995). Moreover, whereas *T. sumatranus* from Borneo, *T. hageni, T. schultzei, T. f. flavomaculatus* and *T. f. mcgregori* are rarely found above 300m (David & Vogel, 1996; Cox *et al.*, 1998; Stuebing & Inger, 1999), *T. malcolmi* is found only in montane rainforest over 1000m (Stuebing & Inger, 1999), and the Sumatran population of *T. sumatranus* occurs between 650m and 800m (unpublished data). The species also differ in prey utilisation; *T. sumatranus, T. hageni, T. malcolmi* and *T. f. mcgregori* feed mainly on mammals, whereas *T. f.*

flavomaculatus seems to feed exclusively on amphibians and the diet of *T. schultzei* includes lizards and frogs (unpublished data).

The systematics of the group, and the precise distribution of certain species, is an area of long-standing confusion. The distinction between *T. sumatranus* and *T. hageni* is especially problematic (Sanders *et al.*, 2002), and most workers assign both species to *T. sumatranus* by default (Tweedie, 1983; Lim, 1991; Jintakune, 1995; David & Vogel, 1996). The status of *T. malcolmi* is also an area of contention; this taxon was recently elevated from a subspecies of *T. sumatranus* on the basis of scalation and colour pattern (Stuebing & Inger, 1998). *T. flavomaculatus* occurs over much of the Philippine Islands and comprises three subspecies: *T. f. halieus*, *T. f. mcgregori* and *T. f. flavomaculatus* (Leviton, 1963). *T. f. halieus* is not generally recognised as a valid subspecies. However, many workers treat *T. f. mcgregori* as a full species due to striking differences in colour pattern (individuals are frequently yellow or grey, always lacking green pigmentation) (Gumprecht, 2002) and extreme isolation on the Batanes islands, which lie 130 miles north of Luzon and have never been connected to the mainland (Leviton, 1963).

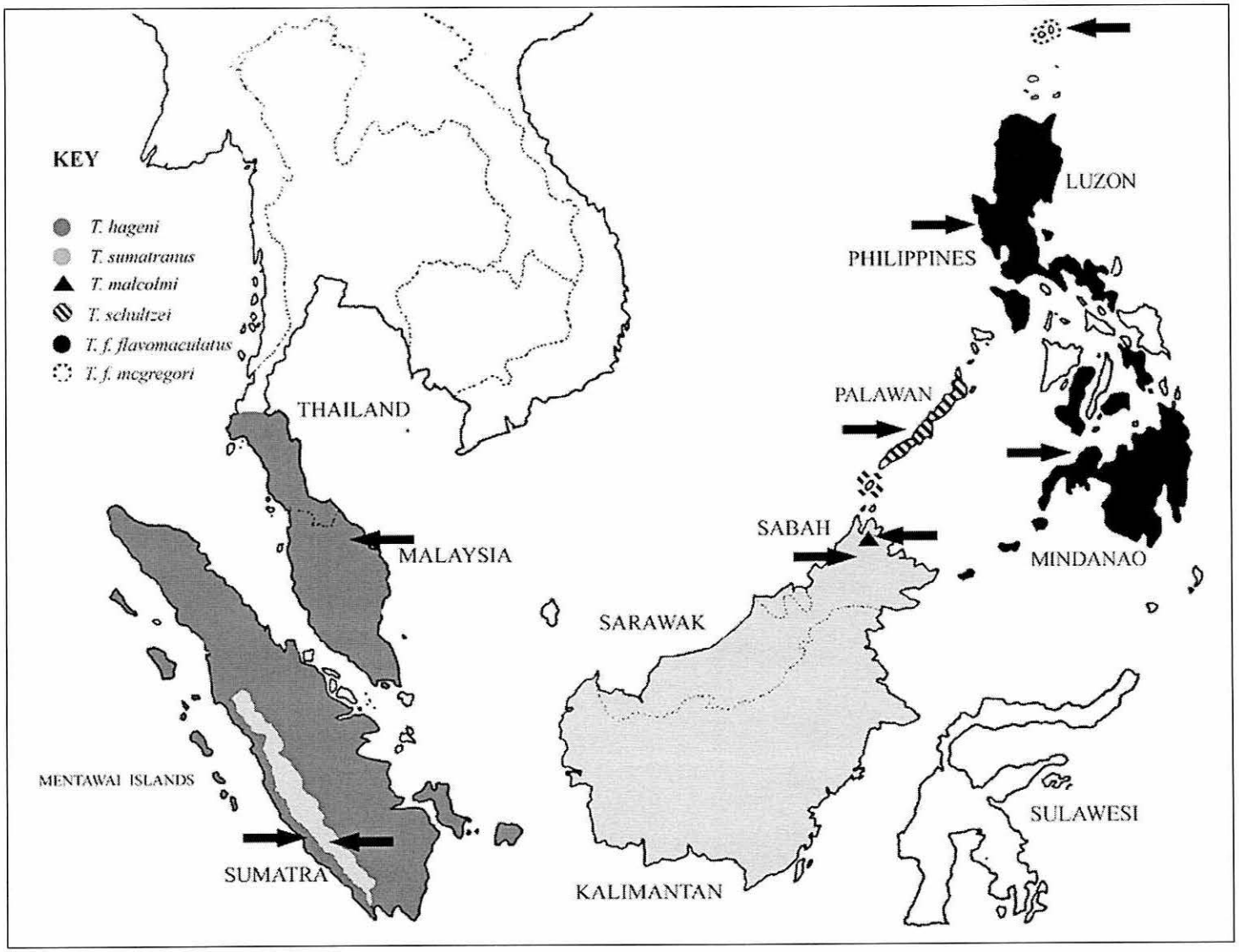

Figure 3.1: Distribution of the *Trimeresurus sumatranus* group in the Indomalayan archipelago. Black arrows indicate geographic origin of specimens used in phylogenetic analysis.

3.3 Materials and Methods

3.3.1 DNA preparation, amplification and sequencing

Blood or tissue samples were obtained from wild-caught and captive specimens (of known locality) of *T. sumatranus* from Sabah and west Sumatra, *T. hageni* from West Malaysia and west Sumatra, *T. malcolmi* from Mt. Kinabalu (Sabah), *T. schultzei* from Palawan, *T. f. flavomaculatus* from Luzon and Mindanao and *T. f. mcgregori* from Batanes (Appendix 2.1 & Figure 3.1). Blood samples were taken from the caudal vein, placed in 1ml 0.1M EDTA, and stored in SDS-Tris buffer (2% SDS, 100mM Tris); liver and muscle tissue was preserved in 80% ethanol. Whole genomic DNA was extracted using standard proteinase K protocols (Sambrook *et al.,* 1989).

Four mitochondrial genes were amplified via polymerase chain reaction: these were cytochrome *b* (750bp), NADH4 dehydrogenase subunit 4 (650bp), 12S ribosomal RNA (400bp) and 16S large subunit ribosomal RNA (500bp). Cytochrome *b* (cyt*b*) sequences were obtained as described in Malhotra & Thorpe (2000). NADH dehydrogenase subunit 4 (ND4) sequences were obtained as described in Parkinson *et al.* (2000), 12S rRNA as described in Knight & Mindell (1993), and 16S rRNA as in Parkinson *et al.* (1997).

Thermal cycling parameters for cyt*b* and ND4 were denaturation at 94°C for 3min followed by 35 cycles of: denaturation at 93°C for 1min, annealing at 48°C for 2min and extension at 72°C for 2min. At the end of the reaction there was a final extension step at 72°C for 3min. Amplification conditions for 12S and 16S were denaturation at 94°C for 2min followed by 30 cycles of: denaturation at 94°C for 2min, annealing at 45°C for 0.5min and extension at 72°C for 1min, with a final extension step at 72°C for 3min.

PCR products were concentrated and purified using Wizard minicolumns (Promega). Single-stranded sequencing was then carried out using dye-labelled

terminators (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit) and run on an ABI 377 DNA sequencer.

3.3.2 Phylogenetic analyses

Outgroups were selected to represent three *Trimeresurus* species groups (*sensu* Malhotra and Thorpe, 2000); these were *T. insularis, T. stejnegeri* and *T. vogeli*. Alignment of cyt*b* and ND4 was trivial as there were no indels. The 12S and 16S sequences were aligned by eye following Parkinson (1999) with the exception of minor changes, which were required in one region of 12S and one region of 16S due to insertions found in some of the new sequences obtained. The coding genes were translated into amino acid sequences to check for the presence of stop codons that might indicate that pseudogenes had been amplified. The four mitochondrial genes were combined into a single data set; mitochondrial genes are inherited as a single linkage group and as such do not provide independent estimates of phylogeny (Moore, 1995; Page, 2000). In addition, an increased number of genes are likely to provide a higher number of potentially variable sites for phylogenetic analysis (Chippindale & Wiens, 1994; Cummings *et al.*, 1995). A model of molecular evolution was assigned to the data using the log likelihood function of MODELTEST 3.0 (Posada & Crandall, 1998). MODELTEST compares 56 different nested substitutional models and uses log likelihood scores to determine which model best fits the data. Corrected pairwise sequence comparisons were then made in MEGA version 2.1 (Kumar *et al.*, 2001) using the model of evolution identified by MODELTEST.

PAUP* 4.0b8 (Swofford, 1998) was used for all further phylogenetic analysis. Maximum Likelihood (ML) analysis, using a heuristic search with a starting tree obtained by 10 random additions of taxa and tree-bisection-reconnection (TBR) branch swapping, was conducted on the parameters identified by MODELTEST. The parameters were then re-estimated from the resulting tree and used in a new ML analysis. 500 bootstrap replicates using the same options were performed to assess

node support for the resulting tree. A maximum parsimony (MP) tree was also produced using the same search options as in the ML analysis. All sites were equally weighted, and 1000 bootstrap replicates were performed.

3.3.3 Multivariate morphometrics

We examined 143 specimens from museum collections in the United States, Europe, the Philippine Islands, Indonesia and Malaysia. Specimens were grouped into operational taxonomic units (OTUs) on the basis of allopatric populations. *T. hageni* was represented by two OTUs, one comprising specimens from Thailand, West Malaysia and Singapore and another comprising specimens from south Sumatra. *T. sumatranus* was represented by OTUs from Borneo (Sabah and Sarawak) and west Sumatra. *T. schultzei* was represented by specimens from Palawan and Balabac island. *T. f. flavomaculatus* was represented by separate OTUs of specimens from Luzon and Mindanao. *T. f. mcgregori* was represented by specimens from the Batanes islands. *T. malcolmi* corresponds to specimens from Mt. Kinabalu (Sabah). The OTUs used and their sample size for each sex are listed in Table 3.2.

A total of 93 characters relating to scalation and colour pattern were recorded for each specimen. Ventral scales were counted from head to vent, with the first ventral identified according to the method of Dowling (1951). The positions of scale reductions along the body (recorded as the number of the ventral or subcaudal scale opposite which it was situated) were transformed to percentage ventral scale (%VS) or subcaudal scale (%SC) position, in order to compensate for variation in ventral and subcaudal scale number. Male and female specimens were treated separately in all analyses to avoid bias caused by sexual dimorphism.

Variation between OTUs was tested for individual characters by means of one-way analysis of variance (ANOVA). Only characters showing significant between-OTU variation were used in subsequent analyses. These are highlighted in Appendix 2.4. Principal components analysis (PCA) was used to check for the possible inclusion of

sympatric species within OTUs. Canonical variate analysis (CVA) was then used to investigate multivariate patterns of geographic variation between OTUs (Thorpe, 1976; Thorpe, 1983). Characters that are invariable in some OTUs and variable in others violate the assumptions of CVA, and were excluded from further analyses at this stage. CVA maximises the separation between groups relative to variation within groups, and has been applied successfully to numerous models of geographic variation.

Species	Sample Size	
	Males	Females
T. hageni (Thailand, West Malaysia, Singapore)	16	15
T. hageni (Sumatra)	5	3
T. sumatranus (Borneo)	7	23
T. sumatranus (Sumatra)	5	10
T. schultzei	8	4
T. malcolmi	3	3
T. flavomaculatus (Mindanao)	3	4
T. flavomaculatus (Luzon)	9	9
T. f. mcgregori (Batanes Is)	4	11
Total	61	82

Table 3.1: OTUs used in morphometric analyses and sample size for each sex.

3.3.4 Independent contrasts

We analysed our morphological data in a phylogenetic context, using Felsenstein's (1985) method of independent contrasts in the PDTREE program of Garland *et al.* (1999). Due to the hierarchical nature of phylogenetic relationships, closely related taxa are more likely to share characteristics through descent from common ancestors than distantly related species (Garland, 1992). The method of independent contrasts attempts to account for this lack of independence in cross-species data sets; correlations between ecology and phenotype are estimated using information on the amount of character change between pairs of closely related species since their most recent shared ancestor. The values of N species are transformed into N - 1 statistically independent and identically distributed contrasts (Garland, 1992).

Overall phenotype (derived from all characters generalised on CV1), maximum SVL, the characters most important in the canonical variate analyses and the characters presently used to distinguish the species were subjected to independent contrasts analysis. These data were analysed for nine allopatric populations (*T. hageni* from West Malaysia; *T. hageni* from Sumatra; *T. sumatranus* from Sumatra; *T. sumatranus* from Borneo; *T. malcolmi*; *T. schultzei*; *T. f. flavomaculatus* from Luzon; *T. f. flavomaculatus* from Mindanao; and *T. f. mcgregori*), using the ML tree. Mean values were used for scalation characters; maximum snout-to-vent length (SVL) was estimated from our data and the published literature (Taylor, 1919; Loveridge, 1938). Mean annual precipitation and temperature data were obtained from on-line world climate databases (Buttle & Tuttle Ltd, 2001; Quikcast, 2002). The upper limit of the range of each population was used for analyses involving altitude; this was taken from data collected in the field and the published literature (Taylor, 1919; Loveridge, 1938; Stuebing & Inger, 1999).

Reported correlation coefficients and significance values refer to those obtained from regression of standardised contrasts based on the ordinary least-squares method, using phylogenetically independent contrasts. Statistical tests are two-tailed. Results are Bonferroni corrected non-sequentially by row. Diagnostic plots (Garland

et al., 1992) of the absolute values of standardized contrasts vs. their standard deviations (square roots of sums of branch lengths) were checked to assess the adequacy of branch lengths for standardisation of independent contrasts. The adequacy of the branch lengths used is confirmed in all analyses other than those involving precipitation, in this case branch lengths are $\log_{10}$ transformed to properly standardise independent contrasts (Garland, 1992; Garland, 1999).

3.4 Results

3.4.1 Preliminary sequence analysis

A total of 2424 bp was used to represent thirteen ingroup accessions and four outgroup taxa in the final phylogenetic analysis. (GenBank accession numbers are given in Table 3.1). These contained 638 variable sites, of which 401 (16.5% of all sites) were parsimoniously informative. There was no indication that pseudogenes had been amplified as no stop codons or indels were found in either of the protein coding genes. The substitutional model of evolution assigned by MODELTEST was TrN (Tamura & Nei, 1993) with a gamma distance shape parameter of 0.8838.

3.4.2 Phylogenetic relationships

Maximum likelihood and maximum parsimony analyses of the combined data set resulted in identical tree topologies, with generally high bootstrap support that is similar in the two analyses (Figure 3.2). The log likelihood score for the final ML tree was –Ln 8200.22, and was obtained using the Tamura-Nei model with gamma distributed rates. MP analysis revealed a single most parsimonious tree with a length of 1071, a consistency index (CI) of 0.65, a retention index (RI) of 0.71 and a rescaled consistency index (RC) of 0.47.

Our analyses reveal five clearly distinct lineages, corresponding to currently recognised species. *T. hageni* diverges earliest in the group, followed by *T. flavomaculatus*, and then *T. malcolmi*; *T. schultzei* and *T. sumatranus* are sister species. *T. f. flavomaculatus* from Mindanao diverges earlier than *T. f. flavomaculatus* from Luzon and *T. f. mcgregori*, which are weakly supported as sister taxa.

Pairwise sequence differences were estimated using the Tamura Nei model with Gamma correction (Tamura and Nei, 1993). Mean levels of sequence divergence

between ingroup species ranged from 7.9% ± 0.9 (between *T. schultzei* and Bornean *T. sumatranus*) to 12.8% ± 1.4 (between *T. hageni* and *T. sumatranus*), and from 13.0% ± 1.0 to 15.4% ± 1.2 between ingroup and outgroup taxa. Within-locality sequence divergence was low: 0.3% ± 0.2 between *T. hageni* from West Malaysia, 0.2% ± 0.1 between *T. hageni* from Sumatra, 0.1% ± 0.1 between *T. sumatranus* from Sumatra, 0.6% ± 0.2 between *T. sumatranus* from Borneo, and 0.1% ± 0.01 between *T. malcolmi*. Sequence differences between allopatric populations were 1.0% ± 1.3 between *T. hageni* from West Malaysia and Sumatra; 3.3% ± 1.5 between *T. sumatranus* from Borneo and Sumatra; 1.9% ± 1.4 between *T. f. flavomaculatus* from Luzon and Mindanao, and 1.6% ± 1.4 between *T. f. flavomaculatus* from Luzon and *T. f. mcgregori*.

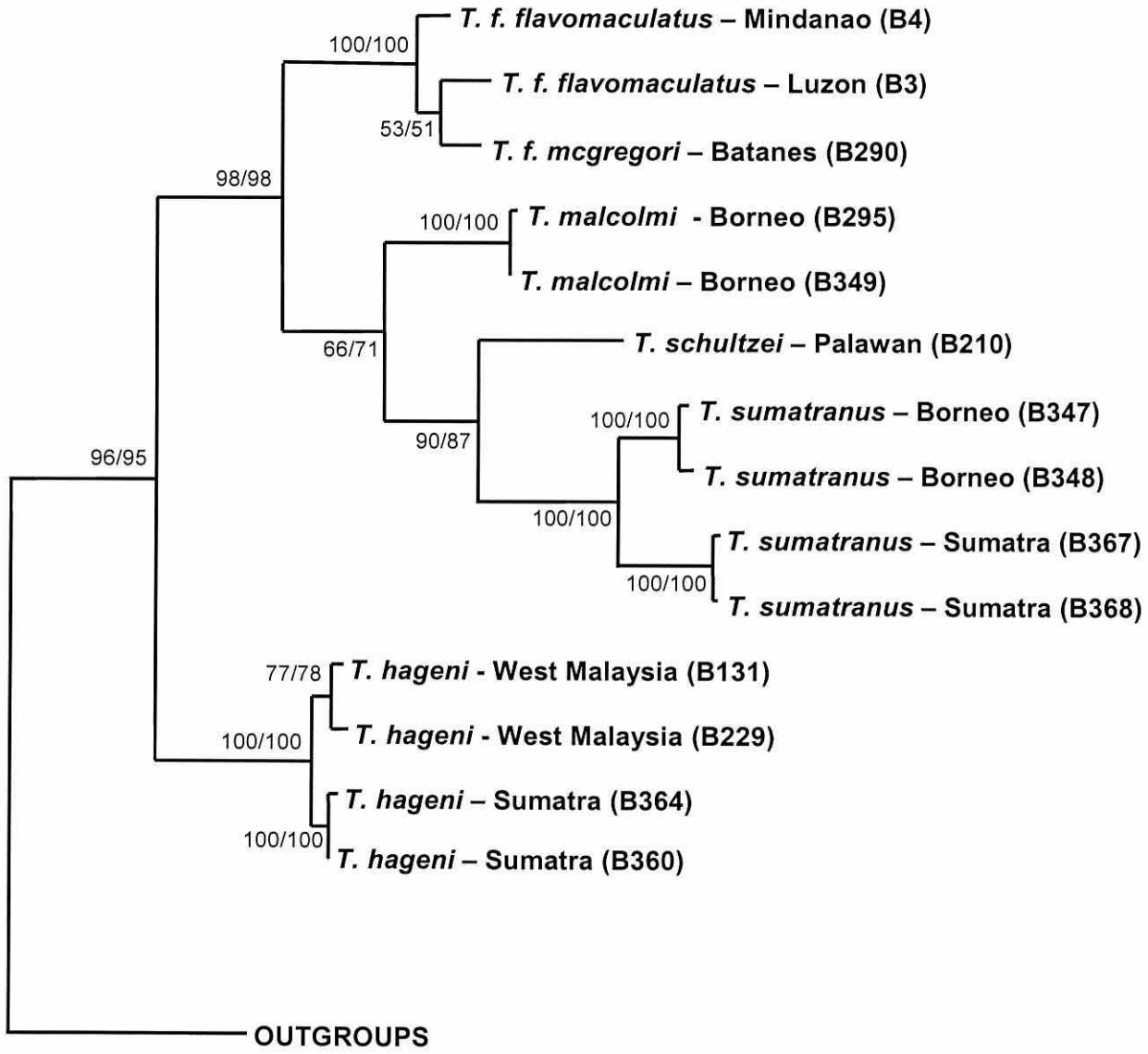

Figure 3.2: Mitochondrial gene tree derived from maximum-likelihood analysis of combined data (2424 bp), with bootstrap support values from maximum-likelihood and maximum parsimony trees respectively, reading left to right and top to bottom.

3.4.3 Morphological variation

CVA analysis revealed a pattern of morphological variation that is generally congruent between males and females (Figure 3.3). Most taxa are clearly distinct, with strong separation between *T. hageni* and *T. sumatranus*, and between these two species and *T. flavomaculatus*. *T. malcolmi* is most similar to *T. sumatranus*, and *T. schultzei* is phenotypically closest to *T. f. flavomaculatus*.

Conspecific OTUs of *T. hageni*, *T. sumatranus* and *T. f. flavomaculatus* show weak differentiation in this analysis, although the separation between these groups is clearer in males than in females. *T. f. mcgregori* is strongly differentiated from *T. f. flavomaculatus* in males, but is weakly differentiated in the analysis of females.

CVA analysis can be used to identify the characters that account for most variation between groups (Table 3.3). The characters with highest eigenvector coefficients for the first two canonical variables are the minimum and maximum number of scales separating the supraocular scales, the number of scales between the nasal scale and the shield bordering the pit anteriorly, the number of internasal scales, the position of scale reductions (on the body) from 17 to 15 scale rows, and (on the tail) from 10 to 8 rows, the number of scales bordering supraoculars, the number of scales separating the fourth supralabial from the subocular scale, and the presence of dark edging on head and body scales.

Character	MALES		FEMALES	
	CV1	CV2	CV1	CV2
Proportion of total variation explained	50%	28%	56%	22%
Maximum no. of scales separating the supraocular scales	0.32	**-0.62**	**0.38**	-0.01
Minimum no. of scales separating the supraocular scales	**0.38**	**0.65**	**-0.34**	**-0.29**
No. of internasal scales	**-0.51**	0.21	-007	**-0.29**
%CS position of reduction from 10 to 8 tail scale rows	**0.48**	**-0.36**	**0.40**	0.18
%CS position of reduction from 6 to 4 tail scale rows	-0.36	0.29	0.06	**0.40**
%VS position of reduction from 17 to 15 body scale rows	0.11	**0.50**	**-0.49**	-0.12
No. of sublabial scales	0.12	0.12	**-0.49**	-0.26
No. of supralabial scales	-0.37	**-0.37**	-0.23	0.10
No. of scales separating the fifth supralabial scale from the subocular scale	0.29	0.29	0.13	-0.04
No. of scales separating the fourth supralabial scale from the subocular scale	**0.83**	**-0.46**	**0.42**	-0.01
No. of scales bordering the supraocular scales	**-0.62**	0.04	**0.37**	0.07
No. of scales between the nasal scale and the shield bordering the pit anteriorly	**-0.65**	**0.69**	-0.04	-0.12
No. of spots on dorsal scale row one	0.03	-0.32	0.21	**-0.68**
Presence of dark edging on head scales	**0.67**	-0.13	0.02	**-0.40**
Presence of dark edging on body scales	-0.21	-0.13	-0.21	**0.43**

Table 3.2: Standardised canonical variate coefficients for CV1 and CV2. Characters with the highest eigenvector coefficients on each CV are highlighted in bold

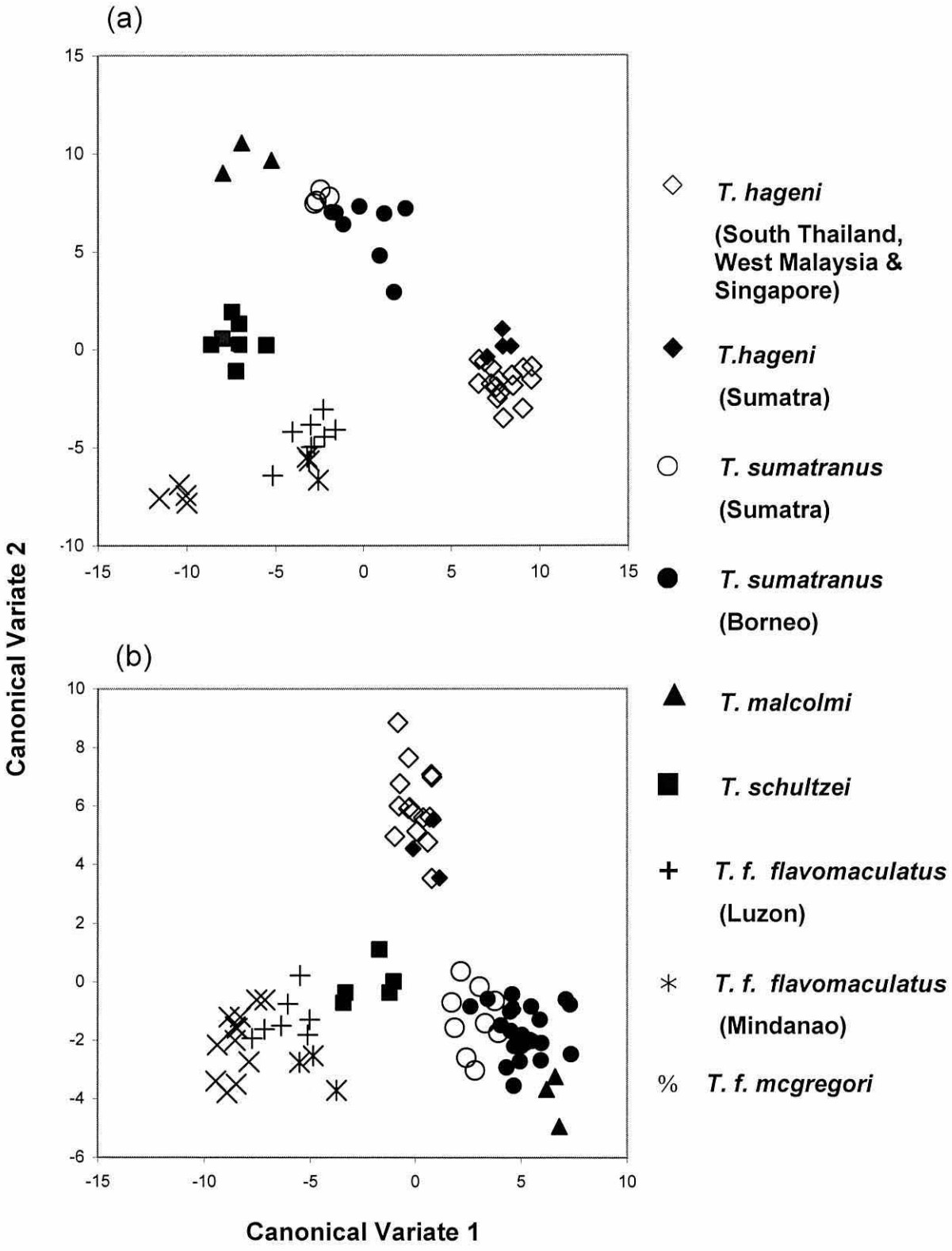

Figure 3.3: Canonical variate analysis of the *T. sumatranus* group (a = males; b = females)

3.4.4 Independent contrasts

Phylogenetically independent contrasts reveal significant correlations between all three ecological factors and most of the characters tested in males and females (Table 3.3). In addition, generalised phenotype is significantly correlated with precipitation in males (Table 3.3). Mean annual precipitation has a negative influence on head scale number, tail scale reduction formula and the presence of a stripe, but is positively correlated with dark edges on head and body scales. Altitude is negatively correlated with scale counts and the presence of a stripe, and is positively correlated with maximum snout-to-vent length in males, although not in females. Altitude is also positively correlated with dark edges on head and body scales. Mean annual temperature is positively correlated with scale number for several head scale characters, scale reduction on the body and the presence of a stripe, but is negatively correlated with dark edges on head and body scales. In general, reduced precipitation and altitude, and increased temperature, are correlated with higher numbers of scales on the head and cause scale reductions to occur closer to the head and vent, resulting in fewer scales for more of the body and tail length. Dark edges on head and body scales increase with precipitation and altitude, and decrease with temperature; the opposite trend is found for the presence of a stripe on dorsal scale row one.

TRAIT	PRECIPITATION				ALTITUDE				TEMPERATURE			
	Males		Females		Males		Females		Males		Females	
	r	P	r	P	r	P	r	P	r	P	r	P
Minimum no. of scales separating the supraocular scales	-	-	-	-	-	-	-	-	-	-	-	-
Maximum no. of scales separating the supraocular scales	-0.8012	<0.05	-	-	-	-	-	-	-	-	-	-
No. of internasal scales	-	-	-	-	-	-	-	-	-	-	-	-
%SC position of reduction from 10 to 8 tail scale rows	-	-	-	-	-	-	-	-	-	-	-	-
%SC position of reduction from 6 to 4 tail scale rows	-0.6929	<0.05	-	-	-	-	-	-	-	-	-	-
%VS position of reduction from 17 to 15 body scale rows	-	-	-	-	-	-	-	-	-	-	0.6469	<0.05*
No. of sublabial scales	-	-	-0.6739	<0.05*	-0.7546	<0.05*	-	-	0.8020	<0.01	0.7511	<0.05*
No. of supralabial scales	-	-	-	-	-	-	-	-	-	-	-	-
No. of scales separating the 5th supralabial scale from the subocular scale	-0.6634	<0.05*	-	-	-0.7738	<0.05*	0.6654	<0.05*	0.7743	<0.05*	-	-
No. of scales separating the 4th supralabial scale from the subocular scale	-	-	-	-	-	-	-	-	-	-	-	-
No. of scales bordering the supraocular scales	-	-	-	-	-	-	-	-	0.6929	<0.05*	-	-
No. of scales between the nasal scale and shield bordering the pit	-	-	-	-	-	-	-	-	-	-	-	-
Presence of dark edging on head scales	0.7843	<0.05*	0.6406	<0.05*	0.8663	<0.01	0.8032	<0.01	-0.8691	<0.01	-0.7936	<0.05*
Presence of dark edging on body scales	0.8115	<0.01	-	-	0.8296	<0.01	0.9089	<0.01	-0.8368	<0.01	-0.8877	<0.01
Presence of stripe on dorsal scale row one	-0.7466	<0.05*	-0.6401	<0.05*	-0.6904	<0.05*	-0.7277	<0.05*	0.7752	<0.05*	0.7789	<0.05*
No. of dorsal body spots	-	-	-	-	-	-	-	-	-	-	-	-
No. of scales above supralabials covered by ventral colour	-	-	-	-	-	-	-	-	-	-	-	-
Maximum snout-to-vent length	-	-	-	-	0.7662	<0.05*	-	-	-0.7898	<0.05*	-	-
Generalised phenotype	0.7334	<0.05*	-	-	-	-	-	-	-	-	-	-

Table 3.3: r and *P* values from independent contrasts regression of morphological characters and generalized phenotype against ecological variables. Significance values are Bonferroni-corrected by row (*P < 0.05/6).

3.5 Discussion

3.5.1 Phylogeny and systematics

This study reveals a well-resolved phylogeny for the *Trimeresurus sumatranus* group. Distinct interspecific divisions correspond to five well-separated lineages: *T. hageni* diverges earliest in the phylogenetic history of the group, followed by *T. flavomaculatus*, and then *T. malcolmi*; *T. schultzei* and *T. sumatranus* are sister species. Our data are also consistent with the species status of *T. malcolmi*, which was recently elevated from a subspecies of *T. sumatranus* on the basis of having fewer scale rows at mid-body and increased dark edging on scales (Stuebing & Inger, 1998).

A varied level of divergence is found at the intraspecific level. Mainland and Sumatran populations of *T. hageni* represent recently diverged populations that are weakly differentiated by phenotype. A higher level of sequence divergence is found between *T. flavomaculatus* populations. *T. f. mcgregori* and *T. f. flavomaculatus* are phenotypically distinct in males, but weakly differentiated in our analysis of females. However, when *T. flavomaculatus* is analysed separately from the remainder of the group, *T. f. mcgregori* forms the most distinct grouping in both sexes (not shown). Mean sequence divergence between *T. sumatranus* from Borneo and Sumatra is comparatively high (3.3% ± 1.5). These groups also show phenotypic and ecological differences (the Bornean population occupies low altitudes, whereas the Sumatran population is not found below 650m (unpublished data), and with further study they may qualify as separate species under the phylogenetic species concept (Cracraft, 1983) and the cohesion species concept (Templeton 1989, 2001).

3.5.2 Adaptive evolution

The results of our multivariate analyses reveal a high level of morphological differentiation between most of the nominal species in the group. The pattern of phenotypic variation is largely congruent between the sexes, but clearly does not reflect the phylogenetic history of the group revealed by the mitochondrial gene tree. *T. malcolmi* and *T. sumatranus* are phenotypically very similar, despite their strong phylogenetic separation. *T. schultzei* is very similar in phenotype to *T. flavomaculatus*, but is most closely related to *T. sumatranus*, from which it is well differentiated by phenotype.

An adaptive explanation for the observed pattern of differentiation is supported by independent contrasts analysis, which reveals significant correlations between current ecological conditions and most characters. A combination of selective pressures is likely to be involved in the diversification of the group. The skin plays an important role in both heat and water exchange in reptiles (Pough *et al.,* 2001), and our results are consistent with numerous studies that report trends of increasing scale number in drier, warmer environments (Klauber, 1941; Klauber, 1972; Soulé & Kerfoot, 1972; Thorpe & Baez, 1987; Brown & Thorpe, 1991; Malhotra & Thorpe, 1997). The reverse relationship has also been observed, with lizard populations occupying hot, dry habitats having larger (and therefore fewer) scales (Hellmich, 1951; Horton, 1972, Lister, 1976). However, these studies highlighted the surface area of scales in relation to radiation of heat; larger scales tend to be more sculptured and have higher surface areas. Keeling of the head and body scales was not found to vary significantly in the *Trimeresurus sumatranus* group, and given that these species are nocturnal and occupy closed habitats, protection from excess heat and ultra-violet light is unlikely to be an important factor.

Cutaneous evaporation has been reported to be the primary avenue of water loss in reptiles (Bentley & Schmidt-Nielsen, 1966). In the *Trimeresurus sumatranus* group it may be that an increase in scale number results in a tighter fit between scales,

reducing the surface area of exposed interstitial skin, and hence facilitating more efficient water retention in hotter, drier climates. This may be of particular relevance for animals that specialise in consuming large prey by distending the body, consequently increasing the surface area of exposed interstitial skin.

Maximum snout-to-vent length is increased in males at higher altitudes and lower temperatures, reducing sexual size dimorphism in the two montane taxa (the female is larger in all species). Endotherms occupying cooler environments tend to attain larger body sizes, which facilitate improved heat retention due to a reduction of the surface area to body volume ratio. However, a converse trend is observed in ectotherms, in which smaller body sizes are generally associated with cooler environments (Mousseau, 1997). Exceptions are known, including the western rattlesnake, *Crotalus viridis*, which attains larger sizes in cooler, more seasonal environments (Ashton, 2001).

Colour pattern characters also reflect adaptation to the local environment. An increase in the dark edges on head and body scales is most strongly associated with high altitude, cooler habitats in which darker pigmentation may provide a thermoregulatory advantage. Vegetational type is dependent on local climate, and habitat-driven selection for camouflage is also likely to be important given that these species are ambush predators that rely on cryptic colour and pattern.

Adaptation to differences in local climate may account for much of the taxonomic confusion among the taxa. Several of the characters presently used to distinguish the species are correlated with current ecology. These include the traits used to distinguish *T. hageni*, *T. sumatranus* and *T. malcolmi*: the number of scales separating the fifth supralabial from the subocular scale and the presence of dark edging on head and body scales (Lidth de Jeude, 1886; Brongersma, 1933; Stuebing & Inger, 1998); the characters used to distinguish between *T. hageni* and *T. sumatranus* and between *T. schultzei* and *T. flavomaculatus*, including the number of sublabial scales and the number of scales bordering supraoculars (Griffin, 1909; Taylor, 1919; Brongersma, 1933; Leviton, 1963); and the positions of scale

reductions on the body, used to distinguish *T. malcolmi* from *T. sumatranus* (Stuebing & Inger, 1998).

Ecological adaptation has led to convergence between *T. hageni* and *T. sumatranus* where they overlap in range. In Sumatra, *T. hageni* shares traits thought to be characteristic of *T. sumatranus*, including lowered scale counts on the head and dark edges on the head and body scales. Phenotypic convergence is especially pronounced in some of the Mentawai Island populations, and has led to confusion in Siberut where *T. hageni* has dorsal crossbands, and in Nias where the species has head and body scales that are edged in black to the same extent as found in *T. sumatranus* (Sanders *et al.*, 2002).

This example demonstrates that ecological convergence in traits used for classification can confuse species identification. However, by using multivariate methods on a broad range of morphological characters we were able to effectively delimit the species' boundaries. Therefore, although we do not suggest that morphological distinctiveness is an inappropriate criterion for species status, we emphasize the potential for error in basing taxonomic decisions on few morphological characters that are of unknown phylogenetic utility.

In this paper, we have shown that ecological adaptation plays an important role in the phenotypic diversification of the *Trimeresurus sumatranus* group. Most of the scalation and colour pattern characters that best account for the variation between taxa reflect some facet of the current environment, and may involve selection pressures relating to heat and water exchange, camouflage requirements and thermoregulation. Our study also shows that convergence in taxonomically important traits can lead to confusion in species identification, and is of particular relevance to taxonomically problematic groups that are widely distributed over varying environments.

CHAPTER 4

Combining molecular, morphological and ecological data to infer species boundaries in a cryptic tropical pitviper: *Trimeresurus popeiorum*

4.1 Abstract

Few operational methods exist for delimiting species boundaries, and these usually require sampling strategies that are unrealistic for widespread organisms that occur at low densities. Here we apply molecular, morphological and ecological species delimitation criteria to a wide-ranging, fragmented group of Asian green pitvipers, the *Trimeresurus popeiorum* complex. A mitochondrial DNA phylogeny for the group indicates two well-differentiated clades, corresponding mainly to northern and southern parts of its range. Strong phylogeographic structure within each clade suggests isolation in forest refugia during the Pliocene and a southward colonisation of the Sunda islands during the Pleistocene. Multivariate analysis of morphological characters reveals a generally conserved pattern of geographical variation, incongruent with the recovered phylogenetic history. We compare groups delineated by mtDNA variation to morphological and ecological divisions in the complex, and discuss the implications of these for the taxonomy of the group. Discordance between species boundaries inferred from different criteria suggests that combining independent sources of data provides the most reliable estimation of species boundaries in organisms that are difficult to sample in large numbers.

4.2 Introduction

A reliable estimate of species boundaries is of central importance to conservation management (Greene, 1997) and the large body of research that concerns this taxonomic level (Goldstein and Brower, 2002). However, there is no unified operational definition of species (Harrison, 1998). The literature is instead saturated with 'species concepts' promoting one or a combination of species criteria that reflect the diversity of events associated with the speciation process and the differing research interests of authors (de Queiroz, 1998; Hey, 2000).

Approaches to species delimitation can be broadly divided into those based on knowledge of the evolutionary process (categorised as mechanistic species concepts) and those based on historical patterns of evolution (historical species concepts) (Luckow, 1995; de Queiroz, 1998). Mechanistic species concepts emphasise species criteria that influence the future cohesion of populations (de Queiroz, 1998). Under the reproductive isolation criterion, gene exchange is reduced either at the whole genome level (Mayr, 1963; Avise and Wollenberg, 1997) or at loci of differential adaptation (Wu, 2001). The 'non-relational' recognition criterion promotes common fertilisation and shared mate recognition systems (Patterson, 1985). Cohesion species criteria include phenotypic and ecological exchangeability (Templeton, 1989).

Alternatively, historical approaches to species delimitation promote criteria that can be used to infer species status from patterns of variation (de Queiroz, 1998). The phenetic criterion (Sokal and Crovello, 1970) distinguishes species as separate clusters in multivariate morphometric analysis. Phylogenetic species criteria equate species with segments or branches of phylogenetic trees (Cracraft, 1983). Some phylogenetic concepts require strict monophyly (Donoghue, 1985; Mishler, 1985; Smith, 1994; Baum and Shaw, 1995) based on apomorphic characters (Hennig, 1966). Others emphasise diagnosability regardless of whether characters are derived (Eldredge and Cracraft, 1980); or require that diagnostic characters be fixed

combinations of character states (Nixon and Wheeler, 1990; Davies and Nixon, 1992).

Although the theoretical framework underlying species criteria has been developed extensively, very few specific operational methods have been proposed for the practical delimitation of species (summarised by Harrison [1998] and Mallet [2001]). Moreover, the statistical power necessary for their application usually requires a level of population sampling (and knowledge of ecology and reproductive biology) that is unrealistic for many taxa. Organisms that occur at naturally low densities over a wide geographic area are particularly problematic. Political constraints and recent habitat fragmentation (MacKinnon, 1997) further preclude incomplete sampling in the tropics, although a reliable taxonomy is most important for vulnerable populations in areas of high conservation interest.

The *Trimeresurus popeiorum* complex of Southeast Asian pitvipers (*sensu* Malhotra and Thorpe, in press a) provides an excellent example of the practical difficulties of delimiting species. The complex has a wide geographic distribution in Southeast Asia, comprising both transcontinental and island populations (Figure 4.1) that occur almost exclusively in undisturbed rainforests at naturally low densities. *T. popeiorum* is one of numerous, strikingly convergent, 'green pitviper' species whose taxonomic resolution and field identification have proved problematic even to professional herpetologists (for example, see Orlov *et al.* (2002) in which a *Trimeresurus* specimen from Vietnam is incorrectly identified as *T. popeiorum*). However, the recent application of molecular methods has resolved the status and relationships of many of the green *Trimeresurus* species, and in many cases has revealed previously undetected, cryptic species (Malhotra and Thorpe, 1997; Malhotra and Thorpe, 2000; Giannasi *et al.,* 2001; Malhotra and Thorpe, in press b). The most recent taxonomic revision of *T. popeiorum* (Regenass and Kramer, 1981) split the complex into three subspecies on the basis of ventral scale counts and the number of scale rows at mid-body; although the geographic range of these was largely inaccurate. *T. p. popeiorum* is recognised on the mainland and, on the basis of our identification of museum specimens, occurs in northeast India, Myanmar,

Laos, Thailand and Malaysia. *T. p. sabahi* refers to the Borneo population. *T. p. barati* is restricted to the Barisan range in west Sumatra, although specimens referable to *T. popeiorum* have also been collected from north Sumatra (Figure 4.1).

In this study, we attempt to revise the taxonomy of the *T. popeiorum* complex by combining molecular, morphological and ecological pattern-based species criteria. MtDNA sequences were used to reconstruct phylogenies, estimate divergence times and compare between-OTU frequencies of fixed nucleotide differences. Generalised morphological variation was investigated using multivariate ordination methods. Ecological data, including vegetational type and altitudinal range, was collected in the field and from museum records. We discuss concordant support for groups delineated by these data with respect to species boundaries in the complex. Finally, we use our data for *T. popeiorum* to assess the limitations of several pattern-based species criteria and their combined utility with respect to delimiting species boundaries in organisms that are difficult to sample in large numbers.

4.3 Materials and Methods

4.3.1 Sample collection

Fieldwork was carried out in Thailand, Malaysia and Indonesia. Wild-caught specimens provided blood samples and morphometric data (under anaesthesis), allowing correspondence between genetic, morphological and ecological data sets for at least one specimen in most OTUs. Blood and tissues were also obtained from museum and private collections when locality information was available and species identity could be verified. A total of 32 specimens of *T. popeiorum* were sampled from Myanmar, Thailand, Laos, peninsular Malaysia, Borneo and Sumatra (Figure 4.1, Table 4.1). Blood samples were taken from the caudal vein with a hypodermic syringe, placed in 1 ml 0.1M EDTA, and stored in SDS-Tris buffer (2% SDS, 100mM Tris); liver and muscle tissue was preserved in 80% ethanol.

4.3.2 DNA preparation, amplification and sequencing

Whole genomic DNA was extracted from blood and tissues using standard proteinase K protocols (Sambrook *et al.,* 1989). Four mitochondrial genes were amplified via polymerase chain reaction: these were cytochrome *b* (cyt*b*), NADH dehydrogenase subunit 4 (ND4), 12S small subunit ribosomal RNA (12S) and 16S large subunit ribosomal RNA (16S). Cyt*b* sequences were obtained as described in Burbrink *et al.* (2000). ND4 sequences were obtained as described in Parkinson *et al.* (2000), 12S as described in Knight and Mindell (1993), and 16S as in Parkinson *et al.* (1997). Unincorporated nucleotides and primers were removed from PCR products using QIAquick columns (QIAGen). Single stranded product was then sequenced using dye-labelled terminators (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit) and run on an ABI Prism 377 DNA automated sequencer.

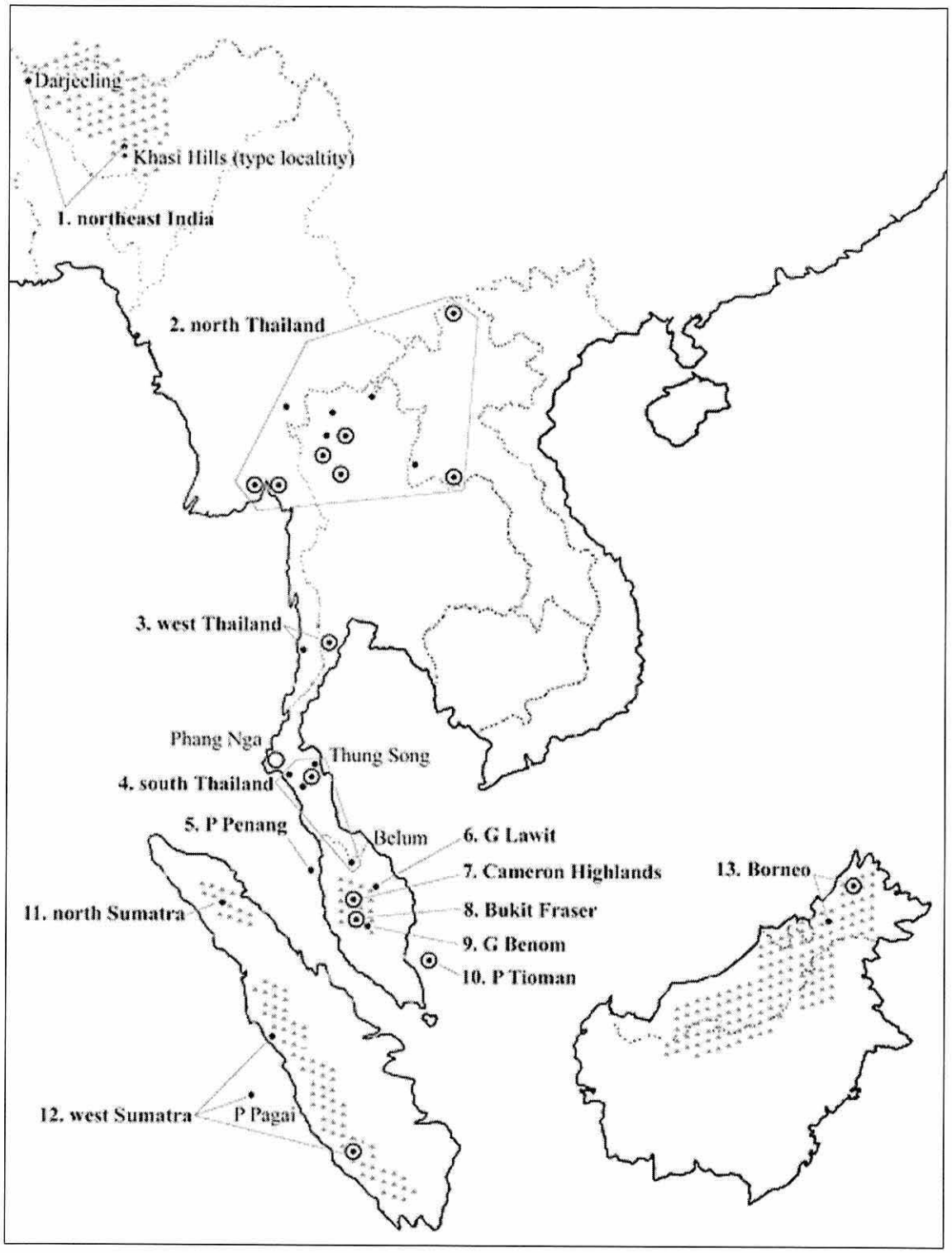

Figure 4.1: Distribution of samples included in morphological (bold dots) and molecular (open circles) analyses. Stippled triangles indicate montane habitats exceeding 1000m. Numbered localities represent OTUs that were used in multivariate morphometric analysis.

4.3.3 Sequence analyses

Outgroups were selected to represent four *Trimeresurus* species groups (*sensu* Malhotra and Thorpe, 2000). These were *T. tibetanus, T. malabaricus, T. vogeli* and *T. septentrionalis.* Alignment of cyt*b* and ND4 was trivial as there were no indels. The 12S and 16S sequences were aligned by eye following Parkinson (1999) with the exception of minor changes, which were required in one region of 12S and one region of 16S due to insertions found in some of the new sequences obtained. Coding genes were translated into amino acid sequences to check for the presence of stop codons that might indicate that pseudogenes had been amplified. The four mitochondrial genes were combined into a single data set under the rationale that they belong to a single linkage group and an increased number of genes are likely to provide a higher number of potentially informative sites for phylogenetic analysis (Chippindale and Wiens, 1994; Cummings *et al.*, 1995). PAUP* 4.0b8 (Swofford, 2003) was used to calculate skewness (g_1) statistics from 10^6 randomly generated trees to evaluate the adequacy of phylogenetic signal in the data (Hillis and Huelsenbeck, 1992).

PAUP* 4.0b10 (Swofford, 2003) was used for maximum parsimony (MP) and maximum likelihood (ML) analyses. A model of molecular evolution was first assigned to the data using the log likelihood function of MODELTEST 3.0 (Posada and Crandall, 2001). MODELTEST compares 56 different nested substitutional models and uses log likelihood scores to determine which model best fits the data. ML analysis, using a heuristic search with a starting tree obtained by 10 random additions of taxa and tree-bisection-reconnection (TBR) branch swapping, was conducted using the parameters identified by MODELTEST. The parameters were then re-estimated from the resulting tree and used in a new ML analysis. The final tree was not bootstrapped to save computational time. A maximum parsimony tree was produced using the same search options as in the ML analysis, but with a starting tree obtained by 100 random additions of taxa. All sites were equally weighted and 1000 bootstrap replicates were performed.

MrBayes v.2.01 (Huelsenbeck and Ronquist, 2001) was used to conduct Bayesian Markov Chain Monte Carlo (MCMC) phylogenetic inference, using the best-fit model indicated by MODELTEST. Substitution model parameters were estimated as part of the analysis. Three heated chains and one cold chain were initiated with a random starting tree and were run for 10^6 generations, these were sampled every 100 generations. The log-likelihood scores of sample points were plotted against generation time to determine when sample points reached stationarity, and samples prior to this point were discarded as 'burn-in' samples. The topologies of all remaining samples were used to generate a majority rule consensus tree. Three additional MCMC phylogenetic reconstructions were performed to confirm convergence of resulting tree topologies, and post burn-in trees were combined in a final majority rule consensus tree, with the percentage of samples that recovered each clade representing posterior clade probabilities (Huelsenbeck and Ronquist, 2001).

4.3.4 Sequence divergence and fixed nucleotide differences

MEGA version 2.1 (Kumar *et al.*, 2001) was used to make between-OTU pairwise sequence comparisons using the model of best-fit indicated by MODELTEST. Only ND4 and cyt*b* were used to allow comparison with the published literature and application of a molecular clock calibrated in New World pitvipers (Wüster *et al.*, 2002). Equality of substitution rates has been tested between the taxa used in the calibration of this molecular clock and the *T. popeiorum* group, and confirmed not to be significantly different (Malhotra and Thorpe, in press a).

We systematically compared fixed nucleotide differences between mtDNA lineages using the diagnostic framework of population aggregation analysis (PAA) (Davis and Nixon, 1992). Only the highly conserved rRNA genes were used (12S and 16S), as character-based delimitation requires that potentially diagnostic characters evolve relatively slowly. Under PAA, sets of populations that have fixed differences at one

or more sites are considered separate species (Davis and Nixon, 1992). However, if too few individuals are sampled the number of species may be overestimated as polymorphic traits may appear to be fixed (Walsh, 2000). This is evidently the case in our data, in which fixed differences separate all *T. popeiorum* OTUs. For this reason, we interpret our results in the context of frequencies of fixed nucleotide differences between OTUs.

4.3.5 Multivariate morphometrics

We examined 74 morphological characters relating to scalation, colour pattern and body proportions (Appendix 2.2) from 88 museum and wild-caught specimens spanning the geographic range of the complex (Appendix 2.1). Male and female specimens were treated separately in all analyses to avoid bias caused by sexual dimorphism. Specimens were grouped geographically into operational taxonomic units (OTUs) on the basis of a preliminary ordination using principal components analysis (PCA). PCA was chosen in preference to canonical variate analysis (CVA) as it summarises multivariate patterns of variation without requiring that specimens be grouped into specific taxonomic units prior to analysis, and does not require homoscedastic data (Thorpe, 1976). In all further analyses, specimens from north Thailand and proximate localities in Myanmar and Laos were grouped separately from localities in northeast India; west Thailand and an adjacent locality in Myanmar; southern Thailand and Belum (Malaysia). All remaining west Malaysian localities were treated as separate OTUs due to high levels of between-locality variation; north and west Sumatra were also treated separately and localities within Borneo were grouped. The OTUs used are illustrated in Figure 4.1 and sample sizes for each sex are listed in Table 4.1.

One-way analysis of variance and covariance (ANOVA/ANCOVA) was used to identify characters showing significant between-OTU variation. Only significant characters were included in subsequent analyses (these are highlighted in Appendix 2.5). Size-correlated characters were adjusted using a pooled within-group

regression coefficient against either snout-to-vent length or head length. PCA analyses were performed on all OTUs for each sex to investigate multivariate patterns of variation in the group.

In order to distinguish between clinal and categorical patterns of variation between parapatric mainland OTUs, these were subjected to separate PCA analyses. This involved male specimens from Thailand and northeast India, and female specimens from mainland peninsular Malaysia (the sex used was decided on the basis of highest available sample size). First principal component (PC1) scores were then plotted against the latitudinal position of OTUs.

OTU	Locality	SAMPLE SIZES			
		Morphological analysis		Molecular analysis	
		Males	Females	Males	& Females
1	northeast India (Darjeeling)	1	0	0	
	northeast India (Khasi Hills)	1	0	0	
2	north Thailand	7	4	3	
	Myanmar (central)	4	1	3	
	Laos	0	2	2	
3	west Thailand	1	2	2	
	Myanmar (south)	1	0	0	
-	south Thailand (Phang Nga)	0	0	1	
4	south Thailand (Thung Song area)	8	4	4	
	Thailand/Malaysia border (Belum)	1	0	0	
5	West Malaysia (Pulau Penang)	1	0	0	
6	West Malaysia (Gunung Lawit)	2	2	0	
7	West Malaysia (Cameron Highlands)	1	7	8	
8	West Malaysia (Bukit Fraser)	3	2	3	
9	West Malaysia (Gunung Benom)	0	1	0	
10	Pulau Tioman	2	1	1	
11	North Sumatra	2	0	0	
12	West Sumatra	9	8	1	
13	Borneo (East Malaysia)	4	6	4	

Table 4.1: OTU sample sizes for morphological and molecular analyses.

4.4 Results

4.4.1 Preliminary sequence analysis

A total of 2419 bp (cyt*b* - 809bp, ND4 - 668bp, 12S - 426bp, 16S - 516bp) was used to represent thirty-two ingroup taxa and four outgroup taxa in the final phylogenetic analysis (GenBank accession numbers are given in Appendix 1.2). These contained 638 variable sites, of which 367 (15.2% of all sites) were parsimoniously informative. There was no indication that pseudogenes had been amplified as no stop codons or indels were found in coding genes. Tree length distribution was significantly skewed to the left, indicating that there was significant structure in the data (g_1 = -0.57, p = <0.01). The substitutional model of evolution assigned by MODELTEST was Tamura-Nei distance (Tamura and Nei, 1993) with gamma correction shape parameter = 0.8448.

4.4.2 Phylogenetic relationships

Bayesian, maximum likelihood and maximum parsimony analyses of the combined data set resulted in identical tree topologies, with generally high support (Figure 4.2). The Bayesian tree had a mean log likelihood score of -7562.72. The final ML tree was obtained using the TrN model with gamma distributed rates and had a log likelihood score of –Ln 8044.23. MP analysis revealed a single most parsimonious tree with a length of 1186, a consistency index (CI) of 0.63, a retention index (RI) of 0.73 and a rescaled consistency index (RC) of 0.46.

Two strongly supported, well-differentiated clades correspond to overlapping lineages in the north and south. The northern clade contains three fairly well-supported clusters. The first comprises north Thailand/Laos, Myanmar and the Cameron Highlands. The second comprises specimens from west Thailand. The Phang Nga specimen (south Thailand) is also supported as being in this clade.

Relationships among these clusters are less well resolved. The southern clade also contains several well-supported clusters, including Bukit Fraser, Borneo and south Thailand (Thung Song area). Relationships among these, and two sequences from west Sumatra and Pulau Tioman, are less well resolved.

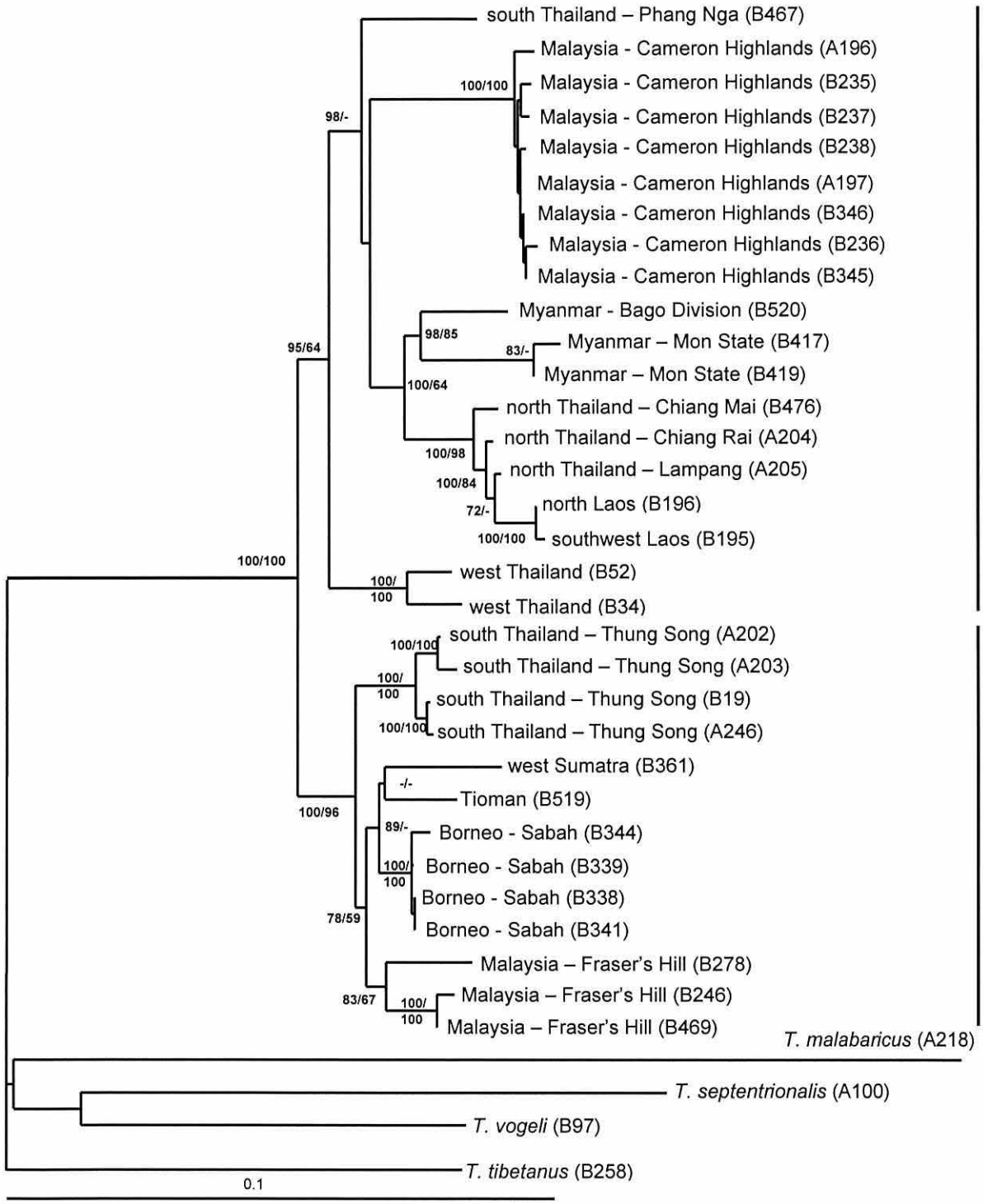

Figure 4.2: Mitochondrial gene tree derived from Bayesian Markov Chain Monte Carlo analysis of combined data from 4 genes, with posterior clade probabilities and bootstrap support values from Bayesian and maximum parsimony trees respectively.

4.4.3 Sequence divergence and fixed nucleotide differences

Levels of average sequence divergence (Tamura-Nei with gamma correction) in ND4 and cyt*b* range from 7.2% ± 1.6 to 14.8% ± 2.4 between ingroup and outgroup taxa; 3.0% ± 1.0 to 5.7% ± 1.4 (mean 4.2% ± 1.1) between northern clade OTUs, and 1.7% ± 0.8 to 2.7% ± 0.7 (mean 2.1% ± 0.8) between southern clade OTUs. Average corrected sequence difference between the northern and southern clades is 5.4% ± 1.4 (Figure 4.5).

Frequencies of between-OTU fixed nucleotide differences were higher within the north clade (mean 9.66 fixed differences) than within the south clade (mean 3.33), and more fixed differences were found between these clades (mean 12.88) than within either clade (Table 4.2). The frequency of fixed differences between parapatric lineages is comparable to that between allopatric lineages. For example, the Cameron Highlands population has a comparable number of fixed differences to the parapatric Bukit Fraser lineage as to geographically isolated populations in Thailand (Table 4.2).

	n	N. THAILAND & LAOS	MYANMAR	W. THAILAND	CAM H'LANDS	S. THAILAND	B. FRASER	BORNEO
NTHAILAND & LAOS	5	**0**	7	11	8	11	9	10
MYANMAR	2	7	**0**	11	14	15	15	17
W.THAILAND	2	11	11	**0**	11	13	12	14
CAM H'LANDS	8	8	14	11	**0**	17	11	15
S. THAILAND	4	11	15	13	17	**0**	3	5
B. FRASER	3	9	15	12	11	3	**0**	2
BORNEO	4	10	17	14	15	5	2	**0**

Table 4.2: Between-OTU frequencies of fixed nucleotide differences for 12S and 16S sequences. The mean between-OTU frequency of fixed differences is 3.33 in the southern clade (above diagonal), and 9.66 in the northern clade (below diagonal). The mean between-clade frequency of fixed differences is 12.88.

4.4 Morphometric variation

PCA plots (Figures 4.3a and b) show a clearer pattern of geographic variation in males than in females, although OTUs do not form distinct clusters in either sex. In both sexes, the specimens from the Cameron Highlands, Sumatra and Borneo are separated from all remaining OTUs on PC1 by the more anterior occurrence of several scale reductions, lower ventral, supralabial and sublabial scale counts, fewer scales between the edge of the mouth and the first ventral scale, and fewer scales between the supraoculars.

Female specimens from Bukit Fraser and Gunung Lawit (Malaysia), north Thailand, Myanmar, Laos, west Thailand, south Thailand and Pulau Tioman are undifferentiated in our multivariate analysis. However, females from north Thailand, Myanmar, Laos and west Thailand can be distinguished from south Thailand, Malaysia and Tioman females by a more distinct lateral stripe that covers at least 100% (versus <50%) of the first dorsal scale row. Females can be further separated by eye colour, which is orange in the Sumatra and Borneo populations, and yellow or green in all other OTUs.

Male specimens from Thailand, Tioman, Bukit Fraser and Gunung Lawit, and northeast India are separated on PC2 primarily by colour pattern characters (they can also be distinguished by eye colour, although this was not included in our analyses), and also by scalation (scale reduction characters and subcaudal scale counts). The presence of dorsal cross-bands along the body and tail and yellow eyes distinguish males from Thailand south of Phang Nga, Belum and Pulau Tioman. Males from west and north Thailand form a separate group on the basis of red eyes and a red lateral stripe that runs above a white stripe from eye to neck, and below a white stripe from neck to vent. A photograph of the Phang Nga specimen includes it in this group due to the presence of red eyes and a red postocular stripe. Males from Bukit Fraser and Gunung Lawit have a red (below) and white (above) lateral stripe from neck to vent, but there is no postocular stripe and the eyes are green (A. Gumprecht, pers. comm.). Cameron Highlands males are distinguished from these groups by a

complete lack of red pigmentation, a reduced white lateral stripe, and green eyes (A. Gumprecht, pers. comm.). Males from Sumatra and Borneo have a single pale orange lateral stripe from neck to vent, and orange eyes.

Plots of PC1 scores against latitudinal position for parapatric OTUs (Figures 4.4a and b) indicate categorical patterns of variation between northern and southern clade populations. Only one south Thailand specimen overlaps with the specimens from west and north Thailand and northeast India, which are weakly differentiated on PC1 despite high latitudinal separation (Figure 4.4a). The morphological discontinuity between the Cameron Highlands and parapatric Malaysian OTUs also indicates categorical variation, with no intermediates present in our analysis (Figure 4.4b).

The clade membership of specimens from localities not represented in the genetic analysis can be deduced by comparison with specimens included in both morphometric and phylogenetic analyses. This includes Malaysian OTUs Gunung Lawit, Pulau Penang and Gunung Benom. Their phenotypic similarity to Bukit Fraser specimens (Figures 4.3a and 4.4b) indicates that these are likely to belong in the southern clade. The Belum specimen clearly groups with southern clade Thailand specimens (Figure 4.3a). North Sumatra specimens show phenotypic similarity to specimens from southern clade OTUs in west Sumatra and Borneo (Figure 4.3a). Specimens from northeast India include the holotype for *T. popeiorum*, and group closely with northern clade specimens from west and north Thailand (Figure 4.3a).

The morphological divisions described here are not concordant with the subspecies diagnosed by Regenass and Kramer (1981). The mainland subspecies (*T. p. popeiorum*) is diagnosed by ventral scale counts higher than 155. However, in our study, female specimens from the Cameron Highlands have an average of 151 ventral scales. The west Sumatran subspecies (*T. p. barati*) is diagnosed by fewer dorsal scale rows at mid-body (19 versus 21). However, the presence of 21 scale rows at mid-body in a specimen from west Sumatra indicates that this is also not a valid character for diagnosing the population.

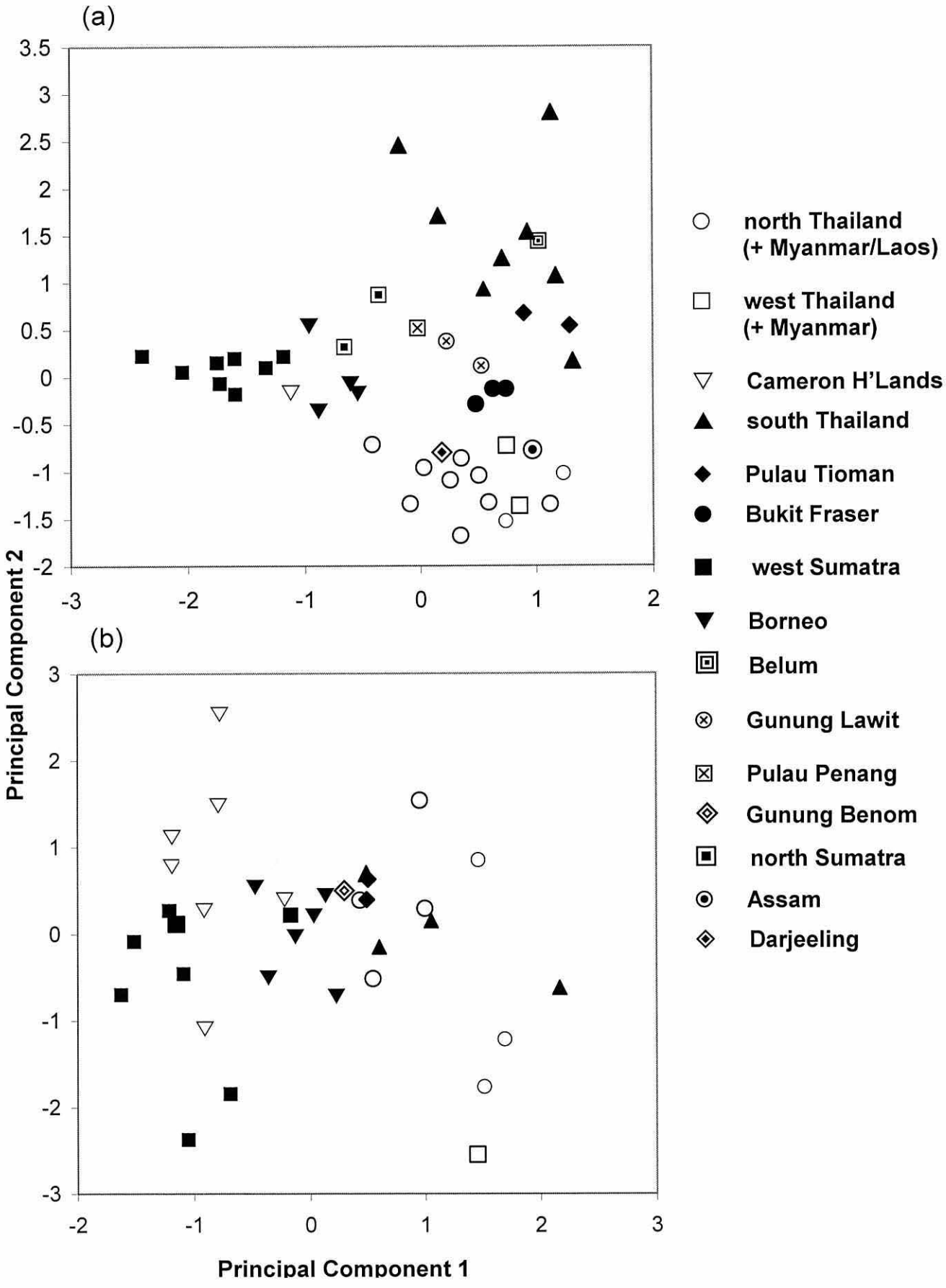

Figure 4.4: Principal component analysis of the *T. popeiorum* complex. (a) males (b) females. Northern clade OTUs are shown as open symbols, southern clade OTUs as bold symbols, and specimens of unknown clade affinity are shown as patterned symbols.

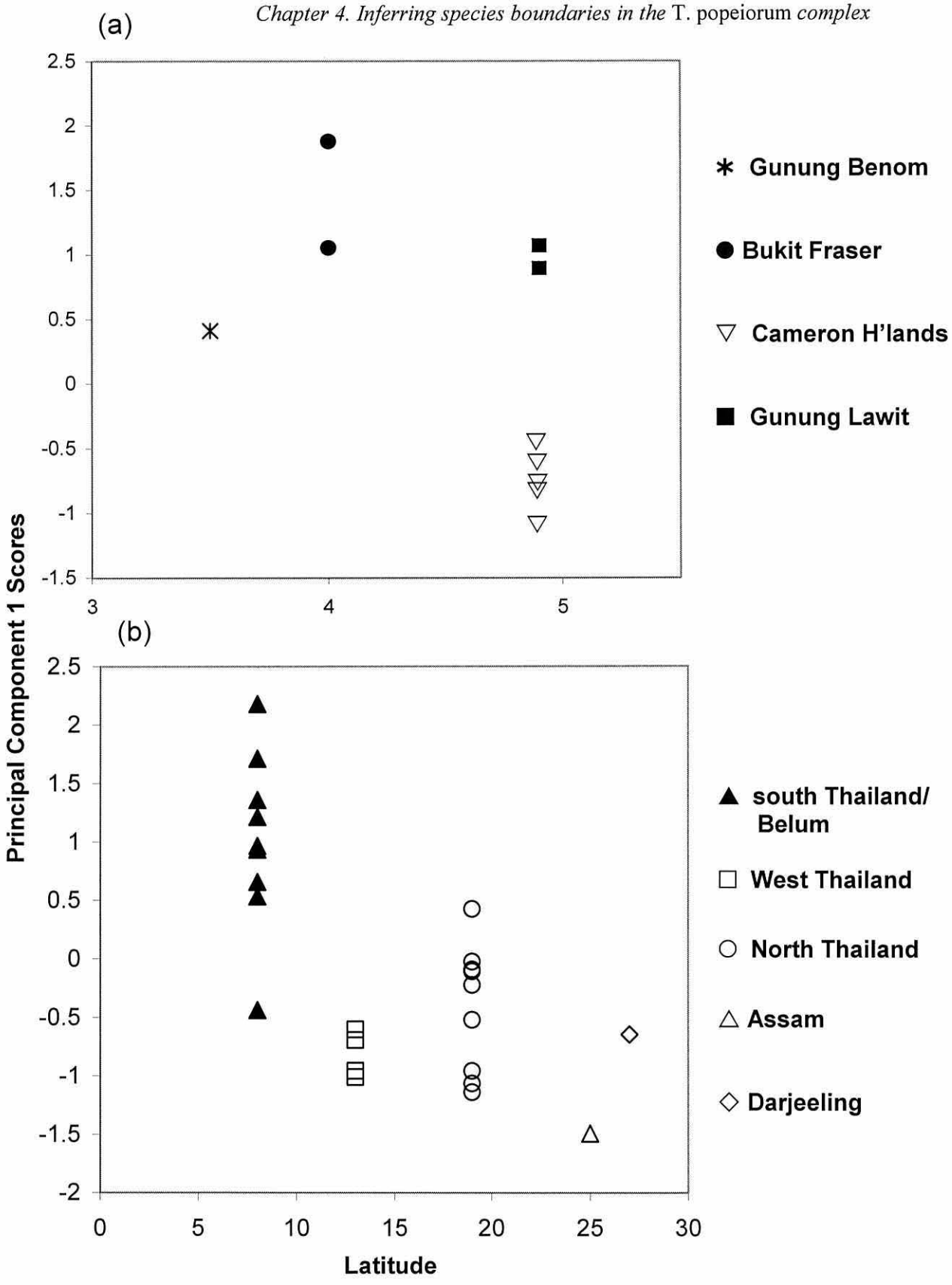

Figure 4.4: Plot of first principal component scores of individual specimens against latitude.
(a) Female specimens from parapatric OTUs in Malaysia;
(b) Male specimens from parapatric OTUs in Thailand.

4.4.5 Ecological divergence

Between-OTU differences in habitat utilisation reflect the latitudinal range of the complex. Semi-evergreen rainforest in northeast India, Myanmar, Laos and Thailand is characterised by lower rainfall (2000-3000mm/yr versus 3000-4000mm/yr) and more pronounced seasons than the equatorial wet-evergreen rainforest in Malaysia, Sumatra and Borneo (Whitmore, 1975; Anon, 1995). *T. popeiorum* populations also vary in altitudinal range. Thailand (both northern and southern lineages), Myanmar and Laos populations occur at moderate altitudes, mostly between 300m and 1000m. The collection of a specimen from Darjeeling (1500m) indicates that *T. popeiorum* may occupy higher altitudes in northeast India. The Cameron Highlands (Malaysia) population occurs between 1500m and 2000m. In Bukit Fraser, Penang and Gunung Lawit (Malaysia), Sumatra and Borneo, *T. popeiorum* occurs between 800m and 2000m, but is most commonly found over 1000m. Specimens from Tioman Island have been collected between 400m and 1050m.

4.5 Discussion

4.5.1 Evolutionary history and biogeography

T. popeiorum mtDNA haplotypes exhibit a strong geographic structure, comprising two well-supported northern and southern clades. The northern clade includes parapatric OTUs in the north that occur at moderate altitudes, and a high altitude allopatric population within the range of the southern clade. The southern clade comprises more closely related, mostly high altitude, allopatric OTUs, including the Sunda island populations. Morphological variation is generally more conserved in the northern clade than in the southern clade island populations, which is unsurprising given that both adaptive and non-adaptive differences are expected to accrue most quickly in isolated founder populations (Berry, 1998; Whittaker, 1998). Ecological convergence is indicated in high altitude populations, whose phenotypic similarity is based on lowered scale counts for characters that were found to be negatively correlated with cool, wet habitats in the related *Trimeresurus sumatranus* Indomalayan species group (Sanders *et al.*, in press). Parapatric northern and southern clade populations in Thailand and Malaysia display categorical patterns of variation despite shared habitat preferences. Figure 4.5 illustrates the geographic distribution of the main phylogenetic and morphological divisions in the complex.

Divergence times can be estimated given a calibrated estimate of the rate of sequence evolution in a comparable taxon. Wüster *et al.* (2002) proposed a rate of sequence evolution for cyt*b* and ND4 of between 1.09 and 1.77% My^{-1} in New World pitvipers. Applying these rates would date the split between the northern and southern *T. popeiorum* clades (using the mean sequence difference of 5.4% ± 1.4) at between 2.29 and 6.25 Mya, i.e. during the late Miocene and Pliocene. Most divergence events within the northern clade (mean sequence difference of 4.2% ± 1.1) are also likely to have taken place in the Pliocene, 1.75-4.94 Mya. Divergence events in the southern clade (mean sequence difference of 2.1% ± 0.8) probably occurred more recently, between 0.75 and 2.63 Mya. This indicates a southward

colonisation of the Indomalayan archipelago during the end of the Pliocene and Pleistocene.

Climatic fluctuations occurred throughout the Miocene, Pliocene and Pleistocene epochs (Morley, 1988), and have been linked to the diversification of numerous species complexes (eg. Wüster and Thorpe, 1990; Schneider *et al.*, 1998). Glacial periods were accompanied by lowered sea levels and increasing aridity and seasonality. In the Indomalayan region, this resulted in the fragmentation of forest habitats on the mainland and the formation of land bridges in the Sunda shelf (Hall and Holloway, 1998). The geographic structuring of *T. popeiorum* haplotypes is suggestive of isolation in mainland forest refugia and subsequent habitat expansion leading to secondary contact. Dispersal to the Sunda shelf is likely to have occurred via land bridges; rising sea levels would have subsequently led to the isolation of island populations, which may have followed their retreating habitats to higher elevations.

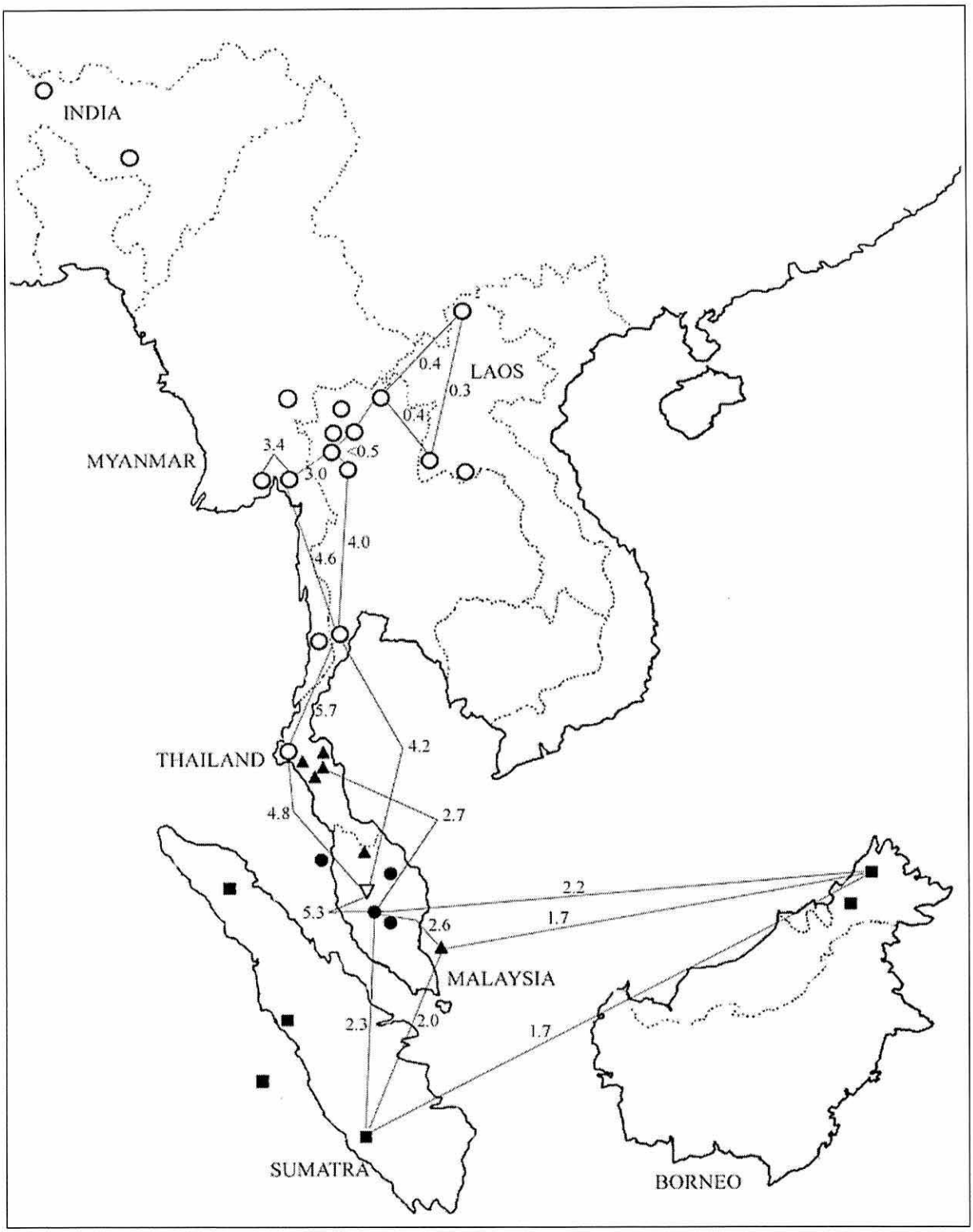

Figure 4.5: Geographic distribution of phylogenetic and morphological divisions in the *T. popeiorum* complex. Corrected pairwise sequence divergence (ND4 and cyt*b*) is shown between OTUs, which are represented by symbols indicating the main morphological groupings. Northern clade OTUs are shown as open symbols, southern clade OTUs are shown as bold symbols.

4.5.2 Delimiting species boundaries

4.5.2a Monophyly

Lineage-based species concepts recognise species on the basis of reciprocal monophyly of gene genealogies (Cracraft, 1987; Baum and Shaw, 1995; Baum and Donoghue, 1995; Donoghue, 1985; Mishler, 1985; Smith, 1994). However, there are a number of problems associated with tree-based species diagnosis. Gene trees may not always be congruent with species trees due to lineage sorting of ancestral polymorphisms (Moore, 1995). Furthermore, the matrilinear non-recombining mode of mtDNA inheritance results in a phylogenetic pattern of descent even among interbreeding organisms (Davis, 1996); so even a gene tree that accurately represents phylogenetic history is of limited value in predicting hybridization between closely related taxa (Mallet, 2001; Schluter, 2001). Paraphyly is particularly common in groups containing peripheral isolates, such as divergent island populations, and is a result of the fundamental process of speciation in these cases (Harrison, 1998).

Monophyly exists at all levels in a phylogeny, and the actual level at which species should be recognised is unclear. We could delimit two monophyletic species in the *T. popeiorum* complex, corresponding to the strongly supported northern and southern clades. Alternatively, we could delimit four species, corresponding to the two monophyletic groups in each primary clade. However, the monophyly criterion is unlikely to lead to reliable species delimitation in the *T. popeiorum* complex. Groupings based on monophyly do not correspond either to the geographic distribution of OTUs or to morphological and ecological divisions in the complex. For example, the southern clade Thailand OTU is closer to northern clade lineages, in terms of geographic and altitudinal range, than to southern clade lineages with which it shares a more recent common ancestor. Furthermore, northern clade Thailand, Myanmar, Laos and Cameron Highlands populations are monophyletic with respect to the west Thailand population. However, the west Thailand population is phenotypically and ecologically undifferentiated from parapatric north Thailand, Myanmar and Laos populations. Therefore, these OTUs are more likely to

represent an independent group with respect to the phenotypically divergent, high altitude allopatric Cameron Highlands population.

4.5.2b Sequence divergence

Mitochondrial pairwise sequence differences, ranging between 1.6-6.2%, are often used to delimit snake species (Kraus *et al.*, 1996; Zamudio and Greene, 1997; Rodriguez-Robles and De Jesús-Escobar, 2000; Ashton and de Queiroz, 2001; Keogh *et al.*, 2001; Rawlings and Donnellan, 2001). Comparable levels of divergence are found between *T. popeiorum* lineages (1.7 ± 0.8 - 5.7% ± 1.6). However, relatively ancient mtDNA haplotypes can co-exist in a single interbreeding population (Thomaz *et al.*, 1996, Ogden and Thorpe, 2002) and can mask high levels of gene flow mediated by dispersing males (Palumbi and Baker, 1994; Thorpe *et al.*, 1996; Stenson *et al.*, 2002). Consequently, mtDNA haplotype distribution alone is an insufficient criterion for recognising species, and should be combined with concordant support from at least one independent source of data. Parapatric northern clade OTUs in Thailand and Myanmar are strongly differentiated by mtDNA haplotype (average divergence = 4.14% ± 1.0), but are phenotypically indistinguishable in both sexes. Genetic exchange between these populations is especially likely given that male pitvipers are known to disperse more widely than females (Shine, 1993).

4.5.2c Fixed nucleotide differences

This diagnostic criterion predicts that differences in inherited character states will most often reach fixation in the context of reproductive isolation, and uses these as evidence for barriers to gene exchange in sympatric and parapatric populations (Davis and Nixon, 1992). In the present study, higher frequencies of fixed differences are found between northern clade lineages than between the more recently diverged southern clade lineages. There are no fewer fixed nucleotide

differences between parapatric lineages than between allopatric lineages. This could be viewed as evidence for reduced gene flow between parapatric populations. However, the extent of introgression can vary across the genome (Harrison, 1986) and gene flow is possible despite fixed differences at some loci (Harrison, 1998). Furthermore, spurious determination of fixed nucleotide differences may result from insufficient sampling within OTUs (Walsh, 2000), or the extinction of haplotypes (Templeton, 1989). Unfortunately the sample sizes currently available do not allow us to distinguish between alternative hypotheses of character fixation.

4.5.2d Phenetic species criteria

The most widely applied morphological method of delimiting species is to base species status on the presence of fixed or non-overlapping character differences between geographical samples (Wiens and Servedio, 2000). Alternatively, multivariate analysis of generalised phenotype can be used to identify groupings (phenetic clusters), which are considered species in the absence of intermediates (Sokal and Crovello, 1970; Mallet, 1995).

The lack of discrete clusters in our multivariate analyses of *T. popeiorum* morphology is incompatible with the phenetic species criterion. However, extreme morphological conservativeness is typical even between distantly related green *Trimeresurus* species (Malhotra and Thorpe, submitted). *T. popeiorum* OTUs can be grouped tentatively on the basis of scalation and colour pattern characters. These groupings correspond to first, northeast India and northern clade Thailand, Myanmar and Laos; second, southern clade Thailand, Belum and Pulau Tioman; third, Bukit Fraser, Gunung Lawit and Pulau Penang (Malaysia); fourth, Borneo, west Sumatra and north Sumatra; and fifth, the Cameron Highlands population, although this OTU partially overlaps with southern clade Sumatra and Borneo specimens in both sexes.

Potentially informative morphological differences also exist between parapatric lineages in Malaysia and Thailand. There are no known colour pattern intermediates

between northern and southern clade males in southern Thailand, and only one intermediate is present in our analysis of generalised phenotype. Morphological discontinuity between the Cameron Highlands and parapatric Malaysian lineages also indicates a categorical pattern of variation. This concordance between morphological and molecular variation in parapatric populations suggests that in Malaysia and Thailand northern clade OTUs represent independent populations with respect to southern clade OTUs.

4.5.2e Ecological species criteria

Although relatively few species concepts promote ecological criteria (Van Valen, 1976; Templeton, 1989), ecology is an important component of current speciation research. Some authors have argued that species delimitation should be treated independently from investigations of the speciation process due to a risk of circularity and compromised generalisability (Rieppel, 1986; Luckow, 1995; Goldstein and DeSalle, 2000). However, given that both sympatric and allopatric populations are more likely to speciate in the context of adaptive divergence (Endler, 1992; Marchetti, 1993; Schluter, 2001), ecological compatibility may provide a useful indication of whether two closely related populations will hybridise (Schluter, 2001; Templeton, 2001).

Overlapping altitudinal range can be used as a basis for ecological compatibility in the *T. popeiorum* group. Southern clade *T. popeiorum* OTUs share moderate and high altitudes, and overlap in altitudinal range with moderate altitude northern clade OTUs in Thailand, Myanmar and Laos. However, the exclusively high altitude Cameron Highlands population is ecologically incompatible with the remaining northern clade populations in Thailand, Myanmar and Laos, from which it is also strongly differentiated by phenotype. Shared habitat may facilitate introgression between the Cameron Highlands and parapatric Malaysian lineages. However, the morphological discontinuity between these OTUs indicates that this is improbable,

and introgression is more likely between northern clade OTUs in Thailand, given their lack of phenotypic differentiation.

4.5.3 Taxonomic recommendations

The current subspecific taxonomy of the *T. popeiorum* complex is clearly inconsistent with the molecular, morphological and ecological divisions revealed in this study. We propose a taxonomic reorganisation of the complex into three species that conservatively represent the evolutionary units delineated by our data. Northern clade OTUs in northeast India, Myanmar, Laos and Thailand are morphologically undifferentiated in females, and group closely in our analysis of males. Furthermore, there is no known biogeographic barrier to introgression of these, mostly parapatric, lineages. Therefore, despite relatively high levels of mtDNA haplotype divergence, we recommend that these populations be considered as a single species. Since it includes the holotype of *T. popeiorum*, this species corresponds to *T. popeiorum sensu stricto.* The Cameron Highlands population represents a morphologically and ecologically divergent allopatric lineage with respect to the remaining northern clade OTUs. In addition, we find no evidence of intergradation with ecologically compatible, parapatric southern clade OTUs, either in terms of mtDNA haplotype distribution or the presence of morphological intermediates. On this basis we propose full species status for the Cameron Highlands population. No previously published names apply to this species, so we propose the name *T. inornatus*. A description of this species that includes analyses of new type material is underway. The southern clade OTUs (south Thailand, Malaysia, Sumatra, Borneo and Pulau Tioman) represent recently diverged populations with compatible habitat preferences. Morphological differentiation is observed within this group, but cannot be interpreted in the context of intrinsic barriers to gene flow between these populations due to their allopatric distribution. Furthermore, intergradation with parapatric, ecologically compatible northern clade populations is unlikely in light of the categorical patterns of variation between these OTUs. Therefore, we suggest a conservative arrangement of the southern clade populations as a single, polytypic,

equatorial species. This newly defined species includes two subspecies (*T. p. barati* and *T. p. sabahi*); we propose to use the name *T. sabahi.* The geographic distributions of the *T. popeiorum* group species delimited by this study are illustrated in Figure 4.6.

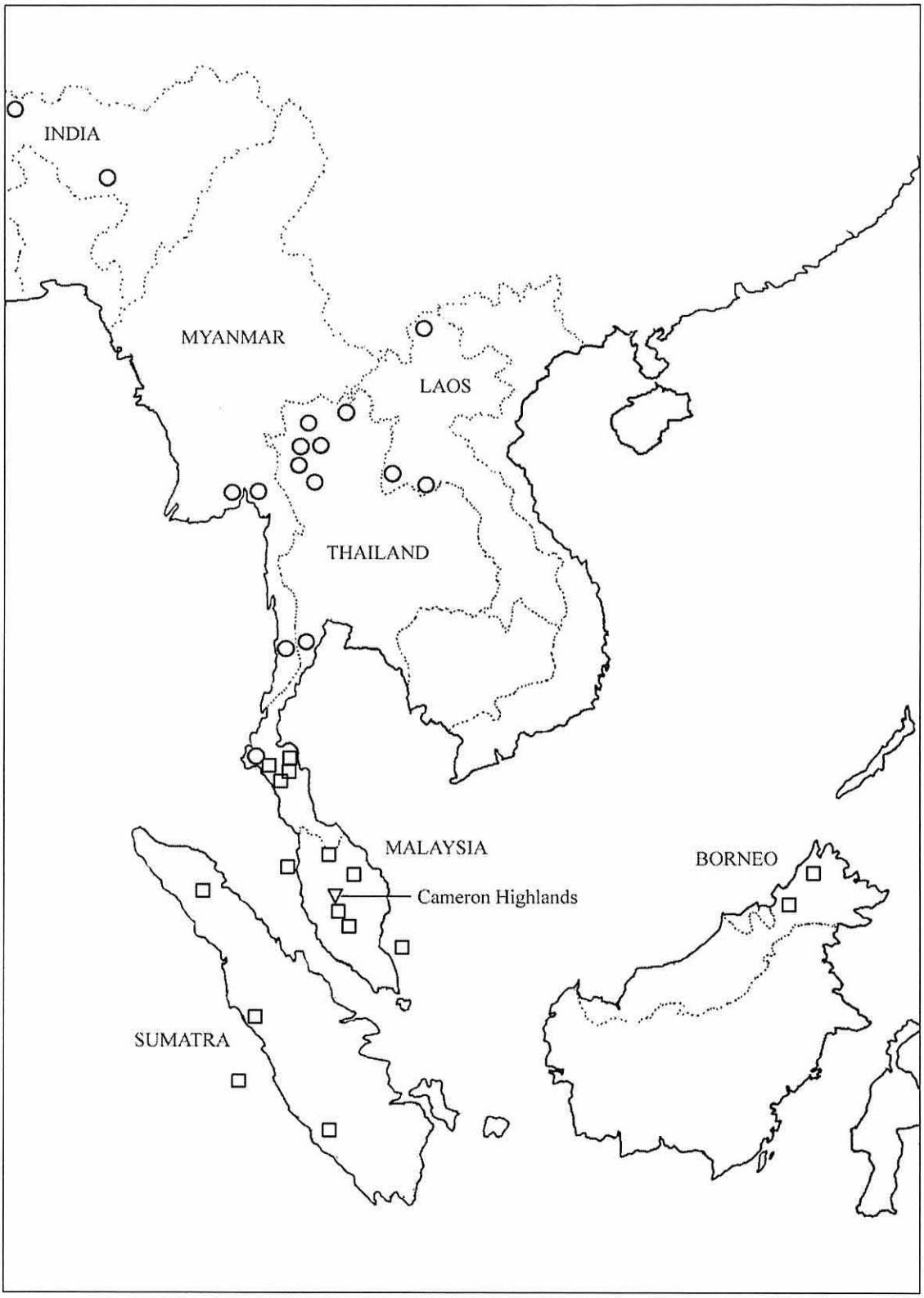

Figure 4.6: Geographic distribution of *T. popeiorum* group species delimited by this study. Circles are used to represent *T. popeiorum sensu stricto*; squares represent *T. sabahi*; and the triangle represents *T. inornatus*.

4.5.4 Implications for the species debate

Our study illustrates the discordance between species boundaries inferred from different pattern-based criteria. This underscores the dichotomy between evolutionary groups and the categories that we use to define them (Hey, 2000) and indicates that taxonomic revisions based on a single species criterion, despite advantages of comparability, are unlikely to lead to a realistic delimitation of species. Therefore, following Mishler and Donoghue (1982), Baum and Shaw (1995), Mallet (2001) and Puorto *et al.* (2001), we recommend an approach to species delimitation that combines as many independent sources of data as are available. We further emphasise the importance of critical analysis of species criteria, particularly with respect to delimiting species on the basis of data sets compromised by incomplete sampling.

CHAPTER 5

The utility of 4-nucleotide extension AFLPs in the *T. albolabris* complex

5.1 Abstract

Phylogenies derived from mitochondrial genes do not provide independent estimates of organismal phylogeny, and may represent gene trees that are incongruent with the species tree. The inclusion of additional, independent, loci from the nuclear genome can provide a more reliable estimate of evolutionary relationships. In this study, mitochondrial gene sequences are used in combination with 4-nucleotide extension AFLP markers to review the systematic relationships and biogeographic history of the *Trimeresurus albolabris* complex of Indomalayan pitvipers. Ordination and phylogenetic analysis of AFLP markers and a mitochondrial DNA phylogeny support previous taxonomic revisions and provide further resolution of systematic relationships within the complex. Phylogeographic patterns show a close relationship between clade distribution and vegetation cover. A sufficient number of polymorphic AFLP markers is generated to distinguish the main clades recovered by phylogenetic analysis of mtDNA sequences, demonstrating the utility of 4-nucleotide extension AFLPs in pitviper systematics.

5.2 Introduction

Studies of animal systematics and phylogeography often rely solely on inferences from mitochondrial DNA sequence variation (Zamudio and Greene 1997; Rodriguez-Robles and De Jesús-Escobar 2000; Ashton and de Queiroz 2001; Keogh *et al.,* 2001; Rawlings and Donnellan 2001). However, a number of limitations are associated with mtDNA due to its unique mode of inheritance, and a more reliable approach to inferring evolutionary relationships can be achieved using additional, independent, loci from the nuclear genome (Avise and Ball, 1990). Amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993; Vos, 1995) has proven to be a reliable source of nuclear markers for inferring relationships and detecting hybridisation between closely related species (Beismann, *et al.,* 1997; Lui et al., 1998; Giannasi *et al.,* 2001b; de Knijff *et al.,* 2001; Teo *et al.,* 2002; Nijman *et al.,* 2003; Semerikov *et al.,* 2003). However, due to the large numbers of fragments resolved, difficulties are sometimes experienced in finding homologous bands on AFLP gels (Tatsuta and Butlin, 2001; Young *et al.,* 2001). This can be solved using a restriction enzyme with a longer (e.g. six base pair) recognition site to reduce the number of fragments generated prior to pre-selective amplification (Hawthorn, 2001). Alternatively, longer selective primers can be used (Young *et al.,* 2001); four-nucleotide extension primers have recently been shown to produce clear bands in sufficient numbers to resolve systematic relationships at low taxonomic levels (Tatsuta and Butlin, 2001).

The present study is part of an ongoing investigation into the systematics and biogeography of the *T. albolabris* complex of Asian green pitvipers. The *T. albolabris* group *sensu* Malhotra and Thorpe (2000) is diagnosed by a fused first supralabial and nasal scale and includes *T. albolabris* (*sensu stricto*), *T. erythrurus, T. purpureomaculatus* and *T. cantori.* Five additional species are now recognised within the complex; these are *T. andersoni*, which was recently elevated from a subspecies of *T. purpureomaculatus* (Malhotra and Thorpe, 1997; Malhotra and Thorpe, 2000), *T. septentrionalis* and *T. insularis,* which were recently elevated from subspecies of *T. albolabris*; *T. labialis* and *T. fasciatus* (Malhotra and Thorpe,

2000; Giannasi *et al.,* 2001b). A distinct monophyletic clade comprised of *T. venustus, T. macrops* and *T kanburiensis* is also included in the *T. albolabris* complex (Malhotra and Thorpe, in press a), however the systematic relationships of these species have been dealt with elsewhere (Malhotra and Thorpe, in press a; Malhotra and Thorpe, in press b) and they are excluded from the present investigation. Figure 5.1 shows the geographic range of the *T. albolabris* complex included in this study.

Previous work on the systematics of *T. albolabris* (*sensu stricto*) has highlighted the presence of cryptic diversity within the species. Multivariate analysis of morphological characters showed clear patterns of geographic variation between mainland populations (Malhotra and Thorpe, 1997), and the presence of distinct clades was revealed by molecular analyses using mitochondrial sequence data (Malhotra and Thorpe, 1997; Malhotra and Thorpe, 2000; Giannasi *et al.,* 2001b) and three-nucleotide extension AFLPs (Giannasi *et al.,* 2001b). A close relationship between *T. purpureomaculatus* and *T. erythrurus* was also observed in previous molecular analyses (Malhotra and Thorpe, 1997; Malhotra and Thorpe, 2000; Giannasi *et al.,* 2001b). Smith (1943) noted a lack of differentiation in the scalation of these species, and suggested that they may represent conspecific colour morphs; however, the status and interrelationships of *T. purpureomaculatus* and *T. erythrurus* have remained unresolved. Also problematic is the distinction between *T. albolabris* and *T. erythrurus* due to apparent morphological intergradation in Myanmar and Bangladesh, where the species overlap in range (Smith, 1943).

The recent collection of *T. erythrurus, T. purpureomaculatus* and *T. albolabris* specimens from previously unsampled localities in Myanmar and Bangladesh presents a promising opportunity to resolve the long-standing confusion surrounding the relationships of these species. The current study uses mitochondrial sequence data from two gene regions, combined with AFLP markers generated using four-nucleotide extension primers, to infer the systematic relationships and biogeographic history of the *T. albolabris* complex. *T. albolabris* species are the leading cause of venomous snakebite in many parts of their range, where they are responsible for

frequent morbidity (Romer, 1963; Hutton *et al.*, 1990; Viravan *et al.*, 1992) and occasional mortality (D. Warrell, pers. comm.). The systematic resolution of the complex will therefore have important implications for the clinical management of envenomings.

5.3 Materials and Methods

5.3.1 Sample collection

Fieldwork was carried out by AM and RST between 1992 and 2000 in Thailand, Vietnam and Indonesia. Blood samples were taken from the caudal vein of wild-caught specimens with a hypodermic syringe, placed in 1ml 0.1M EDTA, and stored in SDS-Tris buffer (2% SDS, 100mM Tris); liver and muscle tissue was preserved in 80% ethanol. Blood and tissues were also obtained from museum and private collections when locality information was available and species identity could be verified. A total of 91 *T. albolabris* group specimens were sampled from across the geographic range of the complex (Table 5.1, Figure 5.1). Specimen information is presented in Appendix 1.3.

5.3.2 DNA preparation, amplification and sequencing

Whole genomic DNA was extracted from blood and tissues using standard proteinase K protocols (Sambrook *et al.* 1989). Two mitochondrial genes were amplified via polymerase chain reaction: these were cytochrome *b* (cyt*b*) and NADH dehydrogenase subunit 4 (ND4). Cyt*b* sequences (750bp) were obtained as described in Burbrink *et al.* (2000) and ND4 sequences (650bp) as described in Parkinson *et al.* (2000). Unincorporated nucleotides and primers were removed from PCR products using QIAquick columns (QIAGen). Single stranded product was then sequenced using dye-labelled terminators (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit) and run on an ABI Prism 377 DNA automated sequencer.

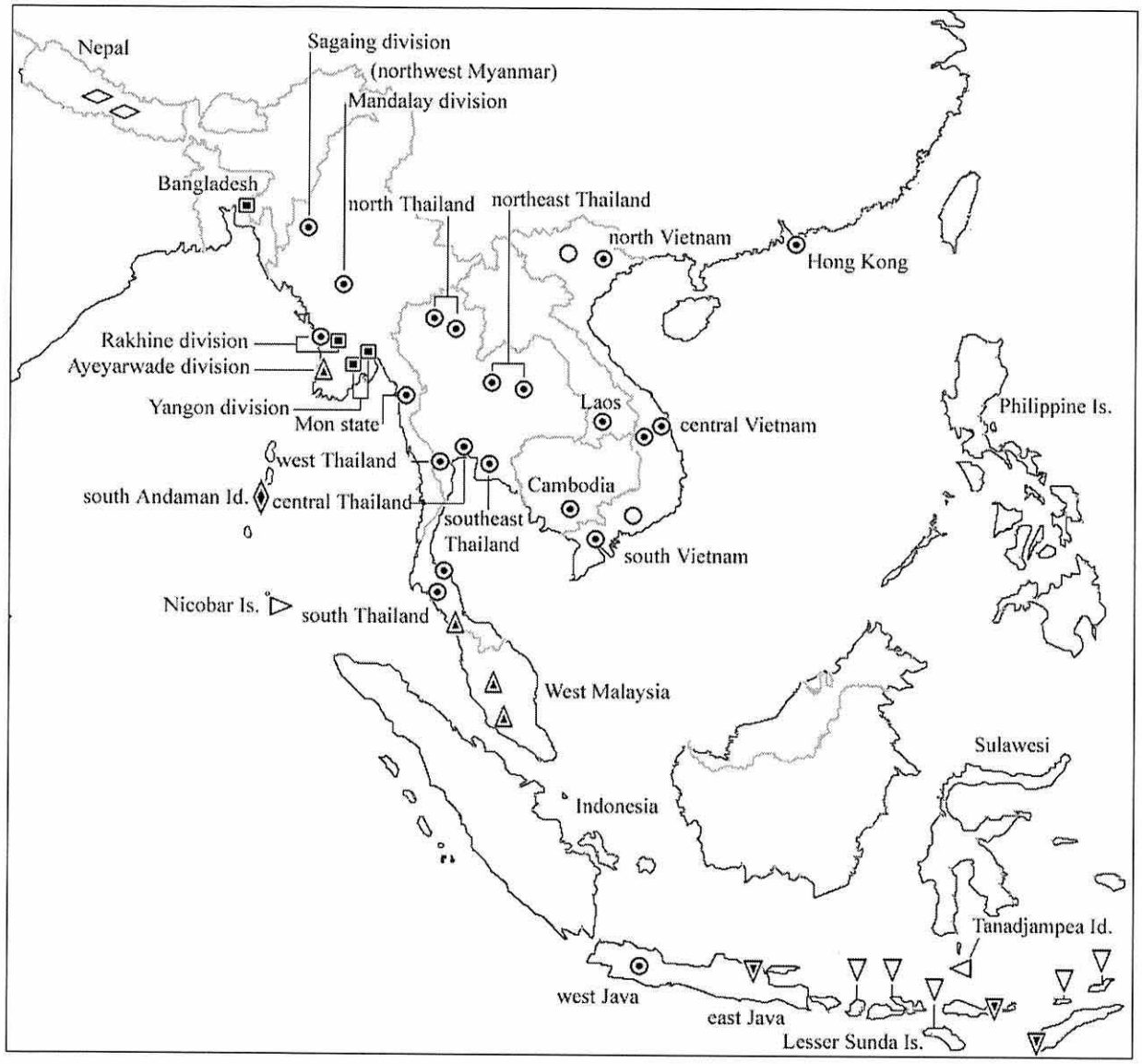

Figure 5.1: Distribution of samples included in AFLP (solid symbols) and mtDNA (open symbols) analyses. Circles = *T. albolabris* (*sensu stricto*); squares = *T. erythrurus*; triangles = *T. purpureomaculatus*; inverted triangles = *T. insularis*; right-pointing triangles = *T. cantori*; left-pointing triangles = *T. fasciatus*; horizontal diamonds = *T. septentrionalis*; vertical diamonds = *T. andersoni*.

5.3.3 Phylogenetic sequence analyses

Sequence alignment was trivial as there were no indels. Coding genes were translated into amino acid sequences to check for the occurrence of stop codons that might indicate that pseudogenes had been amplified. PAUP* 4.0b8 (Swofford 2003) was used to calculate skewness (g_1) statistics from 10^6 randomly generated trees to evaluate the adequacy of phylogenetic signal in the data (Hillis and Huelsenbeck 1992).

T. tibetanus, T. malabaricus, T. vogeli and *T. popeiorum* represent different *Trimeresurus* species groups (*sensu* Malhotra and Thorpe, 2000) and were chosen as outgroups for this study. PAUP* 4.0b8 (Swofford 2003) was used to perform a maximum parsimony (MP) analysis using a heuristic search with a starting tree obtained by 100 random additions of taxa and tree-bisection-reconnection (TBR) branch swapping. Weighting was not used to compensate for saturation of rapidly evolving positions, as several studies have shown that this is ineffective and compromises phylogenetic signal in the data (eg. Milinkovitch and Lyons-Weiler, 1998; Vidal and Lecointre, 1998). 1000 bootstrap replicates were performed.

MrBayes v.3_0b4 (Huelsenbeck and Ronquist 2001) was used to conduct Bayesian Markov Chain Monte Carlo (MCMC) phylogenetic inference, based on the best-fit model indicated by MODELTEST 3.0 (Posada and Crandall 2001). MODELTEST compares 56 different nested substitutional models and uses log likelihood scores to determine which model of molecular evolution best fits the data. Substitution model parameters were estimated as part of the analysis. Three heated chains and one cold chain were initiated with a random starting tree and were run for 500,000 generations, these were sampled every 100 generations. The log-likelihood scores of sample points were plotted against generation time to determine when sample points reached stationarity, samples prior to this point were discarded as 'burn-in' samples. Post 'burn-in' trees were combined in a final majority rule consensus tree, the percentage of samples that recovered each clade represent posterior clade probabilities (Huelsenbeck and Ronquist, 2001).

5.3.4 Sequence divergence

MEGA version 2.1 (Kumar *et al.*, 2001) was used to make between-OTU pairwise sequence comparisons. These data were then used to apply a molecular clock calibrated for cyt*b* and ND4 using divergence events in the *Porthidium* genus of New World pitvipers (1.09 - 1.77% per million years) (Wüster *et al.*, 2002). Equality of substitution rates has been tested between the *Porthidium* genus and the *T. albolabris* group, and confirmed not to be significantly different (Malhotra and Thorpe, in press b).

5.3.5 Amplified Fragment Length Polymorphism (AFLP)

AFLP analysis was performed on sixty *T. albolabris* group samples, including forty-one *T. albolabris* (*sensu stricto*), seven *T. purpureomaculatus*, four *T. erythrurus,* four *T. insularis*, two *T. andersoni* specimens and two control replicates of *T. albolabris* and *T. insularis*. *T. septentrionalis* was excluded as DNA was unavailable for this taxon at the time of analysis. Extracted whole genomic DNA was quantified on 1% agarose electrophoresis gels according to known concentration standards. 250ng of template per individual was pelleted via the standard acetate/ethanol precipitation protocol (Sambrook *et al.,* 1989).

The AFLP assay was generally performed as described by Vos *et al.* (1995) with some modifications. Restriction-ligation products were diluted with 94.5µl of 0.1 x TE prior to pre-selective amplification with *Mse*I and *Eco*RI preselective primers. AFLPs were generated using six 4-nucleotide extension *Eco*RI (fluorescent 5' end-labelled) and *Mse*I selective primer pairs. These are shown in Table 5.3. Selective amplification products (2.5µl FAM, 3.5µl JOE and 4.5µl TAMRA fluorescent end-labelled, to optimise visualisation of each colour) were pelleted and resuspended in 2.25µl of loading buffer (60% formamide, 20%, blue dextran, 20% size marker (PE Applied Biosystems GS500 ROX)). Product was then denatured at 95°C for 2.5 min

and snap chilled on ice. 1.5µl of each was electrophoresed through a 5% polyacrylamide gel on an ABI377 DNA sequencer.

5.3.6 AFLP data analysis

Chromatograms were analysed using Genotyper®. The presence or absence of AFLP markers over 100 rescaled peak height was scored between 100 and 450 base pairs. Categories of polymorphic bands were made from labels with 2 base pair tolerance. The presence or absence of bands within these categories was checked by eye and coded as 1/0 for presence/absence. Data from the 6 primer combinations were combined in a single matrix and subjected to ordination and phylogenetic analysis. The multivariate statistics package MVSP version 3.12d (Kovach, 1999) was first used to perform principal coordinate (PCO) analysis on a dissimilarity matrix generated using Gower's general similarity coefficient (Ogden and Thorpe, 2002). Gower's general similarity coefficient is equivalent to Jaccard's coefficient (Sneath and Sokal, 1973), which omits shared absences from consideration due to reduced confidence that these represent homologous regions in the genome (Parsons and Shaw, 2001). The resulting principal coordinate scores were then plotted to visualise patterns of nuclear genetic distance between *T. albolabris* group populations. Second, a neighbour joining analysis was performed on the raw data matrix using PAUP* 4.0b8 (Swofford 2003); the reliability of the resulting phylogeny was tested by 1000 bootstrap replicates.

5.3.7 Contouring

AFLP data for *T. albolabris* (*sensu stricto*) was subjected to a separate PCO analysis to maximise discrimination between populations. First principal coordinate scores were then contoured onto a map of the Indomalayan mainland using UNIRAS UNIMAP 2000 (European Software Contractors A/S) to visualise the geographic pattern of inter-population genetic distance.

Species	Locality	Sample Size	
		mtDNA	AFLP
T. albolabris	W. Java	3	2
	S. Thailand	5	3
	W. Thailand	4	3
	S.E. Thailand	2	2
	C. Thailand	5	4
	N. Thailand	3	4
	N.E. Thailand	7	5
	S. Vietnam	5	3
	C. Vietnam	2	2
	N. Vietnam	4	2
	S. Laos	2	1
	Hongkong	1	1
	E. Cambodia	1	0
	W. Cambodia	1	1
	Mon State (Myanmar)	1	1
	Rakhine State (Myanmar)	1	0
	N.W. Myanmar	7	8
T. erythrurus	Yangon div (Myanmar)	3	2
	Rakhine State (Myanmar)	3	1
	Bangladesh	2	2
T. purpureomaculatus	S. Thailand	1	1
	W. Malaysia	4	4
	Ayeyarwade div (Myanmar)	1	1
T. andersoni	Andaman Islands (India)	3	2
T. septentrionalis	Nepal	3	0
T. insularis	E. Java	6	2
	W. Timor	2	1
	Flores	4	1
	Sumbawa (Nusa Tenggara)	1	0
	Semau (Nusa Tenggara)	1	0
	Alor (Nusa Tenggara))	1	0
	Wetar (Nusa Tenggara)	1	0
	Komodo (Nusa Tenggara)	1	0
	Bali (Nusa Tenggara)	1	0
T. fasciatus	Tanadjampae Island (Sulawesi)	1	0
T. cantori	Nicobar Islands (India)	1	0

Table 5.1: Sample sizes for localities included in mtDNA and AFLP analyses.

5.4 Results

5.4.1 Preliminary sequence analysis

The final analysis included 1506 base pairs (cyt*b* - 808bp, ND4 - 698bp) from 91 ingroup taxa and 4 outgroup taxa. There was no indication that pseudogenes had been amplified as no stop codons were found and base frequencies conformed to the pattern expected of mtDNA genes. The data contained 553 variable sites, of which 387 (25.7% of all sites) were parsimoniously informative. Tree length distribution was significantly skewed to the left, indicating that there was significant structure in the data ($g1 = 0.57$, $p = <0.01$).

5.4.2 Phylogenetic analyses

Bayesian and maximum parsimony analyses of the combined data set resulted in largely congruent tree topologies, with generally higher support for the Bayesian tree (Figure 5.2). The Bayesian tree had a mean log likelihood score of -7538.39. MP analysis revealed a single most parsimonious tree with a length of 1304, a consistency index (CI) of 0.52, a retention index (RI) of 0.82 and a rescaled consistency index (RC) of 0.43.

Sister taxa *T. insularis* and *T. fasciatus* form the first branching point (Clade A) (Figure 5.2). The next node separates a monophyletic cluster comprising *T. albolabris* from northwest Myanamar and *T. septenrionalis* from Nepal (Clade B). The remaining taxa form two main clades. In one of these (Clade C), *T. cantori* (Nicobar islands) is basal and followed by a group of *T. andersoni* sequences (south Andaman island). The remaining two clusters, which could be defined as a variable number of subgroups, contain a number of examples of paraphyletic taxa. A '*T. albolabris*' specimen groups with several *T. erythrurus* from Rakhine State (Myanmar) and Chittagong (Bangladesh). *T. erythrurus* specimens from Yangon division are sister to a *T. purpureomaculatus* from nearby Ayeyarwade division

(Myanmar), and are nested within a larger clade of *T. purpureomaculatus* from the remainder of its range in south Thailand and West Malaysia. The remaining main clade (Clade D) includes only *T. albolabris* specimens. However, there is considerable substructure and general geographic discordance between haplotype clusters. The first branching point separates a cluster of sequences from Mon State (Myanmar), northwest Thailand and northeast Thailand, and is followed by a group of sequences from south and west Thailand. The next group contains four clusters: southeast Thailand, Cambodia, south Vietnam and a single specimen from northeast Thailand; south and central Vietnam, Cambodia, west Java and a specimen from south Thailand; Laos, northeast Thailand, central Thailand and a specimen from northwest Thailand; and finally, north Vietnam and a specimen from Hong Kong.

5.4.3 Sequence divergence

Sequence comparisons were made using the Kimura 2-parameter model (Kimura, 1980) with gamma correction, as this is the closest model available in MEGA to that indicated by MODELTEST (HKY (Hasegawa *et al.*, 1985)). Levels of mean sequence divergence are 14.1% ± 1.9 between ingroup and outgroup taxa, 3.0% ± 1.7 between *T. albolabris* (*sensu stricto*) clusters (Figure 5.4), and 1.8% ± 0.7 between the paraphyletic clusters corresponding to Rakhine State (Myanmar) and Chittagong (Bangladesh), and Yangon - Ayeyarwade divisions (Myanmar). Mean levels of sequence divergence (and their standard deviations) between the nominal species and primary clades within the group are shown in Table 5.2.

Applying the rates calibrated by Wüster *et al.*, (2002) (1.09 - 1.77% per million years), and considering standard errors of sequence divergence estimates, would date the divergence of clades A, B, C and D between 1.7 and 8.34 Mya, i.e. during the late Miocene and throughout the Pliocene. A relatively deep divergence event may also have occurred between northwest Myanamar *T. albolabris* and *T. septenrionalis*, between 3.28 and 7.7 Mya, during the late Miocene or early Pliocene. Divergence of *T. insularis* and *T. fasciatus* appears to have occurred during the Pliocene, between 1.53 and 5.04 Mya. *T. andersoni* and *T. cantori* are likely to have shared a more recent ancestor, probably in the Pleistocene or late Pliocene, between 0.73 and 2.84 Mya. The clusters corresponding to Rakhine State (Myanmar) and Chittagong (Bangladesh), and Yangon - Ayeyarwade divisions (Myanmar) are also likely to have diverged in the Pleistocene or late Pliocene, between 0.63 and 2.29 Mya. Most divergence events in the *T. albolabris* (*sensu stricto*) clade are likely to have taken place between 0.73 and 4.31 Mya, during the Pleistocene and Pliocene.

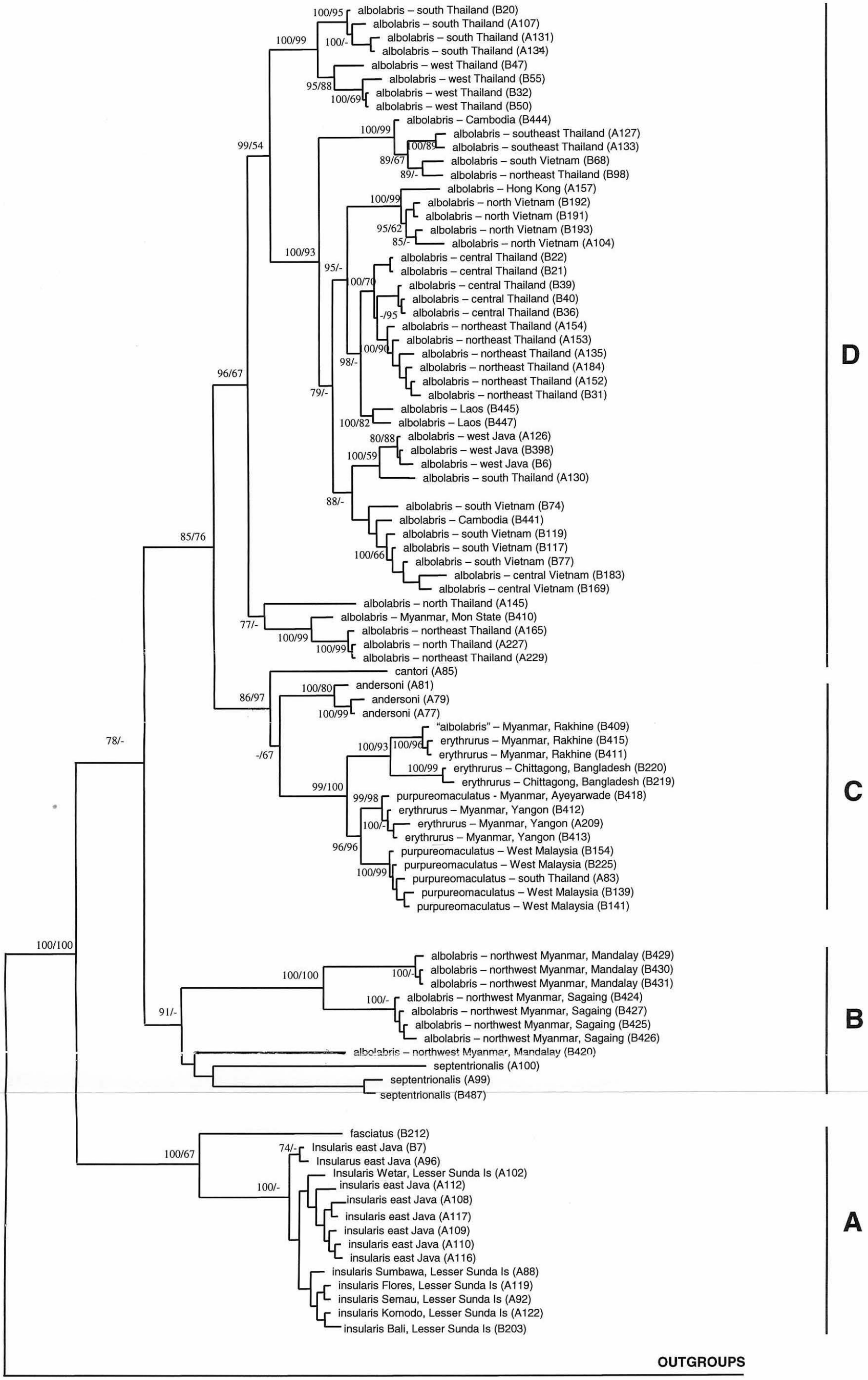

Figure 5.2: Mitochondrial gene tree derived from Bayesian Markov Chain Monte Carlo analysis of combined data from 2 genes, with posterior clade probabilities and bootstrap support values from Bayesian and maximum parsimony trees, respectively.

	Clade A	Clade B	Clade C	Clade D	1	2	3	4	5	6	7	8
Clade A	0											
Clade B	7.3 ± 1.5	0										
Clade C	7.3 ± 1.8	5.9 ± 1.2	0									
Clade D	6.6 ± 1.6	5.3 ± 1.0	4.0 ± 1.0	0								
1 - *albolabris*					0							
2 - *albolabris* (NW Myanmar)					4.7 ± 1.0	0						
3 - *andersoni*					3.7 ± 1.1	5.5 ± 1.3	0					
4 - *cantori*					4.1 ± 1.2	6.0 ± 1.4	2.2 ± 0.9	0				
5 - *erythrurus & purpureomaculatus*					3.7 ± 1.0	5.5 ± 1.3	1.9 ± 0.7	2.4 ± 0.9	0			
6 - *fasciatus*					6.9 ± 1.6	8.4 ± 1.8	6.0 ± 1.5	7.1 ± 1.7	6.9 ± 1.7	0		
7 - *insularis*					6.6 ± 1.6	7.5 ± 1.7	7.1 ± 1.8	7.1 ± 1.8	7.1 ± 1.7	4.1 ± 1.4	0	
8 - *septentrionalis*					6.7 ± 1.3	7.1 ± 1.3	7.2 ± 1.6	6.8 ± 1.5	6.4 ± 1.3	6.8 ± 1.5	6.8 ± 1.5	0

Table 5.2: Mean sequence divergence (Kimura 2-parameter with gamma correction) between primary clades and species.

5.4.4 AFLPs

The six primer pairs generated an average of 28.5 polymorphic bands per primer pair, resulting in a total of 171 markers for 60 individuals including 2 replicates (Table 5.3). A three-dimensional graph was plotted due to the low eigenvalues of principal coordinates (Figure 5.3). First, second and third principal coordinates accounted for 21.8%, 8.7% and 6.5% of the total variation, respectively. The replicate samples of *T. albolabris* can be seen as neighbouring data points, and *T. insularis* as overlapping data points, providing confidence in the data. Separate clusters correspond to *T. albolabris* (*sensu stricto*) from west Java, south, west, central and southeast Thailand, and all localities in north and northeast Thailand, Hong Kong, Laos, Myanmar, Vietnam and Cambodia. Relationships within these clusters are unresolved, with the exception of specimens from northwest Myanmar, which are partially separated from the northern cluster. Two *T. andersoni* specimens are seen to overlap inside a cluster of *T. purpureomaculatus* and *T. erythrurus* specimens from Myanmar and Bangladesh, although relationships within this cluster do not correspond to locality or species groupings. *T. insularis* specimens also form a discrete group.

Neighbour joining analysis of the AFLP data resulted in a poorly resolved phylogeny (Figure 5.5). However, two clades corresponding to the mitochondrial phylogeny were recovered; these are the clade containing *T. insularis* and *T. fasciatus* (A) and the clade containing *T. erythrurus, T. purpureomaculatus, T. andersoni* and *T. cantori* (C). Relationships within these clades were unsupported.

The principal coordinate analysis that included only *T. albolabris* (*sensu stricto*) did not markedly improve discrimination between OTUs and is not shown. The contour map of first principal coordinate scores for *T. albolabris* (*sensu stricto*) illustrates the division between southern and northern localities on the mainland, despite generally equivalent levels of sequence divergence within and between these groups (Figure 5.4). The separation of divergent mtDNA haplotypes in northwest Myanmar

and proximate localities in Myanmar and Thailand is not illustrated on the contour plot as their separation is primarily accounted for on principal coordinate three.

Primer Pair	*Eco*RI +	*Mse*I +	No. polymorphic bands
1	ACCG (FAM)	CTAG	36
2	AGCC (JOE)	CTAG	25
3	AGGG (TAMRA)	CTAG	30
4	ACAG (FAM)	CACC	26
5	AGCC (JOE)	CTGG	28
6	AGGG (TAMRA)	CTGG	26

Table 5.3: *Eco*RI and *Mse*I 4-nucleotide extension primer pairs used in AFLP analysis, and number of polymorphic bands generated by each. Fluorescent end-labels are FAM (blue), JOE (green) and TAMRA (yellow).

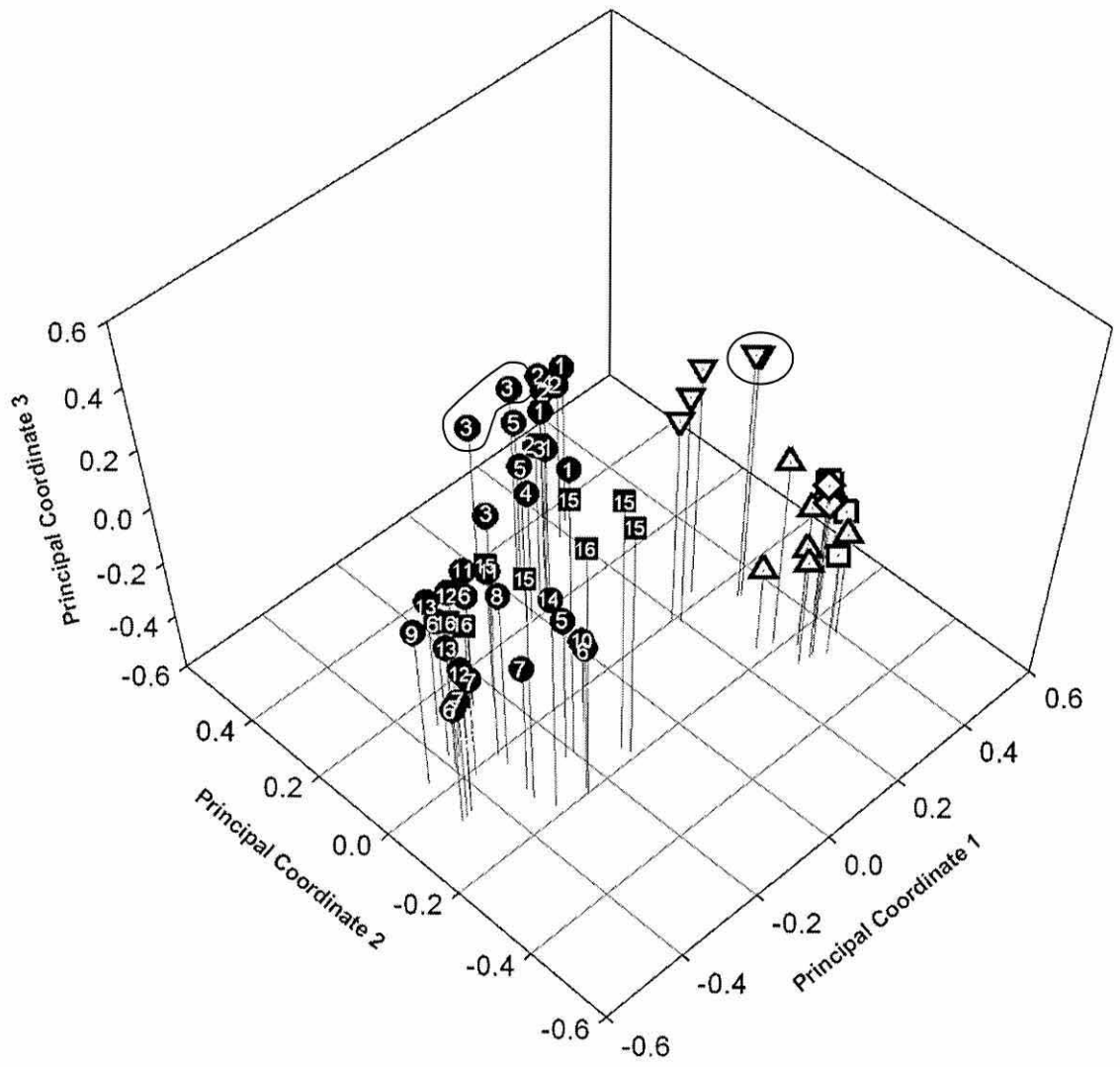

Figure 5.3: Plot of first three principal coordinates from AFLP analysis. Symbols correspond to species groupings. Squares = *T. erythrurus*; triangles = *T. purpureomaculatus*; inverted triangles = *T. insularis*; diamonds = *T. andersoni*; numbered circles = *T. albolabris* Clade D (1 = S Thailand; 2 = W Thailand; 3 = C Thailand; 4 = SE Thailand; 5 = W Java; 6 = N Thailand; 7 = NE Thailand; 8 = Hong Kong; 9 = Cambodia; 10 = Mon State (Myanmar); 11 = S Vietnam; 12 = C Vietnam; 13 = N Vietnam; 14 = Laos; numbered squares = *T. albolabris* Clade B (15 = Sagaing division (northwest Myanmar); 16 = Mandalay division (northwest Myanmar)).Replicate samples are circled.

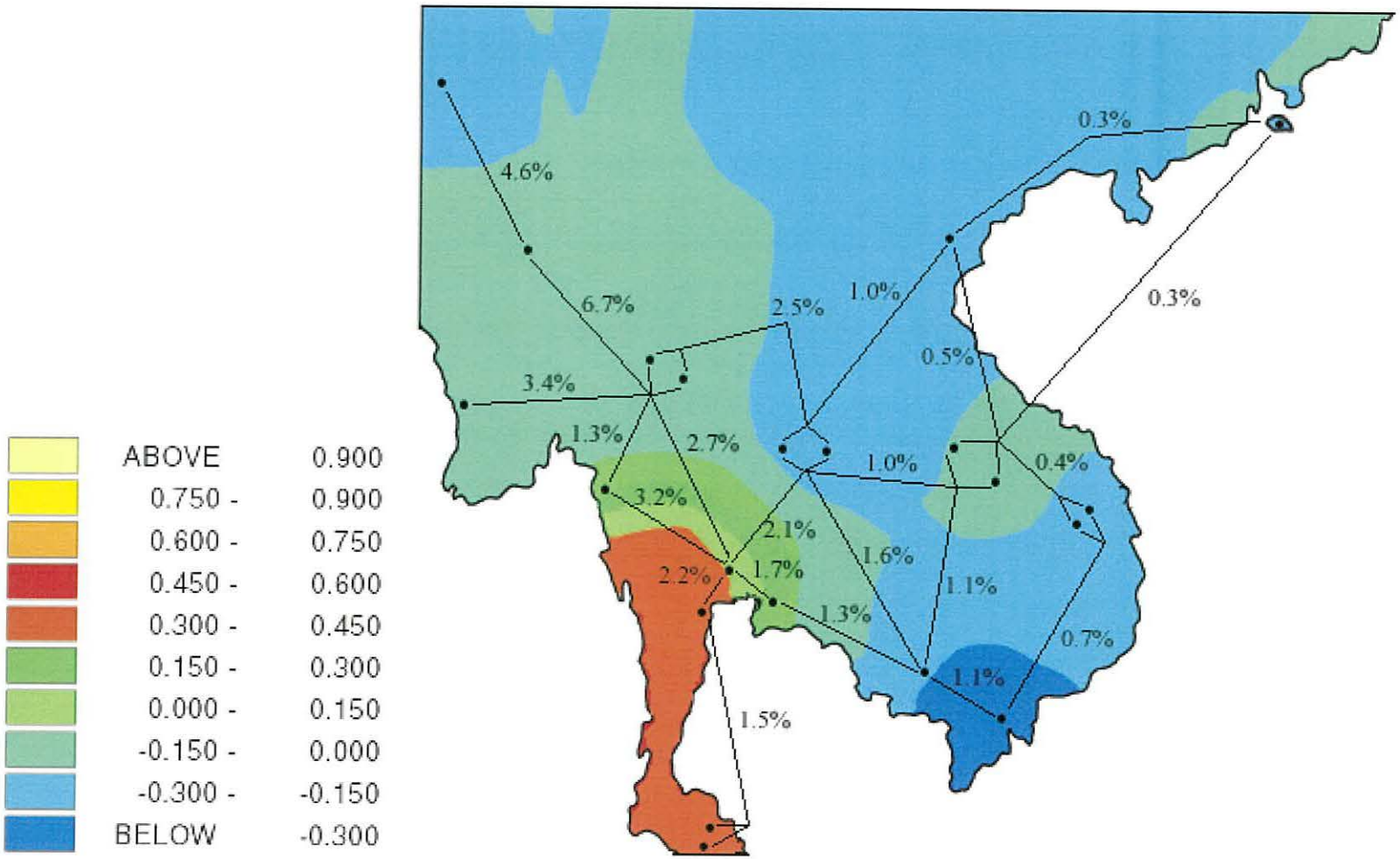

Figure 5.4: First principal coordinate scores of AFLP analysis of *T. albolabris* (*sensu stricto*) contoured onto a map of the Indomalayan mainland. Mean corrected pairwise mtDNA sequence divergence (Kimura 2-P + G) is also shown between locality groupings for comparison.

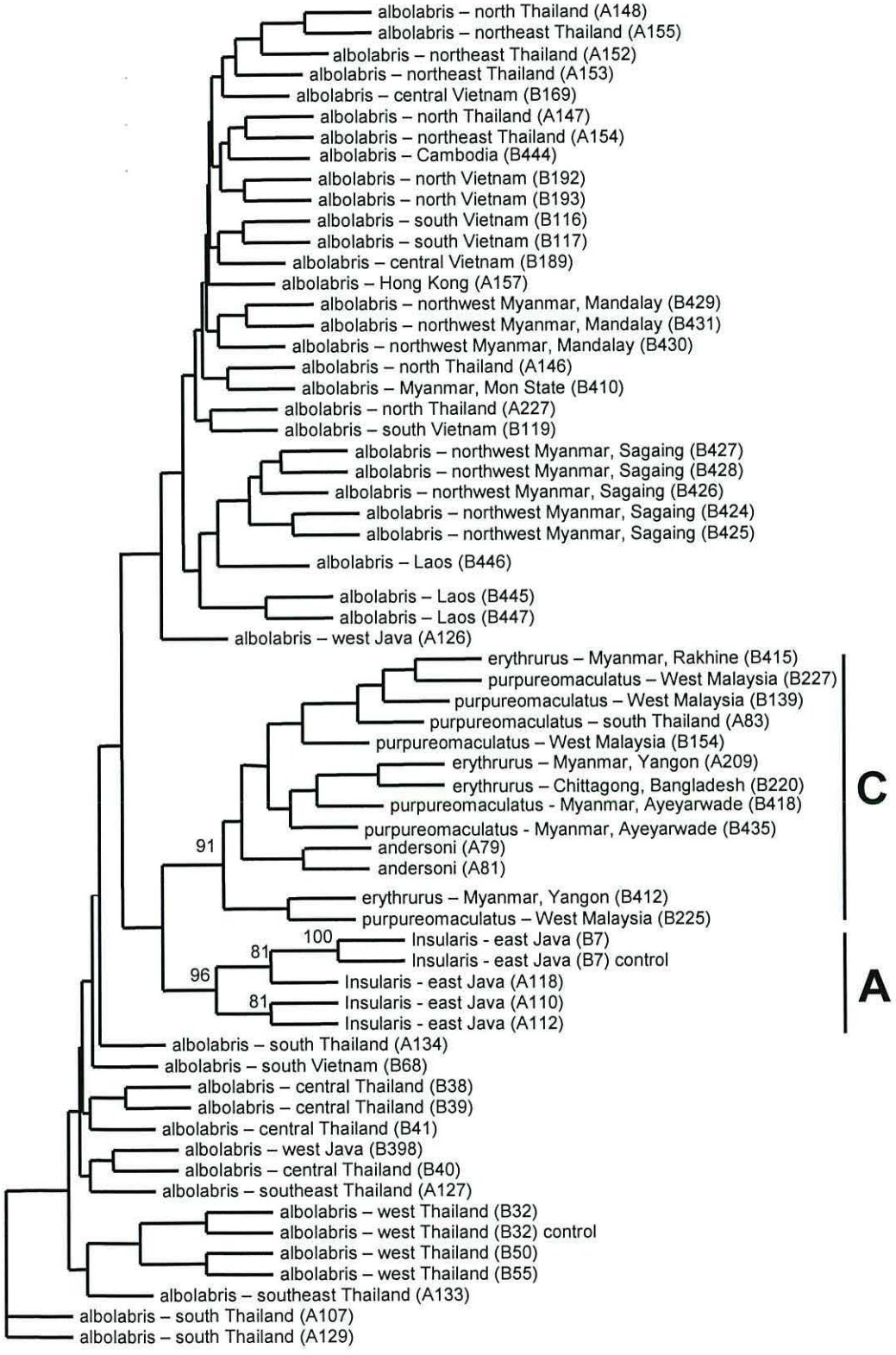

Figure 5.5: Neighbour joining tree derived from AFLP markers.

5.5 Discussion

5.5.1 Biogeography

T. albolabris complex mtDNA haplotypes exhibit a clear geographic structure, comprising four well-supported primary clades that we estimate diverged during the Miocene and Pliocene epochs. Clade D, *T. albolabris sensu stricto*, can be further subdivided into a number of smaller clades, but these are grouped due to the generally low levels of sequence divergence and lack of geographic exclusivity between them. The present geographic distribution of the four clades is closely linked to patterns of habitat distribution (illustrated in Figure 5.5). Clade A contains *T. insularis* and *T. fasciatus*, which occupy seasonal forests in eastern Java and the Lesser Sunda islands. Dispersal via land bridges has been implicated in the disjunct distribution of these species from most of the remainder of the complex (Gianassi *et al.*, 2001). Low levels of sequence divergence within *T. insularis* (0.4% ± 0.3) indicate recent dispersal into the Lesser Sunda Islands. This presumably occurred over water, as the Lesser Sundas have been continually isolated from neighbouring islands Java and Sulawesi (Whitmore, 1998).

Clade B comprises *T. septentrionalis* from Nepal and *T. albolabris* from northwest Myanmar. These specimens were collected over 700m in montane rainforest habitats. Clade C species (*T. erythrurus*, *T. purpureomaculatus, T. andersoni* and *T. cantori*) occur in lowland forests and mangroves in southern Thailand, West Malaysia, Myanmar, Bangladesh and the Andaman and Nicobar islands. Southern Thailand and West Malaysia share a mostly equatorial climate; the Myanmar - Bangladesh coast receives regular rainfall from the Indian Ocean and supports evergreen rainforests. *T. purpureomaculatus* also occurs on the Thai peninsula, but is restricted to the west coast (Smith 1943), which is less seasonal and more densely vegetated than the east coast (Buttle & Tuttle Ltd, 2001; Quikcast, 2002). Successful colonisation of the evergreen

Andaman coast islands, and absence from the seasonal east coast islands, provides further evidence of Clade C's preference for aseasonal habitats. Clade D, *T. albolabris sensu stricto*, shows a disjunct distribution that has been linked to dispersal via land bridges (Gianassi *et al.*, 2001) and is supported by a similar distribution in another pitviper, *Calloselasma rhodostoma* (Daltry, *et al.,* 1996). *T. albolabris* has successfully colonised a diverse range of habitats over a wide geographic area. However, the absence of the species in equatorial areas indicates a preference for seasonal habitats and possibly competition with species better adapted to aseasonal environments (see Briggs, 1987).

Clade B populations represent a monophyletic group whose divergence from the remainder of the complex may be associated with the isolation of wet forests at high elevations during glacial periods. Such vicariance events have been implicated in the strong phylogeographic differentiation uncovered in other montane species groups on the Indomalayan mainland, including *T. popeiorum* (Sanders *et al.*, submitted) and *T. stejnegeri* (Malhotra and Thorpe, submitted). Clade C is geographically separated from sister Clade D by a transition between predominantly evergreen and seasonal habitat types. This phylogeographic pattern is consistent with a parapatric mode of divergence (Brookes and McLennan, 1991; Patton and da Silva, 1998), although isolation in glacial forest refugia may also have contributed to the observed differentiation.

The low levels of sequence difference and lack of geographic exclusivity between haplotype clusters within Clade D indicate introgression between neighbouring populations during the Pleistocene and Pliocene epochs. Similarly, wide geographic distributions and low levels of mtDNA haplotype divergence (<3%) have been observed in other pitviper species, *Crotalus durissus* from central and south America (W. Wüster pers. comm.) and *Calloselasma rhodostoma* from the Indomalayan mainland (J. Daltry, Ph.D. Thesis). These species share a preference for semi-evergreen and deciduous forests, which during glacial periods may have facilitated their dispersal and made them less susceptible to population fragmentation following the contraction

of wet forest habitats and the expansion of seasonal forests. However, our AFLP data indicates cryptic genetic differentiation within Clade D between northern populations in north and northeast Thailand, central Myanmar, Laos, Vietnam, Hong Kong and Cambodia, and southern populations in south, west, central and southeast Thailand and west Java. This could be explained by a present-day range disjunction; however, no known biogeographic barrier exists between central and northern Thailand. Alternatively, the northern and southern clusters may have been historically separated by unsuitable habitat during the Pleistocene glaciations. The centre of the Indomalayan landmass is thought to have been the most arid part of the region during glacial maxima, supporting predominantly savanna or scrub vegetation (Adams, 2001). In this case, the retention of pre-existing genetic variation due to comparatively recent secondary contact would explain the differentiation of northern and southern *T. albolabris* clusters.

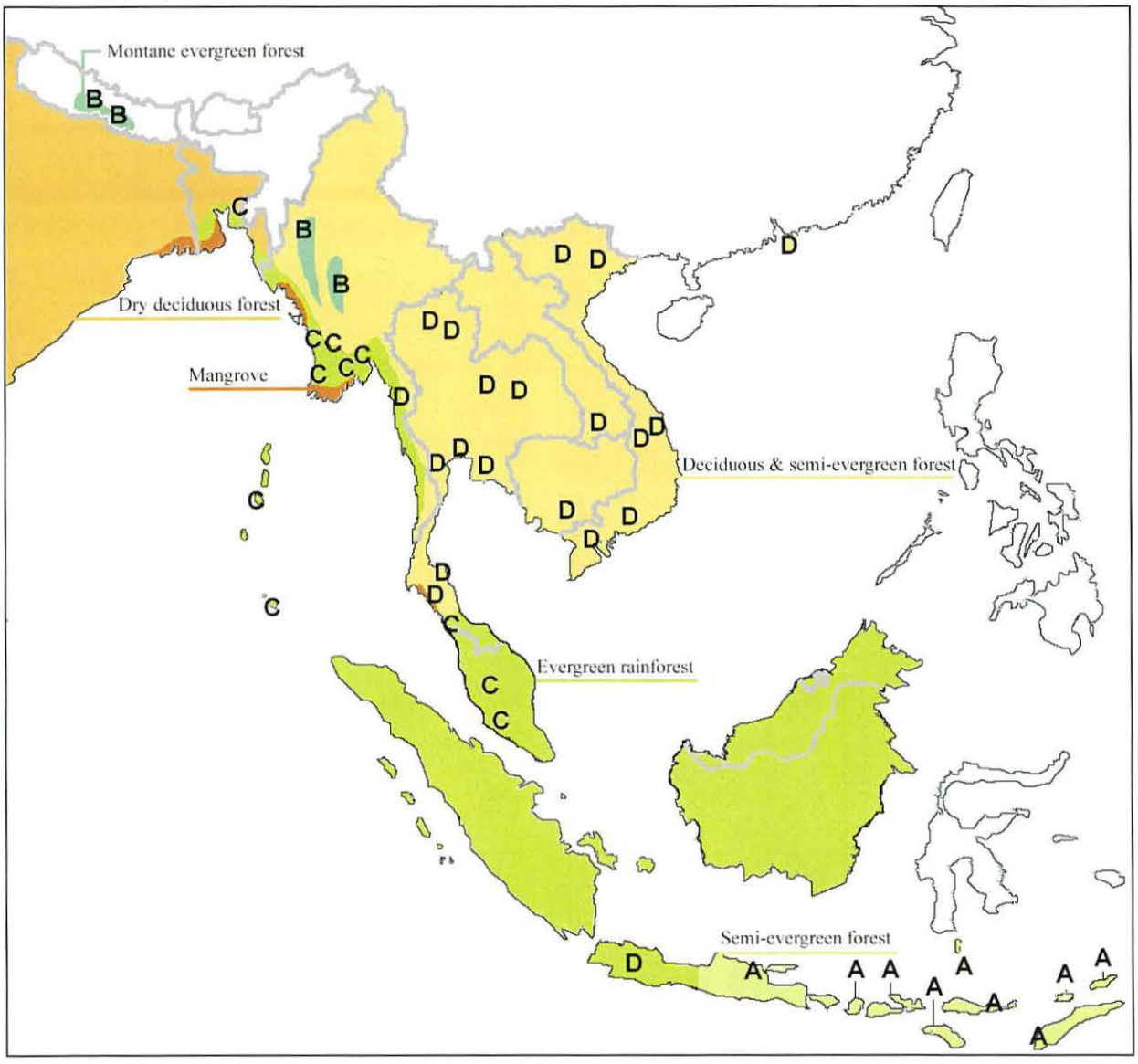

Figure 5.6: Geographic distribution of primary clades within the *T. albolabris* complex in relation to predominant interglacial vegetation cover (from MacKinnon, 1997). Clade A = *T. insularis* and *T. fasciatus*; Clade B = *T. septentrionalis* and *T. albolabris* from northwest Myanmar; Clade C = *T. erythrurus*, *T. purpureomaculatus, T. andersoni* and *T. cantori*; Clade D = *T. albolabris sensu stricto.*

5.5.2 Molecular systematics

A number of parallels between our AFLP and mtDNA sequence data confirm previous findings by Malhotra and Thorpe (1997), Malhotra and Thorpe (2000) and Giannasi *et al.* (2001b). The species status of *T. insularis* is supported by a relatively high level of sequence divergence from sister species *T. fasciatus*, a deep divergence event separating these species from the rest of the complex, and clear separation of *T. insularis* specimens on the AFLP PCO plot. Interrelationships of the *T. insularis* island populations are unresolved in our analyses; however, low levels of sequence divergence between these populations (0.4% ± 0.3) indicate recent divergence.

The close relationship between *T. erythrurus* and *T. purpureomaculatus*, and their recent common ancestry with *T. andersoni* and *T. cantori*, is also confirmed by our AFLP and mtDNA sequence data. The addition of mtDNA sequences for *T. erythrurus* and *T. purpureomaculatus* from previously unsampled localities reveals two monophyletic groups corresponding to Rakhine State (Myanmar) and Chittagong (Bangladesh) in the north, and Yangon and Ayeyarwade divisions (Myanmar), south Thailand and West Malaysia in the south. Since the type locality of *T. purpureomaculatus* is Singapore and *T. erythrurus* is not known to occur south of Mon State in Myanmar (Smith, 1943), the southern clade may refer to *T. purpureomaculatus* and the northern clade to *T. erythrurus*. However, these clades show compatible habitat preferences and the mean sequence difference between them (1.8% ± 0.7) is consistent with intraspecific levels of divergence in snake taxa (Johns and Avise, 1998). Their lack of differentiation in the AFLP analysis, however, cannot be used as evidence of hybridisation given that AFLP markers are not capable of discriminating between weakly divergent populations (Ogden and Thorpe, 2002). Categorical colour pattern differences exist between *T. erythrurus* and *T. purpureomaculatus* (Smith, 1943). Multivariate analysis of morphological characters, including sympatric *T. albolabris*

populations, may be useful in determining the limits of these species and detecting introgression between sympatric populations.

Our results also show relatively deep mtDNA divergence of the Nepalese population, supporting its elevation to *T. septentrionalis*. *T. albolabris* from northwest Myanmar is well-supported within this clade and shows compatible habitat preferences to *T. septentrionalis*. High levels of sequence divergence exist between these populations (7.1% ± 1.3), however this may reflect their considerable geographic separation, especially given that female pitvipers are philopatric (Shine 1993). Northwest Myanmar specimens are also partially separated from other *T. albolabris* specimens on the AFLP plot, although this may reflect their allopatric distribution. Further AFLP or morphometric analysis of these populations, including specimens from geographically intermediate localities, will be required to determine whether the northwest Myanmar *T. albolabris* populations are referable to *T. septentrionalis*.

The remaining *T. albolabris* haplotypes represent fairly recently diverged lineages. Localities in north and northeast Thailand, Myanmar (excluding the northwest), Laos, Vietnam, Hong Kong and Cambodia are mostly separated by relatively low sequence differences (mean between-locality levels range from 0.3% ± 0.2 to 2.5% ± 1.0) and form an undifferentiated cluster on the AFLP PCO plot. Their lack of differentiation in the AFLP analysis is likely to reflect this recent divergence (Ogden and Thorpe, 2002). However, there is a general discordance between mtDNA haplotype clades and the geographic origin of specimens. Northeast Thailand haplotypes cluster with haplotypes from north Thailand, southeast Thailand, Vietnam and Cambodia; likewise, south Vietnam haplotypes cluster with haplotypes from central Vietnam, southeast Thailand, Cambodia and northeast Thailand. The possibility that specimens were assigned incorrect localities is unlikely given that they were wild-caught during fieldtrips. Therefore, in this case, the failure of haplotypes from a given locality to cluster probably indicates introgression with neighbouring populations (Slatkin and Maddison, 1989).

Localities in south, west, central and southeast Thailand and west Java also represent weakly differentiated mtDNA haplotypes (mean between-locality sequence divergence 1.4% ± 0.6 to 3.4% ± 1.2) and form an unresolved cluster in the AFLP analysis. This group is separated from the cluster comprising more northern localities (north and northeast Thailand, Myanmar, Laos, Vietnam, Hong Kong and Cambodia), despite shared clade affinities between these groups. This geographic division of *T. albolabris* populations is generally supported by a previous study based on 3-nucleotide extension AFLP primers (Giannasi *et al.*, 2001b). Given their presently continuous range, these clusters may represent introgressing populations that have recently come into secondary contact following historical separation.

This study has indicated a number of interesting possibilities for the systematics of the complex, such as the inclusion of northwest Myanmar *T. albolabris* within *T. septentrionalis,* the redefinition of *T. purpureomaculatus* and *T. erythrurus*, and cryptic diversity between northern and southern mainland *T. albolabris* populations. However, evidence from independent data sets should be sought before changes to the taxonomic arrangement of the group are recommended. Multivariate analysis of morphological variation within the complex is being undertaken separately, and has proven useful in resolving the systematic relationships of numerous *Trimeresurus* species groups (Malhotra and Thorpe, in press b; Malhotra and Thorpe, submitted; Sanders *et al.,* 2002; Sanders *et al.,* in press).

5.5.3 The systematic utility of 4-nucleotide extension AFLPs in pitvipers

General congruence between the divisions recovered by our AFLP data and mitochondrial phylogeny demonstrates the utility of 4-nucleotide extension AFLPs for generating systematic information within a complex of closely related pitviper species. The number of bands produced was comparable to that of a previous study using 4-

nucleotide extension AFLP primers (Tatsuta and Butlin, 2001). Bands were clearer and could be scored with greater confidence than those generated by previous workers using 3-nucleotide extension primers in the *T. albolabris* group. However, fewer bands were produced overall (average 28.5, compared to 86.8 per primer pair) (Giannasi *et al.,* 2001b). This resulted in a lower level of discrimination from six 4-nucleotide extension primer pairs than was achieved using five 3-nucleotide extension primer pairs (Giannasi *et al.*, 2001b), indicating that a larger number of primer combinations may be required in future applications of 4-nucleotide extension AFLPs in pitvipers.

CHAPTER 6

General Discussion

The work presented in this thesis examined different aspects of geographic variation in three widespread species groups of *Trimeresurus* pitvipers from the Indomalayan region. Analysis of molecular, morphological and ecological data provided clarification of systematic relationships and generated new hypotheses concerning adaptive evolution and biogeographic history. The following chapter summarises the main findings of this study and makes tentative generalisations concerning the systematic and evolutionary biology of *Trimeresurus* pitvipers.

6.1 Systematics

This thesis represents the most comprehensive systematic revision of the *T. sumatranus*, *T. popeiorum* and *T. albolabris* groups to date. Combined sequences from different mtDNA gene regions allowed evolutionary relationships to be reconstructed and revealed clear patterns of phylogeographic variation in each species complex. A combination of maximum likelihood, maximum parsimony and Bayesian methods of phylogenetic inference were used; these produced generally congruent tree topologies, which were mostly well supported. However, the topology of a phylogeny (and the reliability of the conclusions drawn from it) depends critically on the ingroup taxa included (Zwickl and Hillis, 2002; Hillis *et al.*, 2003). Although we were able to include all of the nominal species in each group, the exclusion of OTUs was unavoidable given large gaps in the geographic coverage of DNA samples obtained. This was due to logistic and political constraints that prevented sample collection in many areas and the challenges of finding cryptic animals in arboreal habitats. Sample acquisition for *T. sumatranus*

and *T. popeiorum* group OTUs was further hindered by extensive forest degradation and the reliance of these populations on undisturbed rainforest habitats.

Phylogenies derived from different mitochondrial genes do not provide independent estimates of organismal phylogeny, and may represent gene trees that are incongruent with the species tree (Avise and Ball, 1990; Moore, 1995; Page, 2000). In the *T. albolabris* complex, the fingerprinting technique amplified fragment length polymorphism (Zabeau and Vos, 1993; Vos, 1995) was used to generate nuclear loci for an independent estimation of systematic relationships. Four-nucleotide extension selective primers generated a sufficient number of polymorphic markers to distinguish the main clades recovered by a mitochondrial phylogeny. Thus, demonstrating the utility of 4-nucleotide extension AFLP markers in a complex of closely related pitvipers. The bands produced were clearer and could be scored with greater confidence than those generated by previous workers using 3-nucleotide extension primers in the *T. albolabris* group (Giannasi *et al.,* 2001b). However, fewer bands were produced and this resulted in an overall lower level of discrimination (Giannasi *et al.*, 2001b), indicating that a larger number of primer combinations may be required in future applications of 4-nucleotide extension AFLPs in pitvipers.

Mitochondrial phylogenies were combined with multivariate ordination of morphological variation to infer systematic relationships in the *T. sumatranus* and *T. popeiorum* groups. A well-resolved phylogeny was recovered for the *T. sumatranus* group, with each species represented by a distinct lineage that was effectively delimited using multivariate analysis of morphology. Ecological adaptation to the current environment was found to cause convergence in several taxonomically important characters. This demonstrated the potential for misidentification when species that are widely distributed over varying environments are diagnosed on the basis of only a few phenotypic characters. At the intraspecific level, highest variation within *T. hageni* was found between allopatric mainland and Sumatran populations. However, these were shown to represent recently diverged populations that are weakly differentiated by phenotype. Within *T. flavomaculatus*, the

subspecies *T. f. mcgregori* from Batanes Island showed the highest level of morphological differentiation, although is separated from the mainland population by relatively low sequence divergence. Examination of stomach contents from museum specimens indicated that *T. f. mcgregori* utilises a different diet from *T. flavomaculatus* and differences relating to feeding behaviour have been observed in captive animals (A. Gumprecht, pers. comm.). *T. sumatranus* populations from Borneo and Sumatra are separated by comparatively high sequence divergence, and showed clear phenotypic separation in multivariate analysis. They also differ in habitat utilisation; the Bornean population occupies low altitudes whereas the Sumatran population is not found below moderate altitudes. Descriptions of Bornean *T. sumatranus* and *T. f. mcgregori* as new species are currently underway.

The taxonomy of the *T. popeiorum* complex was revised using a combination of molecular, morphological and ecological pattern-based species criteria. General discordance between the species boundaries inferred from different criteria indicated that taxonomic revisions based on a single species criterion are unlikely to lead to a realistic delimitation of species. It was recommended that attempts to delimit species boundaries should combine as many independent sources of data as are available, particularly with respect to organisms that cannot be comprehensively sampled. The current subspecific taxonomy of the *T. popeiorum* complex was found to be inconsistent with the molecular, morphological and ecological divisions revealed by this study, and a conservative reorganisation of the complex into three species was proposed.

Two well-differentiated *T. popeiorum* clades, corresponding mainly to northern and southern parts of its range, were indicated in the phylogeny. High levels of haplotype divergence were revealed in the northern clade, which included mostly parapatric populations in northeast India, north and west Thailand, Myanmar and Laos. Due to a lack of morphological and ecological differentiation between these populations, it was recommended that they be considered as a single species, corresponding to the nominate form of *T. popeiorum*. The southern clade was found to represent recently diverged allopatric lineages (in southern Thailand, Malaysia,

Sumatra and Borneo) with compatible habitat preferences. Intergradation with parapatric and ecologically compatible northern clade populations was considered unlikely in light of categorical patterns of variation between these OTUs. Therefore, a conservative arrangement of these populations as a single, polytypic, equatorial species with the name *T. sabahi* was proposed. Species status was also recommended for the northern clade lineage from the Cameron Highlands range in West Malaysia. This population is allopatric and morphologically and ecologically divergent with respect to all other northern clade populations, and categorical patterns of morphological variation indicated that intergradation with parapatric southern clade populations is unlikely. A description of this new species, *T. inornatus*, is underway.

Systematic relationships within the *T. albolabris* complex were inferred from ordination and phylogenetic analysis of AFLP markers and phylogenetic reconstruction of mtDNA sequences. Parallels between AFLP and mtDNA analysis support previous taxonomic revisions and provide further resolution of systematic relationships. New material from northwest Myanmar comprised the most divergent *T. albolabris sensu stricto* OTU in both analyses. These specimens formed a monophyletic group with *T. septentrionalis* and displayed similar habitat preferences to this species relative to the remainder of the complex. It was concluded that with further investigation northwest Myanmar *T. albolabris* might be referable to *T. septentrionalis*, leading to a considerable range extension for the later.

Island species *T. andersoni* and *T. cantori* were shown to be closely related and share a recent common ancestor with *T. erythrurus* and *T. purpureomaculatus.* Populations of *T. erythrurus* and *T. purpureomaculatus* formed two paraphyletic clusters on the phylogeny and display compatible habitat preferences. AFLP and sequence analysis revealed a level of divergence between these clusters that is consistent with intraspecific differentiation in snake taxa. Multivariate analysis of morphological variation may provide a better estimation of the boundaries between these species, and is being undertaken separately. Also worthy of further

investigation is the north-south differentiation of mainland *T. albolabris sensu stricto* populations, revealed by AFLP ordination analysis.

6.2 Adaptive Evolution

Trimeresurus pitvipers are well known for their pronounced morphological conservatism, which is sometimes attributed to phylogenetic constraints (e.g. Malhotra and Thorpe, submitted). However, multivariate morphometric analysis of the *T. sumatranus* and *T. popeiorum* groups revealed geographical patterns of phenotypic variation that appeared to reflect current ecology more closely than phylogenetic history. In the *T. sumatranus* complex, an adaptive explanation for the observed pattern of phenotypic differentiation was supported by phylogenetically independent contrasts analysis (Felsenstein, 1985). Significant correlations were found between current environmental conditions and most of the scalation and colour pattern characters that best account for the variation between taxa. In particular, reduced precipitation and altitude, and increased temperature, were correlated with higher numbers of scales on the head, body and tail. It was hypothesised that scale number plays an important role in heat and water exchange by influencing the area of exposed of interstitial skin, and that colour pattern variation reflects selection pressures involving camouflage and thermoregulation.

Ecological adaptation may also explain the apparent convergence of high altitude populations in the *T. popeiorum* group; the phenotypic similarity of these OTUs was based on lowered scale counts for many of the same characters that were found to be negatively correlated with cool, wet habitats in the *T. sumatranus* group. However, it should be acknowledged that the relative importance of genetic inheritance and phenotypic plasticity in morphological variation in pitvipers is currently unknown. Plasticity has been observed in several lizard and colubrine snake species (Madsen and Shine 1993; Queral-Regil, 1998; Qualls and Shine, 1998; Losos, 2001), common-garden studies of pitvipers would therefore be useful.

6.3 Biogeography

The colonisation of new and contrasting environments is an important factor in the diversification of conspecific populations and closely related species (Whittaker, 1998; Mallet, 2001; Schluter, 2001). This can occur *in situ* when parapatric populations become increasingly specialised to adjacent but contrasting environments (Endler, 1977; Jiggins and Mallet, 2000). However, diversification is thought to occur more commonly in allopatry, following the fragmentation of previously contiguous populations due to dispersal or vicariance events (Coyne, 1992; Patton *et al.,* 1998; Jiggins and Mallet, 2000). Ecological shifts between contrasting environments have been implicated in the radiation of a diverse range of taxa. A few examples are fish (Reinthal and Meyer, 2000), lizards (Losos *et al.,* 1997), butterflies (Mallet and Joron, 1999) and plants (Baldwin, 2000). Founder effects (i.e. random drift of reduced genetic variation following a population bottleneck) have been proposed as an alternative explanation for allopatric differentiation that does not invoke natural selection (Avise, 1994), but has yet to be demonstrated empirically (Barton and Charlesworth, 1984; Moya *et al.*, 1995).

The phylogeographic analyses undertaken in this thesis suggest an important role for ecological shifts in the radiation of Indomalayan pitvipers. Patterns of distribution indicated the colonisation of new areas that in some cases support markedly divergent habitats. For example, the ancestors of *T. schultzei* and *T. flavomaculatus* are likely to have colonised the dry forests of the Philippine islands from equatorial Borneo; *T. popeiorum* populations probably shifted to high elevation forest habitats following colonisation of the Sunda islands; and *T. sumatranus* populations colonised montane forests in Sumatra from lowland forests in Borneo, or vice versa. In the *T. albolabris* complex, the geographic distribution of each major clade was seen to be closely linked to original distributions of vegetation types. This included the montane clade in northwest Myanmar and Nepal, and the *T. erythrurus* - *T. purpureomaculatus* clade, which is restricted to relatively aseasonal lowland forests and mangroves near the equator and along the evergreen Andaman coast.

Vicariance associated with glacial climate change has been implicated in the distributions and phylogeographic structure of a wide range of Indomalayan taxa (e.g. Inger, 1966; Lekagul and McNeely, 1977; Heaney, 1985b; Corbet and Hill, 1992; Han and Sheldon, 2000). During glacial periods, land bridges are thought to have facilitated the exchange of mainland and island fauna, preceding the isolation of populations by rising sea levels (Leviton, 1963; Heaney, 1986). This may have allowed the colonisation and subsequent diversification of Indomalayan pitvipers on the Greater Sunda and Philippine islands. However, the occurrence of species on islands that have been continually isolated indicates over water dispersal in some cases, for example, *T. f. mcgregori* on Batanes island and *T. insularis* in the Lesser Sunda islands. On the mainland, strong phylogeographic structure of the montane species *T. popeiorum* suggests population fragmentation in glacial forest refugia. In contrast, the preference of *T. albolabris* for seasonal habitats may have facilitated the dispersal of this species during glacial periods and explain the weak phylogeographic structure of mainland populations. Parapatric divergence is also indicated by the adjoining ranges of ecologically divergent sister clades, *T. albolabris sensu stricto* and *T. erythrurus - T. purpureomaculatus.*

During this study, an attempt was made to statistically test biogeographic hypotheses in the *T. popeiorum* and *T. albolabris* groups using nested clade analysis (Templeton *et al.*, 1987 and 1995). Nested clade uses a haplotype tree to define a nested series of clades that are used to test between alternative hypotheses for the spatial distribution of genetic variation, including restricted gene flow, historical vicariance and population range expansion. The outcomes of these analyses were inconclusive due to a lack of statistical significance associated with the presence of missing haplotypes between highly divergent clades. Nested clade is an intraspecific method restricted to analysing closely related populations that can be comprehensively sampled (Templeton *et al.*, 1995), such as the pitviper *T. stegnejeri* on Taiwan (Creer *et al.*, 2001). With denser geographic sampling this method could be used to test biogeographic hypotheses at local scales, but is unlikely to be of value in uncovering wide-scale biogeographic processes in Indomalayan pitvipers.

6.4 Future work

Pitvipers are rarely studied in the context of adaptive radiation. However, molecular systematic techniques are now standard and can provide a rigorous means of testing causal hypotheses of differentiation and inferring geographical modes of divergence (Brooks and McLennan, 1991). Future work of this kind should provide a more general understanding of pitviper evolution, and lead to insights into the functional significance of traits, competition and community structure, geographical patterns of distribution and the origins of species diversity. The elucidation of factors involved in forming and maintaining *Trimeresurus* species will have important implications for the criteria used to define and diagnose pitviper species, and may lead to more realistic systematic reviews of these animals.

References

Adams, J. 2001. Eurasia during the last 150,000 years. http://www.eds.ornl.gov/projects/qen/nercEURASIA.html.

Anderson, S. G., Gutierrez, J. M. and Ownby, C. L. 1993. Comparison of the immunogenecity and antigenic composition of ten central American snake venoms. *Toxicon* 31:1051-1059.

Anon. 1995. *National Geographic Atlas of the World*, Sixth edition. National Geographic Society, Washington D.C.

Archie, J. W. 1989. A randomization test for phylogenetic information in systematic data. *Systematic Zoology* 28: 239-252.

Arèvalo, E., Davies, S. K. and Sites, J. J. W. 1994. Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in central Mexico. *Systematic Biology* 43 (3): 387-418.

Ashton, K. G. 2001. Body size variation among mainland populations of the western rattlesnake (*Crotalus viridis*). *Evolution* 55(12): 2523-2533.

Ashton, K. G., and de Queiroz, A. 2001. Molecular systematics of the Western Rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-loop in phylogenetic studies of snakes. *Molecular Phylogenetics and Evolution* 21(2): 176-189.

Avise, J. 1989. Gene trees and organismal histories: a phylogenetic approach to population biology. *Evolution* 43: 1192-1208.

Avise, J. C. 1992. Molecular population structure and the biogeographic history of a regional fauna: a case study with lessons for conservation biology. *Oikos* 63: 62-76.

Avise, J. C. 1994. *Molecular markers, natural history, and evolution.* Chapman and Hall, New York.

Avise, J.C. 2000. *Phylogeography: The history and formation of species.* Harvard University Press, USA.

Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A. and Saunders, N. C. 1987. Intraspecific phylogeography: the

mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics* 18: 489-522.

Avise, J. C. and Vrijenhoek, R. C. 1987. Mode of inheritance and variation of mitochondrial DNA in hybridogenetic fishes of the genus *Poeciliopsis*. *Molecular Biology and Evolution* 4: 514-525.

Avise, J., Ball, M. and Arnold, J. 1988. Current versus historical population sizes in vertebrate species with high gene flow: a comparison based on mitochondrial DNA lineages and inbreeding theory for neutral mutations. *Molecular Biology and Evolution* 5(4): 331-344.

Avise, J. C., and Ball, R. M. 1990. Principals of genealogical concordance in species concepts and biological taxonomy. *Oxford Survey of Evolutionary Biology* 7: 45-67.

Avise, J. C., and Wollenberg, K. 1997. Phylogenetics and the origin of species. *Proceedings of the National Academy of Sciences of the USA* 94: 7748–7755.

Baldwin, B. G. 2000. Adaptive radiation of the Hawaiian silversword alliance: congruence and conflict of phylogenetic evidence from molecular and non-molecular investigations. Pages 103-128 *In* Givnish, T. J. and Systsma, K. J., eds. *Molecular Evolution and Adaptive Radiation*. Cambridge University Press, UK.

Barton, N. H. and Charlesworth, B. 1984. Genetic revolutions, founder effects, and speciation. *Annual Review of Ecology and Systematics* 15: 133-164.

Baum, D. 1992. Phylogenetic species concepts. *Trends in Ecology and Evolution* 7: 1-2.

Baum, D. A., and Donoghue, M. J. 1995. Choosing among alternative 'phylogenetic' species concepts. *Systematic Botany* 20: 560-573.

Baum, D. A., and Shaw K. L. 1995. Genealogical perspectives on the species problem. Pages 289-303 *In* Hoch, P. C. and Stevenson, A. G., eds. *Experimental and Molecular Approaches to Plant Biosystematics*. Missouri Botanical Garden, St. Lois.

Bauwens, D., Garland, T. Jr., Castilla, A. M. and Van Damme, R. 1995. Evolution of sprint speed in Lacertid lizards: Morphological, physiological and behavioural covariation. *Evolution* 49(5): 848-863.

Beck, R. A., Burbank, D. W., Sercombe, W. J., Riley, G. W., Barndt, J. K., Berry, J. R., Afzal, J., Khan, A. M., Jurgen, H., Metje, J., Cheema, A., Shafique, N. A., Lawrence, R. D. 1995. Stratigrafic evidence for an early collision between northwest India and Asia. *Nature* 373: 55-58.

Beismann, H., Barker, J. H. K., Karp, A. and 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.

Bently, P. J. and Schmidt-Nielsen, K. 1966. Cutaneous water loss in reptiles. *Science* 151: 1547-1549.

Berry, R. J. 1998. Evolution of small mammals. Pages 35-50 *In* Grant, P. R., ed. *Evolution on islands*. Oxford University Press, Oxford.

Boulenger, G. A. 1896. *Catalogue of the Snakes of the British Museum (Natural History). Volume III. Containing the conclusion of the* Colubridae, Amblycephalidae *and* Viperidae. British Museum of Natural History, London.

Brattstrom, B. H. 1964. Evolution of the pitvipers. *Transactions of the San Diego Society of Natural History* 13:185-268.

Briggs, J. C. 1987. Antitropicality and vicariance. *Systematic Zoology* 36: 206-207.

Brongersma, L. D. 1933. Herpetological notes I – IX. *Zoologische Mededeelingen Leiden* 16: 1-29.

Brooks, D. R. and McLennan, D. A. 1991. *Phylogeny, Ecology and Behaviour: A research program in comparative biology.* University of Chicago Press, Chicago and London.

Brown, L. M., George, M. J., and Wilson, A. C. 1979. Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences of the USA* 76(4): 1967-1971.

Brown, R. P., Thorpe, R. S. and Baez, M. 1991. Lizards on neighbouring islands show parallel within-island micro-evolution. *Nature* 352: 60-62.

Burbrink, F. T., Lawson, R. and Slowinski, J. B. 2000. Mitochondrial DNA phylogeography of the polytypic north American rat snake (*Elaphe obsoleta*): A critique of the subspecies concept. *Evolution* 54(6): 2107–2118.

Buttle and Tuttle Ltd. 2001. http://www.worldclimate.com

Caccone, A., Milinkovitch, M. C., Sbordoni, V. and Powell, J. R. 1997. Mitochondrial DNA rates and biogeography in European newts (Genus *Eurproctus*). *Systematic Biology* 46 (1): 126-144.

Castellano, S. and Giacoma, C. 1998. Morphological variation of the green toad, Bufo viridis in Italy: a test of causation. *Journal of Herpetology* 32 (4): 540-550.

Chipaux, J. P., Williams, V. and White, J. 1991. Snake venom variability: methods of study, results and interpretation. *Toxicon* 29: 1279-1303.

Chippindale, P. T., and Wiens, J. J. 1994. Weighting, Partitioning, and Combining Characters in Phylogenetic Analysis. *Systematic Biology* 43: 278-287.

Corbet, G. B. and Hill, J. E. 1992. *The Mammals of the Indomalayan Region*, Oxford University Press, Oxford.

Courtillot, V., Besse, J., Vandamme, D., Montogny R., Jaeger, J. J., Cappetta, H. 1986. Deccan flood basalts at the Cretaceous/Tertiary boundary? *Earth and Planetary Science Letters* 80: 361-374.

Cox, M. J., van Dijk, P. P., Nabhitabhata, J. and Thirakhupt, K. 1998. *A photographic guide to snakes and other reptiles of Peninsular Malaysia, Singapore and Thailand.* New Holland, UK.

Cox, C. B., Moore, P. D. 2000. *Biogeography; an Evolutionary and Ecological Approach,* 6th edition. Blackwell Science, Oxford.

Coyne, J. A. 1992. Genetics and speciation. *Nature* 355: 511-515.

Coyne, J. A. 1994. Ernst Mayr and the origin of species. *Evolution* 48: 19-30.

Creer, S., Malhotra, A., Thorpe, R. S. and Chou, W. H. 2001. Multiple causation of phylogeographic pattern as revealed by nested clade analysis of the bamboo pitviper (*T. stejnegeri*) within Taiwan. *Molecular Ecology* 10: 1967-1981.

Creer, S., Malhotra, A., Thorpe, R. S., Stocklin, R., Favreau, P. and Chou, W. H. 2003. Genetic and ecological correlates of intraspecific variation in pitviper venom composition detected using Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) and isoelectric focusing. *Journal of Molecular Evolution* 56: 317-329.

Cracraft, J. 1983. Species concepts and speciation analysis. Pages 159-187 *In* Johnston, R. F., ed. *Current Ornithology Volume 1*. Plenum Press, New York.

Cracraft, J. 1989. Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. Pages 28-59 *In* Otte, D. and Endler, J. A., eds. *Speciation and its consequences.* Sinauer Associates, Sunderland, Massachusetts.

Crother, B. I., Campbell, J. A. and Hillis, D. M. 1992. Phylogeny and historical biogeography of the palm-pitvipers, genus *Bothriechis*: Biochemical and morphological evidence. Pages 1-19 *In* Campbell, J. A. and Brodie, E. D., eds. *Biology of the pitvipers.* Selva, Tyler, Texas.

Cummings, M. P., Otto, S. P. and Wakeley, J. 1995. Sampling properties of DNA sequence data in phylogenetic analysis. *Molecular Biology and Evolution* 12: 814-822.

Daltry, J. C., Wüster, W. and Thorpe, R. S. 1996. Diet and snake venom evoluton. *Nature* 379: 537-540.

David, P. and Vogel, J. 1996. *Snakes of Sumatra.* Edition Chimaira, Frankfurt.

Davies, J. I., and Nixon, K. C. 1992. Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology* 41: 421-435.

Davis, J. I. 1996. Phylogenetics, molecular variation and species concepts. *Bioscience* 46: 502-511.

de Knijff, P., Denkers. F., van Swelm, N. D. and Kuiper, M. 2001. Genetic affinities within the *Larus argentatus* assemblage revealed by AFLP genotyping. *Journal of Molecular Evolution* 52: 85-93.

de Queiroz, K. 1998. The general lineage concept of species, species criteria, and the process of speciation. Pages 57-75 *In* Howard, D. J. and Berlocher, S. H.,

eds. *Endless forms: species and speciation*. Oxford University Press, New York.

Dobzhansky, T. 1946. Complete reproductive isolation between two morphologically similar species of *Drosophila*. *Ecology* 27: 205–211.

Donoghue, M. J. 1985. A critique of the biological species concept and recommendations for a phylogenetic alternative. *Bryologist* 88: 172-181.

Dowling, H. G. 1951. A proposed standard system of counting ventrals in snakes. *British Journal of Herpetology* 1: 97-99.

Dowling, T. E., Moritz, C., Palmer, J. D. and Rieseberg, L. H. 1996. Nucleic Acids III: Analysis of fragments and restriction sites. Pages 249-320. *In* Hillis, D. M., Moritz, C. and Mable, B. K., eds. *Molecular Systematics*. Sinauer Associates, Sunderland, Massachusetts.

Dring, J. C. M. 1979. Amphibians and Reptiles from northern Trengganu, Malaysia, with descriptions of two new geckos. *Bulletin of the British Museum (Natural History), Zoology* 34(5): 181-241.

Dring, J. C. M., McCarthy, C. J. and Whitten, A. J. 1989. Herpetofauna of the Mentawai islands, Indonesia. *Indo-Malayan Zoologist* 6: 119-132.

Eldredge, N., and Cracraft, J. 1980. *Phylogenetic patterns and the evolutionary process*. Columbia University Press, New York.

Endler, J. A. 1977. *Geographic Variation, Speciation, and Clines.* Princeton University Press, USA.

Endler, J. A. 1992. Signals, signal conditions, and the direction of evolution. *American Naturalist* 139: 125-153.

Erixon, P., Svennblad, B., Britton, T. and Oxelman, B. 2003. Reliability of Bayesian Posterior Probabilities and Bootstrap Frequencies in Phylogenetics. *Systematic Biology* 52(5): 665–673.

Farris, J. S. 1989. The retension index and the rescaled consistency index. *Cladistics* 5: 417-419.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. *Journal of Molecular Evolution* 17: 368-376.

Felsenstein, J. 1985a. Phylogenies and the Comparative Method. *American Naturalist* 125: 1-15.

Felsenstein, J. 1985b. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783-791.

Felsenstein, J. 1988. Phylogenies from molecular sequences: Inference and reliability. *Annual Review of Genetics* 22: 521-565.

Froehlich, J. W., Supriatna, J. and Froehlich, P. H. 1991. Morphometric analyses of Ateles: systematic and biogeographic implications. *American Journal of Primatology* 25: 1-22.

Gaiotto, F. A., Bramucci, M. and Grattapaglia, D. 1997. Estimation of outcrossing rate in a breeding population of *Eucalyptus urophylla* with dominant RAPD and AFLP markers. *Theoretical and Applied Genetics* 95: 842-849.

Garland, T. Jr. 1992. Rate tests for phenotypic evolution using phylogenetically independent contrasts. *American Naturalist* 140: 509-519.

Garland, T. Jr., Dickerman, A. W., Janis, C. M. and Jones, J. A. 1993. Phylogenetic analysis of covariance by computer simulation. *Systematic Biology* 42: 265-292.

Garland, T. Jr. and Adolph, S. C. 1994. Why not to do two-species comparative studies: limitations on inferring adaptation. *Physiological Zoology* 67: 797-828.

Garland, T. Jr., Midford, P. E. and Ives, A. R. 1999. An introduction to phylogenetically based statistical methods, with a new method for confidence intervals on ancestral states. *American Zoologist* 39: 374-388.

Garland, T. Jr. and Ives, A. R. 2000. Using the past to predict the present: Confidence intervals for regression equations in phylogenetic comparative methods. *American Naturalist* 155: 346-364.

Gavrilets, S., Li, H. and Vose, M. D. 2000. Patterns of parapatric speciation. *Evolution* 54: 1126-1134.

Giannasi, N. C., Thorpe, R. S. and Malhotra, A. 2001a. Nuclear and mitochondrial DNA phylogenies of the *Trimeresurus* complex: Implications for the gene versus species tree debate. *Molecular Phylogenetics and Evolution* 19: 463-471.

Giannasi, N. C., Thorpe, R. S. and Malhotra, A. 2001b. The use of amplified fragment length polymorphism (AFLP) in determining species trees at

fine taxonomic levels: Analysis of a medically important snake, *Trimeresurus albolabris*. *Molecular Ecology* 10: 419-426.

Goebel, A. M., Donnelly, J. M. and Atz, M. E. 1999. PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I and cytochrome b in Bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Molecular Phylogenetics and Evolution* 11: 163-199.

Goldstein, P. Z., and DeSalle, R. 2000. Phylogenetic species, nested hierarchies, and character fixation. *Cladistics* 16: 364–384.

Goldstein, P. Z., and Brower, A. V. Z. 2002. Molecular systematics and the origin of species: new synthesis or methodological introgressions? Pages 147-161 *In* DeSalle, R., Giribet, G. and Wheeler, W., eds. Molecular Systematics and Evolution: Theory and Practice. Birkhauser Verlag, Switzerland.

Gower, J. C. 1966. Some distance properties of latent root and vector methods used in multivariate analysis *Biometrika* 53: 325-338.

Grant, B. R. and Grant, P. R. 1992. Evolution of Darwin's finches caused by a rare climatic event. *Proceedings of the Royal Society of London B, Biological Sciences* 251: 111-117.

Gray, J. E. 1942. Synopsis of the species of rattle snakes, or family of Crotalidae. *Zoological Miscellany*, Wurz Treuttel, London.

Greene, H. W. 1997. Systematics and natural history, foundations for understanding and conserving biodiversity. *American Zoologist* 34: 48-56.

Griffin, L. E. 1909. Snakes of Palawan. *Philippine Journal of Science, Manilla* 5(4): 211-215.

Griffin, L. E. 1910. A list of snakes from the island of Pollilo, P.I., with descriptions of a new genus and two new species. *Philippine Journal of Science, Manilla* 4(6): 595-601.

Gumprecht, A. 2002. Die Bambusottern der Gattung *Trimeresurus* Lacépède Teil VI: Die philippinischen Bambusottern de *Trimeresurus flavomaculatus*-Komplexes II: Die Batanes-Bambusotter *Trimeresurus mcgregori* Taylor, 1919. *Sauria* 24(3): 31-44.

Gyllensten, L., Wharton, D., Josefsson, A. and Wilson, A. C. 1991. Paternal inheritance of mitochondrial DNA in mice. *Nature* 352: 255-257.

Haffer, J. 1969. Speciation in Amazonian forest birds. *Science* 165: 101-137.

Hall, R., and Holloway, J. D. 1998. *Biogeography and geological evolution of SE Asia*. Backhuys Publishers, Leiden.

Han, K. and Sheldon, F. H. 2000. Interspecific relationships and biogeography of some Bornean tree shrews (Tupaiidae: *Tupai*a), based on DNA hybridization and morphometric comparisons. *Biological Journal of the Linnean Society of London* 70: 1–14.

Harris, M. A. and Steudel, K. 1997. Ecological correlates of hind-limb length in the Carnivora. *Journal of Zoology* 241: 381-408.

Harrison, R. G. 1986. Pattern and process in a narrow hybrid zone. *Heredity* 56: 337-349.

Harrison, R. G. 1991. Molecular changes at speciation. *Annual Review of Ecology and Systematics* 22: 281-308.

Harrison, R. G. 1998. Linking evolutionary pattern and process: The relevance of species concepts for the study of speciation. Pages 19-31 *in* Howard, D. J. and Berlocher, S. H., eds. *Endless forms: species and speciation.* Oxford University Press, New York.

Harvey, P. H. and Purvis, A. 1991. Comparative methods for explaining adaptations. *Nature* 351: 619-624.

Hasegawa, M., Kishino, H. and Yano, T. 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22: 160-174.

Hawthorn, D. J. 2001. AFLP-based genetic linkage map of the Colorado potato beetle *Leptinotarsa decemlineata*: sex chromosomes and a pyrethroid-resistance candidate gene. *Genetics* 158: 695-700.

Heaney, L. R. 1985a. Zoogeographic evidence for middle and late Pleistocene land bridges to the Philippine islands. *Modern Quaternary Research in Southeast Asia* 9: 127-143.

Heaney, L. R. 1985b. Systematics of Oriental pygmy squirrels of the genera *Exilisciurus* and *Nannosciurus* (Mammalia : Sciuridae). *Miscellaneous*

Publications of the Museum of Zoology, University of Michigan 170: 1–58.

Heaney, L. R. 1986. Biogeography of mammals in SE Asia: Estimates of rates of colonization, extinction and speciation. *Biological Journal of the Linnean Society of London* 28: 127-165.

Hellmich, W. C. 1951. On ecotypic and autolytic characters, a contribution to the knowledge of the evolution of the genus *Liolaemus* (Iguanidae). *Evolution* 5: 359-369.

Hendy, M. D. and Penny, D. 1982. Branch and bound algorithms to determine minimal evolutionary trees. *Mathematical Biosciences* 59: 277-290.

Hennig, W. 1966. *Phylogenetic systematics*. University of Illinois Press, Urbana.

Hey, J. 2000. The mind of the species problem. *Trends in ecology and evolution* 17(7): 326-329.

Hillis, D. M., and Huelsenbeck, J. P. 1992. Signal, noise, and reliability in molecular phylogenetic analysis. *Heredity* 83: 189-195.

Hillis, D. M. and Moritz, C. and Mable, B. K. 1996. Applications of molecular systematics. Pages 515-543. *In* Hillis, D. M., Moritz, C. and Mable, B. K., eds. *Molecular Systematics*. Sinauer Associates, Sunderland, Massachusetts.

Hillis, D. M., Pollock, D. D., McGuire, J. A. and Zwickl, D. J. 2003. Is spare taxon sampling a problem for phylogenetic inference? *Systematic Biology* 52(1): 124-126.

Hoeh, W. R., Blakly, K. H. and Brown, W. M. 1991. Heteroplasmy suggests limited biparental inheritance of *Mytilus* mitochondrial DNA. *Science* 251: 1488-1490.

Horton, D.R. 1972. Lizard scale size and adaptation. *Systematic Zoology* 21: 441-443.

Howard, D.J., M. Reece, P.G. Gregory, J. Chu, and M.L. Cain. 1998. The evolution of barriers to fertilization between closely related organisms. Pages 279-288 *In* Howard, D. J. and Berlocher, S. H., eds. *Endless forms: species and speciation*. Oxford University Press, New York.

Huelsenbeck, J. P., and Ronquist, F. R. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17: 754-755.

Huelsenbeck, J. P., Larget, B., Miller, R. E. and Ronquist, F. R. 2002. Potential applications and pitfalls of Bayesian inference of phylogeny. *Systematic Biology* 51: 673–688.

Hutchison, C. S. 1989. Geological evolution of South-East Asia. Clarendon Press, Oxford.

Hutton, R. A., Looareesuwan, S. and Ho, M. 1990. Arboreal green pitvipers (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.

Huxley, J. 1942. Evolution: the modern synthesis. Harper and Brothers, New York.

Inger, R. F. 1966. The systematics and zoogeography of the amphibia of Borneo. *Fieldiana Zoology* 52: 1–402.

Jiggins, C. D. and Mallet, J. 2000. Bimodal hybrid zones and speciation. *Trends in Ecology and Evolution* 15(6): 250-255.

Jintakune, P. and Lawan, C. 1995. *Venomous Snakes of Thailand.* The Thai Red Cross Society, Science Division, Bangkok.

Johns, G. C. and Avise, J. C. 1998. A comparative summary of genetic distances in the vertebrates from the mitochondrial Cytochrome *b* gene. *Molecular Biology and Evolution* 15(11): 1481-1490.

Johnson, K. P. and Clayton, D. H. 2000. Nuclear and mitochondrial genes contain similar phylogenetic signal for pigeons and doves (Aves: Columbiformes). *Molecular Phylogenetics and Evolution* 14(1): 141-151.

Joseph, L. Moritz, C and Hugall, A. 1995. Molecular support for vicariance as a source of diversity in rainforests. *Proceedings of the Royal Society of London, Series B* 260: 177-182.

Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S. E., Richmond, K., Adair, D. M., Hugh-Jones, M., Kuske, C. R., and Jackson, P. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *Journal of Bacteriology* 179: 818-824.

Keogh, J. S., Barker, D. and Shine, R. 2001. Heavily exploited but poorly known: systematics and biogeography of commercially harvested pythons (*Python curtus* group) in Southeast Asia. *Biological Journal of the Linnean Society of London* 73: 113–129.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111-120.

Kimura, M. 1986. DNA and the neutral theory. *Philosophical Transactions of the Royal Society of London, Series B.* 312: 343-354.

Klauber, L. M. 1941. Four papers on the applications of statistical methods to herpetological problems. *Bulletin of the Zoological Society of San Diego* 17: 5-93.

Klauber, L. M. 1972. Rattlesnakes: Their habits, life histories, and influence on mankind, Volume 1. University of California Press, USA.

Kludge, A. G. and Farris, J. S. 1969. Quantative phyletics and the evolution of anurans. *Systematic Zoology* 18: 1-32.

Knight, A., and D. P. Mindell. 1993. Substitution bias, weighting of DNA sequence evolution, and the phylogenetic position of Fea's viper. *Systematic Biology* 42: 18-31.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Paabo, S., Villablanca, F. X. and Wilson, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science USA* 86: 6196-6200.

Kohlsdorf, T., Garland, T. and Navas, C. A. 2001. Limb and tail lengths in relation to substrate usage in *Tropidurus* lizards. *Journal of Morphology* 248: 151-64.

Kovach, W. L. 1999. MVSP – a multivariate statistical package for windows, version 3.12d. Kovach Computing Services, Pentraeth, Wales, UK.

Kraus, F., Mink, D. G. and Brown, W. M. 1996. Crotaline intergeneric relationships based on mitochondrial DNA sequence data. *Copeia* 1996: 763-773.

Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA.

Larget, B. and Simon, D. L. 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* 16:750–759.

Leache, A. D. and Reeder, T. W. 2002. Molecular Systematics of the Eastern Fence Lizard *(Sceloporus undulatus)*: A Comparison of Parsimony, Likelihood, and Bayesian approaches. *Systematic Biology* 51(1): 44–68.

Lekagul, B. and McNeely, J. A. 1977. Mammals of Thailand. *Bangkok: Association for the Conservation of Wildlife.*

Lenk, P. and Wink, M. 1997. A RNA-RNA heteroduplex cleavage analysis to detect rare mutations in populations. *Molecular Ecology* 6: 687-690.

Leviton, A. 1963. Contributions to a review of Philippine snakes V. The snakes of the genus *Trimeresurus*. *Proceedings of the California Academy of Science* 31(15): 369-416.

Lewis, P. O. 2001. Phylogenetic systematics turns over a new leaf. *Trends in Ecology and Evolution* 16:30–37.

Lidth de Jeude, T. W. Van. 1886. Note X. On *Cophias wagleri*, Boie and *Coluber sumatranus*, Raffles. *Notes of the Leyden Museum* 8: 43-54.

Lidth de Jeude T. W. Van. 1890. Note VIII. On a collection of snakes from Deli. *Notes of the Leyden Museum* 12: 17-27.

Lim, B. L. 1991. *Poisonous Snakes of Peninsular Malaysia.* The Malayan Nature Society, Malaysia.

Lister, B. C. 1976. The nature of niche expansion in West Indian Anolis lizards. II Evolutionary components. *Evolution* 30: 677-692.

Losos, J. B. 1990. Ecomorphology, performance capability, and scaling of West Indian Anolis lizards: an evolutionary analysis. *Ecological Monographs* 60: 369-388.

Losos, J. B. 2001. Evolution: a lizards tales. *Scientific American* 284(3): 56-61.

Losos, J. B., Irschick, D. J. and Schoener, T. W. 1994. Adaption and constraint in the evolution of specialization of Bahamian *Anolis* lizards. *Evolution* 48: 1786-1798.

Losos, J. B., Irschick, D. J., and Schoener, T. W. 1997. Adaptive differentiation following experimental island colonisation in *Anolis* lizards. *Nature* 387: 70-73.

Loveridge, A. 1938. New snakes of the genus *Calamaria, Bungarus* and *Trimeresurus* from Mount Kinabalu, North Borneo. *Procedings of the Biological Society of Washington, USA* 51: 43-46.

Luckow, M. 1995. Species concepts, assumptions, methods, and applications. *Systematic Botany* 20(4): 589–605.

Lui, G. Q. and Bernatchez, L. 1999. Correlated trophic specialisation and genetic divergence in sympatric lake whitefish ecotypes (*Coregonus clupeaformis*): Support for the ecological speciation hypothesis. *Evolution* 53: 1491-1505.

Lynch, J. D. 1989. The gauge of speciation: on the frequencies of models of speciation. Pages 527-533 *In* Otte, D. and Endler, J. A., eds. *Speciation and its Consequences.* Sinauer Associates, Massachusetts.

MacKinnon, J. and MacKinnon, K. 1986. *Review of the protected areas system in the Indo-Malayan Realm.* IUCN: Gland, Switzerland.

MacKinnon, J. 1997. *Protected areas systems review of the Indo-Malayan realm.* The Asian Bureau for Conservation Limited, Hong Kong.

Madsen, T. and Shine, R. 1993. Phenotypic plasticity in body sizes and sexual size dimorphism in European grass snakes. *Evolution* 47: 321-325.

Malhotra, A., and Thorpe, R. S. 1991a. Microgeographic variation in Anolis oculatus, on the island of Dominica West Indies. *Journal of Evolutionary Biology* 4:321-335.

Malhotra, A., and Thorpe, R. S. 1991b. Experimental detection of rapid evolutionary response in natural lizard populations. *Nature* 353 (6342): 347-348.

Malhotra, A., and Thorpe, R. S. 1993. An experimental field study of a eurytopic anole, *Anolis oculatus*. *Journal of Zoology* 229: 163-170.

Malhotra, A. and Thorpe, R. S. 1997. Microgeographic variation in scalation of *Anolis oculatus* (Dominica, West Indies): A multivariate analysis. *Herpetologica* 53(1): 49-62.

Malhotra, A., and Thorpe, R. S. 1997. New perspectives on the evolution of South-East Asian pit vipers (genus *Trimeresurus)* from molecular studies). Pages 115-128 *In* Thorpe, R. S., Wüster, W. and Malhotra, A., eds. *Venomous Snakes: Ecology, Evolution and Snakebite.* Symposia of the Zoological Society of London 70. Oxford University Press, Oxford.

Malhotra, A. and Thorpe, R. S. 1997. New perspectives on the evolution of south-east Asian pitvipers (genus *Trimeresurus*) from molecular studies. *Symposium of the Zoological Society of London* 70: 115-128.

Malhotra, A., and Thorpe, R. S. 2000a. A phylogeny of the *Trimeresurus* group of pitvipers: new evidence from a mitochondrial gene tree. *Molecular Phylogenetics and Evolution* 16: 199-211.

Malhotra, A., and Thorpe, R. S. 2000b. The dynamics of natural selection and vicariance in the Dominican anole: Patterns of within-island molecular and morphological divergence. *Evolution* 54(1): 245-258.

Malhotra, A. and Thorpe, R. S. A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pit vipers (*Trimeresurus* and *Ovophis*). *Molecular Phylogenetics and Evolution,* in press (a).

Malhotra, A., and Thorpe, R. S. Reassessment of the validity and diagnosis of the pitviper *Trimeresurus venustus* (Vogel, 1991). *The Herpetological Journal*, in press (b).

Malhotra, A., and Thorpe, R. S. Maximising information in systematic revisions: A combined molecular and morphological analysis of a cryptic green pitviper complex (*Trimeresurus stejnegeri*). Submitted.

Mallet, J. 1995. A species definition for the Modern Synthesis. *Trends in Ecology and Evolution* 10: 294-299.

Mallet, J. 2001. Species, concepts of. Pages 427-440 *In* Levin, S. A., ed. *Encyclopedia of Biodiversity, Volume 5*. Academic Press, New York.

Mallet, J. and Joron, M. 1999. Evolution of diversity in warning colour and mimicry: Polymorphisms, shifting balance, and speciation. *Annual Review of Ecology and Systematics* 30, 201-233.

Manly, B. F. J. 1991. Distance matrices and spatial data. *In* Randomisation and Monte Carlo Methods on Biology. Chapman and Hall, London.

Mantel, N. and Valand, R. S. 1970. A technique of nonparametric multivariate analysis. *Biometrics*, 547-558.

Marchetti, K. 1993. Dark habitats and bright birds illustrate the role of the environment in species divergence. *Nature* 362: 149-152.

Martin, A. P. and Palumbi, S. R. 1993. Protein evolution in different cellular environments: cytochrome b in sharks and mammals. *Molecular Biology and Evolution* 10: 873-891

Martins, E. P. 1996. Phylogenies, spatial autoregression and the comparative method: A computer simulation test. *Evolution* 50: 1750-1765.

Martins, E. P. and Hansen, T. F. 1997. Phylogenies and the Comparative Method: A general approach to incorporating phylogenetic information into the analysis of interspecific data. *American Naturalist* 149: 646-667.

Martins, E. P., Diniz-Filho, J. A. F. and Housworth, E. A. 2001. Adaptive constraints and the phylogenetic comparative method: A computer simulation test. *Evolution* 56 (1): 1-13.

Masden, T. and Shine, R. 1993. Phenotypic plasticity in body sizes and sexual size dimorphism in European grass snakes. *Evolution* 47: 321–325.

Maslin, P. 1942. Evidence for the separation of the crotalid genera *Trimeresurus* and *Bothrops,* with a key to the genus *Trimeresurus. Copeia* 1942(1): 18-24.

Masters, J. C. and Bragg, N. P. 2000. Morphological correlates of speciation in bush babies. *International Journal of Primatology* 21: 793-813.

Mau, B., Newton, M. and Larget, B. 1999. Bayesian phylogenetic inference via Markovchain Monte Carlo methods. *Biometrics* 55:1–12.

Mayr, E. 1942. Systematics and the Origin of Species from the veiwpoint of a zoologist. Columbia, New York.

Mayr, E. 1963. *Animal Species and Evolution.* Harvard University Press, Massachusetts.

Mayr, E. and O'Hara, R. J. 1986. The biogeographic evidence supporting the Pleistocene refuge hypothesis. *Evolution* 40: 55-67.

McDiarmid, R. W., Campbell, J. A. and Toure, T. A. 1999. *The snake species of the world. A taxonomic and geographic reference, Volume 1.* Herpetologists League, Washington D. C.

Milinkovitch, M. C. and Lyons-Weiler, J. 1998. Finding optimal ingroup topologies and convexities when the choice of outgroups is not obvious. *Molecular Phylogenetics and Evolution* 9: 348-357.

Mindell, D. P. and Thacker, C. E. 1996. Rates of molecular evolution: phylogenetic issues and applications. *Annual Review of Ecology and Systematics* 27: 279-303.

Mishler, B. D., and Donoghue, M. J. 1982. Species concepts: A case for pluralism. *Systematic Zoology* 31: 491-503.

Mishler, B. D. 1985. The morphological, developmental and phylogenetic species basis of concepts in bryophytes. *Bryologist* 88: 207-214.

Miyamoto, M. M. and Cracraft, J. 1991. Phylogenetic Analysis of DNA sequences. Oxford University Press, New York.

Moore, W. S. 1995. Inferring phylogenies from mtDNA variation: Mitochondrial gene trees versus nuclear gene trees. *Evolution* 49: 718-726.

Morely, R. J. 1998. Palynological evidence for Tertiary plant dispersals in the SE Asian region in relation to plate tectonics and climate. Pages 211-234, *In.* Hall, R. and Holloway, J. D., eds. *Biogeography and Geological Evolution of Southeast Asia.* Blackhuys Publishers, Leiden.

Moreno, E. and Carrascal, L. M. 1993. Ecomorphological pattern of aerial feeding in oscines (Passeriforms: Passeri). *Biological Journal of the Linnean Society of London* 50: 147-165.

Mousaeau, T. A. 1997. Ectotherms follow the converse to Bergmann's rule. *Evolution* 51: 630-632.

Moya, A., Galiana, A. and Ayala, F. J. 1995. Founder-effect speciation theory: failure of experimental corroboration. *Proceedings of the National Academy of Science, USA* 92: 3983-3986.

Mueller, U, G. and Wolfenbarger, L. L. 1999. AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution* 14: 389-394.

Nei, M. and Kumar, S. 2000. *Molecular evolution and phylogenetics*. Oxford University Press, New York.

Nijman, I. J., Bradley, D. G., Hanotte, O., Osten, M. and Lenstra, J. A. 1999. Satellite DNA polymorphisms and AFLP correlate with *Bos indicus-taurus* hybridisation. *Animal Genetics* 30: 265-273.

Nijman, I. J., Otsen, M., Verkaar, E. L. C., Ruijter, C. de., Hanekamp, E., Ochieng, J. W., Shamshad, S., Rege, J. E. O., Hanotte, O., Barwegen, M. W., Sulawati, T., and Lenstra, J. A. 2003. Hybridisation of banteng (*Bos javanicus*) and zebu (*Bos indicus*) revealed by mitochondrial DNA, satellite DNA, AFLPs and microsatellites. *Heredity* 90: 10-16.

Nixon, K. C., and Wheeler, Q. C. 1991. An amplification of the phylogenetic species concept. *Cladistics* 6: 211-223.

Ogden, R. and Thorpe, R. S. 2002. The usefulness of amplified fragment length polymorphism markers for taxon discrimination across graduated fine evolutionary levels in Caribbean *Anolis* lizards. *Molecular Ecology* 11: 437-445.

Ogden, R., and Thorpe, R. S. 2002. Molecular evidence for ecological speciation in tropical habitats. *Proceedings of the National Academy of Sciences of the USA* 99, 13612-13615.

Orlov, N. L., Ananjeva, N. B., Barabanov, A. V., Ryabov, S. A. and Khalikov, R. 2002. Diversity of vipers (Azemiopinae, Crotalinae) in East, Southeast, and South Asia: Annotated checklist and natural history data (Reptilia: Squamata: Serpentes: Viperidae) *In* U. Fritz, ed. *Collectanea Herpetologica: Essays in Honour of Fritz-Jürgen Obst.* Faunistische Abhandlungen, Staatliches Museum fur Tierkunde Dresden.

Page, R. D. M. 2000. Extracting species trees from complex gene trees: reconciled trees and vertebrate phylogeny. *Molecular Phylogenetic and Evolution* 14: 89-106.

Page, R. D. M. and Holmes, E. C. 1998. *Molecular Evolution: A Phylogenetic Approach.* Blackwell Science, Oxford.

Pagel, M. 1999. Inferring the historical patterns of biological evolution. *Nature* 401: 877-884.

Palumbi, S. R. 1996. Nucleic acids III: The polymerase chain reaction. Pages 205-248. *In* Hillis, D. M. and Moritz, C. and Mable, B. K., eds. *Molecular Systematics*. Sinauer Associates, Sunderland, Massachusetts.

Palumbi, S. R., and Baker, C. S. 1994. Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. *Molecular Biology and Evolution* 11: 426-435.

Parkinson, C. L., Moody, S. M. and Ahlquist, J. E. 1997. Phylogenetic relationships of the "*Agkistrodon* Complex" based on mitochondrial DNA sequence data. Pages 63-78 *In* Thorpe, R. S., Wüster, W. and Malhotra, A., eds. *Venomous Snakes: Ecology, Evolution and Snakebite.* Symposium of the Zoological Society of London 70, Clarendon Press, Oxford.

Parkinson, C. L. 1999. Molecular systematics and biogeographical history of pitvipers as determined by mitochondrial ribosomal DNA sequences. *Copeia* 1999, 576-586.

Parkinson, C. L., Campbell, J. A. and Chippindale, P. T. 2002. Multigene phylogenetic analysis of pitvipers, with comments on the biogeography of the group. Pages 93-110 *In* Schuett, G. M., Höggren, M., Douglas, M. D. and Greene, H. W., eds. *Biology of the Vipers*. Eagle Mountain Publishing, Utah.

Parsons, Y. M. and Shaw, K. L. 2001. Species boundaries and genetic diversity among Hawaiian crickets of the genus *Laupala* identified using amplified fragment length polymorphism. *Molecular Ecology* 10: 1765-1772.

Patterson, H. E. H. 1985. The recognition concept of species. Pages 21-29 *In* Vrba, E. S., ed. *Species and speciation*. Transvaal Museum, Pretoria.

Patton J. L., Nazareth, M. and da Silva, F. 1998. Rivers, Refuges, and Ridges. The Geography of Speciation of Amazonian Mammals. Pages 202-213 *In* Howard, D. J. and Berlocher, S. H., eds. *Endless forms: species and speciation*. Oxford University Press, New York.

Platnick, N. I. 1987. An empirical comparison of microcomputer parsimony programs. *Cladistics* 3: 121-144.

Polachan, S., Pradidtan, S., Tongtaow, C., Janmaha, S., Intarawijitr, K. and Sangsuwan, C. 1991. Development pf Cenozoic basins in Thailand. *Marine and Petroleum Geology* 8: 84-97.

Pope, C. H., and Pope, S. H. 1933. A study of the green pitvipers of South-eastern Asia and Malaysia, commonly identified as *Trimeresurus gramineus* (Shaw), with a description of a new species from Peninsular India. *American Museum Novitates* 629:1-12.

Posada, D. and Crandall, K. A. 1998. MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

Posada, D., and Crandall, K. A. 2001. Selecting the best-fit model of nucleotide substitution. *Systematic Biology* 50: 580-601.

Pough, H. F., Andrews, R. M., Cadle, J. E., Crump, M. L., Savitzky, A. H. and Wells, K. D. 2001. *Herpetology.* Prentice-Hall Inc., New Jersey.

Puorto, G., Da Graça Salomão, M., Theakston, R. D. G., Thorpe, R. S., Warrell, D. A. and Wüster, W. 2001. Combining mitochondrial DNA sequences and morphological data to infer species boundaries: Phylogeography of lanceheaded pitvipers in the Brazilian Atlantic forest, and the status of *Bothrops pradoi* (Squamata: Serpentes: Viperidae). *Journal of Evolutionary Biology* 14: 527-538.

Purvis, A., Gittleman, J. L. and Luh, H.-K. 1994. Truth or consequences: Affects of phylogenetic accuracy on two comparative methods. *Journal of Theoretical Biology* 167: 293-300.

Qualls, F. J. and Shine, R. 1998. Geographic variation in lizard phenotypes: importance of the incubation environment. *Biological Journal of the Linnean Society of London* 64: 477-491.

Queral-Regil, A. and King, R. B. 1998. Evidence for phenotypic plasticity in snake body size and relative head dimensions in response to amount and size of prey. *Copeia* 2: 423-429.

Quicke, D. L. J. 1993. *Principles and Techniques of Contemporary Taxonomy.* Blackie Academic & Professional, London.

Raffles, T. S. 1822. Sir T. S. Raffles's Descriptive Catalogue of a Zoological Collection made in Sumatra. *Transactions of the Linnaen Society of London* 3(2): 333-334.

Rand, D.M. 1994. Thermal habit, metabolic rate and the evolution of mitochondrial DNA. *Trends in Ecology and Evolution* 9(4): 125-131.

Rannala, A. B. and Yang, Z. 1996. Probability distribution of molecular evolutionary trees: A new method of phylogenetic inference. *Journal of Molecular Evolution* 43: 304–311.

Rawlings, L., and Donnellan, S. C. 2003. Phylogeographic analysis of the green python, *Morelia viridis*, reveals cryptic diversity. *Molecular Phylogenetics and Evolution* 27: 36-44.

Regenass, U., and Kramer, E. 1981. Zur Systematik der grünen Grubenottern der Gattung *Trimeresurus* (Serpentes, Crotalidae). *Revue Suisse de Zoology* 88: 186-193.

Reinthal P. N. and Meyer, A. 2000. Molecular phylogenetic tests of speciation models in lake Malawi cichlid fishes. Pages 375-390 *In* Givnish, T. J. and Systsma, K. J., eds. *Molecular Evolution and Adaptive Radiation.* Cambridge University Press, UK.

Richard, M. and Thorpe, R. S. 2001. Can microsatellites be used to infer phylogenies? Evidence from population affinities of the western Canary Island lizard (*Gallotia galloti*). *Molecular Phylogenetics and Evolution* 20(3): 351-360.

Rieppel, O. 1986. Species are individuals: A review and critique of and the evolutionarily significant unit in biodiversity and conservation. *Journal of Evolutionary Biology* 20: 283–317.

Rieseberg, L. H. and Wendel, J. F. 1993. Introgression and its consequences in plants. Pages 70-109 *In* Harrison, R. G., ed. *Hybrid zones and the evolutionary process.* Oxford University Press, Oxford.

Rodriguez-Robles, J. A., and De Jesus-Escobar, J. M. 2000. Molecular systematics of New World gopher, bull, and pinesnakes (*Pituophis*: Colubridae), a transcontinental species complex. *Molecular Phylogenetics and Evolution* 14: 35–50.

Romer, J. D. 1963. Notes on the white-lipped pitviper, *Trimeresurus albolabris* Gray, in Hong Kong. Pages 379-384 *In* Keegan, H. L. and MacFarlane, W. V., eds. *Venoms and poisonous animals and noxious plants of the pacific region.* Pergamon Press, Oxford.

Rosendahl, S. and Taylor, J. W. 1997. Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. *Molecular Ecology* 7: 3-10.

Salewski, V. 2003. Satellite species in lampreys: a worldwide trend for ecological speciation in sympatry? *Journal of Fish Biology* 63 (2), 267-279.

Sanders, K. L., Malhotra, A. and Thorpe, R. S. 2002. A contribution to the systematics of two commonly confused pitviper species from the Sunda region: *Trimeresurus hageni* and *T. sumatranus. Bulletin of the Natural History Museum of London* (*Zoology*) 68(2): 107-111.

Sanders, K. L., Malhotra, A. and Thorpe, R. S. Ecological diversification in a group of Indomalayan pitvipers (*Trimeresurus*): convergence in taxonomically important traits has implications for species identification. *Journal of Evolutionary Biology*, in press.

Sanders, K. L., Malhotra, A. and Thorpe, R. S. Combining molecular, morphological and ecological data to infer species boundaries in a cryptic tropical pitviper. Submitted.

Sanderson, M. J. and Kim, J. 2000. Parametric phylogenetics? *Systematic Biology* 49:817–829.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular cloning. A laboratory manual, Second* edition. Cold Spring Harbour Laboratory Press, USA.

Schluter, D. 2001. Ecology and the origin of species. *Trends in Ecology and Evolution* 16(7): 372-380.

Schneider, C. J., Cunningham, M. and Moritz, C. 1998. Comparative phylogeography and the history of endemic vertebrates in the wet tropics of Australia. *Molecular Ecology* 7: 487-498.

Schuett, G. M., Höggren, M., Douglas, M. D. and Greene, H. W., eds. *Biology of the Vipers*. Eagle Mountain Publishing, Utah.

Schluter, D., 1998. Ecological causes of speciation. Pages 114-129 *In* Howard, D. J. and Berlocher, S. H., eds. *Endless forms: species and speciation*. Oxford University Press, New York.

Semblat, J. P., Wajnberg, E., Dalmasso, A., Abad, P. and Castagnone-Sereno, P. 1998. High-resolution DNA fingerprinting of parthenogenetic root-knot nematodes using AFLP analysis. *Molecular Ecology* 7: 119-125.

Semerikov, V. L., Hengqing, Z., Sun, M. and Lascoux, M. 2003. Conflicting phylogenies of *Larix* (Pinaceae) based on cytoplasmic and nuclear DNA. *Molecular Phylogenetics and Evolution* 27: 173-184.

Shine, R. 1993. Sexual dimorphism in Snakes. Pages 49-78 *In* Seigel, R. A. and Collins, J. T., eds. *Snakes: Ecology and Behaviour*. McGraw-Hill, USA.

Simpson, C. Field Museum of Natural History. 2000. http://www.fieldmuseum.org/research_collections/zoology/zoo_sites/seamaps/mapindex.htm

Simpson, G. G. 1951. The species concept. *Evolution* 5: 285-298.

Simpson, G. G. 1953. *The major features of evolution.* Columbia University Press, New York.

Sites, J. W. and Crandall, K. A. 1997. Testing species boundaries in biodiversity studies. *Conservation Biology* 11: 1289-1297.

Slatkin, M. and Maddison, W. P. 1989. A cladistic measure of gene flow inferred from the phylogenies of alleles. *Genetics* 123: 603-613.

Smith, A. B. 1994. *Systematics and the fossil record. Documenting Evolutionary Patterns*. Blackwell Scientific Publications, Oxford.

Smith, M. A. 1943. *The fauna of British India, Ceylon and Burma, including the whole of the Indochinese sub-region. Reptilia and Amphibia. Volume 3, Serpentes*. Taylor and Francis Ltd., London.

Sneath, P. H. A. and Sokal, R. R. 1973. *Numerical Taxonomy. The Principals and Practice of Numerical Classification.* W. H. Freeman and Company, San Francisco.

Sokal, R. R. and Sneath, P. H. A. 1963. *Principles of numerical taxomomy.* Freeman, San Francisco

Sokal R. R., and Crovello, T. J. 1970. The biological species concept: A critical evaluation. *American Naturalist* 104: 127-153.

Stejneger, L. 1927. The Green Pit Viper, *Trimeresurus gramineus*, in China. *Proceedings of the United States National Museum* 72: 1-10.

Stenson, A., Malhotra, A. and Thorpe, R. S. 2002. Population differentiation and nuclear gene flow in the Dominican anole (*Anolis oculatus*). *Molecular Ecology* 11: 1679-1688.

Stuebing, R. B. and Inger, R. F. 1999. *A field guide to the snakes of Borneo*. Natural History Publications, Borneo.

Swofford, D. L. and Olsen, G. J. 1990. Phylogenetic reconstruction. *In* Hillis, D. M. and Moritz, C., eds. *Molecular Systematics*. Sinauer Associates, Sunderland, Massachusetts.

Swofford, D. L., Olsen, G. J., Waddell, P. J. and Hillis, D. M. 1996. Plyogenetic inference. Pages 407-503. *In* Hillis, D. M. and Moritz, C. and Mable, B. K., eds. *Molecular Systematics*. Sinauer Associates, Sunderland, Massachusetts.

Swofford, D. L. 2003. PAUP*: *Phylogenetic Analysis Using Parsimony (and Other Methods), Version 4.0.* Sinauer Associates, Massachussets.

Tamura, K., and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA of humans and chimpanzees. *Molecular Biology and Evolution* 10: 512-526.

Tan, N.H. 1991. The biochemistry of venoms of some venomous snakes of Malaysia: a review. *Tropical Biomedicine* 8: 91-103.

Tapponnier, P., Peltzer, G., Le Dain, A. Y., Armijo, R. and Cobbold, P. 1982. Propagating extrusion tectonics in Asia: new insights from sinple experiments with plasticine. *Geology* 10: 611-616.

Tatsuta, H and Butlin, R. K. 2001. Amplified fragment length polymorphism for the analysis of genetic structure in grasshopper populations. *Journal of Orthoptera Research* 10(2): 117-121.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* 17: 6463-6471.

Taylor, E. H. 1919. The snakes of the Philippine islands. *Proceedings of the California Academy of Science* 14:105-125.

Templeton, A. R., Boerwinkle, E. and Sing, C. F. 1987. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila. Genetics* 117: 343-351.

Templeton, A. R., Routman, E. and Phillips, C. A. 1995. Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the Tiger Salamander, *Ambysoma trigrinum. Genetics* 140: 767-782.

Templeton, A. R. 1989. The meaning of species and speciation: A genetic perspective. Pages 3-27 *In* Otte, D. and Endler, J. A., eds. *Speciation and its Consequences.* Sinauer Associates, Massachusetts.

Templeton, A. 1998. Species and speciation: geography, population structure, ecology and gene trees. Pages 32-43 *In* Howard, D. J. and Berlocher, S. H., eds. *Endless forms: species and speciation.* Oxford University Press, New York.

Templeton, A. R. 2001. Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology* 10: 779-791.

Teo, L. L., Kiew, R., Set, O., Lee, S. K. and Gan, Y. Y. 2002. Hybrid status of kuwini, *Mangifera odorata* Griff. (Anacardiaceae) verified by amplified fragment length polymorphism. *Molecular Ecology* 11: 1465-1469.

Theakston, R. D. G. 1997. The kinetics of snakebite envenoming and therapy. Pages 251-257 *In* Thorpe, R. S., Wüster, W. and Malhotra, A., eds. *Venomous Snakes: Ecology, Evolution and Snakebite.* Symposia of the Zoological Society of London 70. Oxford University Press, Oxford.

Theakston, R. D. G., Warrell, D. A. and Griffiths, E. 2003. Report of a WHO workshop on the standardization and control of antivenoms. *Toxicon* 41: 541–557.

Thomaz, D., Guiller, A. and Clarke, B.. 1996. Extreme divergence of mitochondrial DNA within species of pulmonate land snails. *Proceedings of the Royal Society of London, Series B* 263: 363–368.

Thorpe, R. S. 1975. Biometric analysis of incipient speciation in the ringed snake, *Natrix natrix. Experimentia* 31: 180-182.

Thorpe, R. S. 1976. Biometric analysis of geographic variation and racial affinities. *Biological Review* 51: 407-452.

Thorpe, R. S. 1979. Multivariate analysis of the population systematics of the ringed snake, *Natrix natrix. Proceedings of the Royal Society of Edinburgh* 78B: 1-62.

Thorpe, R. S. 1987. Geographic variation: a synthesis of cause, data, pattern and congruence in relation to subspecies, multivariate analysis and phylogenesis. *Bollettino di Zoologica* 54: 3-11.

Thorpe, R. S. 1989. Microgeographic variation in the colour pattern of the lizard *Gallotia galloti* within the island of Tenerife. Distribution, pattern and hypothesis testing. *Biological Journal of the Linnean Society of London* 38: 303-322.

Thorpe, R. S. 1991. Clines and cause: Microgeographic variation in the Tenerife gecko (*Tarentola delalandii*). *Systematic Zoology* 40: 172-187.

Thorpe, R. S., Brown, R. P., Day, M., Malhotra, A., McGregor, D. P. and Wuster, W. 1994. Testing ecological and phylogenetic hypotheses in microevolutionary studies. Chapter 8 *In* Egglestone, P. and Vane-Wright, R. *Phylogenetics and ecology*. The Linnaen Society of London.

Thorpe, R. S., McGregor, D. P., Cumming, A. M., and Jordan, W. C. 1994. DNA evolution and colonization sequence of island lizards in relation to geological history: mtDNA, RFLP, cytochrome *b,* cytochrome oxidase, 12s rRNA sequence, and nuclear RAPD analysis. *Evolution* 48: 230-240.

Thorpe, R. S., Black, H. and Malhotra, A. 1996. Matrix correspondence tests on the DNA phylogeny of the Tenerife lacertid elucidates both historical causes and morphological adaptation. *Systematic Biology* 45: 35-343.

Turelli, M., Barton, N. H. and Coyne, J. A. 2001. Theory and speciation. *Trends in Ecology and Evolution* 16: 330-343.

Tweedie, M. W. F. 1983. *The Snakes of Malaya*. Singapore National Printers Ltd., Singapore.

Udvardy, M. D. F. 1975. *A Classification of the Biogeographical Provinces of the World.* IUCN Occasional paper 18. Gland, Switzerland.

Van Valen, L. 1976. Ecological species, multispecies and oaks. *Taxon* 25: 233-239.

Vanzolini, P. E. 1970. Zoologia sistématica, geografia e a origem das éspecies. *Institute Geografico São Paulo. Serie Téses e Monografias* 3: 1-56.

Vawter, L. and Brown, W. M. 1986. Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. *Science* 224: 194-196.

Via, S. 2001. Sympatric speciation in animals: the ugly duckling grows up. *Trends in Ecology and Evolution* 16: 381-390.

Vidal, N. and Lecointre, G. 1998. Weighting and congruence: A case study based on three mitochondrial genes in pitvipers. *Molecular Phylogenetics and Evolution* 9: 366-374.

Viravan, C., Looareesuwan, S. and Koskarn, W. 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., Blecker, M., Reijans, M., Lee, T. V. Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407-4414.

Walsh, P. D. 2000. Sample size for the diagnosis of conservation units. *Conservation Biology* 14: 1533-1537.

Whitmore, T. C. 1975. *Tropical rain forests in the Far East.* Clarendon Press, Oxford.

Whitmore, T. C. 1998. *An Introduction to Tropical Rain Forests, Second edition.* Oxford University Press, New York.

Whittaker, R. J. 1998. *Island biogeography: Ecology, Evolution and Conservation.* Oxford University Press, Oxford.

Wiens, J. J., and Servedio, M. R. 2000. Species delimitation in systematics: inferring diagnostic differences between species. *Proceedings of the Royal Society of London, Series B* 267: 631-636.

Wiens, J. J., and Penkrot. 2002, T. A. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). *Systematic Biology* 51(1): 69-91.

Wiley, E. O. 1978. The evolutionary species concept reconsidered. *Systematic Zoology* 27: 17-26.

Wilkinson, M. 1997. Characters, congruence and quality: a study of neuroanatomical and traditional data in caecilian phylogeny. *Biological Reviews* 17: 423–470.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18: 6531-6535.

Williams, V. and White, J. 1990. variation in venom composition and reactivity in two specimens of yellow-faced whipsnake (*Demansia psammophis*) from the same geographical area. *Toxicon* 28:1351-1354.

Williams, V. and J. White. 1997. Snake venom and snakebite in Australia. Pages 205-218 *In* Thorpe, R. S., Wüster, W. and Malhotra, A., eds. *Venomous Snakes: Ecology, Evolution and Snakebite.* Symposia of the Zoological Society of London 70. Oxford University Press, Oxford.

Wilson, A. C., Cann, R. L., Carr, S. M., George, M., Gyllensten, U. B., Helm-Bychowski, K. M., Iguchi, R. G., Palumbi, S. R., Prager, E. M., Sage, R. D. and Stoneking, M. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. *Biological Journal of the Linnean Society* 26: 375-400.

Wink, M. 1995. Phylogeny of Old and New World vultures (Aves: Accipitridae and Cathartidae) inferred from nucleotide sequences of the mitochondrial cytochrome *b* gene. *Zeitschrift für Naturforschung* 50c: 868-882.

Whitmore, T. C. 1975. *Tropical rain forests in the Far East.* Clarendon Press, Oxford.

Wu, C. –I. 2001. The genic view of the process of speciation. *Journal of Evolutionary Biology* 14: 851-865.

Wüster, W., and Thorpe, R. S. 1990. Systematics and biogeography of the Asiatic cobra (*Naja naja*) species complex in the Philippine islands. Pages 333-

341 *In* Peters, G. and Hutterer, R., eds. *Vertebrates in the tropics*. Museum Alexander Koenig, Bonn.

Wüster, W., Otsuka, S., Malhotra, A. and Thorpe, R.S. 1992. Population systematics of Russell's viper: A multivariate study. *Biological Journal of the Linnaen Society* 47: 99-113.

Wüster, W., Thorpe, R. S. and Puorto, G. 1996. Systematics of the *Bothrops atrox* complex (Reptilia: Sepentes: Viperidae) in Brazil: A multivariate analysis. *Herpetologica* 52(2): 263-271.

Wüster, W., Salomão, M. G., Thorpe, R. S., Puorto, G., Furtado, M. F. D., Hoge, S. A., Theakston, R. D. G. and Warrell, D. A. 1997. Ststematics of the *Bothrops atrox complex*: new insights from multivariate analysis and mitochondrial DNA sequence information. Pages 99-113 *In* Thorpe, R. S., Wüster, W. and Malhotra, A., eds. *Venomous Snakes: Ecology, Evolution and Snakebite.* Symposia of the Zoological Society of London 70. Oxford University Press, Oxford.

Wüster, W., Thorpe, R. S., Salomao, M., Thomas, L., Puorto, G., Theakston, R. D. G. and Warrell, D. A. 2002. Origin and phylogenetic position of the Lesser Antillean species of *Bothrops* (Sepentes, Viperidae): Biogeographical and medical implications. *Bulletin of the Natural History Museum of London* (*Zoology*) 68(2): 101-106.

Young, W. P., Ostberg, C. O., Keim, P., Thorgaard, G. H. 2001. Genetic characterisation of hybridisation and introgression between anadromous rainbow trout (*Oncorhynchus mykiss irideus*) and coastal cutthroat trout (*O. clarki clarki*). *Molecular Ecology* 10: 921-930.

Zabeau, M. and Vos, P. 1993. In *European Patent Office.*

Zamudio, K. R., and Greene, H. W. 1997. Phylogeography of the bushmaster (*Lachesis muta*, Viperidae)—Implications for neotropical biogeography, systematics, and conservation. *Biological Journal of the Linnean Society of London* 62: 421–442.

Zevering, C. E. Moritz, C., Heideman, A., and Sturm, R. A. 1991. Parallel origins of duplications and the formation of pseudogenes in mitochondrial DNA

from parthenogenetic lizards (*Heteronotia binoei*, Gekkonidae). *Journal of Molecular Evolution* 33: 431-441.

Zouros, E., Freeman, K. R., Ball, A. O. and Pogson, G. H. 1992. Direct evidence for extensive paternal mitochondrial DNA inheritance in the marine mussel, *Mytilus*. *Nature* 359: 412-414.

Zuckerkandl, E. and Pauling, L. 1965. Evolutionary divergence and convergence. Pages 97-166 *In* Bryson, V. and Vogel, H. J., eds *Evolving genes and proteins*. Academic Press, New York.

Zwickl, D. J. and Hillis, D. M. 2002. Increased taxon sampling greatly reduces phylogenetic error. *Systematic Biology* 51: 588-598.

Appendix 1

Primer and specimen details for phylogenetic analyses

Appendix 1.1: Primers used in phylogenetic analyses.

MtDNA gene	Primer	Sequence	Reference
cytb	MtA	5'-CTCCCAGCCCCATCCAACATCTCAGCATGATGAAACTTCG-3'	Lenk and Wink, 1997
	MtF	5'-AGGGTGGAGTCTTCTGTTTTTGGTTTACAAGACCAATG-3'	Wink, 1995
	L14910	5'-GAC CTG TGA TMT GAA AAC CAY CGT TGT-3'	Burbrink *et al.*, 2000
	GludG	5'-TGACTTGAARAACCAYCGTTG-3'	Palumbi, 1996
ND4	ND4	5'-CACCTATGACTACCAAAAGCTCATGTAGAAGC-3'	Arèvalo *et al.*, 1994
	LEU	5'-CATTACTTTTACTTGGATTTGCACCA-3'	Arèvalo *et al.*, 1994
12S	L1091F	5'-AAACTGGGATTAGATACCCCACTAT-3'	Kocher *et al.*, 1989
	H1557R	5'-GTACACTTACCTTGTTACGACTT-3'	Kocher *et al.*, 1989
16S	L2510F	5'-CGCCTGTTTATCAAAAACAT-3'	Kocher *et al.*, 1989
	H3059R	5'-CCGGTCTGAACTCAGATCACGT-3'	Kocher *et al.*, 1989

Appendix 1.2: Localities and GenBank accession numbers for sequences used in phylogenetic analysis of *T. sumatranus* group

Sample	Catalogue No.	Geographic Origin	Accession nos			
			Cytb	ND4	12S	16S
T. insularus	B7	Java	AY059568	AY059586	AY059534	AY059550
T. vogeli	B9	Taiwan	AY059574	AY059596	AY059546	AY059562
T. septentrionalis	A100	Nepal	AF171909	AY059592	AY059543	AY059559
T. hageni	B131	West Malaysia	AY371826	AY371868	AY371761	AY371787
T. hageni	B229	West Malaysia	AY371827	AY371867	AY371755	AY371794
T. hageni	B360	Bengkulu, Sumatra	AY371829	AY371862	AY371764	AY371789
T. hageni	B364	Bengkulu, Sumatra	AY371825	AY371863	AY371763	AY371790
T. sumatranus	B347	Sabah, Borneo	AY371823	AY371859	AY371759	-
T. sumatranus	B348	Sabah, Borneo	AY371828	AY371866	AY371760	AY371788
T. sumatranus	B367	Bengkulu, Sumatra	AY371864	AY371824	AY371765	AY371791
T. sumatranus	B368	Bengkulu, Sumatra	AY371865	AY371830	AY371762	AY371792
T. malcolmi	B295	Sabah, Borneo	AY371822	AY371860	AY371758	AY371793
T. malcolmi	B349	Sabah, Borneo	AY371832	AY371861	AY371757	AY371786
T. f. flavomaculatus	B3	Luzon, Philippines	AF171916	AY059584	AY059535	AY059551
T. f. flavomaculatus	B4	Mindanao, Philippines	AY352764	AY352830	AY352796	A7352734
T. f. mcgregori	B290/4	Batanes, Philippines	AY371831	AY371858	AY371756	AY371795
T. schultzei	B210	Palawan, Philippines	AY352756	AY352819	AY352785	AY352725

Appendix 1.3: Localities and GenBank accession numbers for mtDNA sequences used in phylogenetic analysis of *T. popeiorum* group.

Locality	Catalogue No.	GenBank Accession Codes			
		Cytb	ND4	12S	16S
Myanmar	B417 (CAS 216609)	AY371805	AY371845	AY371743	AY371776
	B419 (CAS 222195)	AY371806	AY371841	AY371738	AY371777
	B520 (CAS 205847)	AY371816	AY371855	AY371751	AY371783
north Thailand	B476	AY371809	AY371852	AY371745	AY371782
	A204	AF171902	AY371843	AY371742	AY371784
	A205	AF171906	AY371854	AY371741	AY371767
Laos	B195	AY371799	-	-	-
	B196	AY059571	AY059590	AY059538	AY059554
west Thailand	B34	AY059572	AY059591	AY059542	AY059558
	B52	AY371800	AY371836	AY371754	AY371768
south Thailand (Phang Nga)	B467	AY371807	AY371851	AY371744	AY371781
south Thailand (Thung Song)	A202	AF171904	AY371840	AY371739	AY371770
	A203	AY371796	AY059588	AY059537	AY059553
	A246	AY371820	AY371856	AY371749	-
	B19	AY371804	AY371844	-	AY371779
Cameron Highlands (Malaysia)	A196	AF171888	-	-	-
	A197	AY371808	AY371846	AY371746	AY371773
	B235	AY371812	AY371838	AY371740	-
	B236	AY371819	AY371847	AY371747	-
	B237	AY371813	AY371848	AY371748	-
	B238	AY371814	AY371839	AY371737	AY371774
	B345	AY371811	AY371849	-	AY371775
	B346	AY371810	AY371850	-	-
Bukit Fraser (Malaysia)	B246	AY059570	AY059589	AY059540	AY059556
	B278	AY371821	AY371857	AY371750	AY371780
	B469	AY371817	-	-	-
Tioman	B519	AY371818	AY371853	AY371752	AY371778
west Sumatra	B361	AY371801	AY371837	AY371753	AY371769
Borneo (East Malaysia)	B338	AY371798	AY371835	AY371733	AY371785
	B339	AY371802	-	AY371735	-
	B341	AY371803	AY371834	AY371734	AY371772
	B344	AY371815	AY371842	AY371736	AY371771

Appendix 1.4: Specimens used in mtDNA and AFLP analysis of the *T. albolabris* group

Species	Locality	Catalogue no.	mtDNA	AFLP
T. albolabris	W. Java	B6	*	
		A126	*	*
		B398	*	*
	S. Thailand	A107	*	*
		A129		*
		B20	*	
		A134	*	*
		A130	*	
		A131	*	
	W. Thailand	B32	*	*
		B47	*	
		B50	*	*
		B55	*	*
	S.E. Thailand	A127	*	*
		A133	*	*
	C. Thailand	B21	*	
		B22	*	
		B36	*	
		B38		*
		B39	*	*
		B40	*	*
		B41		*
	N. Thailand	A145	*	
		A146		*
		A147		*
		A148		*
		A227	*	*
		A229	*	
	N.E. Thailand	B98	*	
		B31	*	
		A135		*
		A152	*	*
		A153	*	*
		A154	*	*
		A155		*
		A165	*	
		A184	*	
	S. Vietnam	B68	*	
		B74	*	
		B77	*	
		B116		*
		B117	*	*

Species	Locality	Catalogue no.	mtDNA	AFLP
		B119	*	*
	C. Vietnam	B169	*	*
		B183	*	*
	N. Vietnam	B191	*	
		B192	*	*
		B193	*	*
		A104	*	
	S. Laos	B445	*	*
		B446		*
		B447	*	*
	Hongkong	A157	*	*
	E. Cambodia	B441	*	
	W. Cambodia	B444	*	*
	Myanmar (Mon State)	B410	*	*
	Myanmar (Rakhine State)	B409	*	
	N.W. Myanmar	B424	*	*
		B425	*	*
		B426	*	*
		B427	*	*
		B428		*
		B429	*	*
		B430	*	*
		B431	*	*
T. erythrurus	Myanmar (Yangon div)	A209	*	*
		B412	*	*
		B413	*	
	Myanmar (Rakhine State)	B411	*	
		B414	*	
		B415	*	*
	Bangladesh	B219	*	
		B220	*	*
T. purpureomaculatus	S. Thailand	A83	*	*
	W. Malaysia	B139	*	*
		B141	*	
		B154	*	*
		B225	*	*
		B227		*
	Myanmar (Ayeyarwade div)	B418	*	*
		B435		*
T. andersoni	Andaman Islands (India)	A77	*	
		A79	*	*
		A81	*	*
T. septentrionalis	Nepal	A99	*	
		A100	*	
		B487	*	
T. insularis	E. Java	A108	*	
		A109	*	

Species	Locality	Catalogue no.	mtDNA	AFLP
T. insularis	E. Java	A110	*	*
		A112	*	*
		A116	*	
		A117	*	
	W. Timor (NNT)	B7	*	*
		B8	*	
	Flores (NNT)	A118	*	*
		A119	*	
		A120	*	
		A121	*	
	Sumbawa (Nusa Tenggara)	A88	*	
	Semau (Nusa Tenggara)	A92	*	
	Alor (Nusa Tenggara)	A96	*	
	Wetar (Nusa Tenggara)	A102	*	
	Komodo (Nusa Tenggara)	A122	*	
	Bali (Nusa Tenggara)	B203	*	
T. fasciatus	Tanadjampae Island	B212	*	
T. cantori	Nicobar Islands (India)	A85	*	

Appendix 2

Details of specimens and characters used in morphometric analyses

Appendix 2.1 Specimens used in morphometric analyses

Museum acronyms

BMNH	British Museum of Natural History, London
CAS	California Academy of Sciences, San Francisco
FMNH	Field Museum of Natural History, Chicago
HKV	Historisch Museum Zuid-Kennemerland, Netherlands
IMR	Institute of Medical Research, Kuala Lumpur
KSP	Kinabalu NP Museum of Zoology
LSUHC	La Sierra University Herpetology Collection, California
MCZ	Museum of Comparative Zoology, Harvard
MHNG	Museum d'Histoire Naturelle de Geneva, Switzerland
NMBE	Naturhistorisches Museum Basel, Switzerland
NMP	National Museum of the Philippines, Manilla
NMW	Naturhistorisches Museum Wien, Austria
PH	Perhelitan, Kuala Lumpur, Malaysia
PCB	Piboon Collection Bank, Thailand
PCGV	Private Collection of Gernot Vogel
QSMI	Queen Saovabha Memorial Institute, Bangkok
RMNH	Rijksmuseum van Natuurlijke Histoire, Netherlands
SMF	Natur-Museum und Forschungs-institute Senckenberg, Germany
ZRC	Raffles Museum of Biodiversity Research, National University of Singapore

AFS/KLS/RTV indicate wild caught and captive specimens examined under anaesthesia.

T. popeiorum

Northeast India:	BMNH 72.4.17.137; BMNH 72.4.17.377.
North Thailand:	AFS96.3; BMNH 62.7.28.1; BMNH 62.7.28.4; BMNH 1937.2.1.24; BMNH 1937.2.1.25; FMNH 178655; FMNH 178656; FMNH 178658; FMNH 178659; NMW 27947:1; QSMI S1.
West Thailand:	AFS98.16; AFS98.34; RMNH 16715.
Myanmar:	BMNH 1940.39.43; CAS 205847; CAS 216609; CAS 222195; NMW 23923:1; NMW 23923:2.
Laos:	FMNH 258950; HKV 64135.
South Thailand:	AFS97B.13; AFS98.1; QSMI 4 X NO REF; QSMI 13.1; QSMI 13.2; QSMI 17; QSMI JAR 7; QSMI JAR 50.1; QSMI JAR 50.2; QSMI JAR 50.3; QSMI 13.1; QSMI 13.2; QSMI 17; QSMI JAR 7; QSMI JAR 50.1; QSMI JAR 50.2; QSMI JAR 50.3; PCGV 223; PH NO #; PSGV 34.
West Malaysia:	BMNH 1967.2289; BMNH 1974.4995; BMNH 1974.4997; BMNH 1974.4999; BMNH 1974.5000; BMNH (MLD 2007); KLS00.001;

	KLS01.103; LSUHC 4809; QSMI NO REF; ZRC 2.2889; ZRC 2.2891; ZRC 2.2892; ZRC 2.3493; ZRC 2.5361.
Borneo (E. Malaysia):	FMNH 233155; FMNH 243942; NMBA 21026; NMBA 21026; KLS01.104; KLS01.114; KLS01.116; KLS01.117; KLS01.122; KLS01.121; MCZ 43612; MCZ 43614.
Sumatra:	NMBE 210b/197; NMBE 210a/198; NMW 23910:1; NMW 23910:2; NMW 23910:3; NMW 23910:4; NMW 23910:5; NMW 23917:1; NMW 23917:2; NMW 23917:3; NMW 23917:4; NMW 23917:5; NMW 23917:6; NMW 23917:7; NMW 23917:8; NMW 23917:9; NMW 23917:10; SMF 21226.
Cameron H'lands:	AFS00.12; AFS00.13; AFS00.14; AFS00.15; CAS-SU 8863; ZRC2.2884; ZRC 2.2886; ZRC 2.5164.

T. hageni

South Thailand:	QSMI 291190; QSMI 11190-19; MHNG 2072.87; MHNG 2072.89; PCB 19; AFS 9815; AFS97 B20; NMBA 22401; NMBA 22401.
Singapore:	MHNG 1403.95; MHNG 1403.96; BMNH80.9.10.7.
W. Malaysia:	MCZ 132799; MHNG 1403.95; PH unlabelled x4; PH no.79; PH no.76; PH no.134; FMNH 183787; FMNH 183788; FMNH 143948; AFS0005; AFS 99B5; IMR 103649; IMR 104270; IMR 105684; IMR 95995; BMNH 1967-2290; BMNH1936.9.12.5.
Nias:	NMBA 9179; NMW 28160.4; NMW 28160.3; NMW 28160.2; NMW 28160.1; NMW 28157.1; NMW 28156.1; BMNH84.1.8.46; BMNH84.1.8.47; BMNH84.12.31.13; BMNH84.12.31.14
Siberut:	BMNH1977.1237; BMNH1979.267; BMNH1979.268; ZRC2.2938; ZRC2.2937; ZRC2.2936.
Sumatra:	NMBA 5108; NMW 23909.1; NMW 23909.2; NMW 23909.3; NMW KLS 0207; RTV 31; RTV 33; RTV 35.

T. sumatranus

South Thailand:	BMNH 1936.9.12.3
Borneo (E. Malaysia):	FMNH 138690; FMNH 138689; FMNH 148829; FMNH 148830; FMNH 138687; FMNH 239948; FMNH 239959; FMNH 243943; FMNH 230064; FMNH 230063; FMNH 239952; FMNH 239950; FMNH 239958; FMNH 239957; FMNH 239947; FMNH 138688; K-SP04361; K-SP NO; K-SP NO; KLS 01-119; KLS 01-129; RMNH 4696A; RMNH 4696B; BOGOR 2647; BOGOR 2138; BOGOR 1052; BOGOR 1340; BOGOR 2138-2; BOGOR 1035; BMNH1978.1879.
Sumatra:	NMW28159.1; NMW28158.2; NMW28159.4; RMNH 1583; RMNH 4695-4; RMNH 4695-30; BOGOR 2245; BOGOR 1035; BOGOR 2180; BOGOR 457; BOGOR 2166; RTV 31; NMW 28159.2; NMW 23909.4; NMW28158.1.

T. malcolmi:

Sabah:	K-SP04161; K-SP04028; K-SP04089; K-SP04359; K-SP04068; K-SP04055.

T. f. flavomaculatus

Luzon:	FMNH 15044; FMNH 15040; NMP 2096; NMP 2052; NMP 2094; CAS 61545; CAS 61551; CAS 61155; USNM 56014; AFS 02-33; AFS 02-34; AFS 02-35; AFS 02-55; AFS 02-66; AFS 02-68; AFS 02-69; AFS 02-70.
Mindanao:	CAS 23444; USNM 37873; CAS 15356; FMNH 53563; FMNH 53562; FMNH 15354; FMNH15353.

T. f. mcgregori

Batan island:	USNM 266636; USNM 266619; USNM 266613; USNM 266614; USNM 266635; USNM 121408; USNM 266615; AFS 02-25; AFS 02-30; AFS 02-51; AFS 02-54; AFS 02-56; AFS 02-57; AFS 02-59.

T. schultzei

Palawan:	CAS 28532; FMNH 53560; CAR2268; NMP; FMNH 53561; CAS 28533; USNM 37871; NMP 6452; NMP 6424; NMP; FMNH 15045; CAR2266.

Appendix 2.2: Characters used in morphometric analyses.

(A) Scalation:

No. of ventral scales, first ventral identified according to the method of Dowling (1951)

No. of scale rows anterior to the vent

No. of supralabial scales (average on left and right hand sides)

No. of sublabial scales (average on left and right hand sides)

No. of postocular scales

No. of preocular scales

No. of scales bordering the supraocular scales, excluding the preoculars and postoculars

Minimum no. of scales separating the supraocular scales

Maximum no. of scales separating the supraocular scales

No. of sutures dividing supraoculars

No. of scales between nasal scale and shield bordering the pit anteriorly

No. of internasal scales

Minimum no. of scales separating third supralabial and subocular scale

Minimum no. of scales separating fourth supralabial and subocular scale

Minimum no. of scales separating fifth supralabial and subocular scale

Minimum no. of scales bordering suboculars, excluding the preoculars and postoculars

Keeling of temporal scales

Keeling of scales on the back of the head

No. of scales between first ventral and anterior genial shields

No. of scales between edge of mouth and first ventral scale, including last sublabial

(B) Scale reduction formula: Scale reductions along the body were recorded as the number of the ventral scale (VS) or subcaudal scale (SC) opposite which they were situated and the dorso-ventral position (DV) of the merging scale rows. These were transformed to percentage ventral scale (%VS) or caudal scale (%CS) position prior to analysis, to compensate for variation in ventral and subcaudal scale number.

%VS position of reduction from 31 to 29 body scale rows
%DV position of reduction from 31 to 29 body scale rows
%VS position of reduction from 29 to 27 body scale rows
%DV position of reduction from 29 to 27 body scale rows
%VS position of reduction from 27 to 25 body scale rows
%DV position of reduction from 27 to 25 body scale rows
%VS position of reduction from 25 to 23 body scale rows
%DV position of reduction from 25 to 23 body scale rows
%VS position of reduction from 23 to 21 body scale rows
%DV position of reduction from 23 to 21 body scale rows
%VS position of reduction from 21 to 19 body scale rows
%DV position of reduction from 21 to 19 body scale rows
%VS position of reduction from 19 to 17 body scale rows
%DV position of reduction from 19 to 17 body scale rows
%VS position of reduction from 17 to 15 body scale rows
%DV position of reduction from 17 to 15 body scale rows
%SC position of reduction from 12 to 10 tail scale rows
%DV position of reduction from 12 to 10 tail scale rows
%SC position of reduction from 10 to 8 tail scale rows
%DV position of reduction from 10 to 8 tail scale rows
%SC position of reduction from 8 to 6 tail scale rows
%DV position of reduction from 8 to 6 tail scale rows
%SC position of reduction from 6 to 4 tail scale rows
%DV position of reduction from 6 to 4 tail scale rows

(C) Colour pattern:

Presence of stripe on dorsal scale row one (recorded as 0 = absent, 1 = indistinct, 2 = distinct)
No. of scale rows involved in stripe
Presence of postocular stripe (recorded as 0 = absent, 1 = indistinct, 2 = distinct)
No. of scale rows involved in postocular stripe
No. of scales above lip covered by ventral colour
Presence of dark edges body scales (recorded as 0 = none, 1 = narrow, 2 = broad)
No. of spots on the dorsal surface
Mean no. of scales covered by the three largest dorsal spots
Proportion of the first scale row covered by the light area
No. of bands on body
Mean width (in no. of scales) of three half bands at 50% VS length
Mean width (in no. of scales) of three intra-halfband gaps at 50% VS length
% of ventral scales with darker pigmentation

(D) Body dimensions: These were measured on the right side of the head unless damaged, in which case measurements made on the left side.

Snout to vent length, between tip of snout and cloaca
Tail length, measured between first subcaudal and tip of tail
Width of head between the outer edges of the supraoculars
Width of head at the widest point between the jaw bones
Length of head between tip of snout and posterior edge of the lower jaw bone
Diameter of eye measured between outer edges of surrounding scales
Distance between eye and nostril from anterior edge of preoculars to inner edge of nostril
Distance between eye and pit, from anterior edge of preoculars to inner edge of pit
Distance between eye and nostril, between outer edges
Width of internasals at widest part
Width of supraoculars at widest part
Length of supraoculars
Ratio of anterior margin of the rostral scale to the posterior margin

Appendix 2.3: Characters used for multivariate analysis of *T. sumatranus* & *T. hageni*. * indicates significance value p=<0.05 (ANOVA).

Characters	Males	Females
No. of ventral scales	-	*
No. of subcaudal scales	-	*
%VS position of reduction from 21 to 19 body scale rows	*	*
%VS position of reduction from 19 to 17 body scale rows	-	*
%DV position of reduction from 19 to 17 body scale rows	-	*
%VS position of reduction from 17 to 15 body scale rows	*	*
%CS position of reduction from 14 to 12 tail scale rows	-	*
%DV position of reduction from 14 to 12 tail scale rows	-	*
%CS position of reduction from 10 to 8 tail scale rows	-	*
% DV position of reduction from 10 to 8 tail scale rows	-	*
%CS position of reduction from 8 to 6 tail scale rows	*	*
%CS position of reduction from 6 to 4 tail scale rows	-	*
No. of supralabial scales	*	*
No. of sublabial scales	*	*
No. of scales bordering the supraocular scales	*	*
Minimum no. of scales separating the supraocular scales	*	*
Maximum no. of scales separating the supraocular scales	*	*
No. of internasal scales	*	*
No. of scales separating the fourth supralabial scale form the subocular scale	*	*
No. of scales separating the fifth supralabial scale form the subocular scale	*	*
No. of scales contacting the suboculars, excluding the preoculars and postoculars	*	*
Average no. of scales between the first ventral scales and the anterior genial scales	*	*
No. of scales between the last sublabial scales and first vental scales	*	*
Presence of stripe on dorsal scale row one	*	-
No. of scale rows involved in stripe	*	-
Presence of postocular stripe	-	*
No. of scale rows involved in postocular stripe	-	*
Presence of dark edging on body scales	*	*
No. of bands on body	*	*
Mean no. of scales of three half bands on body	-	*
Mean no. of scales between three half bands on body	-	*
32. Presence of dark edging on head scales	*	*

Appendix 2.4: Characters included in multivariate analysis of *T. sumatranus* group *indicates significant between-OTU variation (p = <0.05, ANOVA).

Characters	Males	Females
No. of pairs of subcaudal scales	*	*
%VS position of reduction from 17 to 15 body scale rows	*	*
%CS position of reduction from 10 to 8 tail scale rows	*	*
%CS position of reduction from 8 to 6 tail scale rows	*	*
%CS position of reduction from 6 to 4 tail scale rows	*	*
No. of supralabial scales	*	*
No. of sublabial scales	*	*
No. of scales bordering the supraocular scales	*	*
Minimum no. of scales separating the supraocular scales	*	*
Maximum no. of scales separating the supraocular scales	*	*
No. of internasal scales	*	*
No. of scales separating the fourth supralabial scale form the subocular scale	*	*
No. of scales separating the fifth supralabial scale form the subocular scale	*	*
No. of scales contacting the suboculars, excluding the preoculars and postoculars	*	*
Presence of stripe on dorsal scale row one	*	*
No. of scale rows involved in stripe	*	*
Presence of postocular stripe	*	*
No. of scale rows involved in postocular stripe	*	*
Presence of dark edging on body scales	*	*
No. of bands on body	*	*
Mean no. of scales of three half bands on body	-	*
Mean no. of scales between three half bands on body	-	*
Presence of dark edging on head scales	*	*

Appendix 2.5: Characters included in multivariate analysis of *T. popeiorum* group*indicates significant between-OTU variation (p = <0.05, ANOVA).

Character	Males	Females
No. of ventral scales	*	*
No. of pairs of subcaudal scales	*	-
No. of supralabial scales	*	*
No. of sublabial scales	*	*
No. of postocular scales	*	-
No. of scales bordering the supraocular scales	*	*
Minimum no. of scales separating the supraocular scales	-	*
Maximum no. of scales separating the supraocular scales	*	*
No. of scales between nasal scale and shield bordering the pit anteriorly	-	*
No. of internasal scales	*	*
Minimum no. of scales separating third supralabial and subocular scale	*	*
Minimum no. of scales separating fourth supralabial and subocular scale	*	-
Minimum no. of scales separating fifth supralabial and subocular scale	-	*
Minimum no. of scales bordering suboculars, excluding the preoculars and postoculars	*	*
Keeling of temporal scales	*	-
Keeling of scales on the back of the head	*	-
No. of scales between first ventral and anterior genial shields	*	*
No. of scales between edge of mouth and first ventral scale, including last sublabial	*	*
%VS position of reduction from 23 to 21 body scale rows	*	-
%DV position of reduction from 23 to 21 body scale rows	*	-
%VS position of reduction from 21 to 19 body scale rows	*	*
%VS position of reduction from 19 to 17 body scale rows	*	*
%DV position of reduction from 19 to 17 body scale rows	*	-
%VS position of reduction from 17 to 15 body scale rows	*	*
%SC position of reduction from 12 to 10 tail scale rows	-	*
%SC position of reduction from 10 to 8 tail scale rows	-	*
%SC position of reduction from 8 to 6 tail scale rows	*	*
Presence of stripe on dorsal scale row one	*	*
No. of scale rows involved in stripe	*	*
Presence of postocular stripe	*	*
No. of scale rows involved in postocular stripe	*	*
No. of scales above lip covered by ventral colour	*	*
No. of spots on the dorsal surface	*	*
Mean no. of scales covered by the three largest dorsal spots	*	*
Proportion of the first scale row covered by the light area	*	*
No. of bands on body	*	-
Mean no. of scales between three half bands on body	*	-
Width of head between the outer edges of the supraoculars	*	-
Diameter of eye measured between outer edges of surrounding scales	-	*
Distance between eye and pit, from anterior edge of preoculars to inner edge of pit	-	*
Width of internasals at widest part	*	-

Appendix 3

CD ROM

3.1 *T. sumatranus* group morphometric data

3.2 Sequence alignment for *T. sumatranus* group 12S and 16S RNA genes