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Species delimitation and identification in morphologically cryptic Asian pitvipers

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SPECIES DELIMITATION AND IDENTIFICATION IN MORPHOLOGICALLY CRYPTIC ASIAN PITVIPERS

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SUMMARY

The Asian group of pitvipers represent the quintessential cryptic species. Their complex evolutionary histories and biogeography have resulted in convergent and parallel evolution of morphological characters to the extent that cryptic morphs can be indistinguishable across species and genera. Their classification is highly relevant to modern taxonomy as they present challenges even for contemporary taxonomic methods. Moreover, they inhabit biodiversity hotspots such as southeast Asia and, as venomous species, are also a cause for medical concern in the region to a large extent. The issues addressed in this thesis can be broadly divided into (i) incorporation and evaluation of a new molecular marker and multiple new analysis methods, and (ii) species identification and delimitation in Asian green pitvipers (all former members of *Trimeresurus*).

DNA barcoding, the latest and the most contentious addition to the species debate, offers the simplicity of using a single gene sequence for resolving taxonomies, especially in the case of cryptic taxa. In Chapter 2, the uses of DNA barcoding for species delimitation and identification in cryptic reptiles was explored using Asian green pitvipers. Three closely related and especially cryptic genera, *Viridovipera, Parias* and *Popeia,* were chosen to measure the success of the concepts and protocols of barcoding such as ease of amplification of the barcode gene Cytochrome Oxidase I (COI), obtaining species specific COI barcodes, suitability of the barcode gap for species delimitation, and the congruence of results across phylogenetic methodologies. This study showed that although COI is a useful gene for species identification, the application of a barcode gap for species delimitation may not be straightforward across genera and results could vary depending on the analysis methods used, suggesting that a strong taxonomic framework is key to the success of barcoding.

In situations where two sources of evidence present contradicting results, multiple sources of evidence become a necessity for the identification and definition of new species. *Cryptelytrops macrops,* one of twelve species within the genus, has long been suspected to be a cryptic species complex. Mitochondrial DNA (mtDNA) phylogenies

have suggested the presence of three genetically distinct clades within *C. macrops*. However, morphological analysis failed to robustly resolve corresponding species clusters. To resolve this incongruence, nuclear evidence from Amplified Fragment Length Polymorphisms (AFLP) was used in Chapter 3 to estimate the degree of reproductive isolation between putative species and show that the three lineages were indeed distinct from each other. Being multilocus dominant markers, AFLP genotypes also present conceptual caveats for the application of some of the widely-used methodologies. Hence the data was analysed using a suite of six methodologies, some of which are new and some widely-used, and the results were compared. The multivariate and phylogenetic methods proved to be more sensitive for resolving species clusters. The effect of choice of analysis methods on deriving biological meaningful inferences from AFLPs was highly apparent in this study.

The white-lipped pitviper, *Cryptelytrops albolabris,* has been investigated several times over the past century. Large-scale DNA phylogenies have also demonstrated that *C. albolabris* is closely related to a number of other species in the genus. Two former subspecies (*C. a. septentrionalis, C. a. insularis*) have since been raised to species status as they are paraphyletic with respect to other species such as *C. purpureomaculatus* and *C. cantori*. However, no detailed morphological analysis of this diversity has been presented before. In Chapter 4, morphological data for specimens from the entire range of *"albolabris",* representing five recognized species, was analysed to provide evidence for the presence of morphotypes which correspond to existing taxonomic designations, and to verify the species' ranges. Molecular analysis of four mtDNA genes was also performed to provide a genetic basis of species delimitation and range definition. The results confirm the existence of morphotypes for all named species except *C. purpureomaculatus* and a description of their range is provided. Both genetic and morphological evidence suggest that there may be more than one distinct evolutionary lineage within *C. albolabris*, indicative of the presence of cryptic species. Areas that require further investigation to clarify the taxonomy of *Cryptelytrops* are identified.

CONTENTS

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This thesis is the result of collaborations with: Dr. Anita Malhotra (AM), Prof. Roger S. Thorpe (RST), Dr. Bryan L. Stuart (BLS), Dr. Simon Creer (SC), and Dr. Karen Dawson (KD). All samples used in this study are part of the tissue inventory created over the past 15 years through the combined efforts of members of the Molecular Ecology and Evolution of Reptiles Unit, AM, RST, SC, KD, and BLS, Dr. Catherine Wuster, and Dr. Guo Peng. Along with these, private and museum collections were requested as required by individual projects. For the morphometric analysis, morphological character data which was recorded by AM over the past 20 years was used.

PUBLICATIONS

Published

1. Two new species of pitviper of the genus *Cryptelytrops* Cope 1860 (Squamata:Viperidae: Crotalinae) from southeast Asia. 2011. Malhotra, A., Thorpe, R.S., **Mrinalini**, Stuart, B.L. Zootaxa. 2757:1-23.

   (Submitted as Appendix 4. This publication is the result of AFLP analysis undertaken by Mrinalini, field-work and morphological analysis by AM. Supervision: AM, RST, and SC).
2. Mitochondrial DNA analysis reveals a new member of the Asian pitviper genus *Viridovipera* (Serpentes: Viperidae: Crotalinae). 2008. Dawson. K., Malhotra, A., Thorpe, R.S., Guo, P., **Mrinalini**, Ziegler, T. Molecular Phylogenetics and Evolution. 49:356-361.

   (Submitted as Appendix 5. MtDNA analysis for the non-*V. truongsonensis* species of this publication was undertaken by Mrinalini, morphological work and data analysis by KD. Supervision: AM, RST, and SC).

Under Review

1. Species delimitation using dominant markers: A comparative study of analysis methods using cryptic species of pitvipers (*Cryptelytrops macrops*). 2011. Submitted to Systematic Biology. **Mrinalini**, Thorpe, R.S., Creer, S., Lallias, D., Stuart, B.L., Malhotra, A.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

1.1.1 The Concept Of Species

Species delimitation and identification are key aspects of evolutionary biology and fundamental prerequisites for an organized understanding of biological systems and their functions. Over the last five decades or so, there have been many attempts to clarify what comprises a species (Mayden reviewed 22 species concepts in 1997 and Wilkins listed 26 species concepts in 2002). The main obstacles for arriving at a universally agreeable definition of species have been the complex nature of species, making it difficult to circumscribe it as an entity, and the failings of all species concepts in one way or another. Even the most widely accepted Biological Species Concept (Mayr 1942) has its limitations in that it is applicable only to extant, sexually reproducing organisms, which makes assigning species status to allopatric populations questionable and demonstrating reproductive isolation in the case of sympatric populations difficult (Zink 1996; Boggs 2001).

The Phylogenetic Species Concept (PSC) resolves some of these drawbacks by defining species as the smallest cluster of organisms which is diagnosable by the sharing of unique combination of character states (Eldredge and Cracraft 1980; Nelson and Platnick 1981; Nixon and Wheeler 1990, 1992a; Wheeler and Platnick 2000). PSC addresses the species question from an evolutionary perspective using phenetics, cladistics, and/or statistical likelihoods (Mallet 2007), removing the direct association of reproductive isolation. It has thus becoming one of the most practical and widely-applied species concepts. Some workers have also proposed delimiting populations into Evolutionary Significant Units (ESU) and Management Units (MU) using genetic information (Ryder 1986; Moritz 1994). Establishing reciprocal monophyly in the case of mitochondrial DNA (mtDNA) phylogenies and the level of divergence in the case of nuclear alleles can be useful for the purpose of prioritizing taxa for conservation purposes (Ryder 1986; Moritz 1994). The uses of mtDNA and nuclear DNA (nDNA) data are further discussed in sections 1.2 and 1.3 of this chapter.

Given that most alternate species concepts agree that species are independently evolving units, a general concept to unify diverse contemporary views has been formulated by de Quieroz (2005). This recognizes species as independently evolving metapopulation lineages, which can be differentiated by applying any species concept, and clarifies the species problem into conceptual and species delimitation areas (de Queiroz 2005; 2007). The general unified concept, although not a novel or real species concept, removes drawbacks associated with species delimitation in individual species concepts and is the most comprehensive solution to the species problem in recent times.

1.1.2 Species Delimitation And Identification In Cryptic Taxa

For the planning and management of species conservation and their habitat protection, a comprehensive knowledge of the underlying biodiversity of the given region is a key requirement. Over the past two decades, rising concerns for endangered worldwide biodiversity, together with the development of new and useful tools for speciation research, have led to an exponential increase in the number of studies on hidden or morphologically cryptic species. Consequently, the rate of recovery of cryptic species has also increased to 15,000 – 20,000 per year (Polaszek 2005). In the majority of these cases, it can usually retrospectively be found that morphological stasis, parallel and convergent evolution of phenotypic traits, and/or mimicry are confounding factors for species identification and the establishment of species boundaries (e.g. Duarte et al. 2008; Ferreira et al. 2010; Smith et al. 2011). Evolutionary processes such as ecological adaptation, natural and sexual selection usually drive the persistence of interspecific morphological conservativeness. In such cases, genetic tools play a key role in distinguishing between cryptic morphs, and DNA sequencing technology has contributed significantly by facilitating the routine use of sequence-based phylogenies, in tandem with evolutionary hypotheses, for the discovery of genetically distinct lineages that could be present in such groups of organisms (Hebert et al. 2003a; Wilson 2003; Bickford et al. 2007; Janzen et al. 2009).

Quite often, these discoveries result from investigations of genetic structure of "species". For example, "geographically widespread species" of Southeast Asian

forest frogs and birds (Bain et al. 2003; Stuart et al. 2006; Lohman et al. 2010), South American neotropical frogs (Elmer et al. 2007), and Central American cichlids (Barluenga and Meyer 2004), have repeatedly been found to consist of genetically distinct species complexes. Morphological crypsis has been found to be a feature even in higher vertebrates such as orangutans from Borneo and Sumatra, which are capable of interbreeding to producing fertile offspring, but are genetically highly differentiated (Locke et al. 2011), and sub-Saharan giraffes which have been found to consist of multiple, highly distinct genetic lineages (Brown et al. 2007). In some of these, lineage divergences have been thought to have occurred as a result of allopatric or parapatric speciation events, and in others, through more complex and subtle mechanisms of sympatric diversification, such as high levels of trophic and ecological niche specialization, sexual selection, and assortative mating which are becoming increasingly commonly documented especially in cryptic speciation. In some extreme cases such as the African butterfly fish, extensive interspecific morphological stasis has been found to be prevalent despite tens of millions of years of allopatric divergence, due to stabilizing selection, ecological niche conservatism, genetic and developmental constraints (Erwin 2007; Lavoue et al. 2011).

Among the non-genetic tools for cryptic species research, multivariate morphometrics has been extensively useful (e.g. Klimov et al. 2006; Alencar et al. 2009). Recently, geometric morphometric analysis of landmarks and shapes has emerged as a more powerful method for revealing fine differentiations in cryptic morphology (e.g. Claude et al. 2004; Milankov et al. 2009; Sztencel-Jablonka et al. 2009). Often, two or more independent sources of evidence are used together, such as a combination of molecular phylogenetic inferences and morphological character analyses, for deriving robust taxonomic inferences (Tautz et al. 2003). Other non-genetic tools such as bioacoustics (e.g. Kingston et al. 2001; Ferreira et al. 2010), behavioural differences (e.g Topfer-Hofmann et al. 2000), and biochemical studies (e.g. Griffiths et al. 2011) have also been very useful for species delimitation in certain groups of animals. An introduction to some of the most useful taxonomic tools and methods that are relevant to the study group in this thesis is presented in the following sections.

1.2 TAXONOMIC TOOLS

1.2.1 MtDNA

Organellar DNA in eukaryotic cells includes the mitochondrial genome, a circular molecule with bacterial origins, which in vertebrates generally consists of 37 genes (13 protein-encoding genes, 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes) and a non-coding region. Animal mtDNA has high copy number, and introns and pseudogenes are rare, which usually makes gene amplification fairly straight-forward and reduces the likelihood of phylogenetic errors (Avise 1994; Saccone et al. 1999). Most importantly, unlike nDNA, it does not recombine and is transmitted as a single unit from parent to offspring usually through a maternal mode of inheritance (Avise 1994). Also, the rate of nucleotide evolution of mtDNA is higher than nDNA as its DNA repair mechanism is slower than that of nDNA, allowing for the build-up of neutral mutations (Brown et al. 1979). Hence, lineage sorting occurs faster in mtDNA, with the effective population size for achieving reciprocal monophyly being several times lower than nDNA where ancestral polymorphisms can persist (Moore 1995; Hudson and Turelli 2003; Rosenberg 2003; Kubatko et al. 2011). This renders mtDNA sequence data suitable for systematic studies even at lower (inter-specific and intra-specific) taxonomic levels and mtDNA a highly efficient marker for use in phylogenetic methods to trace genealogical evolutionary histories and divergences in organisms (Avise 1989).

However, mtDNA divergence levels are not necessarily indicative of divergence rates or patterns in nuclear genes (Hudson and Turelli 2003). The maternal mode of inheritance will result in phylogenies that reflect patterns of female-only gene flow and dispersal (Avise 1994). Its inheritance as a single-linkage group provides only one independent estimate of the species tree while nuclear gene trees, obtained via sequencing nuclear genes from distinct chromosomes, could provide independent estimates of species phylogeny (Moore 1995). In some cases, mtDNA could be heterozygous and heteroplasmic (Blaxter 2004) and multiple sampling of the same individual may become necessary, and occasional mitochondrial transfer between sister species may also cause problems for correct species diagnoses (Tautz et al. 2003). MtDNA cannot be ruled out from being subject to selective sweeps due to its

role in oxidative metabolism (Galtier et al. 2009). Allele fixation has sometimes been implicated in the low mtDNA nucleotide diversity as compared to nDNA (Rato et al. 2010). Other potential limitations include selection acting on any mtDNA nucleotide and paralogy due to transfer of mtDNA genes to the nucleus (Tautz et al. 2003). The effects of these drawbacks can be reduced by multilocus sampling and using multiple analysis methods (Mallet and Willmott 2003; Blaxter 2004).

1.2.2 MtDNA Genes

Different parts of the mtDNA genome evolve at different rates and are useful for resolving divergences at different taxonomic levels (Moritz et al. 1987; Avise 1994). In the past, cytochrome *b* (Cytb) has been the most widely-used animal mtDNA marker, having been successfully employed for a variety of phylogenetic and phylogeographic studies in animals (Farias et al. 2001). A protein-encoding gene containing overall conservative and variable regions, Cytb has both slow and rapidly evolving codon positions, and the third codon is especially informative for studying closely-related species (Farias et al. 2001). NADH dehydrogenase 4 (ND4), also a protein-encoding gene, has been found to have very high nucleotide and amino acid substitution rates even at intra-specific and population levels, and has been recommended as a useful gene for resolving cryptic species (Blouin et al. 1998). The Cytochrome Oxidase I gene (COI), has base substitution rates similar to that of Cytb, but the amino-acid sequence evolution is slower, and it therefore provides a deeper and greater range of phylogenetic signal than any other mtDNA gene for assigning taxa to both higher and lower taxonomic groups (Hebert et al. 2003a; Hebert et al. 2003b). COI is thus rapidly becoming the mtDNA gene of choice for systematic studies as a result of the DNA barcoding project which is further discussed in Section 1.2.3 (Hebert et al. 2003a).

The patterns of mtDNA nucleotide evolution vary even between different groups of animals, and this is particularly relevant and interesting in the case of snakes. A recent study has established that the snake mitochondrial genome has the highest evolutionary rate in vertebrates, with the protein-encoding regions having undergone episodic bursts of evolution and rapid remodelling of metabolic proteins to adapt to drastic changes in life-style (Jiang et al. 2007; Castoe et al. 2008; Douglas and Gower

2010). Hence molecular evolution of protein-encoding genes has been found to be dramatically accelerated as compared to the rRNA genes in snakes (Jiang et al. 2007; Douglas and Gower 2010).

While it has to be acknowledged that high substitution rates in the protein-coding genes could present homoplasy issues for phylogenetic reconstructions, a comparative analysis of different gene regions in green pitvipers has shown that Cytb and ND4 offer significant advantages, as opposed to rRNA subunits and nuclear introns, for successfully resolving the taxonomy at lower levels (Creer et al. 2003a). On the other hand, the rRNA subunit genes 12S and 16S, which evolve more slowly, are generally more useful for resolving divergences at higher taxonomic levels (Moritz et al. 1987) and have been employed to infer higher-level snake phylogenies (Heise et al. 1995).

1.2.3 DNA Barcoding

Paul Hebert and colleagues (Hebert et al. 2003a) put forth a proposition for using "DNA barcoding" as a taxonomic tool and global bioidentification system for all animal species. This scheme involved using nucleotide sequences from COI to generate unique, globally-applicable genetic identification tags or "barcodes" for each species. COI was chosen from among other mtDNA genes as it amplifies easily in most animal phyla, with robust "universal" primers being available (Folmer et al. 1994; Zhang and Hewitt 1997; Hebert et al. 2003a), and also as it provides a large range of phylogenetic signal, enabling the detection of sequence divergences at both higher (e.g. phylum, order) and lower (e.g. species) taxonomic levels (Hebert et al. 2003a). More specifically, COI is purported to be a suitable gene for species delimitation purposes as its considerably higher inter-specific sequence divergence, compared to a low intraspecific variability, has the potential to allow differentiation of individuals among and within species, using genetic distances alone (Hebert et al. 2003a; Hebert et al. 2003b).

Although a molecular basis for taxonomy has been advocated in the past (Tautz et al. 2002, 2003), DNA barcoding has attracted intense and wide-spread criticism for being touted as a replacement for traditional and morphology-based taxonomy (Will and Rubinoff 2004; Ebach and Holdrege 2005; Wheeler 2005). The main contention,

however, has been against the use of a single locus to define and circumscribe what comprises a species (Blaxter 2003; Blaxter 2004; Moritz and Cicero 2004). Doubts have also been expressed about the success of barcoding in tropical environments, with higher (and possibly older) diversity, in its ability to differentiate speciose taxa (Moritz and Cicero 2004) and to resolve recently-diverged lineages, closely-related taxa, and detect new species (Mallet and Willmott 2003; Stoeckle 2003). The use of a global "barcode gap" (a threshold difference between intra-specific and inter-specific sequence variation) as a rule of thumb to assign taxa to species has been questioned (Meyer and Paulay 2005), including whether the barcode gap decreases with broader geographical range of sampling (Robinson et al. 2009). In addition, under-representation of taxa within species and incomplete coverage of genera in initial studies could become a fundamental limitation for its use in species identification (Moritz and Cicero 2004). Further, issues such as co-amplification of nuclear mitochondrial pseudogenes (numts) (Song et al. 2008), multiple paralogous mitochondrial copies of COI (Gilmore et al. 2009), and haplotype sharing of COI barcodes between species (Ward 2009) have also been raised as practical difficulties that could make DNA barcoding less than straight-forward.

Despite this, the barcode movement has rapidly gained momentum through the establishment of the Consortium for the Barcode of Life (CBOL) and the Barcode of Life Data (BOLD) System (Ratnasingham and Hebert 2007), creating a niche for COI barcoding as a speedy, easy, and cheap molecular tool for species identification. Numerous arthropods have been barcoded (e.g. Barrett and Hebert 2005; Burns et al. 2008; Sheffield et al. 2009), and barcoding of fishes (e.g. Ward et al. 2005), birds (e.g. Kerr et al. 2007), and mammals (e.g. Lorenz et al. 2005; Clare et al. 2007) has been undertaken across the world, with largely successful results. Although some barcoding studies have been performed on reptiles (e.g. Naro-Maciel et al. 2010), the absence of a reptile barcoding campaign on BOLD Systems is surprising given that there are specialist campaigns such as "Barcoding Earthworms". Moreover, barcode records of snakes are noticeably scant, with less than 10% (252 in total) of the world's species on BOLD Systems (as on May 1st, 2011). Of these, a majority are non-venomous species from the family Colubridae (189 species), and only 39 species (32 from Viperidae and 7 from Elapidae) are venomous. Given that venomous snakes are

medically important and venom variation between snake species has direct implications for antivenom production (Fry et al. 2003), this deficiency is rather striking. Furthermore, DNA barcoding of snake venom (using the 12S rRNA gene) has been successfully explored (Pook and McEwing 2005), which makes an even stronger case for concerted efforts towards barcoding venomous snakes (Creer 2005).

1.2.4 Amplified Fragment Length Polymorphism (AFLP)

Gene sequences from a single locus or genome could bias evolutionary history reconstructions due to locus-specific evolutionary constraints and genomic non-representation (Avise 1994). At the level of recently-diverged species, single-gene nuclear sequences are often phylogenetically uninformative (e.g. Bardeleben et al. 2005; Weisrock et al. 2010). Multilocus nuclear markers, on the other hand, offer quantitative advantages and genome-wide coverage (Zhang and Hewitt 2003; Meyer and Paulay 2005; Brito and Edwards 2009). They are useful for assessing the degree of reproductive isolation and have often revealed surprisingly clear, fine-scale genetic structures which were undetected by mitochondrial genetic analysis (e.g. Brown et al. 2007; Egger et al. 2007; Kingston et al. 2009; Meudt et al. 2009; Mila et al. 2010).

AFLP, developed by Vos et al. (1995), is one such powerful and cost-effective multilocus DNA fingerprinting technique (Figure 1). AFLPs require no *a priori* sequence knowledge for marker-specific primer development. There is also a significant quantitative advantage with this technique since a large number of loci can be sampled in one reaction which provides a more comprehensive coverage of variation as compared to using a single locus The resulting data, although predominantly nuclear, are representative of the entire genome. However, one of the main constraints for AFLPs is that they are dominant markers, i.e., it is not possible to distinguish between homozygous and heterozygous states of an allele. This limits their performance and uses when population genetics models are applied for analysis.

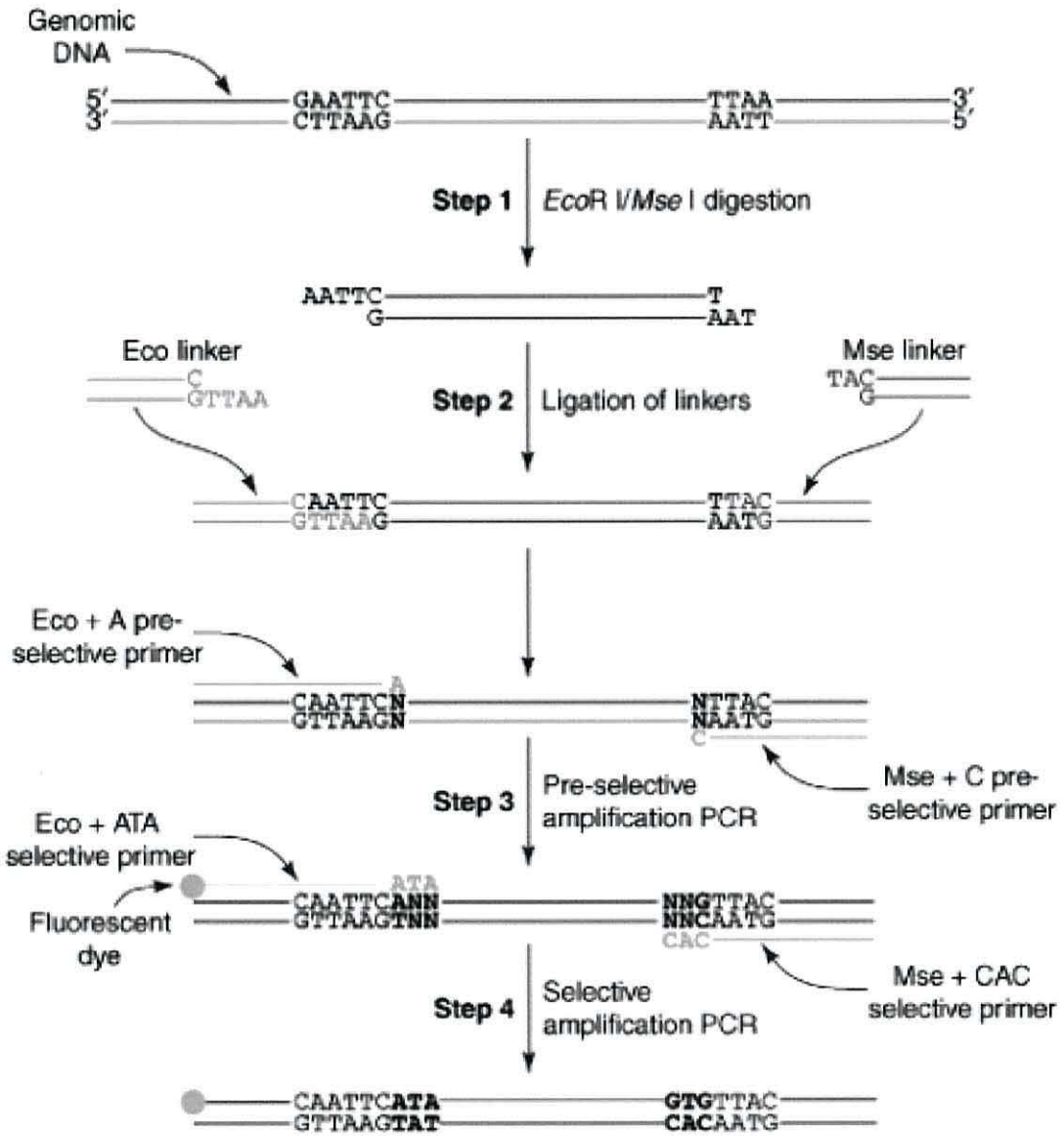


Figure 1. A graphical representation of the AFLP fingerprinting technique (Meudt and Clarke 2007). The method involves digestion of total genomic DNA with restriction endonuclease enzymes to which oligonucleotide adaptors are ligated. PCR amplication is then performed in two stages: first a pre-selective amplification is performed using a single nucleotide extension primer, and secondly a selective amplification is performed using three or more nucleotide extension primers, of which one is labelled using a fluorescent dye. The fragments are then electrophoresed, and the DNA fingerprint unique to each individual can be visualized due to the fluorescent tags on the primers

AFLP technology has, for the most part, been used for plants, fungi, bacteria, with the use in animals being mainly for economically important and model species (Bensch and Akesson 2005; Meudt and Clarke 2007). More recently, it is beginning to consistently prove its usefulness for species delimitation, cryptic species resolution, and population structure analysis of wild populations of wide-ranging animal groups such as butterflies (Kronforst and Gilbert 2008; Quek et al. 2010), cichlids (Albertson et al. 1999), salamanders (Wooten et al. 2010), lizards (Ogden and Thorpe 2002), dolphins (Kingston et al. 2009), and pinnipeds (Dasmahapatra et al. 2009). In green pitvipers, AFLPs have also been successfully used for resolving fine-scale genetic structuring at population and species level and determining evolutionarily significant units within species (Giannasi et al. 2001b; Creer et al. 2004).

1.2.5 Morphometrics In Snakes

In squamates, hemipenial morphology has been one of the most useful characters for distinction at higher taxonomic levels for over a century (Cope 1895), but is rarely used for species-level distinction (e.g. Kohler et al. 2010). Like most taxonomic studies on cryptic species, analysis of multivariate character measurements is an established and powerful method for used in reptilian systematics (Thorpe 1980b). Some of the most informative multivariate characters in snakes are head and body scalation, and mensural characters such as head measurements, snout-vent length, have been extensively used to delimit species (e.g. Lenk and Wüster 1999; Wüster et al. 2001; Sanders et al. 2002; Sanders et al. 2004a; Skinner 2009), and sometimes to rectify over-estimations of species diversity (e.g. Puorto et al. 2001). Colour patterns can also be useful but need to be applied with care as there is evidence for the prevalence of high levels of Batesian and Müllerian mimicry in certain groups of snakes (e.g. Savage and Slowinski 1992; Wüster 2004; Sanders et al. 2006b). Although mobility of skull bones presents an issue for reliable skull morphometrics in snakes, cladistic analysis of skull characters has been found to be a useful source of supplementary systematic evidence at both generic and specific levels (Guo and Zhao 2006; Guo et al. 2009).

1.3 TAXONOMIC METHODS

1.3.1 Molecular Phylogenetics and Phylogenetic Analyses

Phylogenetic trees are used for inferring evolutionary relationships between organisms. The data for tree reconstructions were traditionally derived from sources such as morphology, ontogeny, behaviour, and geographic distribution. Expansion of species concepts to include genetic dimensions and advances in numerical taxonomic methods have now shifted the focus largely to genetic data (Avise 1994). Molecular data is widely useful for producing molecular phylogenies for phylogenetic, phylogeographic, population genetic, and species delimitation studies (Avise 1994; Palumbi and Baker 1994; Schneider et al. 1998; Hebert et al. 2003a). These provide the framework for radiation and colonization studies, allowing identification of recent divergences in sympatric conditions and investigations of historical biogeography (Tautz et al. 2003).

The general principle behind molecular systematic methods is to find a tree that minimizes sequence changes (Hedges 2002). Neighbour-Joining (NJ) (Saitou and Nei 1987) is a popular distance-based method for constructing phylogenetic trees. However, there is loss of character-state information, clustering algorithms are dependent on the order in which sequences are added to the growing tree, and also cluster methods do not allow for the evaluation of measure of fit between alternative trees and the data (Swofford et al. 1996). Among character-based methods which apply sequential evolution of discrete character states for reconstructing evolutionary relationships (Quicke 1993), Bayesian Inference (BI) (Huelsenbeck and Ronquist 2001) is usually the method of choice as it is robust, allows application of complex evolutionary models (e.g. Kimura 1980; Felsenstein 1981; Hasegawa et al. 1985) and estimates the probability of a phylogenetic hypothesis given the observed data (Leache and Reeder 2002; Holder and Lewis 2003).

Phylogenetic trees have several uses such as inferring organismal phylogenies by combining it with analyses of other data sources, studying co-speciation, calibrating rates of molecular evolution, establishing the age of a taxa or lineage, analysis of gene

duplication, estimating rates of diversification, extinction, polymorphism, recombination, and population dynamics (Holder and Lewis 2003). However, certain caveats exist such as gene trees may not correspond to existing species trees due to incomplete lineage sorting of ancestral polymorphisms, gene duplication or loss, or homoplasy, which can potentially confuse the orthology of shared ancestry (Hudson 1992; Avise 2000; Page 2000) and sometimes, horizontal gene transfers can cause species misplacement in trees (Syvanen 1994).

1.3.2 Two New Methods For Analysing Multilocus Dominant Markers

Most studies that use dominant markers (such as AFLPs) employ multivariate statistical tools such as principal component and ordination techniques and distance-based methods such as UPGMA and NJ for data analysis. However, a majority of the studies also regularly implement methods based on population genetics models (designed for co-dominant datasets) necessitating making several assumptions which may not hold good for dominant markers. The population genetics methods are covered in depth in Chapter 3, however, as a general overview, it needs to be noted here that population genetic models use allele-frequency estimates for resolving the genetic structure in a given dataset. In the case of dominant markers, as it is not possible to distinguish between homozygous and heterozygous states of an allele, band absences are treated as double recessives. Clusters of individuals are identified by assuming that they are in Hardy-Weinberg Equilibrium (HWE), and linkage disequilibrium between populations (but not within populations) is also assumed. These assumptions could possibly skew results, and a need for development of more appropriate methods for dominant markers has often been expressed (Hollingsworth and Ennos 2004; Excoffier and Heckel 2006; Bonin et al. 2007; Meudt et al. 2009). Multivariate methods (such as factor and cluster analysis, Principal Component Analysis, Multi-Dimensional Scaling, Molecular Analysis of Variance) have been extensively and routinely used to summarise data and reduce variables in AFLP datasets. Recently, two new methods that use a combination of multivariate procedures have been formally proposed as tools to analyse multilocus genetic data (Hausdorf and Hennig 2010; Jombart et al. 2010).

Discriminant Analysis of Principal Components (DAPC)

DAPC was previously applied in biology for face recognition (Zhao et al. 1998). It has been recently developed in *adegenet*, an *R* library, as a method for inferring genetic clusters and genetic diversity using dominant data (Jombart et al. 2010). *Adegenet* first performs a Principal Component Analysis (PCA) which summarizes the overall genetic variability among individuals in the absence of any population genetic model priors. When the cluster groups (such as populations or species) are unknown, instead of looking for groups of individuals in HWE, a K-means clustering algorithm divides the total variance into between-group and within-group components, and several K-means are run on different numbers of groups. The best number of clusters are determined by decrease in Bayesian Information Criteria (BIC). Discriminant Analysis (DA) of the Principal Components (PCs) then defines a model by which the between group genetic variability is maximised and the within group variability is disregarded. Thus K or the number of groups and sets of individuals belonging to each group can be derived.

Prabclus

Another *R* library called *prabclus* has been specifically developed for species delimitation and uses both dominant and co-dominant multi-locus datasets (Hausdorf and Hennig 2010). *Prabclus* is also based on multivariate statistics and uses ordination-cluster analysis. A Non-Metric Multi-Dimensional Scaling (NMDS) is performed on a distance matrix to derive Euclidean variables of genetic dissimilarity between individuals. As with DAPC, BIC is used as an indicator to estimate the number of clusters/putative species. A Gaussian clustering algorithm implemented in MCLUST (Fraley and Raftery 1998; Fraley and Raftery 2006) is then used to determine clusters of individuals corresponding to mixtures of normal distributions that account for the variation in data, and any datapoints that do not belong to any cluster can also be identified by the estimation of a "noise-component" (Fraley and Raftery 1998, 2002; Hausdorf and Hennig 2010).

1.3.3 Multivariate Morphometrics

Traditionally, morphological character observations have been collected as qualitative data and used in phylogenetic reconstructions to draw direct taxonomic inferences from synapomorphies. This is practical and appropriate only when morphology-based taxonomy is a straightforward exercise, which is rarely the case. In adaptive radiations, inferring phylogenetic relationships at both lower and higher taxonomic levels even when using taxonomically highly-informative characters (such as hemipenes) can be difficult because such radiations, although species-rich, can be morphologically homogeneous and display high levels of character homoplasy which could have possibly arisen numerous times in distantly-related lineages (Keogh 1999). In such cases, the simultaneous analysis of multiple morphological characters, such as morphometric (e.g., body measurements) and meristic characters (e.g., scale counts), using numerical taxonomic methods is more appropriate for morpho-species distinction (Thorpe 1975, 1976, 1980b).

Characters which display significant between-locality differences can be identified using Analysis of Variance (ANOVA) or Covariance (ANCOVA), and using metric ordination, a widely-used phenetic technique in numerical taxonomy, multivariate data can be summarized and presented in fewer dimensions to determine the orientation of Operational Taxonomic Units (OTUs) in space (Thorpe 1980a). The most commonly used ordination techniques are Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA) which summarize the redundancy in information provided by variables correlated across several character variations to arrive at a reduced number of axes (Thorpe 1976, 1980a).

In snakes, multivariate ordination techniques have been successfully used to study speciation, geographic and environmental variation, sexual dimorphism, natural selection, diet, and distribution at both inter-specific and intra-specific levels (Thorpe 1975; Wüster 1992a; Wüster 1992b; Sanders et al. 2002; Malhotra and Thorpe 2004b; Sanders et al. 2004b, 2006a). These methods have also been useful to correlate venom variation with phylogenetic relationships, diet and spatial distribution of populations, and diet and patterns of geographic variation to study adaptive differences (Daltry et al. 1996; Daltry et al. 1997; Creer et al. 2003b). Additionally, morphological data can

also be coded for use in phylogenetic analysis by gap-coding (Thiele 1993), gap-weighting (Chappill 1989), and step-matrix gap-weighting (Wiens 2001) methods which have been used with some success in cladistic morphometrics. However, the application of phylogenetic methods to morphological data is not straightforward as morphometric datasets are usually in the form of continuous variables which, in addition to complicating coding, involves decision-making (Wiens 2001).

1.4 A BRIEF SUMMARY OF THE GEOLOGICAL HISTORY AND BIOGEOGRAPHY OF SOUTHEAST ASIA.

The geological and maritime events that led to the formation of southeast Asia are some of the most complex in the history of the earth. Salomon Müller, in 1846, first observed stark faunal breaks in the Malayan Archipelago which appeared to resolve into the Oriental and Australian regions. This led to Alfred Wallace taking a keen interest in investigating the biogeography of this region, and the faunal divide hence being famously named as Wallace's Line (Wallace 1863; Huxley 1968). Due to difficulties in resolving the faunal breaks in this biogeographically complex area using a single line, there have been several subsequent efforts to draw regional boundary lines (reviewed by Simpson 1977). However Wallace's Line (Figure 2), which traces the Asian Sunda Shelf, running down between Lombok and Bali, dividing the region into Sumatra, Java, Borneo, and Philippines on one side, and Wallacea, a group of islands including Sulawesi and Lesser Sunda Islands, on the other (Dickerson 1928), remains the most widely accepted.

Geological events which occurred over the past 50 million years or so have been key factors in shaping the biogeography on either side of Wallace's Line (Whitmore 1981). These involved plate tectonics of the Indian, Pacific, and Philippines ocean plates and the Eurasian, Indian and Australian land plates, also influenced by the birth and disappearance of various seas (Hall 1998). Although there have been conflicting theories among geologists regarding the individual events and their sequence on the time-scale, mostly due to insufficient data (Rangin et al. 1990; Daly et al. 1991; Lee and Lawver 1994; Hall 1996), it is now widely accepted that three major events occurred in the Cenozoic era. First, the collision of India with Eurasia during the Eocene about 45 mya; second, the collision of the Australian plate with the Philippine sea arc plate during the Miocene about 25 mya, causing further rotation of southeast Asian microcontinental fragments; and finally, the collision of the Philippine arc with Eurasia in the region of Taiwan about 5 mya (Hall 1996, 1997, 1998, 2001). Furthermore, the melting of polar ice in the Quarternary and intermittent glaciation periods in the Pleistocene caused significant sea-level and climatic changes in this region (Morley 2000; Bird et al. 2005; Hanebuth et al. 2009).

These changes are thought to be responsible for major dispersal and vicariance events of the flora and fauna, rendering southeast Asia one of the most biogeographically and taxonomically complex and interesting areas for tracing evolutionary histories and diversification of taxa (Turner et al. 2001). The patterns of species distribution and their molecular evolution have been found to coincide with the Cenozoic tectonic models in various groups of animals such as arthropods, birds, and mammals (Lohman et al. 2011). While dispersal and vicariance events during this period were the driving factors for speciation, the glaciation cycles and sea-level changes during the Pleistocene are thought to have driven intra-specific variation (Lohman et al. 2011).

Present-day southeast Asia is divided into the continental mainland (consisting of Thailand, Myanmar or Burma, Laos, Cambodia, Vietnam, West Malaysia), and the Indomalayan archipelago consisting of thousands of islands (including the Greater and Lesser Sunda Islands) which share political boundaries with different countries and also include several independent countries (Figure 2). The land area, mostly comprising of tropical rainforests and more seasonal monsoon forests, is one of the most biologically diverse regions in the world. Thousands of endemic plants and animals, including a rich herpetofaunal diversity consisting of more than 450 endemic amphibian species and 700 endemic reptilian species, are found here (Myers et al. 2000; Bickford et al. 2010). In recent years, southeast Asia has become a treasure trove for species discovery, with cryptic diversity being regularly uncovered using genetic tools (e.g. Wüster and Thorpe 1994; Bain et al. 2003; Stuart et al. 2006; Lohman et al. 2010). It has also been declared a top biodiversity hotspot as its biodiversity faces high risk due to deforestation and climate change, and catastrophic levels of species extinction have been predicted to occur by the end of the century (Brook et al. 2003; Bickford et al. 2010).

Figure 2. Topological and political map of southeast Asia and some parts of continental Asia showing Wallace's Line.

1.5 THE EVOLUTION AND BIOLOGY OF PITVIPERS

1.5.1 Viperidae

Viperidae is a family of venomous snakes, commonly called vipers or viperids, which originated in Eurasia in the Tertiary Period at least 30 million years ago (mya) (Greene et al. 1992). They are now found distributed in all parts of the world except Australia, New Zealand, Madagascar, Ireland, and Antartica. Members of Viperidae are mostly nocturnal and oviparous and possess the most highly-evolved venom-delivery system among snakes. Their fangs are solenoglyphous or grooved, which fold back when not in use, and their venom consists mainly of haemotoxins. Four subfamilies of Viperidae, Azemiopinae, Causinae, Crotalinae, and Viperinae, are together known to include more than 250 species (McDiarmid et al. 1999), although a more recent validated species catalogue is awaited.

The sub-family Crotalinae is the most species-rich with over 150 species spread across Asia and the New World (McDiarmid et al. 1999; Parkinson et al. 2002). They are referred to as pitvipers due to the presence of advanced thermoreceptive loreal pits between the eyes and nostrils. Fossil evidence has indicated that Crotalinae evolved in Asia from the closest viperid ancestor by the Miocene, c. 18 – 22 mya (Greene et al. 1992). Application of mtDNA divergence rates of closely-related Central and South American pitvipers (Wüster et al. 2002) gave the time of divergence of pitvipers from vipers as 16 – 30 mya, more or less corroborating the estimation from fossil records (Malhotra and Thorpe 2004a). During the upper Miocene, Crotalinae is believed to have spread to the New World across the Bering Land Bridge in a single migration event with no reverse migrations to Asia (Rage 1987; Greene et al. 1992; Kraus et al. 1996; Parkinson 1999; Parkinson et al. 2002).

1.5.2 Asian Pitvipers

Asian crotalines, which originated in early Miocene, further diversified during the mid Miocene (Malhotra and Thorpe 2004a). Among these was the *Trimeresurus* complex which is now widely distributed across southern Asia, ranging from the Indian subcontinent to southern China, Japan, Indochina, the Philippines, and

throughout the Indo-Malayan archipelago, excluding Sulawesi (Malhotra and Thorpe 1997; McDiarmid et al. 1999). Members of *Trimeresurus* were initially considered congeneric as high levels of convergence in important morphological characters long confounded their taxonomy, leading to a conservative arrangement. However cryptic diversity was discovered early on (Stejneger 1927; Pope and Pope 1933) and there have been many investigations for re-classification in the past (e.g. Hoge and Romano Hoge 1978; 1980; Regenass and Kramer 1981). The availability of genetic techniques allowed the investigation of molecular evolution in this group and polyphyletic arrangements of "species" clusters have also been found (Kraus et al. 1996; Parkinson 1999; Malhotra and Thorpe 2000). Investigations of morphology and genetics have led to the recognition of several genera since, and *Trimeresurus sensu lato (s.l.)* now includes the following genera: *Himalayophis, Cryptelytrops, Popeia, Viridovipera, Peltopelor, Parias, Trimeresurus, Garthius* (Malhotra and Thorpe 2004a); *Ovophis* (Burger 1971; Hoge and Romano-Hoge 1978), *Protobothrops* (Hoge and Romano Hoge 1980; Peng et al. 2007), and *Tropidolaemus* (Wagler 1830; Burger 1971). The species under *Trimeresurus s.l.* are of extensive interest to systematists, ecologists, and herpetoculturists, and also of economic and medical importance in the region due to their common occurrence and frequent contact with humans. Their conservative morphology often causes misidentifications even by professional herpetologists, and as venom composition is species dependent (Chippaux and Goyffon 1998), this has caused considerable difficulties for the medical management of envenomations in southeast Asia.

1.5.3 *Trimeresurus sensu stricto*

Seven genera from *Trimeresurus s.l.*, namely *Himalayophis, Cryptelytrops, Popeia, Viridovipera, Peltopelor, Parias,* and *Trimeresurus,* contain species commonly described as the green pitviper or bamboo viper (Malhotra and Thorpe 2004a), and an eighth genus, *Sinovipera,* has recently been described (Guo and Wang 2011). These species occupy a wide range of habitats from low tropical rainforests to hilly regions of Asia and southeast Asia (Greene et al. 1992; Malhotra and Thorpe 1997). They are terrestrial or arboreal, oviparous or viviparous, and consume diverse prey types and share several aspects of morphology, life-style, and habitat range (Greene et al. 1992; Malhotra and Thorpe 1997). Because of their superficial similarity, they were initially

classified as a single species called *Trimeresurus gramineus* (illustrated in Figure 3).

a)

b)

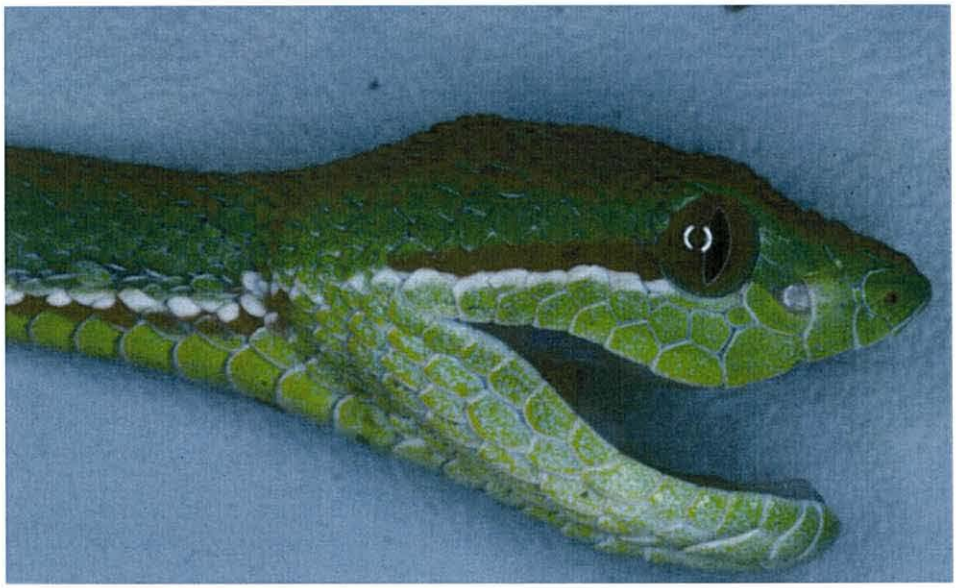
c)

igure 3. Cryptic Species among Asian pitvipers. Identification using morphology is very difficult at both species and generic levels. *Viridovipera*, an Asian and continental southeast Asian genus (a: *V. stejnegeri*, b: *V. vogeli,* photographs by A. Malhotra), and *Cryptelytrops insularis* (c, photograph by ?), an island endemic from the Lesser Sunda, are genetically highly distinct. Juvenile males from two different genera may be virtually identical as illustrated by d) *Viridovipera gumprechti* from

northeast Thailand and e) *Popeia popeiorum* from north Thailand (photographs by A. Malhotra).

d)

e)



Ontogenetic variation and sexual dimorphism also contribute to species misidentification and sytematic confusion. Scale and hemipenial differentiation were discovered in 1927, (Stejneger 1927) and led to the first split into four species: *T. albolabris, T. popeiorum, T. stejnegeri,* and *T. gramineus* (Pope and Pope 1933). Hemipenis type and the state of fusion of the nasal and first supralabial have been found to be the most useful diagnostic characters at the generic level (Malhotra and Thorpe 2004a), but the diagnosis of females and juveniles remains rather difficult due to the absence or underdevelopment of these characters. There is also widespread inter-specific conservativeness in phenotype resulting from repeated evolution of identical morphological characters (Kraus et al. 1996; Malhotra and Thorpe 1997; Malhotra and Thorpe 2000). In addition, inaccurate assessments of key morphological features have frequently led to misidentification of species in published literature and guides for green pitvipers (Malhotra and Thorpe 1997; 2004b). Intraspecifically, a mixture of ontogenetic variation, sexual dimorphism and geographic variation is prevalent in almost every character (Malhotra and Thorpe 1997). Several informative features like eye colour, scale counts, head measurements, dentition, tail colouration, body patterns such as spot, stripes and mottling have been used in multivariate analyses. However, there is widespread ecological convergence in morphological traits which are taxonomically critical, and as a result, the universal applicability of any single morphological feature across species, genera, sexes, age groups, or geographic range for a complete diagnosis has not been possible (Sanders et al. 2004b).

There are other problems such as poor representation of distinguishing features (such as eye colour, tail colour, spots and bands) in preserved specimens deposited in museums due to fading and discoloration over time (Malhotra et al. 2004). Moreover, although phenotypic characters can be described in great detail, a description such as "25% of the tail is rusty red" (David et al. 2001), could be highly subjective assessments even in live specimens, resulting in inaccurate identifications. Internal characters cannot be used for identification under most circumstances (Malhotra et al. 2004). A geographical basis of identification has been used in certain studies where a known species range was used infer species identity, but this subsequently proved to be invalid due to incorrect range definitions in the first instance (Malhotra and Thorpe

1997). All this has contributed to misidentifications, taxonomic inaccuracy, and confounded systematic relationships of green pit vipers, and it is clear that deriving a systematically meaningful arrangement by the independent use of any of these sources of information would not be possible with respect to this group (Sanders et al. 2006a).

With advances in molecular genetic and phylogenetic methods, diverse molecular markers have proved useful at different taxonomic levels of *Trimeresurus sensu stricto (s.s.)* Although some molecular markers are subject to selective forces, their degree of homoplasy is predicted to be lower compared to the morphological convergence prevalent in *Trimeresurus s.s.* (Malhotra and Thorpe 1997). Additionally, multivariate morphometric analyses have also provided very useful evidence for clarifying species boundaries (Malhotra and Thorpe 2000; David et al. 2001; David et al. 2002; Malhotra and Thorpe 2004a; Sanders et al. 2004b, 2006a). The final generic resolution of *Trimeresurus s.s.* has been derived from morphological and mtDNA analyses and has also been supported by nuclear intron analyses (Creer et al. 2003a; Malhotra and Thorpe 2004a; Creer et al. 2006; Malhotra et al. 2010). Subsequently, several cryptic species have been uncovered (e.g.Malhotra and Thorpe 2004a; Sanders et al. 2004a; Grismer et al. 2006; Sanders et al. 2006a; David et al. 2008; Grismer et al. 2008), and the green pitviper complex now consists of at least 46 species placed in the eight genera mentioned above.

1.6 THESIS AIMS

This thesis covers areas of systematics research which can be broadly divided into (i) incorporation and evaluation of new taxonomic tools and methods for use in systematic studies of the *Trimeresurus* group, and (ii) resolution of the systematics of *Cryptelytrops* through confirmation of species designations, clarification of species range, and establishment of the presence of cryptic species.

Chapter 2 reports on a study on DNA barcoding Asian pitvipers. The markers of choice for mtDNA-based phylogenetic studies have mainly been Cytb, ND4, 12S, and 16S in the *Trimeresurus* group. The performance of a relatively newly-proposed barcode gene COI was evaluated against existing taxonomic frameworks derived from these markers, and its uses for species delimitation and identification were explored.

Chapter 3 is a clarification of the systematics of the *C. macrops* complex. It mainly addresses broader issues around the analysis of dominant markers using *C. macrops* as the study group. A comparative study of multiple analysis methods using multivariate statistics, (including two recently proposed methods), and population genetics models was conducted. The usefulness of these methods for species delimitation was evaluated, and the species diversity in *C. macrops* was reassessed using these results.

Chapter 4 explores the basis for the confirmation of the taxonomy and the clarification of distribution ranges of species in the *"albolabris"* group. The species status of five major species and their ranges, which have long been unclear, were evaluated by investigating morphological data for the presence of distinct morphotypes which correspond to species clusters derived from previous and current genetic studies. Both morphological and genetic data were examined for indications of the presence of distinct evolutionary lineages in the *"albolabris"* group.

CHAPTER 2

BARCODING OF SNAKES

2.1 ABSTRACT

DNA barcoding using the COI gene offers the potential for robust species identification of most animal taxa. Although initial testing of COI has been largely successful, there are several caveats which could diminish its robustness. The methodology therefore requires extensive empirical proof which firstly necessitates the development of a comprehensive COI database for various groups of animals, and using this, the efficiency of the technique can then be demonstrated across diverse taxa and at all taxonomic levels. Species identification in the morphologically cryptic Asian green pitviper group is a challenge, and COI barcoding could serve as a particularly useful technique for species distinction. They also provide an ideal testing ground for the ability of COI barcodes in recovering taxonomic information from various lineages. Three genera (*Viridovipera, Popeia,* and *Parias*) were used for this study, the former two having been scrutinized by a number of workers using traditional and modern taxonomic methods. The results show that COI barcoding is only moderately successful at recovering taxonomic information at the intergeneric level, but more useful at species level. While COI barcoding performs best in a well-resolved taxonomic framework, this study raises some important questions for the taxonomy of two genera *Parias* and *Popeia.*

2.2 INTRODUCTION

DNA barcoding has potentially unlimited applications which extend to global biodiversity studies and conservation, wildlife forensics, monitoring cross-boundary shipment of illegal biological material, and medical research such as cell-line identification and antivenin production (Pook and McEwing 2005; Cooper et al. 2007; Dawnay et al. 2007; Jakupciak and Colwell 2009). Given this, it is a key requirement to establish that the barcoding concepts and protocols are robust across most geographic ranges and animal groups. In hotspots such as southeast Asia, barcoding could be a quick and easy tool for biodiversity assessments, and such possibilities have been explored in southeast Asian mammals (Francis et al. 2010). Among reptiles, Asian green pitvipers are an ideal test group in that they are wide-spread, cryptic, tropical in distribution, and consist of a number of closely-related taxa with complex biogeographical and evolutionary histories. In addition, the three genera selected for this study, *Viridovipera, Parias*, and *Popeia* have recently been taxonomically revised with varying degrees of success (Malhotra and Thorpe 2004a, b; Malhotra et al. 2004; Sanders et al. 2004a; Sanders et al. 2004b; Grismer et al. 2006; Sanders et al. 2006a; David et al. 2008). This presents a challenge for the much-debated taxonomic applications of barcoding (Will and Rubinoff 2004; Hajibabaei et al. 2007; Golding et al. 2009; Packer et al. 2009) which are said to be particularly useful in morphologically cryptic groups (e.g. Hebert et al. 2004a; Marshall 2005; Sheffield et al. 2009; Steinke et al. 2009).

The genus *Viridovipera* has a wide distribution with a known geographic range across continental southeast Asia, China, and Taiwan, and consists of six species: *V. gumprechti, V. stejnegeri, V. vogeli, V. medoensis, V. yunnanensis* (Malhotra and Thorpe 2004a), and *V. truongsonensis* (Dawson et al. 2008). The Indomalayan pitviper group, *Parias*, is found in undisturbed forests of the Indomalayan archipelago and consists of allopatrically distributed island taxa (Malhotra and Thorpe 2004a; Sanders et al. 2004b). Widespread phenotypic and ecological diversity resulting from adaptations across ecological gradients have been observed in this group (Sanders et al. 2004b). *Parias,* henceforth designated as *"Pa."* to be able to differentiate from *Popeia*, consists of five species: *Pa. sumatranus, Pa. hageni, Pa. malcolmi, Pa.*

schultzei, Pa. flavomaculatus, and *Pa. mcgregori* (Malhotra and Thorpe 2004a). Some authors have considered *Pa. mcgregori* to be a subspecies of *Pa. flavomaculatus* (Sweeny 1994; Dietz 2003), and morphological analysis of this genus has provided some evidence for the presence of morphological diversity in *Pa. flavomaculatus* (Sanders et al. 2004b).

The taxonomy of the third genus, *Popeia* (also referred to as the *P. popeiorum* complex), is one of the most contentious in the green pitviper group. This genus is widely but exclusively distributed in moderate and high altitude undisturbed rainforests (Sanders et al. 2006a) and alternative arrangements have been proposed for its taxonomy. Vogel et al. (2004) described two new species and divided *Popeia* into five species: *P. barati* (Sumatra), *P. sabahi* (Borneo), *P. popeiorum* (India, Myanmar, Laos, and north and west Thailand), *P. nebularis* (Cameron Highlands), and *P. fucata* (south Thailand, south Myanmar, west Malaysia including Fraser's Hill in Selangor, and Pulau Tioman). However, after extensive sampling and analysis of mtDNA gene sequences, multivariate morphometric analysis, and ecological pattern-based analysis, Sanders et al. (2006a) recognized only three species within the *P. popeiorum* complex. Two well-defined clades, the northern (northeast India, Myanmar, Laos, and Thailand) and the southern (south Thailand, Malaysia, Sumatra, Borneo, and Pulau Tioman), were given species status as *P. popeiorum* and *P. sabahi* respectively (Sanders et al. 2006a), while a third species from Cameron Highlands in west Malaysia, a morphologically and ecologically divergent allopatric lineage with respect to the northern and southern lineages, was described as *P. inornata* (Sanders et al. 2004a). However, according to precedence, this is now a junior synonym of *P. nebularis* (Vogel et al. 2004).

Grismer et al. (2006) rejected the clumping of *P. sabahi* into a single species by Sanders et al. (2006a), but also recognize the problem of polyphyly with Vogel et al's. (2004) taxonomy. Grismer et al. (2006) found that the Pulau Tioman population of *Popeia* differs in at least 10 morphological characters of squamation, body proportions, and colour patterns from other species, irrespective of which of the two classifications was followed, and hence described it as a new species called *P. buniana*. More recently, David et al. (2008) have also described *Popeia toba* from

Utara province and Toba Massif in northern Sumatra as a new species based on morphological character analysis. Cladograms of the two classifications, henceforth referred to as Vogel's and Sanders' taxonomy for convenience, are provided in Figure 4 for an easier understanding of the species arrangements proposed for *Popeia*.

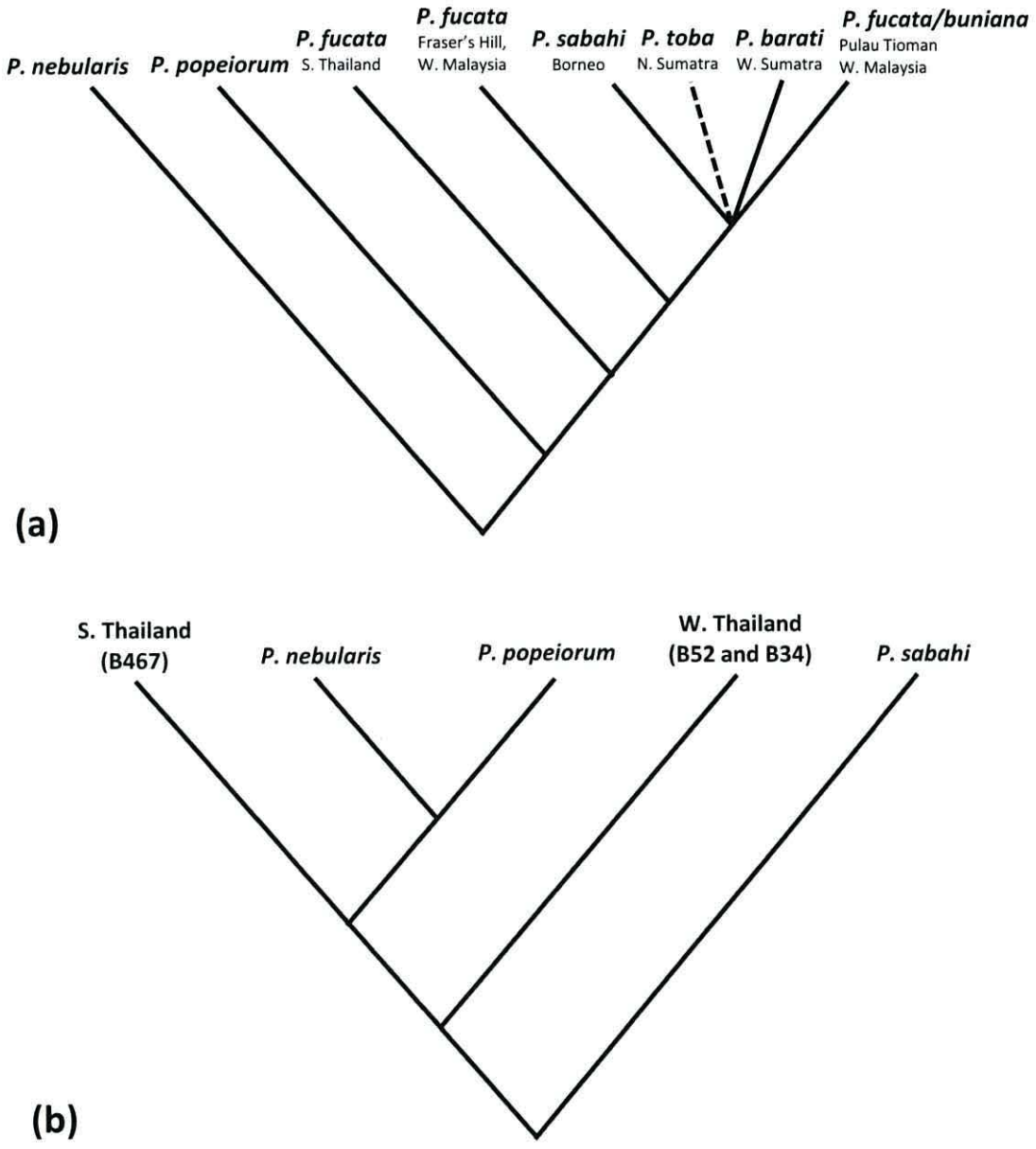


Figure 4. Schematic representation of alternate species classifications proposed for the genus *Popeia*. Branch lengths are not indicative of divergence rates. **(a)** Vogel's Taxonomy: Species representation acording to Vogel et al. (2004) adapted from Grismer et al. (2006) who have renamed *P. fucata* from Pulau Tioman as *P. buniana.* The genetic and taxonomic affiliations of the northern Sumatran *P. toba* is unclear, and it is hence tentatively represented by a dashed line based on morphological similarities (David et al. 2008). **(b)** Sanders' Taxonomy: Species representation according to Sanders et al. (2006).

In this complex scenario with several potentially confounding factors, the DNA barcoding concept was tested for its ability to recover species units; applicability of the barcode gap for within and among species distinction; species clustering efficiency of COI in various phylogenetic analysis methods; and the potential uses of barcoding as a taxonomic tool. Key objectives of this chapter include: evaluation of COI an appropriate gene for generating unique DNA barcodes for each species; the use of percentage of COI sequence divergence to differentiate between taxa is effective in establishing species boundaries; the suitability of the NJ method for use with COI in barcode studies; and the utility of COI barcoding as a tool in taxonomy and species delimitation.

2.3 MATERIALS AND METHODS

2.3.1 Taxon Sampling

Samples were chosen to represent species diversity and geographic range as far as possible. Sanders' taxonomy with the *P. sabahi* nomenclature was retained for the purpose of alternative hypothesis testing for *Popeia*. A full sample list is provided in Appendix 1, Table 1. The putative *P. toba* (David et al. 2008) is not represented since this project was completed before it was proposed as a new species and there was no sample from its range (in northern Sumatra) available in our tissue inventory. Multiple samples of McGregor's pitviper *Pa. mcgregori* from the Batan Islands (nine samples) and the Philippine pitviper, *Pa. flavomaculatus,* Luzon (11 samples from Bicol), Negros (10 samples), and Mindanao (five samples from Davao), were analysed to test for increase in sequence variability of COI with increase in sampling. *Trimeresurus malabaricus* and *T. borneensis* (from the Indian subcontinent group of green pitvipers) were selected as outgroups since they are the most closely-related outgroup clade to *Viridovipera, Parias,* and *Popeia,* and the most basal clade in the former *Trimeresurus s.s* complex (Malhotra and Thorpe 2004a).

2.3.2 Laboratory Protocols

Samples were in the form of liver or muscle tissue in 80% ethanol, clippings from the ventral scales in 80% ethanol, or up to 200µl of blood from the caudal vein preserved in 1mL 5% EDTA and 2mL SDS-Tris buffer (100mM Tris, 3% SDS). Whole genomic DNA was extracted using standard proteinase K protocols (Sambrook et al. 1989). For most samples, COI could be amplified using Folmer's primers (Folmer et al. 1994). However, despite PCR optimization experiments, amplifications were unsuccessful for all the Chinese and one north Vietnamese (from Vinh Phu) specimen of *V. stejnegeri* and also for *V. truongsonensis*. COI sequences from all *Viridovipera* species were aligned and used in PrimerSelect (DNASTAR, Lasergene®) to design non-degenerate primers. These amplicons were however less than the BOLD stipulated minimum (500 bp) for DNA barcodes (Ratnasingham and Hebert 2007). Hence, a consensus sequence from the multiple sequence alignment was used to design degenerate primers using an online primer design tool Primaclade (http://www.umsl.edu/services/kellogg/primaclade.html), which runs Primer3 (http://primer3.sourceforge.net/). Mitochondrial genes 12S, 16S, ND4, and Cytb were amplified (as described in Malhotra et al. 2011a), cleaned with shrimp alkaline phosphatase and Exonuclease I (Werle et al. 1994), and sequenced by Macrogen inc. (http://www.macrogen.com).

2.3.3 Sequence Analysis

Sequences were aligned and analysed in MEGA4 (Tamura et al. 2007). Protein-coding genes were aligned using ClustalW and translated into protein sequences to check for stop codons in the Open Reading Frame (ORF) in the event that pseudogenes were amplified. For 12S and 16S, since the alignment algorithm was found to be less efficient with indel calling, sequences were aligned by eye and indels were edited by double checking against chromatograms. Sequences were divided into COI and total evidence (COI, ND4, Cytb, 16S, 12S) datasets to be able to compare the performance of COI to that of total evidence.

2.3.4 Genetic Distances

Kimura 2-Parameter (K2P) corrected genetic distances (Kimura 1980) were

calculated between sequence pairs to derive intraspecific variation and interspecific divergence rates for COI. Confidence values were obtained from 1000 bootstrap replicates. *Popeia* species were also re-grouped according to Vogel et al's taxonomy, and genetic distances were calculated to compare barcode gaps.

2.3.5 Phylogenetic Reconstructions

NJ Analysis

COI and total evidence NJ trees were reconstructed (Saitou and Nei 1987) in MEGA4 using K2P distance model (Kimura 1980). One thousand bootstraps were performed to estimate the number of replicate trees for each cluster (Felsenstein 1985).

Maximum Parsimony (MP) Analysis

A heuristic search was performed in PAUP* 4b10 (Swofford 2002) with a starting tree obtained by 1000 random additions of taxa followed by tree-bisection-reconnection branch swapping. All sites were equally weighted and gaps were treated as fifth base because they were never more than one base-pair in length and therefore all likely to represent independent evolutionary events (Simmons and Ochoterena 2000). A 50% majority-rule consensus tree was constructed for both datasets. 1000 bootstrap replicates were performed to evaluate branch support using the same settings as above except only 5 random additions were performed per replicate to obtain a starting tree.

Partitioned Bremer Support

For the total evidence MP tree, TreeRot.v3 (Sorenson and Franzosa 2007) commands were used in PAUP* 4b10 (Swofford 2002) to determine the Bremer support indices (Bremer 1988) as a measure of gene-specific contribution to the decay index of each node (Baker and DeSalle 1997; Baker et al. 1998). Twenty heuristic search random addition replicates were performed for each constrained search.

Bayesian Analysis

Nucleotide evolutionary model parameters were estimated using jModelTest (Posada 2008). GTR+I+G substitution model for the COI dataset and GTR+G for each of the five genes in the total evidence dataset were implemented in MrBayes v3.1 (Huelsenbeck and Ronquist 2001) and three independent MCMC runs, of 3 million generations each (sampled every 1000 generations), were performed using one cold chain and three heated chains. *Trimeresurus malabaricus* was used as outgroup. Tracer v1.4 (Rambaut and Drummond 2007) was used to determine stationarity of sample points. The first 300,000 runs were discarded as burnin for both datasets. Post-burnin samples were compiled into a single file and a consensus tree was constructed in MrBayes v3.1 using all compatible groups for the COI dataset, and a 50% majority-rule consensus tree was constructed for the total evidence dataset.

2.4 RESULTS

2.4.1 Sequence Analysis

COI amplifications using degenerate primers (Table 1) were successful for all *Viridovipera* specimens that failed with Folmer's primer. These amplifications generated sequences between 507 and 543bp in length after editing. Haplotypes were unique for all species designations with no cross-overs in either of the taxonomic arrangements. Multiple sampling experiments of COI resulted in two haplotypes for *Pa. mcgregori*, two haplotypes each for *Pa. flavomaculatus* from Luzon and Negros, and three haplotypes for Mindanao specimens, showing that intraspecific sequence variation was not significantly higher with increase in sample size. No pseudogenes were amplified. Sequence length for the complete dataset was 3007 bp with COI consisting of c. 681 bp, ND4 c. 670 bp, Cytb c. 695 bp, 16S c. 513 bp, 12S c. 436 bp. In the total evidence dataset, the alignment of nucleotide positions between 2337 – 2393 bp (16S) and 2727 – 2740 bp (12S) was uncertain and hence these regions were excluded from the analyses.

Table 1. Degenerate primer sequences for COI. F = Forward, R = Reverse.

Primer	Sequence 5' to 3' end
F1	GCCTGCCTAAGCATYCTR
F2	CTGCCTAAGCATYCTRATACG
R1	GAYCCRGTCCTATTCCAACAC
R2	GTCCTATTCCAACACYTRTTCTGAT
R3	TCCTATTCCAACACYTRTTCTGA

2.4.2 Genetic Distances

Within species and among species genetic distances and standard error are given in Table 2a & 2b respectively. All interspecific distances were higher than the highest intraspecific distance of 3.52 ± 0.64 % in *Viridovipera*. This was also true for Sanders' taxonomy of *Popeia,* although the difference between the highest intraspecific, 3.78 ± 0.63 %, and the lowest interspecific distance, 4.23 ± 0.81 %, was less than 1%. In the case of Vogel's taxonomy, interspecific distances fell below the highest intraspecific distance of 3.53 ± 0.51 % in all cases where *P. sabahi* was split, except between *P. buniana* and *P. fucata.* Finally, for *Parias*, the interspecific distance between *Pa. flavomaculatus* and *Pa. mcgregori* was 1.43 ± 0.44 % which was below the highest intraspecific distance of 2.90 ± 0.80 %.

Table 2. Estimates of average K2P evolutionary divergences and Standard Error. (a) Intraspecific Variation. NA denotes cases of COI haplotype sharing intraspecifically (*P. nebularis, Pa. malcolmi*), single samples (*V. medoensis, V. truongsonensis, Pa. schultzei*), and non-availability of taxon (*P. buniana*). **(b) Interspecific Divergence.** These are represented as a matrix including the mean for each genus. Interspecific distances that fall below the highest intraspecific distance in the respective genera are coloured in grey.

a)

Taxon	% K2P (±%SE)
Viridovipera	
V. medoensis	NA
V. truongsonensis	NA
V. gumprechti	1.01 (±0.31)
V. stejnegeri	3.25 (±0.52)
V. vogeli	3.52 (±0.64)
V. yunnanensis	0.22 (±0.20)
Mean	2.0 (±0.42)
Popeia - Sanders' Taxonomy	
P. sabahi	2.48 (±0.41)
P. poperiorum	3.78 (±0.63)
P. nebularis	NA
Mean	3.13 (±0.52)
Popeia - Vogel's Taxonomy	
P. fucata	3.42 (±0.55)
P. barati	0.35 (±0.25)
P. buniana	NA
P. sabahi	0.18 (±0.18)
P. popeiorum	3.53 (±0.51)
P. nebularis	NA
Mean	1.87 (±0.37)
Parias	
Pa. hageni	2.33 (±0.49)
Pa. flavomaculatus	1.07 (±0.32)
Pa. mcgregori	0.22 (±0.21)
Pa. schultzei	NA
Pa. sumatrarus	2.90 (±0.80)
Pa. malcolmi	NA
Mean	1.63 (±0.46)

b)

Viridovipera	*V. medoensis*	*V. truongsonensis*	*V. gumprechti*	*V. stejnegeri*	*V. vogeli*
V. truongsonensis	7.15 (±1.24)				
V. gumprechti	6.09 (±1.09)	5.46 (±1.03)			
V. stejnegeri	6.65 (±1.06)	6.54 (±1.09)	4.30 (±0.72)		
V. vogeli	6.67 (±1.08)	6.69 (±1.08)	5.73 (±0.94)	6.94 (±1.0)	
V. yunnanensis	6.28 (±1.20)	7.79 (±1.35)	7.08 (±1.22)	8.53 (±1.26)	6.04 (±1.02)
Mean	**6.53 (±1.09)**				
Popeia					
Sanders' Taxonomy	***P. sabahi***	***P. poperiorum***			
P. poperiorum	4.90 (±0.73)				
P. nebularis	4.23 (±0.81)	5.60 (±0.90)			
Mean	**4.91 (±0.81)**				
Vogel's Taxonomy	***P. fucata***	***P. barati***	***P. buniana***	***P. sabahi***	***P. popeiorum***
P. barati	3.38 (±0.56)				
P. buniana	3.80 (±0.64)	2.14 (±0.57)			
P. sabahi	3.03 (±0.52)	0.79 (±0.33)	1.87 (±0.55)		
P. popeiorum	5.23 (±0.65)	4.92 (±0.74)	5.34 (±0.80)	4.63 (±0.71)	
P. nebularis	5.30 (±0.84)	4.74 (±0.94)	5.31 (±1.01)	4.08 (±0.86)	5.97 (±0.90)
Mean	**4.04 (±0.71)**				
Parias	***Pa. hageni***	***Pa. flavomaculatus***	***Pa. mcgregori***	***Pa. schultzei***	***Pa. sumatrarus***
Pa. flavomaculatus	13.75 (±1.77)				
Pa. mcgregori	13.71 (±1.78)	1.43 (±0.44)			
Pa. schultzei	12.99 (±1.72)	8.18 (±1.31)	8.31 (±1.35)		
Pa. sumatrarus	11.50 (±1.51)	8.25 (±1.27)	8.41 (±1.30)	7.28 (±1.22)	
Pa. malcolmi	12.83 (±1.71)	9.27 (±1.45)	9.93 (±1.53)	9.45 (±1.53)	8.27 (±1.38)
Mean	**9.57 (±1.42)**				

2.4.3 Phylogenetic Reconstructions

NJ Phylogeny

All taxa clustered to accurate species groups as per Sanders' taxonomic framework in the COI NJ phylogeny (Figure 5a) except for two *P. popeiorum* specimens from west Thailand (B34 and B52) which were paraphyletic with respect to the southern clade *P. sabahi.* Bootstrap support values were mostly moderate to high in the COI NJ tree. The total evidence NJ phylogeny (Figure 5b) showed similar results for *Viridovipera* and *Parias,* except for *Pa. flavomaculatus,* which was polyphyletic as a few samples clustered with *Pa. mcgregori*. The west Malaysian specimens of *P. nebularis* were outside the two monophyletic clusters consisting of *P. popeiorum* and *P. sabahi.* Bootstrap values for the total evidence were generally higher than the COI NJ tree. *P. fucata* from Vogel's taxonomy was polyphyletic in both analyses.

MP Phylogeny

Both the COI and total evidence MP phylogenies (Figure 6a and 6b) were largely congruent. *Pa. flavomaculatus* was polyphyletic as *Pa. mcgregori* clustered with some of these specimens. West Thailand *P. poperiorum* was paraphyletic to *P. sabahi* and *P. fucata* was polyphyletic in both analyses. The total evidence phylogeny showed significantly higher bootstrap support values than the COI phylogeny.

PBS Indices

PBS indices from each gene for the total evidence MP phylogeny are given in Table 3. COI provided the maximum support with a total Bremer support index of 311.6. Interestingly, both COI and Cytb provided the highest indices (20.17 at Node 54 and 64.0 at Node 55 respectively) for the non-monophyletic arrangement of *Pa. flavomaculatus.*

Bayesian Phylogeny

The resolution of the COI 50% majority-rule consensus was poor with fragmentation of *Popeia* and *Parias* into polytomies (Appendix 1 Figure 1). Increasing MCMC sampling rate gave a well-resolved tree but the posterior probability values were inflated. A consensus tree including all compatible groups was constructed for COI. Both COI and total evidence trees were well-resolved with higher support values in

the latter (Figure 7a and 7b). *Pa. mcgregori* continued to cluster with *Pa. flavomaculatus* from north and central Philippines, the west Thailand specimens of *Popeia* with the southern clade, and *P. fucata* was polyphyletic in both analyses.

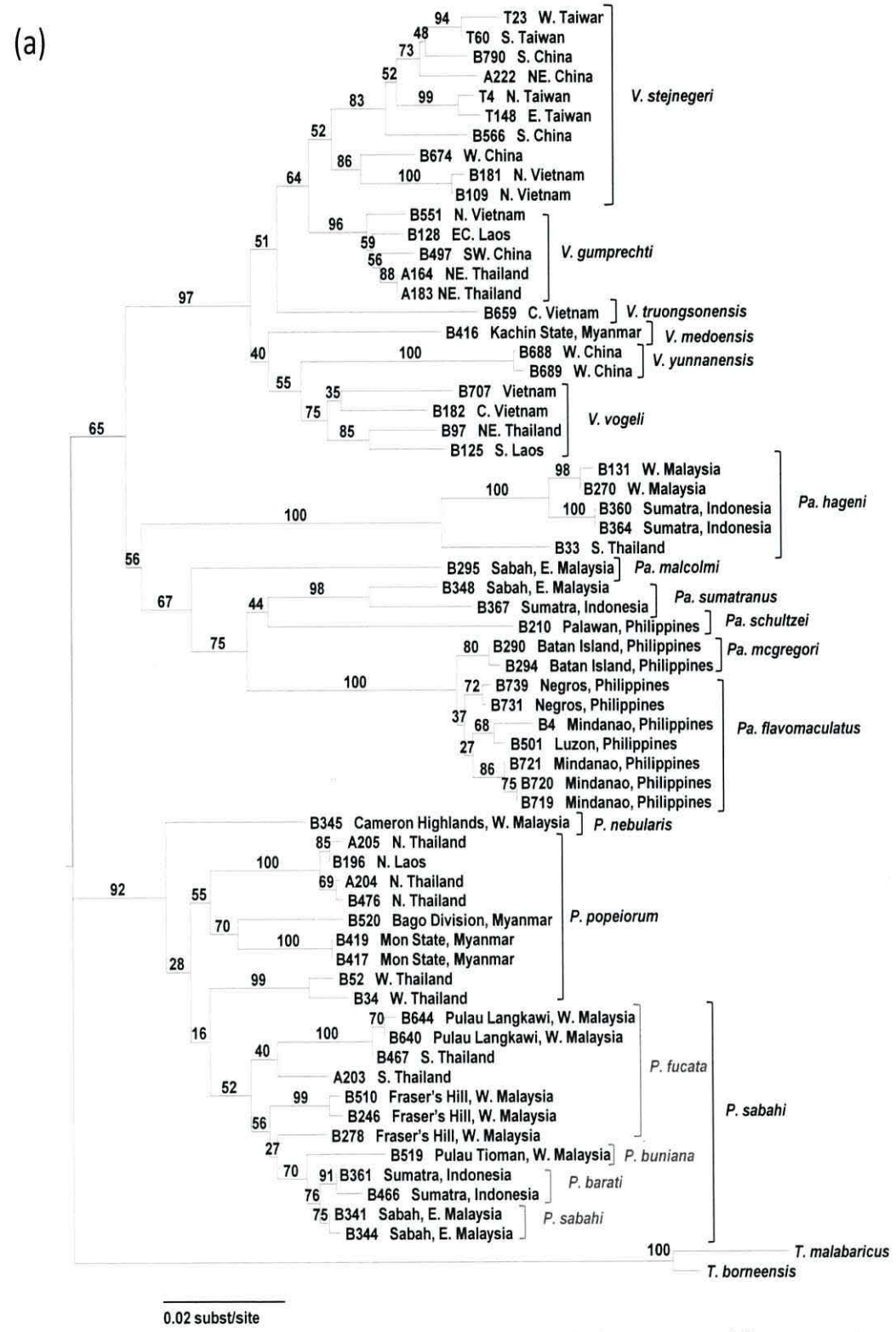
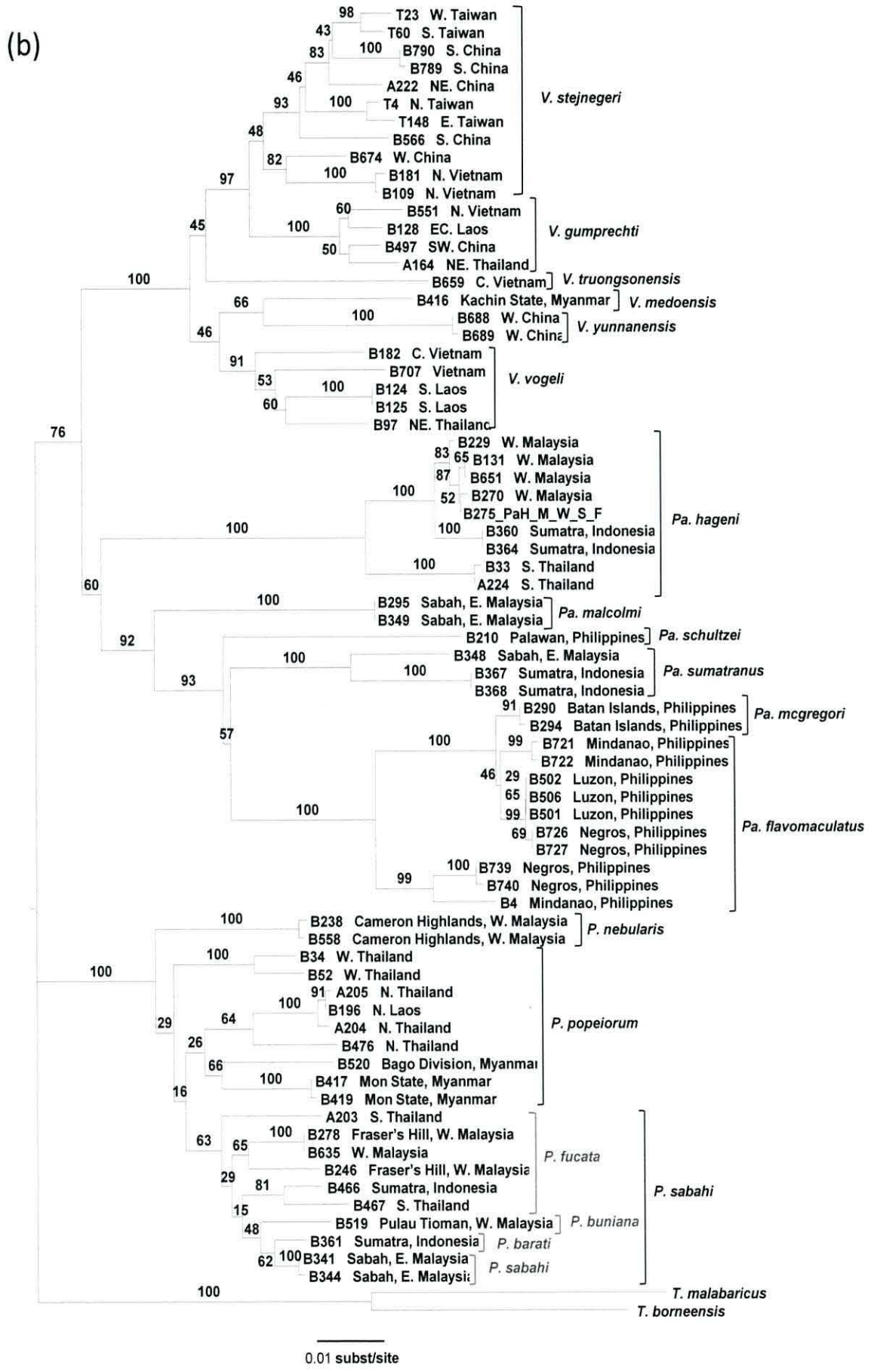


Figure 5. K2P corrected distance Neighbour-Joining trees with support values from 1000 bootstrap replicates. Outgroups are *Trimeresurus malabaricus* and *T. borneensis*. **(a) COI NJ tree.** All species clustered according to Sanders' taxonomy. Species clustering according to Vogel's taxonomy, represented in gray colour, render *P. fucata* polyphyletic. **(b) Total Evidence NJ tree.** Species clusters were accurate only for *Viridovipera,* whereas *Pa. flavomaculatus* and Vogel's taxonomy of *Popeia* were polyphyletic.


(b)
T23 W. Taiwan
T60 S. Taiwan
B790 S. China
B789 S. China
A222 NE. China
T4 N. Taiwan
T148 E. Taiwan
B566 S. China
B674 W. China
B181 N. Vietnam
B109 N. Vietnam
V. stejnegeri
B551 N. Vietnam
B128 EC. Laos
B497 SW. China
A164 NE. Thailand
V. gumprechti
B659 C. Vietnam
V. truongsonensis
B416 Kachin State, Myanmar
V. medoensis
B688 W. China
B689 W. China
V. yunnanensis
B182 C. Vietnam
B707 Vietnam
B124 S. Laos
B125 S. Laos
B97 NE. Thailand
V. vogeli
B229 W. Malaysia
B131 W. Malaysia
B651 W. Malaysia
B270 W. Malaysia
B275_PaH_M_W_S_F
B360 Sumatra, Indonesia
B364 Sumatra, Indonesia
B33 S. Thailand
A224 S. Thailand
Pa. hageni
B295 Sabah, E. Malaysia
B349 Sabah, E. Malaysia
Pa. malcolmi
B210 Palawan, Philippines
Pa. schultzei
B348 Sabah, E. Malaysia
B367 Sumatra, Indonesia
B368 Sumatra, Indonesia
Pa. sumatranus
B290 Batan Islands, Philippines
B294 Batan Islands, Philippines
Pa. mcgregori
B721 Mindanao, Philippines
B722 Mindanao, Philippines
B502 Luzon, Philippines
B506 Luzon, Philippines
B501 Luzon, Philippines
B726 Negros, Philippines
B727 Negros, Philippines
B739 Negros, Philippines
B740 Negros, Philippines
B4 Mindanao, Philippines
Pa. flavomaculatus
B238 Cameron Highlands, W. Malaysia
B558 Cameron Highlands, W. Malaysia
P. nebularis
B34 W. Thailand
B52 W. Thailand
A205 N. Thailand
B196 N. Laos
A204 N. Thailand
B476 N. Thailand
B520 Bago Division, Myanmar
B417 Mon State, Myanmar
B419 Mon State, Myanmar
P. popeiorum
A203 S. Thailand
B278 Fraser's Hill, W. Malaysia
B635 W. Malaysia
B246 Fraser's Hill, W. Malaysia
B466 Sumatra, Indonesia
B467 S. Thailand
P. fucata
B519 Pulau Tioman, W. Malaysia
P. buniana
B361 Sumatra, Indonesia
P. barati
B341 Sabah, E. Malaysia
B344 Sabah, E. Malaysia
P. sabahi
P. sabahi
T. malabaricus
T. borneensis
0.01 subst/site

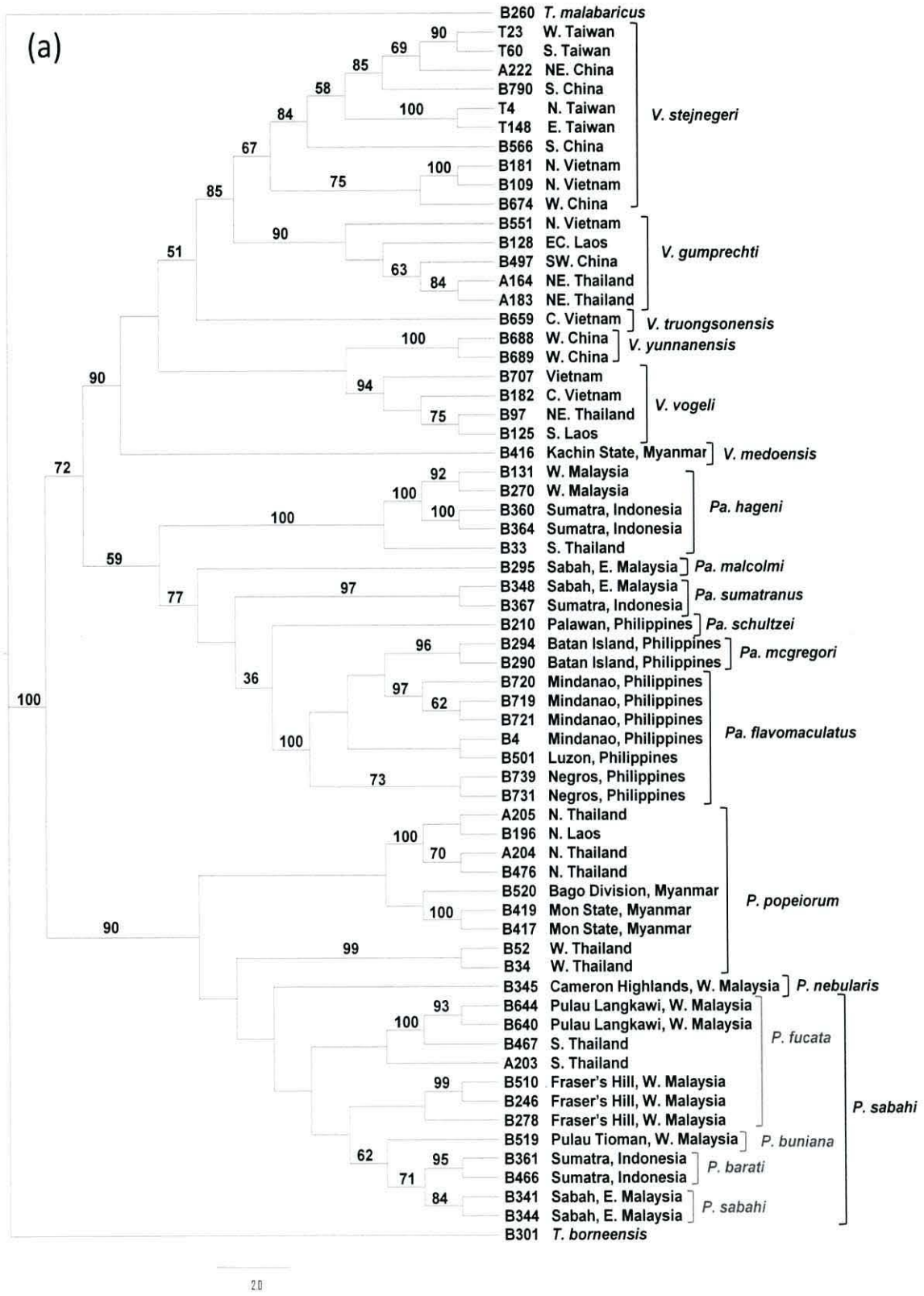


Figure 6. 50% Majority Consensus Maximum Parsimony tree with support values from 1000 bootstrap replicates. Species clusters are monophyletic but trees were not well-resolved and show low to moderate support values at the internal nodes. **(a) COI MP tree. (b) Total Evidence MP tree.** Numbers in black circles indicate node numbers for which Partitioned Bremer Support indices are given in Table 3.

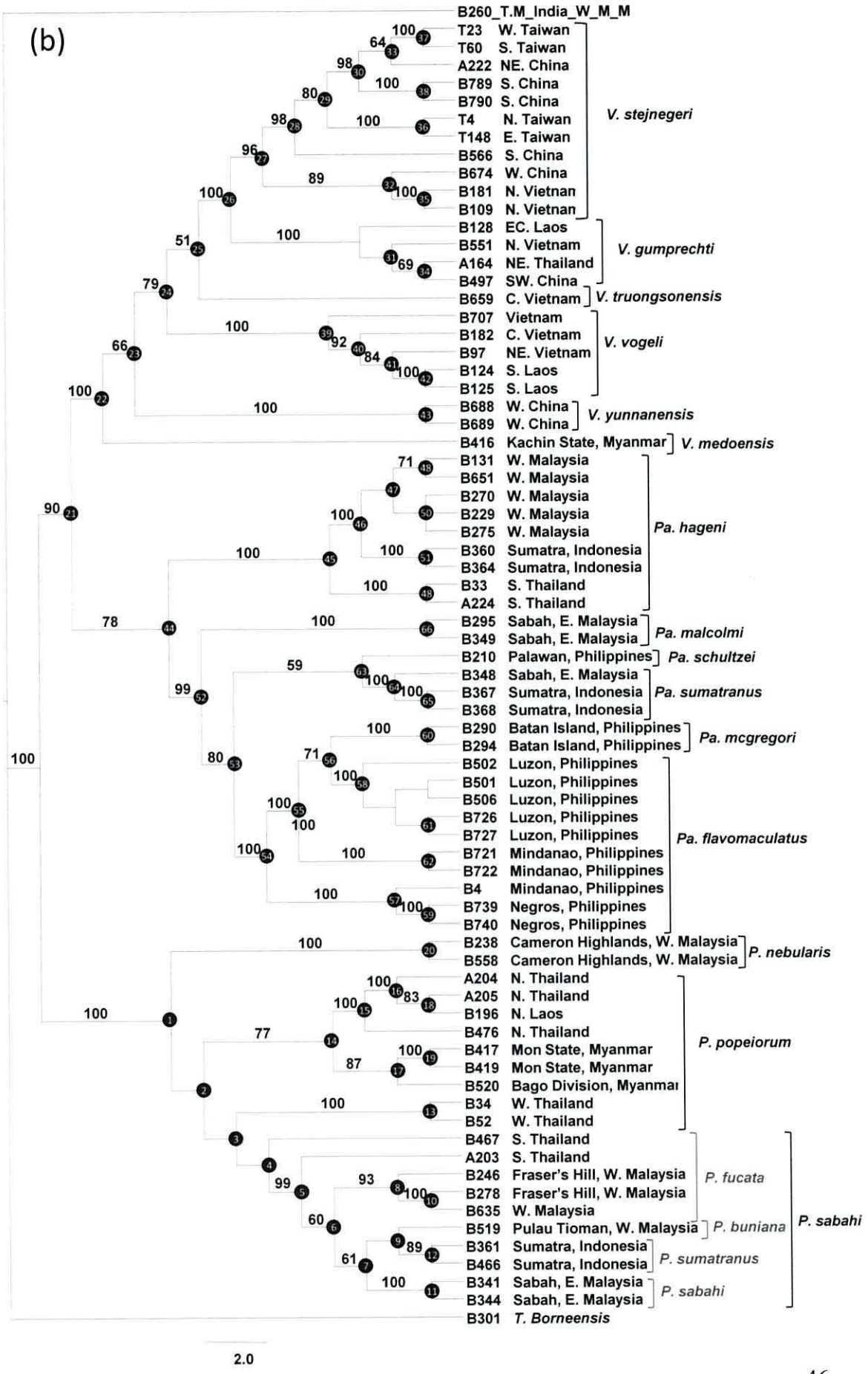

(b)
B260_T.M_India_W_M_M
T23 W. Taiwan
T60 S. Taiwan
A222 NE. China
B789 S. China
B790 S. China
T4 N. Taiwan
T148 E. Taiwan
B566 S. China
B674 W. China
B181 N. Vietnan
B109 N. Vietnan
V. stejnegeri
B128 EC. Laos
B551 N. Vietnam
A164 NE. Thailand
B497 SW. China
V. gumprechti
B659 C. Vietnam
V. truongsonensis
B707 Vietnam
B182 C. Vietnam
B97 NE. Vietnam
B124 S. Laos
B125 S. Laos
V. vogeli
B688 W. China
B689 W. China
V. yunnanensis
B416 Kachin State, Myanmar
V. medoensis
B131 W. Malaysia
B651 W. Malaysia
B270 W. Malaysia
B229 W. Malaysia
B275 W. Malaysia
B360 Sumatra, Indonesia
B364 Sumatra, Indonesia
B33 S. Thailand
A224 S. Thailand
Pa. hageni
B295 Sabah, E. Malaysia
B349 Sabah, E. Malaysia
Pa. malcolmi
B210 Palawan, Philippines
Pa. schultzei
B348 Sabah, E. Malaysia
B367 Sumatra, Indonesia
B368 Sumatra, Indonesia
Pa. sumatranus
B290 Batan Island, Philippines
B294 Batan Island, Philippines
Pa. mcgregori
B502 Luzon, Philippines
B501 Luzon, Philippines
B506 Luzon, Philippines
B726 Luzon, Philippines
B727 Luzon, Philippines
B721 Mindanao, Philippines
B722 Mindanao, Philippines
B4 Mindanao, Philippines
B739 Negros, Philippines
B740 Negros, Philippines
Pa. flavomaculatus
B238 Cameron Highlands, W. Malaysia
B558 Cameron Highlands, W. Malaysia
P. nebularis
A204 N. Thailand
A205 N. Thailand
B196 N. Laos
B476 N. Thailand
B417 Mon State, Myanmar
B419 Mon State, Myanmar
B520 Bago Division, Myanmar
B34 W. Thailand
B52 W. Thailand
P. popeiorum
B467 S. Thailand
A203 S. Thailand
B246 Fraser's Hill, W. Malaysia
B278 Fraser's Hill, W. Malaysia
B635 W. Malaysia
P. fucata
B519 Pulau Tioman, W. Malaysia
P. buniana
B361 Sumatra, Indonesia
B466 Sumatra, Indonesia
P. sumatranus
B341 Sabah, E. Malaysia
B344 Sabah, E. Malaysia
P. sabahi
P. sabahi
B301 T. Borneensis
2.0

Table 3. Total and Partitioned Bremer Support Values from five genes for each node of the Total Evidence MP Tree.

Node	COI	ND4	CytB	16S	12S	TBS
1	10	4	9	4	6	33
2	1	-3.5	0	1	2.5	1
3	1	-3.5	0	1	2.5	1
4	1	-3.5	0	1	2.5	1
5	3	4	4	0	2	13
6	2	1	-1	1	-1	2
7	1	0	1	1	-1	2
8	1	1	1	0	0	3
9	0.5	-1	0.5	0.5	0.5	1
10	5	1	1	1	1	9
11	1	6	3	0	-1	9
12	-1	6	2	2	0	9
13	3.33	4.67	7	5.67	3.33	24
14	-1.03	4.03	-0.03	1.03	0	4
15	-3.01	9.01	3.99	1.01	0	11
16	12	0	0	0	0	12
17	3	0	2	0	0	5
18	1	3	-1	-1	0	2
19	9	8	5	2	4	28
20	11	6	2	3	1	23
21	9	-2.5	-1.5	0.5	2.5	8
22	11.02	1.63	6.37	0.8	3.18	23
23	2	0	4	1	-1	6
24	-2	2	2	1	3	6
25	2	-3	4	0	1	4
26	5	6	7	1.5	0.5	20
27	2.5	6	0.5	2	0	11
28	5	3	-1	0	3	10
29	1	1.5	1.5	0	0	4
30	3.5	-0.5	2.5	-0.5	2	7
31	4	3	8	0	1	16
32	3	1	2	1	1	8
33	0.5	-0.5	0.5	-0.5	0	0

Node	COI	ND4	CytB	16S	12S	TBS
34	1.5	2.5	-3.5	1.5	0	2
35	8	12	9	2	2	33
36	8.5	5.5	3.5	2.5	2	22
37	2.5	2.5	4.5	-0.5	1	10
38	3.5	6.5	3.5	0.5	1	15
39	3	9	6	2	7	27
40	1	4	2	0	-1	6
41	3	0	1	1	0	5
42	9	2	8	5	4	28
43	16	6	14	3	4	43
44	6.02	-8.02	4.02	3.98	3	9
45	23	8.5	8	6	7.5	53
46	9	6.63	8	0.37	0	24
47	1.63	3.16	2	-1.79	1	6
48	10	6	14	3	4	37
49	1	0	0	0	0	1
50	0	1	0	0	0	1
51	6.07	7.4	2	2.87	2.67	21
52	13.4	1	-3	1.6	5	18
53	6	-3	0	1	3	7
54	20.17	6.67	-6.67	3.67	11.17	35
55	0	0	64	0	0	64
56	0	0	0	0	1	1
57	-1	0	11	-1	0	9
58	1	6	0	0	1	8
59	1	5	3	0	2	11
60	3	4	0	1	1	9
61	0	0	1	0	0	1
62	5	3	1	1	1	11
63	0	0	-1	1	2	2
64	12	9	5	3	3	32
65	10	9	8	2	3	32
66	21	12.67	7	3.67	4.67	49
Total	311.6	191.85	250.68	79.38	114.52	948

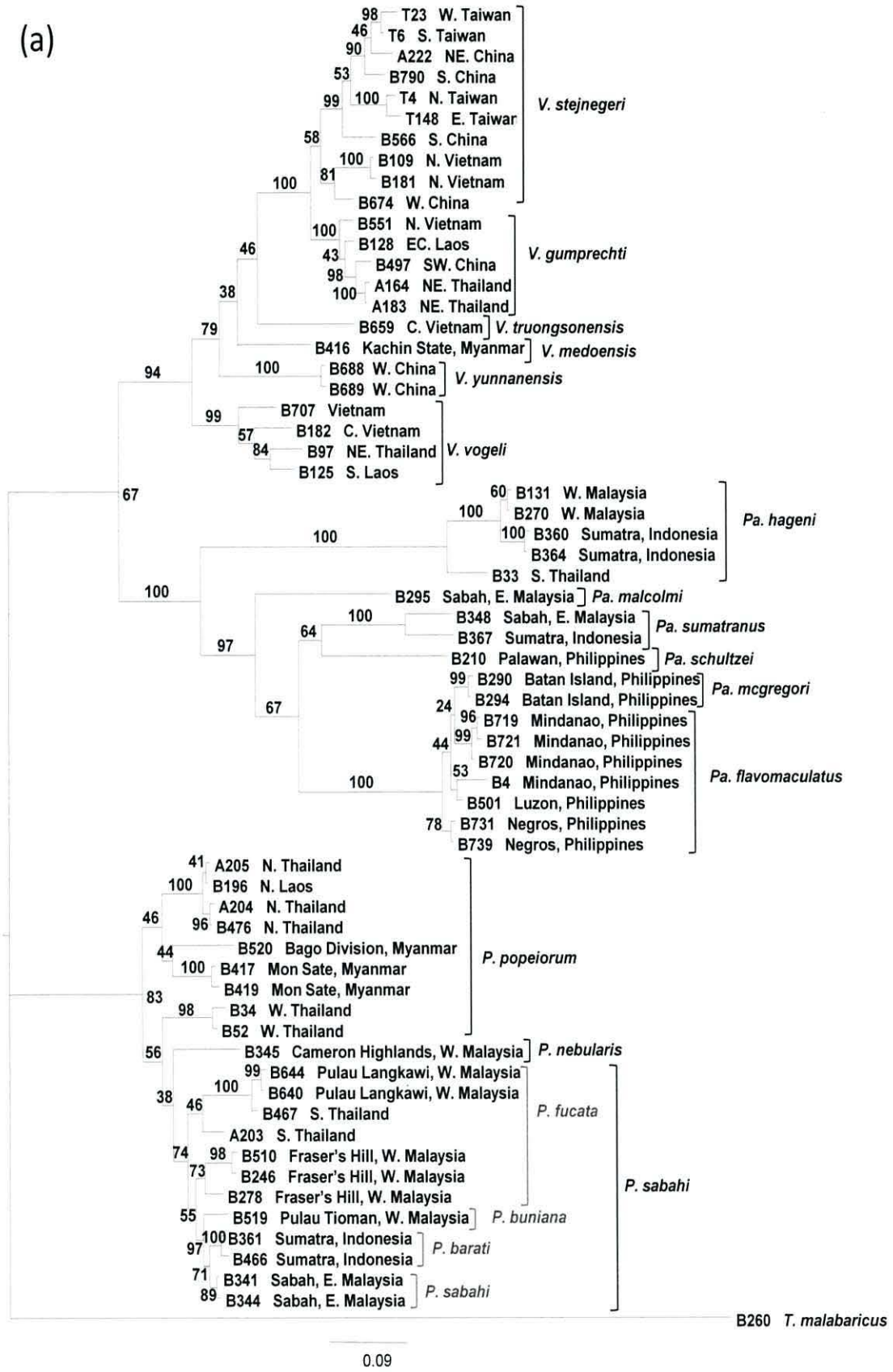
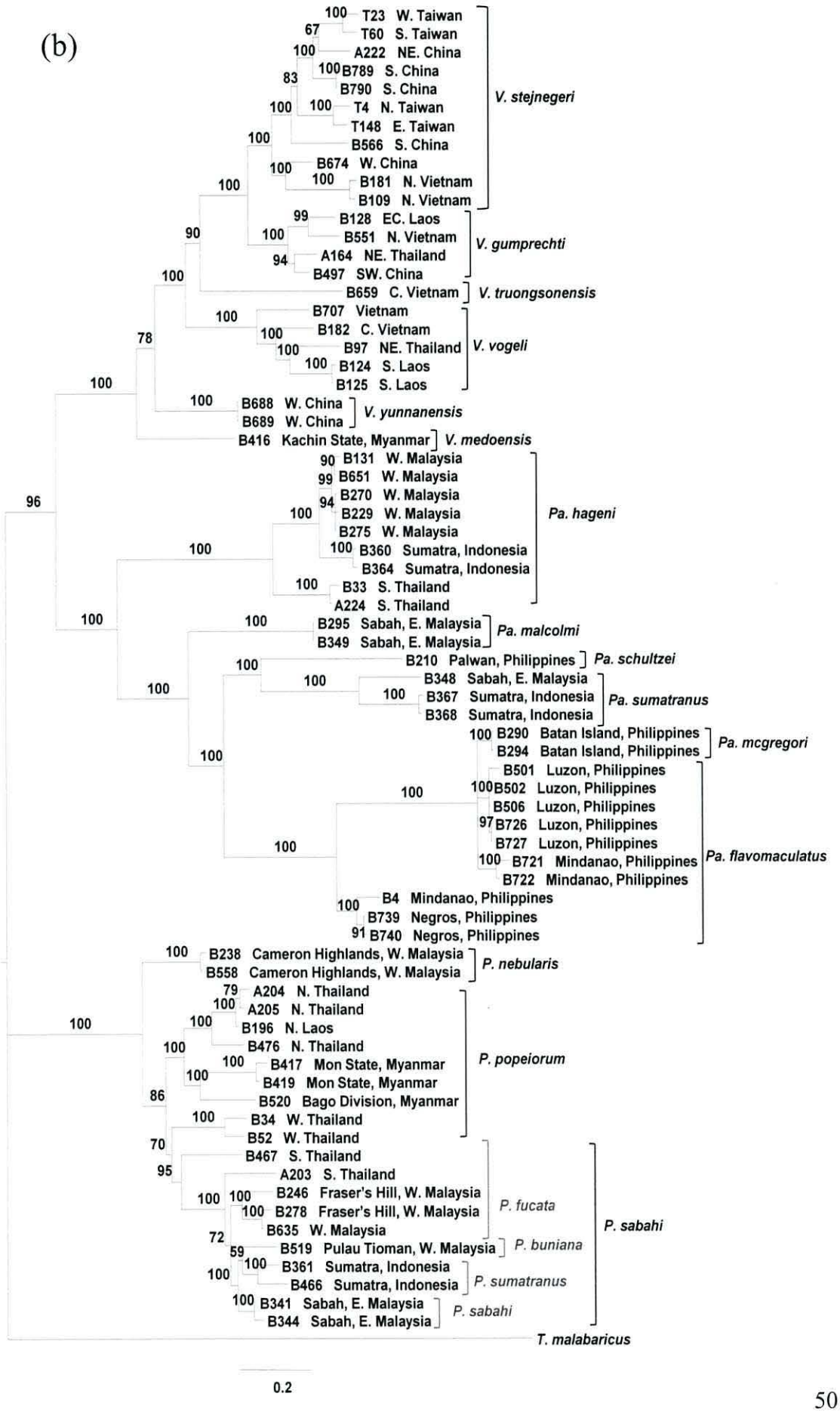


Figure 7. Bayesian MCMC phylogeny of three million generations. **Support values at each node are Bayesian posterior probabilities. (a) COI consensus tree using all compatible groups (b) Total Evidence consensus tree**


(b)
T23 W. Taiwan
T60 S. Taiwan
A222 NE. China
B789 S. China
B790 S. China
T4 N. Taiwan
T148 E. Taiwan
B566 S. China
B674 W. China
B181 N. Vietnam
B109 N. Vietnam
V. stejnegeri
B128 EC. Laos
B551 N. Vietnam
A164 NE. Thailand
B497 SW. China
V. gumprechti
B659 C. Vietnam
V. truongsonensis
B707 Vietnam
B182 C. Vietnam
B97 NE. Thailand
B124 S. Laos
B125 S. Laos
V. vogeli
B688 W. China
B689 W. China
V. yunnanensis
B416 Kachin State, Myanmar
V. medoensis
B131 W. Malaysia
B651 W. Malaysia
B270 W. Malaysia
B229 W. Malaysia
B275 W. Malaysia
B360 Sumatra, Indonesia
B364 Sumatra, Indonesia
B33 S. Thailand
A224 S. Thailand
Pa. hageni
B295 Sabah, E. Malaysia
B349 Sabah, E. Malaysia
Pa. malcolmi
B210 Palwan, Philippines
Pa. schultzei
B348 Sabah, E. Malaysia
B367 Sumatra, Indonesia
B368 Sumatra, Indonesia
Pa. sumatranus
B290 Batan Island, Philippines
B294 Batan Island, Philippines
Pa. mcgregori
B501 Luzon, Philippines
B502 Luzon, Philippines
B506 Luzon, Philippines
B726 Luzon, Philippines
B727 Luzon, Philippines
B721 Mindanao, Philippines
B722 Mindanao, Philippines
B4 Mindanao, Philippines
B739 Negros, Philippines
B740 Negros, Philippines
Pa. flavomaculatus
B238 Cameron Highlands, W. Malaysia
B558 Cameron Highlands, W. Malaysia
P. nebularis
A204 N. Thailand
A205 N. Thailand
B196 N. Laos
B476 N. Thailand
B417 Mon State, Myanmar
B419 Mon State, Myanmar
B520 Bago Division, Myanmar
B34 W. Thailand
B52 W. Thailand
P. popeiorum
B467 S. Thailand
A203 S. Thailand
B246 Fraser's Hill, W. Malaysia
B278 Fraser's Hill, W. Malaysia
B635 W. Malaysia
P. fucata
B519 Pulau Tioman, W. Malaysia
P. buniana
B361 Sumatra, Indonesia
B466 Sumatra, Indonesia
P. sumatranus
B341 Sabah, E. Malaysia
B344 Sabah, E. Malaysia
P. sabahi
P. sabahi
T. malabaricus
0.2

2.5 DISCUSSION

2.5.1 Universal Primers and Unique Haplotypes

The non-amplification of COI using Folmer's primers (Folmer et al. 1994) in *V. stejnegeri* and *V. truongsonensis* is clear evidence that universal COI primers are not robust across all animal taxa. The barcoding protocol includes primer-design recommendations (Hajibabaei et al. 2005), and mini-barcodes from amplicons of size 100 – 250bp (Hajibabaei et al. 2006b; Meusnier et al. 2008) have been successfully used for species identification including in snakes (Dubey et al. 2011). However, barcode studies are approved only with a minimum sequence length of 500bp from the Folmer's primer region of COI (Ratnasingham and Hebert 2007), and obtaining sequences of this length is not always straightforward. Consequently, universal primer designs specific to animal groups, such as fish (Ivanova et al. 2007), birds (Patel et al. 2010), are becoming increasingly common. The case of Asian green pitvipers is another example for group-specific universal primers being the only effective solution for ensuring global uniformity in barcoding projects. Intrageneric haplotype comparisons did not show cross-overs between pre-ordained species which is a key requirement for successfully barcoding species. Double-checking species identity against BOLD Systems and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was possible only for *V. stejnegeri* since no COI barcodes have been submitted for the green pitviper group apart from this species.

2.5.2 The Barcode Gap

A global barcode gap (e.g. 10X intraspecific variation) to distinguish between species of all animal taxa (Hebert et al. 2004b) is problematic (Meyer and Paulay 2005), and the barcode gap itself has been considered an artefact of sampling by some (Wiemers and Fiedler 2007). Multiple sampling of *Pa. flavomaculatus* and *Pa. mcgregori* did not increase intraspecific variation, but since, as isolated island species, their distribution ranges are relatively smaller, it could be argued that they are not really appropriate for testing this. However, these results are also consistent with a previous study of continental species *V. gumprechti* and continental and island species *P. sabahi,* where 10 samples resulted in only two haplotypes for each (Mrinalini unpublished). Further, despite the Chinese specimens not being included in the above

study, intraspecific variation of *V. stejnegeri* was higher (3.4 ± 0.5 %) and the barcode gap was lower than in the current study, where the species sampling range was extended. With respect to green pitvipers, it appears that intraspecific variation estimates do not vary significantly enough with multiple sampling or with geographic range (Robinson et al. 2009) to be able affect the barcode gap. While the scale of this study is relatively small, larger studies with hundreds of species and wider geographic ranges have also reported similar results with a few exceptions which were attributable to the underestimation of species diversity in the first instance (Robinson et al. 2009; Steinke et al. 2009).

Application of the barcode gap concept for species identification and delimitation is based on the presence of a significant difference between intraspecific variation and interspecific divergence in a majority of species in question (Hebert et al. 2003a). Among the three genera, barcode gaps varied drastically, being highest in the island-archipelago genus *Parias*, moderate in the continental genus *Viridovipera,* and lowest in *Popeia* which consists of both continental and island taxa. It is impossible to derive a specific percentage of sequence divergence as a rule-of-thumb for within and among species distinction across genera. The most important factors for this extreme inter-generic variability appear to be the complex and fragmented biogeography of southeast Asia and the closely-related, recent evolutionary histories of green pitviper genera. Failure of barcode gap in *Popeia* was due to low interspecific divergence, and similar cases have also been reported even in closely-related temperate and polar taxa (e.g. Wiemers and Fiedler 2007; Allcock et al. 2011). In most situations, this can be attributed to incomplete lineage-sorting, hybridization, or oversplitting of species within genera (Steinke et al. 2009). In Vogel's taxonomy of *Popeia,* barcode gaps were non-existent, consistent with a study on spiders where barcode gaps were absent in c. 91% of paraphyletic and intermingled clades (Robinson et al. 2009). While this correlation holds good for *Pa. mcgregori* and *Pa. flavomaculatus* as well, species status in all these cases cannot be dismissed based solely on this criteria. A more comprehensive discussion on species designations within these two genera is presented in the next few sections. In summary, the barcode gap is a function of the evolutionary history of the study species and therefore of little use as a quantified or strictly-defined species identification or delimitation tool especially in closely-related

and recently-diverged species.

2.5.3 Species Identification In A Resolved Taxonomic Framework

The resolutions in COI phylogenies of *Viridovipera* and *Parias* were generally similar to those of total evidence, although the nodal support values were also generally lower. NJ analysis of Cytb and ND4 genes individually was also performed (not shown) and resulted in fragmented species and generic clusters which were incongruent with the taxonomy in the three genera. There is no doubt that COI is the best mtDNA gene for deriving taxonomically congruent monophyletic species clusters, and this is further corroborated by the PBS index which was highest for the COI partition in the total evidence MP analysis. *Pa. mcgregori* formed a clear monophyletic cluster only in the NJ phylogeny, which is the standard methodology used in barcoding studies. This difference from other genes and analyses is important, as it can easily be interpreted as support for the use of K2P-corrected distance phylogenies in barcoding studies (Hebert et al. 2003a). However, a closer look at the taxonomic resolution in *Parias* raises questions about the species designations in the *Pa. flavomaculatus – Pa. mcgregori* group.

Initially considered a subspecies within *Pa. flavomaculatus, Pa. mcgregori,* found exclusively on the Batan Islands (about 130 miles north of Luzon), was raised to species level due to its distinct yellow and grey colouration and complete lack of green pigmentation (Gumprecht 2002), its isolation from mainland Philippines (Leviton 1963), its morphological distinctiveness (Sanders et al. 2004b), and mtDNA phylogenetic affiliations (Malhotra and Thorpe 2004a; Sanders et al. 2004b). However, despite forming a distinct cluster from *Pa. flavomaculatus* in Luzon in a multivariate scalation and colour pattern character analysis (Sanders et al. 2004b), the evolution of its overall colour pattern mapped onto a mtDNA phylogeny showed that *Pa. flavomaculatus,* Luzon, had three colour pattern variations of which, the non-green colour was shared with *Pa. mcgregori* (Sanders et al. 2006b). *Pa. flavomaculatus* from Mindanao in the south, on the other hand, did not share any colour patterns with either of these (Sanders et al. 2006b).

In the mtDNA analysis of four genes in character-based phylogenies (MP and

Bayesian) *Pa. mcgregori* always clustered with *Pa. flavomaculatus* specimens in paraphyletic arrangements (Malhotra and Thorpe 2004a; Sanders et al. 2004b). MtDNA divergence between *Pa. mcgregori* and *Pa. flavomaculatus* from Luzon was found to be lower (1.6 ± 1.4%) than between conspecifics of *Pa. flavomaculatus* from Luzon and Mindanao (1.9 ± 1.4%) (Sanders et al. 2004b). Although the sample size used in these studies was low, these results are consistent with the character-based COI phylogenies and COI divergence rates in the current study. Furthermore, in the entire total evidence MP phylogeny of this study, the PBS values were highest for a paraphyletic arrangement (nodes 54 and 55, Figure 6b) of *Pa. flavomaculatus*. With low intraspecific genetic variability and its close relationship with *Pa. flavomaculatus,* the designation of *Pa. mcgregori* as separate species appears to be a result of either over-splitting *Pa. flavomaculatus flavomaculatus* into *Pa. flavomaculatus* and *Pa. mcgregori* or not splitting the Luzon and Mindano populations of *Pa. flavomaculatus* into two distinct species. Hence, the monophyly of *Pa. mcgregori* in distance-based NJ analysis needs to be interpreted as the effect of the loss of character-state information and the lack of alternative phylogenetic hypothesis-testing to fit the data (Swofford et al. 1996) rather than as a 100% success of COI in monophyletic species clustering of *Parias*.

2.5.4 Species Delimitation In The Taxonomically Contentious Genus *Popeia*

Popeia is one of the most recently-diverged genera in the *Trimeresurus s.s.* group (Malhotra and Thorpe 2004a), with the divergence between the northern and southern clades estimated to be c. 2.29 – 6.25 mya (Sanders et al. 2006a). Species delimitation in this genus has always been problematic, with alternative arrangements having been considered due to non-correspondence of monophyletic mtDNA clusters to morphological groups or geographical distributions (Sanders et al. 2006a). Hence it is not surprising that monophyly of species clusters failed in both Sanders' and Vogel's taxonomic frameworks for all analyses. The Cameron Highlands species *P. nebularis,* the west Thailand specimens from Phetburi, and one specimen from Phang Nga in south Thailand (B467), clustered with *P. poperiorum* in previous studies of four mtDNA genes (Malhotra and Thorpe 2004a; Sanders et al. 2006a). In addition, the

phenotype and habitat usage of these specimens is congruent with the northern clades (Sanders et al. 2006a). It has been hypothesized that *Popeia* populations could have spread southward from continental Asia into the Sunda region (Sanders et al. 2006a).

With the inclusion of COI however, the Phang Nga specimen clustered with the southern clade *P. sabahi* and the position of *P. nebularis* and west Thailand specimens became uncertain. For these specimens, DNA was re-extracted and all the five genes were re-amplified and re-sequenced to rule out the possibility of human error. The results confirmed that the sequences were indeed accurate and the mtDNA affiliations changed after adding COI to the dataset. Although the support for the external nodes is low, the change in relationships and tree topologies suggests that COI, as a gene, carries a substantial amount of genetic information for species-level taxonomy, and that the evolutionary and biogeographical history of *Popeia* could possibly be more complicated, involving vicariance and recolonization events.

During the glacial and inter-glacial periods of Pleistocene, extensive sea-level changes in southeast Asia led to the isolation and secondary contact of flora and fauna through the formation of refugia and subsequent habitat expansion (Haffer 1969; Voris 2000). Relatively recent hybridization events and/or incomplete lineage-sorting are the most plausible explanations for the lack of complete resolution in this genus. Moreover, convergence in molecular evolutionary patterns across a wide-variety of animals such as mosquitoes (Morgan et al. 2009), rodents (Gorog et al. 2004), and monkeys (Ziegler et al. 2007) have also indicated that this has been a particularly important instrument in determining high intra-specific variability and the low inter-specific diversity in the fauna of continental Asia, Sunda mainland and island regions. In the case of Vogel's taxonomy, *P. fucata* has always been polyphyletic when other mtDNA genes were used (Sanders et al. 2006a). Considering that there is no further resolution for the monophyly of species clusters in Vogel's taxonomy and the variation in results between analysis methods for COI in the Sanders taxonomic framework, it can be concluded that COI sequences are, in general, more useful for species identification in a well-resolved taxonomic framework rather than as a tool for species delimitation (Hajibabaei et al. 2006a; Golding et al. 2009).

2.5.5 Recommendations For The Taxonomy Of *Popeia* And *Parias*

Although the overall uses of COI for DNA barcoding appear to be moderate in the southeast Asian green pitviper group, this study has raised some important taxonomic issues. *Popeia* is an evolutionarily complex genus in which both mtDNA phylogenetic and phenetic species criteria have been useful only to a limited extent (Sanders et al. 2006a). Divergent morphologies but conservative arrangement of mtDNA lineages has been observed in recently-diverged sympatric island species and may not necessarily indicate taxonomic errors (Pestano et al. 2003). However, given that particularly high levels of crypsis are prevalent in *Popeia,* the sub-division of *P. sabahi* into five species based on morphology alone (Vogel et al. 2004; Grismer et al. 2006; David et al. 2008) is difficult to justify. Hence Vogel's taxonomic arrangement will need further validation using another independent source of evidence. Analysis of nuclear markers could possibly provide a better understanding of the degree of reproductive isolation between the populations designated as species.

The Philippine and McGregor's pitvipers of *Parias* are allopatrically distributed and hence reproductive isolation is not a matter of contention. However, as some aspects of morphology and mtDNA evolution raise questions about the degree of their distinctiveness, grouping *Pa. mcgregori* as a putative species under *Pa. flavomaculatus* complex is a better taxonomic arrangement for now. In both these genera, ecological bases of their differentiation can be explored using highly sensitive tools such as geometric morphometrics for shape analyses of skull (Claude et al. 2004) and stable isotope analyses (Markos Alexandrou and Axel Barlow pers. comm.) to gain a better understanding of possible divergences in their evolutionary ecology.

CHAPTER 3

CRYPTIC SPECIES DELIMITATION USING DOMINANT MARKERS:

A Comparative Study of Analysis Methods Using The *Cryptelytrops macrops* Complex.

3.1 ABSTRACT

In recently diverged species, short sequence-based nuclear markers may be relatively uninformative. In such cases, AFLPs have proved to be useful by providing a substantial amount of genome-wide information essential for establishing the degree of reproductive isolation between species. However, the effectiveness of various methods used to analyse dominant data is still unclear. In this study, a comparative analysis of some of the more widely-applied and some new methods of AFLP analysis was conducted using *Cryptelytrops macrops* as a case study. A gene tree was also reconstructed using mtDNA gene sequences. Inferences of population structure and species boundaries from both mtDNA phylogeny and AFLP analyses show that *C. macrops* consists of a complex of three cryptic species which are not easily identifiable by morphological studies alone. Further, these results clearly demonstrate that while AFLP is undoubtedly a useful genetic tool for cryptic species delimitation, the application of appropriate analysis methods and the verification of results by using multiple types of analysis are critical for making robust biological inferences.

3.2 INTRODUCTION

Multilocus nuclear markers such as AFLPs (Vos et al. 1995) are robust and are useful for speciation research as they can provide a better assessment of species diversity where mtDNA sometimes overestimates it (Dasmahapatra et al. 2010). Multilocus markers are generally analysed through genetic clustering and diversity analyses implemented under population genetics models which are based on F-statistics using allele frequency calculations. Spatial priors can be incorporated into cluster analyses, and the uses of both spatial and non-spatial models have been evaluated with respect to co-dominant datasets such as microsatellites (Latch et al. 2006; Chen et al. 2007; Frantz et al. 2009). However, the main constraint for applying these methods to dominant markers such as AFLPs is that it is not possible to distinguish between homozygous and heterozygous states of an allele, necessitating making several assumptions in the implementation of population genetics models. Irrespective of this, the majority of AFLP studies regularly apply allele frequency-based algorithms and derive biological inferences, with rarely any discussion of possible result biases arising from inappropriate analysis methods (Hollingsworth and Ennos 2004; Bonin et al. 2007).

The non-spatial genetic clustering program STRUCTURE, which uses a Bayesian MCMC algorithm, is most popular for AFLP analysis and has been routinely used to infer K (the number of populations). STRUCTURE assumes that the loci are in Hardy-Weinberg Equilibrium (HWE) and linkage equilibrium to infer population structure (Falush et al. 2007). The algorithm has been modified for dominant data by assuming that there are recessive alleles at a subset of loci which provide partial information about the diploid genotypes for the entire dataset (Falush et al. 2007). There are also programs such as TESS, GENECLUST, and GENELAND that perform Bayesian cluster analysis under spatial models (Guillot et al. 2005a; Guillot et al. 2005b; Francois et al. 2006; Chen et al. 2007; Guillot 2008; Guillot et al. 2008). GENELAND, although using a similar algorithm to STRUCTURE and idenfying groups of individuals in Hardy-Weinberg Equilibrium (HWE) with linkage equilibrium between loci, allows for the use of spatial priors (Guillot and Santos 2010). GENELAND has also been recently upgraded to be able to correct allele-

frequency estimates from dominant data by distinguishing between observed and true unobserved genotypes and incorporating simulations of true unobserved genotypes into the MCMC algorithm (Guillot and Santos 2010).

However, both STRUCTURE and GENELAND methods still assume that AFLP null-alleles (i.e., band absences) are recessive alleles when calculating allele frequencies and subsequently estimating K. The common assumptions (such as HWE in a population, linkage disequilibrium between populations, but not within populations) which drive these analyses are conceptually not applicable to dominant data. It has also been explicitly acknowledged that the models used in STRUCTURE for K estimation and their assumptions are less than straightforward, could yield inaccurate results in general, and need to be approached with caution when deriving biological inferences (Pritchard et al. 2000). Furthermore, the accuracy of K estimation for dominant datasets has been found to be lower than that of co-dominant datasets even in the new version of GENELAND (Guillot and Santos 2010). In some cases, tree-building using AFLP data has been found to perform better at cluster identification due to the lack of population genetics model assumptions (Meudt et al. 2009). Taking all this into consideration, it is important that care be exercised in the application of population genetics models to AFLP data, allowing for the possibility that there may be a certain degree of uncertainty in estimating the number of K, the assignment of individuals to each K, and the genetic structure of each K. In addition to this, Bayesian inference performed through sequential MCMC simulations is dependent on analysis parameters and starting point and length of the chain, and is also computationally intensive. All the above issues have initiated a call for the proper use of statistics and the development of new and more appropriate methods for analysing dominant markers (Hollingsworth and Ennos 2004, Excoffier and Heckel 2006, Bonin et al. 2007, Meudt et al. 2009).

The two new multivariate tools DAPC (Jombart et al. 2010) and *prabclus* (Hausdorf and Hennig 2010) (described in section 1.3.3) have given better results than STRUCTURE in the initial clustering/species delimitation studies, and being implemented outside the confines of population genetics models, show considerable promise for cluster analysis, population structure analysis, and speciation research

using dominant markers (Hausdorf and Hennig 2010; Jombart et al. 2010). The true test of performance, however, is by validation of the utility of these methods in real scenarios, where species boundaries and genetic structure still need to be clearly established. This study compared, for the first time, the performance of non-spatial and spatial Bayesian clustering methods (STRUCTURE and GENELAND) with multivariate methods (DAPC and *prabclus*) in successfully delimiting cryptic species using dominant data.

The genus *Cryptelytrops* is one of eight genera resulting from the splitting of *Trimeresurus sensu lato* by Malhotra and Thorpe (2004a). It is estimated to have diverged from other closely-related genera of *Trimeresurus* during early to mid-Miocene, about 10.34 to 19.17 mya (Malhotra and Thorpe 2004a). The members of the genus are distinguished by fully-fused or semi-fused state of the first supralabial scale and the nasal scale and an elongated, non-spiny, hemipenis type (Malhotra and Thorpe 2004a). *Cryptelytrops* currently consists of 12 species of which, *Cryptelytrops macrops* (Figure 8) is known to be distributed across Thailand, Laos, Cambodia, and Vietnam. Although *C. macrops* specimens resemble *C. albolabris* superficially, they are more closely-related to *C. venusuts* and *C. kanburiensis* (Malhotra and Thorpe 2004a). Recently, a combined phylogenetic analysis of mtDNA and nuclear introns showed paraphyletic arrangements of *C. macrops* with respect to *C. venustus,* suggesting that *C. macrops* could be a complex of more than one cryptic species (Malhotra et al. 2010). However, when further samples were subjected to a multivariate morphometric analysis to investigate this, the results were inconclusive (Appendix 2, Figure 1).

Therefore, genetic studies using both mtDNA gene sequences and AFLP genome scans were conducted on the *C. macrops* complex in this project. Strong evidence was found for the presence of three distinct cryptic species from genetic analysis using multivariate statistics and tree-building methods. Further, the AFLP dataset was analysed in STRUCTURE, GENELAND, DAPC, and *prabclus* in order to compare the utility of each of these methods for cryptic species delimitation.

a)

b)

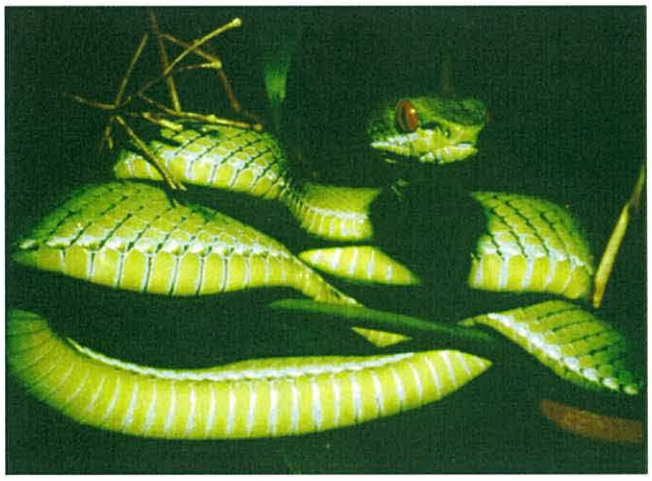


Figure 8. ***Cryptelytrops macrops.*** Specimens from a) southeast Thailand (photo by R. S. Thorpe) and b) from Vietnam (photo by Peter Paul Van Dijk) are part of the *C. macrops* complex suspected to contain cryptic species.

3.3 MATERIALS AND METHODS

3.3.1 MtDNA Amplification And Sequencing

Samples were obtained from field collections, museums and private collections. The geographic distribution of samples covers much of the known range of *C. macrops* (Gumprecht et al. 2004). For many specimens, morphological data were also available. Samples were in the form of liver or muscle tissue in 80% ethanol, clippings from the ventral scales in 80% ethanol, or up to 200µl of blood from the caudal vein preserved in 1mL 5% EDTA and 2mL SDS-Tris buffer (100mM Tris, 3% SDS). Whole genomic DNA was extracted using standard proteinase K protocols (Sambrook et al. 1989). 12S rRNA (12S), 16S rRNA (16S), and NADH4 (ND4) mitochondrial genes were amplified (as described in Malhotra et al. 2011a), cleaned with shrimp alkaline phosphatase and Exonuclease I (Werle et al. 1994), and sequenced using dye-labelled terminators (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit) on an ABI 3730XL automated sequencer.

3.3.2 AFLP Genotyping

A total of 50 individuals, of which 35 samples represented *C. macrops,* with a further 15 individuals of the closely related species *C. venustus* from South Thailand and West Malaysia as an outgroup, were genotyped (Appendix 2, Table 1). Genomic DNA was extracted by standard protocol using GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Extracts were duplicated for six samples using the same tissue type, and three samples using different tissue types (as some tissues differed in storage conditions), for repeatability tests, and negative controls were used to monitor contamination. Extract quality was checked on 1% Agarose-EtBr gels, DNA was quantified on a NanoDrop ND-1000 Spectrophotometer, and corrected to 10 ngμl^{-1} using 0.1M TE. 100 ng DNA was used per sample and 6.9 µl digestion-ligation mix (final concentrations: 1X TA buffer, 0.17 µgμl^{-1} bovine serum albumin, 0.059 Uμl^{-1} each of *Eco*RI and *Mse*I enzymes, 0.3X T4 ligase buffer, 0.03 Uμl^{-1} T4 DNA ligase, 0.74 µm each of *Eco* and *Mse* adaptors with 3 µl d_2H_2O) was added to make up a final volume of 16.9 µl. This was incubated at 16°C for 16 hours in a preconditioned water bath in ThermoFast® 96-well plates (ABgene) and diluted by a

factor of 1:4 (i.e. to a final volume of 50 µl) with d_2H_2O. Pre-selective amplification (PA) reactions were performed using 1 µl diluted ligated product in 10 µl reactions (final concentrations: 1X PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM each of pre-selective *Eco*RI and *Mse*I primers, 0.025 $U\mu l^{-1}$ Thermoprime *Taq*) with 4.15 µl d_2H_2O. Thermocycling parameters were initial warm-up at 94°C for 2 minutes, 20 cycles of denaturing at 94°C for 30 seconds, annealing at 56°C for one minute, extension at 72°C for 2 minutes, and a final extension of 72°C for 10 minutes and 20°C for 5 minutes. PA products were diluted 1:10, and 1 µl of each, along with 5 µl loading buffer, was run on 1.5% Agarose-EtBr gels. Successful PAs resulted in a smear across the whole range of a 500bp ladder. 1 µl of diluted PA product was used for selective amplification (SA) in10 µl reactions with final concentrations same as PA except the primers were replaced by fluorophores and reverse selective primer. Thermocycling parameters were initial warm-up at 94°C for 2 minutes, 12 cycles of denaturing at 94°C for 30 seconds, annealing at 65°C Δ-0.7°C/cycle for 30 seconds, extension at 72°C for 1 minute, 23 cycles of 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 1 minute, and a final extension of 72°C for 10 minutes and 20°C for 5 minutes. SA products were diluted at 1:100 and 1 µl from each primer pair was poolplexed in 10 µl formamide along with 0.5 µl GeneScan™ 500 LIZ® Size Standard and the samples were processed on an ABI 3130XL Genetic Analyzer. Pre-selective and selective primer sequences are given in Appendix 2, Table 2.

3.3.3 Peak Scoring

AFLP profiles were visualized and processed in GeneMapper® Software v4.0, and samples with amplification problems for one or more markers were discarded. Several automated and semi-automated AFLP scoring methods have been proposed in an effort to reduce time, error, and subjectivity of peak calling (reviewed in Meudt and Clarke (2007)). The semi-automated method proposed by Whitlock et al. (2008) was used but it was found that direct application of this method to raw or filtered data (using the specified phenotype-calling threshold) resulted in a high proportion of inaccurate peak-calling from unaccounted false peaks (artefacts, inter-dye pull-ups, shoulder peaks, saturation peaks), peak mobility, and clear peaks failing to get called. Therefore, the data was first checked by eye and corrected for peak mobility, false

peaks and uncalled peaks. Mean Peak Height (MPH) was calculated at each locus, and a locus-selection threshold of 100 relative fluorescence units was applied. A relative phenotype-calling threshold of 20% of MPH was applied, ie., all peaks ≥ 20% MPH were marked as present (1) and peaks ≤ 20% MPH were marked absent (0). This modified method, albeit time-consuming, significantly enhanced genotyping accuracy. Repeatability was measured as the number of loci with corresponding band presences across duplicated samples compared to the total number of loci scored.

3.3.4 Phylogenetic Reconstructions

MtDNA

Sequences were aligned by eye and maximum parsimony (MP) analysis was performed in PAUP* 4b10 (Swofford 2002). *Viridovipera vogeli, Cryptelytrops kanburiensis, Cryptelytrops purpureomaculatus,* and *Cryptelytrops albolabris* were selected to represent outgroups (Malhotra and Thorpe 2004a). A heuristic search was performed with a starting tree obtained by 1000 random additions of taxa followed by tree-bisection-reconnection branch swapping. Gaps were treated as a fifth base because they were never more than one base-pair in length. All sites were equally weighted and 1000 bootstrap replicates were performed to evaluate branch support (using the same settings as above except only 10 random additions were performed per replicate to obtain a starting tree).

Mixed-model Bayesian analyses were performed using MrBayes v3.1 (Ronquist and Huelsenbeck 2003), using *Viridovipera vogeli* as the outgroup. The dataset was partitioned into 12S, 16S, and first, second and third codon positions of ND4, and models of sequence evolution were inferred by Modeltest 3.7 using the Akaike Information Criterion (Posada and Crandall 1998) were TrN+G for the 12S partition, GTR+I+G for the 16S partition, GTR+G for the ND4 first codon position partition, HKY+G for the ND4 second codon position partition, and GTR for the third codon position partition. The model TrN+G is not implemented in MrBayes v3.1, and so the next more complex model available in the program, GTR+G, was used for this partition.Four independent MCMC analyses of 15,000,000 generations each (sampled every 3,000 generations) were

performed in Mr. Bayes 3.1 with one cold chain and three heated chains. The first 25% of trees were discarded as burn-in, the 50% majority-rule consensus tree was constructed from combined post-burn in trees of converged runs, and trace plots of clade probabilities were viewed using AWTY (Wilgenbusch et al. 2004).

AFLP and Total Evidence

Bayesian phylogenetic inference was performed on both AFLP and combined (mtDNA and AFLP) datasets to provide a further source of evidence from AFLP analyses in addition to population genetics models. A Bayesian model has been developed for AFLP evolution and phylogenetic reconstruction (Luo et al. 2007), but there is no easy method available to implement it. MrBayes v3.1 was used under the restriction data model by setting the coding bias to 'noabsencesites' for AFLP data to correct data for unobserved all-absence sites (Ronquist and Huelsenbeck 2003). The combined dataset was partitioned into AFLP, 12S, 16S, and ND4 partitions and model parameters for the mtDNA genes were estimated using jModelTest (Posada 2008). Substitution models GTR+G for 12S, TrN for 16S, and K80+G for ND4, were implemented in MrBayes v3.1 (Huelsenbeck and Ronquist 2001), and four independent MCMC analyses were performed with 3 million generations (sampled every 1000 generations) using one cold chain and three heated chains. Tracer v1.4 (Rambaut and Drummond 2007) was used to determine when sample points reached stationarity with an initial run. The first 300,000 and 500,000 runs were discarded as burnin for AFLP and total evidence datasets respectively. Post-burnin runs were compiled into a single file and a consensus tree was constructed in MrBayes v3.1 using all compatible groups. A final 50% majority rule consensus trees were constructed and re-rooted using the basal clade from the mtDNA reconstruction, using FigTree v1.1.2 (Rambaut and Drummond 2008).

3.3.5 Population Genetics Methods

Descriptive Statistics

Estimating genetic diversity from dominant data under non-HWE is possible by incorporating population specific inbreeding co-efficients (F_{is}) into calculations of diversity indices such as F_{st} (Yeh et al. 1997; Foll et al. 2008). Although F_{is} values

from small populations (less than 10 individuals) could be unreasonable (Holsinger and Lewis 2007), the difficulty of estimating allele frequencies from small populations could be overcome by analysing a large number of loci (Krauss 2000). Given that the sample sizes for two of the putative species were small (six and seven individuals), F_{is} values from ABC4F were incorporated (Foll et al. 2008) into F_{st} calculations in POPGENE (Yeh et al. 1997) and also calculated F_{st} in the *f* - free model in HICKORY 1.1 (Holsinger and Lewis 2007). An AMOVA was also performed in *GenAlex v6.3* (Peakall and Smouse 2006)to calculate the percentage genetic variance and Φ_{PT} (a distance-based analog of F_{st}) of populations, based on 9999 permutations.

Detection Of Outliers

BayeScan v1.0 (Foll and Gaggiotti 2008), a hierarchical Bayesian method for calculating Bayes Factors (BF) from posterior probabilities, was used to test for outlier loci in the AFLP dataset as recommended by Pérez-Figueroa et al. (2010). The model parameters were automatically estimated based on 10 pilot runs (length = 5,000), using default chain parameters (sample size = 5,000, thinning interval = 20, and additional burnin = 50,000). Jefferey's scale of evidence was set to maximum (decisive) by which the loci with $\log_{10}$ (BF) = 2.0 (corresponding p = 0.99) were considered as outliers.

STRUCTURE

Cluster analysis for dominant data was implemented in the admixture model using correlated allele frequencies, which is more accurate in assigning individuals to closely-related groups (Pritchard et al. 2000; Falush et al. 2003, 2007). Ten runs of 100,000 iterations each were performed with K ranging from 1 to 10, and a burnin of 10,000 iterations. Since estimating the probability of K from the data, Pr(X|K), is computationally difficult, two *ad hoc* methods were used: ln Pr(X|K) as prescribed by Pritchard et al. (2000), and ΔK based on the second order rate of change of the likelihood function with respect to K, as proposed by Evanno et al. (2005). ln Pr(X|K) and ΔK for each value of K were plotted using STRUCTURE HARVESTER v0.56.4 (Earl 2007, http://taylor0.biology.ucla.edu/struct_harvest/). Assignment tests were performed to obtain the accuracy of assignment of individuals to putative species by

including prior population membership information for each sample, and setting K = 3 and 4 based on ΔK and ln Pr(X|K) plots. The Q matrices of population membership from 10 replicates were permuted in the GREEDY_OPTION of CLUMPP v1.1.2 for a mean permuted matrix (Jakobsson and Rosenberg 2007) and the results were visualized in *Distruct* (Rosenberg 2004).

GENELAND

Preliminary test runs of 200,000 iterations were implemented to check for appropriateness of correlated and uncorrelated allele frequency model assumptions under both spatial and non-spatial priors. Based on these results, four independent MCMC runs of 500,000 iterations each were performed using a spatial prior with coordinate uncertainty fixed at 1km, uncorrelated allele frequencies, minimum and maximum K fixed at 1 and 16 respectively, and a burnin of 50,000 generations. Another run of 1 million iterations and burnin set at 100,000 generations, with other parameters as above, was also performed to check for any difference in K estimation with an increase in the number of iterations.

3.3.6 Multivariate Statistics

Principal Co-ordinate Analysis (PCoA)

PCoA, also called Metric Multidimensional Scaling, is a distance-based ordination method. It calculates a distance matrix and produces a graphical representation of the data in reduced Euclidean dimensions. The advantage of PCoA is that it is not based on correlation and covariation coefficients (used in Principal Component Analysis) which may not always be appropriate, and can use any measure of association between scores (Zuur et al. 2007). In a PCoA, the distance between points reflect the original distances. A PCoA was performed in MVSP© Kovach Computing Services v3.13n using Gower General Similarity Co-efficient (Gower 1966) and the resulting clusters were plotted .

DAPC

DAPC was implemented in *adegenet* in R using the *find.clusters* function to perform a PCA and estimate the overall genetic variance. When groups (such as species) are unknown, instead of looking for groups of individuals in HWE, a K-means clustering

algorithm divides the total variance into among-group and within-group components. K-means clustering was run several times, allowing K to vary from 1 to 49 in the first instance, and the best number of clusters/putative species was determined by the lowest Bayesian Information Criteria (BIC). As many principal components as necessary were retained in the preliminary data transformation step to represent 75% of the total genetic variation. The optimal number of PCs to obtain a robust discrimination is estimated in *optim.a.score,* and the quality of discrimination is indicated by *a.score* for each cluster. 30 DAPC simulations were performed in *optim.a.score* for each of the 10 PCs retained. A second PCA, with K allowed to vary from 1 to 10, was performed to better visualize the BIC results, and a DAPC was performed using the appropriate number of PCs to maximize the *a.scores* according to the *optim.a.score* result.

Gaussian Clustering

In *prabclus,* a Non-Metric Multi-Dimensional Scaling (NMDS) is performed on a distance matrix to derive Euclidean variables of genetic dissimilarity between individuals. Jaccard distances (Jaccard 1908) between individuals were calculated from the binary matrix using the *prabinit* function. As with DAPC, BIC was used as an indicator to estimate the number of clusters/putative species and a Gaussian mixture model determined the clusters of individuals corresponding to mixtures of normal distributions that account for the variation in data. Ten permutations of NMDS were performed on the distance matrix by *kruskal* method in three dimensions using *prabclust* function. The clusters object, showing the assignment of individuals to each cluster, was exported to *Rcmdr* (Fox 2005) to visualize the scatterplot of clusters.

3.4 RESULTS

3.4.1 Phylogenetic Reconstruction

The geographic distribution of samples used are given in Figure 9. Of a total of 1450 bp from the mtDNA sequences (350 bp of 12S, 474 bp of 16S, and 626 bp of ND4), 210 bp were parsimony informative. The mtDNA MP analysis was identical to the Bayesian inference tree, with *C. macrops* samples being split into three distinct clades (Figure 10). The first clade consisting of samples from Thailand, Laos and Northeastern Cambodia was assigned to the nominate species *C. macrops sensu stricto* (*C. macrops s.s.*) as it included specimens from the type locality (Bangkok, Thailand). The second clade, consisting of samples from southeastern provinces of Thailand and southwestern provinces of Cambodia, was found to be the sister group to *C. venustus*, and both of these together formed the sister group to *C. macrops s.s.* The third clade contained samples from southern Viet Nam and eastern Cambodia and was sister group to all above clades, and *C. kanburiensis*. These relationships were strongly supported, with parsimony bootstrap values > 95% and Bayesian posterior probabilities of 100%.

Recognizing the second and the third clades as *C. macrops* would render this species polyphyletic. The lack of concordance with morphological patterns (Appendix 2, Figure 1) suggests, however, that such a pattern does not reflect the species tree. Therefore, a tree using the final AFLP dataset, consisting of 298 polymorphic loci was also constructed. The Bayesian AFLP tree (Figure 11) was poorly resolved at the deeper nodes with low support values, and the second clade and *C. venustus* were nested within two sub-divisions of *C. macrops s.s.* In contrast, the total evidence tree using both mtDNA and AFLP data (Figure 12) was both superior in terms of topology and robust in terms of support values. The three clades of *C. macrops* group were well-differentiated with 100% support values at the deeper nodes and with mostly high support values at the tips. Therefore, the two differentiated clades were designated *C. macrops sp nov 1* and *2* respectively and

will henceforth be referred to as such.

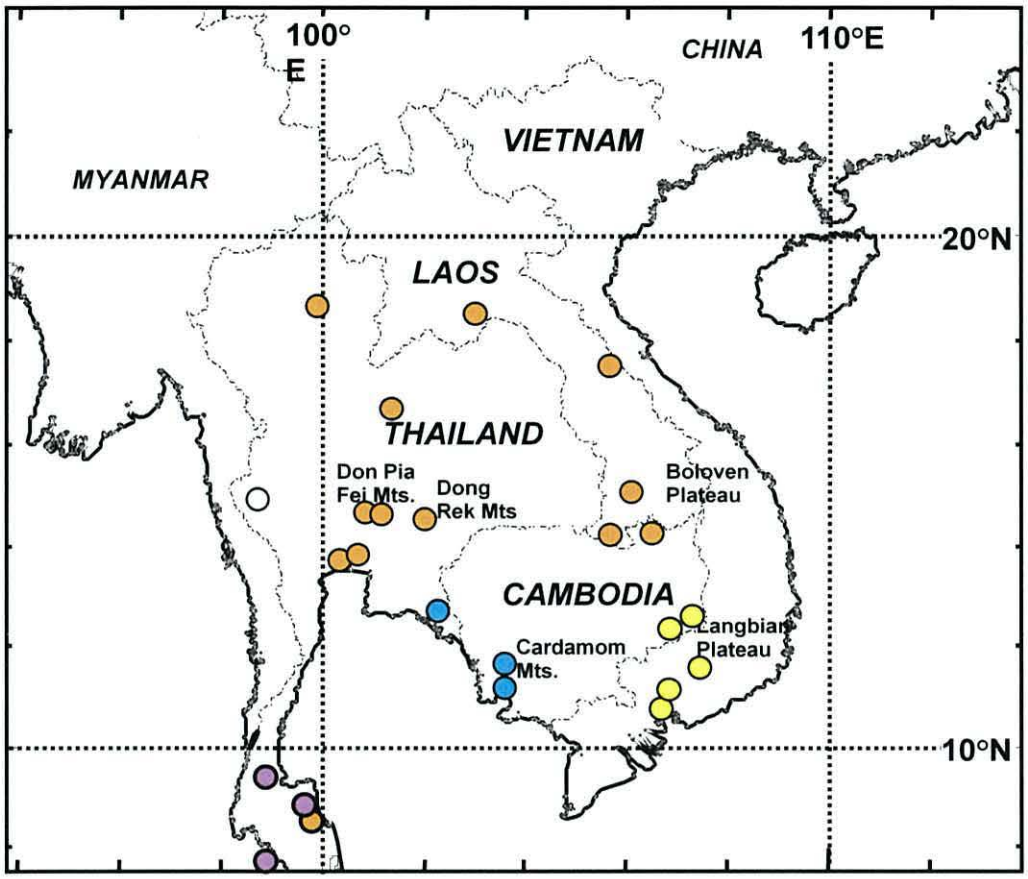


Figure 9. Map of southeast Asia showing the geographic distribution of samples. Different colours represents distinct lineages in the mtDNA and AFLP phylogenetic reconstructions and putative species and species clusters in the STRUCTURE analysis. Orange = *C. macrops sensu stricto*; Blue = *C. macrops sp nov 1*; Yellow = *C. macrops sp nov 2*; Pink = *C. venustus*. The empty circle from west Thailand represents *C. kanburiensis* which could be used only in the mtDNA phylogenetic analysis as AFLPs were unsuccessful for this sample.

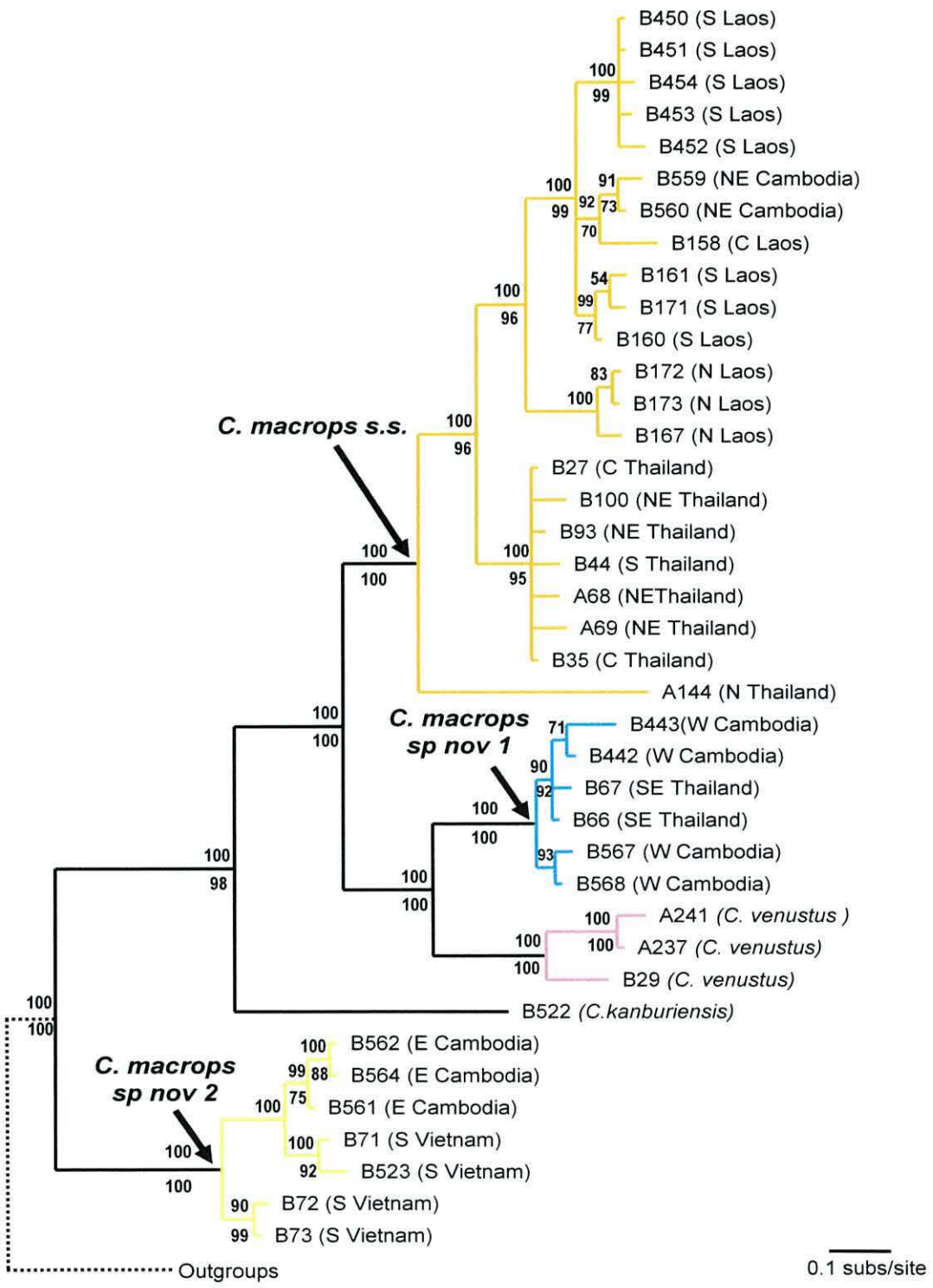


Figure 10. Fifty percent majority-rule consensus phylogram resulting from mixed-model Bayesian analysis of mitochondrial DNA. Strict consensus of all most parsimonious trees recovered the same topology. Support values are Bayesian posterior probabilities and parsimony bootstrap values above and below the branch, respectively. Specimens identified as *Cryptelytrops macrops* fall into three distinct clades, labelled *C. macrops sensu stricto* in Orange; *C. macrops sp nov 1* in Blue; and *C. macrops sp nov 2* in Yellow; and *C. venustus* is in Pink.

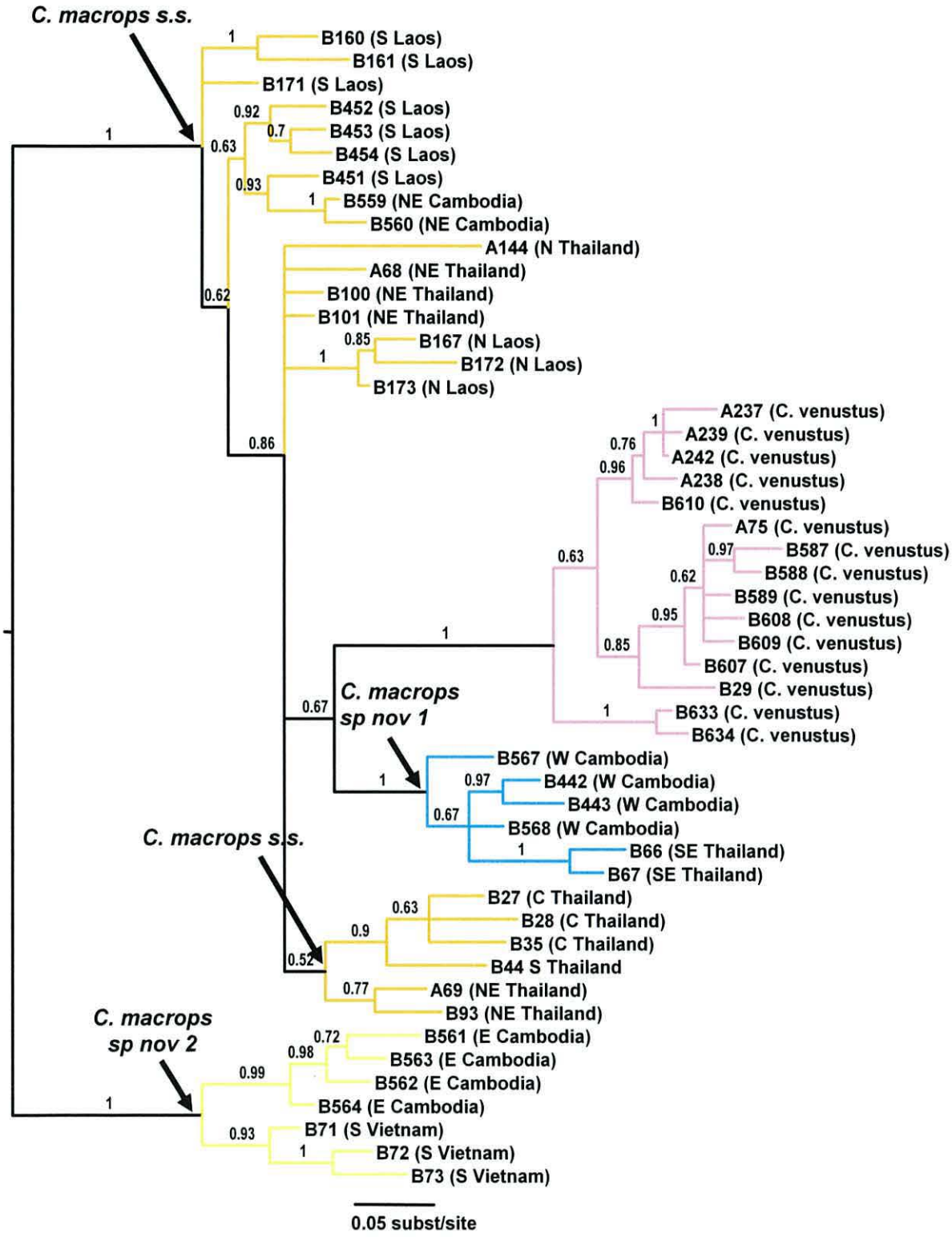


Figure 11. Fifty percent majority-rule consensus phylogram resulting from analysis of AFLPs. *C. macrops sensu stricto* is non-monophyletic cluster due to lack of resolution at the deeper nodes.

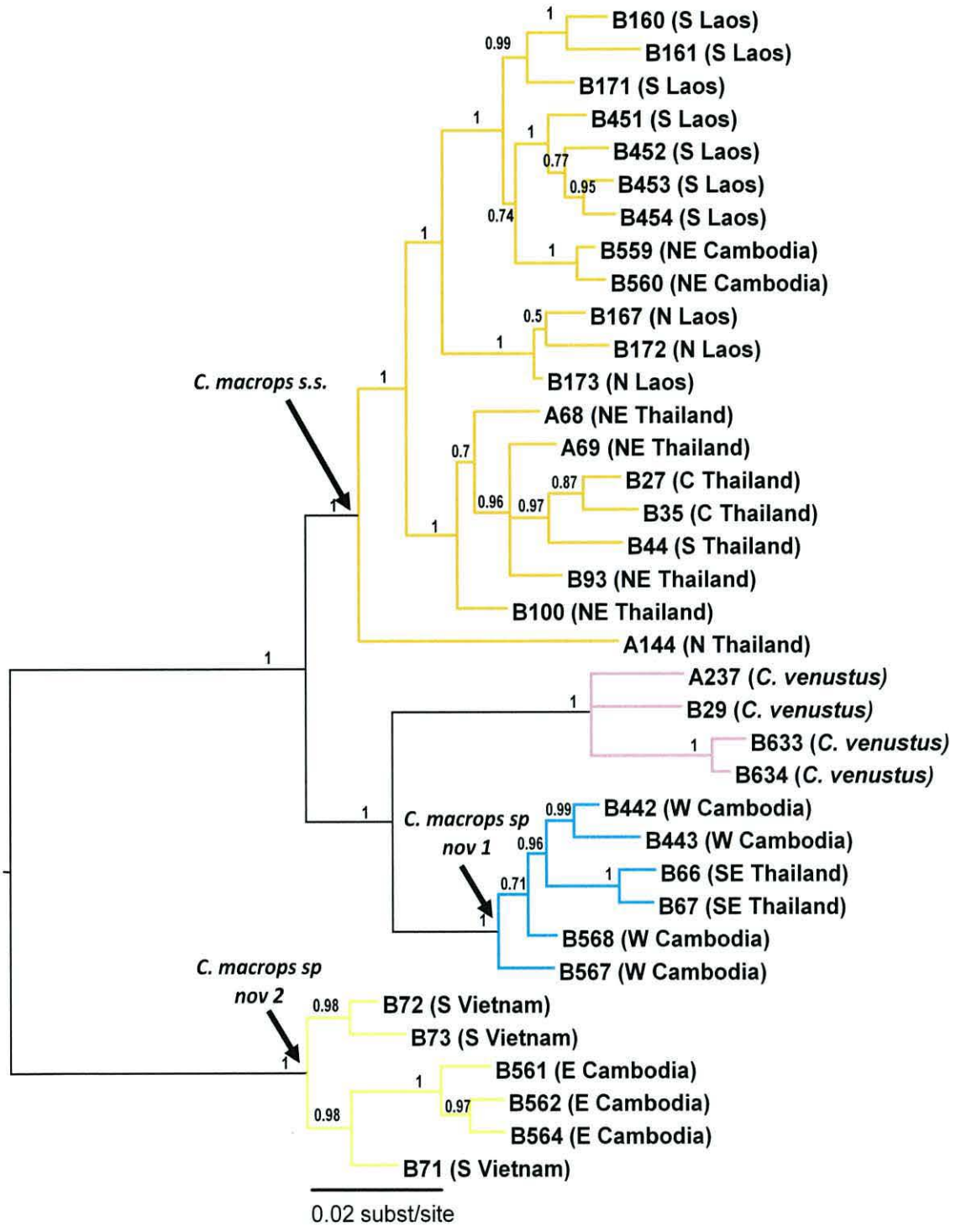


Figure 12. Fifty percent majority-rule consensus phylogram resulting from mixed-model Bayesian analysis of mitochondrial DNA and AFLPs. Support values at each node are Bayesian posterior probability values. Specimens identified as *Cryptelytrops macrops* fall into three distinct clades corresponding to *C. macrops sensu stricto*, *C. macrops sp nov 1* and *C. macrops sp nov 2*.

3.4.2 Population Genetics Methods

Descriptive Statistics

Repeatability score for AFLPs was 97% from a total of 330 loci of which 298 were polymorphic. F_{st} across all samples was 0.5 on average for both POPGENE and HICKORY. The percentage variation within and among putative species from the AMOVA was 46% and 54% respectively. The overall Φ_{PT} was 0.538 and the Φ_{PT} between populations were as given in Table 4. The percentage of polymorphic loci for each of the three putative species (*C. macrops s.s., C. macrops sp nov 1* and *2*) were 54%, 21% and 25% respectively. The plot of $\log_{10}$ (BF) against F_{st} in BayesScan showed no outlier loci indicating neutrality of all loci (Appendix 2, Figure 2).

Table 4. Inter-population Φ_{PT} values from AMOVA

Putative Species	*C. macrops s.s.*	*C. macrops sp nov 1*	*C. macrops sp nov 2*
C. macrops sp nov 1	0.328		
C. macrops sp nov 2	0.555	0.627	
C. venustus	0.494	0.579	0.694

Bayesian MCMC Cluster Analysis

In STRUCTURE, increases in Ln Pr(X|K) were substantial up to K = 4, borderline at K = 5, and insignificant after K = 6 (Figure 13a), while plot of ΔK by Evanno's method clearly peaked at three populations (Figure 13b). The output from *Distruct* (Figure 14) shows striking division of the four species clusters. The probability for each individual belonging to the assigned species for K = 4 (including *C. venustus*) is given in Appendix 2, Table 3. The probabilities of individual assignments when K=3 (Evanno method) were nearly all equal to 1.0. In GENELAND, the number of clusters visualized from the posterior distribution was three. Maps of individual posterior probabilities of membership to each cluster are given in Figure 15; all individuals from the *C. macrops sp nov 1* cluster were assigned to *C. macrops s.s.* The posterior probabilities of cluster membership for individual samples are given in Appendix 2, Table 4.

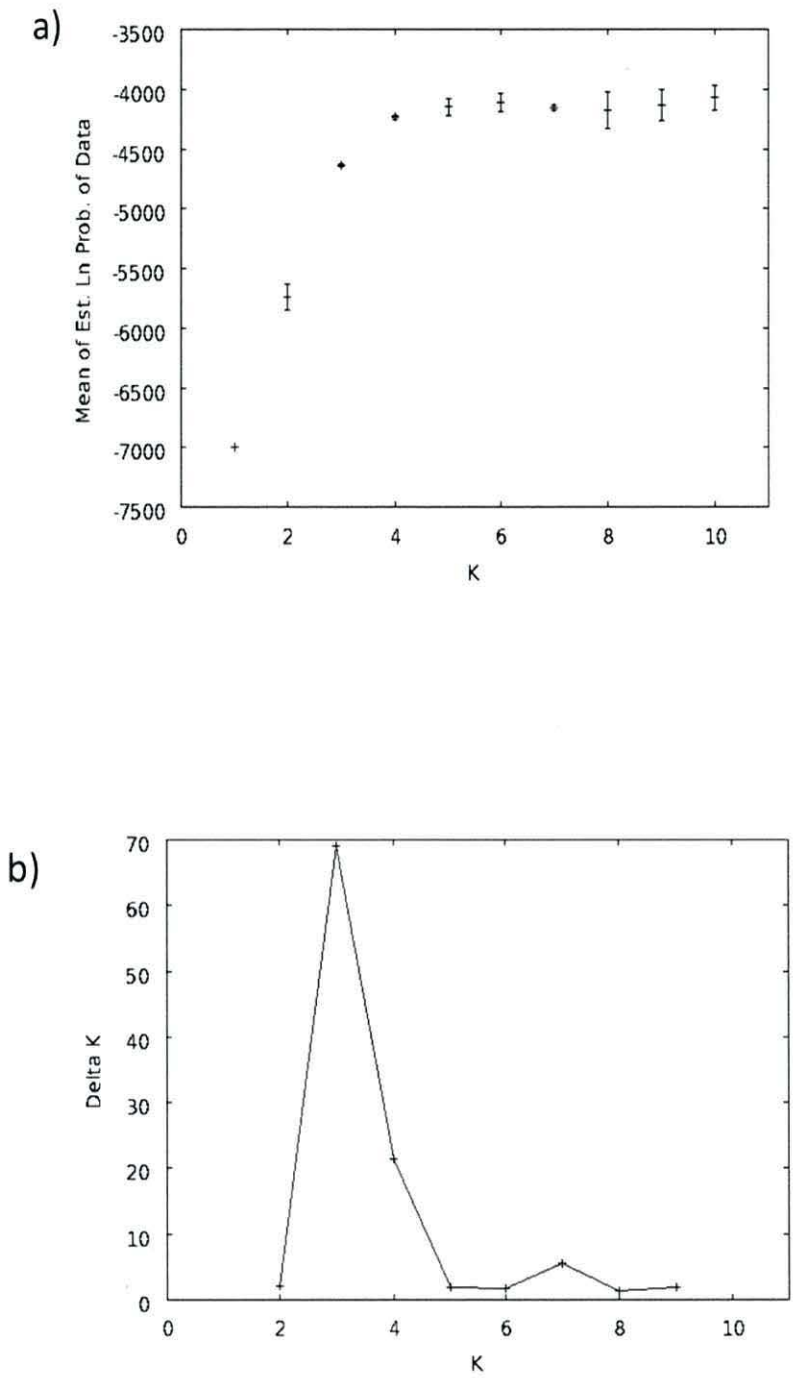


Figure 13. Graphical representation of the average likelihood values for each value of K (from 1 to 10) over 10 runs of 100,000 iterations each in STRUCTURE. a) ln Pr(X|K), where K = 4. b) ΔK by Evanno's method, where K = 3.

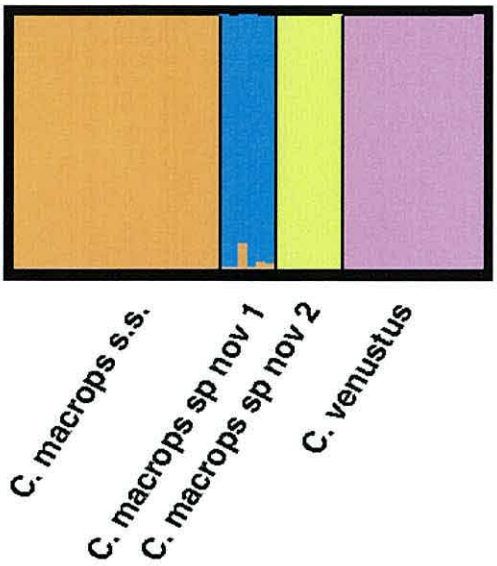


Figure 14. Estimated genetic structure for *K* = 4 obtained with the STRUCTURE program. The four colours represent the proportion of individual polymorphic loci assigned to each cluster: Orange = *C. macrops sensu stricto;* Blue = *C. macrops sp nov 1;* Yellow = *C. macrops sp nov 2*; and Pink = *C. venustus.*

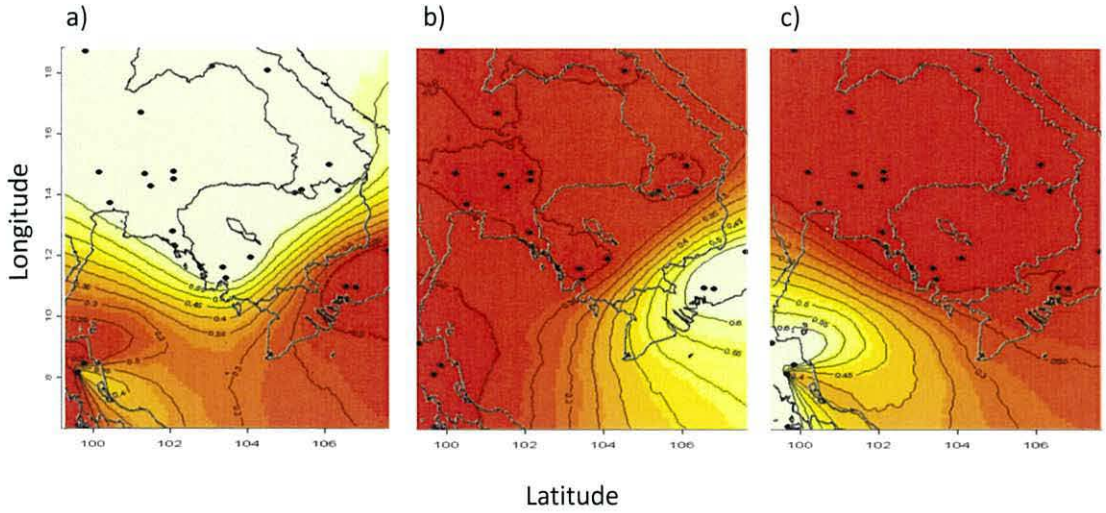


Figure 15. Map of posterior probabilities of species membership and spatial location of genetic discontinuities for K = 3 from GENELAND. The three plots represent the assignment of pixels to each cluster: a) *C. macrops sensu stricto* and *C. macrops sp nov 1;* b) *C. macrops sp nov 2*; and c) *C. venustus.* Lightest colours indicate highest probabilities of membership and contour lines represent the spatial position of genetic discontinuities between species.

Multivariate Statistics

The PCoA showed that about 50% of the total variation was explained by the first three axes which accounted for 24%, 18% and 7% of the observed variation. The scatterplot showed the three *C. macrops* putative species to be well separated on axes 1 and 3 (Figure 16b). A scatterplot of the DAPC showed clear demarcation between the three clusters of *C. macrops,* with *C. venustus* as the fourth cluster (Figure 16c). Assignment of individuals to each of the three clusters in *C. macrops* group agreed completely with the geographic distributions of the three mtDNA clades (Figure 10). The *a.score* for each DAPC cluster was: *C. macrops sensu stricto* = 0.15, *C. macrops sp nov 1* = 0.96, *C. macrops sp nov 2* = 0.97, and *C. venustus* = 0.68. *Prabclus* detected four species clusters which were clearly separated in dimensions 1 and 3, three in the *C. macrops* group and *C. venustus,* with 100% accuracy of individual assignment to respective clusters (Figure 16d). No datapoints were classified as noise components in this analysis (Fraley and Raftery 1998, 2002; Hausdorf and Hennig 2010).

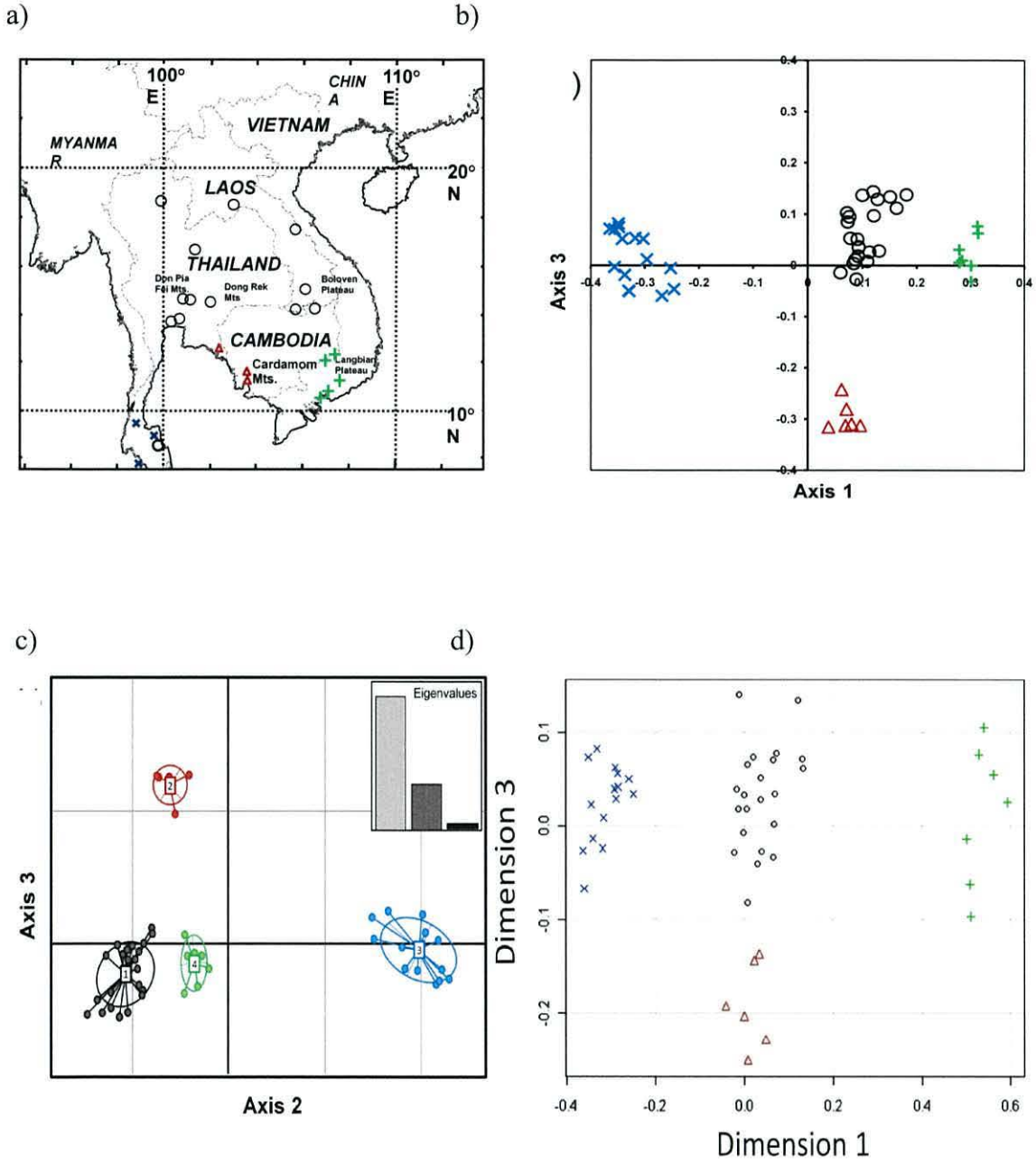


Figure 16. a) Map showing the geographic distribution of samples used in the AFLP multivariate analyses. Black = *C. macrops sensu* stricto; Green = *C. macrops sp nov* 1; Red = *C. macrops sp nov* 2; Blue = *C. vensutus.* **b) PCoA eigenvector plot of the *C. macrops* group and *C. venustus* using Gower General Similarity Coefficients.** Four clear clusters are seen on axes 1 and 3. **c) Scatterplot of four clusters showing PCs two and three of the DAPC. d) Gaussian clusters identified from Non-metric Multi-Dimensional Scalings in *prabclus*.** Four clear clusters are seen on dimensions 1 and 3.

3.5 DISCUSSION

3.5.1 Phylogenetics

Tree-building and systematic studies using AFLPs have been largely confined to distance-based methods such as Neighbour-Joining and UPGMA. However, despite homology and non-independence of fragments being the main contentions, phylogenetic reconstructions using AFLPs are becoming popular and have been successfully used to delimit species, sometimes performing better than STRUCTURE (Meudt et al. 2009) and even mtDNA reconstructions (Mendelson and Simons 2006; Kingston et al. 2009). Total evidence from combined AFLP and mtDNA datasets have been particularly recommended as they yield robust phylogenies and can provide evidence for interspecific hybridization (Pelser et al. 2003; Despres et al. 2004; Meudt and Clarke 2007; Kingston et al. 2009). The resolution of the total evidence Bayesian phylogenetic reconstruction in this study matched that of the traditionally used mtDNA analysis, producing a robust phylogeny. As opposed to this, the AFLP tree showed poor resolution of species and low support values at deeper nodes. This difference in results from the two datasets is interesting, given that there have been conflicting reports in the past for the utility of AFLPs at deeper phylogenetic or interspecific levels, which appear to be affected by a drastic increase in non-homologous shared fragments at the interspecific level, resulting in the loss of phylogenetic signal (Althoff et al. 2007; Dasmahapatra et al. 2009; Graves 2009; Kingston et al. 2009). Moreover, the choice of bands, tree-building method, and the application of restriction sites model could potentially over-simplify the complex evolutionary processes of AFLP as a marker and exert an effect on the resolution of deeper nodes (Dasmahapatra et al. 2009; Graves 2009). With no easy method to implement the new phylogenetic techniques developed for AFLPs (Luo et al. 2007; Koopman et al. 2008; Dasmahapatra et al. 2009), the failure of the AFLP reconstruction reinforces the need for new and better phylogenetic methods exclusive to AFLPs (Graves 2009).

3.5.2 Comparative Assessment Of AFLP Analysis Methods

Given that AFLPs have been extensively used to answer biological questions, it is surprising that there are very few studies which have compared multiple analysis

methods for dominant markers (e.g. Meudt et al. 2009; Reeves and Richards 2011), and that most comprehensive comparative studies have only assessed genetic similarity co-efficients and multivariate clustering methods such as UPGMA, NJ etc. (Meyer et al. 2004; Kosman and Leonard 2005; Dalirsefat et al. 2009). Apart from the preliminary evaluation conducted for DAPC and *prabclus* (Hausdorf and Hennig 2010; Jombart et al. 2010), and the comparative analysis of dominant and co-dominant datasets in GENELAND (Guillot and Santos 2010), this is the first time that the performance of both spatial and non-spatial Bayesian clustering methods and multivariate statistical techniques has been assessed in the exclusive context of dominant markers. Additionally, this study also included two new multivariate methods, DAPC and *prabclus*, which show considerable promise and urgently require additional empirical testing.

Both the Bayesian MCMC methods, STRUCTURE and GENELAND, assign individuals probabilistically to populations based on allele frequencies, and cluster groups of individuals into populations by assuming that they are in HWE and linkage equilibrium. In the estimation of the number of clusters (K), both failed to differentiate *C. macrops sp nov 1* as a separate species cluster and returned K = 3 by grouping *C. macrops sp nov 1* specimens with *C. macrops s.s.* in a single cluster. Recently, applying Evanno's correction has become the norm for K estimation in STRUCTURE, since it is more formal and has also been endorsed by Pritchard et al. (2007). Applying Evanno's method gave an extremely clear result of K = 3 (Figure 13b), while the Pritchard et. al.'s (2000) method was inconclusive whether K equalled 3, 4, or even 5, depending on what cut off was applied to the ln Pr(X|K) increase. However, Pritchard et. al.'s (2000) method of K estimation, which is said to be unreliable, subjective, with a tendency to be biologically meaningless in certain scenarios, proved to be more realistic when the ln Pr(X|K) values were compared. The ln Pr(X|K) from K = 3 to 4 was 8.57 %, which is approximately 50% of the value of the ln Pr(X|K) increase for both K = 1 to 2, and K = 2 to 3, and sharply dropped to 2.1% for K = 4 to 5 (Appendix 2, Table 5). There are several recorded cases where K has been underestimated and STRUCTURE results have been said to be conservative when Evanno's method has been applied (e.g. Frantz et al. 2009; Blanquer and Uriz 2010), and the result from this study appears to support this.

The individual assignment probabilities were calculated by assigning individuals in the *C. macrops* group to three clusters and *C. venustus* individuals to a fourth cluster. This was done on the basis of the phylogenetic results, and also taking into consideration that K = 4 proved a more sensible result in the Pritchard et al.'s (2000) method of K estimation. The mean permuted assignment probability for *C. macrops sp nov 1* individuals to a separate cluster (as output from CLUMPP) was only slightly lower than the rest (p = 0.969), which further increased confidence that Evanno's method was an underestimation. In GENELAND, all individuals of *C. macrops sp nov 1* were given a probability of assignment of 0.644 to *C. macrops s.s.* Interestingly, one *C. macrops s.s.* specimen (B44), reportedly from Nakhon Si Thammarat in southern Thailand, had a lower probability (p = 0.441) of belonging to *C. macrops s.s.* and a higher probability of belonging to *C. venustus* (p = 0.363) as compared to other *C. macrops s.s.* samples. In the STRUCTURE analysis, this specimen was assigned to *C. macrops s.s.* with a maximum mean permuted probability of 1.0 by CLUMPP. As B44 is the only *C. macrops s.s.* specimen reported from southern Thailand (the specimen was obtained from a dealer and its presence at the reported locality is unconfirmed), the origin of the majority of *C. venustus* specimens, this appears to be the effect of spatial location. To test this, the geographic co-ordinates of B44 was swapped with another sample (A144) from north Thailand and the analysis was re-run under the same parameters. The results showed that the probability of membership of B44 to *C. macrops s.s.* increased to p = 0.693 and to *C. venustus* decreased to p = 0.158, while the probability of membership of A144 to *C. macrops* decreased to p = 0.550 (from p = 0.664) and to *C. venustus* increased to p = 0.304 (from p = 0.158) clearly demonstrating the influence of spatial priors on individual assignment probabilities.

Allele frequency estimations from small populations are said to be compensated by analysing large number of loci (Krauss 2000). The AFLP dataset consisted of 298 loci, yet the genetic diversity indices show a clear bias caused by small sample sizes of *C. macrops sp nov 1* and *2,* since the percentage of polymorphic loci decreased considerably relative to population size. The F_{st} values were much lower (0.0507 and 0.00729 for *C. macrops sp nov 1* and *2* as opposed to 0.9 in the other two groups with

p<0.00001), whereas the Φ_{PT} values showed that *C. macrops sp nov 1* is less genetically distinct from *C. macrops s.s.* than *C. macrops sp nov 2* (Table 4). These estimates appear to be non-representative, with a combined bias arising from sampling deficiency, probably insufficient number of loci, as well as the dominant nature of the marker, and hence deriving any strong biological inferences from the diversity indices would be highly dubious. Despite this, it is difficult to predict whether increasing the sample size may have provided better resolution for *C. macrops sp nov 1* in the Bayesian cluster analysis because *C. macrops sp nov 2* (represented by only seven individuals), was still sufficiently genetically diversified to form a separate cluster as well as achieve 100% individual assignment success in all analysis methods.

Multivariate techniques, on the other hand, proved to be superior to Bayesian MCMC clustering in terms of sensitivity and confidence as PCoA, DAPC and *prabclus* all split *C. macrops* group into three clean clusters and also assigned individuals to their corresponding mtDNA clades with 100% success. In the DAPC analysis, *optim.a.score* recognized that the first three PCs would give highest *a.scores*. Although they represented only 45% of the total variance, the first three PCs were used to obtain a strong and stable DAPC solution (Thibaut Jombart, pers. comm.). The *a.scores* were very high for *C. macrops sp nov 1* and *2* (0.96 and 0.97) which increases the confidence that these two groups are genetically distinct. *C. macrops s.s.* had an *a. score* of 0.15, but given that *optim.a.score* and *a.score* functions are still under development and some issues exist (e.g., with repeatability), a more critical review of these scores is not justified at this stage. Gaussian clustering in *prabclus* which has been specifically designed for species discrimination using multi-locus dominant and co-dominant markers, was also highly successful, defining three clusters of *C. macrops* and assigning individuals correctly to all three clusters. It is important to note that the performance of both DAPC and Gaussian clustering was not affected by probable sampling deficiency if any, or the lower level of genetic diversity with respect to *C. macrops sp nov 1,* as this was always identified as a separate cluster in contrast to the Bayesian methods.

3.6 CONCLUSIONS

The utility of AFLP markers for cryptic species delimitation, and the greater accuracy and sensitivity of multivariate analyses of these dominant markers compared to population genetics methods is evident from thist study. Sampling of natural populations is generally not easy in most groups of animals and is inherently difficult in animals such as snakes which lead a cryptic life-style. This can be a major drawback in population genetics analyses (in this study, genetic distinction between *C. macrops s.s.* and *C. macrops sp nov 1* was not clear due to lower levels of genetic divergence and sampling issues). If, in addition to this, the methods exert fundamental biases because of non-recognition of marker properties, the results can become unreliable, as clearly demonstrated here. With results from three different multivariate analyses, phylogeny of the combined data, and (to a certain degree) STRUCTURE, suggesting that *C. macrops sp nov 1* is indeed a distinct species, the contradictory GENELAND results can be disregarded as being unreliable. This makes a clear case for the importance of analysing a given dataset using different analytical tools, and assessing their performance comparatively to be able to obtain meaningful conclusions to answer biological questions. Based on this study, *C. macrops sp nov 1* and 2 have now been raised to full species, *C. cardamomensis* and *C. rubeus* respectively (Malhotra et al. 2011b).

CHAPTER 4

MORPHOLOGICAL AND MOLECULAR EVIDENCE FOR CRYPTIC SPECIES IN THE WHITE-LIPPED PITVIPER *Cryptelytrops albolabris*

4.1 ABSTRACT

The *"albolabris"* species group within the genus *Cryptelytrops* has been subjected to much study over the past century, and several subspecies have been raised to species status using molecular and morphological evidence. However, its species diversity and their geographic ranges have continued to remain unclear since morphological studies, for the most part, have been limited to several individual reports which describe specimens from various regions. Furthermore, evidence from mtDNA and nuclear markers have suggested the presence of at least one cryptic species. In this chapter, a multivariate morphometric analysis was performed using specimens from the entire geographic range of *"albolabris"*, with emphasis on five major species, to confirm the presence of morphotypes which correspond to existing species and to establish their geographic distribution. Additionally, four mtDNA genes were used to infer phylogenetic relationships at the inter-specific level. The results show congruence between morphological clusters and established taxonomic designations to a large extent. The results also show the presence of morphologically and genetically distinct groups which indicate that additional cryptic species maybe occuring in what is currently recognised as *C. albolabris*.

4.2 INTRODUCTION

The genus *Cryptelytrops* is broadly divided into the *"kanburiensis"* and the *"albolabris"* species groups. It is widely distributed across Asia and southeast Asia with a known range of India, Bangladesh, Myanmar, China, Thailand, Cambodia, Laos, Vietnam, Malaysia, Sumatra, and the Lesser Sunda islands (Regenass and Kramer 1981; David and Vogel 2000; Gumprecht et al. 2004). The genus is diagnosed by the fully-fused or semi-fused state of the first supralabial scale and the nasal scale and the possession of an elongated, non-spiny, hemipenis type (Malhotra and Thorpe 2004a). Over the past few years, evidence from morphological analysis, mtDNA phylogenies, and AFLPs, has helped to resolve 12 *Cryptelytrops* species (Vogel 1991; Giannasi et al. 2001b; Malhotra and Thorpe 2004a, c; Grismer et al. 2008), and an additional two species, *C. cardamomensis* and *C. rubeus,* in the *C. macrops* complex of the "*kanburiensis*" group more recently (Malhotra et al. 2011b). Using the fusion state of the nasal and supralabial scales, hemipenal morphology, and mtDNA evidence, the taxonomy of *V. truongsonensis*, initially incorrectly allocated to *Cryptelytrops* (Orlov et al. 2004), was also rectified (Dawson et al. 2008).

Commonly referred to as white-lipped pitvipers due to the whitish-coloured labial and ventral head regions of *C. albolabris,* the members of *"albolabris"* are considered to be quite dangerous to humans and have been implicated in causing the most number of snake-bites in certain regions of Asia (Cockram et al. 1990; Yang et al. 2007). The systematics of this group has been investigated many times over the last century, and as part of the revision of members of the *Trimeresurus* group, several subspecies were raised to species level (Giannasi et al. 2001b; Malhotra and Thorpe 2004a). The group now consists of: *C. albolabris, C. septentrionalis, C. purpureomaculatus, C. erythrurus, C. andersonii, C. cantori, C. labialis, C. insularis,* and *C. fasciatus*. However, there are many issues with respect to the species diversity and their distribution that still require clarification and confirmation.

Among the continental and southeast Asian mainland species, the distribution of *C. septentrionalis* is thought to be limited to Nepal (Giannasi et al. 2001b), and it is not

clear whether this population is distinct from the east Indian and Bangladeshi specimens and whether its range extends into the western Himalayan region. The early records of *C. purpureomaculatus* indicate its range as Singapore, west Malaysia, Sumatra, and some parts of Thailand (Regenass and Kramer 1981). This was later extended to include Ayeyarwade division in central Myanmar, and Moulmein (Mawlamyaing) and Kayin state in southwest Myanmar (Gumprecht et al. 2004).

Initially described from the Ganges Delta and Myanmar (Regenass and Kramer 1981), the range of *C. erythrurus* is now thought to include Yangon and Ayeyarwade divisions in central Myanmar, northern parts of Myanmar, the Arakan Yoma mountain range in west Myanmar, extending northwards into east Bangladesh and to northeast India (Gumprecht et al. 2004). It is unclear whether *C. erythrurus* is present in Thailand, whether the ranges of *C. purpureomaculatus* and *C. erythrurus* overlap, or even whether their designation as separate species is justified.

The distribution of *C. albolabris* has been reported as China, Thailand, Vietnam, Laos, Cambodia, India, Bangladesh, Myanmar, and west Java (Gumprecht et al. 2004), with the type locality being China (Regenass and Kramer 1981). However, mtDNA phylogenies have shown one monophyletic clade that includes *C. albolabris* from north Thailand (Lampang, Chiang Mai, Chiang Rai, and Pha Yao provinces), northeast Thailand (Loei), and one specimen from Moulmein district in Myanmar, as paraphyletic to the rest of the *C. albolabris* representatives (from Thailand, Vietnam, Laos, Cambodia, and China) (Sanders 2003). Specimens from the North Thailand region were noted by Malhotra and Thorpe (1997) as being different in appearance from other Thai specimens of *C. albolabris* as they had deep red eyes rather than the normal yellow. PCA analysis of AFLPs proved inconclusive as to whether these two were indeed genetically distinct lineages (Sanders 2003).

Additionally, evidence from both mtDNA and AFLP analysis suggests that a population in the Mandalay and Sagaing divisions of upper Myanmar, which is geographically proximal to the ranges of *C. purpureomaculatus* and *C. erythrurus*, could be distinct from both of these species (Sanders 2003). MtDNA phylogenies using two fast-evolving genes have shown that this population is more closely related

to the Nepalese *C. septentrionalis* (Sanders 2003). However, in the mtDNA phylogeny, support values for the division of deeper nodes were not high (Sanders 2003), and the possibility that inter-specific relationships could be altered with the addition of rRNA subunit genes, as they evolve more slowly, needs to be investigated. Although former subspecies in the *"albolabris"* group have been elevated to species (Malhotra and Thorpe 2004a) on the grounds of paraphyly in mitochondrial phylogenies, a detailed multivariate morphological assessment of the species from the *"albolabris"* group has not yet been conducted. Moreover, with respect to the upper Myanmar and the north and northeast Thailand populations, it needs to be investigated whether they are indeed distinct morphotypes.

Among the insular endemic species, *C. insularis* is found in east Java and the Lesser Sunda islands (Giannasi et al. 2001b); *C. fasciatus* is confined to a single island, Tana Djampea (south of Sulawesi and north of Nusa Tenggara Timor (NTT)); *C. andersonii,* initially designated as a subspecies of *C. purpureomaculatus,* from the Andaman islands; and *C. cantori* and an unconfirmed species *C. labialis* from the Nicobar islands. It is unclear whether the Andaman and Nicobar species live on both islands. A few reports (see Reptile database) indicate that the Andaman and Nicobar species may be found on both island groups.

This chapter forms a part of the ongoing investigation of this group and aims to present morphological evidence to support or refute the existing systematic arrangements, determine the true species diversity and geographic ranges of these species, to investigate the presence of cryptic species, and to clarify deeper phylogenetic relationships between species/putative species by the addition of slow-evolving genes to the existing mtDNA dataset. Due to the range of taxonomic units investigated (*C. albolabris, C. erythrurus, C. purpureomaculatus,* and *C. septentrionalis*), it is not within the scope of this chapter to discuss the morphology for each of these in great depth.

The specific aims of this study are as follows: 1. Establishing the sympatric distribution and morphological distinctiveness of two taxonomic units in the *"C. purpureomaculatus – C. erythrurus"* complex and clarifying their geographic range; 2. Providing evidence of morpho-species clusters corresponding to *C. albolabris, C. insularis,* and *C. septentrionalis*; 3. Identifying geographic areas in continental southeast Asia where the presence of cryptic *"albolabris"* may warrant further investigation.

4.3 MATERIALS AND METHODS

4.3.1 Taxon Sampling

Meristic morphological data for 354 specimens (160 males and 194 females) from across Myanmar, Nepal, India, Thailand, Laos, Cambodia, Vietnam, China, Malaysia, and the Sunda islands, which were recorded over the past 20 years by AM, was made available. Re-recordings have been performed and compared to original recordings on numerous occasions to check for discrepancies arising due to drift in recording methodology. A full list of samples used for the morphological analysis is given in Appendix 4, Table 1a and 1b for males and females respectively. The geographic origins of the samples used in the morphological analysis and their putative species designations are represented in Figure 17 (samples from western Himalayas are not shown). DNA sampling (Appendix 4, Table 2) was performed for over 120 samples to maximize locality representation for *C. albolabris* using the existing tissue inventory.

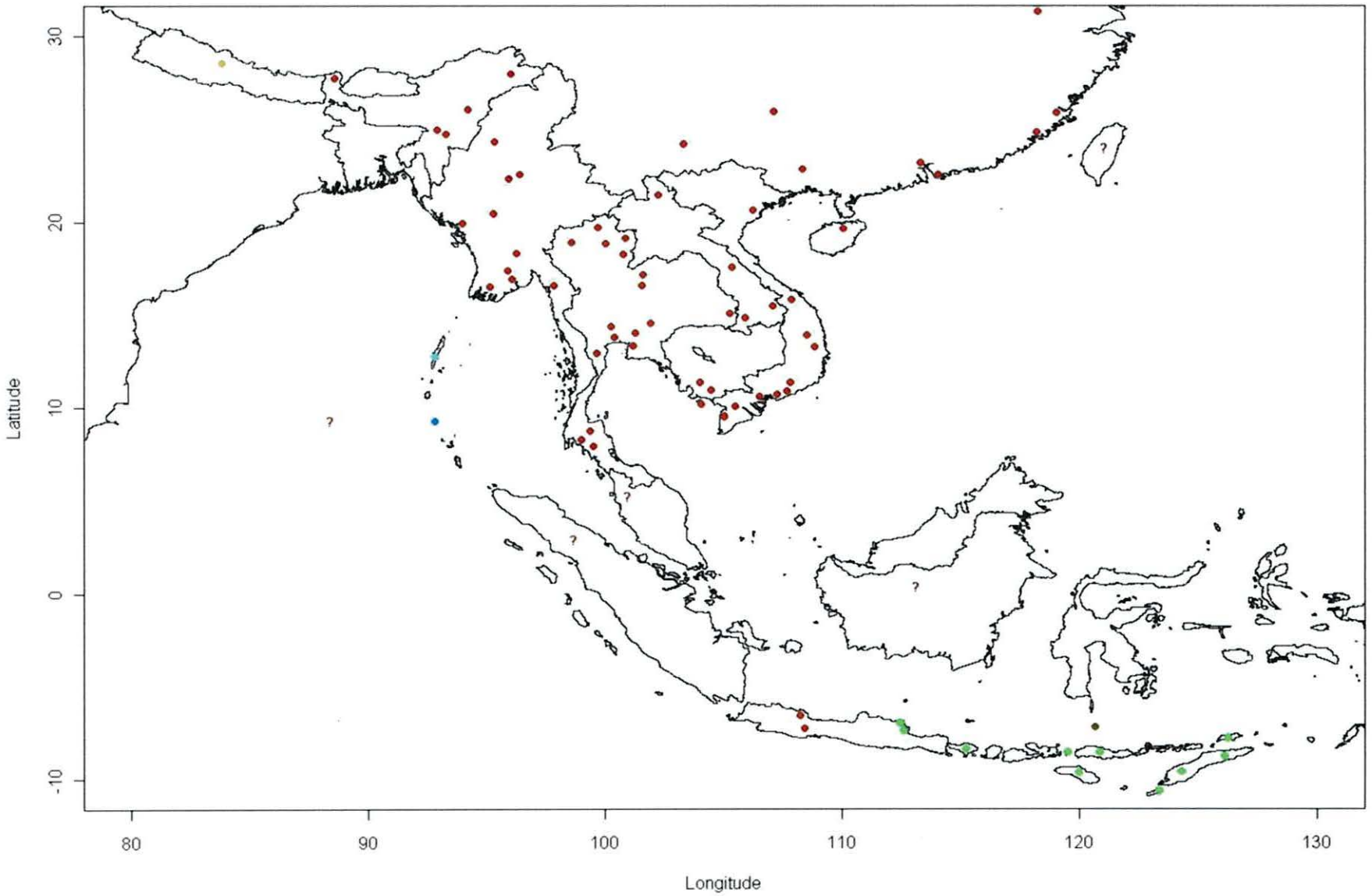


Figure 17. Map showing the estimated and sampling ranges for putative species in the *"albolabris"* group.
Red = *C. albolabris* + *C. erythrurus* + *C. purpureomaculatus*. (The species status of *C. erythrurus* and *C. purpureomaculatus* is unclear). Green = *C. insularis;* Orange = *C. septentrionalis*; Dark Blue = *C. cantori*; Light Blue = *C. andersonii; and* ? = unconfirmed localities.

4.3.2 Laboratory Protocols

Samples were in the form of liver or muscle tissue in 80% ethanol, clippings from the ventral scales in 80% ethanol, or up to 200µl of blood from the caudal vein preserved in 1mL 5% EDTA and 2mL SDS-Tris buffer (100mM Tris, 3% SDS). Whole genomic DNA was extracted using standard proteinase K protocols (Sambrook et al. 1989). 12S, 16S, ND4, and Cytb genes were amplified (as described in Malhotra et al. 2011a), cleaned with shrimp alkaline phosphatase and Exonuclease I (Werle et al. 1994), and sequenced by Macrogen inc. (http://www.macrogen.com).

4.3.3 Morphometric Analysis

Morphometric Characters

42 meristic characters were used from the following main categories: ventral scale counts, subcaudal scale counts, dorsal and caudal position of scale reductions, head scalation, degree of scale keeling, and colour patterns on the head. Individual characters under each category, recording methodology, and scale reduction calculations are as described in Malhotra et al. 2011b. All variables were subjected to preliminary statistical tests to check for errors which were then rectified using original datasheets and photographs. All scale reductions characters were converted to percentage of total ventral scale counts for body scale rows and percentage of total subcaudal scale counts for caudal scale rows.

Grouping

Sexual dimorphism is a well-documented phenomenon in the *Trimeresurus* group, and hence males and females were used in separate analyses. For selecting characters that display significant between-group variability, initial OTU grouping decisions were based on sympatric distributions. MtDNA phylogeny was also consulted but decisions for grouping specimens were taken only if the exact specimens involved were represented in the phylogram. Decisions to replace missing character recordings with group means were taken on a case-by-case basis and performed only in very few situations where the within-group

variation was not significant and only when the missing character value was not used in the calculation of any other character measures. Some groups which had fixed character states for certain characters were excluded from the analysis of that particular character.

Selection of characters

Characters were analysed for correlations and highly correlated characters were compared and then selected or removed depending on their correlations with other characters. Levene's test for the homogeneity of variances (Levene 1960) was used to select characters which displayed equality of variances. ANOVA was performed, and all characters which did not show significant between-group variation were dropped from the analysis. In cases where the null hypothesis of equal variances was rejected, Brown-Forsythe's robust test of equality of means (Brown and Forsythe 1974) was applied.

PCA

Initial analysis consisted of several sets of PCAs to optimize the preliminary groups. A number of coloration and head scalation characters, which tended to take a limited range of values and were frequently invariant within groups, were first combined using PCA, and the first PC from each analysis used as a "colour pattern" and "headscales" index in subsequent analyses. Scree plots and eigenvalues were examined and the most useful characters were identified. The initial smaller groups which clustered together and corresponded to larger areas of geographic distribution were combined into bigger groups after verification with mtDNA phylogeny. Clearly clustered groups were removed as and when they formed and remaining data used into the next stages of analysis. A more detailed account of grouping decisions is presented as part of the results for PCA.

CVA

All OTUs were assigned to new groups based on PCA-optimized groups and mtDNA phylogeny. Levene's test, ANOVA, and Brown-Forsythe tests were

performed to select the most useful variables for between-group discrimination. In some cases OTUs which could not be confidently assigned to any other group were included in the analysis as ungrouped specimens. Clusters were removed as they formed and the remaining data reanalysed. A more detailed account of grouping decisions for the CVA is presented as part of the CVA results. Morphological characters which contributed most to group separation were investigated using eigenvalue coefficients for the Canonical Variates.

4.3.4 Sequence Analysis

Sequences were checked for quality, aligned, and analysed in CodonCode Aligner v2.0.6. Protein-coding genes were aligned using ClustalW and translated into protein sequences to check for stop codons in the ORF in case of pseudogene amplifications. For 12S and 16S, since the alignment algorithm was found to be less efficient with indel calling, sequences were aligned by eye and indels were edited by double checking against chromatograms. The data from all genes were combined into one total evidence dataset for phylogenetic analysis.

4.3.5 Phylogenetic Analysis

MtDNA data was partitioned by gene and nucleotide evolutionary model parameters were estimated for each partition using jModelTest (Posada 2008). *Viridovipera stejnegeri* and *Trimeresurus borneensis* were selected as outgroups as they form the most closely-related genus and the most basal clade to *Cryptelytrops* respectively. GTR+G model was applied to all paritions and a Bayesian phylogeny constructed in MrBayes v3.1 (Huelsenbeck and Ronquist 2001) by performing three independent MCMC runs of 3 million generations each (sampled every 1000 generations) using one cold chain and three heated chains. Tracer v1.4 (Rambaut and Drummond 2007) was used to determine stationarity of sample points and check for convergence of runs. The first 300,000 runs were discarded as burnin, and the post-burnin generations were compiled into a single file and a consensus tree was constructed in MrBayes v3.1 using all compatible groups.

4.4 RESULTS

4.4.1 Morphometric Analysis

PCA

The first PCA for males and females (Figure 18a and b) together showed the separation of Arakan Yoma, Ayeyarwade, Moulmein, central Myanmar, east India and Bangladesh, Phetburi, and Sikkim specimens. The holotype for *C. erythrurus*, a male specimen from the Ganges Delta, also differentiated at this stage. All these samples were analysed in a second PCA (Figure 19a and b) and, based on these results, Arakan Yoma specimens were combined into one group and east Indian and Bangladeshi specimens were combined into the second group for males. In the case of females, only Arakan Yoma specimens were retained in one group since one sample from east India and Bangladesh did not separate from the rest of the *"albolabris"* specimens at this stage. Hainan, Hong Kong, northeast China, north Vietnam, and Taiwan specimens emerged together in the third PCA (Figure 20a and b) and were combined together into one group. In the case of females, visualization of alternative axes showed the separation of Nepal and Mekong Delta specimens (Figure 21) which were therefore grouped into separate groups. PCA of the remaining specimens separated *C. insularis* from Komodo, Flores, Sumba, east Java, west Timor, and Wetar (Figure 22a and b), which were combined into one group for both males and females. Several localities with scattered distribution of specimens (central Thailand, Laos, Vietnam, northeast Thailand, north Thailand, south China, west Java, China, and upper Myanmar) were left ungrouped.

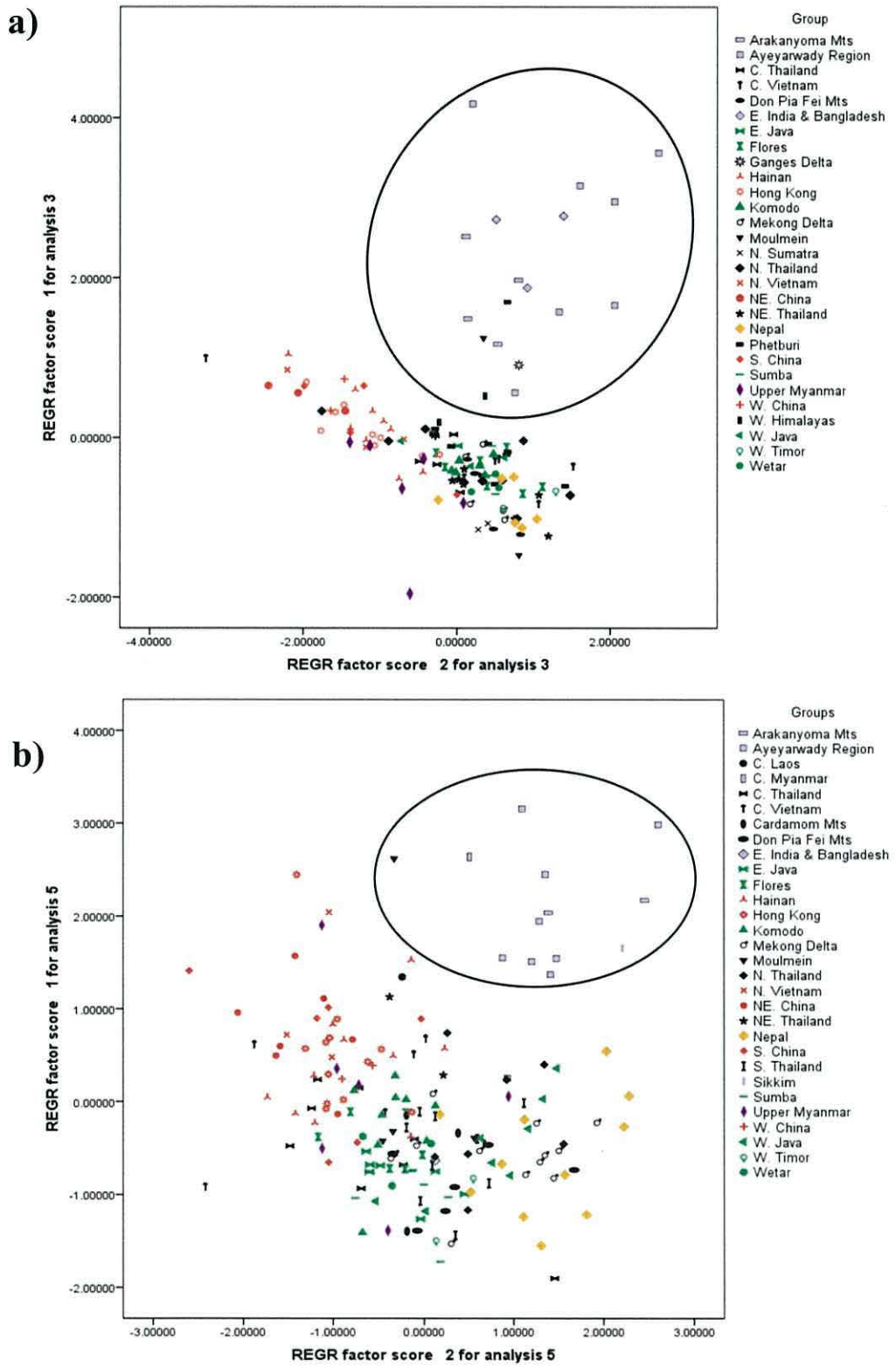
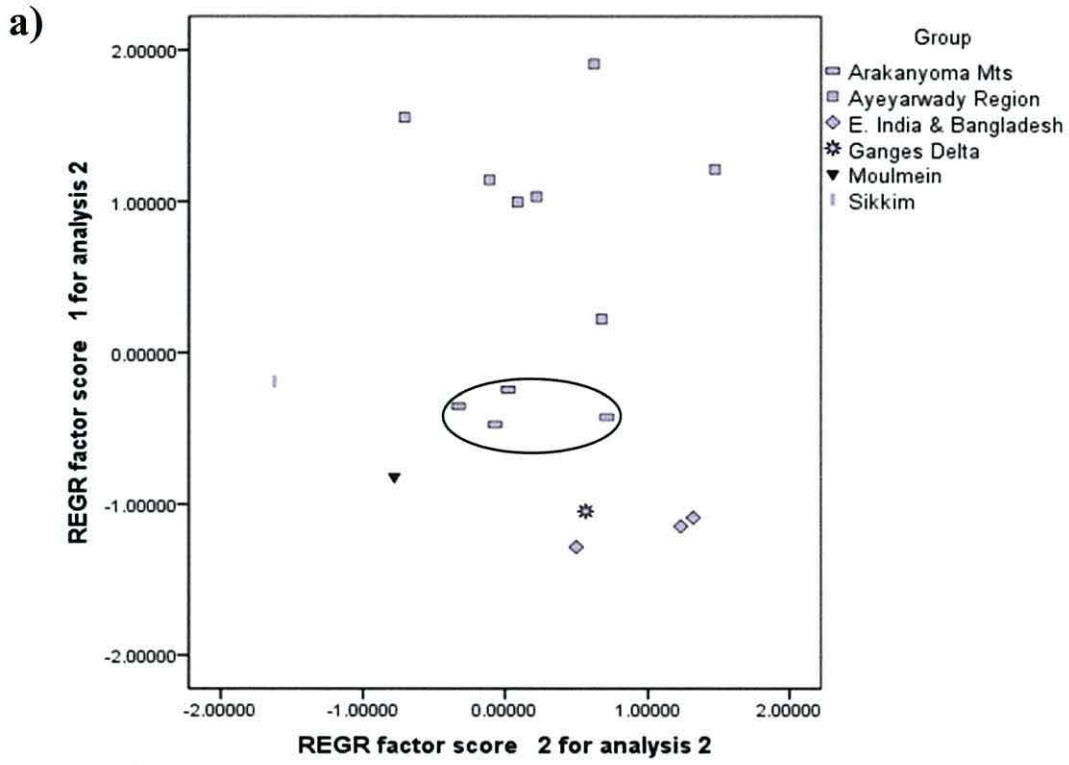


Figure 18. PCA plots for males (a) and females (b) with all specimens of the *"albolabris"* group included. Circled specimens separating from the main cluster are *C. erythrurus,* and possibly *C. purpureomaculatus,* mainly from east, west, and central Myanmar, east India, and Bangladesh, and a few specimens from Moulmein in south Myanmar, Phetburi in west Thailand, and western Himalayas.



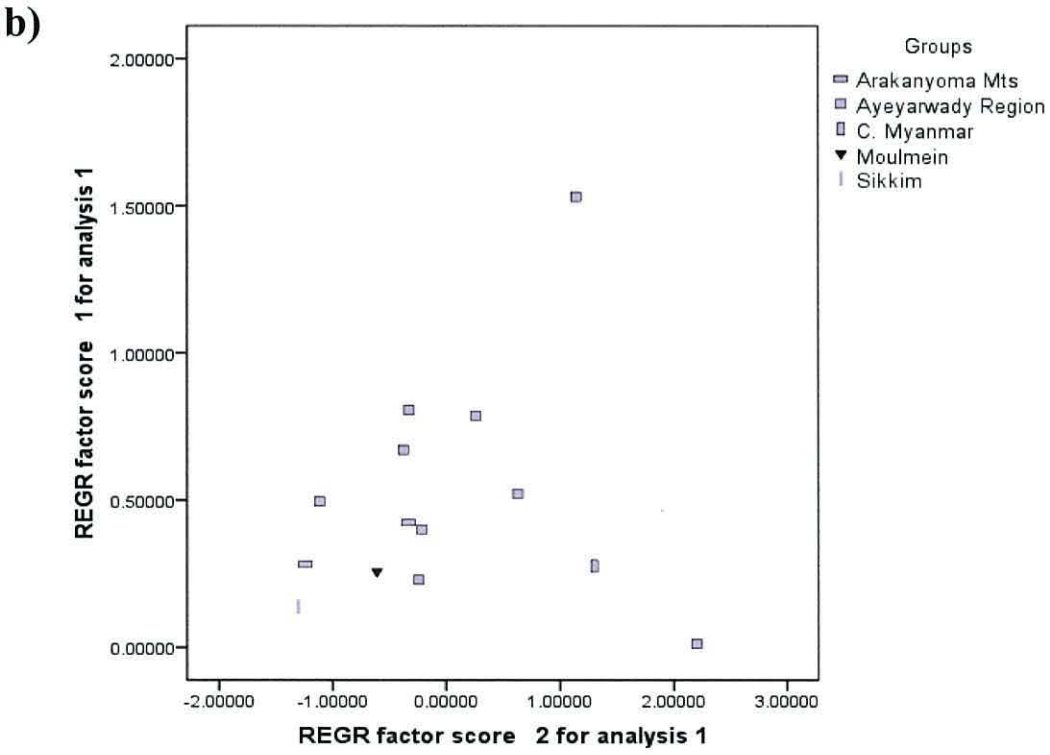


Figure 19. PCA plots for males (a) and females (b) with all specimens from the range of *C. erythrurus* and *C. purpureomaculatus*. Except for the Arakan Yoma mountain range specimens from west Myanmar which clustered together in males, there was not much structure in this subgroup.

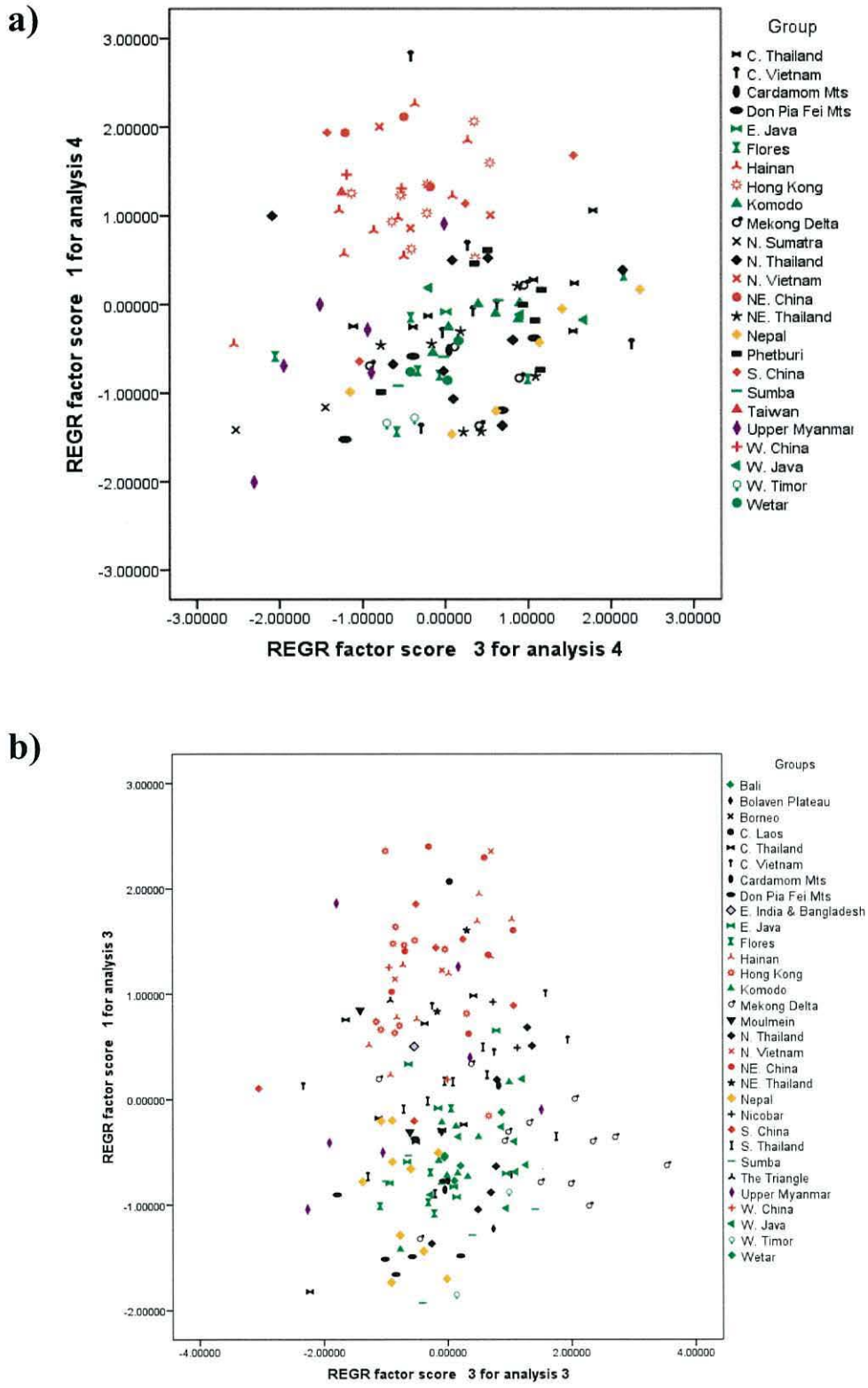


Figure 20. PCA plots for males (a) and females (b) showing clustering of specimens representing *C. albolabris* (coloured in red), mainly from Hainan, Hong Kong, northeast, west, and south China, and north Vietnam.

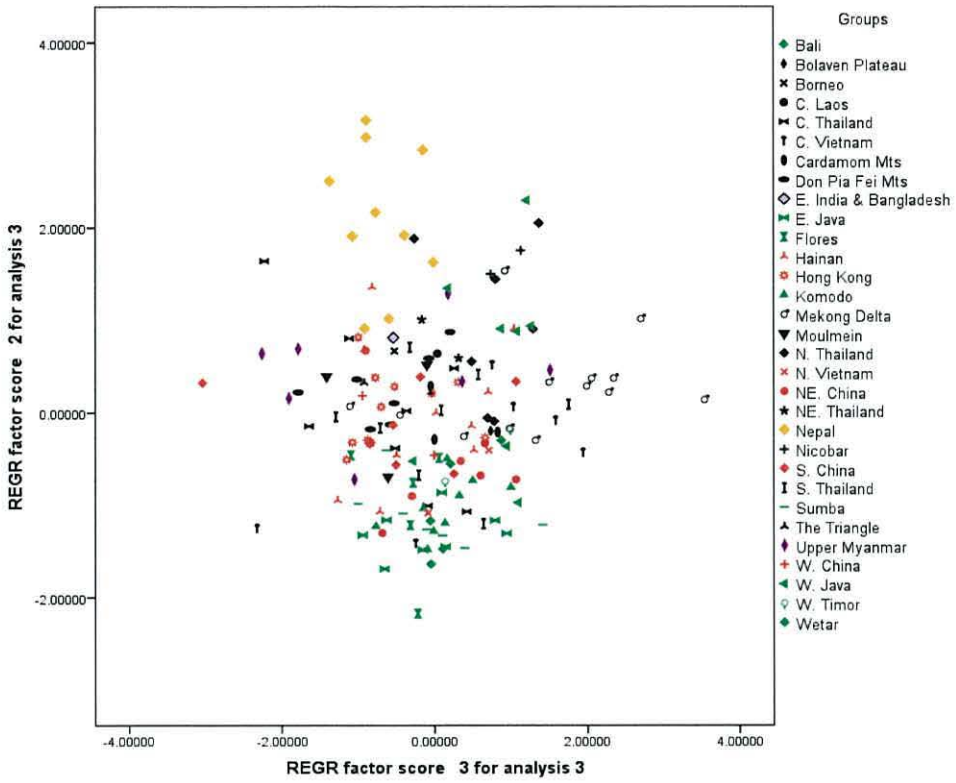


Figure 21. PCA plots for females showing clustering of some specimens from Nepal and Mekong Delta localities.

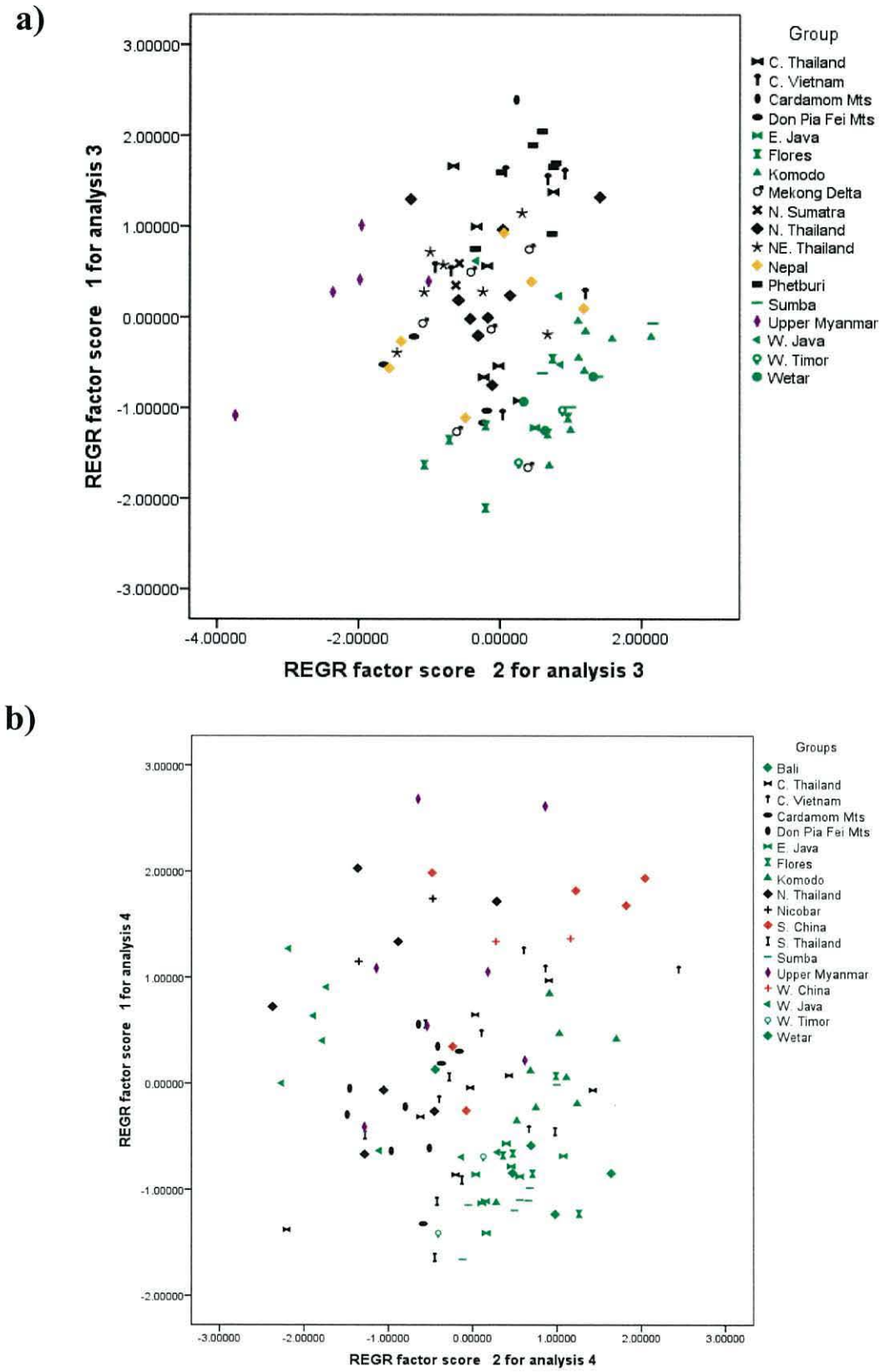


Figure 22. PCA plots for males (a) and females (b) showing clustering of specimens representing *C. insularis* (in green) from the Lesser Sunda islands and a few specimen from west Java.

CVA

The first CVA plots (Figure 23a and b) showed clear separation of two clusters from the main *"albolabris"* group in both males and females. These collectively represented specimens from west, central, southeast Myanmar, east India, Bangladesh, and Nicobar. Ventral scale counts, the position of the dorsal scale reduction from 25 to 23 and 23 to 21 rows, caudal scale reduction from 6 to 4 rows, and head scalation were the most important characters distinguishing these two clusters from the rest of *"albolabris"* group.

In the second CVA (Figure 24a and b), one Chinese/north Vietnamese cluster and a second cluster with specimens from the Lesser Sunda islands were formed both in males and females. In the case of females, the second CVA showed separation of the Nepalese specimens *C. septentrionalis,* whereas in males this separation was unclear. In males, a distinct cluster with specimens from west, central, and northeast Thailand, Cardamom Mountains in Cambodia, and central Vietnam was seen, but was not apparent in females. All other localities were fragmented in distribution, showing no obvious pattern. The species ranges derived from the CVA and phylogenetic analyses are represented in Figure 25.

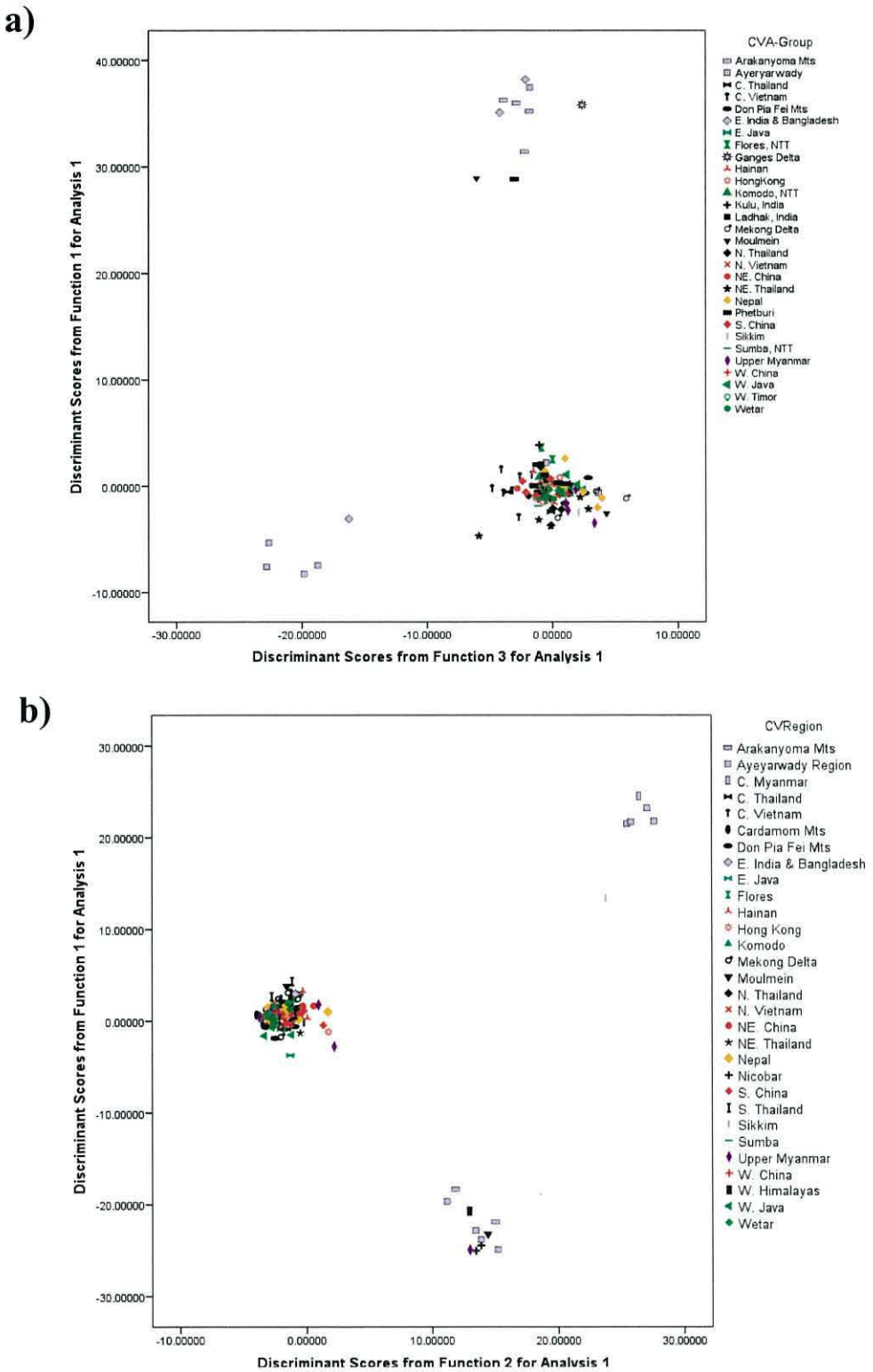


Figure 23. Canonical Variate Analysis with specimens from full range of *"albolabris"* included showing two smaller clusters clearly separating from the main cluster, one corresponding to *C. erythrurus* and the other possibly to *C. purpureomaculatus* (see text for further discussion). a) Males: The first canonical variate represents 58% of variance and the second a further 15%. **b) Females:** The first canonical variate represents 54% of variance and the second a further 43%.

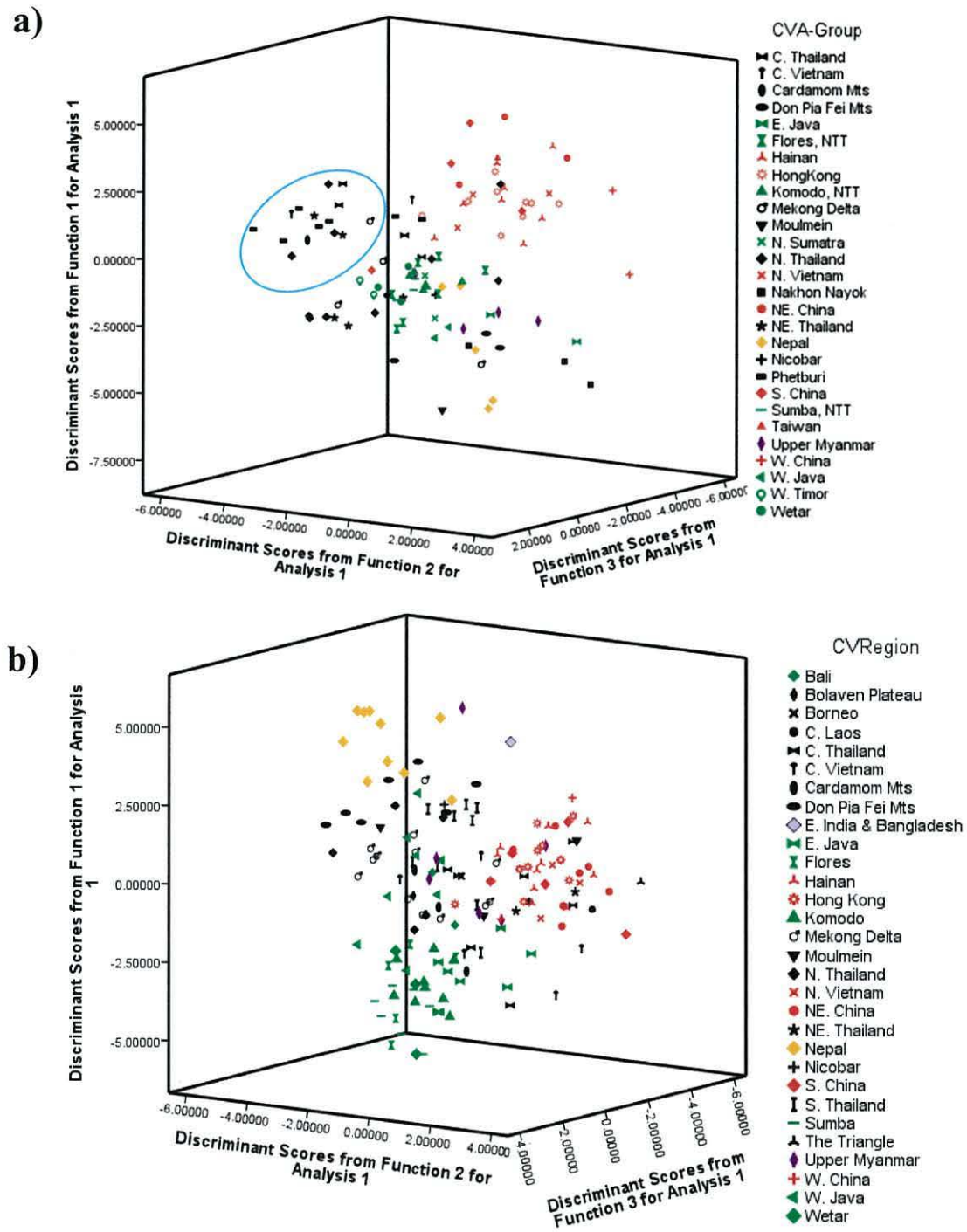


Figure 24. Second Canonical Variate analysis showing clusters which are indicative of the main species of the *"albolabris"* group: *C. septentrionalis* from Nepal, *C. insularis* from the Lesser Sunda islands, and *C. albolabris* mainly from northeast China and north Vietnam. **a) Males:** The first canonical variate represents 24% of variance, and separates a distinct cluster circled in blue which includes specimens from Cambodia, southcentral Vietnam, and southcentral Thailand. CV2 and CV3 represent a further 19% and 15% respectively. b**) Females:** The first, second, and third canonical variates represent 28%, 20%, and 11% of variance respectively.

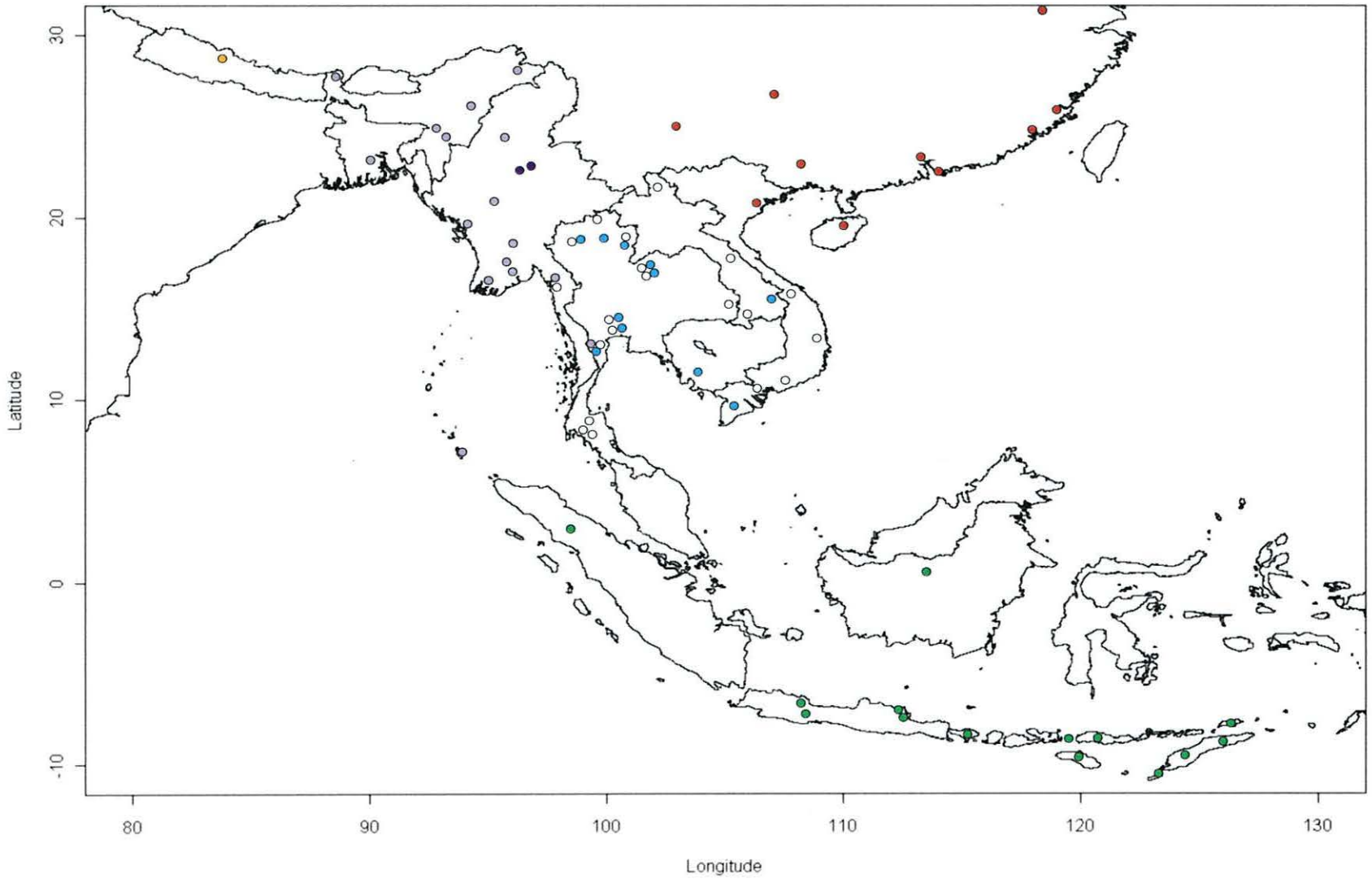


Figure 25. Map showing the resolved species ranges in the *"albolabris"* group based on multivariate morphological analysis and mtDNA phylogeny. Red = *C. albolabris*; Light Purple=*C. erythrurus* and *C. purpureomaculatus* complex; Green = *C. insularis;* Orange = *C. septentrionalis*. Specimens in blue and dark purple are possible cryptic species, morphologically distinct in males as well as in the mtDNA phylogeny. Empty circles represent samples which could not be assigned to any definite morphological cluster in the present analysis and require further investigation.

4.4.2 Phylogenetic Analysis

The overall topology was altered significantly (compared to previous studies) at the internal nodes with the addition of 12S and 16S rRNA genes (Figure 26). For ease of interpretation, the monophyletic clades are named as Clade A, B, and C. Clade A consists of *C. albolabris* specimens from Vietnam, China, Laos, Cambodia, and W. Java. Clade B is subdivided into B1 consisting of south and west Thailand specimens; B2 consisting of north and northeast Thailand; and B3 consisting of *C. purpureomaculatus, C. erythrurus, C. andersoni, C. cantori, C. septentrionalis,* and the upper Myanmar cluster in a monophyletic arrangement (which interestingly also includes two north Thailand specimens from Chiang Mai in paraphyly). *C. insularis* and *C. fasciatus* formed Clade C and continued to be the most basal clade. The species clusters resolved from the morphological analysis are represented in different clusters in each of these clades.

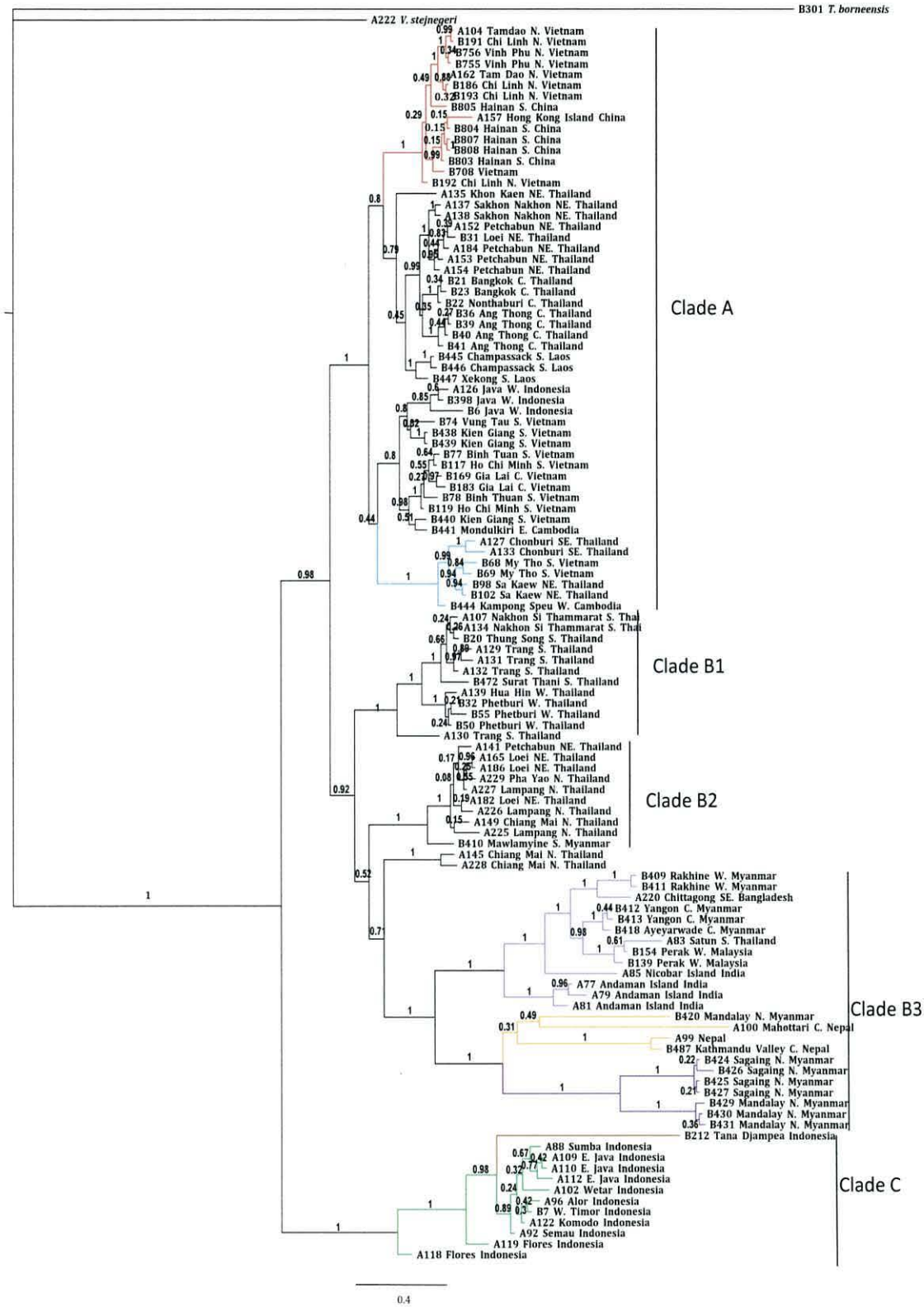


Figure 26. Bayesian mixed-model phylogeny of *"albolabris"* using four mtDNA genes. *T. borneensis* and *V. stejnegeri* are the outgroups and support values at the nodes are posterior probabiliies. Clade A comprises of *C. albolabris sensu stricto* specimens. The monophyletic clades corresponding to morphological species clusters are represented as: Red = *C. albolabris* and Blue = Possible cryptic species. Clades B3 and C represent the main species clusters which are in correspondence with the morphological analysis: Light purple = *C. erythrurus* and *C. purpureomaculatus* complex; Orange = *C. septentrionalis*; Green = *C. insularis*; Brown = *C. fasciatus*, which was not used in the morphological analysis but is shown to distinguish it from *C. insularis*; Dark Purple = Possible cryptic species from upper Myanmar.

4.5 DISCUSSION

4.5.1 Confirmation of the species status and range definitions

C. erythrurus and *C. purpureomaculatus*

The first CVA shows two morphologically distinct forms in the distribution range described for *C. erythrurus* and *C. purpureomaculatus* (Gumprecht et al. 2004). In males, the Arakan Yoma mountain range specimens aligned with the *C. erythrurus* holotype from the Ganges Delta. Based on this, the range for *C. erythrurus* can be defined as Arakan Yoma in Rakhine state, west Myanmar, extending into Assam and Nagaland, and the Ayeyarwade region in central Myanmar, extending into west Thailand. While it might be tempting to assign the two morphologically distinct clusters to *C. purpureomaculatus* and *C. erythrurus*, which would confirm them as two distinct species (Malhotra and Thorpe 2004a), and describe their individual geographic ranges, there are several points of note which indicate that a further investigation focusing on these two "species" is required.

In females, it is difficult to decide which of the morpho-clusters represents *C. erythrurus*. Two specimens represented in the mtDNA phylogeny, B412 from Yangon and B409 from Arakanyoma, are paraphyletic to each other but were found in one morpho-cluster, whereas one specimen B418 which has been thought to be *C. purpureomaculatus* from Ayeryarwade is monophyletic with B412 and was found in the second morpho-cluster. If the designation of B418 as *C. purpureomaculatus* is accurate, then it is appears that this species is represented by only one known specimen in the morphological analysis and would also render *C. purpureomaculatus* paraphyletic, as the specimens from Perak in west Malaysia and Satun in south Thailand are definitely representative of *C. purpureomaculatus* (AM pers. comm.). Although two specimens from the Nicobar islands were found in the same morpho-cluster as B418, without any DNA from these specimens, it is difficult to know which species to assign them to. It is possible that they represent *C. andersonii* (previously a sub-species of *C. purpureomaculatus*). Hence it is impossible to assign a morpho-cluster to either *C. pupureomaculatus* or *C. erythrurus* in the case of females. When results from a previous study using AFLPs was referred to, the degree of isolation

between specimens designated as *C. purpureomaculatus* and *C. erythrurus* was not found to be very clear and there appeared to be intermingling of clusters (Sanders 2003).

Photographs of specimens which appear to be morphologically dissimilar but occuring in similar geographic ranges have been posted on the web as *C. erythrurus* by different herpetologists (Figure 27a and b). One male specimen B413 from Yangon which was retained with the rest of the *"albolabris"* group in the morphological analysis was found to be in the same monophyletic clade as a female assigned to one of the distinct morpho-clusters (B412).

Considering all this, it appears that *C. purpureomaculatus* may have been unrepresented in the morphological analysis and there could be another cryptic species in the geographic range of *C. erythrurus* and *C. purpureomaculatus*. On the other hand, the incongruence of morphological patterns between males and females could be attributed to the high degree of sexual dimorphism in Asian pitvipers. Alternatively, if the two morpho-clusters are indeed *C. erythrurus* and *C. purpureomaculatus,* the mtDNA paraphyly could be explained as incomplete lineage-sorting, whereas the lack of resolution in previous AFLP analysis (Sanders 2003) could be attributed to the two "species" possibly hybridizing. In any of these three situations, with the distribution of *C. erythrurus* having been confirmed, it is evident that further sampling is required.

a)

b)

Figure 27. Very different pitvipers are identified as *Cryptelytrops erythrurus* by different herpetologists. a) Yangon, Myanmar (photo by W. Wüster) b) Sunderbans (photo by S. Bhattacharya).

Cryptelytrops septentrionalis

Cryptelytrops septentrionalis has been found to be genetically distinct from other *"albolabris"* species (Giannasi et al. 2001b; Sanders 2003). A distinct morpho-cluster which included the majority of the Nepalese specimens and one specimen from Mandalay in upper Myanmar (B420) was found in the case of females. This is congruent with the placement of B420 in monophyly with Nepalese specimens in the mtDNA analysis. However, one Nepalese female specimen could not be assigned to the same morpho-cluster. In males, the separation of Nepalese population was not as obvious and only two specimens along with one specimen from Moulmein were placed togehter. Morphological distinctiveness of at least some of the Nepalese population, and a high level of congruence with mtDNA in females, confirms *C. septentrionalis* as a distinct species. A "paratype" recorded from western Himalayas in Kashmir was later found to be *C. albolabris* (Regenass and Kramer 1981) and none of the western Himalayan specimens clustered with *C. septentrionalis* in this analysis indicating that this species may be restricted to Nepal. *C. albolabris* has also been suspected to be present in Nepal (Gumprecht et al. 2004) and the non-assignment of all Nepalese specimens to one single cluster in both males and females lends support to this possibility.

Cryptelytrops albolabris

Both mtDNA phylogeny and mophological analysis of males and females show that the Chinese and north Vietnamese populations are evolutionarily highly distinct. The type locality for *C. albolabris* is China (Regenass and Kramer 1981), and with the confirmation of *C. albolabris* as a morphologically and genetically distinct species, a preliminary range can be defined from this study as: Hong Kong, Fujian, Amoy, Wuhu Anhui in northeast China; Guangxi, Guizhou, Tung Kun in south China; Hainan; and Tam Dao in North Vietnam. The origin of the Taiwanese specimen is unconfirmed (AM pers. comm.) and hence Taiwan cannot be included in this range description.

The mtDNA phylogeny was significantly altered at deeper nodes with the addition of 12S and 16S rRNA subunits. Specimens from the south, west, north, and northeast of

Thailand are now found to be paraphyletic with respect to other species of *Cryptelytrops*, and the majority of specimens from each of these regions formed more or less distinct morpho-clusters. Two specimens (A145 and A228) from Chiang Mai in north Thailand were found to be genetically distinct and paraphyletic to the other specimens from this region. Analyses using only ND4 and Cytb in this study and in previous study showed A145 to group with other north and northeast Thailand specimens (Sanders 2003). On visual inspection, the 12S and 16S rRNA subunit gene sequences were found to be highly distinct from all other *"albolabris"* species, but these specimens were not found to be significantly different in morphology from the other north Thailand specimens. The genetic distinctiveness may be the result of pseudogene amplification, some nucleotides becoming fixed, or due to incomplete lineage sorting, but human error, although highly unlikely, cannot be entirely ruled out. Future investigations of *C. albolabris* will require using the preliminary range definition as a reference point to confirm the presence of *C. albolabris sensu stricto* in other regions (such as Thailand, Laos, Myanmar). This and the diversity of morpho-types in the initial *C. albolabris* group need to be re-examined by analysing the morphological data on a finer scale.

Cryptelytrops insularis

The species status of *C. insularis* is now confirmed from three sources: mtDNA, AFLP, and morphological evidence (Giannasi et al. 2001b; Sanders 2003). The existing estimated range of this island endemic is supported by morphology and it can now be re-defined as: Komodo, Sumba, Flores, Alor, Semau, west Timor, Wetar, Gresik and Sidoarjo in east Java, and most probably extending to Tasikmalaya and Indramayu in west Java. Further sampling may be required to confirm the presence of *C. insularis* in west Java as the specimens used in the morphological analysis were not represented in the genetic analysis.

4.5.5 Areas of interest for the investigation of cryptic species

The upper Myanmar specimens, which form a distinct mtDNA sister clade to *C. septentrionalis,* were seen to differentiate more or less as a single cluster (not shown) and did not align with any of the Nepalese specimens in males or with *C.*

septentrionalis specimens in females indicating that there is a high possibility that this population could be evolutionarily distinct. Additionally, a highly distinct morpho-cluster which included specimens from Cardamom Mountains in Cambodia, central and south Vietnam, and central and northeast Thailand was found. A distinct mtDNA clade more or less representing these regions was also found, although only the Cardamom Mountain specimen was common to both datasets. Although this pattern was not apparent in females, this needs further investigation as other cryptic *Cryptelytrops* species have been found from these regions (Malhotra et al. 2011).

4.6 CONCLUSIONS

With confirmation of the presence of morphologically distinct forms for *C. erythrurus, C. septentrionalis, C. albolabris,* and *C. insularis,* the systematic resolution for these species is now complete. The relationship of the distinct morphological type to *C. purpureomaculatus* will require further sampling with the possible addition of morphological data from the type specimen (from Singapore) and a combined morphological and genetic analysis including *C. erythrurus*. The complete range definition for *C.* albolabris, the confirmation of possible cryptic species in this group, and the establishment of the degree of morphological distinctiveness of the upper Myanmar population, will require re-analysis of morphological data using only a few well-defined species clusters and the addition of more characters (such as body dimensions and internal characters).

CHAPTER 5

GENERAL DISCUSSION

"Fortunately, this species concept problem is not as serious as it appears."

– Kevin De Queiroz

5.1 INTRODUCTION

Using the notoriously cryptic Asian pitvipers as an example, this thesis addresses some of the important and practical issues of species delimitation. The main areas of concern that are investigated are reliability of methods, relevance of particular data, and cases of incomplete lineage separation (De Queiroz 2007). The need to distinguish and delimit species encourages biologists to develop new methods (De Queiroz 2007), and cryptic species research has also led to the development of an array of genetic and non-genetic tools whose uses are wide and commonplace in many animal groups.

In snakes, some exploration of non-genetic methods such as bioacoustics (Young 2003), behavioural studies (Roth and Johnson 2004), and biochemical studies (LeMaster and Mason 2001; LeMaster et al. 2001) have been undertaken but mainly for studying their ecology at microevolutionary levels. Consequently, in cases such as Asian pitvipers where morphological crypsis has been identified as a common and recurring feature that frequently confounds species resolution, the use of genetic tools such as mtDNA genes, nuclear introns, and AFLPs, and of non-genetic tools such as multivariate morphometrics, is more popular. However, it is important that every now and again new concepts and molecular markers are incorporated, new methods are applied to tried and tested well-established markers, and their usefulness assessed to update taxonomic methods and enhance the efficiency of species discrimination and identification. This thesis has been an attempt towards this goal and has, in the process, contributed to the discovery of three new species of Asian pitvipers, one possible new species, and questioned some of the existing species designations.

5.2 DNA BARCODING IN SNAKES

This is a pilot study for DNA barcoding of snakes and provides the most comprehensive assessment of the uses of barcoding in reptiles to date. Full-length barcodes that have been generated for 15 new species during this project will be submitted to BOLD, significantly enhancing the number of venomous snake species representation in BOLD records, especially for Viperidae (c. 30%) which will then include a total of 46 species. Detailed morphological examination of snakes is often not possible, or recommended, due to the dangerous nature of the subjects. Given that DNA extractions and gene amplifications are successful using even shed skin or a single scale clipping, this study reinforces the idea that barcoding could be particularly useful for species identification of venomous snakes. Another consequence of this study is that some of the conflicting taxonomic issues in the genus *Popeia* have been addressed and the species status of *Pa. mcgregori* has been reassessed, using evidence from COI phylogenies.

5.3 UTILITY OF AFLPs FOR CRYPTIC SPECIES DELIMITATION

Although AFLPs have been widely employed for establishing the presence of distinct evolutionary units in a wide variety of animals, to date there has been no in-depth exploration of analysis methods to optimize its use as a marker. The AFLP project of this thesis forms the first comprehensive assessment of multiple analysis methodologies for dominant markers. In the genus *Cryptelytrops*, AFLPs have been used to establish distinct OTUs (Giannasi et al. 2001a; Sanders 2003). It has been found that three-nucleotide extension primers produce higher number of bands but have lower levels of discrimination (Giannasi et al. 2001b) as opposed to four-nucleotide extension markers, which are more selective in their amplifications resulting in higher levels of discrimination (Sanders 2003). Like most AFLP studies, PCA has been the method of choice for analysing these AFLP datasets, and it was concluded that the number of markers may need to be increased to increase the sampling rate across the genome and generate highly discriminatory datasets.

In the case of the current study group, *C. macrops*, the same four-nucleotide extension primers developed by Sanders (2003) were incorporated into different laboratory protocols and carefully optimized. A semi-automated band-scoring method for reducing genotyping errors (Whitlock et al. 2008) was adapted by incorporating a manual peak correction step This further increased band-inclusion and band-scoring precision and provided 298 polymorphic loci as opposed to 171 by Sanders (2003). The dataset was analysed using multiple methodologies (including some recent and advanced procedures) and successfully provided an independent source of genetic information and valuable evidence for resolving the disparity in morphological and mtDNA analyses for *Cryptelytrops macrops*. As a result of this study, the existence of three genetically distinct units within *C. macrops* was confirmed beyond doubt and a taxonomic revision of this complex has since followed with the distinct genetic lineages having been recognized as new species (Malhotra et al. 2011b). The clarity in the resolution of species clusters in multivariate analyses, as well as in the total evidence phylogenetic tree, shows that by tailoring protocols and methods to specific datasets, AFLPs can be robust markers for establishing genetic distinctiveness and delimiting cryptic species, and establishes the superiority of multivariate analysis for for use with dominant markers.

The repeatability tests which showed 97% accuracy for AFLP genotyping (both for duplicates of a given sample and for different tissue types under different storage conditions for a given sample), also significantly reinforced the confidence in this procedure as a dependable multilocus genetic sampling technique. Furthermore, considering that AFLP genome scans have not been as extensively applied to wild populations of animals as compared to plants (Bensch and Akesson 2005), this study has provided further support for AFLPs as robust markers, with power to infer species boundaries and resolve fine genetic structure even in closely related, recently diverged, non-model animal populations.

Next-generation high-throughput sequencing technology, which is rapidly gaining popularity in speciation research as it produces large multilocus datasets (Fonseca et al. 2010; Morin et al. 2010), is not yet widely applicable to taxonomic studies. With several issues such as high cost, high error rates, unreliability for taxonomic purposes

at genus and species level due to short sequence reads, and irregularities in the classification efficacy and resolution between sequencing types (Schuster 2008; Claesson et al. 2010), this technology requires further development before finding routine application in taxonomic studies. In view of this, this study demonstrates that AFLP technology (albeit relatively time-intensive), is undoubtedly a tested, cheap, and powerful technique, which can easily provide hundreds of informative genomic markers at lower taxonomic levels even in non-model organisms, for which we tend to have little knowledge of genomic sequence. The recently renewed interest and increase in the number of studies using AFLPs are also proving that this marker still has a future in speciation and biodiversity studies (Bensch and Akesson 2005; Meudt and Clarke 2007).

5.4 SYSTEMATICS OF *CRYPTELYTROPS*

Deriving a robust taxonomy, especially in the case of complex groups such as the Asian green pitvipers, is particularly difficult due to the complicated biogeographical history of southeast Asia. For establishing evolutionary inter-relationships and for classifying them as independently evolving units (De Queiroz 2007), it is important to take this into account rather than applying various species "concepts" to find distinctive populations. It needs to be borne in mind that although discovering new and cryptic species in biodiversity hotspots is important, cryptic species are only a "biodiversity wildcard" (Bickford et al. 2007) and not a real solution to what is now confirmed to be the sixth mass extinction (Barnosky et al. 2011). Incorrect and overzealous taxonomic revisions are niggling, circular, and will only serve to complicate and confuse the management of conservation practices. Therefore, it needs to be reinforced that at least two or three independent sources providing corroborating evidence should be provided for the circumscription and the definition of new species in this taxonomically challenging group.

The fourth chapter has attempted to achieve this, by evaluating evidence derived from mtDNA, multivariate morphometrics, and AFLPs for the taxonomy of the genus

Cryptelytrops (Giannasi et al. 2001b; Sanders 2003; Malhotra and Thorpe 2004a). Given the number of independent studies conducted on what is now the genus *Cryptelytrops* over more than a century (Gumprecht et al. 2004), a compilation of species diversity and range definitions from these contributions was also urgently required. This study is the first to present a combined analysis of multivariate morphological characters as evidence of the taxonomy of five major species in the *"albolabris"* group. This represents a big step forward in clarifying species boundaries and gives direction to new research by identifying areas that need further investigation. In particular, the confirmation of the morphological and genetic characteristics of *C. albolabris sensu stricto,* and the identification of its geographic range, is particularly important as it will form the basis for gaining a better understanding of species diversity in the rest of the *"albolabris"* group. *C. erythrurus* was the first to be proposed as a species in this group, and its designation, characteristics, and range which have been debated for several decades, are now clarified. The presence of a possible additional cryptic species within the range of *C. erythrurus* is a novel and unexpected discovery. A need for further sampling effort in the range described for the *"C. erythrurus – C. purpureomaculatus"* complex, including Andaman and Nicobar islands as a priority, has been identified. Asian pitvipers are a particularly difficult group to work with using morphology, and with the magnitude of conflicting and confusing literature on this genus, the power of multivariate statistics for deriving broad-scale taxonomic clarifications is evident from this study, especially as it in the only method currently available by which information from type specimens can be included.

5.5 DIRECTIONS FOR FUTURE WORK

This study presented an evaluation of the uses of a few tools and methods for towards resolving the taxonomy and investigating the biodiversity in Asian green pitvipers. However, it also presented several cases where these methods were unsuccessful in clarifying species affinities, and identified their limitations. Compared to other groups of animals, snakes are particularly difficult to work with as their cryptic lifestyles makes it difficult to observe them and document their ecology, and this has been a

significant drawback for understanding their evolution. However, technological advances in recent years have made available a variety of new tools and markers for speciation research, and the uses of these for resolving the taxonomy of Asian pitvipers needs to be evaluated.

Analysis of anonymous nuclear loci and multiple nuclear protein-coding loci (NPCL) have been found to be useful for species-level phylogenetic research (Townsend et al. 2008; Gibbs and Diaz 2010). Geometric morphometrics using landmarks is an established, powerful, and highly sensitive tool which is being increasingly employed in morphometric analysis of biological forms, and its uses have been evaluated in squamates (Baszio and Weber 2002) Initial analysis of stable isotopes using snake skin has presented interesting patterns of congruence with phylogenetic relationships and geographic distributions (Axel Barlow pers. comm.). With head shape being indicative of the evolutionary ecology of reptiles (Claude et al. 2004; Smith and Collyer 2008) and snake skin being very easy to source, a test of these two tools for distinguishing species in Asian pitvipers would be highly recommended. The initiation of a "Barcoding Snakes" movement on BOLD Systems to compile DNA barcodes for snake species is also urgently required. In conclusion, incorporation of new and possibly more efficient tools for optimizing taxonomic methods is the future direction for Asian pitviper taxonomy.

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APPENDIX 1

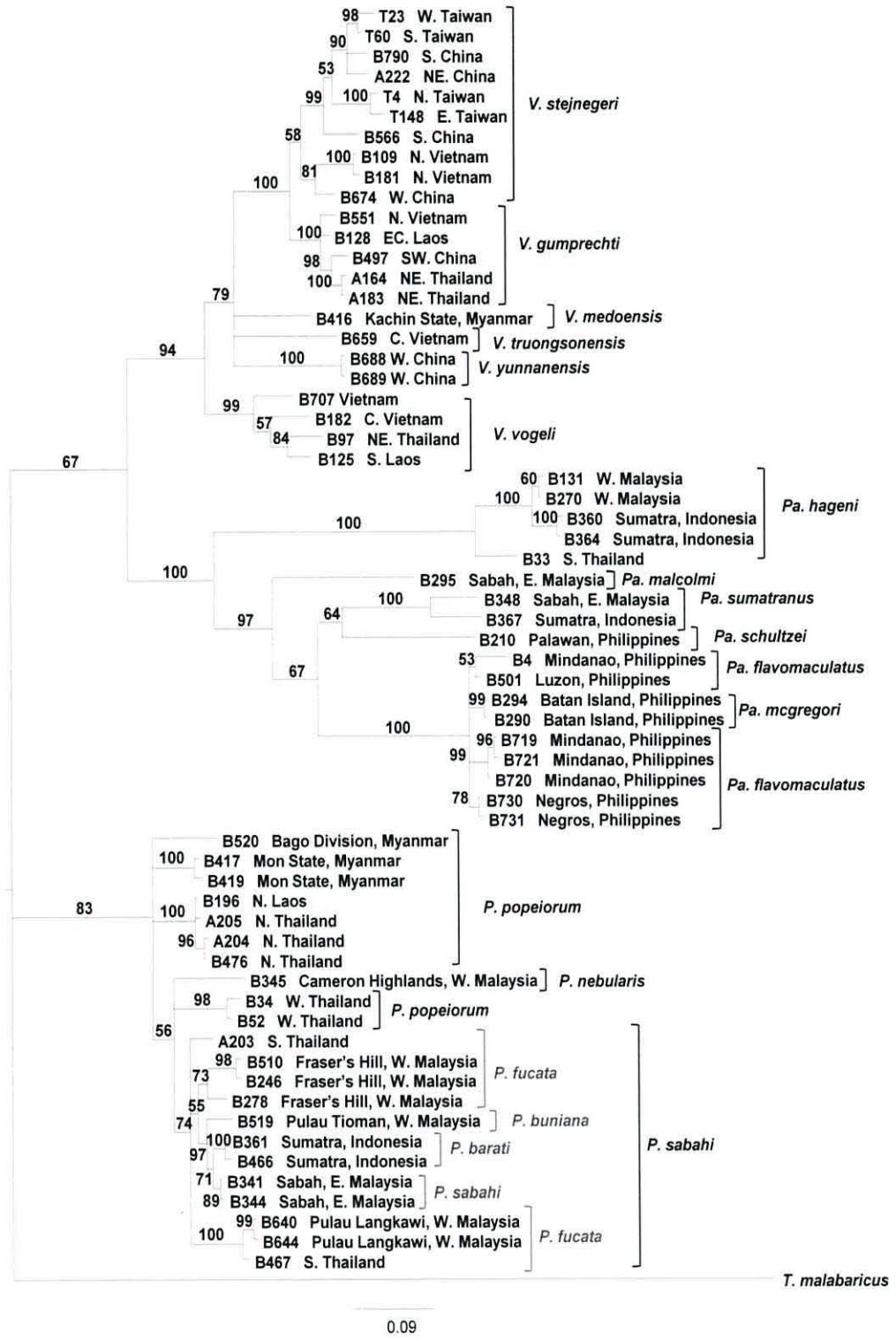


Figure 1. COI 50% majority-rule concensus Bayesian MCMC phylogeny of three million generations. Support values at each node are Bayesian posterior probabilities. The resolution for *Parias* and *Popeia* is poor with *Pa. flavomaculatus* fragmented into a polytomy and *Pa. mcgregori* nested between the north and central/south specimens. *P. popeiorum* clade is similar, with the two west Thailand specimens formed a polytomy with *P. nebularis* and the southern *P. sabahi.*

Table 1. Sample list used in mtDNA phylogeny

Taxa	Geographic Location	Sample No.	Field/Mus No.	Cytb	ND4	12S	16S	COI
V. gumprechti	NE. Thailand	A164	AFS99.1	AY352766	AF517224	AF517168	AF517181	M. Unpublished
	C. Laos	B128	FMNH 256419	AY059579	EU443787	EU443788	EU443789	M. Unpublished
	SW. China	B497	AFS02.37/BMNH 2002.53	AY321489	EU443790	EU443791	EU443792	M. Unpublished
	N. Vietnam	B551	NA	Dr. AM	Dr. AM	Dr. AM	Dr. AM	M. Unpublished
V. vogeli	NE. Thailand	B97	AFS99.5	AY059574	AY059596	AY059546	AY059562	M. Unpublished
	S. Laos	B125	FMNH 258945	AY059581	AF517225	AF517170	AF517183	M. Unpublished
		B124	FMNH 258946	AY059580	EU443808	EU443809	EU443810	M. Unpublished
	Vietnam	B707	RH060142	Dr. Dawson	Mrinalini	Dr. Dawson	Mrinalini	Mrinalini
	C. Vietnam	B182	ROM 34565	AY059578	EU443805	EU443806	EU443807	M. Unpublished
V. stejnegeri	N. Vietnam	B181	ROM 35321	AF278711	EU443793	EU443794	EU443795	M. Unpublished
		B109	AFS99.17	AF278709	Dr. Peng	Dr. AM	Dr. AM	Mrinalini
	W. Taiwan	T23	2671 (4109)	AF277689	EU443799	EU443800	EU443801	M. Unpublished
	E. Taiwan	T148	3599 (12221)	AF277690	EU443796	EU443797	EU443798	M. Unpublished
	N. Taiwan	T4	2558 (3782)	AF277700/AF171896	AY059593	AY059539	AY059555	M. Unpublished
	S. Taiwan	T60	2684 (4160)	AF277676/AF171880	EU443802	EU443803	EU443804	M. Unpublished
	NE. China (Fujian)	A222	F12 (NMNS 3651)	AF277677	AY059594	AY059541	AY059557	Mrinalini
	S. China (Guangdong)	B790	GP422	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B789	GP421	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
	S. China (Hainan)	B566	NA	Dr. Dawson	Dr. Dawson	Dr. Dawson	Dr. Dawson	Mrinalini
	W. China (Sichaun)	B674	GP49	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
V. medoensis	Myanmar (Kachin State)	B416	CAS 221528	AY352765	AY352831	AY352797	AY352735	M. Unpublished
V. yunnanensis	W. China	B688	GP37	EF597522	EF597527	EU443811	EU443812	M. Unpublished
		B689	GP38	EF597523	EF597528	EU443813	EU443814	M. Unpublished
V. truongsonensis	C. Vietnam	B659	NA	EU443815	EU443816	EU443817	EU443818	Mrinalini
P. sabahi	S. Thailand	A203	AFS96.19	AY371796	AY059588	AY059537	AY059553	M. Unpublished
		B467	AFS02.05	AY371807	AY371851	AY371744	AY371781	M. Unpublished
	W. Malaysia	B246	KLS00-01	AY059570	AY059589	AY059540	AY059556	M. Unpublished
		B278	KLS01-61	AY371821	AY371857	AY371750	AY371780	M. Unpublished
		B519	LHSUHC 4809	AY371818	AY371853	AY371752	AY371778	M. Unpublished
		B635	AFS06.69	M. Unpublished	M. Unpublished	M. Unpublished	M. Unpublished	M. Unpublished
		B640	AFS06.74	NA	NA	NA	NA	Mrinalini
		B644	AFS06.78	NA	NA	NA	NA	Mrinalini
	E. Malaysia (Sabah)	B341	KLS01-116	AY371803	AY371834	AY371734	AY371772	M. Unpublished
		B344	KLS01-122	AY371815	AY371842	AY371736	AY371771	M. Unpublished
	Indonesia (Sumatra)	B361	KLS02-06	AY371801	AY371837	AY371753	AY371769	M. Unpublished
		B466	AFS02.04	M. Unpublished	M. Unpublished	M. Unpublished	M. Unpublished	M. Unpublished
P. popeiorum	W. Thailand	B34	AFS98.16	AY059572	AY059591	AY059542	AY059558	M. Unpublished
		B52	AFS98.34	AY371800	AY371836	AY371754	AY371768	M. Unpublished
	N. Thailand	A204	AFS96.17	AF171902	AY371843	AY371742	AY371784	M. Unpublished
		A205	AFS96.3	AF171906	AY371854	AY371741	AY371767	M. Unpublished
		B476	AFS02.14, AFS06.28	AY371809	AY371852	AY371745	AY371782	M. Unpublished
	N. Laos	B196	FMNH 258950	AY059571	AY059590	AY059538	AY059554	M. Unpublished
	Myanmar (Mon State)	B417	CAS 216609	AY371805	AY371845	AY371743	AY371776	M. Unpublished
		B419	CAS 222195	AY371806	AY371841	AY371738	AY371777	M. Unpublished
	Myanmar (Bago Div)	B520	CAS 205847	AY371816	AY371855	AY371751	AY371783	M. Unpublished
P. nebularis	W. Malaysia	B345	KLS01-128	NA	NA	NA	NA	M. Unpublished
		B238	AFS00.15	AY371814	AY371839	AY371737	AY371774	M. Unpublished
		B558	NA	M. Unpublished	M. Unpublished	M. Unpublished	M. Unpublished	M. Unpublished
Pa. hageni	S. Thailand	B33	AFS98.15	AY059567	AY059585	AY059536	AY059552	M. Unpublished
		A224	AFS97B.20	AF171911	Mrinalini	Dr. Sanders	Dr. Sanders	Mrinalini
	W. Malaysia	B131	AFS99B.5	AY371826	AY371868	AY371761	AY371787	Mrinalini
		B651	AFS06.85	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B270	KLS00-08	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B229	AFS00.6	AY371827	AY371867	AY371755	AY371794	Mrinalini
		B275	KLS01-37	Mrinalini	Dr. Sanders	Mrinalini	Mrinalini	Mrinalini
	Indonesia (Sumatra)	B360	KLS02-05	AY371829	AY371862	AY371764	AY371789	Mrinalini
		B364	KLS02-09	AY371825	AY371863	AY371763	AY371790	Mrinalini
Pa. flavomaculatus	Philippines (Mindanao)	B4	NMP/CMNH 1798	AY352764	AY352830	AY352796	AY352734	Mrinalini
		B719	MD1	NA	NA	NA	NA	Mrinalini
		B721	MD3	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B720	MD4	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B722	MD4	Dr. AM	Dr. AM	Dr. AM	Dr. AM	Mrinalini
		B723	MD5	NA	NA	NA	NA	Mrinalini
	Philippines (Negros)	B731	NE2	NA	NA	NA	NA	Mrinalini
		B739	NE10	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B732	NE3	NA	NA	NA	NA	Mrinalini
		B733	NE4	NA	NA	NA	NA	Mrinalini
		B735	NE6	NA	NA	NA	NA	Mrinalini
		B736	NE7	NA	NA	NA	NA	Mrinalini
		B737	NE8	NA	NA	NA	NA	Mrinalini
		B738	NE9	NA	NA	NA	NA	Mrinalini
		B730	NE10	NA	NA	NA	NA	Mrinalini
		B740	NE11	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
	Philippines (Luzon)	B501	AFS02.57	Mrinalini	Mrinalini	Dr. Sanders	Mrinalini	Mrinalini
		B502	AF202.58	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B506	AFS02.62	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B507	NA	NA	NA	NA	NA	Mrinalini
		B725	BC2	NA	NA	NA	NA	Mrinalini
		B726	BC3	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B727	BC4	Mrinalini	Mrinalini	Mrinalini	Mrinalini	Mrinalini
		B728	BC5	NA	NA	NA	NA	Mrinalini
		B729	BC6	NA	NA	NA	NA	Mrinalini
		B724	BC1	NA	NA	NA	NA	Mrinalini
		B603	AFS06.35	NA	NA	NA	NA	Mrinalini
		B13	NA	NA	NA	NA	NA	Mrinalini

Taxa	Geographic Location	Sample No.	Field/Mus No.	Cytb	ND4	12S	16S	COI
		B14	NA	NA	NA	NA	NA	Mrinalini
Pa. mcgregori	Philippines (Batanes)	B290	NA	Mrinalini	AY371858	AY371756	Mrinalini	Mrinalini
		B292	CR-449	NA	NA	NA	NA	Mrinalini
		B294	CR-510	Mrinalini	Mrinalini	Dr. Sanders	AY371795	Mrinalini
		B289	CR-487	NA	NA	NA	NA	Mrinalini
		B293	CR-485	NA	NA	NA	NA	Mrinalini
		B488	AFS02.25	NA	NA	NA	NA	Mrinalini
		B493	AFS02.30	NA	NA	NA	NA	Mrinalini
		B503	AFS02.59	NA	NA	NA	NA	Mrinalini
		B620	AFS06.52	NA	NA	NA	NA	Mrinalini
Pa. schultzei	Philippines (Palawan)	B210	NA	AY352756	AY352819	AY352785	AY352725	Mrinalini
Pa. sumatranus	E. Malaysia (Sabah)	B348	KLS01-129	AY371828	AY371866	AY371760	AY371788	Mrinalini
	Indonesia (Sumatra)	B367	KLS02-14	AY371824	AY371864	AY371765	AY371791	Mrinalini
		B368	KLS02-15, RTV31	AY371830	AY371865	AY371762	AY371792	Mrinalini
Pa. malcolmi	E. Malaysia (Sabah)	B295	KLS 0185	AY371822	AY371860	AY371758	Dr. Sanders	Mrinalini
		B349	KLS01-108	AY371832	AY371861	AY371757	AY371786	Mrinalini
T. malabaricus	W. India	B260	AFS01.1	AY352763	AY352829	AY352795	AY352733	Mrinalini
T. borneensis	E. Malaysia (Sabah)	B301	KLS01-120	AY352754	AY352817	AY352783	AY352722	Mrinalini

APPENDIX 2

MORPHOMETRIC ANALYSIS OF *Cryptelytrops macrops*.

Morphometric and meristic characters were measured as described in Malhotra and Thorpe (2004a). (A list of characters used follows). Two-way analysis of variance and covariance (ANOVA/ANCOVA) was used to identify characters showing significant between-sex and among-OTU variation. No significant sexual dimorphism was found in many characters, so to maximize sample sizes, which were small for many critical populations, sexes were pooled for further analyses. Characters that were not significantly different between-OTUs were excluded from subsequent analyses. Some sexually dimorphic characters, which showed significant between-OTU differences, also had to be excluded from the analysis (number of subcaudal scales, number of supralabial scales and length of head). A canonical variate analysis (CVA) of morphological characters (between individual localities rather than clades) was then performed (Fig. 1). It was noted that some characters (particularly colour pattern characters) showed some heteroscedascity, which may perturb CVA. Alhough this should be apparent in the results (e.g., if heteroscedastic characters dominate the axes), the presence of potential perturbation due to heteroscedascity was also checked by carrying out a principal component analysis (PCA) on individuals (i.e., not assigned to groups). This has much less discriminatory power, but is less affected by departures from the assumptions of the model of homoscedascity (Thorpe 1976, 1983). All size–related characters used in the PCA were first adjusted to a common size using the pooled within–group slope, with either snout–vent length (SVL) or head length (LHEAD) as the covariate.

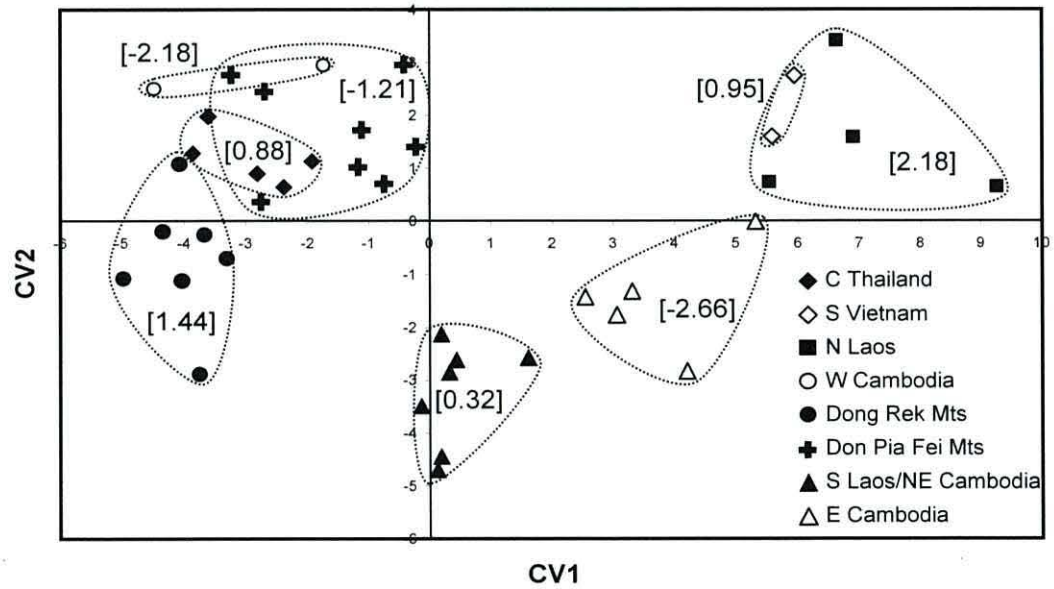


Figure 1. Canonical variate analysis of all specimens. Sexes were pooled and sexually dimorphic characters were excluded. The first three axes summarise 16.6 %, 4.8 % and 3.0 % of the total variation respectively. Black symbols correspond to populations assigned to *C. macrops sensu stricto* in phylogenetic analysis, which divide into two main clusters corresponding roughly to an eastern (right hand side) and western (left-hand side) group. The other two clades can be distinguished from each of these clusters on the third axis (the group centroid on CV3 is indicated in square brackets). However, the extent of the morphological differentiation between putatively different species seems smaller than that (presumably reflecting geographic variation) within *C. macrops sensu stricto*.

A list of characters used in the morphometric analysis of *C. macrops*.

Scale reductions are recorded as a series of characters, each referring to a specific reduction. Each position will have two characters, the dorso-ventral (DV) position of the reduction (the lowest of the two merging scale rows), and the ventral scale or subcaudal scale (VS or CS) position (counted from the head and vent respectively), which is the ventral scale to which the scale reduction traces diagonally. Before analysis, the VS position was transformed into the percentage of the total number of ventral scales (%VS) or subcaudal scales (%SC), to control for variation. All measurements are made on the right side of the head only unless this was damaged, in which case they were done on the left.

VSC: the number of ventral scales (VS), not including anal scale, recorded by the Dowling (1951) method (i.e. the first VS is the one which contacts the first dorsal scale row on both sides).
POSTOC: number of postocular scales.
BORSUPOC: the number of scales bordering the supraocular scales (average of right and left), not counting pre- or post-oculars.
LABNAS: the degree of fusion of the first labial and nasal scale.
KTEMP: the keeling of the temporal scales.
KHEADSC: the keeling of the scales on the back of the head.
KSIDESC: the keeling of the scales on the side of the head, between the temporals and the rear supralabial scales.
BSCK: the keeling of the dorsal scales at mid body.
VS19to17: ventral scale position of the reduction from 19 to 17 scale rows.
DV19to17: dorsoventral position of reduction from 19 to 17 scale rows.
VS17to15: ventral position of the reduction from 17 to 15 scale rows.
SVL: distance between the tip of the snout and the cloaca (only used to size-adjust head dimensions).
WSUPOC: the width of the supraoculars measured in mm, at the widest part.
STRIPE: presence of lateral stripe (0, absent; 1, indistinct; 2, distinct).
SCR1: the proportion of the first scale row covered by the light area.
DENT: the number of dentary teeth.
LKPOST: VS position of the posterior tip of the left kidney.

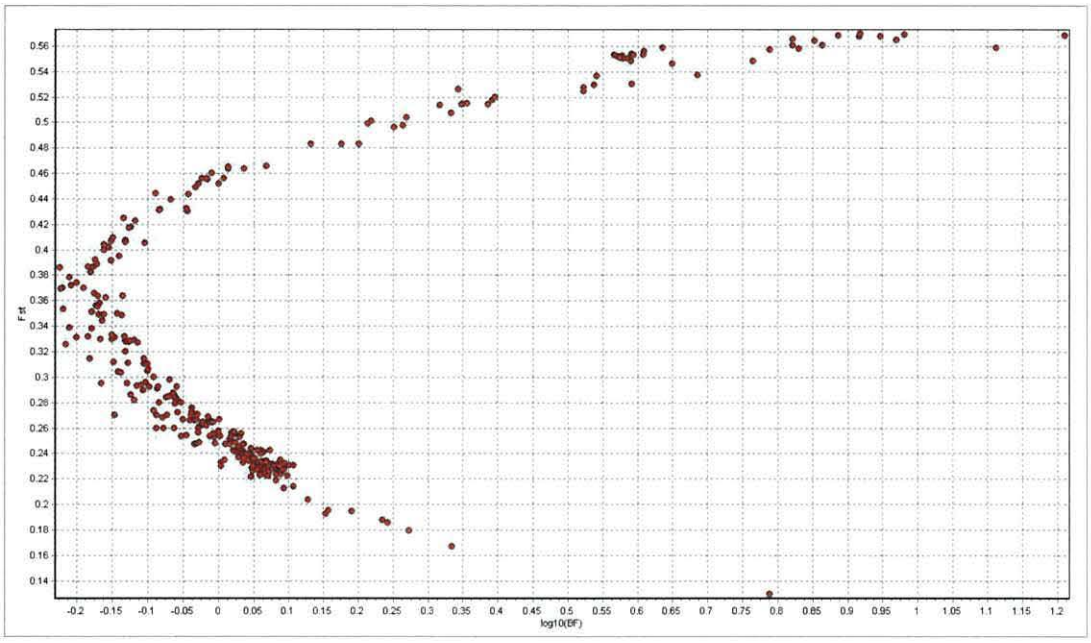
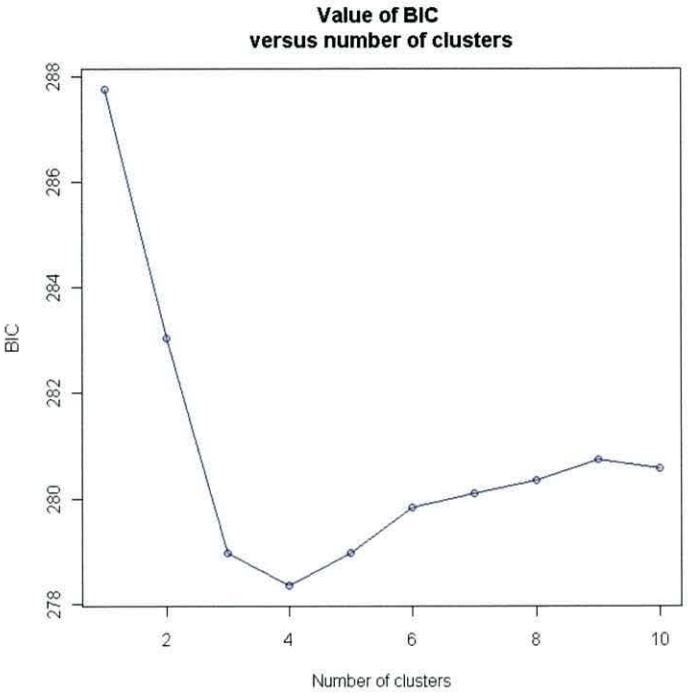


Figure 2. Plot of Log_{10} Bayes Factor (BF) versus locus specific F_{st} for 298 loci from BayesScan, showing lack of evidence for loci affected by selection (with Jefferey's scale set to decisive where log_{10} (BF) = 2.0).

a)



b)

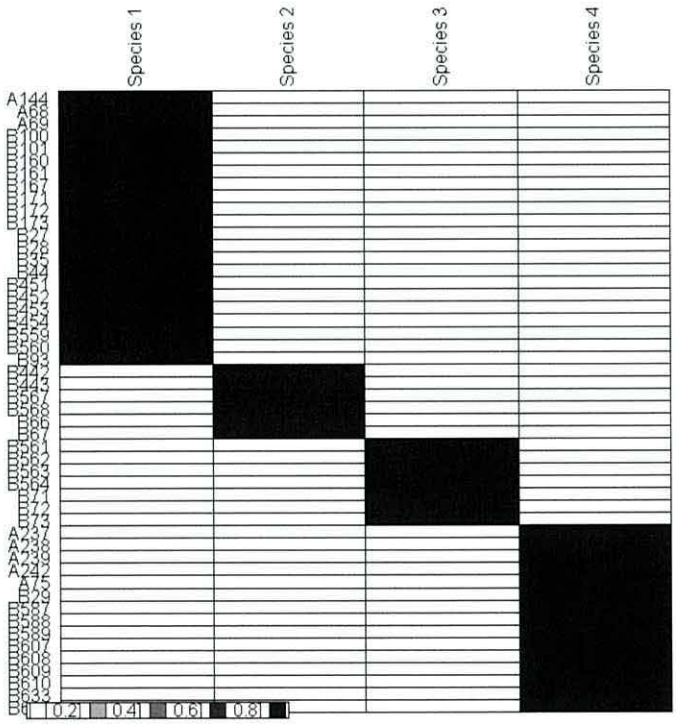


Figure 3. Discriminant Analysis of Principal Components. a) Inference of the number of clusters. Bayesian Information Criteria (BIC) is shown for each value of K from 1 to 10, with K=4 being the chosen number of clusters. b) Summary plot of individual assignment probabilities. Each individual was clearly assigned to one of the four clusters with no ambiguities. Species 1 = *C. macrops sensu stricto*, Species 2 = *C. macrops sp nov 1*, Species 3 = *C. macrops sp nov 2* and Species 4 = *C. venustus*.

Table 1. List of samples used for AFLP genotyping. Sample ID number refers to tissue collection catalogue numbers (AM). Where voucher specimens exist, museum codes are given.

Sample ID	Museum Voucher No.	Putative Species	Country	State
A68		*C. macrops s.s.*	Thailand	Nakhon Ratchasima
A69		*C. macrops s.s.*	Thailand	Petchabun
A75		*C. venustus*	Thailand	Nakhon Si Thammarat
A144		*C. macrops s.s.*	Thailand	Lampang
A237		*C. venustus*	Thailand	Nakhon Si Thammarat
A238		*C. venustus*	Thailand	Nakhon Si Thammarat
A239		*C. venustus*	Thailand	Nakhon Si Thammarat
A242		*C. venustus*	Thailand	Nakhon Si Thammarat
B27		*C. macrops s.s.*	Thailand	Bangkok
B28		*C. macrops s.s.*	Thailand	Bangkok
B29		*C. venustus*	Thailand	Surat Thani
B35		*C. macrops s.s.*	Thailand	Ang Thong
B44		*C. macrops s.s.*	Thailand	Nakhon Si Thammarat
B66		*C. macrops sp nov 1*	Thailand	Chantaburi
B67		*C. macrops sp nov 1*	Thailand	Chantaburi
B71		*C. macrops sp nov 2*	Viet Nam	Ho Chi Minh City
B72		*C. macrops sp nov 2*	Viet Nam	Bienhoa
B73		*C. macrops sp nov 2*	Viet Nam	Bienhoa
B93		*C. macrops s.s.*	Thailand	Nakhon Ratchasima
B100		*C. macrops s.s.*	Thailand	Nakhon Ratchasima
B101		*C. macrops s.s.*	Thailand	Nakhon Ratchasima
B160	FMNH 255252	*C. macrops s.s.*	Laos	Champassak Province
B161	FMNH 255249	*C. macrops s.s.*	Laos	Champassak Province
B167	FMNH 255254	*C. macrops s.s.*	Laos	Bolikhamxay Province
B171	FMNH 255251	*C. macrops s.s.*	Laos	Champassak Province
B172	FMNH 255255	*C. macrops s.s.*	Laos	Bolikhamxay Province
B173	FMNH 255253	*C. macrops s.s.*	Laos	Bolikhamxay Province
B442	FMNH 259191	*C. macrops sp nov 1*	Cambodia	Koh Kong Province
B443	FMNH 259192	*C. macrops sp nov 1*	Cambodia	Koh Kong Province
B451	FMNH 258955	*C. macrops s.s.*	Laos	Champassak Province
B452	FMNH 258956	*C. macrops s.s.*	Laos	Champassak Province
B453	FMNH 258957	*C. macrops s.s.*	Laos	Champassak Province
B454	FMNH 258958	*C. macrops s.s.*	Laos	Champassak Province
B559	FMNH 262715	*C. macrops s.s.*	Cambodia	Stung Treng, Siem pang
B560	FMNH 262716	*C. macrops s.s.*	Cambodia	Stung Treng, Siem pang

Sample ID	Museum Voucher No.	Putative Species	Country	State
B562	FMNH 262718	*C. macrops sp nov 2*	Cambodia	Mondolkiri, O'Rang
B563	FMNH 262719	*C. macrops sp nov 2*	Cambodia	Mondolkiri, O'Rang
B564	FMNH 262720	*C. macrops sp nov 2*	Cambodia	Mondolkiri, O'Rang
B567	FMNH 263387	*C. macrops sp nov 1*	Cambodia	Koh Kong Province
B568	FMNH 267732	*C. macrops sp nov 1*	Cambodia	Koh Kong Province
B587		*C. venustus*	Thailand	Nakhon si Thammarat
B588		*C. venustus*	Thailand	Nakhon si Thammarat
B589		*C. venustus*	Thailand	Nakhon si Thammarat
B607		*C. venustus*	Thailand	Nakhon si Thammarat
B608		*C. venustus*	Thailand	Nakhon si Thammarat
B609		*C. venustus*	Thailand	Nakhon si Thammarat
B610		*C. venustus*	Thailand	Nakhon si Thammarat
B633		*C. venustus*	Malaysia	Pulau Langkawi
B634		*C. venustus*	Malaysia	Pulau Langkawi

Table 2. Details of AFLP primers used.

Pre-Selective Amplification Primers	
*Eco*RI 5' GACTGCGTACCAATTCA 3'	
*Mse*I 5' GATGAGTCCTGAGTAAC 3'	
Selective Amplification Primer Combinations	
5' *Eco*RI + CCG 3'	5' *Mse*I + TAG 3'
5' *Eco*RI + GCC 3'	5' *Mse*I + TAG 3'
5' *Eco*RI + GGG 3'	5' *Mse*I + TAG 3'
5' *Eco*RI + CAG 3'	5' *Mse*I + ACC 3'
5' *Eco*RI + GCC 3'	5' *Mse*I + TGG 3'
5' *Eco*RI + GGG 3'	5' *Mse*I + TGG 3'

Table 3. Sample ID is as explained in Table 1. Mean permuted individual assignment probabilities to populations obtained from CLUMPP (10 runs of 100,000 iterations with K=4 in STRUCTURE).

Sample ID	Assigned Species	*C. macrops s.s.*	*C. macrops sp nov 1*	*C. macrops sp nov 2*	*C. venustus*
A68	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
A69	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
A75	*C. venustus*	0.0000	0.0000	0.0000	1.0000
A144	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
A237	*C. venustus*	0.0000	0.0000	0.0000	1.0000
A238	*C. venustus*	0.0000	0.0000	0.0000	1.0000
A239	*C. venustus*	0.0000	0.0000	0.0000	1.0000
A242	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B27	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B28	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B29	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B35	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B44	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B66	*C. macrops sp nov 1*	0.0281	0.9719	0.0000	0.0000
B67	*C. macrops sp nov 1*	0.0242	0.9758	0.0000	0.0000
B71	*C. macrops sp nov 2*	0.0000	0.0000	1.0000	0.0000
B72	*C. macrops sp nov 2*	0.0000	0.0000	1.0000	0.0000
B73	*C. macrops sp nov 2*	0.0001	0.0000	0.9999	0.0000
B93	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B100	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B101	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B160	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B161	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B167	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B171	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B172	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B173	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B442	*C. macrops sp nov 1*	0.0095	0.9905	0.0000	0.0000
B443	*C. macrops sp nov 1*	0.0098	0.9902	0.0000	0.0000
B451	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B452	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B453	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B454	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000

Sample ID	Assigned Species	*C. macrops s.s.*	*C. macrops sp nov 1*	*C. macrops sp nov 2*	*C. venustus*
B560	*C. macrops s.s.*	1.0000	0.0000	0.0000	0.0000
B561	*C. macrops sp nov 2*	0.0000	0.0000	1.0000	0.0000
B562	*C. macrops sp nov 2*	0.0000	0.0000	1.0000	0.0000
B563	*C. macrops sp nov 2*	0.0000	0.0000	1.0000	0.0000
B564	*C. macrops sp nov 2*	0.0000	0.0000	1.0000	0.0000
B567	*C. macrops sp nov 1*	0.1027	0.8973	0.0000	0.0000
B568	*C. macrops sp nov 1*	0.0095	0.9905	0.0000	0.0000
B587	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B588	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B589	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B607	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B608	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B609	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B610	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B633	*C. venustus*	0.0000	0.0000	0.0000	1.0000
B634	*C. venustus*	0.0001	0.0000	0.0000	0.9999

Table 4. Individual assignment probabilities to putative species in *Cryptelytrops* from 1 million iterations in GENELAND, using spatial priors. Co-ordinates are given in decimal degrees.

Sample ID	Latitude	Longitude	*C. macrops s.s. & sp nov 1*	*C. macrops sp nov 2*	*C. venustus*
A68	101.41	14.7	0.644	0.197	0.158
A69	101.31	16.75	0.644	0.197	0.158
A75	99.87	8.45	0.197	0.158	0.644
A144	99.88	18.78	0.644	0.197	0.158
A237	99.68	8.14	0.197	0.158	0.644
A238	99.68	8.14	0.197	0.158	0.644
A239	99.68	8.14	0.197	0.158	0.644
A242	99.68	8.14	0.197	0.158	0.644
B27	100.53	13.76	0.644	0.197	0.158
B28	100.53	13.76	0.644	0.197	0.158
B29	99.31	9.16	0.197	0.158	0.644
B35	100.24	14.76	0.644	0.197	0.158
B44	99.68	8.15	0.441	0.196	0.363
B66	102.13	12.8	0.644	0.197	0.158
B67	102.13	12.8	0.644	0.197	0.158
B71	106.52	10.98	0.158	0.644	0.197
B72	106.78	10.96	0.158	0.644	0.197
B73	106.78	10.96	0.158	0.644	0.197
B93	102.15	14.79	0.644	0.197	0.158
B100	102.14	14.54	0.644	0.197	0.158
B101	101.55	14.3	0.644	0.197	0.158
B160	105.4	14.17	0.644	0.197	0.158
B161	105.4	14.17	0.644	0.197	0.158
B167	103.1	18.27	0.644	0.197	0.158
B171	105.22	14.08	0.644	0.197	0.158
B172	103.1	18.27	0.644	0.197	0.158
B173	104.53	18.14	0.644	0.197	0.158
B442	103.47	11.26	0.644	0.197	0.158
B443	103.39	11.62	0.644	0.197	0.158
B451	106.1	15.02	0.644	0.197	0.158
B452	106.1	15.02	0.644	0.197	0.158
B453	106.1	15.02	0.644	0.197	0.158
B454	106.1	15.02	0.644	0.197	0.158
B559	106.31	14.15	0.644	0.197	0.158
B560	106.31	14.15	0.644	0.197	0.158

Sample ID	Latitude	Longitude	*C. macrops s.s. & sp nov 1*	*C. macrops sp nov 2*	*C. venustus*
B562	107.6	12.19	0.158	0.644	0.197
B563	107.6	12.19	0.158	0.644	0.197
B564	107.6	12.19	0.158	0.644	0.197
B567	104.1	11.95	0.644	0.197	0.158
B568	104.1	11.95	0.644	0.197	0.158
B587	99.68	8.16	0.197	0.158	0.644
B588	99.68	8.16	0.197	0.158	0.644
B589	99.68	8.16	0.197	0.158	0.644
B607	99.68	8.16	0.197	0.158	0.644
B608	99.68	8.16	0.197	0.158	0.644
B609	99.68	8.16	0.197	0.158	0.644
B610	99.68	8.16	0.197	0.158	0.644
B633	99.82	6.37	0.197	0.158	0.644
B634	99.7	6.42	0.197	0.158	0.644

Table 5. Average and percentage changes in the likelihood values for K=1 to 10 from 10 runs of 100,000 each from STRUCTURE.

K Change	Average	Percentage
1 to 2	-1259.96	18.00
2 to 3	-1107.80	19.30
3 to 4	-397.07	8.57
4 to 5	-88.90	2.10
5 to 6	-32.77	0.79
6 to 7	43.36	-1.05
7 to 8	20.76	-0.50
8 to 9	-44.96	1.08
9 to 10	-60.01	1.45

APPENDIX 3

Table 1. Sample list used in morphological analysis. Corresponding mtDNA number used in the phylogenetic analysis are also given.
a) Males

Sample No.	MtDNA No.	Region	Museum No.
8		Hong Kong	MCZ_173525
10		Komodo, NTT	AMNH_31965
11		Komodo, NTT	AMNH_31964
18	A118	Flores, NTT	IM1
19	A119	Flores, NTT	IM2
21		Flores, NTT	IM4
30		Sidoarjo, E. Java	ING1
32		Baaguia, E. Timor	NMB_12902
38		Sumba, NTT	NMB_14894
45		Sumba, NTT	NMW_14889
46		NikiNiki, Timor	NMBA_12772
52		Flores, NTT	UF_30144
53		Komodo, NTT	UF_28698
54		Flores, NTT	UF_30150
55		NakhonRatcha, Thai	FMNH_180241
68		LangBiahPeah,Annam, Vietnam	FMNH_26454
69		LangBiahPeah,Annam, Vietnam	FMNH_26452
70		ChiangMai, Thai	FMNH_179414
71		NakhonRatcha, Thai	FMNH_180240
73		Cochinchina, Vietnam	FMNH_11535
74		Flores, NTT	UF_30149
75		Flores, NTT	UF_30127
76		Komodo, NTT	UF_28701
79		Komodo, NTT	UF_28690
80		Komodo, NTT	UF_287.4
81		Komodo, NTT	UF_28703
82		Komodo, NTT	UF_28691
83		Komodo, NTT	UF_28694
84		Tasikmalaya, W. Java	NMW_23902:2
85		Tasikmalaya, W. Java	NMW_23902:5
88		Penang, W. Malaysia	NMW_23920:2
90		Tasikmalaya, W. Java	NMW_23902:10
91		Hong Kong	MCZ_176065
92		Hong Kong	MCZ_176074
95		Hong Kong	MCZ_177101
96		Hong Kong	MCZ_173512
97		Hong Kong	MCZ_172785
98		Hong Kong	MCZ_172602
99		Flores, NTT	UF_30141
102		Sumba, NTT	NMBA_14886
103		Sumba, NTT	NMBA_14894
106		Pokhra, Nepal	NMBA_21012
107		RotiIsland, Timor	NMBA_12930
108		Cochinchina, Vietnam	NMBA_2588
110		Borneo?	NMBA_18667
114		QuangNam, Vietnam	USNM_163961
117		PhuLaung,Loei, Thai	PLC_920407
118	A165	PhuLaung,Loei, Thai	AFSPL_2
119	A182	LomSak,Loei, Thai	AFSPL_19
131		Pokhra, Nepal	MHNG_1404.3
133		Pokhra, Nepal	MHNG_1400.31
134		Pokhra, Nepal	MHNG_1404.4
135		Pokhra, Nepal	MHNG_1400.24
136		Pokhra, Nepal	MHNG_1400.29
137		Guangxi, China	MHNG_1400.68
138		Karengates, Java	MHNG_2221.35
140		Tung Kun, China	MHNG_1464.69
144		NakhonRatcha, Thai	FMNH_180275
145		Hainan, China	FMNH_6705
146		Hainan, China	FMNH_6707
150		ChiangMai, Thai	FMNH_188921
151		ChiangMai, Thai	FMNH_188922
152		NakhonNayok, Thai	FMNH_180266

Sample No.	MtDNA No.	Region	Museum No.
153		NakhonNayok, Thai	FMNH_180252
156		NakhonNayok, Thai	FMNH_180271
158		Hainan, China	FMNH_6706
159		Hainan, China	FMNH_6704
160		Hainan, China	FMNH_6708
163		NakhonRatcha, Thai	FMNH_180249
164		Amoy, China	FMNH_8922
167		Darsai,Loei, Thai	FMNH_122227
170		Fukien,Fujian, China	FMNH_25192
171		Hong Kong	CAS_12919
176		Saigon, Vietnam	BMNH_1937.2.1.27
177		Sumatra?	BMNH_84.3.7.5
178		Taiwan	BMNH_66.6.8.69
181		Triangle?, Myanmar	BMNH_1940.6.5.85
186		Andaman	BMNH_1936.7.7.48
187		Nicobar	BMNH_1936.7.7.47
192		Sumprabum, Myanmar	BMNH_1974.907
193		ChangYong, Myanmar	BMNH_?
194		Kulu, India	BMNH_1937.3.1.15
195		Shimla, India	BMNH_1940.3.9.44
197		Darjeeling, India	BMNH_72.4.17.379
199		ChiangMai, Thai	CUB_1996.08.07.1
200		Lampang, Thai	JSCOLL_26
203		Hong Kong	CAS_12918
204		NamNaoNatPark, Thai	AFSPL_22
205	A184	NamNaoNatPark, Thai	AFSPL_21
209		Chonburi, Thai	CUB1996.08.07.2
212	A225	Lampang, Thai	AFS96.1
213		ChiangMai, Thai	COXCOLL_2
214		ChiangMai, Thai	COXCOLL_1
216		???	NMBA_12461
217		Wetar, E. Java	WAM_R117634
221		Wetar, E. Java	WAM_R117590
222		Wetar, E. Java	WAM_R117631
237	B21	Bangkok, Thai	AFS98.3
238	B22	Bangkok, Thai	AFS98.4
245	B31	Phu Laung, Loei, Thai	AFS98.13
246	B32	Phetburi, Thai	AFS98.14
251	B40	AngThong, Thai	AFS98.22
252	B41	AngThong, Thai	AFS98.23
253		AngThong, Thai	AFS98.24
257		Phetburi, Thai	AFS98.29
258		Phetburi, Thai	AFS98.30
259		Phetburi, Thai	AFS98.33
260		Phetburi, Thai	AFS98.35
261		Phetburi, Thai	AFS98.36
262		Phetburi, Thai	AFS98.38
263		Phetburi, Thai	AFS98.39
265		Nan, Thai	FMNH_186707
271		BinhThuan, Vietnam	AFS98.58
272	B77	BinhThuan, Vietnam	AFS98.59
277		Randau, Sumatra	SMF_37833
278		Randau, Sumatra	SMF_37834
279	B410	Moulmein, Myanmar	CAS_222595
285	B427	Sagaing, Myanmar	CAS_210287
288		Mandalay, Myanmar	CAS_214113
289	B431	Mandalay, Myanmar	CAS_208444
290	B429	Mandalay, Myanmar	CAS_204846
294	B169	GiaLai, Vietnam	FMNH_252070
298		Tamdao, Vietnam	ROM_30867
303		Yangon, Myanmar	UF_48828
304		Yangon, Myanmar	UF_48830
307		Yangon, Myanmar	UF_48833
308		Yangon, Myanmar	UF_48834
312		Guangxi, China	YBU_091064
321		WuhuAnhui, China	AMNH_23529
322		Hainan, China	AMNH_27922
323		Hainan, China	AMNH_27924

Sample No.	MtDNA No.	Region	Museum No.
324		Hainan, China	AMNH_27918
326		Hainan, China	AMNH_27916
327		Yunnan, China	CIB_13604
328		Guizhou, China	CIB_13608
332	A162	Tamdao, Vietnam	ROM_30872
333		Krong Pa, Vietnam	ROM_30872
335		Krong Pa, Vietnam	ROM_34546
344	A228	ChiangMai, Thai	AFS96.7
345	A145	ChiangMai, Thai	AFS96.6
348	B439	Vinh Thuan, Vietnam	FMNH_259116
350	B447	Xe Kong, Laos	FMNH_258951
351	B444	KiriromNP, Cambodia	FMNH 259189
352	B430	Mandalay, Myanmar	CAS_204847
353		Ganges Delta	BMNH_1946.1.19.99
356		Ladhak, India	BMNH_60.3.19.1330A
358		Chumukedima, Nagaland	BMNH_1940.3.9.25
359		Chumukedima, Nagaland	BMNH_1940.3.9.26
360		Moulmein, Myanmar	BMNH_1940.3.9.28
361		GWA,Rakhine, Myanmar	CAS_216575
362		GWA,Rakhine, Myanmar	CAS_216451
363		GWA,Rakhine, Myanmar	CAS_220254
366		GWA,Rakhine, Myanmar	CAS_216423
369		Hailakandi, Assam	BMNH_1937.3.1.13
370	B413	Yangon, Myanmar	CAS_213587
371		AyeyarwadyRegion, Myanmar	CAS_206604
372		AyeyarwadyRegion, Myanmar	CAS_219762
373		AyeyarwadyRegion, Myanmar	CAS_219783
377		Tamdao, Vietnam	ROM_30994

b) Females

Spec No.	MtDNA No.	Region	Museum No.
280	B409	GWA,Rakhine, Myanmar	CAS_221954
292		GWA,Rakhine, Myanmar	CAS_221997
364	B412	Yangon, Myanmar	CAS_213410
374	B418	AyeyarwadyRegion, Myanmar	CAS_212246
300		Yangon, Myanmar	CAS_8864
301		Yangon, Myanmar	RTV7
305		Yangon, Myanmar	UF_48831
306		Yangon, Myanmar	UF_48832
365		Pegu, Myanmar	BMNH_68.4.3.18
375		AyeyarwadyRegion, Myanmar	CAS_212245
291		Yangon, Myanmar	CAS_213412
274		Bali, Indonesia	SMF_78735
275		Bali, Indonesia	SMF_78734
276		Bali, Indonesia	SMF_73324
297	B445	Bolaven Plateau, Lao	FMNH_258947
293	B446	Bolaven Plateau, Lao	FMNH_258948
109		Borneo???	NMBA_18741
296		Khammouane, Laos	FMNH_255250
357		Taungoo, Myanmar	BMNH_93.1.16.14
143		Bangkok, Thailand	FMNH_179412
198		No Locality, Thailand	FMNH_180262
207		Chonburi, Thai	Thai Rdkill Coll 1
208		Chonburi, Thai	Thai Rdkill Coll 2
239	B23	Bangkok, Thai	AFS98.5
240		Bangkok, Thai	AFS98.6
248	B36	Ang Thong, Thai	AFS98.18
249		Ang Thong, Thai	AFS98.19
250		Ang Thong, Thai	AFS98.20
254		Ang Thong, Thai	AFS98.25
256		Ang Thong, Thai	AFS98.28
72		LangBiahPeah,Annam, Vietnam	FMNH_26453
113		QuangNam, Vietnam	USNM_163956
115		QuangNam, Vietnam	USNM_163959
116		QuangNam, Vietnam	USNM_163954

Spec No.	MtDNA No.	Region	Museum No.
334	B183	Krong Pa, Vietnam	ROM_34544
336		Krong Pa, Vietnam	ROM_34552
142		Kompot Chhuk, Cambodia	MHNG_11534
172		Battambang, Cambodia	CAS_111407
224		Ko Chang Isl, Thai	CAS_132864
12		NakhonRatcha, Thai	AMNH_14168
62		NakhonRatcha, Thai	FMNH_180245
63		NakhonRatcha, Thai	FMNH_180239
154		NakhonNayak,Thai	FMNH_180250
155		NakhonRatcha, Thai	FMNH_180253
157		NakhonRatcha, Thai	FMNH_180254
162		NakhonRatcha, Thai	FMNH_180270
180		DibrugarhAssam, India	BMNH_1908.6.23.99
302		Baramchal, Bangla	MCZ_58277
22		Sidoarjo, E. Java	ISJ3
23	A110	Lakasantri/Gresik, E. Java	IGK2
24	A109	Gresik, E.Java	IGK1
27	A112	NearGresik, E.Java	IGK4
25		Sidoarjo, E. Java	ISJ3
26		Sidoarjo, E. Java	ISJ1
28		Lakasantri/Gresik, E. Java	IGK3
29		GunungSari,Indonesia	IGS1
20		Flores, NTT	IM3
56		Flores, NTT	UF_30138
58		Flores, NTT	UF_30137
100		Flores, NTT	UF_30151
104		Flores, NTT	NMBA_10681
147		Hainan, China	FMNH_6711
148		Hainan, China	FMNH_6712
149		Hainan, China	FMNH_6709
315		Hainan, China	AFS08.11
316	B803	Hainan, China	AFS08.01
317	B804	Hainan, China	AFS08.02
318	B805	Hainan, China	AFS08.03
319	B807	Hainan, China	AFS08.06
320	B808	Hainan, China	AFS08.07
376		Hainan, China	SCUM_035055
1		Hong Kong	MCZ_173524
2		Hong Kong	MCZ_173522
3		Hong Kong	MCZ_173518
4		Hong Kong	MCZ_173523
5		Hong Kong	MCZ_173517
6		Hong Kong	MCZ_173520
7		Hong Kong	MCZ_173521
9		Hong Kong	MCZ_173519
31		Hong Kong	MCZ_173526
93		Hong Kong	MCZ_176551
94		Hong Kong	MCZ_176553
202		Hong Kong	CAS_8865
48		Komodo, NTT	UF_28697
49		Komodo, NTT	UF_28696
50		Komodo, NTT	UF_28692
51		Komodo, NTT	UF_28687
57		Komodo, NTT	UF_28699
60		Komodo, NTT	UF_28700
77		Komodo, NTT	UF_28688
78		Komodo, NTT	UF_28707
101		Komodo, NTT	UF_28695
269	B74	Xuyen Moc, Vietnam	AFS98.56
295	B440	AnMinh, Vietnam	FMNH_259117
341	B117	Ho Chi Minh, Vietnam	AFS99.25
343	B119	Ho Chi Minh, Vietnam	AFS99.27
349	B438	VinThuan, Vietnam	FMNH_259115
161		Da Lat, Vietnam	FMNH_71716
270		Xuyen Moc, Vietnam	AFS98.57
273		langBianPlateauVietnam	BMNH_1921.4.
337		Ho Chi Minh, Vietnam	AFS99.26
338		Ho Chi Minh, Vietnam	AFS99.30

Spec No.	MtDNA No.	Region	Museum No.
339		Ho Chi Minh, Vietnam	AFS99.24
340		Ho Chi Minh, Vietnam	AFS99.28
342		Ho Chi Minh, Vietnam	AFS99.29
87		Moulmein, Myanmar	NMW_23920:1
182		Moulmein, Myanmar	BMNH_66.7.10.3
355		Moulmein, Myanmar	BMNH_1940.3.9.27
368		Moulmein, Myanmar	BMNH_1938.8.7.62
66		ChiangMai, Thai	FMNH_179417
67		ChiangMai, Thai	FMNH_179416
111		ChiangMai, Thai	FMNH_179415
112		ChiangMai, Thai	FMNH_179413
210	A227	Lampang, Thai	AFS96.4
211	A226	Lampang, Thai	AFS96.2
215	A229	Phayao, Thai	AFS97B_1
330		Tamdao, Vietnam	ROM_30869
331	A104	Tamdao, Vietnam	ROM_30854
347		Tamdao, Vietnam	ROM_30870
13		Fukien,Fujian, China	AMNH_33774
14		Fukien,Fujian, China	AMNH_34286
15		Fukien,Fujian, China	AMNH_33768
16		Fukien,Fujian, China	AMNH_33767
165		Fujian, China	FMNH_25194
166		Fujian, China	FMNH_25193
173		Amoy, China	CAS_74580
346		JingdeAnhui, China	SNHM_729160
120		NamNaoNatPark, Thai	NAMNAOCOLL
168		ChongMek,Loei, Thai	FMNH_143158
206	A186	Phu Luang,Loei, Thai	AFSPL_23
40		Pokhra, Nepal	NMB_21011
124		Pokhra, Nepal	MHNG_1400.25
125		Pokhra, Nepal	MHNG_1400.30
126		Pokhra, Nepal	MHNG_1404.18
127		Pokhra, Nepal	MHNG_1404.13
128		Pokhra, Nepal	MHNG_1400.35
129		Pokhra, Nepal	MHNG_1404.16
130		Pokhra, Nepal	MHNG_1400.28
132		Pokhra, Nepal	MHNG_1400.39
174		Pokhra, Nepal	MHNG_1400.37
175		Pokhra, Nepal	MHNG_1400.38
223		No Locality, Nepal	NMP6V_34500
185		Car Nicobar	BMNH_1936.7.7.42
188		Car Nicobar	BMNH_1936.7.7.41
189		Car Nicobar	BMNH_1936.7.7.40
139		Tung Kun, China	MHNG_1464.88
141		Guangxi, China	MHNG_1400.69
309		Guizhou, China	CIB_13609
310		Guizhou, China	CIB_13610
313		Guangxi, China	YBU_091065
314		Guangxi, China	YBU_091067
17	A131	Trang, Thailand	AFS7
121		Krabi, Thai	PBCOLL_14
122		Krabi, Thai	PBCOLL_8
123		Krabi, Thai	PBCOLL_23
190		Trang, Thailand	BMNH_1988.788
191		Trang, Thailand	BMNH_1988.780
201		Trang, Thailand	BMNH_1988.787
236	B20	Thung Song, Thai	AFS98.2
329		Trang, Thailand	BMNH_1988.789
367		Sikkim, India	BMNH_1946.1.19.53
34		Sumba, NTT	NMB_14896
35		Sumba, NTT	NMB_14887
36		Sumba, NTT	NMB_14898
37		Sumba, NTT	NMW_14897
41		Sumba, NTT	NMB_14888
47		Sumba, NTT	NMBA_14885
105		Sumba, NTT	NMBA_14895
169		PhongSaly, Laos	FMNH_14429
184		Mogok, Myanmar	BMNH_1907.4.26.6

Spec No.	MtDNA No.	Region	Museum No.
281	B420	PyinOoLwin, Myanmar	CAS_216144
282	B426	Sagaing, Myanmar	CAS_210148
283	B424	Sagaing, Myanmar	CAS_210108
284		Mandalay, Myanmar	CAS_214110
286		Sagaing, Myanmar	CAS_210301
287	B425	Sagaing, Myanmar	CAS_210109
179		Triangle?, Myanmar	BMNH_1940.6.5.81
183		Triangle?, Myanmar	BMNH_1940.6.5.82
354		Ladakh, India	BMNH_60.3.19.1330B
42		Tasikmalaya, W.Java	NMB_9477
43		Tasikmalaya, W.Java	NMBA_9478
44		TenggerUsulMts,Java	NMBA_2595
61		Tasikmalaya, W.Java	NMW_23902:1
86		Tasikmalaya, W.Java	NMW_23902:4
89		Tasikmalaya, W.Java	NMW_23902:7
196		Tasikmalaya, W.Java	NMW_23902.6
266		Indramayu, W. Java	ZRC_2.2862
33		Amarasi, W. Timor	NMB_12771
39		Amarasi, W. Timor	NMB_12770
218		Wetar, E. Java	WAM_R117636
219		Wetar, E. Java	WAM_R117648
220		Wetar, E. Java	WAM_R117592

Table 2. Sample list used in mtDNA phylogeny

CAT NO	COUNTRY	REGION	STATE	MUSEUM NO.
A104	VIETNAM	NORTH		ROM 16752/30854
A107	THAILAND	SOUTH	NAKHON SI THAMMARAT	AFS97B.15
A126	INDONESIA	JAVA	WEST	
A127	THAILAND	SE	CHONBURI	
A129	THAILAND	SOUTH	TRANG	AFS6
A130	THAILAND	SOUTH	TRANG	AFS3
A131	THAILAND	SOUTH	TRANG	AFS7
A132	THAILAND	SOUTH	TRANG	AFS2
A133	THAILAND	SE	CHONBURI	
A134	THAILAND	SOUTH	NAKHON SI THAMMARAT	AFS4
A135	THAILAND	NE	KHONKAEN	
A136	THAILAND	SOUTH	RANONG	AFS8
A137	THAILAND	NE	SAKHON NAKHON	
A138	THAILAND	NE	SAKHON NAKHON	
A139	THAILAND	WEST	HUA HIN	
A141	THAILAND	NE	PETCHABUN	
A145	THAILAND	NORTH	CHIANG MAI	AFS96.6
A149	THAILAND	NORTH	CHIANG MAI	
A152	THAILAND	NE	PETCHABUN	
A153	THAILAND	NE	PETCHABUN	
A154	THAILAND	NE	PETCHABUN	
A157	CHINA	HONGKONG		
A162	VIETNAM	NORTH		ROM 16715/30872
A165	THAILAND	NE	LOEI	AFS99.2
A182	THAILAND	NE	LOEI	AFSPL19

CAT NO	COUNTRY	REGION	STATE	MUSEUM NO.
A184	THAILAND	NE	PETCHABUN	AFSPL21
A185	THAILAND	NE	LOEI	AFSPL22
A186	THAILAND	NE	LOEI	AFS99.23
A225	THAILAND	NORTH	LAMPANG	AFS96.1
A226	THAILAND	NORTH	LAMPANG	AFS96.2
A227	THAILAND	NORTH	LAMPANG	AFS96.4
A228	THAILAND	NORTH	CHIANG MAI	AFS96.7
A229	THAILAND	NORTH	PHA YAO	AFS97B.1
B6	INDONESIA	JAVA	WEST JAVA	
B20	THAILAND	SOUTH	NAKHON SI THAMMARAT	AFS98.2
B21	THAILAND	CENTRAL	BANGKOK	
B22	THAILAND	CENTRAL	NONTHABURI	AFS98.4
B23	THAILAND	CENTRAL	BANGKOK	AFS98.5
B31	THAILAND	NORTH-EAST	LOEI	AFS98.13
B32	THAILAND	WEST	PHETBURI	AFS98.14
B36	THAILAND	CENTRAL	ANG THONG	AFS98.18
B39	THAILAND	CENTRAL	ANG THONG	AFS98.21
B40	THAILAND	CENTRAL	ANG THONG	AFS98.22
B41	THAILAND	CENTRAL	ANG THONG	AFS98.23
B47	THAILAND	WEST	PHETBURI	AFS98.29
B49	THAILAND	WEST	PHETBURI	AFS98.31
B50	THAILAND	WEST	PHETBURI	AFS98.32
B55	THAILAND	WEST	PHETBURI	AFS98.37
B68	VIETNAM	SOUTH	MY THO	AFS98.50
B69	VIETNAM	SOUTH	MY THO	AFS98.51
B74	VIETNAM	SOUTH	VUNG TAU	AFS98.56
B77	VIETNAM	SOUTH	BINH TUAN = BINH THIEN?	AFS98.59
B78	VIETNAM	SOUTH	BINH TUAN = BINH THIEN?	AFS98.60
B98	THAILAND	NORTH-EAST	SA KAEW	AFS99.6
B102	THAILAND	NORTH-EAST	SA KAEW	AFS99.10
B117	VIETNAM	SOUTH	HCM CITY	AFS99.25
B119	VIETNAM	SOUTH	HCM CITY	AFS99.27
B164	LAOS	CENTRAL	BOLIKHAMXAY PROV	FMNH 255256
B169	VIETNAM	CENTRAL	GIA LAI	FMNH 252070
B183	VIETNAM	CENTRAL	GIA LAI	ROM 34544
B186	VIETNAM	NORTH		ROM 35300
B191	VIETNAM	NORTH		ROM 35299
B192	VIETNAM	NORTH		ROM 35302
B193	VIETNAM	NORTH		ROM 35304
B398	INDONESIA	JAVA	WEST	
B409	MYANMAR	RAKHINE STATE	GWA TOWNSHIP	CAS 221954
B410	MYANMAR	MON STATE	MAWLAMYINE DISTR, MUDON TOWNSHIP	CAS222595
B420	MYANMAR	MANDALAY DIV	PYIN OO LWIN DISTRICT	CAS 216144
B424	MYANMAR	SAGAING DIV	ALAUNDAW KATHAPA NATL PK	CAS 210108
B425	MYANMAR	SAGAING DIV	ALAUNDAW KATHAPA NATL PK	CAS 210109

CAT NO	COUNTRY	REGION	STATE	MUSEUM NO.
B426	MYANMAR	SAGAING DIV	ALAUNDAW KATHAPA NATL PK	CAS 210148
B427	MYANMAR	SAGAING DIV	ALAUNDAW KATHAPA NATL PK	CAS 210287
B429	MYANMAR	MANDALAY DIV	96 KM S OF MANDALAY ON YANGON RD; 21 19 47 N, 96 4 23 E	CAS 204846
B430	MYANMAR	MANDALAY DIV	96 KM S OF MANDALAY ON YANGON RD; 21 19 47 N, 96 4 23 E	CAS 204847
B431	MYANMAR	MANDALAY DIV	KYAUK SE TOWNSHIP	CAS 208444
B438	VIETNAM	SOUTH	KIEN GIANG	FMNH 259115
B439	VIETNAM	SOUTH	KIEN GIANG	FMNH 259116
B440	VIETNAM	SOUTH	KIEN GIANG	FMNH 259117
B441	CAMBODIA	EAST	MONDOLKINI	FMNH 259190
B444	CAMBODIA	WEST	KAMPONG SPEU	FMNH 259189
B445	LAOS	SOUTH	CHAMPASSAK PROV	FMNH 258947
B446	LAOS	SOUTH	CHAMPASSAK PROV	FMNH 258948
B447	LAOS	SOUTH	XE KONG	FMNH 258951
B472	THAILAND	SOUTH	SURAT THANI	AFS02.10, AFS06.32
B708	VIETNAM			RH060125
B755	VIETNAM	nORTH	VINH PHU	MVZ 226618
B756	VIETNAM	nORTH	VINH PHU	MVZ 226619
B803	CHINA	SOUTH	HAINAN	AFS08.02
B804	CHINA	SOUTH	HAINAN	AFS08.03
B805	CHINA	SOUTH	HAINAN	AFS08.04
B807	CHINA	SOUTH	HAINAN	AFS08.06
B808	CHINA	SOUTH	HAINAN	AFS08.07