A Taxonomic Study of *Rhinolophus affinis* Horsfield, 1823 (Rhinolophidae: Chiroptera) in Oriental Region

Saveng Ith

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

Prince of Songkla University

2014

Thesis Title	A Taxonomic Study of *Rhinolophus affinis* Horsfield, 1823 (Rhinolophidae: Chiroptera) in Oriental Region
Author	Mr. Saveng Ith
Major Program	Biology

Major Advisor

…….....
(Assist. Prof. Dr. Sara Bumrungsri)

Co-advisor

…….....
(Dr. Nikky M Thomas)

…….....
(Dr. Neil M Furey)

…….....
(Dr. Monwadee Wonglapsuwan)

Examining Committee

…….....
(Assist. Prof. Dr. Supiyanit Maiphae)

…….....
(Assist. Prof. Dr. Sara Bumrungsri)

…….....
(Dr. Singtoe Boonrotpong)

…….....
(Assist. Prof. Dr. Nantasak Pinkaew)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Biology.

….……..
(Assoc. Prof. Dr. Teerapol Srichana)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……

(Assist. Prof. Dr. Sara Bumrungsri)

Major Advisor

……

(Mr. Saveng Ith)

Candidate

I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

……

(Mr. Saveng Ith)

Candidate

Thesis Title	A Taxonomic Study of *Rhinolophus affinis* Horsfield, 1823 (Rhinolophidae: Chiroptera) in Oriental Region
Author	Mr. Saveng Ith
Major Program	Biology
Academic Year	2014

ABSTRACT

Rhinolophus affinis sensu lato, Horsfield has a broad distribution in the Indomalayan zoogeographical region, extending from northern India, Nepal, Myanmar, southern China, Vietnam, Thailand and Cambodia to Malaysia and Indonesia. The current taxonomic study is limited to mainland Southeast Asia, Borneo and Sumatra where four subspecies namely *R. a. tener*, *R. a. macrurus*, *R. a. superans* and *R. a. nesites* are recognized. The study is based on morphological, genetic and acoustic data. In result, three subspecies namely *R. a. macrurus*, *R. a. superans* and *R. a. nesites* were confirmed while *R. a. tener* was not encountered in the study. Two outlying forms were proposed, an east Myanmar (Shan state)/lower north Vietnam (Nghe An Province) form and a south Sumatra form (Lampung). Variations within subspecies were also observed; in *R. a. macrurus*, such as a central/south Vietnam form (supported by morphology, echolocation and genetic), north Cambodia form (supported by morphology and genetic); in *R. a. superans*, such as the deviation along the Kangar Pattani Line (supported by morphology and echolocation).

The divergence between subspecies was congruent with the geographical demarcation proposed in the literature. The distribution range of the two continental forms (*R. a. marcrurus* and *R. a. superans*) was limited at the join between the mainland and the peninsula. The two morphological forms of *R. a. superans* (also supported by echolocation) were divided by the Kangar Pattani Line. *R. a. nesites* and *R.* cf. *affinis* (south Sumatra) were divided from the continent by the South China Sea.

The diversification of the subspecies was driven by the effects of Pleistocene climatic cycles which directly shaped the habitats of the animals through time. The divergence of the insular populations was relatively deeper than between the continental forms.

Discovering additional phylogroups of *R. affinis* are definitely likely throughout its distribution range as multiple ancient refugia were recognized yet studies have not been conducted for hypothesis testing for the species.

Contents

Contents (continued)

Contents (continued)

Contents (continued)

List of Tables

List of Tables (continued)

List of Figures

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction to taxonomy

Wherever we look in nature, we find uniqueness of organisms and uniqueness means diversity. No two individuals in sexually reproductive populations are the same, nor are any two populations, species, or higher taxa. The task of taxonomy is to determine the nature of this diversity. There is no general agreement as to the definitions of systematics and of related words, such as taxonomy, biological system, and classification (Minelli, 1993; Winston, 1999). The term systematics stems from the Latinized Greek word "sistema" which was developed by the early naturalists, the most well-known of which is Linnaeus. Simpson (1961) defined "systematics" as the scientific study of the kinds and diversity of organisms and of any and all relationships among them. "Taxonomy" is derived from the Greek words *taxis* meaning arrangement and *nomos* meaning law. It was first proposed in French form by De Candolle for the theory of plant classification (Mayr & Ashlock, 1991). It refers to the subset of systematics consisting of three associated activities, including identification (referring a specimen to a previously classified and named group), classification (ordering organisms into group based on perceived similarities or differences), and nomenclature (naming groups of organisms according to rules developed for the process) (Winston, 1999), and as such, taxonomy is the theory and practice of classifying organisms (Mayr & Ashlock, 1991).

One of the major tasks of systematics is to determine by means of comparison what the unique properties of each species and higher taxon are. Another is to determine what properties certain taxa have in common and what the biological causes of the differences or shared characters are. Finally, systematics is concerned with variation within taxa. Systematics is one of the major subdivisions of biology, serving not only for the identification and classification of specimens in collections but also the comparative study of all type of organisms as well as understanding the place of taxa in nature and evolutionary history. Taxonomy is the synthesis of knowledge, theories and methods applied to all aspects of classification; therefore systematics is not for only describing the diversity of the living world but to

contribute to its understanding (Mayr & Ashlock, 1991). Modern taxonomists are naturalists who study various branches of biology including ecology, animal behaviour, genetics, molecular biology, laboratory techniques and so forth. They provide most of the information needed for the phylogenetic reconstruction of life, reveal evolutionary phenomena of organisms and provide background knowledge available for other branches of biology such as evolutionary, biochemistry, immunology, ecology, genetics, ethology and biogeography.

Due to its breadth, taxonomy is a complex mixture of biology, philosophy and mathematics (Quicke, 1993). Taxonomy is an ever-changing, controversial and exciting field of biology. Early advances were made by Aristotle and Linnaeus, and subsequently major advances have been made, particularly in the last two or three decades (Minelli, 1993). The discipline continues to advance in leaps and bounds, both in procedure and in philosophy (Quicke, 1993). This is largely due to the technical progress in molecular biology and computer techniques. The use of molecular techniques has opened a new dimension to the comparative study of living organisms, whereas computers have allowed the development of powerful numerical techniques and their use for straight forwards handling of large data matrices of descriptive data (Minelli, 1993). Minelli (1993) reviewed modern systematic methods and suggested that with the development of cladistics, computers and new molecular techniques, the study of biological systematics is advancing rapidly. Taxonomists are now able to gain more data from living animals for taxonomic research such as behaviour, vocalisations, ecological requirements, physiology and biochemistry and even laboratory techniques (Mayr & Ashlock, 1991; Thomas, 1997) which further strengthens the study of population systematics and evolutionary biology (Mayr & Provine, 1980).

1.2. Order Chiroptera

Chiroptera, in Greek means ‘Hand Wing’, the term for classifying bats, the only true flying mammals on earth. This Order is the second most diverse after rodents, comprises of 18 families, 202 genera and > 1,116 species worldwide (Simmon, 2005).

Bats are very diverse in body mass (2-1000g), being very small in insectivorous bats and largest in fruit bats (Neuweiller, 2000). Different species groups evolve to consume different food sources such as nectar, pollen, flowers, fruit, fish, blood and insects.

Bats are broadly distributed throughout most of the world, in tropical and temperate habitats. They are absent only from Polar Regions and from some remote islands (Altringham, 1996; Kunz, 2003; Mickleburgh, 2002). Although bats are moderately common in temperate regions, their greatest diversity is in tropical and subtropical regions (Corbet & Hill, 1992). According to the IUCN Red List, there are 10 families, 72 genera, and 326 specie in the countries of South and Southeast Asia (Mickleburgh, 2002). Among the countries Indonesia has the most species of all, 175 species (Hutson, 2001).

1.2.1. Systematics

Bat systematics is complex, especially when considering their relationships in higher-level classification. It is partly due to the difficulty in identifying fossil records and the uncertainty of chronology of divergence events and its biogeography (Czaplewski *et al.*, 2008). Genetic data is used widelty in classification studies, such as work by Teeling *et al.*, (2005), however it is still almost impossible to obtain DNA sequences for fossil taxa (Springer *et al.*, 2001). In higher-level phylogeny bats were previously placed in the Superorder Archonta (Novacek, 1992) based on morphological data which also comprises of others orders including Primates (primates), Scandentia (tree shrews) and Dermoptera (flying lemurs). However, based on molecular data, bats were instead placed in the Superorder Laurasiatheria which also comprises of Orders Carnivora (carnivores), Cetartiodactyla (certartiodactyls), Eulipotyphla (eulipotyphlan), Perissodactyla (perissodactyls) and Pholidota (pangolins) (Madsen *et al.*, 2001; Murphy *et al.*, 2001; Waddell *et al.*, 1999).

The recent morphological study supported the monophyly of the Order Chiroptera, and subdivided this order into two monophyly suborders, Megachiroptera (megabats) and Microchiroptera (microbats) (table 1.1) (Simmons & Geisler, 1998) therefore suggesting a single origin of laryngeal echolocation and flight in bats.

Molecular data also supported the monophyly and single origin of this order, however rejected the microbat monophyly, implying microbats are paraphyletic (Springer *et al.*, 2001; Teeling *et al.*, 2002) (table 1.1, fig. 1.1). The Superfamily Rhinolophoidae previously comprised of families Nycteridae, Megadermatidae and Rhinolophidae are now known as polyphyletic (based on molecular data) (Teeling *et al.*, 2002) and were placed in the suborder Yinpterochiroptera together with rhinopomatids (Craseonycteridae and Rhinopomatidae) and megabats (Pteropodidae) while the family Nycteridae were placed in the suborder Yangochiroptera together with vespertilionoids, noctilionoids and emballonuroids. The classification comparing both morphology and genetics is summarized in table 1.1.

Table 1.1. The summary of traditional classification based on morphology (Simmons & Geisler, 1998) and modern classification based on genetics (Springer *et al.*, 2001; Teeling *et al.*, 2005).

Classification based on morphology	Classification based on genetics
Order Chiroptera	Order Chiroptera
Suborder Megachiroptera	Suborder Yinpterochiroptera
Family Pteropodidae	Superfamily Pteropodoidea
Suborder Microchiroptera	Family Pteropodidae
Superfamily Emballonuridea	Superfamily Rhinolophoidea
Family Emballonuridae	Family Rhinolophidae
Infraorder Yinochiroptera	Family Hipposideridae
Superfamily Rhinopomatoidea	Family Megadermatidae
Family Rhinopomatidae	Family Craseonycteridae
Superfamily Rhinolophoidea	Family Rhinopomatidae
Family Nycteridae	Suborder Yangochiroptera
Family Megadermatidae	Superfamily Emballonuroidea
Family Rhinolophidae	Family Emballonuridae
Subfamily Hipposiderinae	Family Nycteridae
Subfamily Rhinolophinae	Superfamily Noctilionoidea
Infraorder Yangochiroptera	Family Phyllostomidae
Superfamily Noctilionoidea	Family Mormoopidae
Family Noctilionidae	Family Noctilionidae
Family Phyllostomidae	Family Furipteridae
Superfamily Nataloidea	Family Thyropteridae
Family Natalidae	Family Mystacinidae
Superfamily Molossoidea	Family Myzopodidae
Family Antrozoidae	Superfamily Vespertilionoidea
Family Molossidae	Family Vespertilionidae
Superfamily Vespertilionoidea	Family molossidae
Family Vespertilionidae	Family Natalidae
	Family Miniopteridae

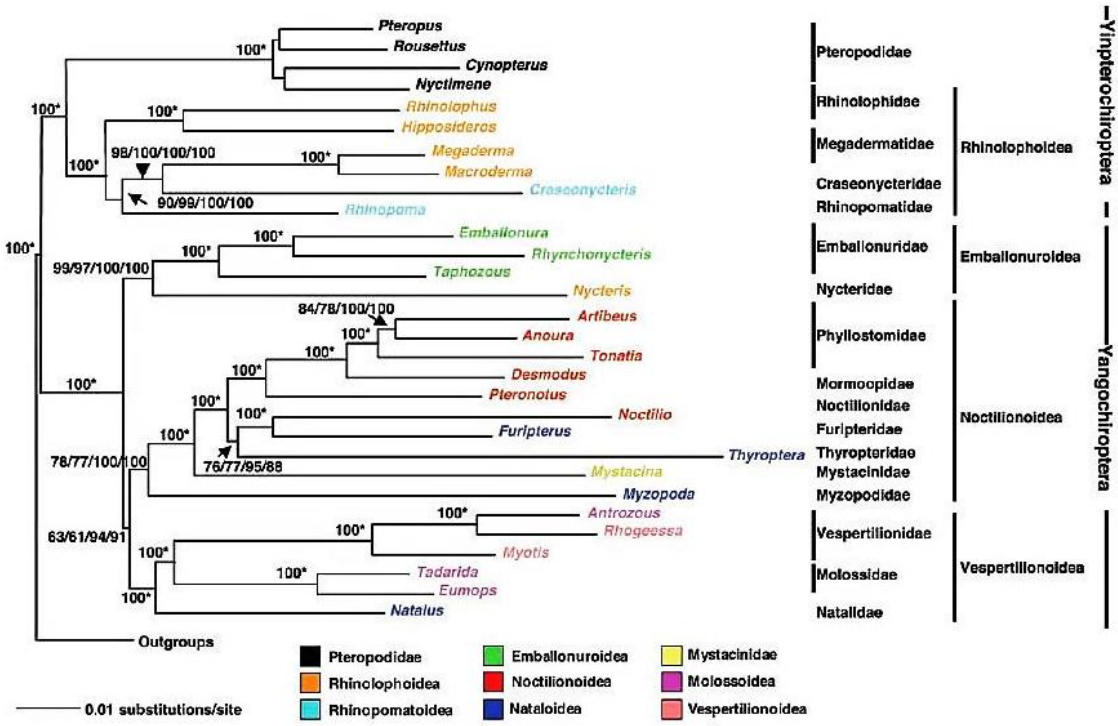

Figure 1.1. The classification of bats based on genetic data (in Teeling *et al.*, 2005).

1.3. Family Rhinolophidae

Rhinolophidae Gray is a family of horseshoe bat consisting of a single genus *Rhinolophus* Lacépède, (Bogdanowicz & Owen, 1992; Corbet & Hill, 1992; Csorba *et al.*, 2003; Simmon, 2005; Thomas, 1997). The number of species included in the genus *Rhinolophus* has increased in recent years, from 64 species in Koopman (1993) to 71 species in Csorba *et al.* (2003) and 77 species currently in Simmons (2005). In addition, Soisook *et al*. (2008) elevated *R. microglobusus* to be the full species and recently Wu & Thong (2011) described a new species, *R. schnitzleri* from China and Taylor *et al.* (2012) described four new species from Africa, *R. cohenae, R. mabuensis, R. smithersi and R. mossambicus*. In total, there are at least 83 bat species in this family.

Previously, additional genera were proposed due to the degree of morphological variation observed between different species, e. g. *Phyllorhina* (Leach, 1816) for *Rhinolophus hipposideros minutus*; *Aquias* (Gray, 1847, 1866) for *R.*

trifoliatus and *R. luctus* and *Phyllotis* for *R. philippinensis*; *Coelophyllus* (Peters, 1867) for *R. coelophyllus*. Dobson (1876) ignored all the previously proposed genera and proposed a single genus *Rhinolophus* and also separated rhinolophid and hipposiderid bats into two different subfamilies Rhinolophinae and Phyllorhininae as previously *Rhinolophus* also included *Hipposiderid* bats. The two subfamilies were elevated to family rank later by Miller (1907). Later, other genera were also proposed including *Rhinophyllotis* (Iredale & Troughton, 1834) for *R. megaphyllus* and *Rhinomegalophus* (Bourret, 1951) for *R. paradoxolophus*. However the additional genera were dropped by subsequent authors and *Rhinolophus* was kept as the only genus for the family Rhinolophidae.

All bats in this family are characterized by having a noseleaf consisting of an erect posterior lancet, a lower horizontal horseshoe-shaped expansion surrounding the nostrils, a perpendicular median sella and connecting process. The ears are moderate to large and lack a tragus. The tail is well developed and is completely enclosed in the uropatagium. There are two additional teat-like processes on the abdominal region of adult females beside the two functional mammae on the chest. This family is also characterized by having highly specialized auditory systems which allow bats to use echolocation calls for communication and for navigation and the detection of prey. In general, echolocation calls are characterized by a strong constant frequency (CF) component with a short beginning or terminal frequency-modulated (FM) component.

The bats of this family are distributed in tropical and temperate portions the Old World, extending from Western Europe and Africa to Japan, South-east Asia, New Guinea, the Bismack Archipelago and Australia (Bogdanowicz & Owen, 1992; Corbet & Hill, 1992; Csorba *et al.*, 2003).

The first comprehensive review of the family was undertaken in a series of papers by Andersen (1905a, 1905b, 1905c, 1905d, 1905e, 1918); he was the first to construct a phylogenetic tree for the family. He also described new species and advanced the first phylogenetic hypotheses on evolution and biogeography of the group. In his systematic arrangement, he employed some characters including the size and degree of displacement from the toothrow of minor teeth, size and shape of noseleaves and ears, length of palate, and relative length of finger bones of the wing.

Csorba *et al.* (2003) grouped all the species within the genus into 15 groups and a category as follows: *adami*, *capensis*, *euryale*, *euryotis*, *ferrumequinum*, *fumigatus*, *hipposidero*, *landeri*, *maclaudi*, *megaphyllus*, *pearsoni*, *philippinensis*, *pusillus*, *rouxi*, and *trifoliatus*, and a category *incertae sedis*.

1.3.1. *Rhinolophus affinis* Horsfield, 1823

Common name: Intermediate Horseshoe Bat.

R. affinis affinis, Horsfield 1823: Indonesia, Java.

R. a. andamanensis Dobson, 1872: South Andaman Islands, India.

R. a. himalayanus Andersen, 1905a: Mussoories, Kumaon, north-west India.

R. a. tener Andersen, 1905a: Pegu, Myanmar.

R. a. macrurus Andersen, 1905a: Taho, Karenee, Myanmar.

R. a. superans Andersen, 1905a: Pahang, Malaysia.

R. a. nesites Andersen, 1905a: Bunguran Island, north Natuna Islands, Indonesia.

R. a. princeps Andersen, 1905a: Lombok, Lesser Sunda Islands, Indonesia.

R. a. hainanus Allen, 1906: Pouten, Hainan Island, China.

In total, there are 9 subspecies of *Rhinolophus affinis* sensus lato. This species has a wide distribution range, extending from northern India (including Andaman Islands), Nepal to southern China, mainland Southeast Asia, Borneo, Java and East Java (Francis, 2008; Simmon, 2005) (figure 1.2) and exhibits considerable geographical variation in terms of morphometrics and acoustics (Andersen, 1905a; Csorba *et al.*, 2003; Kingsada *et al.*, 2011)

External characters: *R. affinis* sensus lato is a medium to fairly large bat (Csorba *et al.*, 2003); the forearm is small, 45.0-55.0 mm; the ears are small, 18.4-25.8 mm and do not reach the tip of the nose when laid forward. The horseshoe is relatively broad but does not cover the whole muzzle; its width is 7.5-11.2 mm. The sella is pandurate and its lateral margins vary from slightly concave to almost parallel sided. The connecting process is variously rounded and sparsely haired; the lancet is always high, straight sided and pointed to varying degrees. The lower lip has three mental grooves. The tail is 17.5-30.7 mm in length. The third metacarpal measures (3MT) 35.5-43.7 mm and the fourth metacarpal (4MT) is 36.4-44.3 mm, shorter than

or subequal to the fifth metacarpal (5MT) which is 36.6-44.8 mm in length. In the pelage, the upper side of the body is darker or lighter brown, sometimes golden yellow or light yellow brown while the belly ranges from brown to cream-buff (Csorba *et al.*, 2003).

Cranial characters: The skull is robust, with a fairly long rostrum. The mastoid width exceeds zygomatic width. The anterior median swellings are less inflated and semicircular in outline while the posterior swellings are well defined (Csorba *et al.*, 2003). The sagittal crest is medium or strong, extending posteriorly almost to the lambda. The frontal depression is moderately developed; the supraorbital ridges are quite prominent. The palatal bridge (PB) is short (1.63-2.67 mm) and measures 23-29% of upper toothrow length (8.20-9.38 mm) (Csorba *et al.*, 2003).

Dental characters: The upper canine is usually massive, and not in contact with the posterior upper premolar (P^4). The anterior upper premolar (P^2) is small or medium and in the toothrow or slightly displaced. The second lower premolar (P_3) is small or very small and usually extruded from the toothrow, sometimes partly out or rarely in the toothrow. The first (P_2) and the third (P_4) lower premolars are always in contact or nearly so (Andersen, 1905a; Csorba *et al.*, 2003).

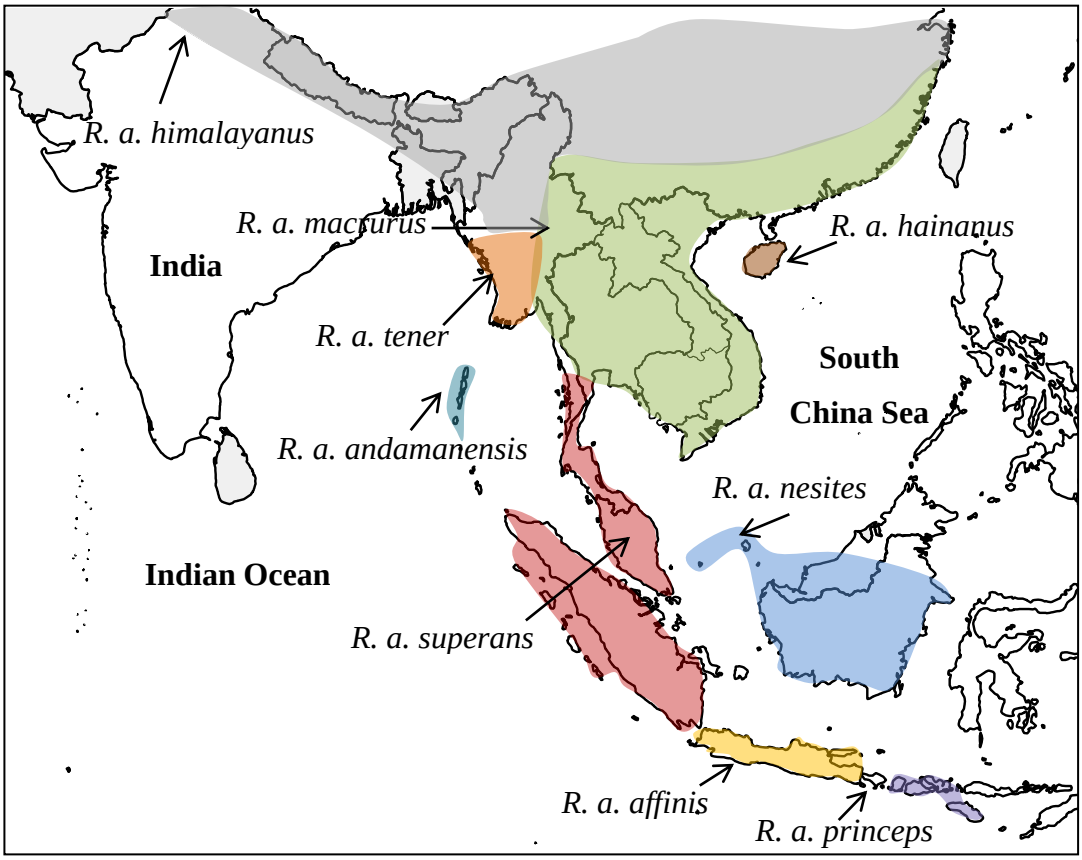

Figure 1.2. Distribution range of *R. affinis* sensus lato. The arrows indicate the type localities of subspecies whereas colored boundaries represent the approximate distribution range of the subspecies.

Figure 1.3. Noseleaf structure of *Rhinolophus affinis* from Chumphorn Pro., Thailand; (a): frontal view, (b): lateral view of the noseleaf and (c): structure of the sella.

Baculum: According to Thomas (1997), the shaft of the baculum is long, parallel-sided and essentially straight, thickening towards the base. In lateral view, the shaft curves ventrally; the base is expanded and angled ventrally in lateral profile. The tip is simple and unexpanded. The length (GLB) is 2.1 mm and width (GWB) is 0.8 mm.

Echolocation call: The call comprises of an initial upward FM component, followed by a CF component, and ends with a downward FM component. The peak call frequency (FMAXE) of *R. affinis* sensus lato shows a considerable degree of variation, ranging from 62.3 kHz to 88.5 kHz between the extreme.

1.3.1.1. *Rhinolophus affinis affinis* Horsfield, 1823

The specimen (type form) was collected from Java, Indonesia and deposited in British Museum. However, the original description of the species indicated no type specimen (Horsfied, 1823). Three specimens were assigned as the syntypes, one is in British Museum (labelled as holotype) and other two (only skulls) are in the National Museum of Natural History, Leiden (RMNH 25236 and 25237). The two specimens in Leiden were proved to be *Hipposideros larvatus* by their skull morphology (Csorba *et al.*, 2003). The specimen of typical form deposited in British Museum is poor condition, very old skin and a fragment of the skull, representing the facial portion and the toothrows which unable to derive the definite diagnosis from the specimens, but sufficient to show the sella shape (panduate character), second phalanx of the third metacarpal, dentition and so on (Andersen, 1905a). Recently, Csorba *et al.*, (2003) designated a specimen (BMNH79.11.21.70) from Java as a lectotype to the British Museum, UK.

1.3.1.2. *Rhinolophus affinis andamanensis* Dobson, 1872

The subspecies was originally described by Dobson (1872), and collected by Mr. Homfray from South Andaman Island (Andersen, 1905a; Sinha, 1973). Holotype (specimen code: 15561) is deposited in Zoological Survey of India, Calcutta, India (Thomas, 1997). This form resembles to *R. affinis* species, the horseshoe is very broad and flat, concealing the muzzle in dorsal view (as in *R. yunanensis*). The lancet is long and produced backwards between the ears (Dobson, 1872). Dobson (1872) also noted that the wings began from the ankle or slightly up the tibia, and described the interfemoral membrane as square, with the tip of the tail projecting. Some external measurements were included such as head and body, tail, ears, forearm, thumb and tibia (Dobson, 1872).

1.3.1.3. *Rhinolophus affinis himalayanus* Andersen, 1905

Holotype held in British Museum (Natural History), London, UK (BMNH.79.11.21.148). It was firstly described by Andersen (1905a), the type locality

is Mussoorie, Kumaon Division, northern India. Andersen (1905a) described this form as the largest size form with small ears (17.2-18.5 mm), narrow horseshoe (8.0-8.0 mm), short tail (21.8-25.0 mm) and tibia (22.8-23.8 mm). He also pointed out that, the form has moderate skull length (22.7-23.9 mm), braincase width (9.2-10 mm), and toothrows-upper toothrow (9.0-9.4 mm), lower toothrow (9.7-10.2 mm). Anderson (1881) recorded the species occurred in Ceylon (Sri Lanka) but the locality was excluded by Ellerman and Morrison-Scott (1951). However, due to a specimen of *R. himalayanus* form in his collection, Sinha (1973) confirmed the present of the species occurred in the region. Ellerman and Morrison-Scott (1951) reported the species ranged from Kumaon (northern India), Nepal, Bhutan Duars (northern of India), Darjeeling (southern of Bhutan), eastwards to Myanmar (Burma) and China (Hunan, Szechwan, and Yunnan). Kurup (1968) recorded the species from Meghalaya State (north-eastern India) and Bangladesh (Sylhet).

1.3.1.4. *Rhinolophus affinis tener* Andersen, 1905

An adult male of type specimens is deposited in British Museum (Natural History), London, UK (BMNH.87.3.4.11). The specimen was collected by W. Theobald from Pegu (recently known as Bago region), Myanmar and described by Andersen (1905a). Andersen (1905a) described the species as a small size form, with small ears (18.8 mm), short tail (23 mm) but fairly long tibia (24mm) and broad horseshoe (9.5 mm). The skull was shorter than other subspecies of *R. affinis* examined, with narrow nasal swellings (5.7 mm) and braincase (9 mm), and short toothrows-upper toothrow (8.7 mm) and lower toothrow (9.2 mm). Only a single female holotype was available for the description (Sinha, 1973).

1.3.1.5. *Rhinolophus affinis macrurus* Andersen, 1905

The subspecies was described by Andersen (1905a) from Taho, Karenni, spelled as “Karenee” in other publications (recently known as Kayah State), Myanmar (formely Burma). The holotype of male adult held in British Museum (Natural History), London, UK (BMNH.90.4.4.7). The species is moderate in size,

larger ears (20.0-20.7 mm), broader horseshoe (9.0-9.8 mm), long tail (26.0-29.3) but shorter tibia (23.9-25.4 mm). And moderated in skull length (22.5-23.2 mm), brain case width (9.3-9.8 mm), toothrows-upper toothrow (8.8-9.2 mm), lower toothrow (9.6-9.9 mm) and nasal swellings (5.8-6.2 mm) (Andersen, 1905a).

1.3.1.6. *Rhinolophus affinis superans* Andersen, 1905

Female adult holotype held in British Museum (Natural History), London, UK (BMNH.0.7.3.3). This subspecies was described by Andersen (1905a) from Pahang, Malaysia. It was morphologically similar to *R. a. macrurus* but distinguished by its shorter tail (21.5-25.2 mm), longer skull (22.8-23.8mm), broader nasal swellings (6.2-6.7 mm), brain case (9.8-10.2 mm) and toothrows-upper toothrow (9.0-9.7 mm), lower toothrow (9.7-10.1 mm) (Andersen, 1905a). This subspecies recorded to distribute in lower Siam (Trong-probably Trang Province, peninsula Thailand) peninsula Malaysia (Pahang) and Sumatra (Andersen, 1905a).

1.3.1.7. *Rhinolophus affinis nesites* Andersen, 1905

This subspecies was described by Andersen (1905a) from Bunguran Island, North Natunas, Indonesia. The adult female holotype (AMNH.104753) is deposited in American Museum of Natural History, New York, USA. The species described as being close related to *R. a. superans* but differ by its smaller size and shorter tibia (22.8 mm). Skull morphology was not available for description due to its bad damage (Andersen, 1905a).

Andersen (1905a) gave remark that, *R. a. nesites* was an offshoot of the Malacca forms *R. a. superans*, isolated by the outlying north Natunas. *R. a. nesites* shows some shared characters to *R. a. superans* such as large ears (20.2 mm), broad horseshoe (9.8 mm), short tail (22.0 mm) and short tibia.

1.3.1.8. *Rhinolophus affinis princeps* Andersen, 1905

This subspecies was initially described by Andersen (1905a) and the type locality is Lombok, Lesser Sunda Islands, Indonesia. Male adult holotype held in

British Museum (Natural History), London, UK (BMNH.97.4.18.13). Andersen (1905a) described this form as being moderate in general size with short tail (21.0 mm), but broadest in horseshoe (11.1 mm) and ears (21.3 mm); and longest in tibia (26.0 mm), skull (24.1 mm), nasal swellings (6.8 mm), upper toothrow (9.9 mm) and lower toothrow (10.5 mm).

1.3.1.9. *Rhinolophus affinis hainanus* Allen, 1906

Adult female holotype was collected from Pouten, Island of Hainan, China and deposited in American Museum of Natural History (AMNH.26748). The type was initially described by Allen (1906) as being large, broad pointed ears with large antitragus; noseleaf was described as "rather small, pointed", sella is "nearly quadrate, about twice as high as broad, the basal anterior extension forming an oval cut." And the color phases observed varied between individuals.

External measurement, head and body of type specimens (dry skin) 55 mm; tail, 16 mm; ear, 18 mm; forearm, 50 mm; third metacarpal, 35 mm; fifth metacarpal, 37 mm; tibia, 22.3 mm. Craniodental measurements, skull length, 22 mm; zygomatic width, 10.5 mm; mastoid width, 10 mm; width of nasal protuberance (nasal swellings), 5.5 mm; palatal bridge width, 4 mm; upper toothrow, 9 mm; lower toothrow, 9.5 mm and mandible length 15.5 mm (Allen, 1906).

In brief, Andersen (1905a) described and proposed the subspecific forms *R. a. himalayanus* from Kumaon, north-western India; *R. a. macrurus* from Karennee, south-eastern Myanmar; *R. a. tener* from Pegu, Myanmar; *R. a. superans* from Pahang, Malaysia; *R. a. nesites* from Natuna Island, Indonesia; and *R. a. princeps* from Lombok, Indonesia. Dobson (1872) described and proposed a subspecies of *R. a. andamanenis* from southern Andaman Island, India. Allen (1906) proposed a sub form *R. a. hainanus* from Pouten, Island of Hainan, China. Review work was followed by Tate and Archbold (1939), they kept all the subspecies proposed by Andersen and erected *R. andamanensis* as a distinct species (Thomas, 1997). In their checklist, Ellerman & Morrison-Scott (1951) agreed with Tate and Archbold (1939) to the status of *R. andamanensis* but noted that, it was similar to *R. affinis* and "may be a representation of it" (Thomas, 1997). Sinha (1973) judged *R. andamanensis* as

back to the subspecies level as it resembled *R. a. superans* in all characters except the length of ears, toothrows and mandible (Thomas, 1997). Corbet & Hill (1992) listed *R. andamanensis* and *R. hainanus* as synonym of *R. affinis* but did not give taxonomical description. Kooman (1993), Simmons (2005) recognized *R. andamanensis, R. hainanus, R. himalayanus, R. macrurus, R. nesites, R. princeps, R. superans* and *R. tener* as being synonyms of *R. affinis* but with no explanation. Thomas (1997), based on phenetic and molecular analysis, proved the four subspecific forms proposed by Andersen (1905a) and Allen (1938). The four forms were *R. a. affinis*, Java; *R. a. himalayanus*, northern India; *R. a. tener*, from Myanmar to Malaysia and *R. a. hainanus*, China; other forms were not able to be examined due to a lack of material.

Andersen (1905a) proposed the morphological transition rule for the subspecies of *R. affinis* but gave the exception to the type species, *R. affinis* (from Java) and *R. a. nesites* (north Natuna Islands). The rule is, "the more southern or south-eastern the habitat, the longer ears, the broader horseshoe, the longer tibia, the larger skull, the broader nasal swellings, and the longer toothrows." Csorba *et al.* (2003) based on the mean value of skulls and toothrows of *R. a. superans* proved the agreement to the rule proposed by Andersen (1905a); but considered that the rule does not suitable for the population in Sunda Islands and Kangean Islands (Kepulauan Kangean), Indonesia. They found that, the population from those habitats in average is smaller than other subspecies recognized by Andersen. Thomas (1997) suggested that Kangean Islands population may represent a distinct subspecies, a view firstly proposed by Bergmans & Van Bree (1986).

Lekagul & McNeely (1977) recognized two subspecific forms *R. a. macrurus*, recognized by longer tail and larger ears and *R. a. superans* recognized by shorter tail, occurring in Thailand. *R. a. macrurus* distributed in Chiang Mai, Mae Sariang and Mae Hong Son while *R. a. superans* distributed in the south. This proposal seems congruent to the work of Kingsada *et al.* (2011), they provisionally referred the specimens from north peninsula Thailand, Cambodia and Vietnam to *R. a. macrorus*, originally known from eastern Myanmar and the average larger form from peninsula Thailand to *R. a. superans* which as described from Pahang, Malaysia. However, Thomas (1997) proposed only *R. a. tener* ranging from Myanmar to Malaysia.

Kingsada *et al.* (2011) due to numerous data of *R. affinis* from Cambodia, Loa PDR, Myanmar, Thailand and Vietnam proposed further detail study on intraspecific variation of this species which claimed that the taxonomic story of this taxa is not well understood to taxonomists (Andersen, 1907b; Bergmans & Van Bree, 1986; Csorba *et al.*, 2003; Kingsada *et al.*, 2011).

1.3.2. Taxonomic Status of *R. affinis* sensu lato

Due to its considerable range, *R. affinis* sensus lato shows many geographical forms throughout its distribution range and classification of each subspecies still remains unsatisfactory, owing primarily to poor sample sizes and studies to date being based mainly on classical systematic (morphology), particularly comparing the characters, e.g. ear, noseleaf, tibia, tail, skull, tooth row and so on.

Corbet and Hill (1992) proposed two subspecies *R. a. andamanensis* and *R. a. hainanus* to be a synonym of *R. affinis* but without giving any reasons for their decision. Koopman (1993) as cited by Thomas (1997) proposed that all recognized subspecies be considered synonyms of *R. affinis* without commenting on their status. Thomas (1997) reviewed the classification of *R. affinis* based on a combination of classical and modern systematics, confirmed that four subspecies *R. a. affinis*, *R. a. himalayanus*, *R. a. tener* and *R. a. hainanus* occur throughout the region, and that *R. a. tener* is distributed from Myanmar to Malaysia. Csorba *et al.*, (2003) based mainly on morphology and morphometric data, accepted all the recognized subspecies proposed by Andersen (1905a), Allen (1906) and Dobson (1872), then described variation of the species according to Koopman (1994). Similarly, Simmons (2005) accepted all the recognized subspecies referring mainly to the works of Sinha (1973), Bergmans and van Bree (1986), Bates and Harrison (1997) and Csorba *et al*., (2003). Most recently, Kingsada *et al*., (2011) based on morphology and acoustic data recorded *R. affinis* from Cambodia for the first time. They provisionally confirmed the occurrence of two subspecies (*R. a. macrurus* and *R. a. superans*) from Thailand which is concordant with the findings of Lekagul and McNeely (1977), yet contrary to the conclusion of Thomas (1997) which proposed *R. a. tener* instead.

Echolocation calls of *R. affinis* sensus lato show considerable variation across its distribution, with a difference of up to ~20 kHz between the extremes. In Cambodia the call frequencies of maximum energy (FMAXE) range from 76.1-79.9 kHz (Kingsada *et al.*, 2011), which is similar to calls recorded from northern Thailand and Lao PDR, which range from 70.0-76.1 kHz (Francis, 2008). Calls of populations from peninsula Thailand represent one extreme, with populations below $7^{o}00$ south emitting very low calls ranging from 66.7-71.3 kHz. However, one population nearby (Hala Bala, Narathiwat Province) emits higher calls of 78 kHz (Kingsada *et al.*, 2011) which is comparable to calls from peninsula Malaysia which range from 77.0-78.0 kHz (Francis, 2008; Kingston *et al.*, 2009). In Vietnam, calls range from 69.5-73.8 kHz for the species collected in the north of the country (Furey, Mackie, *et al.*, 2009), with high frequency call (81.2-84.5 kHz) recorded from central and south central Vietnam (O' Shea & Gore, 2011; Thong, 2011). Such variation can indicate morphological variation as a result of the presence of cryptic species, as was shown in the case study of *R. stheno* and *R. microglobosus* (Soisook *et al.*, 2008).

Molecular techniques allow the rapid and effective identification of most taxa and also allow investigation of the evolutionary patterns of biodiversity (Blaxter, 2004). For example, a combination of molecular and morphological techniques were employed by Anwarali *et al*. (2010) to predict the evolutionary relationships of Malaysian woolly bats, *Kerivoula*. Thomas (1997) used a combination of phenetic and molecular analyses to assess the taxonomic status of Afro-Asiatic Rhinolophidae in the *ferrumequinum* group. Thomas (2000) subsequently used morphological data and the cytochrome gene *b* of mitochondrial DNA to elevate *Rhinolophus rouxi sinicus* from subspecies to species level, *R. sinicus* . Francis *et al*. (2010) used the DNA barcodes of mitochondrial COI gene to predict bat diversity in Southeast Asia, and confirmed that nearly all species of his sample, which were morphologically and acoustically distinct could be discriminated by DNA barcodes. Although morphological variation in *R. affinis* has long been recognised, molecular evidence is still very poor, and the degree of intraspecific variation remain unclear. It is understood that widespread species always show considerable genetic differentiation across their distribution (Francis *et al.*, 2010). However, the picture appears to be more complex in *R. affinis*. Thomas (1997), based on morphological and molecular

data, confirmed the recognition of four subspecies of *R. affinis.* However, due to a lack of specimen material from the region, the author also proposed further study to confirm these subspecific divisions.

In conclusion, the taxonomic status of *R. affinis* sensus lato is insufficiently understood as the study of the morphological, acoustic and molecular characters is incomplete.

1.4. Objectives:

The present study aims:

- To assemble morphological data on the species and define diagnostic characters for identification.
- To assemble echolocation data on the species and define the call frequency zones.
- To construct phylogenetic trees and define the relationships between geographical populations, subspecies and possible cryptic species within the *Rhinolophus affinis* species complex.

CHAPTER2

Geographical Variation of *Rhinolophus affinis* (Chiroptera: Rhinolophidae) in Mainland Southeast Asia

ABSTRACT

Rhinolophus affinis sensu lato is distributed throughout Southeast Asia. The taxonomic status of forms attributed to the species is unclear due to variation in morphology and echolocation call frequency. The aim of the study was to review the distribution and taxonomic status of the subspecific forms of *R. affinis* in mainland Southeast Asia using multiple datasets, including morphological, acoustic and genetic data, both to elucidate taxonomic relationships and to test for congruence between these datasets. Three morphological forms were confirmed within the region; two concur with previously recognized taxa, namely *R. a. macrurus* and *R. a. superans*, and are strongly supported by morphological and genetic data. The third form is morphologically distinct but its taxonomic status remains unclear. It is probable that this third form represents a distinct taxonomic entity however more data is required to confirm this. *R. a. macrurus* is known from north of peninsula Thailand, Cambodia, Myanmar, Lao, and Vietnam (Indochinese subregion); *R. a. superans* is found throughout Malay Peninsula (Sundaic subregion); whilst the third form is presently known from east central Myanmar (Shan state) and lower north Vietnam (Nghe An Province). Our results suggest that at least three morphological forms occur in mainland Southeast Asia including one form which appears to be undescribed to science. Echolocation call data for *R. affinis* is not a robust taxonomic tool as it shows a significant degree of variation which is not explained or supported by genetic and morphological findings. This study highlights significant levels of morphological variation in mainland Southeast Asia and provides an essential basis for further studies aiming to understand the population genetics, phylogeography and taxonomy of the species.

2.1. INTRODUCTION

Rhinolophus Lacépède is the only genus in the Old World family Rhinolophidae Gray (Corbet & Hill, 1992). All members of this monogeneric family are characterized by the presence of a horseshoe-shaped anterior noseleaf, the morphology of which can be diagnostic between species. Other characters commonly used to distinguish rhinolophid species include external and cranio-dental measurements, the presence or absence and position of the anterior upper premolar and the number of mental grooves in the lower lip (Csorba *et al.*, 2003; Hill, 1959; Hill & Schlitter, 1982). Constant frequency of the echolocation call emitted by this bats also has been proposed as a mean for species-level distinction for this genus (Csorba *et al.*, 2003; Ith *et al.*, 2011; Kingston & Rossister, 2004; Soisook *et al.*, 2008; Thong, 2011). However, in many cases data for these characters overlap, making genetic analysis an important additional tool for resolving species identifications (Cooper *et al.*, 1998; Li *et al.*, 2006; Maharadatunkamsi *et al.*, 2000; Patrick *et al.*, 2013).

The Intermediate Horseshoe Bat, *Rhinolophus affinis* Andersen, is a medium sized bat (forearm length 45-56 mm) distributed widely in South and Southeast Asia, including northern India (including Andaman Islands), Nepal to southern China, mainland Southeast Asia, Borneo, Java and East Java (Francis, 2008; Simmon, 2005). The taxon includes nine recognized subspecies throughout its range: *Rhinolophus affinis affinis* Horsfield (type locality Java), *R. a. andamanensis* Dobson (type locality South Andaman Island), *R. a. himalayanus* Andersen (type locality Mussoorie, Kumaon Division, north India), *R. a. tener* Andersen (type locality Pegu Division, recently known as Bago, Myanmar), *R. a. macrurus* Andersen (type locality Taho, Karennee, Kyah State, Myanmar), *R. a. superans* Andersen (Pahang, peninsula Malaysia), *R. a. nesite* Andersen (type locality Bunguran Island, North Natunas, Indonesia), *R. a. princeps* Andersen (type locality Lombok, Lesser Sunda Island) and *R. a. hainanus* Allen (type locality Pouten, Hainan Island). The geographical scope of the present study is limited to mainland Southeast Asia and as such includes the range of three of these forms (Andersen, 1905a): *R. a. tener, R. a. macrurus* and *R. a. superans. R. a. tener* is a small rhinolophid with a short tail and a relatively large horseshoe; *R. a. macrurus* is described as being more moderate in size with large ears,

a long tail and a broad horseshoe (Sinha, 1973); while *R. a. superans* is described as similar as *R. macrurus*, but with short tail (Andersen, 1905a).

Lekagul and McNeely (1977) proposed two subspecific forms from Thailand: *R. a. macrurus* and *R. a. superans. R. a. macrurus* is originally known from southeastern Myanmar and characterized by a long tail and large ears. Its range in Thailand includes Chiang Mai, Mae Sariang and Mae Hong Son in the north of the country (Lekagul & McNeely, 1977). *R. a. superans* was described from Pahang State, peninsula Malaysia, is characterized by a short tail and small ears, and occurs in south Thailand (Andersen, 1905a; Lekagul & McNeely, 1977). Consistent with this, Kingsada *et al.,* (2011) referred specimens from north of peninsula Thailand, Cambodia and Vietnam to *R. a. macrurus*, and the form from peninsula Thailand to *R. a. superans* on the basis of the peninsula form being larger on average and having a lower echolocation call frequency (70.0-76.1 kHz versus 66.7-71.3 kHz). The findings of Kingsada *et al*., (2011) broadly agree with the morphological transition rule proposed by Andersen (1905a) for *R. affinis* subspecies, namely: "the more southern or south-eastern the habitat, the longer the ears, the broader the horseshoe, the longer the tibia, the larger the skull, the broader the nasal swellings, and the longer the toothrows". The third form known from the region, *R. a. tener*, is very poorly known, with no further information being available regarding status and distribution since the original description by Andersen (Andersen, 1905a).

The current study is motivated by the extensive variation recorded in the frequency of maximum energy across the species' distribution range, with a difference of almost 20 kHz between extremes: 66.7 kHz recorded from peninsula Thailand (Kingsada *et al.*, 2011) and 84.5 kHz from central Vietnam (Thong, 2011). Acoustic analysis has revealed the existence of cryptic taxa among Asian bat species that are morphologically similar but acoustically divergent (Kingsada *et al.*, 2011; Kingston *et al.*, 2001; Kingston & Rossister, 2004; Soisook *et al.*, 2008; Thabah *et al.*, 2006) and such variation has yet to be fully explored in *R. affinis*. As such, this paper reviews the distribution and taxonomic status of forms of the species in mainland Southeast Asia using multiple datasets, including morphological, acoustic and genetic data, both to elucidate taxonomic relationships and to test for congruence between these datasets.

2.2. MATERIALS AND METHODS

2.2.1. Sample collection and study sites

A total of 170 specimens were examined from mainland Southeast Asia. Samples examined were from existing museum collections and those arising from recent surveys. Specimens were examined from collections held at Princess Maha Chakri Sirindhorn Natural History Museum, Prince of Songkla University, Thailand [PSU collection]; Zoological Collection, Centre for Biodiversity Conservation, Cambodia (CBC); Harrison Institute, UK (HZM); Institute of Ecology and Biological Resources (IEBR), Vietnam; Natural Science and Research Laboratory at Museum of Texas Tech University; Zoological Museum at University Malaysia Sarawak and Kim Hy Nature Reserve Collection (NF), Vietnam.

Specimens were collected by Saveng Ith and team (Small Mammals and Birds Research Unit Team, PSU, Thailand) between November 2010 and March 2012 from survey sites in Thailand. Animals were captured in the field using a combination of harp traps, mist net and hand nets. Field surveys were conducted in several localities in Thailand namely, Chiang Dao Wildlife Sanctuary, Hala Bala Wildlife Research Station, Kaeng Kra Chang National Park, Khao Namkhang National Park, Khao Ban Tad Wildlife Sanctuary, Krom Luang Chumpon Wildlife Sanctuary, Rajjaprabha Dam and Ton Nga Chang Wildlife Sanctuary; study localities are illustrated in fig. 2.1.

Many specimens from Cambodia, Thailand and Vietnam were previously included in Kingsada *et al.* (2011). All specimens and surveyed localities and habitats for the current study are listed below.

Cambodia

Siem Reap Province: [C1] Phnom Kbal Spean, Banteay Srei District, Phnom Kulen National Park (14°21’ N 107°22’ E). Six males (five adult and one sub-adult) and one nulliparous female were collected by Ben Hayes, Sarith Pen and Sophany Pauk between January and July 2010 on the mountain of evergreen forest. [C2] Ka Kek, Preah Vihear Protected Forest (14°04’ N 105°17’ E).

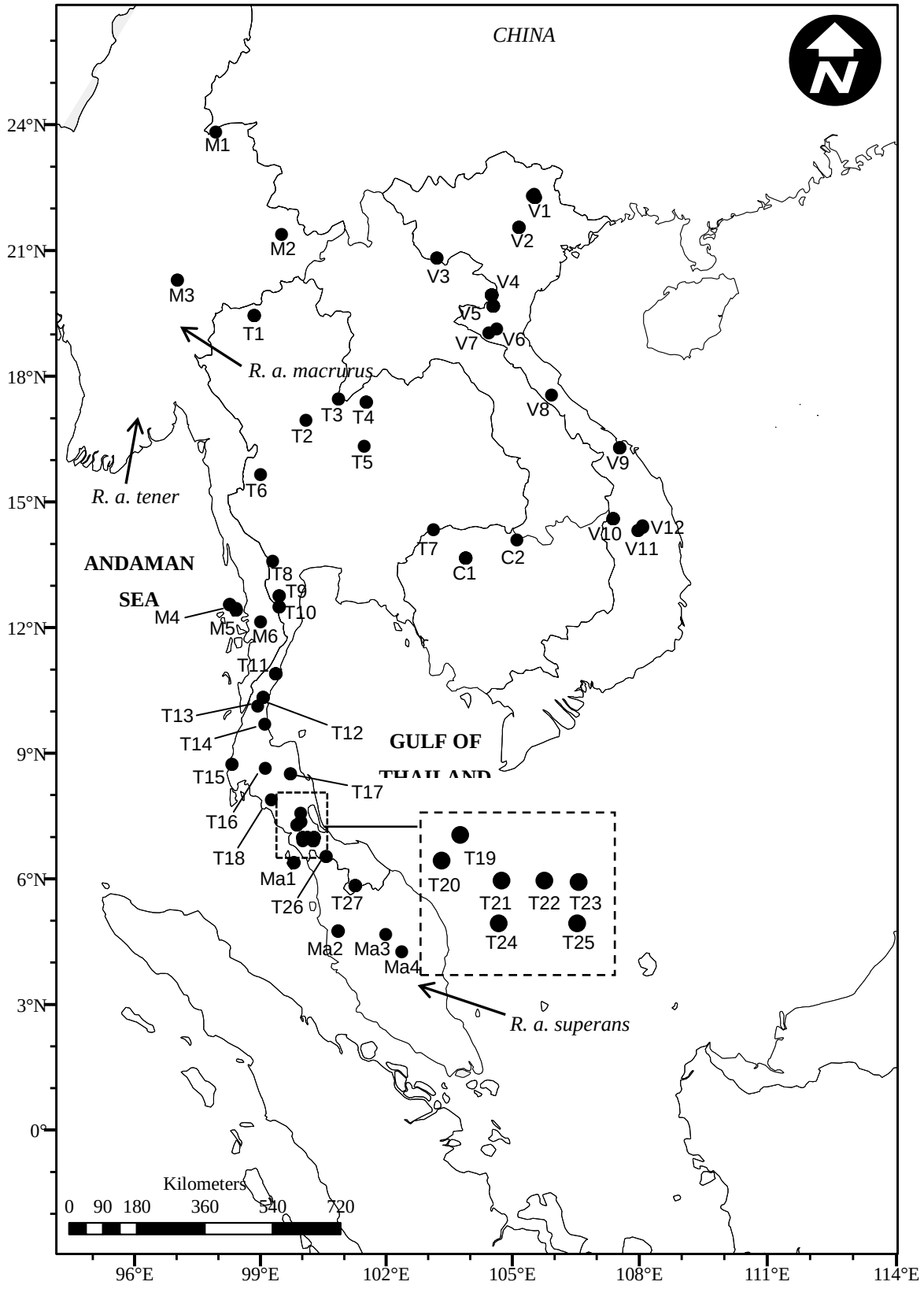

Figure 2.1. Research localities of Rhinolophus affinis from Mainland Southeast Asia.-

C=Cambodia, M=Myanmar, Ma=Malaysia, T=Thailand and V=Vietnam. Black circles are localities where materials were examined (based on sample collection & study sites in methodology). The arrows indicate the approximate localities of subspecific forms in the research area.

One nulliparous female was captured by Gabor Csorba, Neil Furey and Saveng Ith on 17 February 2011 in semi-evergreen forest.

Peninsula Malaysia

Kedah State: [Ma1] Langkawi Island (approx. 6°23.204' N, 99°47.831' E). An adult male was collected by Mohd Isham Mohd Azhar; Penang State [Ma2] (05°15.795' N, 100°29.076' E). A nulliparous female was collected by Faisal Ali Anwarali Khan on 9 August 1988; Kelantan State: [Ma3] Gua Madu, Gua Musang Division (approx. 5°10.462' N, 101°54.191' E). A parous female was captured by Faisal Ali Anwarali Khan; Pahang State: [Ma4] Nature Study of Kuala Atok, Taman Negara National Park (04°16.281' N, 102°22.316' E). One adult male and one nulliparous female were collected by Faisal Ali Anwarali Khan on 19-22 May 2008.

Myanmar

Shan State: [M1] Mant Hai Village, Muse Twonship (23°54.962' N, 97°49.000' E); [M2] Holin Village, Keng Taung (21°27.483' N, 99°32.000' E); [M3] Taung Pauk Village, Inle Lake (20°21.175' N, 96°53.189' E). Three adult males and one female were collected by Paul Bates and Iain Mackie between March 2002 and December 2003. All areas were on the Shan plateau in areas of limestone karst, comprising limestone outcrops, deforested agricultural land and small patches of deciduous forest. Taninthary Division: [M4] Katalu Village, (12°28.436' N, 98°24.191' E); [M5] Kyi Village (12°30.113' N, 98°24.333' E), Kadan ID; specimens were collected in mist nets over a stream in open heavily degraded forest and agricultural land and from a roost in granite boulders in secondary forest; [M6] Hnedchey Khan Cave, Kyauk Taun Village (12°11.400' N, 99°00.600' E). The cave is in a limestone outcrop and surrounded by patches of degraded evergreen forest and

agricultural land. One adult male and four females were collected by Paul Bates and Iain Mackie between June and November 2003.

Thailand

Chiang Mai Province: [T1] Khun Mae Ngai Ranger Station, Chiang Dao Wildlife Sanctuary (approx.19°30.556’ N, 98°49.956’ E). A sub-adult female was collected in a harp trap on 28 June 2011 in hilly evergreen forest; (approx. 19°31’55” N 98°50’26” E; 864 m a.s.l), two adult males, one parous female and one nulliparous female were captured by Pipat Soisook between August 2005 and October 2006. Bats were captured from the limestone cave surrounded by orchards, mixed deciduous and bamboo forest. Petchaboon Province: [T2] Nhong Mae Na, Thung Sa Lang Luang National Park (16°34’17’’ N 100°52’35’’ E). One adult male was captured by Charles Francis and Sara Bumrungsri on 16 May 2006 in semi-evergreen forest. Loei Province: [T3] Na Haeo District, Phu Suan Sai National Park (17°30’19” N 100°56’18” E, 620 m, 975 m a.s.l). Two adult males were captured by Sara Bumrungsri and Charles Francis on 18-20 May 2006. Bats were captured using harp traps set across the trails within evergreen forest mixed with bamboo; [T4] Phu Ruea District, Phuluang Wildlife Sanctuary (17°25.742’ N, 101°38.006’ E). Three adult males and one nulliparous female were collected by Sara Bumrungsri and team on 17-18 March 1993. The habitat is unknown. Chaiyapum Province: [T5] Thung Kamang, Khon San District, Phukieo Wildlife Sanctuary (16°18’ N 101°52’ E). One Nulliparous female was captured by Pipat Soisook on 08 April 2006 in hilly semi evergreen forest. Tak Province: [T6] Kavackee, East Thung Yai Naresuan Wildlife Sanctuary (15°42’26’’ N 98°59’28’’ E). One adult male was captured by Sara Bumrungsri on 11 March 2003 in semi evergreen forest. Surin Province: [T7] Ta Muen Thom, Huai Thap Than-Huay Sumran Wildlife Sanctuary (14°21’08’’ N 103°15’54’’ E). One adult male was captured by Sara Bumrungsri on 28 January 2000 in dry semi evergreen forest. Ratchaburi Province: [T8] Mae Nam Pha Chi Wildlife Sanctuary (13°18.142’ N, 99°25.009’ E). A male adult was captured by a harp trap set over a seasonal stream in dry evergreen forest by Pipat Soisook on 20 January 2008. Petchaburi Province: [T9] Kaeng Kra Chan National Park (approx. 12°47.965’ N, 99°27.812’ E). Two adult males and one nulliparous female were collected by Saveng

Ith and team in August 2011. Three harp traps and two nets were set in bamboo forest, across a stream and a trail in evergreen forest. Prachuap Kiri Khan Province: [T10] Pa La-ou Ranger Station, Kaeng Kra Chan National Park (approx. 12°32.228’ N, 99°27.812’ E). Two adult males and one nulliparous female were collected by Saveng Ith in August 2011. Three harp traps were set on forest trails in evergreen forest. Ranong Province: [T11] Klong Sai On Waterfall, Krom Luang Chumpon Wildlife Sanctuary (10°22.21’ N, 99°04.27’ E). Three adult males were collected in August 2011. Three harp traps were set on forest trails of evergreen forest and surrounded rubber plantation and fruit orchards. Chumphon Province: [T12] Khao Kram cave, Patiew District (10°55’08” N 99°22’26” E, 67 m a.s.l). Three adult males and three nulliparous females were captured by Sara Bumrungsri and team on 10 October 2006. The harp trap was set across the entrance of the cave surrounded by rubber plantation; [T13] Huay Wang Cave, Tumbon Khao Talu, Sawi District (10°10’00’’ N 98°55’11’’ E, 55 m, a.s.l). One adult male was captured by Sara Bumrungsri and team on 10 January 2007. The harp trap was set across the entrance of a limestone cave surrounded by deciduous forest and rubber plantation; [14] Klao Plu Cave, Lamae District (09°43’36’’ N 99°06’30’’ E). One adult male was captured by Sara Bumrungsri and team on 09 January 2007. Harp traps were set across the trails in rubber plantation and fruit orchards. Pang Nga Province: [T15] North Surin Island (approx. 8°46.200’ N, 98°18.600’ E). Two adult males were collected by Sara Bumrungsri on 02 February 2006. The specimens were captured in a harp trap set over the trail on hill side surrounded by evergreen forest and close to the beach. Surat Thani Province: [T16] Rajjaprabha Dam and Khlong Saeng Wildlife Sanctuary (approx. 8°58.885’ N, 97°47.706’ E). One adult male was collected by Saveng Ith on 31 August 2011 and one adult male was collected by Sara Bumrungsri on 17 January 2012. The harp traps and mist nets were set on small trails and streams surrounded by disturbed evergreen forest, rubber plantations and a mixed fruit orchard. Nakhon Si Thammarat Province: [T17] Khao Phlu Cave, Khao Ro Commune, Ron Piboon District (8°32.250’ N, 99°43.396’ E). One adult male and nulliparous female were collected by Sara Bumrungsri from the cave on 15 October 2011. The cave is located in a limestone outcrop surrounded by rubber and oil palm plantations. Krabi Province: [T18] Khao Pra Bang Kram Wildlife Sanctuary (7°55.31’N, 99°15.47’ E). One adult

male was collected by Pipat Soisook on 04 May 2012. A harp trap was set across forest trail surrounded by lowland evergreen forest. Pattalung Province: [T19] Khao Ban Tad Wildlife Sanctuary (approx. 7°23.48’ N, 99°58.40’ E). Two adult males, one parous female and one nulliparous female were collected by Pipat Soisook in March 2012 using harp traps and mist net set in evergreen forest across a stream and forest trail. Trang Province: [T20] Sai Rung Waterfall, Khao Ban Tad Wildlife Sanctuary (7°18.080’ N, 99°41.988’ E). One adult male and two nulliparous females were collected by Pipat Soisook on 09 January 2011. Three harp traps and a mist net were set on forest trails and across a stream. Songkhla Province: [T21] Khuan Khao Wang Forest Park, Rattaphum District (7°00.776’ N, 100°01.259’ E). Four adult males and two nulliparous females were captured by Saveng Ith in August 2011 and February 2012. Mist nets and harps were set on the forest trails and across the small streams in evergreen forest surrounded by rubber plantation and fruit orchards; [T22-25] Ton Nga Chang Wildlife Sanctuary (approx. 6°55.783’ N, 100°16.299’ E) including Boripatr Waterfall, Pha Dam Ranger Station, Makling Waterfall and Hin Sam Kon Waterfall. 13 adult males and four nulliparous females were collected using harp traps and mist nets by Saveng Ith in February 2012 and Sara Bumrungsri between October 2006 and January 2007. The traps and nets were set on small trails and streams surrounded by evergreen forests and a rubber plantation; [T26] Khao Namkhang National Park (6°33.108’ N, 100°16.299’ E). Two adult males were captured by hoop net in a man-made tunnel by Saveng Ith on 16 May 2012. Narathiwat Province: [T27] Hala Bala Wildlife Sanctuary (05°47’54’’ N 101°49’30’’ E). Six adult males and two nulliparous females were collected by Saveng Ith in January 2012. Harp traps were set on forest trails in evergreen forest.

Vietnam

Bac Kan Province: [V1] Kim Hy Nature Reserve (22°11.320’ N 106°03.530’ E). One immature male and seven parous females were captured by Neil Furey between June 2006 and February 2007. Bats were captured using mist net set in primary forest ridge. Vinh Phuc Province: [V2] Tam Dao National Park (21°30.448’ N, 105°36.4924’ E). Five adult males were collected by Vu Dinh Thong on 24 November 2009. Son La Province: [V3] Tin To Area, Sop Cop Nature Reserve

(20°49.758’ N, 103°29.519’ E). Three adult males were collected in November 2004 by Pham Duc Tien. Nghe An Province: Four sites were surveyed including [V4] Que Phong District, Pu Hoat Nature Reserve (approx. 19°54.221’ N, 104°50.243’ E); [V5] Ban Khom Cave, Que Phong District, Pu Hoat Nature Reserve (approx. 19°54.221’ N, 104°50.243’ E); [V6] Phu Nong Mount, Pu Mat National Park (19°01.340’ N 104°44.726’ E); [V7] A cave at Khe Mat ridge, Pu Mat National Park (approx. 19°01.340’ N, 104°44.726’ E). Nine adult males, 10 females were collected between August 1998 and October 2008 by Pham Duc Tien, Vu Dinh Thong, Thomas Howard and Ben Hayes. Quang Binh Province: [V8] Hoa Son Village, Ke Bang, Phong Nha National Park (17°28.200’ N, 105°31.200’ E). One adult male was collected on 18 August 1998 by Ditte Hendrichsen. Thua Thien Hue Province: [V9] Bach Ma National Park (16°10.989’ N, 107°52.496’ E). Three adult males were collected between June and October 2001 by Pham Duc Tien and Vu Dinh Thong. Kon Tum Province: [V10] Chu Mom Ray National Park (14°29.021’ N, 107°38.139’ E). Two parous females and seven nulliparous females were collected by Vu Dinh Thong between May and August 2005. Gia Lai Province: Two sites were surveyed including [V11] Kon Cha Rang Nature Reserve (14°17.400’ N, 108°21.600’ E) and [V12] Kon Ka Kinh Nature Reserve (14°11.400’ N, 108°15.000’ E). Two males and two females were collected in March 1999 by Ben Hayes.

2.2.2. Morphological measurements

Multiple external and craniodental characters of each specimen were measured following Bates and Harrison (1997), Csorba *et al*., (2003), Furey *et al*., (2009) and Thomas (1997) (figs. 2.2 – 2.6). Wet specimens were measured using a pair of dial calipers to the nearest 0.1mm, whereas craniodental characters were measured to the nearest 0.01mm using a digital caliper under stereo microscope. Bacular morphology was also observed using a stereo microscope.

External characters measured included FA: forearm length – from the extremity of the elbow to the extremity of the carpus with the wings folded; EL: ear length – from the lower border of the external auditory meatus to the tip of the pinna; TL: tail length – from the tip of the tail to its base adjacent to the anus; HF: hind foot

length – from the extremity of the heel behind the os calcis to the extremity of the longest digit, not including the hairs or claws; TIB: tibia length – from the knee joint to the extremity of the heel behind the os calcis; 2MT, 3MT, 4MT, 5MT: length of metacarpals – taken from the extremity of the carpus to the distal extremity of the second, third, fourth and fifth metacarpals respectively; 1P3D, 2P3D, 1P4D, 2P4D, 1P5D, 2P5D: length of the first and second phalanges of the third, fourth and fifth digits respectively – taken from the proximal to the distal end of the phalanx; GWN: greatest width of noseleaf – greatest diameter across the horseshoe; GHN: greatest height of noseleaf – from the base of the horseshoe to the tip of the lancet, not including the hairs.

External measurement illustration (figs. 2.2 – 2.3): External characters of wet specimen were measured follow Csorba *et al*., (2003) and Bates and Harrison (1997).

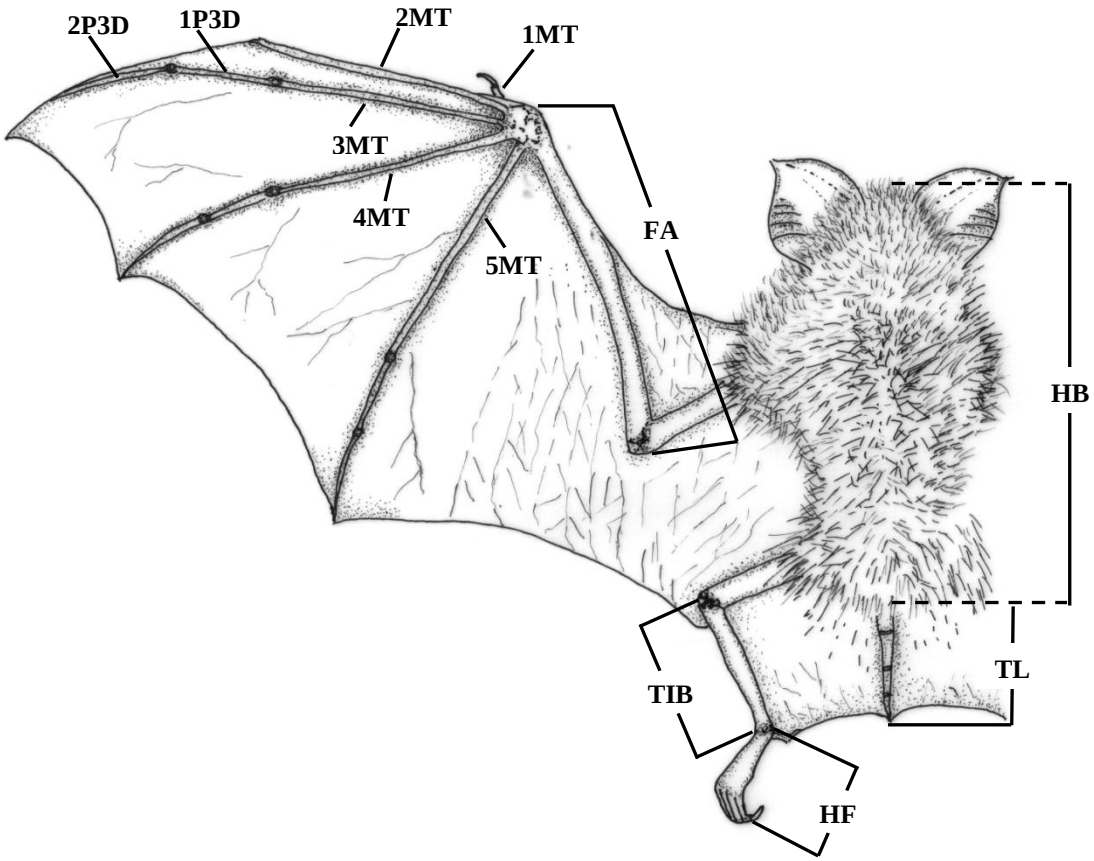

Figure 2.2. Left wing and body of *Rhinolophus affinis*.

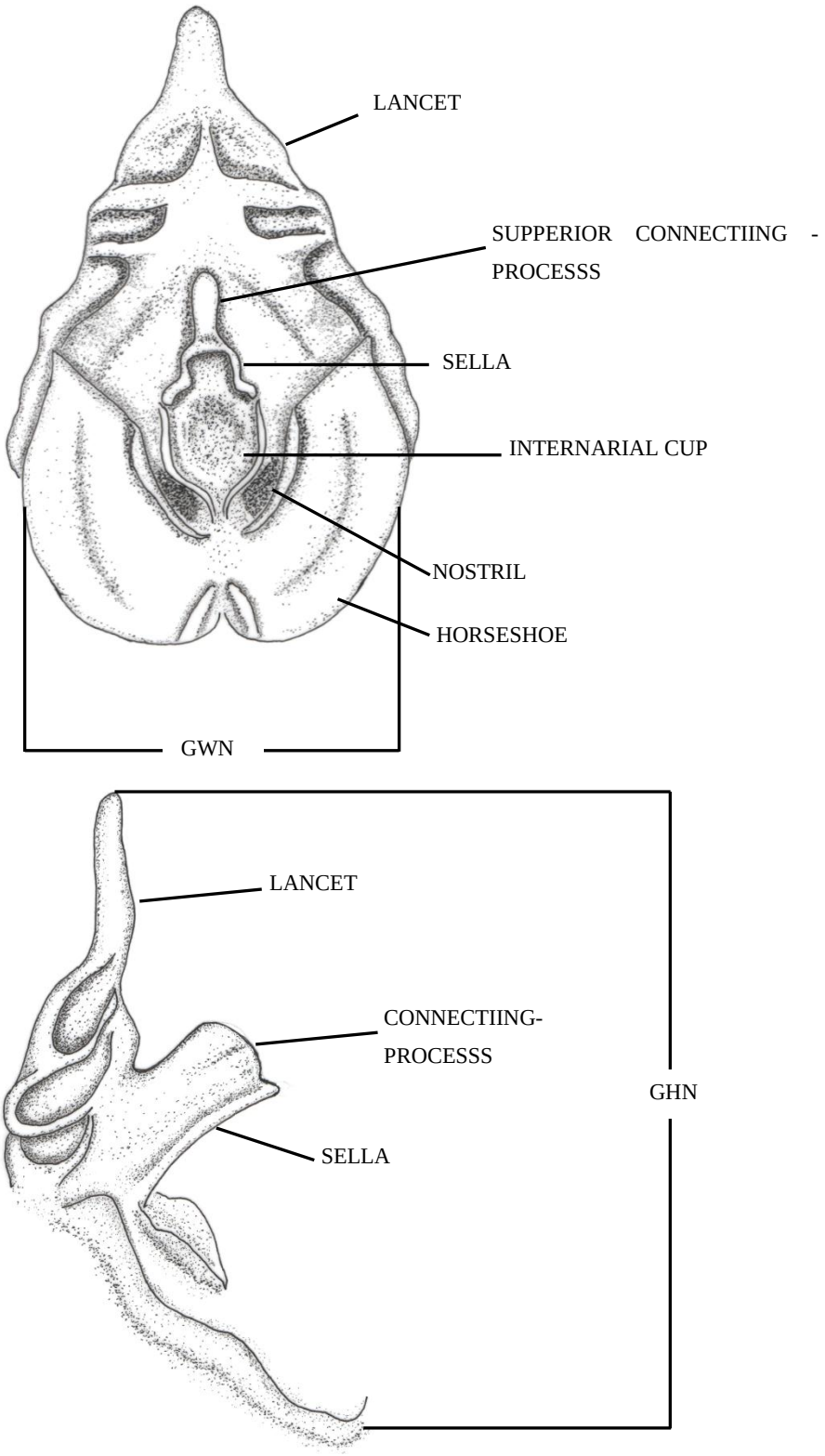

Figure 2.3. Noseleaf of *Rhinolophus affinis* in frontal and lateral views.

Craniodental measurement illustrations (figs. 2.4 – 2.6): Cranial and dental characters were measured follow Csorba *et al*. (2003), Bates and Harrison (1997), Furey *et al.* (2009) and Thomas (1997).

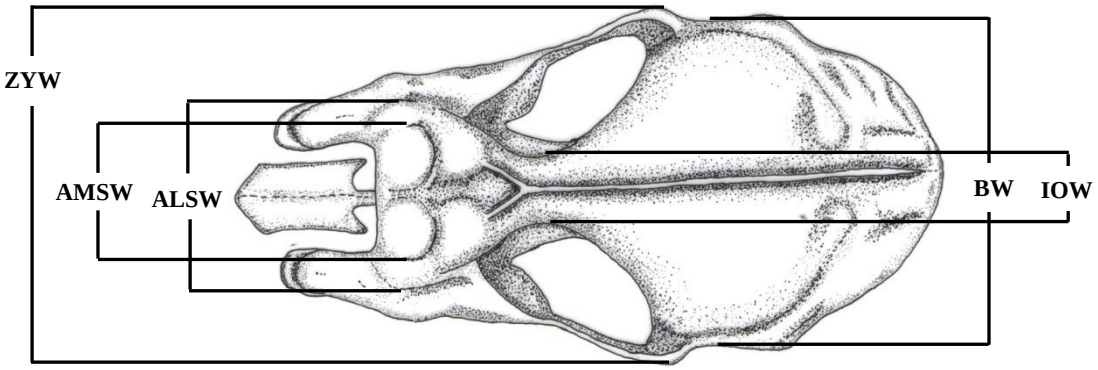

Figure 2.4. Dorsal view of the skull of *Rhinolophus affinis*.

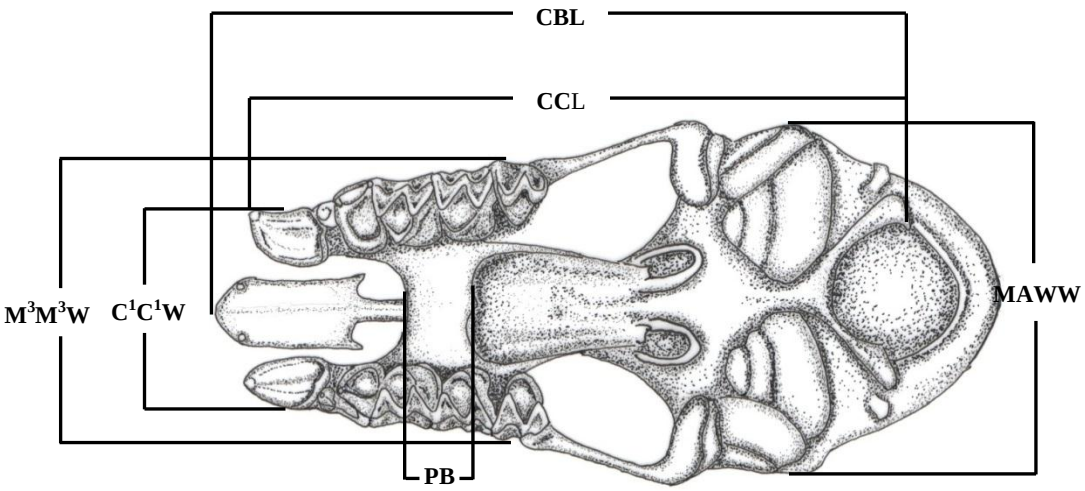

Figure 2.5. Ventral view of the skull of *Rhinolophus affinis*.

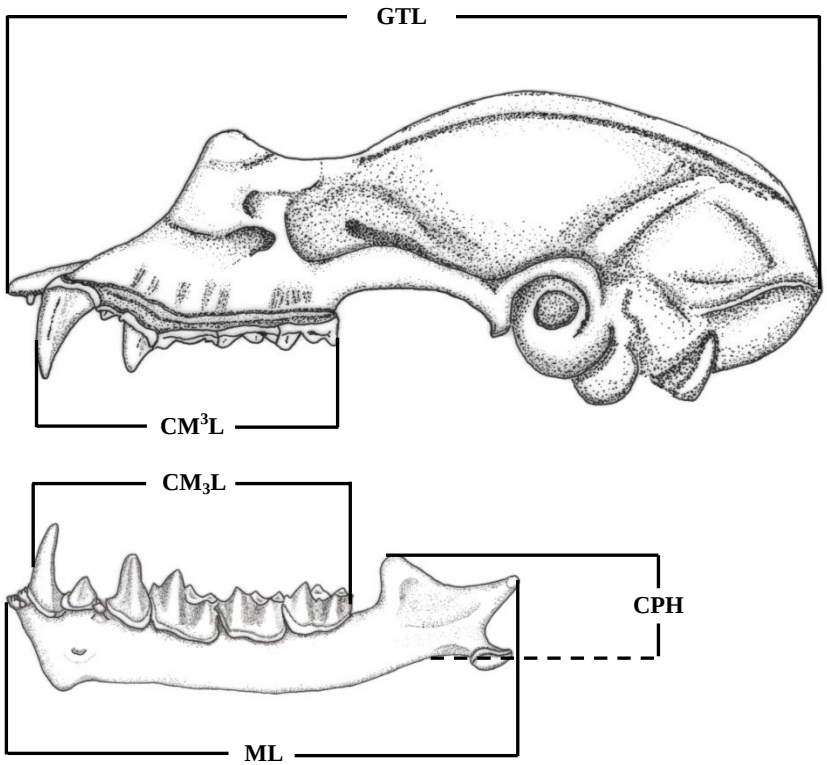

Figure 2.6. Lateral view of the skull of *Rhinolophus affinis*.

Craniodental characters measured included, SL: skull length – the greatest length from the occiput to the front of the canine; CCL: condyle-canine length – from the exoccipital condyle to the anterior alveolus of the canine; ALSW: the greatest width across the anterior lateral compartments of the rostrum; AMSW: anterior median swellings width – the greatest width across the median swellings in dorsal view; ZYW: zygomatic width – the greatest width of the skull across the zygomata; BW: braincase width – the width of the braincase at the posterior roots of the zygomatic arches; GBW: braincase width–the greatest width across the braincase; MAW: mastoid width – greatest width of the braincase taken across the mastoid region; IOW: interorbital width – the narrowest width of the interorbital constriction; PB: palatal bridge – length of bony palate excluding the posterior spike; M^3M^3W: posterior palatal width – taken across the widest part of the outer borders of the third upper molar; C^1C^1W: anterior palatal width – taken across the widest part of the outer border of the upper canine; CM^3L: upper toothrow length – from the front of the upper canine to the back of the crown of the third upper molar; CM_3L: lower toothrow

length – from the front of the lower canine to the back of the crown of the third lower molar; ML: mandible length – from the most posterior part of the condyle to the most anterior part of the mandible, including the lower incisors; CPH: least height of the coronoid process – from the tip of the coronoid process to the apex of the indentation on the inferior surface of the ramus adjacent to the angular process.

2.2.3. Morphological analysis

Statistical analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, U.S.A.) and PC-ORD 5.10 (MjM Software, Oregon, U.S.A.) for windows. Descriptive statistics (minimum, maximum, mean and standard deviation) were calculated for external and cranio-dental measurements. Normality of data and homogeneity of variances were explored prior to parametric *t*-tests, to determine sexual dimorphism within the taxa. Non-parametric tests (Mann-Whitney U-test) were used for characters that did not show normality of data (HF, $p<0.05$) and/or homogenous variances (ALSW, $p<0.05$). Multiple comparisons of characters between populations and colonies were calculated using a multivariate analysis of variance (MANOVA). Linear regression was used to examine the correlation between morphology and echolocation call frequencies. Principal component analysis (PCA) on the correlation matrix was used for multivariate comparisons.

2.2.4. Echolocation call recording and measurement

Values for the frequency of maximum energy (FMAXE) for *R. affinis* in this study were largely obtained from survey work, with some additional data published by Kingsada *et al.,* (2011) and Furey *et al.,* (2009). Echolocation calls were recorded using a Pettersson D-240X bat detector set in 10x time-expansion mode and call data was stored on a digital iRiver iHP-120 Multi Codec Jukebox recorder. When available, a Pettersson D1000X was also used, and call storage was then on a built in Compact Flash card (type I). The detector was set to manual recording mode with the maximum sampling rate at 768 kHz. A time expansion factor of 10 was used. Sound files were recorded and saved in ‘wav’ format then transferred to a laptop computer

for analysis. Echolocation calls from Vietnam were recorded using the PCTape system, which was custom-made by the University of Tuebingen, Germany. Call components were displayed using spectrograms and oscillograms in BatSound Pro 3.31 (Pettersson Elektronik, AB, Sweden) in which sampling frequency was 44.10 kHz; spectrograms were set as 1024 sampling size in Fast Fourier Transforms with Hanning windows. The constant frequency portion of the call was selected for measuring FMAXE (kHz) from the power spectrum feature in BatSound Pro 3.31 (fig. 2.7). Multiple calls were measured for individuals where this data was available.

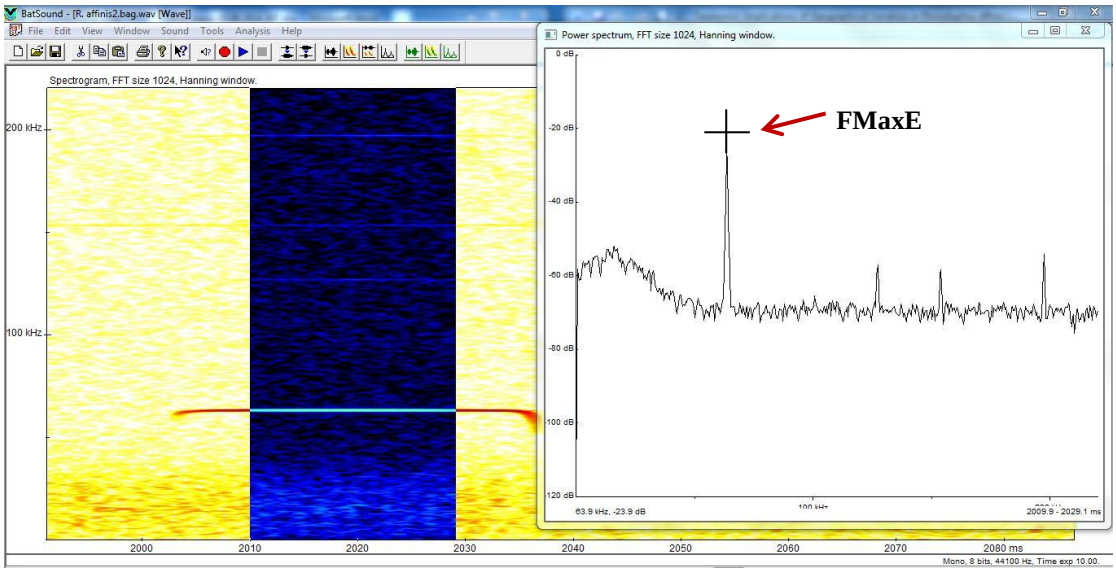

Figure 2.7. Oscillogram, spectrogram and power spectrum illustrated the selection and measuring of the maximum energy frequency (FMaxE) of the *Rhinolophus affinis* from Khao Nam Khaeng National Park, south of Songkhla, Thailand.

2.2.5. Molecular systematics

2.2.5.1. Tissue collection, DNA extraction and analysis

Tissue (liver, tongue and wing membrane) was collected from voucher specimens and preserved in 95% concentration ethanol. Two mitochondrial DNA (mtDNA) gene fragments were selected for analysis. A 657 base pair segment of COI analysis was carried out at the Canadian Center for DNA Barcoding (CCDB) using

standardized barcoding protocols (Ivanova *et al.*, 2012), while a 517 base pair segment of control region (D-loop gene) was analyzed at the Department of Biotechnology and Molecular Biology, Prince of Songkla University, Thailand. For comparison, sequences from the Genbank were also accessed and included [six sequences of D-loop gene (accession numbers: GQ265988, GQ265994-GQ265995, GQ265998, GQ266002-GQ266003) from south China. Fifty sequences of COI gene were included for comparison, 11 sequences were from peninsula Malaysia (accession numbers: HM541330-HM541332, HM541407-HM541409, HM541410-HM541414) and 39 sequences were from the Indochinese subregion (accession numbers: HM541326, HM541341, HM541364, HM541366-HM541367, HM541382-HM541384, HM541347-HM541351, HM541398-HM541406, HM541395-HM541397, HQ580330-HQ580331, JF444035-JF444036, JF444039-JF444043, GU684791-GU684794, GU684798, GU694801)].

Genomic DNA was extracted using DNeasy Tissue Kits (QIAGEN). The tRNA-proline end of mitochondrial DNA control region containing the hypervariable domain (HVI) was amplified (Chen et al. 2006) by polymerase chain reaction (PCR) using the primers DL-H 16750 (5'-CCTGAAGTAGGAA-CCAGATG-3') (Wilkison and Chapman 1991) and Thr-L 16272 (5'-CCCGGTCTTGTAAAC C-3') (Stanley *et al.* 1996). PCRs were carried out in 25 µl volumes on a DNA Engine thermal cycler (BIORAD). Each reaction contained 7.5 µl of water, 2 µl of reach primers (10 µm), 12.5 µl of Top Taq Master Mix Kit (QIAGEN) and1-2 µl of DNA template (50 ng/µl). The amplification was run under the thermal conditions of an initial denaturation at 95 °C for 5 min followed by 34 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s and a final extension cycles at 72 °C for 10 min. Possible contamination was checked by gel electrophoresis of 6 µl of PCR reaction including a negative control (containing all reagents, but no DNA template). DNA present in a 1.5% agarose gel was stained with ethidium bromide and visualized under UV using gel analysis equipment (UVITEC, Cambridge). PCR product was purified using QIA quick Gel Extraction Kit (QIAGEN) before sequencing. The ABI PRISM^{TM} Big Dye Terminator Cycle Sequencing Kit was used to prepare the DNA samples for sequence analysis. Sequencing was performed on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Framingham, MA, USA). The chromatograms were edited using

Geneious Pro 5.6-trial version and BIOEDIT 7.0.0 (Hall, 1999) and aligned using CLUSTAL_X 1.83 (Thompson *et al.*, 1997) and Mega5.2.2 (Tamura *et al.*, 2011).

Phylogenetic relationships among sequences were reconstructed using maximum-likelihood in the program MEGA5.2.2 (Tamura *et al.*, 2011). The most appropriate substitution model was determined using BIC as implemented in jModel Test 2.14 (Darriba *et al.*, 2012). Among the 88 models in the 100% confidence interval, the Hasegawa-Kishino-Yano substitution model (HKY) with proportion of invariant sites (I) was the best-fit model selected for D-loop and Kimura 2-parameter (K80) was the best-fit model for COI. We also performed Bayesian Analysis using Mr Bayes 3.2.2 (Huelsenbeck & Ronquist, 2001). In Bayesian Analysis, convergence stationary was searched by two independent Metropolis-coupled Markov chain Monte Carlo (MCMC), each comprising three incrementally heated chains and one cold chain, run for six million generations, with parameters sampled every 1000 generations. Convergence stationary of the MCMC chains was evaluated by inspecting whether the standard deviation of split frequencies approached zero and the potential scale reduction factor (PSRF) reached 1.0 for all parameters. We also investigated the convergence using Tracer 1.5 (Rambaut & Drummond, 2009), and the 25% initial phase of the Markov chain was discarded as a burn-in. A congeneric *R. pearsoni* was used as an out group in the phylogenetic analysis of D-loop gene in order to examine the monophyletic lineage of *R. affinis*.

To estimate the time to the most recent common ancestor (TMRCA) among the observed clades, D-loop gene was analyzed in BEAST version 1.8 (Rambaut and Drummond 2007). Based on jModel Test, HKY + I were selected as the best substitution model and relaxed-clock model with an uncorrelated lognormal distribution was used to estimate the substitution rate. We performed two independent runs of MCMC chains with 60 million generation each with parameters logged every 1000 generation. Tracer version 1.5 (Rambaut & Drummond, 2009) was used to combine the two runs as well as to examine the effective sample size (ESS) for the parameters. Trees were collated using Tree Annotator version 1.8 where Maximum clade credibility tree and Median heights were selected; and 10% (6000 trees) sample trees were selected as burn-in. To convert the estimates scaled by mutation rate to calendar years, we used the divergence rate of 20%/Myr for control region which was

previously calibrated in the noctule bat (Petit *et al.*, 1999) and used in *R. affinis* (Mao *et al.*, 2010) and other bats (Chen *et al.*, 2006; Salgueiro *et al.*, 2004).

2.3. RESULTS

2.3.1. Morphometrics

External and cranial measurements were available for 170 specimens. No significant size variation in 34 external and cranial characters was observed between the sexes (table 2.1). A total of 19 external and cranial characters were retained for multivariate analysis, these characters being selected on the basis of their eigenvector values in the preliminary PCA. A multivariate analysis (PCA) using these 19 external and cranial characters from the total 168 specimens from continental Southeast Asia formed two relatively distinct groups (fig. 2.8). These represent the two recognized zoogeographic subregions (Indochinese subregion and Sundaic subregion) and correspond to *R. a. macrurus* and *R. a. superans* respectively. Although a well-known zoogeographic boundary between the two subregions occurs at the Isthmus of Kra ($10^{o}30$'N), the Sundaic morphological characters appear to extend north of this to Kanchanaburi province (fig. 2.9). Based on the 19 external and cranial characters analyzed, northern Cambodian specimens largely overlap with the Sundaic group (fig. 2.8). However, a further PCA performed on 11 selected characters with high loading scores, separated this population from the peninsula group (fig. 2.10).

In the Indochinese subregion, specimens have significantly larger forearm and wing measurements ($p < 0.001$ for most characters). The tail and hind foot are also longer but the horseshoe is significantly smaller ($p<0.05$) (table 2.2). In terms of skull characters, this population is significantly smaller ($p < 0.001$ for most characters) compared to individuals from the Sundaic subregion. Sundaic specimens generally have a broader cranial dimensions and larger rostral chambers. Additionally, the anterior lateral swellings, anterior median swellings and posterior median swellings are more enlarged.

Table 2.1. Morphometric comparison between male and female of *R. affinis* from Thailand, Myanmar and Vietnam. External and craniodental measurements, values are given as min-max, mean ± standard deviation (in mm). Acronyms and definitions for measurements are given in the text. Sex. dim. = sexual dimorphism; ns = not significant (p>0.05).

n	Sex	FA	HB	TL	EL	TIB	HF	2MT	3MT	4MT	5MT
39	♀♀	51.3±1.7	51.7±2.4	24.0±2.6	22.2±1.4	24.3±0.9	10.50±0.4	41.7±1.4	39.1±1.3	40.2±1.3	40.8±1.5
		48.3-54.8	46.5-59.1	19.3-29.3	19.7-25.4	22.2-26.4	9.4-11.2	39.4-44.5	36.8-42.4	37.6-42.8	38.1-44.0
60	♂♂	51.0±1.5	52.1±2.8	23.3±2.4	21.9±1.1	24.3±1.0	10.3±0.5	41.3±1.4	38.8±1.4	39.9±1.4	40.5±1.4
		48.3-54.4	42.7-57.8	18.8-30.7	19.6-25.8	21.8-26.0	9.0-11.3	38.3-44.7	35.7-43.0	36.7-43.5	37.7-44.5
Sex. Dim.		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

n	Sex	3D1P	3D2P	4D1P	4D2P	5D1P	5D2P	GHN	GWN
39	♀♀	15.2±0.6	25.9±1.5	10.4±0.4	15.4±0.8	11.8±0.5	13.7±1.3	13.6±1.0	9.9±0.6
		13.7-16.6	18.3-27.9	9.7-11.5	13.5-17.2	10.7-12.9	9.0-15.5	10.4-15.6	8.3-11.1
60	♂♂	15.0±0.6	26.1±1.2	10.3±0.4	15.4±0.8	11.7±0.6	13.6±1.0	13.8±0.9	9.9±0.5
		13.7-16.5	23.5-30.0	9.5-11.4	13.8-17.6	10.5-13.2	9.6-15.8	11.8-16.1	8.5-11.2
Sex. Dim.		ns	ns	ns	ns	ns	ns	ns	ns

Table 2.1. Continued.

n	SL	CCL	ZYW	MAW	BW	ALSW	AMSW	IOW
39	22.55±0.4	19.90±0.3	11.22±0.2	10.58±0.2	10.19±0.1	6.07±0.1	4.22±0.1	2.18±0.2
	21.92-23.53	19.11-20.68	10.80-11.87	10.17-11.15	9.89-10.60	5.65-6.36	3.81-4.72	1.72-2.60
60	22.55±0.4	19.89±0.4	11.21±0.2	10.63±0.2	10.18±0.2	6.12±0.2	4.20±0.2	2.27±0.2
	21.47-23.33	18.78-20.78	10.53-11.91	9.81-11.16	9.54-10.67	5.56-6.72	3.59-4.67	1.70-2.81
Sex. Dim.	ns	ns	ns	ns	ns	ns	ns	$P = 0.047$

n	PB	CM^3L	C^1C^1W	M^3M^3W	ML	CM_3L	CPH
39	2.15±0.1	8.91±0.1	5.75±0.1	8.20±0.1	15.48±0.2	9.33±0.1	3.13±0.1
	1.82-2.67	8.53-9.34	5.23-6.05	7.77-8.86	14.91-15.96	8.98-9.74	2.52-3.42
60	2.19±0.1	8.96±0.2	5.79±0.1	8.23±0.2	15.51±0.3	9.34±0.2	3.14±0.1
	1.63-2.61	8.36-9.38	5.22-6.13	7.73-8.72	14.59-16.07	8.75-9.82	2.86-3.63
Sex. Dim.	ns	ns	ns	ns	ns	ns	ns

Table 2.2. External and craniodental measurements of *R. affinis* forms within Southeast Asia. Values are given as min-max, mean ± standard deviation (in mm). Acronyms and definitions for measurements are given in the text. Values marked with * are based on literature. The forms (A, B, C) were assigned based on PCA (fig. 2.17).

n	Sex	FA	HB	TL	EL	TIB	HF	2MT	3MT	4MT	5MT	3D1P
						Rhinolophus a. tener Holotype						
1	♂♂	49.3	--	23.00*	18.85	23.85	11.51	--	37.04	38.02	39.33	14.77
						Rhinolophus a. macrurus Holotype						
1	♂♂	53.03	--	--	--	24.76	11.60	--	40.94	41.86	42.82	16.67
						R. a. macrurus-form A						
9	♀♂	45.7-50.0 47.8±1.4	51.3-7.1 53.8±1.8	20.4-25.1 23.0±1.7	18.4-23.3 21.2±1.6	19.4-22.9 21.9±1.0	8.8-10.7 9.7±0.5	38.3-40.9 39.3±0.7	35.5-39.0 37.0±1.0	36.4-39.6 37.7±1.0	36.6-40.2 38.0±1.1	13.4-14.7 14.1±0.4
						R. a. macrurus-form B						
14	♀♂	50.6-54.2 52.6±0.8	45.7-52.3 49.7±1.9	22.0-27.7 24.0±1.5	18.4-21.9 20.3±1.0	21.7-23.8 23.2±0.6	10.0-11.0 10.4±0.2	41.0-44.3 42.4±0.8	39.3-43.7 40.5±1.0	40.3-44.3 41.6±1.0	41.5-43.3 42.4±0.6	14.3-15.9 15.0±0.4
						R. a. macrurus-form C						
60	♀♂	48.3-55.9 52.2±1.4	42.7-59.1 52.2±2.6	20.3-30.7 25.8±2.2	19.7-25.8 22.4±1.3	22.5- 27.2 24.9±0.8	9.0-11.6 10.4±0.5	38.6-46.1 42.5±1.4	36.2-43.0 40.0±1.3	37.9-44.3 41.2±1.2	38.9-44.8 41.8±1.2	14.3-17.8 15.6±0.6
						R. a. macrurus-form C-central Vietnam						
18	♀♂	49.0-53.2 50.6±1.1	48.2-57.5 52.5±2.7	17.5-27.5 23.7±3.2	19.0-22.7 21.2±0.9	21.7-25.0 23.6±0.8	10.1-11.0 10.5±0.2	39.9-43.2 41.5±0.9(17)	37.3-41.1 38.9±1.0(17)	38.4-41.9 40.1±0.9(17)	39.4-43.4 41.0±1.0(17)	14.1-16.1 15.2±0.6(17)
						Rhinolophus a. superans Holotype						
1	♀♀	50.97	--	--	20.36	25.35	11.66	--	39.52	40.67	41.45	15.10
						R. a. superans-Malay Peninsula						
66	♀♀	48.3-52.9 50.6±1.2	46.9-57.8 51.9±2.4	18.8-25.8 22.1±1.6	18.4-24.4 21.6±1.1	22.4-26.4 24.2±0.8	8.5-11.6 10.5±0.5	38.5-44.0 40.9±1.1	35.7-40.3 38.4±1.0	37.1-42.0 39.4±1.0	38.1-42.3 40.0±1.0	13.7-16.5 14.9±0.6

Table 2.2. Continued.

n	Sex	3D2P	4D1P	4D2P	5D1P	5D2P	GHN	GWN
				Rhinolophus a. tener Holotype				
1	♂♂	25.32	--	--	--	--	13.8*	9.50*
				Rhinolophus a. macrurus Holotype				
1	♂♂	26.69	--	--	--	--	--	--
				R. a. macrurus-form A				
9	♀♂	23.7-26.2 24.6±0.87	9.8-10.4 10.0±.1	13.0-15.0 14.4±0.6	10.8-12.0 11.4±0.4	12.6-14.4 13.4±0.5	12.7-14.7 13.6±0.7	9.8-10.5 10.0±0.2
				R. a. macrurus-form B				
14	♀♂	26.5-29.8 28.1±1.0	9.8-11.3 10.3±0.4	16.7-18.0 17.2±.3	12.0-13.4 12.6±0.4	13.5-14.5 14.0±.3	11.0-13.8 12.6±0.8(13)	7.5-9.0 8.2±0.4
				R. a. macrurus-form C				
60	♀♂	23.5-29.0 26.7±1.1	9.5-12.1 10.7±0.5	13.8-17.8 16.1±0.8	10.5-13.8 12.2±0.5	12.7-16.3 14.7±0.8	11.8-16.1 13.8±0.8	8.3-11.2 9.7±0.5
				R. a. macrurus-form C-central Vietnam				
17	♀♂	25.1-30.0 27.3±1.0	9.7-11.90 10.5±0.6	15.5-17.6 16.4±0.6	11.5-13.1 12.2±0.5	13.1-15.8 14.5±0.83	12.0-15.0 13.5±0.9(18)	8.2-9.7 9.0±0.3
				Rhinolophus a. superans Holotype				
1	♀♀	25.38	--	--	--	--	--	--
				R. a. superans-Malay Peninsula				
66	♀♂	23.8-27.7 25.7±0.8	9.2-11.5 10.3±0.5	11.2-16.3 14.9±0.8	10.6-12.6 11.5±0.5	9.6-14.6 13.1±0.7	10.4-15.9 14.0±0.9	8.7-11.0 10.0±0.5(63)

Table 2.2. Continued.

n	Sex	SL	CCL	ZYW	MAW	BW	GBW	ALSW	AMSW	IOW
					Rhinolophus a. tener Holotype					
1	♂♂	21.34	18.80	10.64	--	--	8.83	5.56	3.72	--
					Rhinolophus a. macrurus Holotype					
1	♂♂	22.91	20.11	11.38	10.78	--	9.44	5.60	3.71	--
					R. a. macrurus-form A					
9	♀♂	21.13- 22.53 22.11±0.44	18.46-19.75 19.40±0.42	10.69-11.51 11.05±0.25	10.04-10.55 10.30±0.16	9.77-10.38 9.99±0.20	9.27-9.49 9.40±0.06	5.72-6.20 5.94±0.17	3.90-4.45 4.10 ±0.19	1.95-2.32 2.18±0.11
					R. a. macrurus-form B					
8	♀♂	21.52-22.08 21.77±.21	18.85-19.37 19.07±0.19	10.38-11.14 10.71±0.24	9.97-10.36 10.14±0.14	9.54-9.95 9.77±0.12	9.01-9.42 9.18±0.15	5.31-5.66 5.44±0.11	3.63-4.06 3.78±0.15	1.83-2.42 2.00±0.19
					R. a. macrurus-form C					
60	♀♂	21.47-23.40 22.64±0.38	18.78-20.64 19.94±0.37	10.53-11.49 11.08±0.20	9.81-11.15 10.47±0.20	9.54-10.57 10.06±0.16	9.00-9.85 9.41±0.20	5.63-6.35 6.01±0.16	3.70-4.72 4.17±0.18	1.70-2.50 2.08±0.17
					R. a. macrurus-form C-central Vietnam					
19	♀♂	21.61-22.35 22.02±0.21(17)	18.96-19.78 19.34±0.20(17)	10.78-11.34 11.03±0.15	10.15-10.64 10.38±0.15	9.81-10.28 10.03±0.10	9.12-9.73 9.49±0.13	5.59-6.05 5.81±0.12	3.93-4.31 4.07±0.10	1.94-2.49 2.20±0.14
					Rhinolophus a. superans Holotype					
1	♀♀	22.38	19.65	11.32	10.84	--	9.41	5.60	3.71	--
					R. a. superans-Thai Peninsula					
66	♀♂	21.59-23.27 22.51±0.40	19.08-20.78 19.87±0.37	10.84-11.91 11.37±0.22	10.39-11.16 10.76±0.18(65)	9.80-10.67 10.32±0.20	9.27-10.14 9.77 ±0.20	5.82-6.72 6.15±0.19	3.76-4.67 4.26±0.21	2.00-2.81 2.35±0.17

Table 2.2. Continued.

n	Sex	PB	CM^3L	C^1C^1W	M^3M^3W	ML	CM_3L	CPH
				Rhinolophus a. tener Holotype				
1	♂♂	2.22	8.52	5.51	8.26	15.04	9.00	--
				Rhinolophus a. macrurus Holotype				
1	♂♂	2.42	9.01	5.77	8.76	15.92	9.67	--
				R. a. macrurus-form A				
9	♀♂	1.94-2.28 2.09±0.11	8.60-9.20 8.91±0.20	5.27-6.35 5.74±0.37	7.90-8.74 8.36±.28	14.81-15.95 15.40±0.35	8.94-9.65 9.3±0.23	2.95-3.21 3.06±.09
				R. a. macrurus-form B				
8	♀♂	1.70-2.20 1.89±0.15	8.20-8.69 8.46±0.15	5.26-5.84 5.56±0.19	7.56-8.03 7.78±0.18	14.57-14.90 14.71±0.12	8.53-9.06 8.80±0.18	2.83-3.06 2.93±0.06
				R. a. macrurus-form C				
60	♀♂	1.89-2.67 2.17±0.15	8.39-9.22 8.87±0.19	5.22- 6.13 5.79±0.18	7.73-8.48 8.06±0.15	14.80-15.94 15.50±0.23	8.75-9.67 9.27±0.19	2.52-3.42 3.12±0.15
				R. a. macrurus-form C-central Vietnam				
19	♀♂	1.63-2.52 2.04±0.18	8.45-8.87 8.67±0.11	5.21-5.99 5.74±0.18	7.77-8.33 8.05±0.14	14.70-15.50 15.10±0.18	8.87-9.31 9.08±0.11	2.83-3.29 3.03±0.11(18)
				Rhinolophus a. superans Holotype				
1	♀♀	2.63	8.63	5.66	8.46	15.52	--	--
				R. a. superans-Thai Peninsula				
66	♀♂	1.89-2.61 2.22±0.13	8.48-9.38 8.99±0.19	5.23-6.20 5.78±0.17(65)	7.92-8.86 8.34±0.18(65)	14.79-16.07 15.51±0.30(65)	9.03-9.82 9.41±0.20	2.87-3.63 3.18±0.14

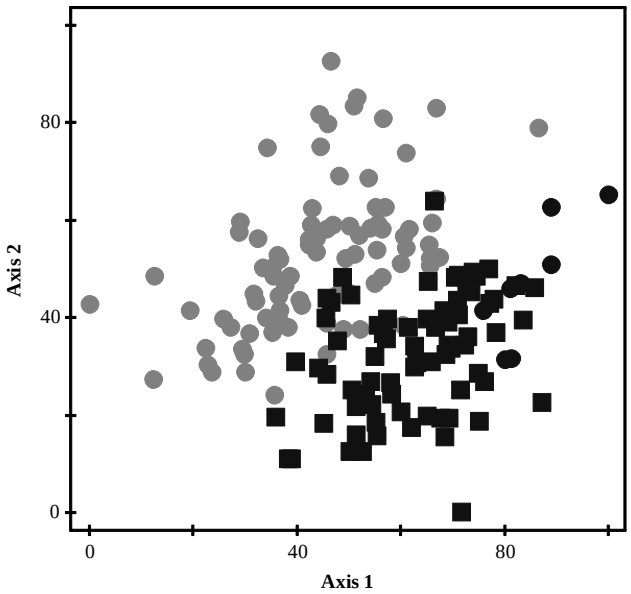

Figure 2.8. Principle component analysis (PCA) of nineteen external and cranial characters of 168 specimens. The morphological comparison between Sundaic subregion (black square) and Indochinese subregion (grey and black circle). Specimens from northern Cambodia (black circle) are largerly overlapped with Sundaic specimens.

Fifty-one bacula (27 bacula from Sundaic subregion, 24 from Indochinese subregion) were extracted for examination. All bacula observed were symmetrical (in dorsal view and ventral views), the basal part being bulbous with a long slender curved shaft. The basal portion is typically emarginated in the dorsal view and lateral views, with the dorsal aspect more compressed than the ventral aspect. Bacular characters showed a relatively divergent pattern between zoogeographic subregions with specimens from the Indochinese subregion (including Cambodia, Vietnam and central Myanmar) having a smaller and more curved shaft baculum, while Sundaic specimens have a larger and straighter shafted baculum (fig. 2.11A – C).

Table 2.3. Eigenvectors and eigenvalues of principle component analysis of nineteen external and cranial characters of 168 specimens; the values explain figure 2.8, the morphological comparison between Sundaic subregion and Indochinese subregion.

	Eigenvector		
Characters	**1**	**2**	**3**
FA	-0.8942	-0.0053	-0.0409
HF	-0.3719	-0.1768	-0.7725
2MT	-0.9173	0.0721	-0.0830
3MT	-0.9002	0.1358	-0.0529
4MT	-0.9259	0.1528	-0.0406
5MT	-0.9268	0.1707	-0.0332
1P3D	-0.7995	0.0340	-0.1200
2P3D	-0.6992	0.2868	0.1212
2P4D	-0.6856	0.4231	0.2013
1P5D	-0.7253	0.3758	-0.0026
2P5D	-0.7145	0.2948	0.1864
SL	-0.5323	-0.7665	0.1739
CCL	-0.4869	-0.7989	0.1734
MAW	-0.0733	-0.8317	-0.2550
BW	-0.0452	-0.8306	-0.2277
ALSW	-0.0425	-0.8672	-0.0504
CM^3L	-0.1204	-0.8925	0.1818
ML	-0.3408	-0.8288	0.2264
CM_3L	-0.1089	-0.8837	0.1187
Eigenvalue	7.613	6.222	0.993
% of total variation explained	40.068	72.815	78.044

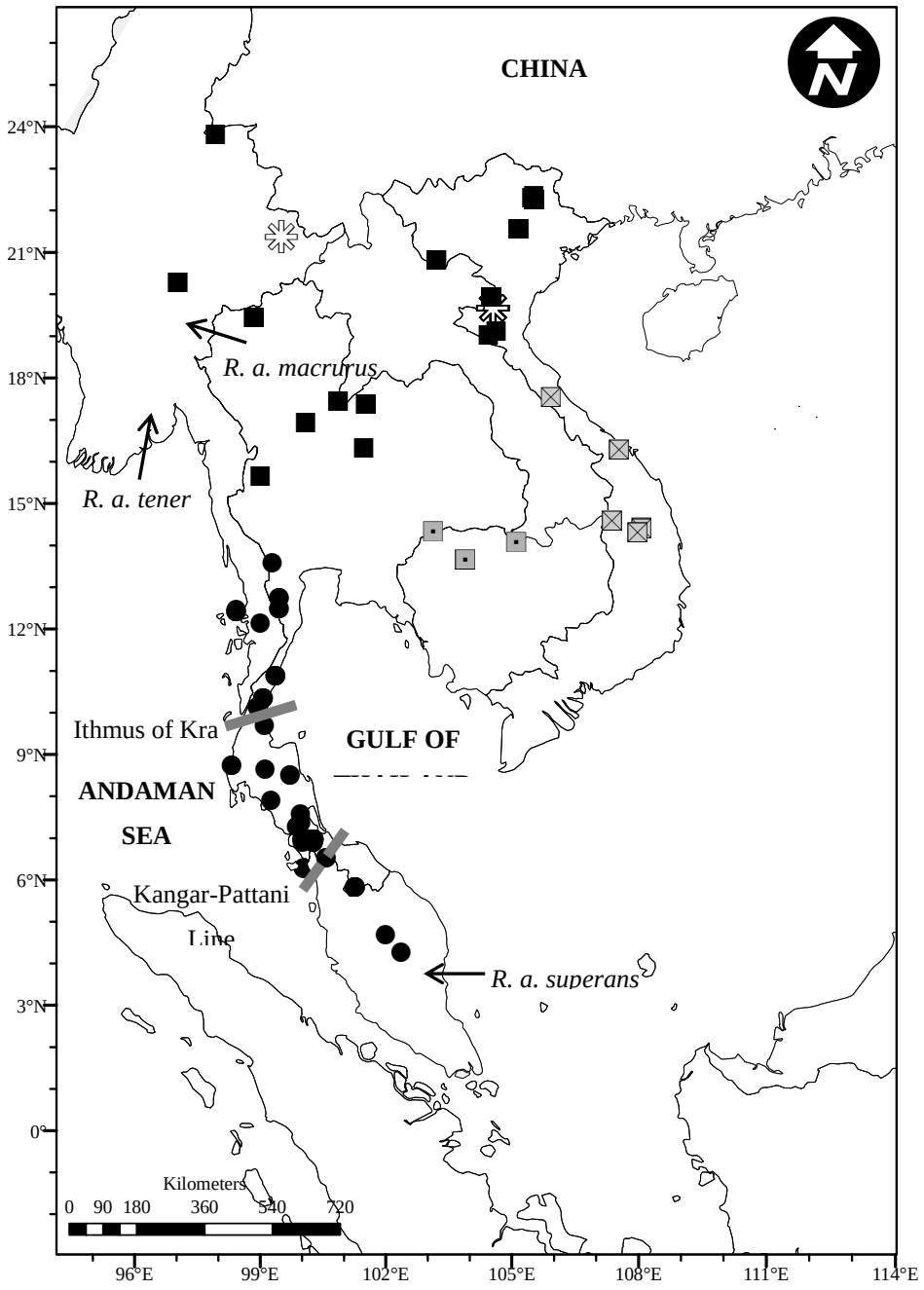

Figure 2.9. Morphological distribution of *Rhinolophus affinis* from mainland Southeast Asia. Circle (●) represents morphological form from Malay Peninsula; solid square (■) and crossed square (☒) represent morphological variation of form C; dot square (⊡) represents morphological form A whereas asterisk (✱) represents the un-described form B (*R.* cf. *affinis*). The defined forms (A, B, C) are corresponded to fig. 2.17. Grey solid line (Ithmus of Kra) and dashed lines (Kangar-Pattani Line) are the biota transition zones proposed in the peninsula. The arrows indicate the approximate localities of subspecific forms in the research area.

The Indochinese population generally has shorter and smaller sized bacula, characterized by a bulbous and more rounded basal portion abruptly depressed to a slender shaft (fig. 2.11B – C). The shaft is more curved, slender to the tip with no enlarged-portion at shaft (fig. 2.11B – C). The basal portion is broader and deeper in dorsal view. In Sundaic specimens, bacula are generally longer with less curved shaft (fig. 2.11A). The basal portion is broader and more elongated, gradually becoming slender at the tip which is rather pointed and typically shows enlargement characteristic of specimens from the Sundaic region (15 of 19 bacula from the peninsula Thailand have this character). The emargination of the basal portion in the dorsal view is not obvious, being mostly narrow and shallow, while ventral emargination is comparable to the Indochinese shaft (fig. 2.11A).

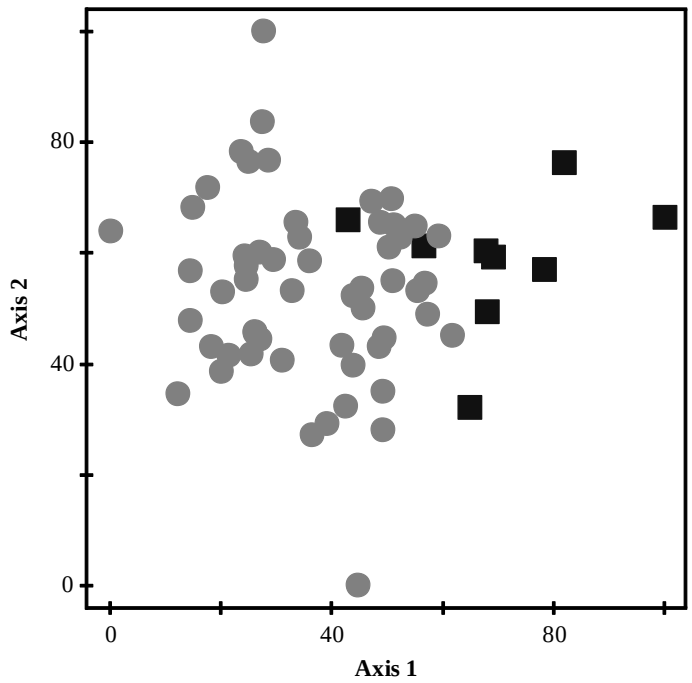

Figure 2.10. PCA based on eleven external and cranial characters of sixty-four specimens from Malay Peninsula (circle), northern Cambodian (square).

Table 2.4. Eigenvectors and eigenvalues of PCA of eleven external and cranial characters of sixty-four specimens from Malay Peninsula and northern Cambodian; the values explain figure 2.10.

	Eigenvector		
Characters	**1**	**2**	**3**
FA	-0.9139	0.1463	-0.1636
TIB	-0.8970	-0.0528	-0.2291
2MT	-0.8714	0.3612	0.1029
3MT	-0.8263	0.3825	0.0265
4MT	-0.8536	0.4281	-0.0247
5MT	-0.8971	0.3108	-0.0712
1P3D	-0.7018	0.3296	0.3255
SL	-0.7728	-0.5100	-0.1588
CCL	-0.7469	-0.5373	-0.2982
MAW	-0.7008	-0.5513	0.3228
BW	-0.7209	-0.5700	0.3041
Eigenvalue	7.274	1.867	0.513
% of total variation explained	66.125	83.100	87.763

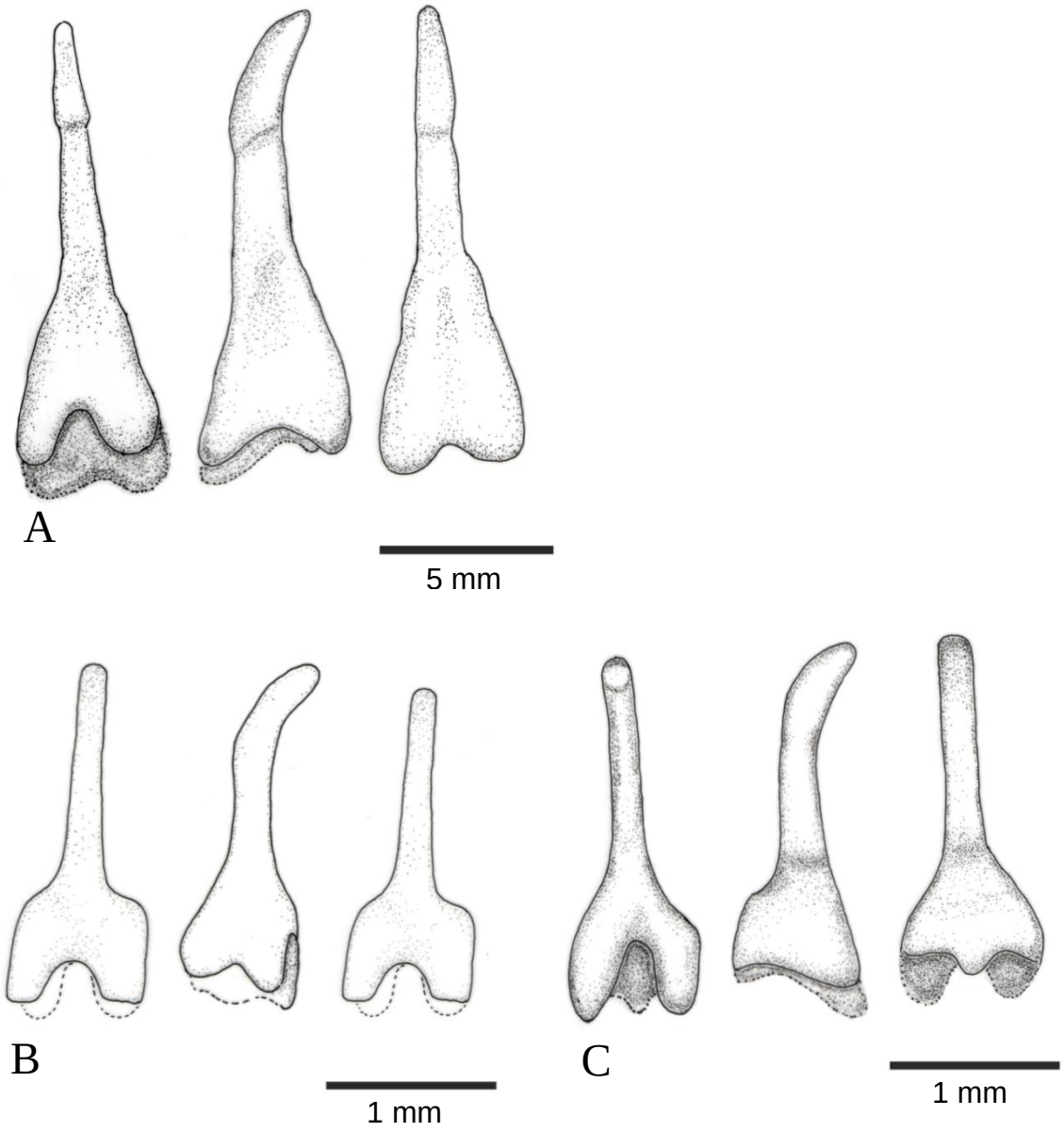

Figure 2.11. Baculum morphology of *R. a. superans* (A) and *R. a. macrurus* (B, C). A: specimen from Songkhla, central Malay Peninsula, B: specimen from Siem Reap, north-west Cambodia and C: specimen from Chiang Mai, north Thailand).

2.3.2. Echolocation

In total 47 echolocation calls were available from 21 localities in Cambodia, Thailand and Vietnam. These comprised 33 calls recorded from 12 localities in the current study and 14 calls published in Kingsada *et al*., (2011). In total, five calls were excluded from the analysis as they were all from male individuals from the same locality. As such, a *t*-test was run on 42 calls (29 males, 13 females) from the central Malay Peninsula. No significant variation in call frequency was found between the sexes ($p = 0.932$).

Correlations between peak call frequency (FMAXE) and size were explored. No correlation was found between FA and FMAXE ($y= 0.010x + 73.064$, $r = 0.004$, $P = 0.978$). However, a significant negative relationship was found between skull size (SL, $y= -3.789x + 158.839$, $r = 0.400$, $P < 0.002$; CCL, $y= -6.476x + 202.112$, $r = 0.629$, $P = 0.001$) and sound generating organs and chambers (GWN, $y= -4.319x + 116.610$, $r = 0.731$, $P = 0.001$; ALSW, $y= -9.493x + 131.790$, $r = 0.544$, $P = 0.002$; AMSW, $y= -6.142x + 99.649$, $r = 0.394$, $P < 0.002$).

Call frequency showed considerable variation throughout the region, ranging from 69-84 kHz. Four call frequency zones were designated; A = Indochinese low frequency (69-74 kHz) (upper north Vietnam and low south China); B= Indochinese high frequency (75-84 kHz) (north Thailand down to south Vietnam, Cambodia and upper peninsula Thailand) (table 2.5 and fig. 2.12); C= Sundaic low frequency (69-72 kHz) (Songkhla up to Chumphon); D = Sundaic high frequency (77-78 kHz) (Thai-Malay border down peninsula Malaysia). A mixture of low and high call frequencies (69-76 kHz) were recorded from zone C, around Chumphon and Ranong Provinces between 9^{o}-11^{o}N (fig. 2.12).

Table 2.5. The summary data for frequency maximum energy (FMAXE) of *R. affinis* from mainland Southeast Asia.

Locality	**No. bats**	**No. calls**	**Frequency (kHz)**	**Source**
North Vietnam		13	72.1±0.9 71.4–73.4	Current study & Furey *et al.*, (2009)
South Vietnam	3	36	81.9±0.23 81.2–82.4	O'Shea & Gore (2011) (personal communications)
North Thailand		13	76.4±0.5 75.8–77.7	Current study & Kingsada *et al.*, (2011)
Cambodia			-- 76.1–79.9	Kingsada *et al.*, (2011)
Central Malay Peninsula		31	70.9±0.7 69.5–72.6	Current study & Kingsada *et al.*, (2011)
Southern Malay Peninsula	10	10	78.7±0.7 77.3–79.3	Current study

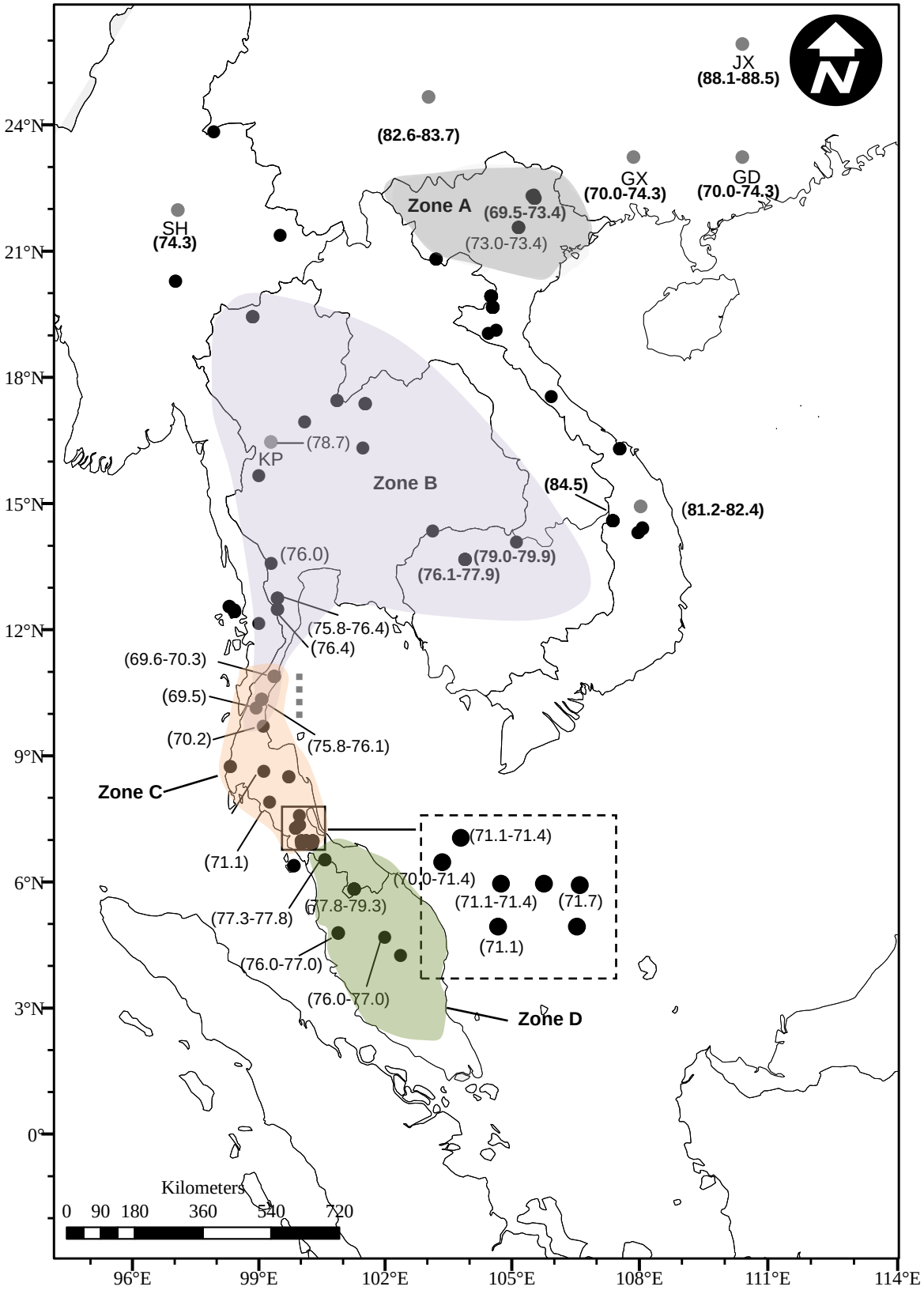

Figure 2.12. Patterns of echolocation call frequencies of *Rhinolophus affinis* within mainland Southeast Asia. Black circles respond to research locality map in figure 2.1-

whereas grey circles are approximate localities from literature, KP=Kamphaeng Phet, JX=Jiangxi, GD=Guangdong, GX=Guangxi, QN=Quang Nam, SH=Shan and YN=Yunnan. Grey shades are FMAXE zones and a vertical dashed-line demarks a locality where zone B and C frequencies were found overlapped. Values in parentheses are peak frequency (FMAXE) in kHz which are bold figures are based on literature.

2.3.3. Genetics

In total 26 sequences of hyper-variable gene (control region) and 20 sequences of cytochrome oxidase subunit I (COI) were available for genetic analysis.

Results from both maximum likelihood (ML) and Bayesian analysis (BA) showed similar topologies. For both genes analyzed, three well supported clades were recovered, clade I, II (D-loop and COI) and III (COI) (fig. 2.13, 2.14). Clade I comprised of all sequences from the peninsula region including peninsula Myanmar, peninsula Thailand and peninsula Malaysia, and was therefore defined as the Sundaic clade, representing *R. a. superans*. Clade II and III comprised sequences from north of the peninsula to the north, and these combined were defined as the Indochinese clade, representing *R. a. macrurus*. Clade II comprised sequences from North Vietnam (Hoang Lien Son and Vinh Phuc), north Lao (Vientiane and Xiangkhouang), north Thailand (Chiang Mai), central Myanmar (Shan), north-west Cambodia (Siem Reap), south Vietnam (Thua Thien-Hue and Kon Tum) and lower south China (Guangxi) while clade III comprised sequences which broadly overlapped geographically with clade II, comprising sequences from south Vietnam (Lam Dong and Quang Nam), North Vietnam (Hoang Lien Son and Lao Cai) and upper south China (Hunan) (fig. 2.15, 2.16). The uncorrected pairwise sequence distances (p-distance) between the Indochinese subregion and the Sundaic subregion were 9.1% (D-loop) and 2.4% (COI).

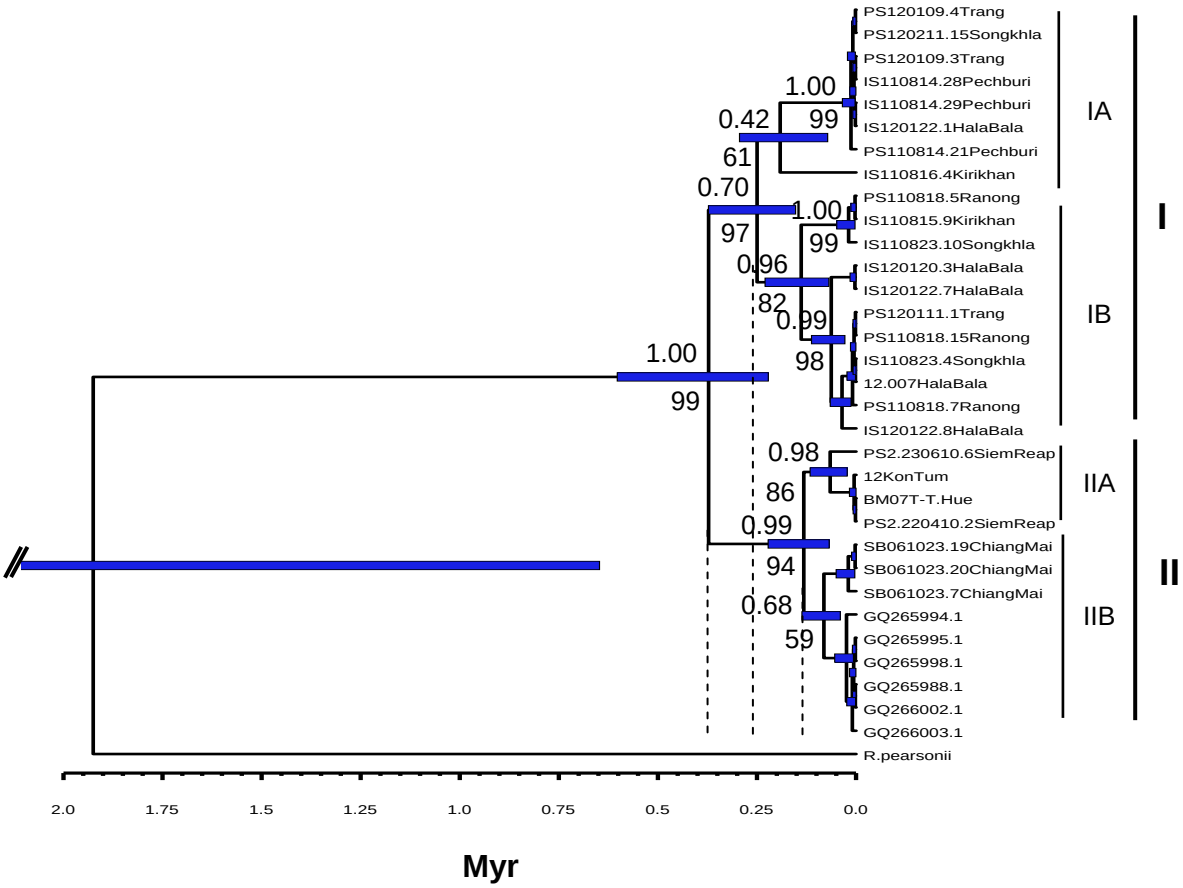

Figure 2.13. Bayesian Phylogenetic tree based on control region. Numbers above and below the branches are posterior probabilities and bootstrap support values (1,000 iterations) respectively. Two recovered clades, I and II represent two subspecies *R. a. superans* and *R. a. macrurus* respectively. Two subclades were recovered within each main clade. TMRCA are scaled by Beast, for which the 95% credible intervals are shown in blue bars.

In analyzing D-loop data, two subclades were recovered within each main clade: Subclade IA, IB and IIA and IIB nested within clades I and II respectively. Subclade IA and IB were both from the peninsular Thailand area of the Sundaic region (fig. 2.16) yet showed rather high genetic distance (8.3%) with a bootstrap support of 97%. The genetic distance between IIA and IIB was lower (5.0%). Sequences of clade IIA were from south Vietnam (Kontum and Thua Thien-Hue-provinces) and northwest Cambodia (Siem Reap province), while clade IIB comprised

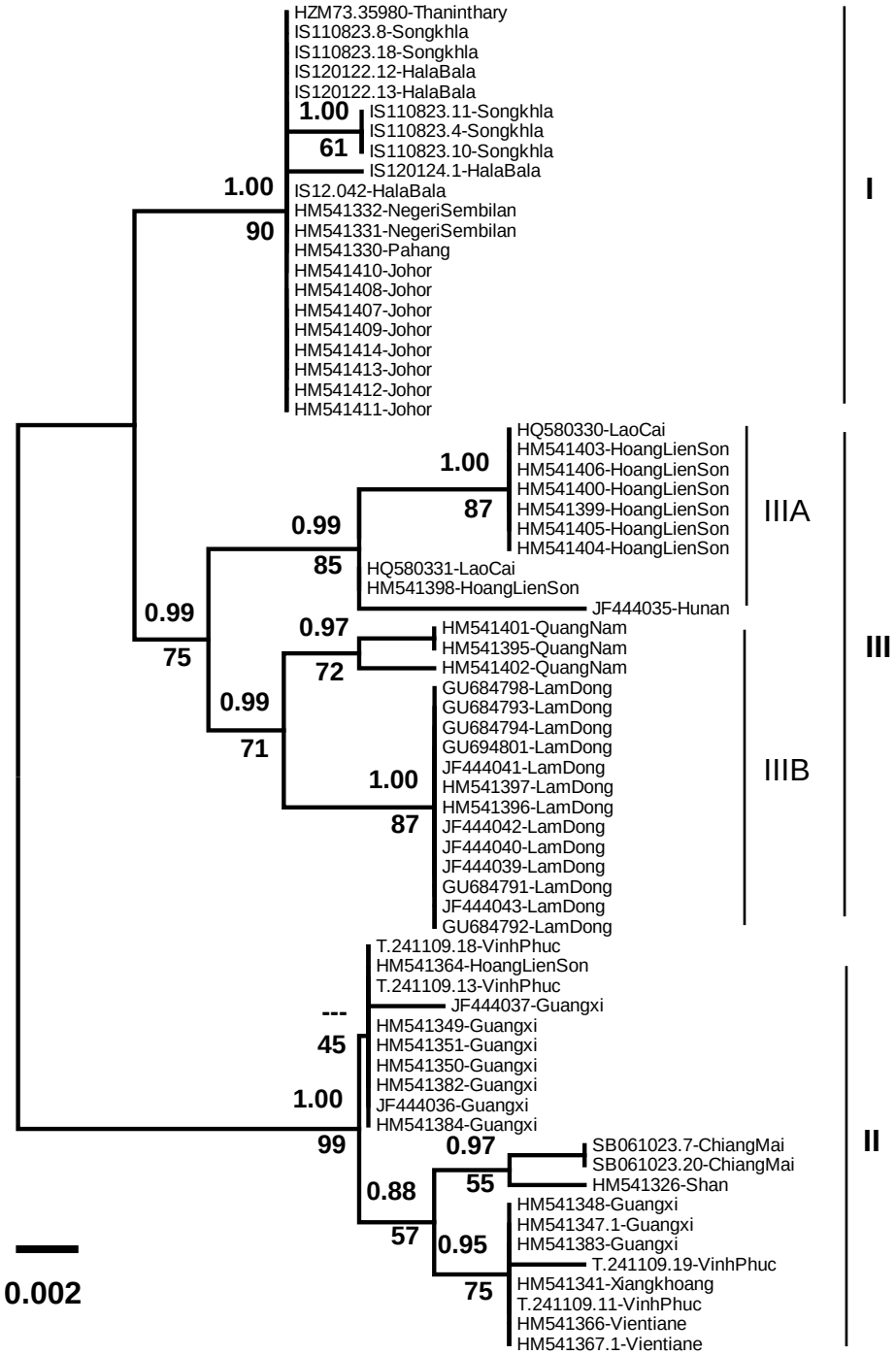

Figure 2.14. Maximum Likelihood tree based on COI gene. Numbers above and below the branches are posterior probabilities and bootstrap support values (1,000 iterations) respectively. An out group clade (*Rhinolophus stheno*) was excluded in order to enlarge spaces between the tree branches, therefore allowing the manipulation of support values.

sequences from Chiang Mai province and south China. The split between IIA and IIB was supported by a bootstrap value of 94%. In analysis of COI, two subclades were recovered within clade III (subclades IIIA and IIIB) with a genetic distance of 1.7% between them. Clade IIIA comprised sequences from upper north-west Vietnam (Hoang Lien Son and Lao Cai) and upper south China (Hunan), and clade IIIB comprised sequences from central Vietnam (Thua Thien-Hue) and South Vietnam (Lam Dong) (fig. 2.15).

Among the Indochinese clades (clades II and III combined), clade III shares a recent common ancestor with the Sundaic clade (clade I) rather than to its closest geographical clade (clade II). This is also reflected by the genetic distance 1.7% (clade I versus II) and 2.9% (clade II versus III). Bayesian estimates of time to the most recent common ancestor (TMRCA) provided effective sample size values >500 for all parameters. The inferred TMRCA for all recovered clades, including Sundaic and Indochinese clades (I versus II) was 391 000 years BP (95% CI 222 000-603 000) (fig. 2.13), corresponding to a period of Pleistocene glacial cycling. The TMRCA for IA versus IB was 256 000 years BP (95% CI 152 000-372 000), whereas the TMRCA for IIA versus IIB was slightly more recent at 139 000 years BP (95% CI 68 000-222 000).

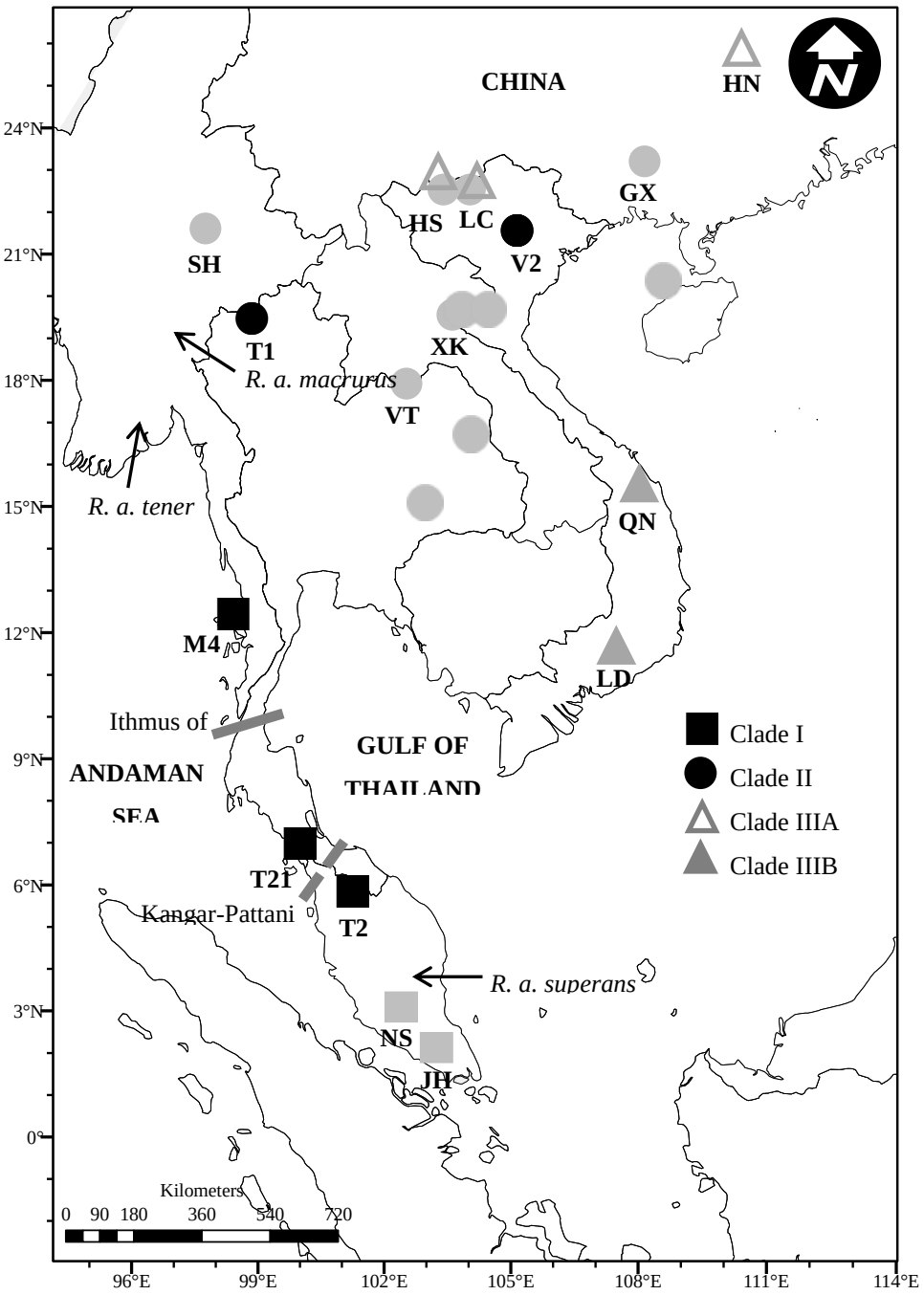

Figure 2.15. Cytochrome c oxidase subunit I (COI) distribution of *Rhinolophus affinis*. The shape of the symbols corresponds to the clades defined in figure 2.14. Black symbols are sequences from particular localities of the current study whereas grey symbols are sequences from genbank (HN=Hunan, HS=Hoang Lien Son, QN=Quang Nam, GX=Guangxi, LC=Lao Cai, LD=Lam Dong, SH=Shan, VT=Vientiane, XK=Xiangkhouang). Grey solid line (Ithmus of Kra) and dashed lines (Kangar-Pattani Line) are the biota transition zones proposed in the peninsula. The arrows indicate the approximate localities of subspecific forms in the research area.

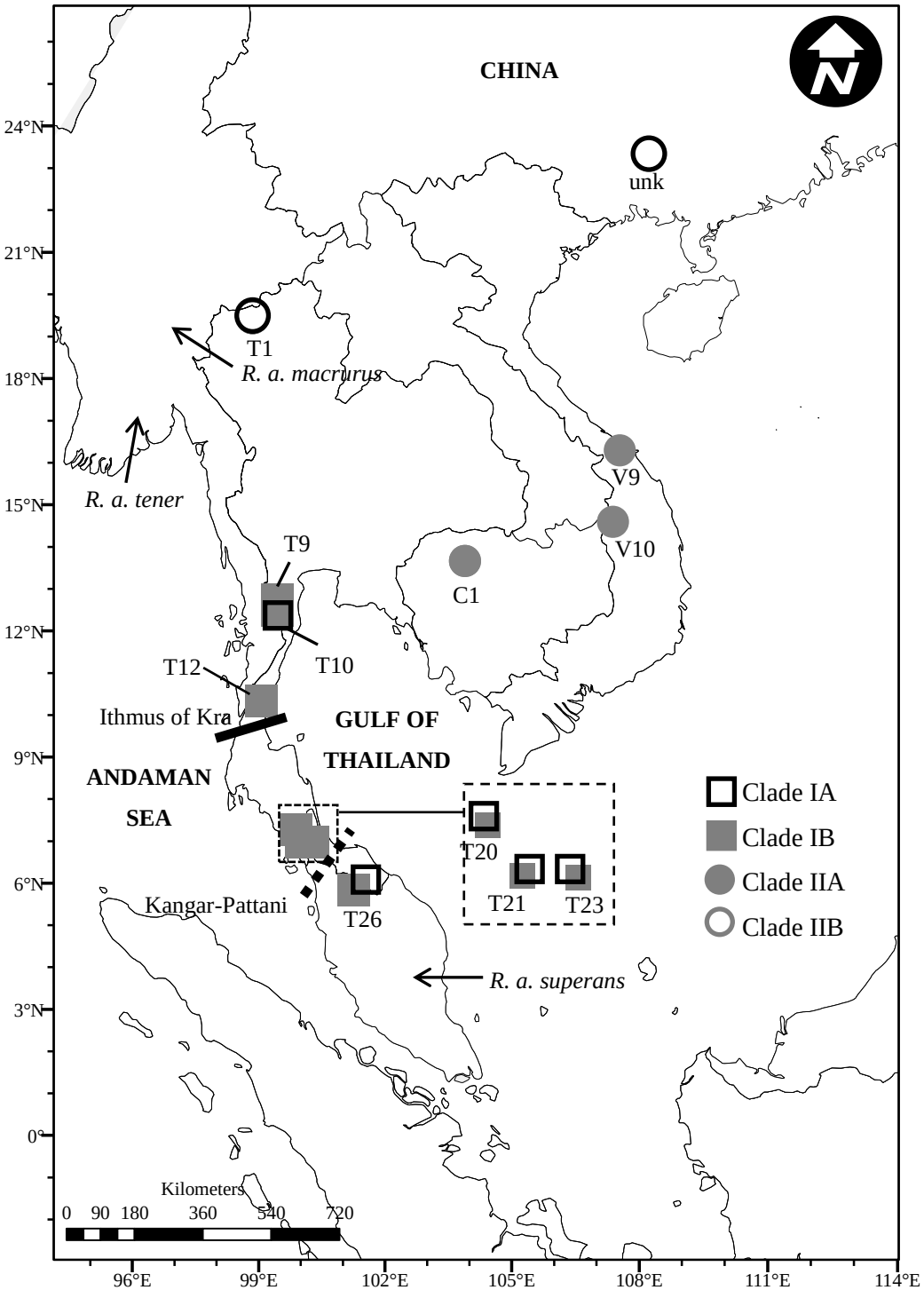

Figure 2.16. Control region gene (D-loop) distribution of *Rhinolophus affinis* from mainland Southeast Asia. The shape of the symbols corresponds to the clades defined in figure 2.13. Unk=unknown locality in China where sequences were accessed from the genbank. Black solid line (Ithmus of Kra) and dashed lines (Kangar-Pattani Line) are the biota transition zones proposed in the peninsula. The arrows indicate the approximate localities of subspecific forms in the research area.

2.3.4. Variation within Indochinese subregion population

In the Indochinese subregion, noteworthy morphometric variations were observed. A multivariate analysis based on 15 external and cranial characters classified the Indochinese specimens (referred to *R. a. macrurus*) into three forms (fig. 2.17, 2.9). Northern Cambodia and south-eastern Thailand (Surin Province) specimens (form A) were smaller in forearm, tail, foot and wing measurements (table 2.2). This variation was supported genetically with D-loop gene results (sub clade II A). Sequences nested with those from central Vietnam (Kon Tum and Thua Thien-Hue) forming a sister clade to Chiang Mai and south China sequences (II B). The second form (form B) was found from lower North Vietnam (Nghe An province) and east Myanmar and was characterized by a smaller noseleaf and smaller skull measurements (table 2.2). Unfortunately, genetic data was not available for specimens of this form as only old tissue was available for analysis which did not sequence well. The third form (form C) was more widespread, found from central Myanmar, northern Thailand, northern and southern Vietnam. This form has comparable skull morphology to form A, which in turn is generally larger than form B. Form C was supported by available COI sequences from Chiang Mai and Vinh Phuc, and forms its own clade (subclade IIIB) as a sister clade to III A (sequences from Lao Cai and Hoang Lien Son, Vietnam and Hunan, China).

Based on noseleaf characteristics, form B has a notably small noseleaf (fig. 2.18C) with a less rounded horseshoe (rather elongated posteriorly) with the anterior median emargination of the horseshoe being rather deep and narrow. The sella is narrow and moderately high (fig. 2.18G). The base of the sella is always enlarged, being about 30% reduced in size compared to the typical forms of A and C. The lateral margin varies from slightly concave in the middle to almost parallel sided, with the tip varying from rounded to almost squared-off in some individuals. The internarial cup is small, the lateral margin of the cup being well defined and raised which results in a deeper median internarial cup. The connecting process is small, slender, rounded and almost pointed. The lancet is slender and narrower with an elongated tip. The basal part is not obviously larger than the middle and tip respectively, resulting in a less triangular shaped lateral margin. Forms A and C have comparable noseleaf morphology, however specimens generally have a wider

horseshoe, with the anterior median emargination being deeper and with a well-defined notch. The sella is broader and higher; the connecting process is larger and more rounded; the lancet is broader and more enlarged at the base and the internarial cup is also broader.

In skull morphology, form B has smaller skull measurements in general. This population was found to have a smaller braincase and rostrum, narrower inter-orbital width, shorter palatal bridge and smaller nasal depressions (fig. 2.19F and 2.21E, F). This population has more compressed rostral compartments (anterior lateral swellings, anterior median swellings and posterior median swellings) whereas form A and C have broader palatal bridges (fig. 2.19D), larger nasal inflations and more bulbous compartments (fig. 2.21C, D). The central Vietnam population (form C) is supported genetically by COI results (clade IIIB).

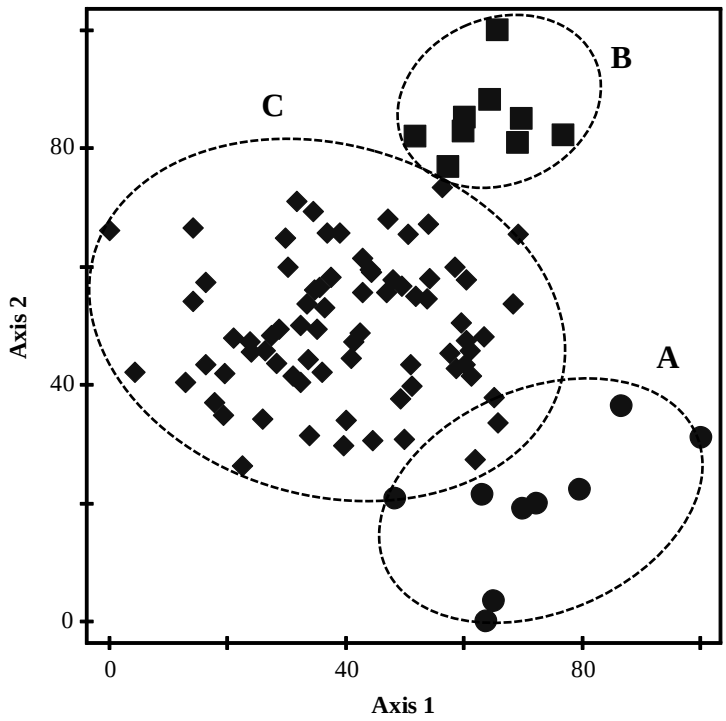

Figure 2.17. PCA based on fifteen external and cranial characters of specimens from Indochinese subregion. A total of ninety-four specimens were classified into three groups. Specimens from Shan (Muse and Taung Pauk), northern Thailand, north, central and southern Vietnam clustered as group C (diamond); central Vietnam and an individual from east central Myanmar (Keng Tung) formed group B (square) while Cambodian specimens formed the third group A (circle).

Table 2.6. Eigenvectors and eigenvalues of PCA of nineteen external and cranial characters of ninety-four specimens from Indochinese subregion; the values explain figure 2.17.

	Eigenvector		
Characters	**1**	**2**	**3**
FA	-0.8098	0.4285	0.1554
TIB	-0.8852	0.1377	-0.1298
2MT	-0.8089	0.4695	0.0456
3MT	-0.7820	0.5071	0.2177
4MT	-0.8079	0.5109	0.2081
5MT	-0.7801	0.5336	0.1844
1P3D	-0.7565	0.3615	-0.3457
2P5D	-0.6667	0.2083	-0.6033
SL	-0.8609	-0.3938	0.0183
CCL	-0.8627	-0.3977	0.0455
MAW	-0.6885	-0.3162	0.0332
ALSW	-0.6116	-0.5559	-0.0739
CM^3L	-0.5818	-0.7068	0.1711
ML	-0.7184	-0.6070	-0.0530
CM_3L	-0.5560	-0.7184	0.0713
Eigenvalue	8.481	3.506	0.697
% of total variation explained	56.537	79.912	84.561

2.4. SUBSPECIES DIAGNOSIS

2.4.1. *Rhinolophus macrurus*, Andersen 1905

R. a. macrurus Andersen, 1905: Taho, Karenee, Myanmar

In comparison to *R. a. superans*, this subspecies can recognized by being average larger in external measurements and smaller in cranio-dental measurements.

External: Forearm (FA) is larger, 45.7 – 55.9 mm (min – max); metacarpal and phalanx are larger (table 2.2).

Cranio-dental: The condyle-canine length (CCL) is smaller, 18.46 – 20.64 mm (table 2.2); the zygomatic width (ZYW) is narrower, 10.53 – 11.51 mm; the greatest braincase width (GBW) is narrower, 9.00 – 9.85 mm; anterior lateral swellings (ALSW) are narrower, 5.59 – 6.35 mm and anterior median swellings are narrower (AMSW), 3.70 – 4.72 mm.

Baculum: The baculum is shorter and more curve dorsal ward (fig. 2.11).

2.4.2. *Rhinolophus* cf. *affinis* - form B

Pu Hoat Nature Reserve, Nghe An Province, Vietnam and Keng Taung Shan State, Myanmar

In comparison to *R. a. macrurus*, this taxon has similar external measurements, however can be recognized by being average smaller in noseleaf characters and cranio-dental measurements.

External: The size of the horseshoe (GWN) is smaller, 7.5 – 9.0 mm (table 2.2). The sella is small, at least 30% reduce in size; the connecting process is smaller. The secondary horseshoe is more developed, as it projects anteriorly and clearly visible. The lancet is narrower in width (fig. 2.18).

Cranio-dental: The skull length (SL) is smaller, 21.52 – 22.08 mm (table 2.2); zygomatic width (ZYW) is narrower, 10.38 – 11.14 mm; ALSW are narrower 5.31 – 5.66 mm; AMSW are narrower, 3.63 – 4.06 mm; the palatal bridge (BP) is smaller 1.70 – 2.20 mm; the upper toothrow (CM^3L) is shorter, 8.20 – 8.69 mm; the lower toothrow (CM_3L) is shorter, 8.53 – 9.06 mm; and the mandible length (ML) is shorter, 14.57 – 14.90 mm.

Baculum: The baculum is similar in size to *R. a. macrurus*.

2.5. DISCUSSION

Our results indicate that there are at least three forms of *R. affinis* distributed within mainland Southeast Asia; the Sundaic form, the Indochinese form (comprising of forms A & C), and form B. Since the Sundaic and Indochinese forms are clearly differentiated by stable external, craniodental and baculum characters that strong supported by genetic data as well as being geographically isolated, we refer the Indochinese form here to *R. a. macrurus* and the Sundaic form to *R. a. superans* following Andersen (1905a) and as also recognized by Lekagul and McNeely (1977), Csorba *et al.*, (2003) and Kingsada *et al.*, (2011). The Indochinese form B is an undescribed form, and additional echolocation and genetic data are required to establish its status.

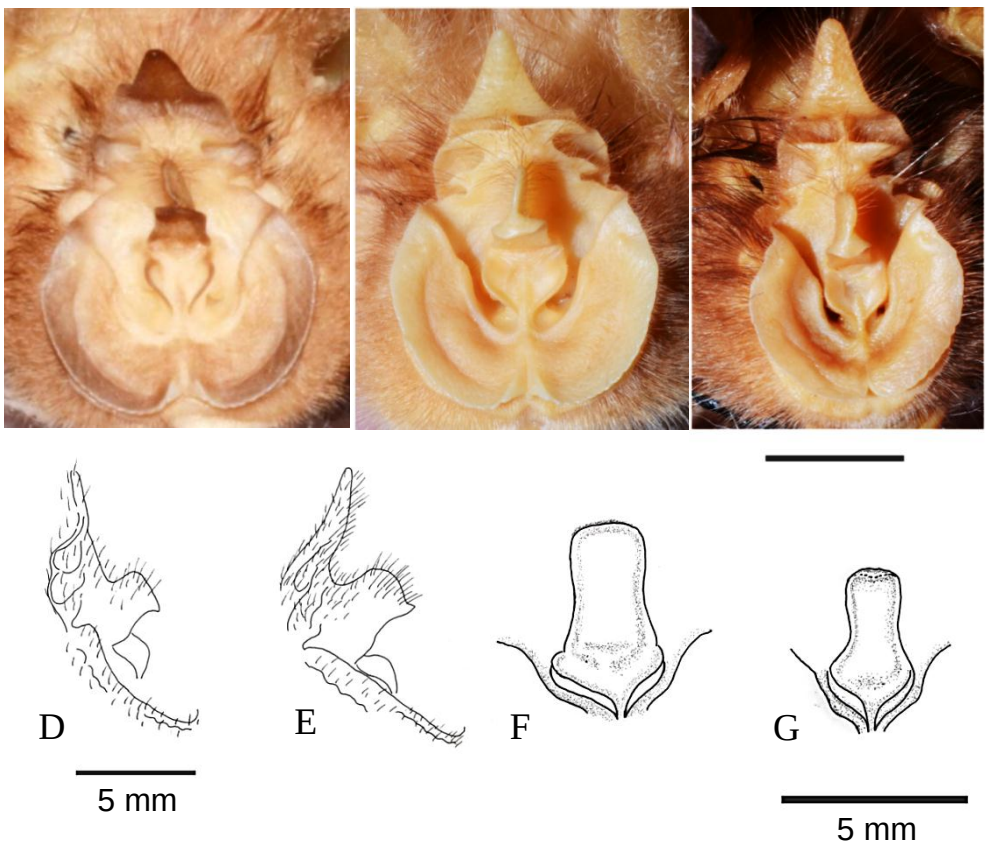

Figure 2.18. Noseleaf variation of *Rhinolophus affinis*. A: *R. a. superans* (specimen from Songkhla, central peninsula Thailand); B, E, F: *R. a. macrurus* (specimen from Vinh Phuc and Nghe An, north Vietnam); C, D, G: form B, (specimen from Nghe An, north Vietnam).

Based on morphological and genetic data, it is clear that *R. a. macrurus* and *R. a. superans* meet north of the Kra Isthmus to Ratchaburi province, which is a similar pattern to that observed in snakes (Pauwels *et al.*, 2003; Pauwels *et al.*, 2002), other bats (Hughes *et al.*, 2011; Woodruff & Turner, 2009) and non-volant mammal species (Woodruff & Turner, 2009). Form B is known from a small disjunct distributional area including lower north Vietnam and east Myanmar.

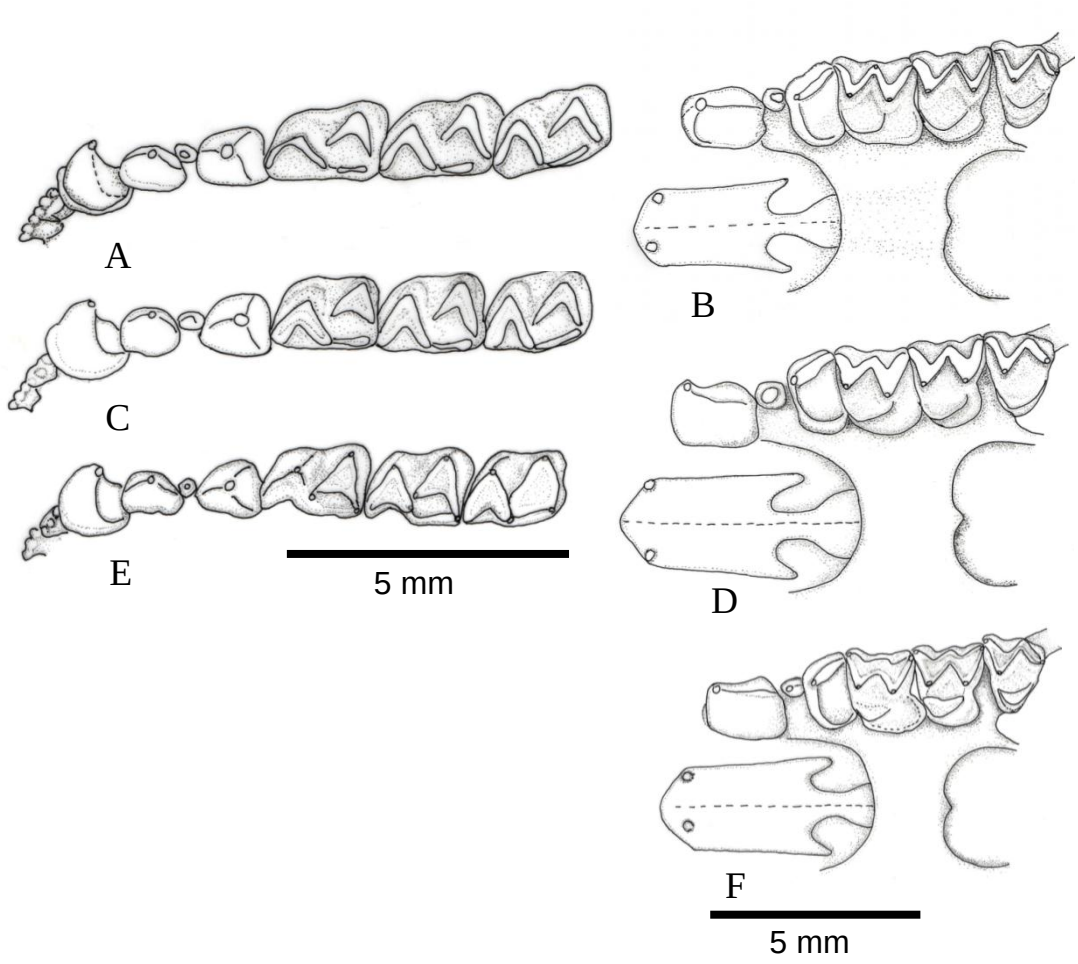

Figure 2.19. Dental and palatal bridge variation of *Rhinolophus affinis*. A, B: *R. a. superans* (specimen from Surathani, central peninsula Thailand); C, D: *R. a. macrurus* (specimen from Vinh Phuc, north Vietnam); E, F: form B, (specimen from Nghe An, lower north Vietnam).

As mentioned in the introduction, we were not able to define the subspecific form of *R. a. tener* which was described from Pegu, south west Myanmar (see fig. 2.1) due to lack of available material. This subspecies was described as being small in size (Andersen, 1905a), with small ears, less than 20 mm (Sinha, 1973); a narrow horseshoe (Sinha, 1973); a short tail and rather long tibia; a short skull; and narrow nasal swellings and brain-case and a short tooth-row (Andersen, 1905a). Although *R. a. tener* is smaller in size compared to average measurements of *R. a. macrurus*, measurement ranges overlap (table 2.2). Only ear length, skull length, braincase and anterior lateral swellings of holotype specimens appeared to be smaller than minimum values for *R. a. macrurus*. Accurately defining *R. a. tener* is therefore difficult due to insufficient samples and high degree of morphological variation in *R. affinis.*

The morphological transition rule "the more southern or south-eastern the habitat, the longer the ears, the broader the horseshoe, the longer the tibia, the larger the skull, the broader the nasal swellings, and the longer the toothrows" proposed by Andersen (1905a) was incongruent with form B recorded from lower northern Indochinese subregion. This form has an overlapping distribution with *R. a. macrurus* yet appears to be smaller in most noseleaf and craniodental characters. In addition, *R. a. macrurus* from central and southern Vietnam are smaller in ears, tibia, skull and nasal swellings compared to more northerly populations within the Indochinese subregion (fig. 2.20; table 2.7). Therefore the morphological rule is unlikely to be generally accepted since *R. affinis* shows high intraspecific variation in morphology. In general we found that the horseshoe and nasal swellings size are negatively correlated with the echolocation call frequencies. The rule is likely true when observing broader distribution ranges, e. g. comparisons between *R. a. himalayanus*, *R. a. macrurus*, *R. a. superans* and *R. a. princeps*, but exceptions to the rule occur when more samples are examined from each region, such as the small form from Kangean Islands (Bergmans & Van Bree, 1986; Thomas, 1997).

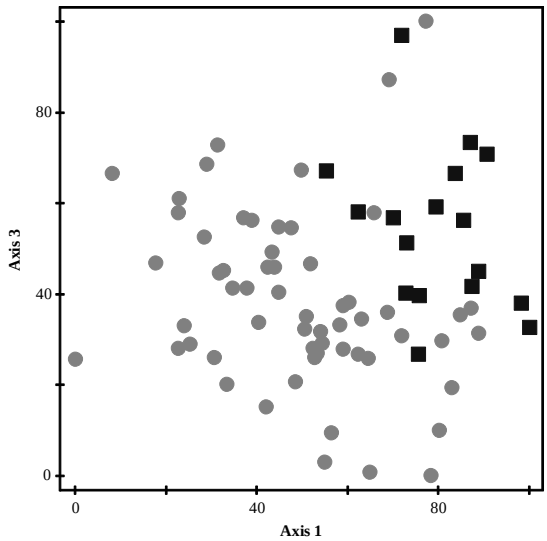

Figure 2.20. Seventeen individuals of *R. a. macrucrus* from southern Vietnam (square) shows the patterns of being relatively isolated from others fifty-three specimens from the region (circle). PCA based on eleven characters of seventy specimens from Indochinese subregion.

Table 2.7. Eigenvectors and eigenvalues of PCA of eleven external and cranial characters of seventy specimens from Indochinese subregion; the values explain figure 2.20.

	Eigenvector		
Characters	**1**	**2**	**3**
FA	-0.8749	0.1734	-0.1865
TIB	-0.8378	0.1779	-0.3676
2mt	-0.8088	0.4436	0.0432
3mt	-0.8274	0.4357	0.1751
4mt	-0.8686	0.4168	0.1188
5mt	-0.8315	0.3993	0.2261
SL	-0.8690	-0.3465	-0.1358
CCL	-0.8717	-0.3623	-0.0873
CM3L	-0.7189	-0.5483	0.3042
ML	-0.8516	-0.3602	-0.1925
CM3L	-0.6825	-0.5805	0.2045
Eigenvalue	7.475	1.800	0.465
% of total variation explained	67.955	84.320	88.549

2.5.1. Genetics

Phylogenetic analysis of COI and control region gave comparable results, supporting the separation of the Sundaic and Indochinese forms, which are referred to *R. a. superans* and *R. a. macrurus* respectively, following current taxonomy (Csorba *et al.*, 2003; Kingsada *et al.*, 2011; Koopman, 1994). Genetic divergences observed were also supported by morphometric characteristics, namely cranial and bacular data.

The genetic separation observed generally agrees with existing biogeographical demarcations for the region (de Bruyn *et al.*, 2005; Hughes *et al.*, 2011; Hughes *et al.*, 2003; Khan *et al.*, 2010; Woodruff & Turner, 2009). The genetic split in *R. affinis* is very recent (*c.* 400, 000 before present[BP]), just falling within the glacial period of the Pleistocene epoch when sea levels fluctuated to between 60 m to 80 m below present sea level. At this time large areas of the peninsula emerged and connected many present-day islands (Woodruff & Turner, 2009). Therefore, the rapid fall in Pleistocene sea levels (Woodruff & Turner, 2009), climatic zones (Hughes *et al.*, 2011) and phytogeographical transitions (Baker *et al.*, 1998; Good, 1964; Keng, 1970; Richards, 1996; van Steenis, 1950; Whitmore, 1984; Wikranmanayake *et al.*, 2002) likely explain the genetic variation observed in *R. affinis* rather than the high sea level hypothesis when marine waters (100 m, 150-220 m above the present level) breached the peninsula during the Neogene period (Hughes *et al.*, 2003; Hutchison, 1989). Therefore, Pleistocene climate may have played an important role in shaping the genetic profile of *R. affinis* from the peninsula (Mao *et al.*, 2010).

The two subclades (control region, IA & IB) observed from the Sundaic region which represent *R. a. superans* are of interest as the genetic cline was not supported by morphometric data, bacular morphology, echolocation call frequencies or biogeographical demarcations. The split of the subclades (IA versus IB) was more recent (*c.* 200, 000 years BP) and also falls within the glacial period of Pleistocene. However, seven sequences (IS11.65, IS16.8, IS11.64, IS14.16, IS15.10, IS14.17 and IS12.042) which were available for COI analysis did not show the separation pattern (clade I of COI). Further population research is therefore recommended to clarify this cryptic genetic variation, and fast-mutating genes such as D-loop gene and microsatellites would be appropriate for such studies (Chen *et al.*, 2006; Mao *et al.*, 2010).

The genetic variation within the Indochinese subregion is also of note, with clades II and III of COI suggesting there may be two lineages present. The clades of both lineages showed large genetic distances despite their geographical overlap. The separation was partially supported by morphology, with specimens from clade III (south Vietnam) relatively smaller in many characters (fig. 2.20, table 2.2). Echolocation calls from south Vietnam were also higher in frequency (O' Shea & Gore, 2011; Thong, 2011). This lineage is distributed in the eastern part of Indochinese subregion extending from upper south China (a sequence from Hunan, China) down to central and southern Vietnam and Cambodia (recovered IIA of control region). It may have connected with the Sundaic lineage during the glacial period of the Pleistocene, resulting in closer genetic relationships with the Malaysia Peninsula clade (I versus III) rather than the Indochinese clade II. This linkage may be attributable to the Pleistocene climate during glacial periods, when the sea level dropped around 100 m below present levels, exposing vast areas of shallow seabeds on the Sunda shelf which formed migration ways between two subregions (Voris, 2000; Woodruff, 2003). For instance, the peninsula-restricted rhinolophid, *R. stheno*, was recorded as having an isolated population in central Vietnam (Bach Ma National Park) yet shares similar morphological characters with the peninsula population (Soisook *et al.*, 2008). A similar pattern was also observed within the giant fresh water prawn *Macrobrachium rosenbergii* (de Bruyn *et al.*, 2005). Lineage clade II occurs from central to westward areas within the Indochinese subregion, extending from the coast of south China (Guangxi) to north Vietnam (Vinh Phuc and Hoang Lien Son), northern Lao PDR (Vientiane and Xiangkhong) central Myanmar (Shan) and northern Thailand (Chiang Mai). This clade overlaps in distribution with subclade IIIA yet showed highest the genetic distance (3%) among the recovered clades, strong evidence which supports the hypothesis that two distinct taxa are present.

2.5.2. Echolocation and morphology

The variation in echolocation call frequency of *R. affinis* throughout the region resulted in the taxonomic status of this widespread species being re-examined (Kingsada *et al.*, 2011). Call frequency has been found to be a useful tool for

classification in bats, particularly among cryptic species (Thabah *et al.*, 2006). Echolocation call frequencies of *R. affinis* in mainland Southeast Asia were comprehensively documented by Kingsada *et al.*, (2011), but do not appear to be congruent with morphology or genetics.

The analysis of call frequencies showed a cline between both subregions to the lower south area of the peninsula, just around the Isthmus of Kra. High call frequencies (≥ 75 kHz) were found to have a southern limit at Ranong province while lower call frequencies (≤ 71 kHz) were found to have their northern limit at Chumphon province. In Ranong, both high and low frequencies were recorded. However, the high frequencies recorded from north of the Isthmus of Kra (Ranong, Prachuap Kiri Khan and Phetchaburi) were not supported by morphology and genetic data as belonging to the Indochinese subregion, but grouped with material from the Sundaic subregion. This highlights the general need for examination of morphological and genetic data in tandem with echolocation call data.

Here we conclude that both the Sundaic and Indochinese forms are recognized and supported by morphological and genetic data, but share similar call frequencies in provinces to the north of the Isthmus of Kra. The Sundaic form of *R. a. superans* occurs from Ratchaburi province to the south, while the Indochinese form of *R. a. macrurus* occurs from Tak, Chaiyaphum and Surin to the north. Variation observed in call frequency within the Indochinese subregion was partially supported by morphological and genetic data. Though the high call frequency (>80 kHz) from central Vietnam was supported, lower call frequencies recorded northern Vietnam and southern China were not. This was also the case with variations in call frequency observed in peninsula Thailand.

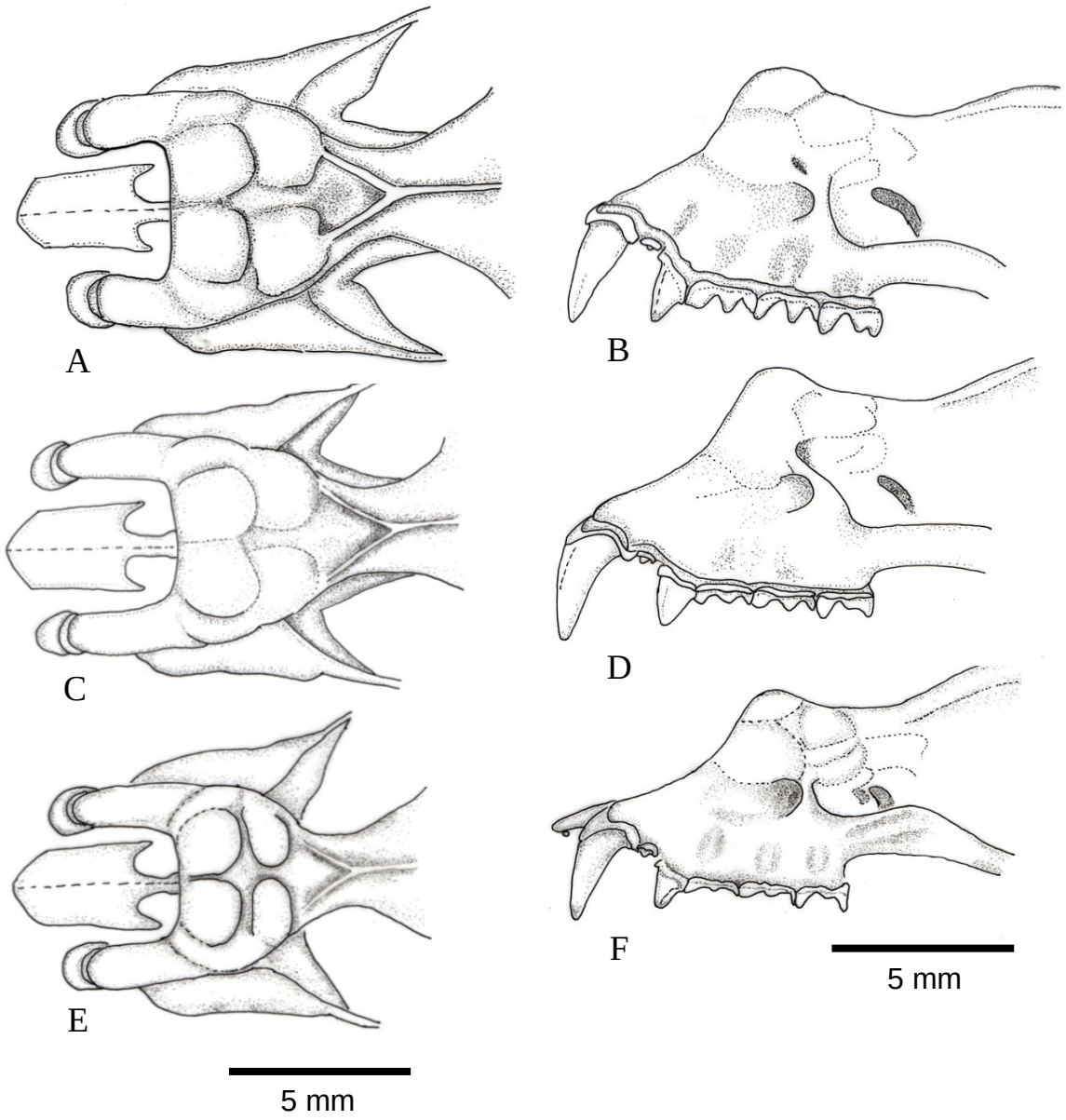

Figure 2.21. Rostrum variation of *Rhinolophus affinis* from mainland Southeast Asia. A, B: *R. a. superans* (specimen from Surathani, central peninsula Thailand); C, D: *R. a. macrurus* (specimen from Vinh Phuc, north Vietnam); E, F: form B, (specimen from Nghe An, lower north Vietnam).

A significant negative relationship was observed between call frequencies and size of the rostrum and horseshoe within *R. affinis*, which is supported by previous research (Barclay & Brigham, 1991; Barclay *et al.*, 1999; Francis & Habersetzer,

1998; Guillén *et al.*, 2000; Heller & v. Helversen, 1989; Jacobs *et al.*, 2007; Jones *et al.*, 1993; Kingston & Rossister, 2004; Robinson, 1996; Soisook *et al.*, 2008). This suggests adaption of populations to local environments, and during the evolutionary history of this species, shifts in echolocation frequency may have occurred prior to changes in their body size.

The degree of morphological variation in *R. affinis* is highlighted by the morphology of the as yet undesignated form B from Nghe An province, North Vietnam and east Shan, Myanmar. No call frequencies or genetic data were available, however morphologically this population had the smallest cranial and noseleaf morphology of all the material examined. Based on our findings relating to relationships between size and call frequency, this population possibly emits a call frequency higher than 80 kHz. By comparison, this form agrees closely with individuals from Mussoorie, northern India, albeit smaller overall. This suggests that *R. a. himalayanus* (or its immediate descendants) may have spread southward down to northern Vietnam. In Vietnam, the specimens were captured in Nghe An province where *R. a. macrurus* (form C) was also found. In Myanmar, the specimen was from Shan where many *R. a. macrurus* were also captured. Based on sympatric speciation, two morphologically different populations with sympatric distributions are considered to represent distinct species. On current knowledge, we suspect the cryptic clade III A of COI gene represents form B. It is more likely, because (1) this population has cranial and noseleaf characteristics which produce the highest call frequency, (2) in the north, the highest call frequency populations are found in upper south China, and (3) clade IIIA comprises only sequences from North Vietnam and upper south China where *R. a. himalayanus* was recorded. It would be of interest to access call frequency and morphological data of specimens from Hoang Lien Son (extreme north Vietnam) to compare with form B.

2.6. CONCLUSION

In conclusion, echolocation call data for *R. affinis* is not a robust taxonomic tool when considered in isolation as there is a significant degree of variation which is not explained or supported by genetic and morphological findings. *R. affinis* shows strong divergence between the zoological subregions, which is supported by morphology and molecular sequence data. The transition zone of the Sundaic form extends up to northernmost peninsula area at least to Ratchaburi province which known to be the transition zone for many other bat (Hughes *et al.*, 2011; Woodruff & Turner, 2009) and non-volant mammal species (Woodruff & Turner, 2009). The Sundaic form represents *R. a. superans* and the Indochinese form represents *R. a. macrurus.* Form B from north Vietnam and east of central Myanmar is of interest and may represent a distinct taxon, although more data, including echolocation call frequency and genetic sequence data is needed. This study has highlighted significant levels of variation in *R. affinis* throughout its distribution in mainland Southeast Asia. As the species has an extensive distribution throughout the continental and insular regions of Southeast Asia, it is likely that taxonomic revision is required for the species throughout its range.

CHAPTER 3

Geographical Variation of *Rhinolophus affinis* (Chiroptera: Rhinolophidae) in the Sundaic Sub-Region, including the Malay Peninsula, Borneo and Sumatra

ABSTRACT

Rhinolophus affinis sensu lato is a widespread species in South and Southeast Asia and shows high geographical variation in their morphology, call frequency and genetics. However the taxonomic status of the taxon in the Sundaic subregion is uncertain as the limited studies to date have been largely based on morphology. The aim of the present study was to evaluate the taxonomic status of subspecific forms recognized in the Sundaic subregion and to evaluate phylogeographic distinctiveness between Borneo and the Malay Peninsula using genetic, morphological and acoustic datasets. Two forms were confirmed including *R. a. nesites* from Borneo and *R. a. superans* from the peninsula. The recognition of a population from southernmost Sumatra as *R. a. superans* is not valid however as this form is likely *R. a. affinis*. Genetic divergence between these three forms is rather deep and estimated to have occurred during the arid climatic period of the Pleistocene when forests size was reduced resulting in isolated forest pockets. Our results support the phylogeographic distinctiveness hypothesis as *R. affinis* lato shows discrete affinities between Borneo and Malay Peninsula. Discovery of new forms of *R. affinis* is likely in large sample size from the region. Our study also demonstrates the importance of multiple datasets in taxonomic evaluations, as morphological and/or acoustic datasets alone can lead to erroneous conclusions.

3.1. INTRODUCTION

The Intermediate Horseshoe Bat, *Rhinolophus affinis* Andersen is a medium sized bat (forearm length 45-56 mm) distributed widely in South Asia, ranging from northern India (including Andaman Islands), Nepal to southern China, mainland Southeast Asia, Borneo, and Java (Francis, 2008; Simmon, 2005). The taxon exhibits considerable morphological and acoustic variation across its range (Andersen, 1905a;

Csorba *et al.*, 2003; Kingsada *et al.*, 2011) (Ith *et al.*, in review). Nine subspecies are traditionally recognized: *Rhinolophus affinis affinis* Horsfield (type locality Java), *R. a. andamanensis* Dobson (type locality South Andaman Island), *R. a. himalayanus* Andersen (type locality Mussoorie, Kumaon Division, north India), *R. a. tener* Andersen (type locality Pegu Division, recently known as Bago, Myanmar), *R. a. macrurus* Andersen (type locality Taho, Karennee, Kyah State, Myanmar), *R. a. superans* Andersen (Pahang, peninsula Malaysia), *R. a. nesite* Andersen (type locality Bunguran Island, North Natunas, Indonesia), *R. a. princeps* Andersen (type locality Lombok, Lesser Sunda Island) and *R. a. hainanus* Allen (type locality Pouten, Hainan Island) (Csorba *et al.*, 2003; Simmon, 2005).

The status of two subspecies, *R. a. macrurus* and *R. a. superans*, has recently been confirmed in continental Southeast Asia (Ith *et al*., in review). The geographical boundary of these two forms is in north peninsula Thailand, which accords with biogeographical demarcations within the region (de Bruyn *et al.*, 2005; Hughes *et al.*, 2011; Hughes *et al.*, 2003; Woodruff & Turner, 2009). *R. a. macrurus*, the Indochinese form, exhibits considerable variation in its genetics, morphology and echolocation call frequencies (Ith *et al*., in review). In contrast, the taxonomic status of the Sundaic form, *R. a. superans*, remains problematic, particularly in relation to populations on the island of Sumatra. Though Andersen (1905a) described the Sumatra form as resembling specimens from the Malay Peninsula in cranial, dental and external morphology, the taxon has not been evaluated since this publication and its genetic and acoustic variation is unknown.

The taxonomic status of *R. a. nesites* Andersen is similarly little evaluated. This form was proposed by Andersen (1905a) as an offshoot of *R. superans* in Bungaran Island, north Natunas (adjacent to Borneo at the north). The comparison was mainly based on the remaining parts of a damaged holotype which showed *R. nesites* has large ears, a broad horseshoe and a short tail. Though the form is recognized in recent literature (Csorba *et al.*, 2003; Koopman, 1994; Medway, 1977; Simmon, 2005), very little taxonomic work has actually been done to confirm its status.

R. a. superans may have similar morphology and genetic variation to that found in the Indochinese form of *R. affinis*: *R. a. macrurus* (Ith *et al*., in review).

Francis *et al.,* (2010) have shown that widespread taxa often have substantial geographic variation in their barcode sequences and that populations from peninsula Malaysia and Borneo are often genetically distinct (e.g. Khan *et al.,* 2008, Khan *et al.,* 2010). As a consequence, the aim of the current study was to evaluate the taxonomic status of *R. a. superans* and *R. a. nesites* and to evaluate the phylogeographic distinctiveness of *R. affinis* from Borneo and the Malay Peninsula using genetic, morphological and acoustic datasets.

Multiple datasets help putting more weight for taxonomic decision, e. g. *R. a. superans* from northernmost the Malay Peninsula would be mistakenly assigned to *R. a. macrurus* if echolocation call or morphology alone was used, due to this colony showed intermediate craniodental characters which similar to *R. a. macrurus* and also has similar call frequency but genetically different (Ith *et al.,* in review). The morphological cryptic *Hipposideros bicolor* (Kingsada *et al.,* 2011) may have not been discovered without the incorporation from genetic and ecological data. However, based on genetic alone would not be helpful to evaluate the taxonomic status of *Rhinolophus macrotis* and *R. siamensis* as they showed very shallow genetic differences (Francis *et al.,* 2010). Similar cases were observed *Miniopterus schreibersii* (Furman *et al.,* 2010), *Eptesicus serotinus* and *E. nilssonii* (Mayer & von Helversen, 2001), and *Myotisan namiticus* (Francis *et al.,* 2010; Kruskop & Tsytsulina, 2001).

3.2. METHODS AND MATERIALS

3.2.1. Study specimens and sampling sites

Seventy-six specimens were available for morphological study, including five from southern Sumatra, seven from Sarawak, north-western Borneo and 64 from the Malay Peninsula. Two specimens from central Java and two specimens from Musoorie, northern India were also included for comparison. Samples examined were from existing museum collections and those arising from recent surveys. Specimens were examined in collections held at the Princess Maha Chakri Sirindhorn Natural History Museum, Prince of Songkla University, Thailand (PSUZC); Harrison

Institute, UK (HZM); Museum Zoologicum Bogoriense, Research Center for Biology-Indonesian Institute of Science (MZB); Museum of Texas Tech University, USA (TTU collection); and Zoological Museum of Universiti Malaysia Sarawak.

Specimens from the Malay Peninsula were captured by Saveng Ith and the Small Mammals and Birds Research Unit Team of PSU between August 2011 and May 2012. Animals were captured in the field using a combination of harp traps, mist nets and hand nets. Field surveys were conducted in several localities in Thailand including Hala Bala Wildlife Research Station, Khao Namkhang National Park, Khao Ban Tad Wildlife Sanctuary, Rajjaprabha Dam and Ton Nga Chang Wildlife Sanctuary. All study localities where the 76 specimens were collected are illustrated in fig. 3.1 and collection information is given below.

Borneo

Sarawak: [Sa1] Mount Penrisen (1^{o}7.884'N, 110^{o}13.124'E) – two adult males and three females collected by R. J. Baker and P. A. Larsen from August 2006 to May 2010.

Indonesia

Sumatra: [S1] Way Canguk and Way Heni, Lampung (approx. 4^{o}33.585'N, 105^{o}24.410'E) – two adult males and one female collected by Bahri Syaiful, Hesti, Karlina and Joe Chun-Chia from July 2007 to May 2012. One adult male and one female were also collected by Joe Chun-Chia Huang in August 2010.

Malaysia

Kedah State: [Ma1] Langkawi Island (approx. 6^{o}23.204'N, 99^{o}47.831'E) –adult male collected by Mohd Isham Mohd Azhar. Penang State: [Ma2] (05^{o}15.795'N, 100^{o}29.076'E) – nulliparous female collected by F. A. A. Khan on in August 1988. Kelantan State: [Ma3] Gua Madu, Gua Musang Division (approx. 5^{o}10.462'N, 101^{o}54.191'E) – parous female captured by F. A. A. Khan. Pahang State (Tingga *et al.*, 2012): [Ma4] Nature Study of Kuala Atok, Taman Negara National Park

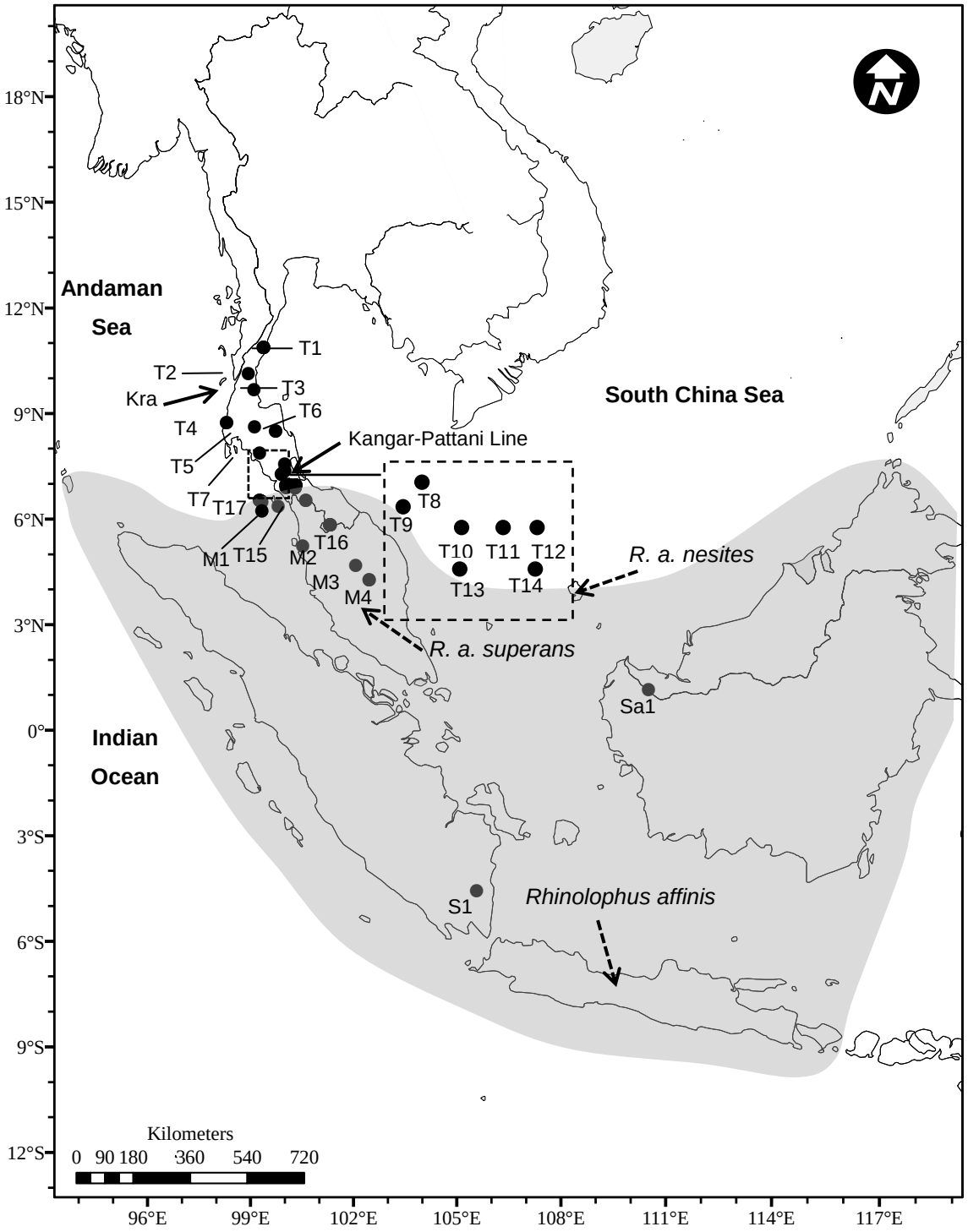

Figure 3.1. Sample localities for *R. affinis* in the Sundaic subregion. Sa=Sarawak, S=Sumatra, and T=Thailand. Abbreviations for localities are given in the methods and materials. Dashed arrows indicate type localities and subspecies names. The grey shading indicates the Sundaic biogeographic subregion following Woodruff (2010)-

and the solid arrows the transition zone of biota within the peninsula. Note: the northern boundary of the region is sometimes placed at the Isthmus of Kra (e.g. Lekagul & McNeely 1988; and Corbet & Hill, 1992).

(04°16.281'N, 102°22.316'E) – adult male and nulliparous female collected by F. A. A. Khan in May 2008.

Thailand

Chumphon Province: [T1] Khao Kram Cave, Patiew District (10°55.133'N 99°22.433'E); [T2] Huay Wang Cave, Tambon Khao Talu, Sawi District (10°10.00'N 98°55.183'E); and [T3] Klao Plu Cave, Lamae District (09°43.600'N 99°06.500'E) – Five adult males and three nulliparous females collected by Sara Bumrungsri from October 2006 to January 2007.

Pang Nga Province: [T4] Koh Surin (approx. 8°46.200'N, 98°18.600'E) – two adult males collected by Sara Bumrungsri in February 2006.

Surat Thani Province: [T5] Ratchabrapha Dam and Khlong Saeng Wildlife Sanctuary (8°58.885'N, 97°47.706'E) –adult male collected by Saveng Ith in August 2011 and adult male collected by Sara Bumrungsri in January 2012.

Nakhon Si Thammarat Province: [T6] Khao Phlu Cave, Khao Ro Commune, Ron Piboon District (8°32.250'N, 99°43.396'E) –adult male and nulliparous female collected by Sara Bumrungsri in October 2011.

Krabi Province: [T7] Khao Pra Bang Kram Wildlife Sanctuary (7°55.517'N, 99°15.790'E) –adult male collected by Pipat Soisook in 4 May 2012.

Pattalung Province: [T8] Khao Ban Tad Wildlife Sanctuary (approx. 7°23.800'N, 99°58.682'E) – two adult males, one parous female and one nulliparous female collected by Pipat Soisook in March 2012.

Trang Province: [T9] Sai Rung Waterfall, Khao Ban Tad Wildlife Sanctuary (7°18.080'N, 99°41.988'E) –adult male and two nulliparous females collected by Pipat Soisook in January 2011.

Songkhla Province: [T10] KuanKhao Wang Park, Rattaphum District (7°00.776'N, 100°01.259'E) – Four adult males and two nulliparous females captured by Saveng Ith in August 2011 and February 2012. [T11-14] Ton Nga Chang Wildlife Sanctuary (approx. 6°55.783'N, 100°16.299'E) including Pha Dam Ranger Station, Makling Waterfall and Hin Sam Kon Waterfall – nine adult males and two females collected by Saveng Ith in February 2012. [T15] Khao Namkhang National Park (6°33.108'N, 100°16.299'E) – two adult males captured by Saveng Ith in May 2012.

Narathiwat Province: [T16] Hala Bala Wildlife Sanctuary (05°47.900'N, 101°49.500'E) – six adult males and two nulliparous females collected by Saveng Ith in January 2012.

Satun Province: [T17] A-Dang Island (6°30.878'N, 99°19.040'E) and Rawee Island (6°33.496'N, 99°15.033'E), Tarutao National Park – three adult males, one nulliparous female and one parous female collected from A-Dang Island and three adult males collected from Rawee Island in February 2012 by Saveng Ith.

3.2.2. Morphological measurement

Totally, 33 external and craniodental characters of each specimen were measured following Bates and Harrison (1997), Csorba *et al.*, (2003), Furey *et al.*, (2009) and Thomas (1997). External characters were measured using a pair of analog calipers to the nearest 0.1mm and craniodental characters were measured to the nearest 0.01mm using a digital caliper under a stereo microscope. Definitions for external measurements were as follows, FA: forearm length – from the extremity of the elbow to the extremity of the carpus with the wings folded; EL: ear length – from the lower border of the external auditory meatus to the tip of the pinna; TL: tail length – from the tip of the tail to its base adjacent to the anus; HF: from the extremity of the

heel behind the os calcis to the extremity of the longest digit, not including the hairs or claws; TIB: tibia length – from the knee joint to the extremity of the heel behind the os calcis; 2MT, 3MT, 4MT, 5MT: length of metacarpals – taken from the extremity of the carpus to the distal extremity of the second, third, fourth and fifth metacarpals respectively; 1P3D, 2P3D, 1P4D, 2P4D, 1P5D, 2P5D – length of the first and second phalanges of the third, fourth and fifth digits respectively – taken from the proximal to the distal end of the phalanx; GWN – greatest width of noseleaf – greatest diameter across the horseshoe; GHN: greatest height of noseleaf – from the base of the horseshoe to the tip of the lancet, not including the hairs.

All skulls were extracted for examination. Definitions for craniodental measurements were as follows: SL: skull length – the greatest length from the occiput to the front of the canine; CCL: condyle-canine length – from the exoccipital condyle to the anterior alveolus of the canine; ALSW: the greatest width across the anterior lateral compartments of the rostrum; AMSW: anterior median swellings width – the greatest width across the median swellings in dorsal view; ZYW: zygomatic width – the greatest width of the skull across the zygomata; BW: braincase width – width of the braincase at the posterior roots of the zygomatic arches; GBW: greatest braincase width – width of the braincase, the greatest width across the braincase; MAW: mastoid width – greatest width of the braincase taken across the mastoid region; IOW: interorbital width – the narrowest width of interorbital constriction; PB: palatal bridge – length of bony palate excluding the posterior spike; M^3M^3W: posterior palatal width – taken across the widest part of the outer borders of the third upper molar; C^1C^1W: anterior palatal length – taken across the widest part of the outer border of the upper canine; CM^3L: upper toothrow length – from the front of the upper canine to the back of the crown of the third upper molar; CM_3L: lower toothrow length – from the front of the lower canine to the back of the crown of the third lower molar; ML: mandible length – from the most posterior part of the condyle to the most anterior part of the mandible, including the lower incisors; CPH: least height of the coronoid process – from the tip of the coronoid process to the apex of the indentation on the inferior surface of the ramus adjacent to the angular process.

Baculum characters were measured to the nearest 0.01 mm using a digital caliper under a stereo microscope. Thirty bacula were available for examination, comprising 27 from the Malay Peninsula, two from Sumatra and one from Borneo.

3.2.3. Echolocation call measurement

Values for the frequency of maximum energy (FMAXE) for *R. affinis* in this study were obtained from field work. In total, 71 calls (from 71 bats) were available for measurement. Fifty-nine calls were from the Malay Peninsula, one from north-western Borneo, six from central Java and five from southern Sumatra.

Echolocation calls were recorded from bats held in the hand using a Pettersson D-240X bat detector and in some instances, a Pettersson D1000X bat detector. The Pettersson D-240X detector was set in x10 time-expansion mode and call data was recorded to a digital iRiver iHP-120 Multi Codec Jukebox recorder. Where a Pettersson D1000X was used, calls were stored on a built in Compact Flash (CF) card (type I). The detector was set to manual recording mode (MAN) and the maximum sampling frequency (fs) to 7680 kHz. A time expansion factor of x10 was also used. All sound files were recorded and saved in 'wav' format for analysis. Call components were displayed using spectrogram, oscillograms and power spectrums in BatSound Pro 3.31 (Pettersson Elektronik, AB) in which sampling frequency was formatted as 44.10 kHz and spectrograms were set to 1,024 sampling size using Fast Fourier Transforms (FFT) with Hanning windows. In all cases, FMAXE (kHz) was measured from the constant frequency portion of a call and the mean value was used in analysis. To avoid pseudo-replication, one echolocation call per bat was used in analysis.

3.2.4. Morphological and acoustic analysis

Statistical analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, U.S.A.) and PC-ORD 5.10 for windows (MjM Software, Oregon, U.S.A.). Descriptive statistics (minimum, maximum, mean and standard deviation) were calculated for external and craniodental measurements. Normality of data and

homogeneity of variances were tested prior to using parametric *t*-tests and non-parametric Mann-Whitney U-tests to evaluate sexual dimorphism. Multiple comparisons of characters between populations were calculated using a multivariate analysis of variance (MANOVA). Principal component analysis (PCA) on the correlation matrix was used for multivariate comparisons.

3.2.5. Molecular analysis

Tissue was collected from different organs of voucher specimens such as liver, tongue and wing membrane and preserved cold in 95% concentration ethanol. Two mitochondrial DNA gene fragments were used for phylogenetic analysis. A 657 base pair segment of 17 COI sequences was analyzed at the Canadian Center for DNA Barcoding (CCDB) using the barcoding protocols (Ivanova *et al.*, 2012). Nineteen sequences of *Cytochrome b* (*cyt b*) gene was generated and analyzed in collaboration with the Coral Triangle Partnerships in International Research and Education Project (https://sci.odu.edu/impa/ctpire.html). Genomic DNA was isolated from bat tissue samples using the QiagenDNeasy mini kit (Qiagen, Valencia, CA) following manufacturer's instructions and *Cytb* sequences were generated, aligned and proofread as described in (Willette & Padin, 2014) using the primers *Cytb*07 (5'-AATAGGAGGTATCATTCGGGT-3') and *Cytb*09 (5'-GTGACTTGAAAAACCAC-CGTT-3'). While other 17 *cyt b* sequences were analyzed (DNA extraction, PCR amplifications, and sequencing reaction) by F.A.A.K. following (Khan *et al.*, 2013) using primer set LGL765 (5'-GAAAAACCAYCGTTGTWATTCAACT-3'), LGL766 (5'- GTTTAATTA GAATYTYAGCTTTGGG-3') with an annealing temperature of 50˚C.

In total, 37 sequences of *cytochrome b* gene (*cyt b*) and 7 sequences of cytochrome oxidase I (COI) were available. Sequences from Genbank and BOLDSYSTEMS Databases were also accessed, eight sequences of *cyt b* gene (accession number: EF108156-EF108160, EU521607, JN106274 and JN106280) from Borneo and peninsula Malaysia were included for comparison. Twenty-one sequences of COI gene were included, 11 sequences were from peninsula Malaysia

(accession number: HM541330-HM541332, HM541407-HM541414) and 10 sequences from peninsula Thailand.

Phylogenetic relationships among sequences were reconstructed using maximum-likelihood in the MEGA5.2.2 program (Tamura *et al.*, 2011). The most appropriate substitution model was determined using Akaike Information Criterion (AIC) and Bayesian Information Criterion Bickham *et al*., (2004) as implemented in jModelTest 2.14 (Darriba *et al.*, 2012). Among the 88 models in the 100% confidence interval, Hasegawa-Kishino-Yano substitution model (HKY) was the best-fit model selected COI. While General Time Reversible model (GTR) with proportion of invariant sites (G) were the best-fit model selected for *cyt b*. We also performed Bayesian Analysis using MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2001). In Bayesian Analysis, convergence stationary was searched by two independent Metropolis-coupled Markov chain Monte Carlo (MCMC), each comprising three incrementally heated chains and one cold chain, run for 24 million generations, with parameters sampled every 500 generations. Convergence stationary of the MCMC chains was evaluated by inspecting whether the standard deviation of split frequencies <0.01 and the potential scale reduction factor (PSRF) reached 1.0 for all parameters. We also investigated the convergence using Tracer 1.5 (Rambaut & Drummond, 2009). 12, 000 trees of initial phase of the Markov chain was discarded as 25% burn-in. A congeneric *Rhinolophus stheno* was used as an out-group in the phylogenetic analysis of *cyt b* gene in order to examine the monophyletic lineage of *R. affinis*.

To estimate the time to the most recent common ancestor (TMRCA) among the observed clades, *cyt b* gene was analyzed in BEAST 1.8 (Rambaut & Drummond, 2007). GTR + Gwas selected as the best substitution model based on jModelTest and relaxed-clock model with an uncorrelated lognormal distribution was selected for the substitution rate. We performed two independent runs of MCMC chains with 60 million generation with parameters logged very 1000 generation. Tracer 1.5 (Rambaut & Drummond, 2009) was used to combine the two runs as well as to examine the effective sample size (ESS) for the parameters. Trees were collated using Tree Annotator 1.8 where Maximum clade credibility tree and Median heights were selected; and 10% (6000 trees) sample trees were selected as burn in. To convert the estimates scaled by mutation rate to calendar years, we used the mean substitution rate

of 1.30 x 10-8 subs/site/year which was previously used in hipposiderid bats (Lin *et al.*, 2013; Thong *et al.*, 2012).

3.3. RESULTS

3.3.1. Morphology

No significant differences were found in 33 external and cranial characters between the sexes (P> 0.05). A total of 18 external and cranial characters were retained for multivariate analysis, these being selected on the basis of their eigenvector values in a preliminary PCA. A PCA using these 18 characters for 74 specimens from the Sundaic subregion generated two relatively isolated groups (fig. 3.2). Specimens from Borneo formed a relatively isolated group while those from Sumatra and the Malay Peninsula formed an overlapping group.

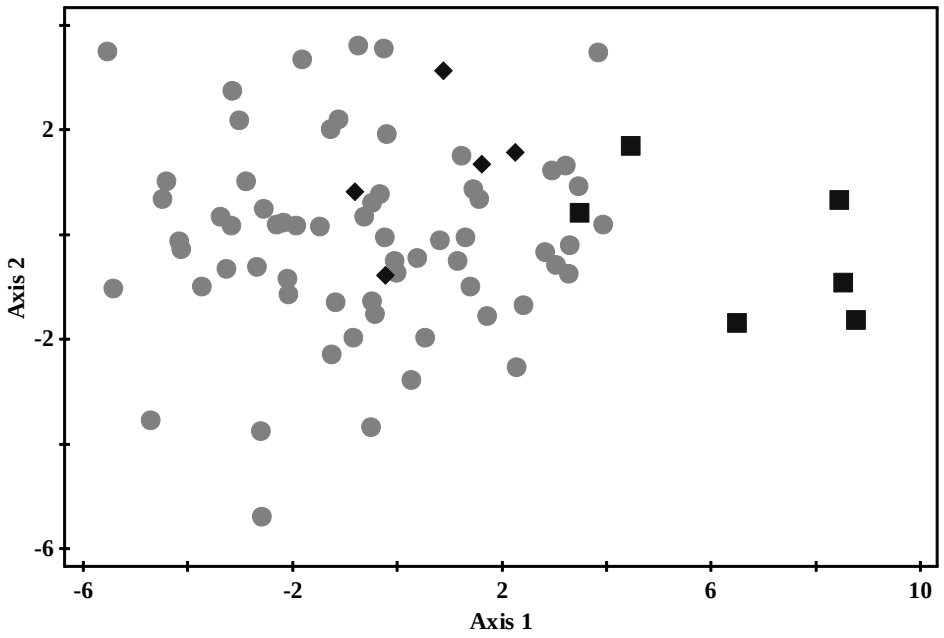

Figure 3.2. PCA of eighteen external and cranial characters for *R. affinis* specimens from Borneo (black squares), Sumatra (black diamonds) and Malay Peninsula (grey circles).

Table 3.1. Eigenvectors and eigenvalues of PCA of eighteen external and cranial characters of specimens from Sudaic subregion; the values explain figure 3.2.

	Eigenvector		
Characters	1	2	3
FA	-0.7290	0.5095	0.0933
TIB	-0.7933	0.3270	0.1361
HF	-0.3202	0.0108	0.8136
2MT	-0.6305	0.6541	-0.0915
3MT	-0.5364	0.6973	-0.0997
4MT	-0.6572	0.6647	-0.1076
5MT	-0.6658	0.6357	-0.0683
SL	-0.9238	-0.1301	-0.0277
CCL	-0.8648	-0.1394	-0.1155
ZYW	-0.7302	-0.3682	0.0983
MAW	-0.8284	-0.3365	0.1256
BW	-0.8296	-0.2915	0.1369
ALSW	-0.7323	-0.5120	-0.0123
PB	-0.2948	-0.3961	-0.4832
CM^3L	-0.8611	-0.2616	-0.1395
M3M3W	-0.6946	-0.3580	0.0340
ML	-0.8759	-0.1347	-0.1298
CM_3L	-0.8428	-0.1980	-0.0135
Eigenvalue	9.657	3.170	1.053
% of total variation explained	53.652	71.262	77.114

Specimens from Borneo were distinguished from Sumatra and Malay Peninsula specimens in their generally smaller external and cranial measurements and noseleaves. Specifically, Borneo specimens were smaller on average in FA, TL, TIB and HF ($p<0.05$) and several wing measurements (2MT, 3MT, 4MT, 5MT and P1D3, all $p<0.05$). Several skull characters were also significantly smaller including SL, ZYW, CM^3L, C^1C^1W, M^3M^3W, CM_3L and CPH (all $p<0.05$) (table 3.2). The skull of these specimens has a short frontal depression and the canines and teeth are smallest overall (fig. 3.3). The noseleaf is small, as is GWN with an average width of 9.1 mm, while GHN is also small, at 12.9 mm. The median emargination of the horseshoe is narrow (fig. 3.4). The rudimentary secondary noseleaf is less developed and completely concealed by the horseshoe and surrounding dense hair (fig. 3.4). The

sella is small and slender, rounded off on the top and the lateral margin is more strongly constricted in the middle (fig. 3.5). The internarial cup is moderate in size and the margin is developed (fig. 3.4). The connecting process is small, slender, rather pointed and covered with numerous short hairs and shows the notch pattern on the top. The lancet is small, slender, triangular-shaped and straight-sided.

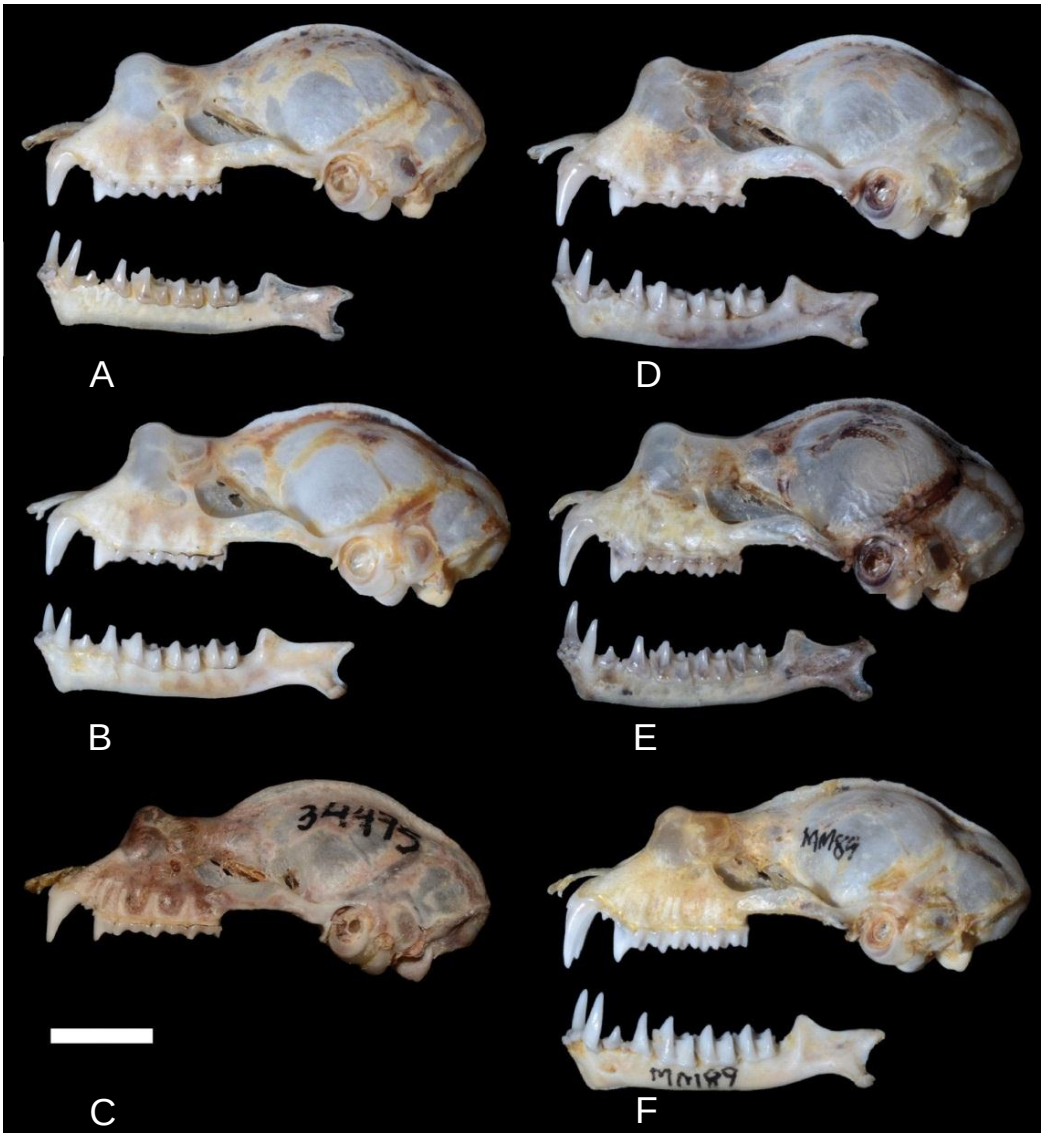

Figure 3.3. Lateral view of *R. affinis* skulls from Borneo (A: TK168483, ♀), Sumatra (B: MZB35882, ♀), Central Java (C: MZB34475, ♀), Malay Peninsula (D: IS110823.10, ♂; E: IS120122.1, ♂) and India (F: HZM4.28148, ♂). Scale = 5 mm.

Specimens from Sumatra also formed a relatively isolated group from peninsula populations (particularly the northern peninsula) when 12 characters with high eigenvector values (from the preliminary PCA) were analyzed (fig. 3.6). Compared with specimens from the northern peninsula, Sumatran specimens are externally smaller in TIB, P2D3, P1D4 and P2D5 ($p<0.05$) but larger in GHN (fig. 3.4, Table 3.2). The skulls of Sumatran specimens also have significantly smaller MAW, GBW, ALSW, AMSW, IOW, CM^3Land CM_3L ($p<0.05$) (fig. 3.3, 3.7, 3.8, 3.9; Table 3.2). Compared with specimens from the southern peninsula, Sumatran specimens are similar in size with only two external (TIB and P2D5) and one craniodental character (AMSW) significantly smaller, and three characters significantly larger (CCL, PB, and C^1C^1W) ($p<0.05$). Sumatran specimens were found to have a more developed sagittal crest (fig. 3.3B) however, which is well built and visible from the supraorbital ridges to the lambda.

Table 3.2. External and craniodental measurements of *R. affinis* forms within the Sundaic subregion. Values are given as min-max, mean ± standard deviation (in mm). Acronyms and definitions for measurements are given in the text.

n	Sex	FA	TL	EL	TIB	HF	2MT	3MT	4MT	5MT	3D1P
						Java					
2	♀♀	49.8–50.1	23.4–24.4	19.3–21.5	23.0–23.3	10.3–10.6	41.5–42.8	38.6–40.2	40.2–40.4	40.0–40.1	14.8–15.0
		49.9±0.2	23.9±0.7	20.4±1.5	23.2±0.2	10.5±0.2	42.2±0.9	39.4±1.1	40.3±0.1	40.1±0.1	14.9±0.1
						Borneo					
6	♀♂	46.7–49.5	19.2–21.8	20.0–21.6	20.0–21.8	9.2–10.8	38.2–40.6	35.8–38.5	36.5–38.8	37.1–39.5	13.3–15.0
		48.1±0.9	20.6±1.1	20.7±0.7	21.1±0.7	9.9±0.6	39.4±0.9	37.2±1.1	37.5±1.0	38.3±0.9	14.1±0.6
						Sumatra					
5	♀♂	48.9–50.6	21.5–23.0	21.7–23.6	22.6–24.5	10.0–11.0	41.0–42.3	38.5–39.4	39.2–40.3	39.3–41.0	14.7–15.5
		49.8±0.7	22.4±0.6	22.4±0.8	23.3±0.8	10.5±0.4	41.4±0.5	39.1±0.4	39.7±0.4	40.0±0.7	15.0±0.3
						Southern Malay Peninsula					
15	♀♂	48.8–51.8	20.4–26.0	18.4–24.2	21.2–25.7	10.0–11.6	39.4–42.5	37.3–40.0	37.9–40.5	38.4–41.3	13.7–15.0
		50.6±0.9	22.4±1.5(14)	21.5±1.3(14)	24.2±1.0	10.6±0.4	40.8±0.9	38.6±0.8	39.5±0.8	40.0±0.9	14.6±0.3
						Northern Malay Peninsula					
50	♀♂	48.3–52.9	18.8–25.8	19.6–24.4	22.6–26.4	9.1–11.3	38.5–44.0	35.7–40.3	37.1–41.8	38.1–42.3	13.7–16.2
		50.5±1.2	22.0±1.7	22.0±1.1	24.2±0.8	10.4±0.4	40.8±1.1	38.3±1.0	39.2±1.0	39.9±1.0	14.8±0.5

Table 3.2. Continued

n	Sex	3D2P	4D1P	4D2P	5D1P	5D2P	GHN	GWN
				Java				
2	♀♀	25.0–26.4	10.0–10.3	14.4–15.2	12.0–12.1	13.5–13.5	10.7–11.5	8.2–8.4
		25.7±1.0	10.2±0.2	14.8±0.6	12.1±0.1	13.5±0.1	11.1±0.5	8.3±0.1
				Borneo				
6	♀♂	22.8–24.8	8.2–10.3	14.0–15.8	9.9–12.1	11.8–12.4	11.5–14.0	8.5–10.3
		23.9±0.7	9.5±0.7	14.6±0.7	11.0±0.7	12.1±0.2	12.9±1.1(4)	9.1±0.6
				Sumatra				
5	♀♂	23.7–26.0	9.7–10.0	13.7–15.1	11.3–11.8	9.8–13.1	14.0–16.1	8.9–10.6
		24.7±0.9	9.8±0.2	14.6±0.7	11.5±0.2	12.2±1.4	15.0±0.8	9.9±0.7
				Southern Malay Peninsula				
15	♀♂	23.8–27.0	9.2–10.9	11.2–15.2	10.6–11.9	12.4–13.7	12.8–14.9	8.9–10.2
		25.3±0.8	10.2±0.5	14.4±1.0	11.4±0.4	13.0±0.4	13.8±0.6(14)	9.6±0.4(14)
				Northern Malay Peninsula				
50	♀♂	24.2–27.7	9.5–11.5	13.8–16.3	10.6–12.6	9.6–14.6	10.4–15.9	9.6–11.0
		25.8±0.8	10.3±0.5	15.0±0.6	11.4±0.5	13.0±0.8	13.9±1.1	10.3±0.4

Table 3.2. Continued

n	Sex	SL	CCL	ZYW	MAW	BW	GBW	ALSW	AMSW	IOW
					Java					
2	♀♀	22.18–22.47	19.50–19.69	10.95–11.71	10.16–10.71	9.85–10.17	9.31–9.48	5.88–5.95	3.74–4.01	2.00–2.09
		22.33±0.21	19.60±0.13	11.33±0.54	10.44±0.39	10.01±0.23	9.40±0.12	5.92±0.05	3.88±0.19	2.05±0.06
					Borneo					
7	♀♂	21.36–22.31	19.06–19.75	10.83–11.20	10.22–10.58	9.81–10.26	9.38–9.82	5.76–6.02	4.00–4.29	2.18–2.33
		21.82±0.40	19.36±0.26	11.04±0.15	10.38±0.15	10.03±0.16	9.57±0.18	5.88±0.10	4.18±0.12	2.27±0.05
					Sumatra					
5	♀♂	22.14–22.64	19.70–20.02	11.03–11.60	10.45–10.78	9.96–10.34	9.15–9.79	5.79–6.01	3.65–4.13	2.10–2.34
		22.48±0.20	19.88±0.12	11.33±0.23	10.57±0.14	10.17±0.14	9.56±0.24	5.89±0.10	3.93±0.18	2.22±0.09
					Southern Malay Peninsula					
12	♀♂	21.59–22.62	19.08–19.92	11.07–11.49	10.39–10.89	9.80–10.44	9.34–10.01	5.86–6.18	3.96–4.36	2.11–2.53
		22.21±0.36	19.51±0.27	11.26±0.15	10.70±0.15	10.22±0.19	9.74±0.19	6.00±0.09	4.22±0.14	2.33±0.15
					Northern Malay Peninsula					
50	♀♂	21.97–23.27	19.36–20.78	10.84–11.91	10.41–11.16	9.84–10.67	9.27–10.14	5.91–6.72	3.81–4.67	2.13–2.81
		22.63±0.35	20.03±0.32	11.40±0.22	10.81±0.18(49)	10.35±0.19	9.79±0.21	6.22±0.17	4.34±0.18	2.40±0.14

Table 3.2. Continued

n	Sex	PB	BOW	CM^3L	C^1C^1W	M^3M^3W	ML	CM_3L	CPH
					Java				
2	♀♀	1.97–2.23	1.29–1.38	8.90–9.01	5.70–5.79	8.28–8.61	15.49–15.74	9.31–9.42	2.79–3.38
		2.10±0.18	1.34±0.06	8.96±0.08	5.75±0.06	8.45±0.23	15.62±0.18	9.37±0.08	3.09±0.42
					Borneo				
7	♀♂	1.93–2.20	0.94–1.24	8.41–8.73	5.12–5.64	7.65–7.96	14.58–15.29	8.72–9.07	2.72–2.99
		2.11±0.09	1.05±0.13	8.54±0.12	5.45±0.17	7.82±0.10	14.99±0.24	8.86±0.13	2.87±0.09
					Sumatra				
5	♀♂	2.22–2.43	1.11–1.26	8.77–9.07	5.71–6.04	7.98–8.43	15.33–15.67	9.02–9.44	2.99–3.24
		2.30±0.08	1.19±0.06	8.89±0.13	5.85±0.14	8.22±0.20	15.47±0.14	9.22±0.18	3.10±0.10
					Southern Malay Peninsula				
12	♀♂	2.05–2.38	1.12–1.53	8.48–9.03	5.47–5.79	7.92–8.49	14.79–15.61	9.06–9.45	2.99–3.28
		2.12±0.10	1.34±0.13	8.78±0.14	5.65±0.11	8.18±0.14	15.21±0.31	9.25±0.13	3.14±0.09
					Northern Malay Peninsula				
50	♀♂	1.89–2.61	1.04–1.65	8.69–9.38	5.23–6.11	7.98–8.86	15.09–16.07	9.05–9.82	2.87–3.63
		2.26±0.12	1.23±0.11(49)	9.07±0.16	5.78±0.18(49)	8.37±0.18(49)	15.60±0.24(49)	9.46±0.19	3.16±0.14

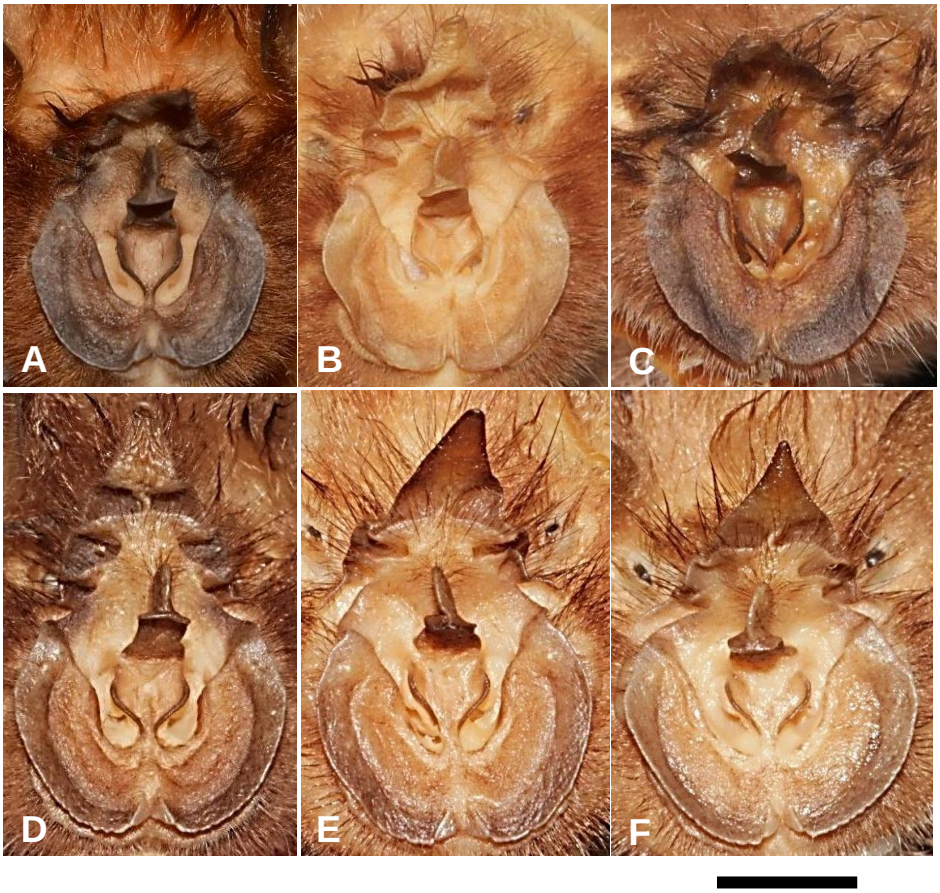

Figure 3.4. Noseleaf variation of *R. affinis* in the Sundaic subregion (specimen from northern India included for comparison). Kabumen, central Java (A: MZB34475, ♀); Musoorie, northern India (B: HZM.4.28148, ♂); Sarawak, northwest Borneo (C: TK152216, ♀); Lampung, southern Sumatra (D: MZB34965, ♀); Narathiwat, southern Thai Peninsula (E: IS120122.1, ♂); Songkhla, southern Malay Peninsula (F: IS110823.10, ♂). Scale = 5mm.

The noseleaf of Sumatran specimens is medium sized in general and shares many characters with specimens from the Malay Peninsula. GWN in Sumatran specimens is slightly smaller than Malay Peninsula specimens with an average 9.9 mm; GHN is highest in Sumatran population, averaging 15.0 mm. The median emargination of the horseshoe is as wide as specimens from central Java and Malay Peninsula (fig. 3.4) and differs from specimens from Sarawak (fig. 3.4) and India (fig. 3.4).

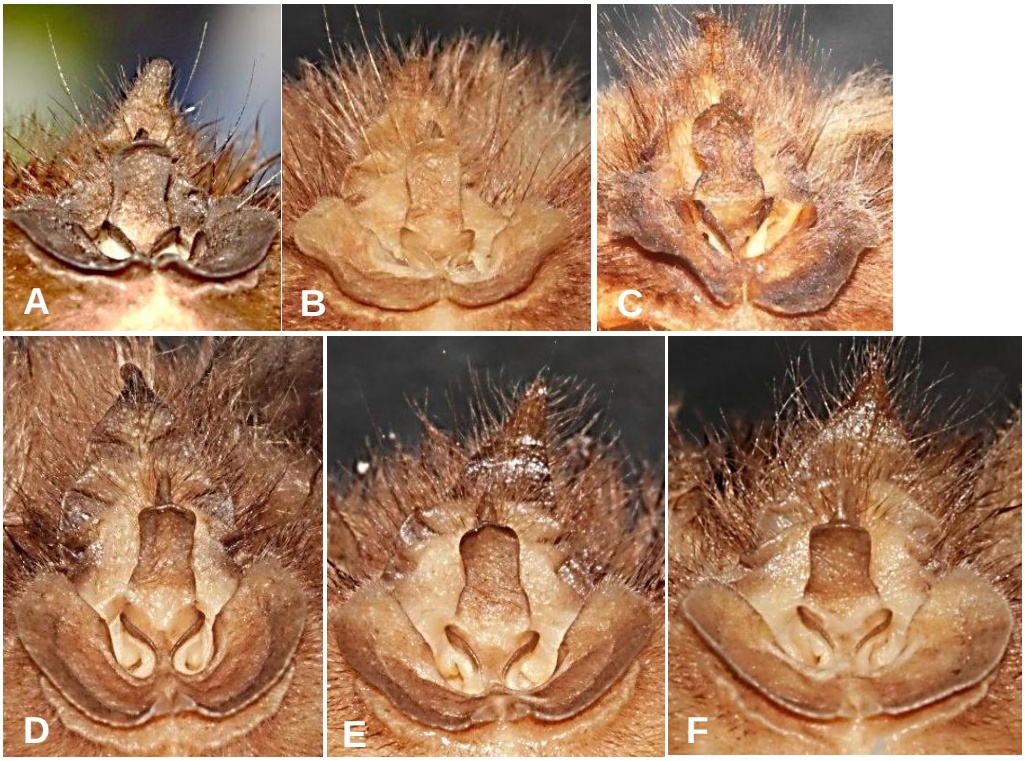

Figure 3.5. Sella variation of *R. affinis* in the Sundaic Subregion (specimen from northern India included for comparison). Kabumen, central Java (A: MZB34475, ♀); Musoorie, northern India (B: HZM.4.28148, ♂); Sarawak, northwest Borneo (C: TK152216, ♀); Lampung, southern Sumatra (D: MZB34965, ♀); Narathiwat, southern Malay Peninsula (E: IS120122.1, ♂); Songkhla, southern Malay Peninsula (F: IS110823.10, ♂). Scale = 5mm.

The rudimentary secondary noseleaf is visible in dorsal view, with fewer hairs (fig. 3.4) compared to Sarawak and Java specimens (fig. 3.4). The sella is large, tall and rounded off on the top, and the lateral margin is only slightly constricted in the middle (fig. 3.5). The internarial cup is moderate in size and the margin is less developed compared to specimens from Sarawak (fig. 3.4). The connecting process is typically round and the lancet is triangular, straight-sided and high.

Specimens from the Malay Peninsula had the largest craniodental measurements overall (table 3.2). The rostrum chambers are large (fig. 3.7) and ALSW and AMSW are broad, averaging 6.15 mm and 4.26 mm, respectively. The

anterior median swellings are inflated (fig. 3.3) and rounded in the dorsal view (fig. .7). The frontal depression (fig. 3.3) and supraorbital ridges (fig. 3.7) are elongated and the palatal bridge is long (fig. 3.8), with CM^3L, ML (fig. 3.9) and CM_3L (fig. 3.8) also large. Similarly, the noseleaf is relatively large with the largest GWN, averaging 10.0 mm. The rudimentary secondary noseleaf is developed but almost invisible in the dorsal view being largely concealed by horseshoe (fig. 3.4). The sella is very broad and lacks an obvious middle constriction as the lateral margins gradually constrict (fig. 3.5). The tip of the sella is always rounded off. The internarial cup is broad with well-defined but not especially developed lateral margins (fig. 3.4). The connecting process is typical of the species, large and rounded off and covered with many short hairs. The lancet is broad and high with elongate tip, and its lateral margins are normally straight-sided or slightly convex at the base for some individuals.

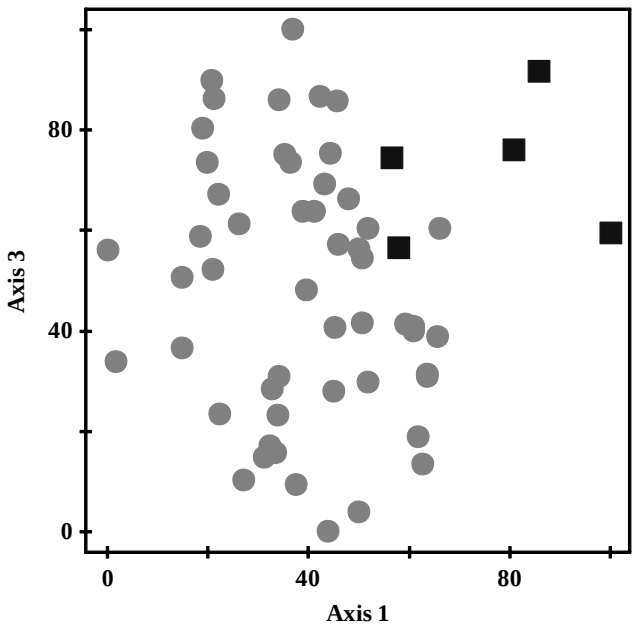

Figure 3.6. PCA of twelve external and cranial characters for *R. affinis* specimens from Sumatra (black squares) and Malay Peninsula (grey circles).

Table 3.3. Eigenvectors and eigenvalues of PCA of twelve external and cranial characters of specimens from Sumatra and Malay Peninsula; the values explain figure 3.6.

Characters	Eigenvector 1	2	3
TIB	-0.6426	-0.5447	0.1594
2P3D	-0.4378	-0.7258	-0.2028
1P4D	-0.5003	-0.4900	-0.0341
2P5D	-0.4303	-0.5248	-0.1019
GWN	-0.4359	0.1383	-0.5515
MAW	-0.7988	0.3567	-0.0911
GBW	-0.7433	0.2750	0.2308
ALSW	-0.7665	0.4181	-0.1277
AMSW	-0.7090	0.1306	-0.1699
IOW	-0.4879	0.1357	-0.5019
CM^3L	-0.8200	0.0644	0.3816
CM_3L	-0.7554	0.0755	0.4489
Eigenvalue	4.992	1.781	1.088
% of total variation explained	41.599	56.442	65.508

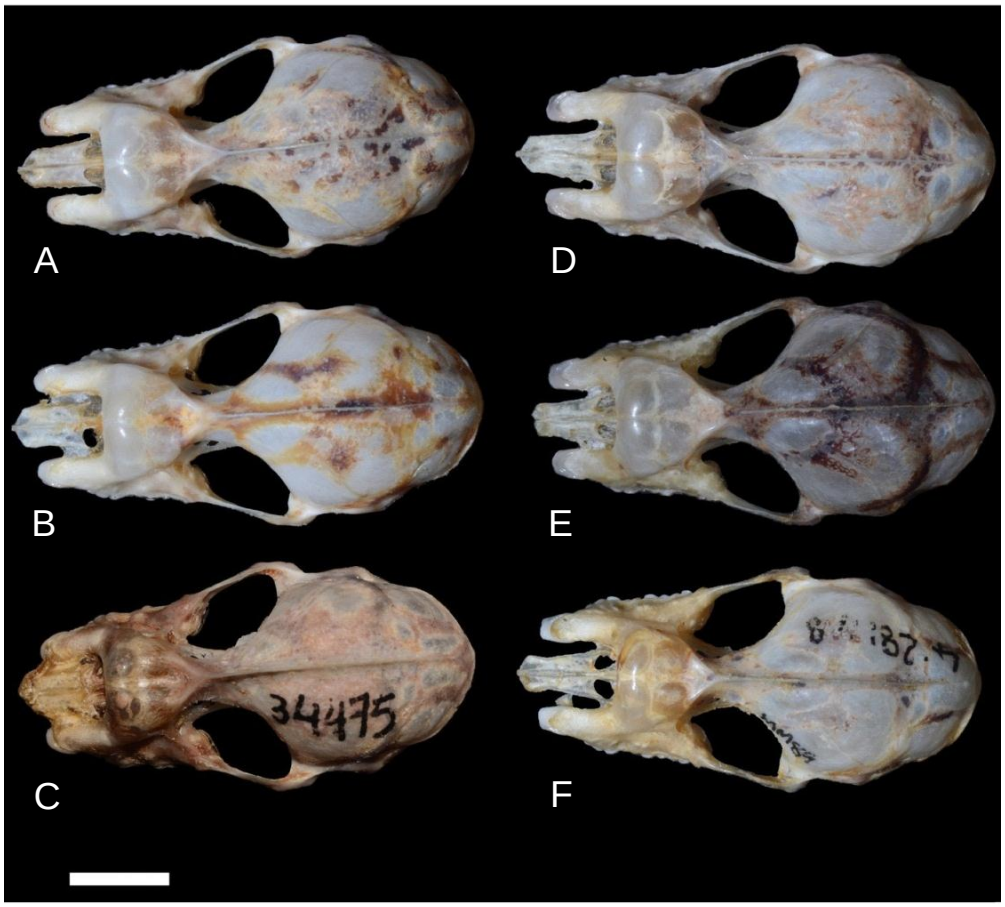

Figure 3.7. Dorsal view of *R. affinis* skulls from Borneo (A: TK168483, ♀), Sumatra (B: MZB35882, ♀), Central Java (C: MZB34475, ♀), Malay Peninsula (D: IS110823.10, ♂; E: IS120122.1, ♂) and India (F: HZM4.28148, ♂). Scale = 5 mm.

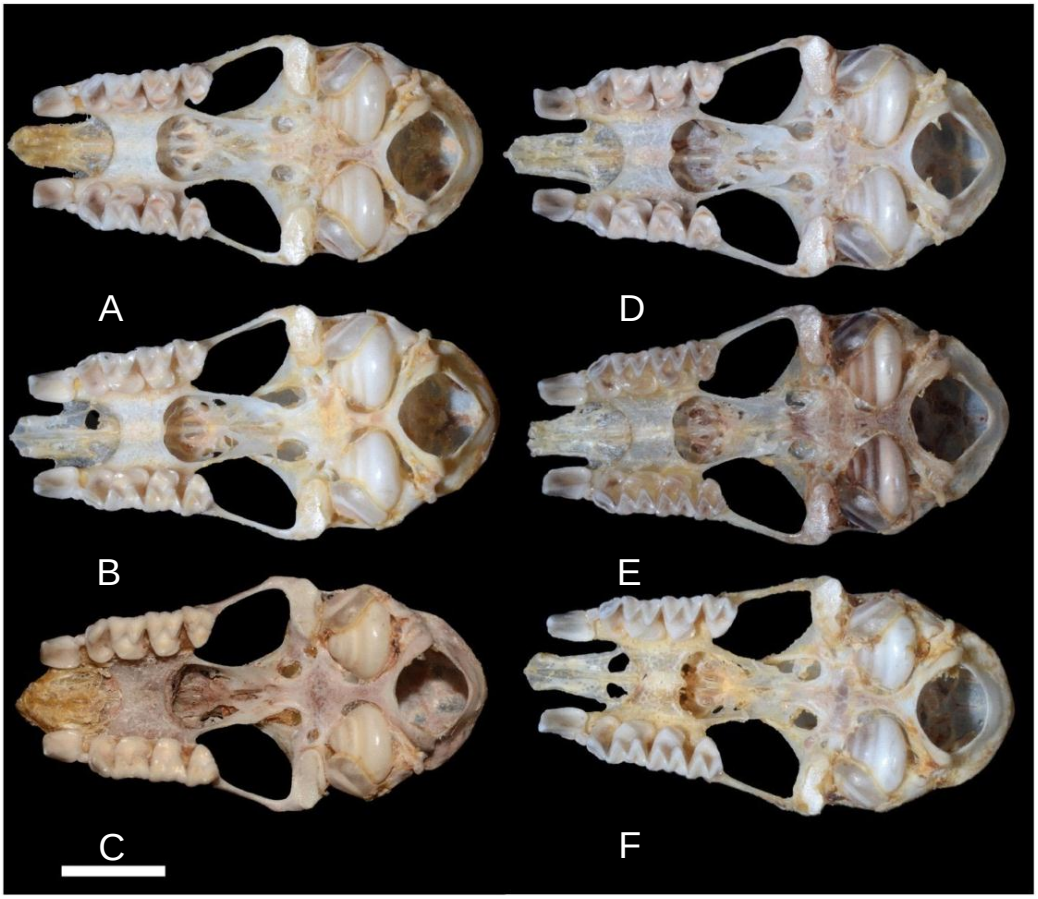

Figure 3.8. Ventral view of *R. affinis* skulls from Borneo (A: TK168483, ♀), Sumatra (B: MZB35882, ♀), Central Java (C: MZB34475, ♀), Malay Peninsula (D: IS110823.10, ♂; E: IS120122.1, ♂) and India (F: HZM4.28148). Scale = 5 mm.

3.3.1.1. Baculum

The bacula of Sumatran specimens is similar to that of specimens from the Malay Peninsula, although some differences are apparent. Overall, the bacula of Sumatran specimens is slightly shorter and the basal portion is more inflated and rounded (fig. 3.9B versus 3.9C). In the lateral view, the bacula of Sumatran specimens has a larger shaft and an enlarged and less pointed tip. An enlarged tip is also found in many but not all Malay Peninsula specimens. In the dorsal view, the vertical ridges along either side of the basal part are almost invisible and sometimes absent in Sumatra specimens but are well developed in Malay Peninsula specimens.

The baculum of specimens from Sarawak is similar to that of Sumatran specimens, just slightly slender overall with a less inflated basal portion (fig. 3.9A versus 3.9B). In the lateral view, the tip of the shaft also shows enlarged character but elongated and less prominent compared to Sumatran specimens. In the dorsal view, the basal emargination is deeper and narrower.

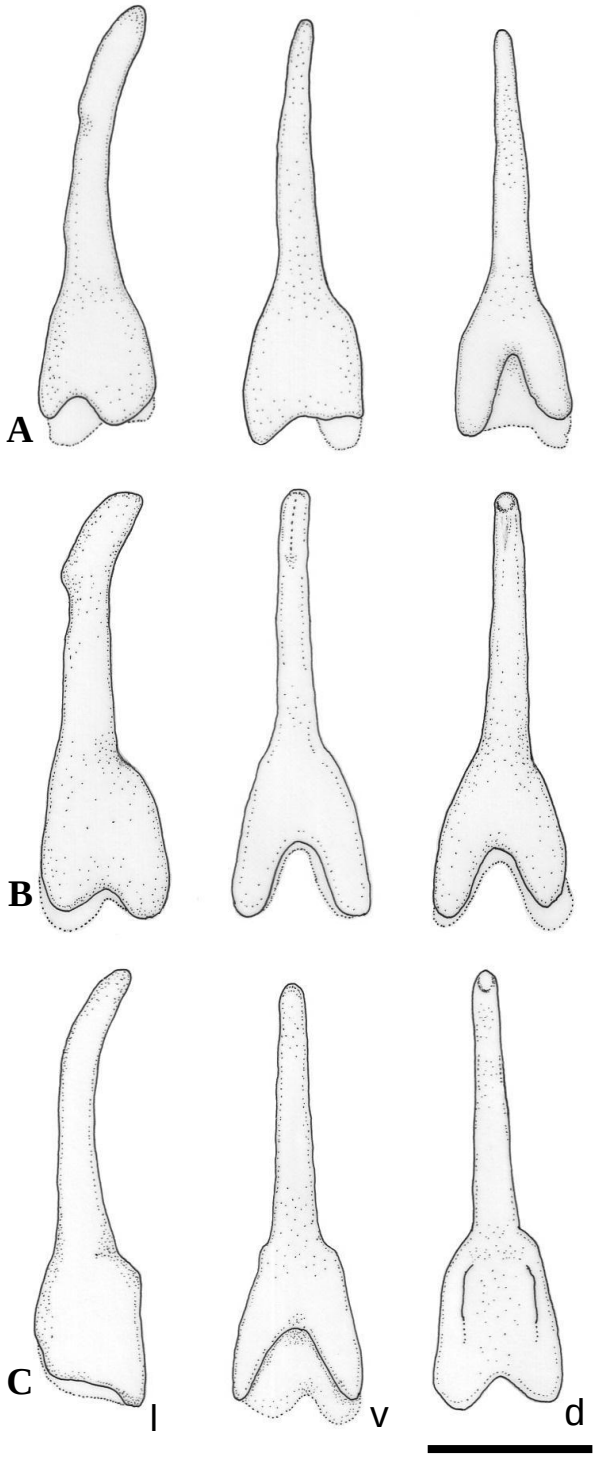

Figure 3.9. Bacula of *R. affinis* from the Sundaic Subregion in lateral view (l), ventral view (v) and dorsal view (d); Sarawak, north-western Borneo (A: TK152217); Lampung, southern Sumatra (B: MZB31501); Narathiwat, southern Malay Peninsula (C: IS120124.1). Scale = 1mm.

3.3.2. Echolocation

Extensive variation in call frequencies occur within the Sundaic subregion, with differences of 20 kHz recorded across the range (62.3 – 82.3 kHz). Average frequencies observed are recorded as: central Java 81.8 kHz, Sarawak 68.9 kHz, Sumatra 74.2 kHz, southern Malay Peninsula 77.8 kHz and northern Malay Peninsula 71.2 kHz (table 3.4, fig. 3.10). Bats from islands adjacent to the peninsula emit lower call frequencies compared to mainland populations. For instance, mean frequencies produced by bats from the Tarutao Island group (Tarutao, Andang and Rawi islands) on the west coast of the Thai part of the peninsula [T15] are 65.1 kHz compared to 71.2 kHz from the central area of the peninsula. Similarly, bats on Taman Negara Pulau Pinang [M2] emit mean frequencies of 72.8 kHz compared to 77.8 kHz on the peninsula Malaysia.

Table 3.4. The summary data for frequency maximum energy (FmaxE) of *R. affinis* from Sundaic subregion. Values are given as min-max, mean ± standard deviation.

Locality	**No. bats**	**No. calls**	**Frequency (kHz)**	**Source**
Java	6	6	81.8±0.4 81.2–82.3	Current study
Borneo	1	1	68.9 -- --	Current study
Sumatra	5	5	74.2±0.5 73.2–74.6	Current study
Southern peninsula	16	27	77.8±1.3 75.4–79.3	Current study & Ith *et al*. (in review)
Taman Negara Pulau Penang (island)	2	11	72.8±0.5 72.6–73.1	Current study
Northern Peninsula	31	49	70.8±0.7 69.5–72.6	Ith *et al.* (in review)
Tarutao islands	10	21	65.1±1.3 63.6–66.6	Current study
KohSurin, PhangNga	1	1	62.3 – -- --	Current study

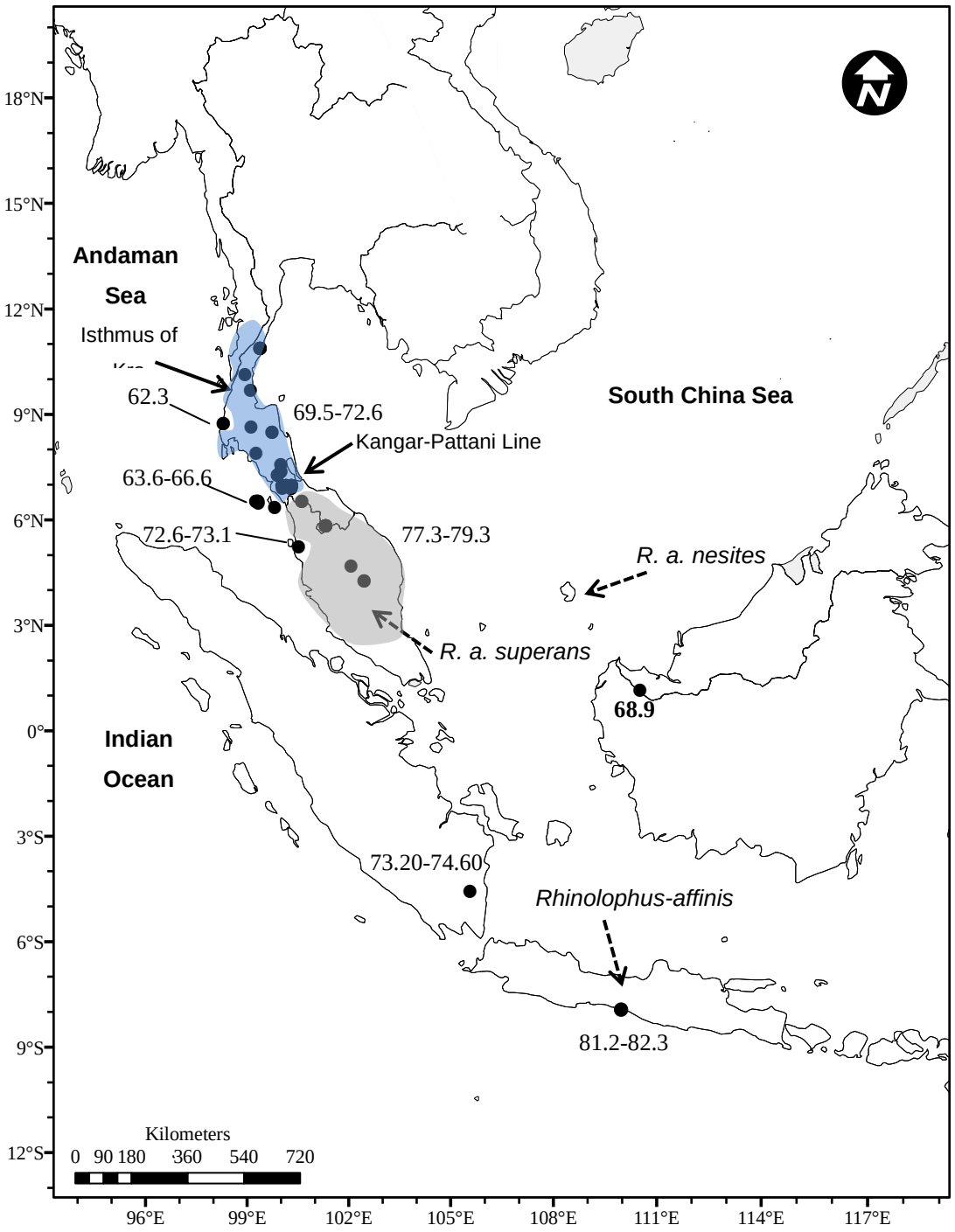

Figure 3.10. Echolocation call frequencies of *R. affinis* in the Sundaic subregion. Values are given in kHz and the different color shades represent the different call frequency zones. Dashed arrows indicate the type locality of subspecies whereas solid arrow indicates transition zones of biota within the peninsula.

3.3.3. Genetics

Results from both maximum likelihood (ML) and Bayesian analysis (BA) showed similar topologies in phylogenetic trees. Three clades were recovered based on *cyt b* genes (fig. 3.11). Clade A and C lineages were supported by high bootstrap values (BT = 90 – 99%) and posterior probabilities (PP = 100%) while clade B supported by lower BT = 60% but rather high PP = 94%. The recovery of the three lineages were very consistent in analysis, however the recovery of basal linage was inconsistent. Clade A was a basal lineage to clade B and C (fig. 3.11), however in some cases clade C was basal to clade A and B. In such cases, sister relationships between clade A and B or clade B and C were always supported by lower statistical values (e.g. BT = 30%, PP = 75%).

Clade A comprised sequences from Borneo, whereas clade B comprised sequences from Borneo, Java and Sumatra and clade C comprised sequences from the Malay Peninsula (fig. 3.12). Pair-wise genetic distances within clades were low at 0.01%, 0.00 – 0.03 (mean, range) for clade A, 0.06%, 0.00 – 1.30 for clade B and 0.05%, 0.00 – 0.10 for clade C. Mean genetic distance between Borneo and Java-Sumatra were lower (clade A versus B: 2.8%, 2.6 – 3.3), and relatively higher between the Malay Peninsula and Borneo (clade C versus A: 3.7%, 3.7 – 4.4) and the Malay Peninsula and Java (clade C versus B: 3.6%, 3.0 – 4.4). Based on the mean genetic distance, the Java and Borneo clades (B and A) shared a more recent ancestor than the Malay Peninsula clade (C). Clade C was therefore assumed to be basal to clade A and B.

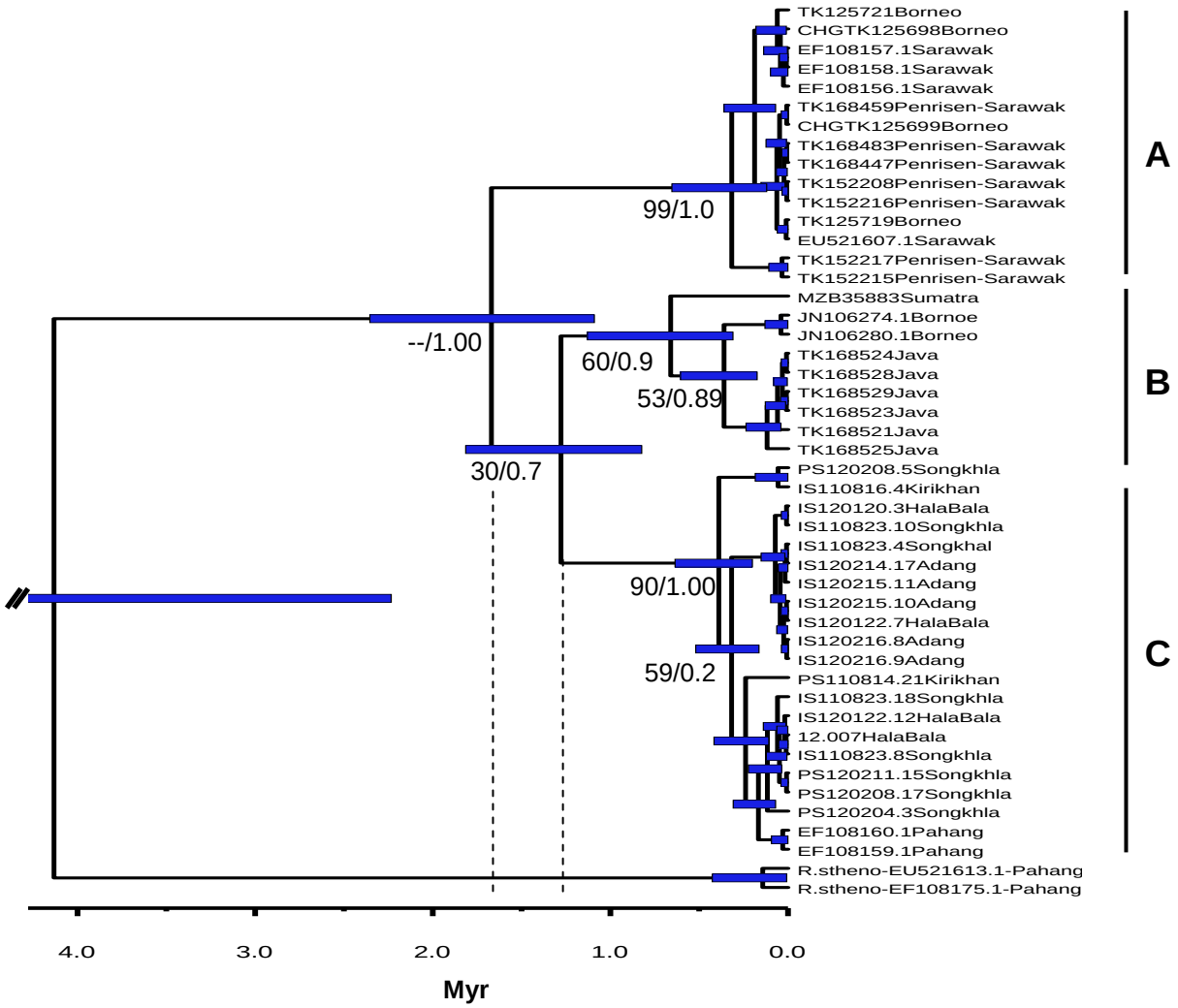

Figure 3.11. Baysian phylogenetic tree based on cytochrome-b (Cytb) gene. Scores on the branches refer to bootstrap support values (1,000 iterations) derived from maximum likelihood (1st score) and Bayesian posterior probabilities (2nd score); -- = no support value. The blue bars represent the 95% highest posterior density intervals for the divergence estimates. Specimens are labeled by specimen codes (CHGTK, EF, EU, JN, IS, MZB, PS and TK) and collecting localities.

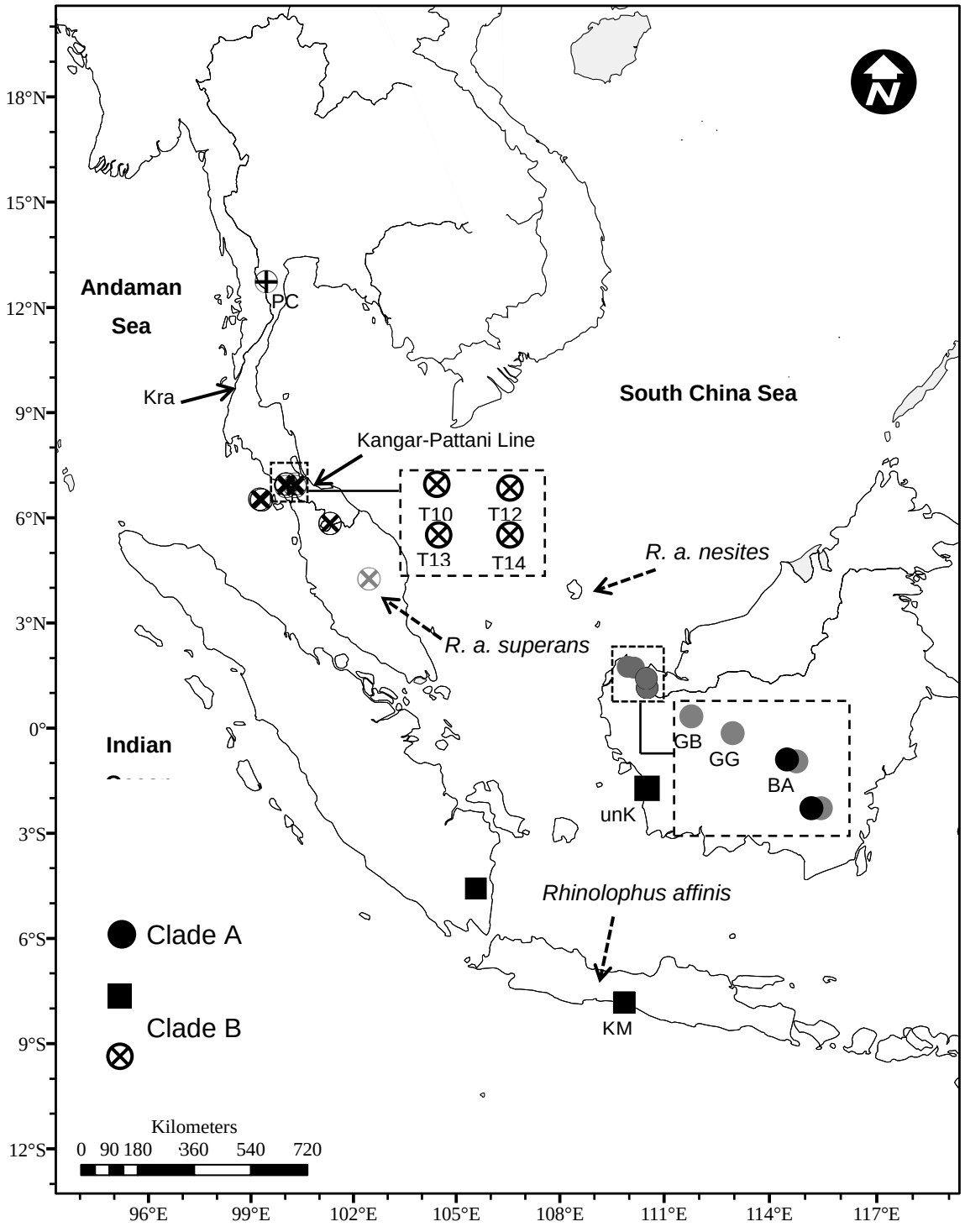

Figure 3.12. Distribution of cytochrome b (Cytb) clades of *R. affinis* within the Sundaic subregion. The shape of the symbols corresponds to clades defined in figure 3.11. Black symbols are sequences from the current study whereas grey symbols are sequences from the genbank. Localities of sequences not listed in the methods and materials of the-

current study are listed for the first time as following, BA=Jambusan- Cave, Bau, Sarawak; GB=Gunung Berumput, Sarawak; GG=Gunung Gading NP, Sarawak; KM=Kabumen, Central Java and PC=Prachuap Kiri Khan. unK=unknown specific locality from Borneo (sequences from genbank). Dashed arrows indicate the type localities of subspecies. Black solid arrows indicate the transition zones of biota in the peninsula.

Results from both ML and BA illustrated similar topologies, with two clades recovered for COI gene (fig. 3.13). Clade A (BT=99%, PP=100%) comprised all sequences from the Malay Peninsula whilst clade B (BT=59%, PP=100%) comprised sequences from Sumatra (fig. 3.14). Pairwise genetic distance within clades were low at 0.02%, 0.00 – 0.07 (mean, range) for clade A and 0.03%, 0.00 – 0.05 for clade B. Mean genetic distance between the clades was 2.2%, 1.7 – 2.7 (A versus B). As both clades were consistently recovered with strongly supported values and observed in *cytb* analysis (clade B and C, fig. 3.12), these populations were recognized as two isolated lineages.

Bayesian estimates of time to the most recent common ancestor (TMRCA) provided effective sample size (ESS) values >500 for all parameters. The inferred TMRCA for all recovered clades, including the Borneo and Java and Malay Peninsula clades (A versus B, C) was 1.7 million years before present (MyrBP) (95% CI 1.09 – 2.35) (fig. 3.12), corresponding to an early stage of the Pleistocene epoch. The TMRCA for B versus C was more recent at 1.30 Myr BP (95% CI 0.82 – 1.81) which corresponds to the mid Pleistocene period. However, as recovery of basal lineages was inconsistent (switching between clade A and C), we assume TMRCA between lineages is more or less the same (ca. 1.30 – 1.70 Myr BP).

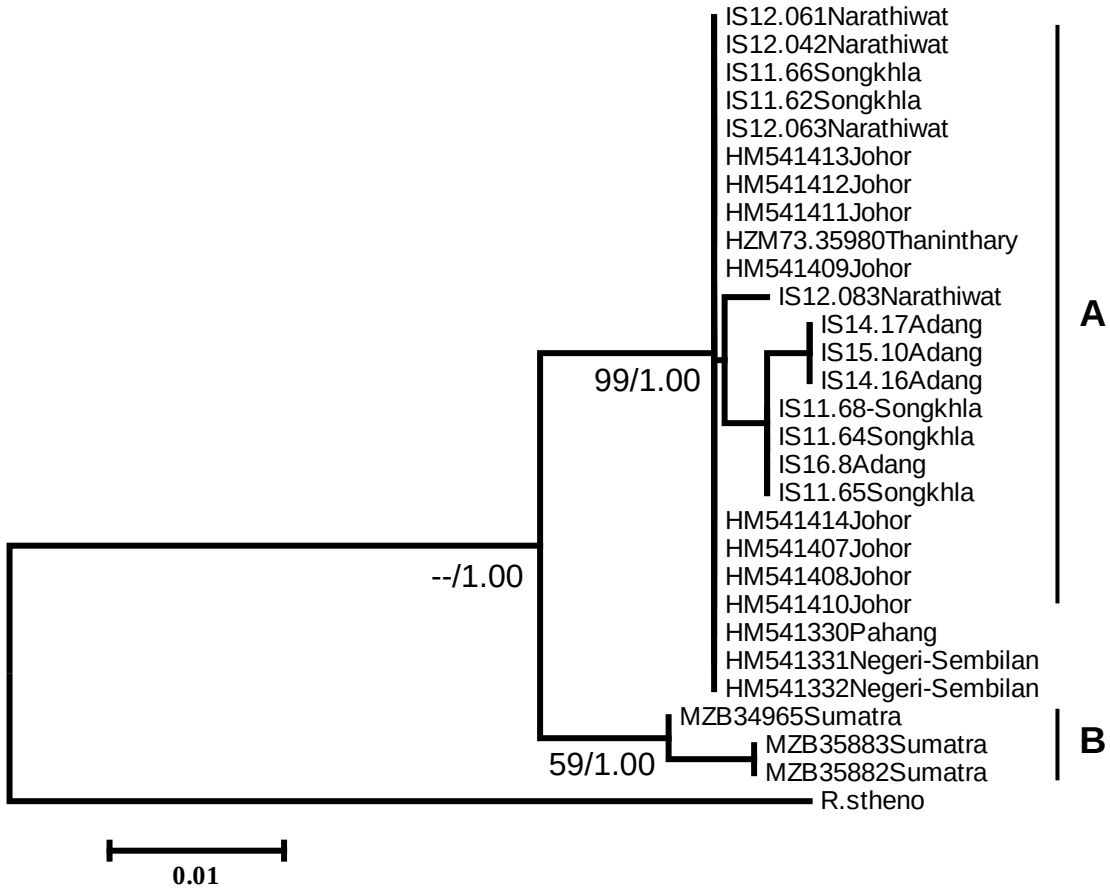

Figure 3.13. Maximum likelihood tree based on based on cytochrome C oxidase subunit I (COI). Scores on the branches refer to bootstrap support values (1, 000 iterations) derived from maximum likelihood (1st score) and Bayesian posterior probabilities (2^{nd} score); -- = no support value. Specimens are labeled by specimen codes (IS, HM, HZM and MZB) and collecting localities.

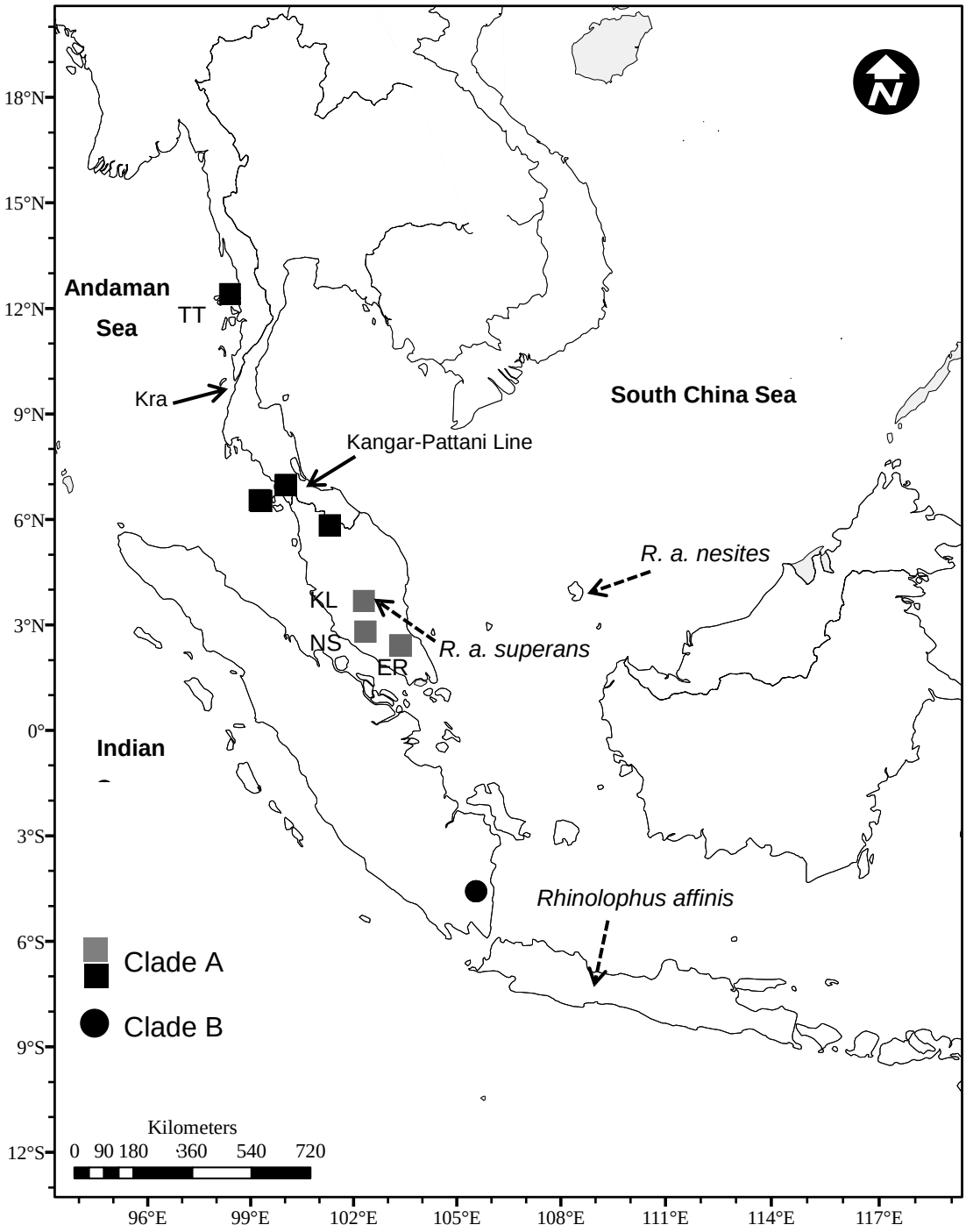

Figure 3.14. Distribution of cytochrome c oxidase subunit I (COI) clades of *R. affinis* in the Sundaic subregion. The shape of the symbols corresponds to clades defined in figure 3.13. Black symbols are sequences from the current study and Ith *et al*. (in review) whereas grey symbols are sequences from genbank. Localities of the sequences not listed in the methods and materials of the current study are listed for the first time as following, ER=Endau Rompin National Park, Peninsula Malaysia;-

KL=Kuala Lompat, Pahang; NS=Negeri-Sembilan; TT=Thaninthary Div, Myanmar. Dashed arrows indicate the type localities of subspecies. Black solid arrows indicate the transition zones of biota in the peninsula.

3.3.4. Variation within the Malay Peninsula

Intraspecific variation was also found within the Malay Peninsula. Specimens from high call frequency zone (A: 77.3 – 79.3 kHz, fig. 3.10) found to be smaller in many instances compared to specimens northwards of Khao Namkhang National Park (T15) (the lower call frequency zone B: 69.5 – 72.6 kHz, fig. 3.10) particularly in cranial characters (table 3.2). The former have significantly smaller horseshoes, SL, CCL, ALSW, PB, C^1C^1W, M^3M^3W, CM^3L, CM_3L and ML ($p<0.05$) (fig. 3.4E, 3.4F, table 3.2). Moreover, zone A specimens have slightly smaller teeth overall (fig. 3.8D, 3.8E). However, both populations have similar baculum morphology. A PCA using 9 external and cranial characters of all specimens from Malay Peninsula illustrated two relatively isolated groups (fig. 3.15).

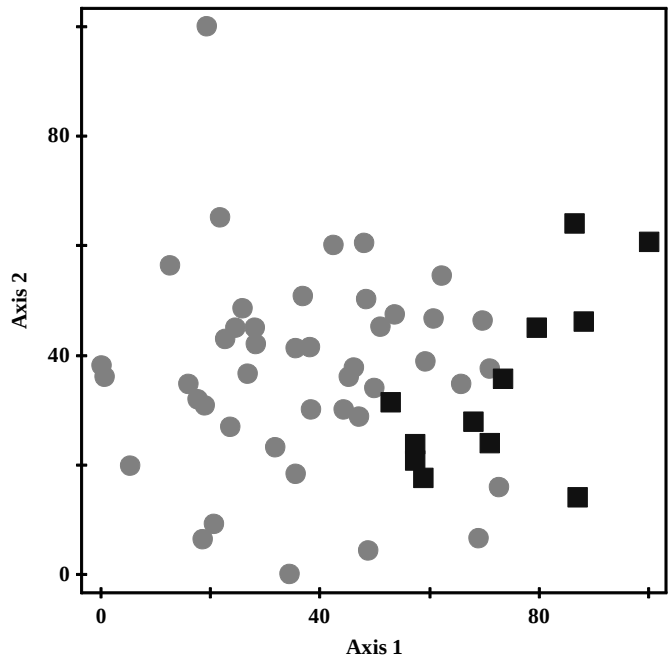

Figure 3.15. PCA of nine external and cranial characters of *R. affinis* specimens from southern Malay Peninsula (black squares) and northern Malay Peninsula (grey circles).

Table 3.5. Eigenvectors and eigenvalues of PCA of nine external and cranial characters of specimens from southern Malay Peninsula and northern Malay Peninsula; the values explain figure 3.15.

	Eigenvector		
Characters	**1**	**2**	**3**
EL	-0.4334	-0.5876	0.5309
GWN	-0.6082	-0.0374	0.2000
SL	-0.9222	-0.1007	-0.1878
CCL	-0.9275	-0.1860	-0.1091
ALSW	-0.7831	0.4187	0.0095
PB	-0.4084	0.5368	0.6846
CM_3L	-0.8633	-0.0139	-0.0482
C^1C^1W	-0.5806	0.3772	-0.3354
ML	-0.8843	-0.2389	-0.1447
Eigenvalue	4.913	1.054	0.974
% of total variation explained	54.585	66.300	77.117

3.4. SUBSPECIES DIAGNOSIS

3.4.1. *Rhinolophus affinis superans*, Andersen 1905

R. a. superans Andersen, 1905: Pahang, Malaysia

Rhinolophus affinis superans is closely similar to *R.* cf. *affinis* from south Sumatra, distinguishable by being average larger in tibia (TIB), 21.2 – 26.4 mm and ALSW, 3.83 – 4.67 mm. The frontal depression and supraorbital ridges are more elongated in *R. a. superans*, but *R.* cf. *affinis* appears to have more developed sagittal crest.

In comparison with *R. a. nesites*, *R. a. superans* is average larger in many external, cranio-dental measurements.

External: *R. a. superans* is larger in FA, tail length (TL), TIB, hint foot (HF) and several wing measurements including second metacarpal (2MT), 3MT, 4MT and 5 MT (table 3.2).

Cranio-dental: *R. a. superans* is larger in SL, ZYW, CM^3L, C^1C^1W, posterior palatal width (M^3M^3W), CM_3L and least height of the coronoid process (CPH) (table 3.2).

Baculum: Distinguishable by being rectangular shape at base, in lateral view and the basal emargination is shallower, in dorsal view (fig. 3.9).

3.4.2. *Rhinolophus* cf. *affinis*

R. cf. *superans*: south Sumatra

This taxon is closely similar to *R. a. superans*, only few characters are smaller in average (see the description of *R. a. supernas* above).

R. cf. *affinis* is larger in many external and cranio-dental characters compares *R. a. nesites*.

External: *R.* cf. *affinis* is larger in FA, TL, TIB, HF and several wing measurements including 2MT, 3MT, 4MT and 5 MT (table 3.2).

Cranio-dental: *R.* cf. *affinis* is larger in SL, ZYW, CM^3L, C^1C^1W, M^3M^3W, CM_3L and CPH (table 3.2).

Baculum: *R.* cf. *affinis* has slightly larger build in overall and the tip shows obvious swollen characters (fig. 3.9).

3.4.3. *Rhinolophus affinis nesites*, Andersen 1905

R. a. nesites Andersen, 1905: Bunguran Island, North Natuna Islands

This taxon appears to be the smallest form among the Sundaic taxa. In comparison with *R. a. superans* and *R.* cf. *affinis*, *R. a. nesites* is smaller in many external and cranio-dental characters (see the comparison of *R. a. superans* and *R.* cf. *affinis* with *R. a. nesites* above).

In comparison with *R. a. superans* and *R.* cf. *affinis*, *R. a. nesites* has smaller noseleaf. The median emargination of the horseshoe is narrower (fig. 3.4). The secondary noseleaf is less developed and concealed by the horseshoe in dorsal view. The sella is small and obviously constricted in the middle (fig. 3.5). The skull has shorter frontal depression, the canines and teeth are smaller in overall (fig. 3.3.).

3.5. DISCUSSION

On the basis of morphology, baculum and genetic evidence, three geographical forms of *Rhinolophus affinis* were recognized from Sundaic subregion. *R. a. suprans* distributes within Malay Peninsula, *R. a. nesites* from Borneo and *R.* cf. *affinis* from Sumatra. The discrete morphology and genetic of this widespread species from the region reflects the phylogeographic distinctiveness between the locations particularly between Borneo and Peninsula. Many bat species examined between Borneo and the peninsula were also found to have similar the patterns (Francis *et al.*, 2010; Khan *et al.*, 2010). Similar pattern was also found in murine rodents examined from Sunda shelf (Gorog *et al.*, 2004). The call frequencies are highly fluctuated within the region and lack of supporting for the morphology and genetic differences. This is similar to the previous study on this species from mainland Southeast Asia (Ith *et al*. in review). Poor evolutionary supporting by echolocation was also known in Southeast Asian bats, *R. malayanus* (Soisook *et al.*, 2008) and *Hipposideros larvatus* (Thabah *et al.*, 2006).

3.5.1. Morphology

R. a. nesites was first described from Bunguran islands, north Natunas (Andersen, 1905a) and described to distribute in Borneo from Sarawak (Bau, Kuap) to

west and south Kalimantan (Medway, 1977). Recently, the form was also recognized by Csorba *et al*., (2003) followed Koopman (1994). According to the current examination on specimens from Sarawak, the specimens agreed closely with the first description of the holotype of *R. a. nesites* by Andersen (1905a) as being short TIB, TL and MT. However, our specimens appeared to have relatively smaller EL and GWN which is different from the first description, describing *R. a. nesites* has comparable EL and GWN to *R. a. superans*. It is likely as the holotype of *R. a. nesites* was from the island (type locality: Bunguran Island) and we notice that *R. affinis* specimens collected from the islands tend to have larger ears and noseleaf characters (e.g. horseshoe, sella and connecting process) and emit lower call frequencies.

Sumatra population was described as *R. a. superans* by Andersen (1905a) and also recognized in Csorba *et al*., (2003). The only specific locality recorded from the region is Sirambas, central Sumatra (Andersen, 1907). In the current study, *R.* cf. *affinis* from southern Sumatra appeared to have many craniodental and baculum characters which were different from Malay Peninsula specimens as well as genetic data (COI, *cytb*). This form shares many craniodental characters and genetic with Java population. Based on the result we strongly suggest this southern population to be separated from the peninsula population however would not reject any populations from Sumatra (particularly from the central and northern Sumatra) to be part of peninsula population. This is due to our sample from Sumatra is constrain to only the southern part of the island. Therefore, the possibility that the adjacent areas of the two islands share morphological and genetic characters is expected (e. g. *R. affinis* from Wallacea archipelago [Maharadatunkamsi *et al.*, 2000])

The morphological cline of *R. a. superans* from the peninsula aligned closely to the historical plant transition zone (Kangar Pattani Line) (Baker *et al*., 1998; Good 1964; Keng, 1970; Richards, 1996; van Steenis, 1950; Whitmore, 1984; Wikranmanayake *et al*., 2002) and climatic zones (Hughes *et al*., 2011). This morphological cline was reflected by call frequencies but not in genetic, suggesting the two forms evolved very recently. We assumed the deviation of the morphology was associated with the adaptation for different habitat types, climate and or preys availability as discussed below (echolocation and genetic).

3.5.2. Echolocation

The call frequencies of *Rhinolophus affinis* from the current study showed relatively high fluctuated patterns which are similar to *R. affinis* (Ith *et al*., in review) and *R. malayanus* from the region (Soisook *et al*., 2008). The call frequencies was just below 20 kHz difference between the extremes (62.3-81.8 kHz), the lowest frequency was from Tarutao Island populations and highest from central Java population.

Call frequencies appeared to show clear patterns as comparing between insular populations. However, the call frequency differences about 7 kHz between Sumatra and Java did not reflect in genetic and overall craniodental morphology, except noseleaf characters as Sumatra specimens have overall larger horseshoe, sella, and lancet. In Borneo, the call frequency was lowest among the insular populations (12.9 kHz lower than Java population and 5.3 kHz lower than Sumatra population) and the frequency difference was reflected by morphology and genetic therefore it seems a useful tool for classification. However, more calls from Borneo are needed to see its overall patterns and variations as we have only one call available in the current study.

In the Malay Peninsula two clear frequency patterns were observed if the island populations were excluded (T4, T15 and M2). These frequency patterns correspond very well to the morphological deviation discussed above, therefore aligned closely to the phytogeographical transition line, Kangar Pattani Line (Baker *et al*., 1998; Good 1964; Keng, 1970; Richards, 1996; van Steenis, 1950; Whitmore, 1984; Wikranmanayake *et al*., 2002) and climatic zones (Hughes *et al*., 2011) of the peninsula. Higher frequency lied to the tropical evergreen rain forest with higher humidity (south of Kangar Pattani Line) while lower frequency lied to the semi-evergreen rain forest with lower humidity (north of the line). Though, this call frequencies patterns were reflected by some craniodental characters (fig. 3.11) but not supported by the genetic (*cytb* and COI [fig. 3.12, 3.14]), also D-loop (Ith *et al.*, in review). It suggests that these two phonic populations have just initiated very recent. Based on the current result, we assume that the call frequency patterns were associated with the habitat types, climatic conditions and or preys available of the peninsula. The current forest patterns and climatic conditions have been shaping as recently as the end of the last glacial maxima when the climate was under today

condition, therefore left little time (~9500 years ago, after the breakup of Sunda Shelf land bridge for gene to evolve (Inger & Voris, 2001; Voris, 2000).

Humidity seems not affect to the call frequency emitted by *R. affinis*. High humidity (south of transition line) attenuates high frequency sound more than lower frequencies (Guillén *et al.*, 2000; Hartely, 1989) (Griffin, 1971), therefore lower call frequencies would be expected to find from southern peninsula and higher call frequencies would be expected from northern peninsula but our result was contradict to the expectation. It is likely that the foraging habitat may be a controlling factor. It is known that in cluttered habitat bats use higher call frequency to increase their detection efficiency while lower call frequency has better detection range suitable for less-cluttered habitat (Kingston *et al.*, 2001; Schnitzler & Kalko, 1998). The higher cluttered spaces are the characters of tropical rain forest therefore it is likely that frequency differences in *R. affinis* may the result from microhabitat preferences. Similar call frequency patterns were also found in *R. malayanus* (Soisook *et al.*, 2008).

Here we observed that the island populations adjacent to the peninsula emitted lower call frequencies than the mainland populations. Adang and Rawee islands [T15] and Koh Surin [T4] emitted lower call frequencies compare to the mainland populations adjacent to these islands (65.1, 62.3 versus 70.8 kHz respectively). It is similar to Penang population [M2] compare to its adjacent mainland population (72.8 versus 77.8 kHz). Islands north of Kangar Pattani Line (Adang, Rawee and Koh Surin) have seasonal forest similar to northern peninsula as its position is north of the Kangar Pattani Line, in reverse evergreen rain forest is from Penang island as its position is just south of the line. Therefore, other factors rather than just forest types may have been driving the evolution of these island populations such as humidity, preys and or island size. Alternatively, it may reflect the random drift of distance-isolated populations. It would be of interest for further researches determining relevant factors on such frequency drift of *R. affinis* from the island habits (e. g. insect prey species and abundant).

3.5.3. Genetics

Phylogroups which were genetically defined have been documented in many vertebrate species (Avise & Walker, 1999) including mammals (Baker & Bradley, 2006) (da Silva & Patton, 1998; Gorog *et al.*, 2004; Khan *et al.*, 2010; Lin *et al.*, 2013; Mao *et al.*, 2010). Phylogenetic analysis of COI and *cytb* inferred tree lineages of *R. affinis* from Sundaic subregion distinguishable by levels of genetic divergence between Borneo (*R. a. nesites*), Java/Borneo/Sumatra (*R.* cf. *affinis*) and Malay Peninsula (*R. a. superans*). This result was congruent to genetic barcoding analysis of bats from Southeast Asia (Francis *et al.*, 2010) which reported that 21 widespread species of bats (out of approx. 100 species which occurs in both peninsula and Borneo) sampled from peninsula Malaysia and Borneo, only three showed less than 1% genetic divergence between locations, while eight differed by more than 6%. Similar genetic structure was also observed in *Kerivoula* species (Khan *et al.*, 2010) and murine rodents (Gorog *et al.*, 2004). However, an absence of phylogeographic structure between the locations was known in a species wooly bat *Kerivoula pellucida* (Khan *et al.*, 2010).

The results of the study contribute for the understanding of paleoenvironments across the Sundaic subregion. The consistent lineages constructed, associated with geographical regions within our samples of *R. affinis* indicate that at least the islands (Java, Borneo, Sumatra) and peninsula populations were isolated prior to the breakup of the last Pleistocene land bridges the event that took place until ~ 9,500 years ago (Inger & Voris, 2001; Voris, 2000). Based on relaxed molecular clocked, mean diversification for *R.* cf. *affinis*, *R. a. nesites* and *R. a. superans* were all in the early Pleistocene epoch (1.7-1.3 mya). This is similar to the initial diversification time of *Hipposideros amiger* lineages (1.35 mya) which might have caused by refugial isolation prior to the coldest time of Pleistocene (Lin *et al.*, 2013). This pattern is closely congruent with many other taxonomic groups throughout the region, including gymnures (Ruedi & Fumagalli, 1996), murine rodents (Gorog *et al.*, 2004), bats (Khan *et al.*, 2010), snakes and frogs (Inger & Voris, 2001) and termites (Gathorne-Hardy *et al.*, 2002).The result reflects the early Pleistocene isolation, therefore does not support the hypothesis that Pleistocene land bridges would have allowed periodic migrations between the peninsula and Borneo in the Late Pleistocene (Inger & Voris,

2001; Voris, 2000) and suggest a deeper history of earlier vicariance in the region (Gorog *et al.*, 2004; Khan *et al.*, 2010).

The formation of *R. affinis* linages on Sunda shelf may be partly explained by its ecology associated with the habitat characters. *R. affinis* principally is a forest species (Kingston *et al.*, 2009), roosting in the cave, depends on forest as the feeding grounds (Medway, 1969). The repeated contracting and expanding of the forest throughout the region might have fragmented the populations and limited the gene flow. Southeast Asia experienced perhumid climate during the Miocene (Gorog *et al.*, 2004), at the Miocene-Pliocene boundary (Dersch & Stein, 1994), followed a sudden deteriorated condition in the early to mid-Pliocene (~3 mya) (Kashiwaya *et al.*, 2001). In Pleistocene epoch (~1.8 mya), the world experienced more extreme climate. Sunda shelf experienced cool climate, arid and covered in large part by savanna-like or steppe vegetation (Heaney, 1991; Morley, 1998, 2000; van der Kaars *et al.*, 2001). The historical climatic conditions of relatively stable tropical environment and humid through the Miocene followed by Plio-Pleistocene deterioration of condition resulted forest contraction and refugia in Southeast Asia may explain for the vicariant patterns of *R. affinis* populations. Other taxa concerning the occurrence of rain forest refugia during Pleistocene including leaf monkey (*Presbytis* and *Pygathrix*), proboscis monkey (*Nasalis*), loris (*Loris* and *Nycticebus*), gibbon (*Hylobates*) and termite community (Brandon-Jones, 1996, 1998; Gathorne-Hardy *et al.*, 2002).

Cytb analysis discovered a lineage (clade B) comprising of sequences from different islands of Sunda shelf (Java, Borneo and Sumatra) (fig. 3.12). This lineage distantly split from Borneo clade Asine early Pleistocene (~1.7 mya), indicating animal was migrating between the islands and form their own genetic affinity population followed the time of initial split (between clade A, B). We do not have the specific localities of sequences from Borneo in clade B (genbank sequences). Therefore, we assume localities in Borneo adjacent to Java or Sumatra would be more likely as it facilitates the dispersal success during Pleistocene between the localities. Multiple lineages in Borneo were also known from woolly bat, genus *Kerivoula* (Khan *et al.*, 2010) and murine rodents (Gorog *et al.*, 2004). Similar scinaro would be expected in *R. affinis* from Sumatra and Java if large sample size from different localities are observed. This is due to multiple rain forest refugia were recognized

from the region such as northern Sumatra, western Java, northern and eastern Borneo (Brandon-Jones, 1996, 1998; Gathorne-Hardy *et al.*, 2002).

The concordance between genetic and phenetic relationships was found in *R. affinis* populations from Wallacea islands (Maharadatunkamsi *et al.*, 2000). Our results support the previous finding when comparing between islands and between islands and peninsula. However, genetic structure was not found in the peninsula where two morphological forms with different call frequencies were observed. This peculiar may due to the recent colonization of the animal to the peninsula and experienced rapid morphological divergence (Mayer & von Helversen, 2001) to adapt to different habitats and food available. This peninsula form (*R. a. superans*) initially diverged from its sister, Indochinese form (*R. a. macrurus*) rather recently (~400,000 years ago) (Ith *et al*., in review). The two morphological forms aligned closely to the phytogeogrphical transitions (Kangar Pattani Line [fig. 3.1]) and climatic zones (Hughes *et al.*, 2011) which reported to have different plant composition (Baker *et al*., 1998; Good 1964; Keng, 1970; Richards, 1996; van Steenis, 1950; Whitmore, 1984; Wikranmanayake *et al*., 2002). The region south of the transition line described as a tropical evergreen rain forest while north of the line described as semi-evergreen rain forest (Whitmore, 1984). The evergreen rain forest was not established during the last glacial maxima (LGM) (~18,000 years ago), instead seasonal forest and savannah were dominated as the weather was cooler and driver (Heaney, 1991). The present-like forest pattern could be assumed during the last interglacial period (120,000-140,000 years ago) when the similar climatic condition was predicted (Hughes *et al.*, 2011). However, due genetic structure was not detected between the two forms, we assumed that the deviation of the morphology was shaped by the current forest patterns which have been shaping followed the end of the LGM when the world began to have the present-like climate. Evidences of population expansion during warm climate of Pleistocene when feeding habitats are suitable and abundant food were also known form *Hipposideros armiger* (Lin *et al.*, 2013), *Presbytis* and *Semnopithecus* monkeys (Brandon-Jones, 1996), dipteran (Morgan *et al.*, 2011) and coleopteran (Li *et al.*, 2012). Lack of genetic different could be associated with genetic drift which caused by the peninsula affect, e.g. *Myotis muricola* was found to decrease genetic difference and variation in eastern end distribution of Wallacea archipelago (Hisher *et*

al., 2004). Other bat taxa which likely to experienced similar evolutionary history (shallow genetic differences) including *Miniopterus schreibersii* (Furman *et al.*, 2010), *Eptesicus serotinus* and *E. nilssonii* (Mayer & von Helversen 2001), *Rhinolophus macrotis* and *R. siamensis* (Francis *et al.*, 2010) and *Myotis annamiticus* (Francis *et al.*, 2010; Kruskop & Tsytsulina, 2001). The ability to rapidly fit to the novel environment (flexible in morphology and call frequency) prior to the deviation in genetic characters would be the benefit for animal. This strategy would help both maintaining the high genetic diversity and successfully occupying novel habitat types therefore would be existed in widespread species like *R. affinis*.

3.6. CONCLUSION

Three subspecific forms of *R. affinis* were recognized from Sundic subregion, two forms (*R. a. supperans* and *R. a. nesites*) were recognized follow the previous finding while the third form (*R.* cf. *affinis*) was firstly described by this study and rather deserves as the immediate descendent of *R. a. affinis* (described from Java). The three forms diverged during the arid climate with forest depressed period of Pleistocene therefore suggests this phylogeographical structure was the result of refugia isolation. The evidences of discrete morphology and genetic between *R. a. nesties* and *R. a. supernas* supported the phylogeographical distinctiveness between Borneo and peninsula while the deep divergence between *cytb* sequences from Borneo (clade A and B [fig. 3.12]) suggested multiple lineages of *R. affinis* from Borneo as well as ancient migration occurred between Borneo, Java and Sumatra (Clade B comprises of sequences from Java, Borneo and Sumatra [fig. 3.12]). The overall characters of being smaller in *R. a. nesites* from Borneo, again (discussed once in Ith *et al*., in review) appears to contradict to the morphological transition rule of Andersen (1905a), as he gave the exception to only the type locality of this species, N. Natunas Island and Java. This study confirmed the taxonomic status of previous recognized forms and described a new form by employing multiple dataset. We predict the high possibility of describing new forms of *R. affinis* from the region (e.g. *R.* cf. *affinis* from Sumatra) if larger effort is input throughout various localities in the

region. We also recommend using the multi dataset for taxonomic work, as depending on morphology and or echolocation alone would lead to wrong taxonomic decision.

CHAPTER 4

GENERAL DISCUSSION

The current study confirmed the subspecific status of *Rhinolophus affinis* sensus lato with three subspecies being confirmed from the Oriental region, namely *R. a. macrurus*, *R. a. superans* and *R. a. nesites* (Andersen, 1905a). The study also discovered two hitherto undescribed populations; one is an east Myanmar/low-north Vietnam population which occurs sympatrically with *R. a. macrurus*. Another population is a south Sumatra population which has potentially been mis-identified as *R. a. superans* by previous workers (Andersen, 1905a; Csorba *et al.*, 2003) yet shared closer craniodental morphology and genetic with Java population. *R. a. tener* was not considered in the current study, due to lack of sample data from Myanmar particularly from around the type locality of the type specimen.

Variations within the subspecies were also recognized. *R. a. macrurus* from central/south Vietnam showed morphology, genetic and echolocation structure deviates from others localities of the same subspecies. Cambodian specimens showed smaller external characters (similar to the peninsula form), yet shared closer genetic relationship to central Vietnam and north Thailand respectively, than the peninsula form. The two morphological forms of *R. a. superans* from Malay Peninsula, approximately divided by Kangar Pattani Line (also supported by echolocation but not by genetics). This suggests that the taxon has a complex evolutionary history which may explain its taxonomic difficulties.

The large range (~20 kHz between the extremes) and highly fluctuated peak frequencies (Kingsada *et al.*, 2011) which previously caused doubt in taxonomic decision were clarified in the current study. Based on the support evidence from morphology and genetic, it suggests that the peak frequency is not a good taxonomic tool for in *R. affinis*, as each subspecies (at least *R. a. macrurus* and *R. a. suparans*) emits different peak frequencies locally with approximately over 10 kHz between the extremes. This was not supported by genetics though it significantly correlated with sound emiting apparatus and organs. It is concluded that the call frequency has just

radiated recently and rapidly along with sound emitted apparatus and organs to adapt to recent forming habitat or preys, therefore left short time for gene to evolve.

The two subspecific forms from mainland Southeast Asia (*R. a. macrurus* and *R. a. supperans*) were divided by the transition zone which was also observed in other mammal species by Woodruff & Turner (2009), including bats (Woodruff & Turner, 2009; Hughes *et al.*, 2011). This zone aligned upper north of the zoological transition zone Isthmus of Kra, 10°30'N (Corbet & Hill, 1992; de Bruyn *et al.*, 2005; Lekagul & McNeely, 1977; Woodruff & Turner, 2009) reach to ~13.5°N which lay closely to the transition line proposed by Wallace (1876) (12-13.5°N). The two subspecies of *R. affinis* sensus lato split as recently as late Pleistocene (~400,000 years ago, 95% CI 222 000-603 000) suggesting the subspecies were initiated by the Pleistocene climate (Mao *et al.*, 2010). The time of split just fell within the warmest period of Pleistocene (Lin *et al.*, 2013), implying the two forms resulted from multi-directional dispersal during the warm period of Pleistocene.

The phylogenetic structures observed within Sunda Shelf showed relatively deeper vicariant population compared to between mainland populations, particularly between Borneo and the peninsula and Java/Sumatra populations (~1.7 Myr BP) while slightly more recent between Java/Sumatra population and peninsula populations (1.3 Myr BP). The current result reflected the geographical distinctiveness between Borneo and peninsula which was proposed by Francis *et al.*, (2010). South China Sea may act as the significant geographical barrier resulted in allopatric populations between Borneo and the peninsula (Francis, 2007). Moreover, *R. affinis* is probably one of a weak fliers among others species of the genus *Rhinolophus* (Patrick *et al.*, 2013). Similar phylogenetic structures were also observed in other mammal species (Ruedi & Fumagalli, 1996; Gorog *et al.*, 2004) and snakes and frogs (Inger and Voris, 2001).

It is possible to elevate populations from Sunda Shelf (*R. a. nesites* from Borneo and *R.* cf. *affinis* from southern Sumatra) to be the full species differ from the mainland as they showed relatively large genetic distance (*cyt b*) compared to the peninsula population (Borneo versus peninsula: mean, 3.7%, range: 3.7-4.4%; Java/Sumatra/Borneo versus peninsula: 2.8%, 2.6-3.3% respectively). It is greater than 2% distance proposed by Bradley & Baker (2011), indicative of conspecific

population or valid species. *Rhinolophus arcuatus* and *R. euryotis* from Sulawesi had only 2.2% genetic distance (*cyt b*) yet considered as the distinct species based on nasal sella morphology (Patrick *et al.*, 2013). Similar findings were observed in *Rhinolophus macrotis* and *R. siamensis* although they are different in size and echolocation call frequency (Francis *et al.*, 2010). However, the current study proposed for additional study before considering them as the different taxa separating from the mainland forms; especially to ascertain reproductive isolation between the two subregions.

The current study helped to improve the understanding of the taxonomy of *R. affinis sensus* lato, by employing large sample sizes from numerous localities in the region. The current study confirmed the taxonomic status of the species for the first time employing multiple datasets. The outlier forms (east Myanmar/low-north Vietnam [form B, figure 3.3] and south Sumatra [clade B: *cytb* and COI (figure 3.12, 3.14)] were highlighted by this study and further evaluation of these forms is a priority. The study clarified taxonomic uncertainties from previous studies e. g. the morphological transition rule by Andersen (1905a) and the doubt of call frequency patterns highlighted by Kingsada *et al.*, (2011). Morphology, echolocation and genetic variation within the subspecific forms was thoroughly researched throughout the region, and this data will form the basis for further study, especially for workers who are interested in systematics, population structure and biogeography.

REFERENCES

Allen, G. M. (1938). *The mammals of China and Mongolia* (Vol. 22). New York: American Museum of Natural History.

Allen, J. A. (1906). Mammals from the Island of Hainan, China. *Bulletin of th American Museum of Natural History, 22*, 463-490.

Altringham, J. D. (1996). *Bats: Biology and Behaviour*. New York: Oxford University Press.

Andersen, K. (1905a). On some bats of genus *Rhinolophus*, with remarks on their mutual affinities and descriptions of twenty-six new forms. *Proceeding of Zoological Society of London, 2*, 75-145.

Andersen, K. (1905b). List of the species and subspecies of the genus Rhinolophus, with some notes on their geographical distribution. *Annals and Magazine of Natural History, 7*(16), 648-662.

Andersen, K. (1905c). On the bats of the *Rhinolophus arcuatus* group, with description of the five new forms. *The Nannals and Magazine of Natural History, 16*, 281-288.

Andersen, K. (1905d). On the bats of the *Rhinolophus macrotis* group. *The Nannals and Magazine of Natural History (Ser. 7), 16*, 289-292.

Andersen, K. (1905e). On the bats of the *Rhinolophus philippinensis* group. *The Nannals and Magazine of Natural History (Ser. 7), 16*, 243-257.

Andersen, K. (1907). Chiroptera notes. *Annali del Museo civico di storia naturale di Genova, (Ser:3)3*, 5-45.

Andersen, K. (1907b). Chiroptera notes. *Annali del Museo civico di storia naturale di Genova, (Ser:3)3*, 473-478.

Andersen, K. (1918). Diagnoses of new bats of the families Rhinolophidae and Megadermatidae. *Annals and Magazine of Natural History, 2*, 374-384.

Anderson, J. (1881). *Catalogue of the Mammalia in the Indian Museum, Culcutta, Part I*. Culcutta: Indian Museum.

Anwarali, F. A., Solari, S., Swier, V. J., Larsen, P. A., Abdullah, M. T., & Baker, R. J. (2010). Systematics of Malaysian woolly bats (Vespertilionidae: *Kerivoula*)

inferred from mitochondiral, nuclear, karyotypic, and morphological data. *Journal of Mammalogy, 91*(5), 1058-1072.

Avise, J. C., & Walker, D. (1999). Species realities and numbers in sexual vertebrates: perspectives from an asexually transmitted genome. *Proceedings of the National Academy of Sciences, 96*, 992-995.

Baker, R. J., & Bradley, R. D. (2006). Speciation in mammals and the Genetic Species Concept. *Journal of Mammology, 87*(643-662).

Baker, W. J., Coode, M. J. E., Dransfield, J., Dransfield, S., Harley, M. M., Hoffmann, P., *et al.* (1998). Patterns of distribution of Malesian vascular plants. In R. Hall & J. D. Holloway (Eds.), *Biogeography and geological evolution of Southeast Asia.* Leiden: Backhuys.

Barclay, R. M. R., & Brigham, R. M. (1991). Prey Detection, Dietary Niche Breadth, and Body Size in Bats: Why are Aerial Insectivorous Bats so Small? *The American Naturalist, 137*(5), 693-703.

Barclay, R. M. R., Fullard, J. H., & Jacobs, D. S. (1999). Variation in the echolocation calls of the hoary bat (Lasiurus cinereus): influence of body size habitat structure, and geographical location. *Canadian Journal of Zoology, 77*, 530-534.

Bates, J. J. P., & Harrison, D. L. (1997). *Bats of Indian subcontinent.* Sevenoaks, Kent, England: Harrison Zoological Museum.

Bergmans, W., & Van Bree, P. J. H. (1986). On a collection of bats and rats from the Kangean Islands, Indonesia (Mammalia: Chiroptera and Rodentia). *Zeitschrift für Säugetierkunde, 51*, 329-344.

Bickham, J. W., Patton, J. C., Schlitter, D. A., Rautenbach, I. L., & Honeycutt, R. L. (2004). Molecular phylogenetics, karyotypic diversity, and partition of the genus *Myotis* (Chiroptera: Vespertilionidae). *Molecular Phylogenetics and Evolution, 33*, 333-338.

Blaxter, M. L. (2004). A promise of a DNA taxonomy. *The Royal Society, 359*, 669-679.

Bogdanowicz, W., & Owen, R. D. (1992). Phylogenetic analysis of the bat family Rhinolophidae. *Zeitschrift fur Zoologische Systematik und Evolutionsforschung, 30*, 142-160.

Bourret, R. (1951). Une nouvelle chauve-souris du Tonin, *Rhinomegalophus paradoxolophus*. *Bulletin de la Musee d'Histoire Naturelle de Paris, 33*, 607-609.

Brandon-Jones, D. (1996). The Asian Colobinae (Mammalia: Cercopithecidae) as indicators of Quaternary climatic change. *Biological Journal of the Linnean Society, 59*, 327-350.

Brandon-Jones, D. (1998). Pre-glacial Bornean primate impoverishment and Wallace's line. In J. D. Holloway & R. Hall (Eds.), *Biogeography and geological evolution of SE Asia* (pp. 393-404). Leiden: Backhuys.

Chen, S. F., Rossister, S. J., Faulkes, C. G., & Jones, G. (2006). Population genetic structure and demographic history of the endemic Formosan lesser horshoe bat (*Rhinolophus monoceros*). *Molecular Ecology, 15*, 1643-1656.

Cooper, S. J. B., Reardon, T. B., & Skilins, J. (1998). Molecular systematics of Australian rhinolophid bats (Chiroptera: Rhinolophidae). *Australian Journal of Zoology, 46*, 203-220.

Corbet, G. B., & Hill, J. E. (1992). *The mammals of the Indomalayan region*. London: The Natural History Museum Publications, Oxford University Press.

Csorba, G., Ujhelyi, P., & Thomas, N. M. (2003). *Horseshoe bats of the world (Chiroptera: Rhinolophidae)*. Bishops Castle: Alana Books.

Czaplewski, N. J., Morgan, G. S., & McLeod, S. A. (2008). Chiroptera. In C. G. Gunnell & M. Uhen (Eds.), *Evolution of Tertiary mammals of North America. Vol. 2. Marine mammals and smaller terrestrial mammals* (Vol. 2, pp. 174-197). New York: Cambridge University Press.

da Silva, M. N. F., & Patton, J. L. (1998). Molecular phylogeography and the evolution and conservation of Amazonian mammals. *Molecular Ecology, 7*, 475-486.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods, 9*(8), 772.

de Bruyn, M., Nugroho, E., Hossain, M. M., Wilson, J. C., & Mather, P. B. (2005). Phylogeography evidence for the existence of an ancient biogeographic barrier: the Isthmus of Kra of Kra Seaway. *Heredity, 94*, 370-378.

Dersch, M., & Stein, R. (1994). Late Cenozoic records of eolian quartz flux in the Sea of Japan (ODP Leg 128, Sites 798 and 799) and paleoclimate in Asia. *Palaeogeography, Palaeoclimatology, Palaeoecology, 108*, 523-535.

Dobson, G. E. (1872). Brief description of five new species of Rhinolophus bats. *Journal of the Asiatic Society of Bengal, 41*, 336-338.

Dobson, G. E. (1876). *Monograph of the Asiatic Chiroptera and catalogue of the species of bats in the collection of the Indian Meuseum, Calcutta*. London: Trustees of the Indian Meuseum and Taylor & Francis.

Ellerman, J. R., & Morrison-Scott, T. C. S. (1951). *Checklist of Palaearctic and Indian Mammals 1758-1946*. London: Trustees of the British Museum.

Francis, C. M. (2008). *A field guide to the mammals of Thailand and South-East Asia.* Bangkok: Books Co., Ltd.

Francis, C. M., Borissenko, A. V., Ivanova, N. V., Eger, J. L., Lim, B. K., Guillén-Servent, A., *et al.* (2010). The Role of NDA Barcodes in Understanding and Conservation of Mammal Diversity in Southest Asia. *PLos one, 5*(9), 1-12.

Francis, C. M., & Habersetzer, J. (1998). Interspecific and intraspecific variation in echolocation call frequency and morphology of horseshoe bats, *Rhinolophus* and *Hipposideros.* In T. Kunz & P. A. Racey (Eds.), *Bat biology and conservation*: Smithsonian Institute Press.

Furey, M. N., Mackie, I. J., & Racey, P. A. (2009). The role of ultrasonic bat detectors in improving inventory and monitoring surveys in Vietnamese karsts bat assemblages. *Current Zoology, 55*, 327-345.

Furey, M. N., Thong, V. D., Bates, P. J. J., & Csorba, G. (2009). Description of a new species belonging to the Murina 'suilla-group' (Chiroptera: Vespertilionidae: Murininae) from north Vietnam. *Acta Chiropterologica, 11*(2), 225-236.

Furman, A., Öztunç, T., Postawa, T., & Çoraman, E. (2010). Shallow genetic differentation in Miniopterus schreibersii (Chiroptera: Vespertilionidae) incates a relatively recent-colonization of Europe from a single glacial refugium. *Acta Chiropterologica, 12*(1), 51-59.

Gathorne-Hardy, F. J., Syaukani, Davies, R. G., Eggleton, P., & Jones, D. T. (2002). Quaternary rainforest refugia in south-east Asia: using termites (Isoptera) as indicators. *Biological Journal of the Linnean Society, 75*, 453-466.

Good, R. (Ed.). (1964). *The geographiy of the flowering plants.* (3 ed.). London: Longman.

Gorog, A. J., Sinaga, M. H., & Engstrom, M. D. (2004). Vicariance or dispersal? Historical biogeography of three Sunda shelf murine rodents (*Maxomys surifer, Leopoldamys sabanus and Maxomys whiteheadi*). *Biological Journal of the Linnean Society, 81*, 91-109.

Gray, J. E. (1847). Characters of six new genera of bats. *Proceedings of the zoological society of London, 1847*, 14-16.

Gray, J. E. (1866). A revision of the genera of Rhinolophidae. *Proceedings of the zoological society of London, 1866*, 81-83.

Griffin, D. R. (1971). The important of atmospheric attenuation for the echolocation of bats (Chiroptera). *Animal Behaviour, 19*, 55-61.

Guillén, A., Just, J. B., & Ibàñez, C. (2000). Variation in the frequency of the echolocation calls of *Hipposideros ruber* in the Gulf of Guinea: an exploration of the adaptive meaning of the constant frequency value in rhinolophoid CF bats. *Journal of Evolutionary Biology, 13*, 70-80.

Hall, T. A. (1999). BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser, 41*, 95-98.

Hartely, D. J. (1989). The effect of atmospheric sound absorption on signal bandwidth and energy and some consequence for bat echolocation. *Journal of Acoustical Society of America, 85*, 1338-1347.

Heaney, L. R. (1991). A synoptic climatic and vegetational change in Southeat Asia. *Climatic Change, 19*, 53-61.

Heller, K.-G., & v. Helversen, O. (1989). Resource partioning of sonar frequency bands in rhinolophid bats. *Oecologia, 80*, 178-186.

Hill, J. E. (1959). Bates in limestone of Sarawak. *The Annals Magazine of Natural History, 13*, 85-91.

Hill, J. E., & Schlitter, D. A. (1982). A record of *Rhinolophus arcuatus* (Chiroptera: Rhinolophidae) from New Guinea, with the description of a new subspecies. *Annals of Carnegie Museum, 51*, 455-464.

Hisher, S., How, R. A., Suyanto, A., & Schmitt, L. H. (2004). Implications of contrasting patterns of genetic variability in two vespertilionid bats from the Indonesian archipelago. *Biological Journal of the Linnean Society, 83*, 421-431.

Horsfied, T. (1823). *Zoological researches in Java*. London: Kingsbury, Parbury & Allen.

Huelsenbeck, J. P., & Ronquist, F. (2001). "MRBAYES: Bayesian inference of phylogeny." *Bioinformatics, 17*, 754-755.

Hughes, A. C., Satasook, C., Bates, P. J. J., Bumrungsri, S., & Jones, G. (2011). Explaining the casuses of the zoogeographic transition around the Isthmus of Kra: using bats as a case study. *Journal of Biogeography*, 1-11.

Hughes, J. B., Round, P. D., & Woodruff, D. S. (2003). The Indochinese-Sundaic faunal transition at the Isthmus of Kra: an analysis of resident forest bird species distributions. *Journal of Biogeography, 30*, 569-580.

Hutchison, C. S. (1989). *Geological evolution of South-east Asia.* Oxford: Clarendon Press.

Hutson, A. M. M., S.P; Racey, P.A. (2001). *Microchiropteran bats: global status survey and conservation action plan*. Gland, Switzerland and Cambridge, UK: IUCN/SSC Chiroptera Specialist Group.

Inger, R. F., & Voris, H. K. (2001). The biogeographical relations of the frogs and nakes of Sundaland. *Journal of Biogeography, 28*, 863-891.

Iredale, T., & Troughton, E. I. G. (1834). A checklist of the mammals recorded from Australia. *Memoirs Australian Museum, 6*, 1-122.

Ith, S., Soisook, P., Bumrumgsri, S., Kingston, T., Puechmaille, S. J., Struebig, M. J., *et al.* (2011). A taxonomic review of *Rhinolophus coelophillus* Peters 1867 and *R. shameli* Tate 1943 (Chiroptera: Rhinolophidea) in continental Southeast Asia. *Acta Chiropterologica, 13*(1), 41-59.

Ivanova, N. V., Clare, E. L., & Borisenko, A. V. (2012). DNA Barcoding in Mammals. In W. J. Kress & D. L. Erickson (Eds.), *DNA Barcodes: Methods and Protocols.* (pp. 485). New York: Humana Press.

Jacobs, D. S., Barclay, R. M. R., & Walker, M. H. (2007). The allometry of echolocation call frequencies of insetivorous bats: why do some bats deviate from the pattern? *Oecologia, 152*, 583-594.

Jones, G., Morton, M., Hughes, P. M., & Budden, R. M. (1993). Echolocation, flight morphology and foraging strategies of some West African hipposiderid bats. *Journal of Zoology London, 230*, 385-400.

Kashiwaya, K., Ochiai, S., Sakai, H., & Kawai, T. (2001). Orbit related long-term climate cycles revealed in a 12-myr continental record from Lake Baikal. *Nature, 410*, 71-74.

Keng, H. (1970). Size and affinities of the flora of the Malay Peninsula. *Journal of Tropical Geography, 31*, 43-56.

Khan, F. A. A., Phillips, C. D., & Baker, R. J. (2013). Timeframes of Speciation, Reticulation, and Hybridization in the Bulldog Bat Explained Through Phylogenetic Analyses of All Genetic Transmission Elements. *Systematic Biology, 63*(1), 96-110.

Khan, F. A. A., Solari, S., Swier, V. J., Larsen, P. A., Abdullah, M. T., & Baker, R. J. (2010). Systematics of Malaysian woolly bats (Vespertilionidae: *Kerivoula*) inferred from mitochondrial, nuclear, karyotypic, and morphological data. *Journal of Mammalogy, 91*(5), 1058-1072.

Khan, F. A. A., Swier, V. J., Larsen, P. A., Solari, S., Besar, K., Wahap, M., *et al.* (2008). Using Genetics and Morphology to Examine Species Diversity of Old World Bats: report of a recent Collection from Malaysia. *Occasional Papers of the Museum of Texas Tech University, 281*(1-28).

Kingsada, P., Douangboubpha, B., Ith, S., Furey, N., Soisook, P., Bumrungsri, S., *et al.* (2011). A checklist of bats from Cambodia, including the first record of the intermediate horseshoe bat Rhinolophus affinis (Chiroptera: Rhinolophidae) with additional information from Thailand and Vietnam. *Cambodian journal of natural history* (1), 49-59.

Kingston, T., Boo Liat, L., & Akbar, Z. (2009). *Bats of Krau Wildlife Reserve*. Bangi: Penerbit Universiti Kebangsaan Malaysia.

Kingston, T., Lara, M. C., Jones, G., Akbar, Z., Kunz, T. H., & Schneider, C. J. (2001). Acoustic divergence in two cryptic Hopposideros species: a role for social selection? *The Royal Society, 268*, 1381-1386.

Kingston, T., & Rossister, S. J. (2004). Harmonic hopping in Wallacea's bats. *Nature, 429*, 654-657.

Koopman, K. F. (1993). Chiroptera. In D. E. Wilson & D. M. Reeder (Eds.), *Mammal species of the world: A taxonomic and Geographic reference* (2 ed., pp. 137-241). Washington and London: Smithsonian Institution Press.

Koopman, K. F. (1994). *Chiroptera: Stystematics. Handbook of Zoology. Mammalia, part 60.* Berlin: Walter de Gruyter.

Kruskop, S., & Tsytsulina, K. A. (2001). A new big-footed mouse-eared bat Myotis annamiticus sp. nov. (Vespertilinoidae, Chiroptera) from Vietnam. *Mammalia, 65*(1), 63-72.

Kunz, T. H., Fenton, M. B. (2003). *Bat Ecology*. Chicago, London: The University of Chicago Press.

Kurup, G. U. (1968). Mammals of Assam and ajoining areas. A distributional list. *Proceedings of the Zoological Society (Calcutta), 21*, 79-99.

Leach, W. E. (1816). *Systematic catalogue of the specimens of the indigenous of the Mammalia*. London: British Museum.

Lekagul, B., & McNeely, J. A. (1977). *Mammals of Thailand.* Bangkok: Assoication for the Conservation of Wildlife, Saha Karn Bhaet Co.

Li, G., Jones, G., Rossister, S. J., Chen, S.-F., Parsons, S., & Zhang, S. (2006). Phylogenetics of small horseshoe bats from East Asia based on mitochondrial DNA sequence variation. *Journal of Mammalogy, 87*(6), 1234-1240.

Li, X., Yin, H., Li, K., & Gao, X. (2012). Population genetic structure and historical demography of the ground beetle *Pheropsophus jessoensis* from the Tsinling-Dabashan Mountains, Central China based on mitochondrial DNA analysis. *Zoological Science, 29*, 238-246.

Lin, A.-Q., Csorba, G., Li, L.-F., Jiang, T.-L., Lu, G.-J., Thong, V. D., *et al.* (2013). Phylogeography of *Hipposideros armiger* (Chiroptera: Hipposideridae) in the Oriental Region: the contribution of multiple Pleistocene glacial refugia and

intrinsic factors to contemporary population genetic structure. *Journal of Biogeography*.

Madsen, O., Scally, M., Douady, C. J., Kao, D. J., DeBry, R. W., Adkins, R., *et al.* (2001). Parallel adaptive radiations in two major clades of placental mammals. *Nature, 409*, 610-614.

Maharadatunkamsi, Hisheh, S., Kitchener, D. J., & Schmitt, L. H. (2000). Genetic and Morphometric Diversity in Wallacea: Geographical Patterning in the Horse Shoe Bat, Rhinolophus affinis. *Journal of Biogeography, 27*(1), 193-201.

Mao, X. G., Zhu, G. J., Zhang, S., & Rossister, S. J. (2010). Pleistocene climatic cycling drives intra-specific diversification in the intermediate horseshoe bat (*Rhinolophus affinis*) in Southern China. *Molecular Ecolocy, 19*, 2754-2769.

Mayer, F., & von Helversen, O. (2001). Cryptic Diversity in European Bats. *The Royal Society, 268*, 1825-1832.

Mayr, E., & Ashlock, P. D. (Eds.). (1991). *Principles of systematic zoology* (2 ed.). New York: McGRAW-HILL, INC.

Mayr, E., & Provine, W. (1980). *The evolution synthesis.* Cambridge: Harvard Univeristy Press.

Medway, L. (1969). *The Wild Mammals of Malaya*: Oxford University Press.

Medway, L. (1977). *Mammals of Borneo. Field keys and an annotated checklist.* (Vol. 6). Kuala Lumpur: Monograph of the Malaysian Brance Royal Asiatic Society.

Mickleburgh, S. P. H., A.M; Racey, P.A (2002). A view of global conservation status of bats. *Oryx, 36*(1), 18-34.

Miller, G. S. (1907). The families and genera of bats. *Bulletin of the United States Natational Museum, 57*(xxvii), 282.

Minelli, A. (1993). *Biological systematics.* London: HALL & CHAPMAN.

Morgan, K., O'Loughlin, S. M., Chen, B., Linton, Y., Thongwat, D., Somboon, P., *et al.* (2011). Comparative phylogeography reveals a shared impact of Pleistocene environmental change in shaping genetic diversity within nine *Anopheles* mosquito species across the Indo-Burma biodiversity hotspot. *Molecular Ecology, 20*, 4533-4549.

Morley, R. J. (1998). Palynological evidence for Tertiary plant dispersals in the SE Asian region in relation to plate tectonics and climate. In J. D. Holloway & R. Hall (Eds.), *Biogeography and geological evolution of SE Asia* (pp. 211-234). Leiden: Backhuys.

Morley, R. J. (2000). *Origin and evolution of tropical rain forests*. Chichester: J. Wiley.

Murphy, W. J., Eizirik, E., Johnson, W. E., Zhang, Y. P., Ryder, O. A., & O'Brien, S. J. (2001). Molecular phylogenetics and the origins placental mammals. *Nature, 409*, 614-618.

Neuweiller, G. (2000). *The Biology of Bats* (E. Covery, Trans.). New York, Oxford: Oxford University Press.

Novacek, M. J. (1992). Fossils, topology, missing data and the higher level phylogeny of Eutherian mammals. *Systematic Biology, 41*, 58-73.

O' Shea, T. J., & Gore, J. A. (2011). *Preliminary report on a survey for bats, Quang Ngai Province, Vietnam.*

Patrick, L. E., McCulloch, E. S., & Ruedas, L. A. (2013). Systematics and biogeography of the arcuate horseshoe bat species complex (Chiroptera, Rhinolophidae). *Zoologica Scripta, 42*(6), 553-590.

Pauwels, O. S. G., David, P., Chimsunchart, C., & Thirakhupt, K. (2003). Reptiles of Phetchaburi Province, Western Thailand: a list of species, with natural history notes, and a discussion on the biogeography at the Isthmus of Kra. *The Natural History Journal of Chulalongkorn University, 3*(1), 23-25.

Pauwels, O. S. G., Laohawat, O.-A., Naaktae, W., Puangit, C., Wisutharom, T., Chimsunchart, C., *et al.* (2002). Reptile and Amphibian Diversity in Phang-nga Province, Southern Thailand. *The Natural History Journal of Chulalongkorn University, 2*(1), 25-30.

Peters, W. (1867). On some mammals collected by Capt A. C. Beavan, C. M. Z. S., at Mulmein, Burma. *Proceedings of zoological society of London, 1866*, 426-430.

Petit, E., Excoffier, L., & Mayer, F. (1999). No evidence of bottleneck in the postglacial recolonization of Europe by the noctule bat (*Nyctalus noctula*). *Evolution, 53*, 1247-1258.

Quicke, D. L. J. (1993). *Principles and techniques of contemporary taxonomy.* London: CHAPMAN & HALL.

Rambaut, A., & Drummond, A. J. (2007). BEAST: Bayesian evolutionary analysis by sampling trees., *BMC Evolutionary Biology* (7), 214.

Rambaut, A., & Drummond, A. J. (2009). Tracer (Version 1.5): Available from the BEAST site: http://beast.bio.ed.ac.uk/.

Richards, P. W. (Ed.). (1996). *The tropical rain forest: an ecological study.* (2 ed.). Cambridge: Cambridge University Press.

Robinson, M. F. (1996). A relationship between echolocation calls and noseleaf widths in bats of the genera *Rhinolophus* and *Hipposidero. Journal of Zoology London, 239*, 389-393.

Ruedi, M., & Fumagalli, L. (1996). Genetic structure of gymnures (genus Hylomys: Erinaceidae) on continental islands of Southeast Asia: historical effects of fragmentation. *Journal of Zoological Systematics and Evolutionary Research, 34*, 153-162.

Salgueiro, P., Coelho, M. M., Palmeirim, J. M., & Ruedi, M. (2004). Mitochondrial DNA variation and population structure of the island endemic Azorean bat (*Nyctalus azoreum*). *Molecular Ecology, 13*, 3357-3366.

Schnitzler, H.-U., & Kalko, K. M. (1998). How echolocating bats search and find food. In T. H. Kunz & P. A. Racey (Eds.), *Bat biology and conservation* (pp. 183-196). Washington D. C: Smithsonian Institute Press.

Simmon, N. (2005). Order Chiroptera. In D. E. Wilson & D. M. Reeder (Eds.), *Mammal species of the world: A taxonomic and geographical reference.* (3 ed., pp. 312-529). Baltimore: Jhons Hopkin University Press.

Simmons, N. B., & Geisler, J. H. (1998). Phylogenetic relationships of Icaronycteris, Archaeonycteris, Hassianycteris, and Palaeochiropteryx to extant bat lineages, with comments on the evolution of echolocation and foraging strategies in Microchiroptera. *Bulletin of th American Museum of Natural History, 235*, 1-182.

Simpson, G. G. (1961). *Principle of animal taxonomy.* New York: Columbia University Press.

Sinha, Y. P. (1973). Taxonomic studies on the Indian horseshoe bats of the genus *Rhinolophus*. *Mammalia, 37*(4), 603-630.

Soisook, P., Burungsri, S., Satasook, C., Thong, V. D., Hla Bu, S. S., Harrison, D. L., *et al.* (2008). A taxonomic review of *Rhinolophus stheno* and *R. malayanus* (Chiroptera: Rhinolophidae) from continental Southeast Asia: An evaluation of echolocation call frequency in descrimating between cryptic species. *Acta Chiropterologica,* 10(2), 221-242.

Springer, M. S., Teeling, E. C., Madsen, O., Stanhope, M. J., & de Jong, W. W. (2001). Integrated fossil and molecular data reconstruct bat echolocation. *Proceeding of the National Academy of Science., 98*, 6241-6246.

Tamura, K., Peterson, D., Peterson, N., G., S., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution, 28*, 2731-2739.

Tate, G. H. H., & Archbold, R. (1939). Results of Archbold Expidition no. 24. Oriental *Rhinolophus*, with special reference to material from Archbold collections. *American Museum Novitates, 1036*, 1-12.

Taylor, P. J., Stoffberg, S., Monadjem, A., Schoeman, M. C., Bayliss, J., & Cotterill, F. P. D. (2012). Four New Bat Species (Rhinolophus hildebrandtii Complex) Reflect Plio-Pleistocene Divergence of Dwarfs and Giants across an Afromontane Archipelago. *PLoS ONE, 7*(9), 1-23.

Teeling, E. C., Madsen, O., Van Den Bussche, R. A., De Jong, W. W., Stanhope, M. J., & Springer, M. S. (2002). Microbat paraphyly and the convergent evolution of a key innovation in Old World rhinolophoid microbats. *PNAS, 99*, 1431-1436.

Teeling, E. C., Springer, M. S., Madsen, O., Bates, P., O'Brien, S. J., & Murphy, W. J. (2005). A Molecular Phylogeny for Bats Illuminates Biogeography and the Fossil Record. *Sciene, 307*, 580-583.

Thabah, A., Rossister, S. J., Kingston, T., Zhang, S., Parsons, S., Mya, K. M., *et al.* (2006). Genetic divergene and echolocation call frequency in cryptic species of Hipposideros larvatus s. l. (Chiroptera: Hipposideridae) from the Indo-Malayan region. *Biological Journal of Linnean Society, 88*, 119-130.

Thomas, N. M. (1997). *A systematic review of selected Afro-Asiatic Rhinolophidae (Mammalia: Chiroptera): An evaluation of taxonomic methodology.* University of Aberdeen.

Thomas, N. M. (2000). Morphology and mitochondrial-DNA variation in Rhinolophus rouxii (Chiroptera). *Bonner Zoologische Beitrage, 49*, 1-18.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876-4882.

Thong, V. D. (2011). *Systematics and echolocation of rhinolophoid bats (Mammalia: Chiroptera) in Vietnam.* University of Tubingen, Tubingen, Germany.

Thong, V. D., Puechamaille, S. J., Denzinger, A., Bates, P. J. J., Dietz, C., Csorba, G., *et al.* (2012). Systematics of the *Hipposideros turpis* complex and a description of a new subspecies from Vietnam. *Mammal Review, 42*, 166-192.

Tingga, R. C. T., Khan, F. A. A., Mohd-Ridwan, A. R., Senawi, J., & Abdullah, M. T. (2012). Small mammals from Kuala Atok, Taman Negara Pahang, Malaysia. *Sains Malaysiana, 41*, 659-669.

van der Kaars, S., Penny, D., Tibby, J., J., F., C., D. R. A., & Suparan, P. (2001). Late Quaternary palaeoecology, palynology and palaeolimnology of a tropical lowland swamp: Rawa Danau, West-Java, Indonesia. *Palaeogeography, Palaeoclimatology, Palaeoecology, 171*, 185-212.

van Steenis, C. G. G. J. (1950). The delimitation of Malesia and its main plant geographical divisions. *Flora Malesiana, 1*(1), lxx-lxxv.

Voris, H. K. (2000). Map of Pleistocene sea levels in Southeast Asia: shorelines, river systems and time durations. *Journal of Biogeography, 27*, 1153-1167.

Waddell, P. J., Okada, N., & Hasegawa, M. (1999). Towards resolving the Interordinal relationships of Placental mammals. *Systematic Biology, 48*, 1-5.

Wallace, A. R. (Ed.). (1876). *The geographical distribution of animals* (1 ed. Vol. 2). London: Macmillan.

Whitmore, T. C. (Ed.). (1984). *Tropical rainforests of the Far East.* (2 ed.). Oxford: Oxford University Press.

Wikranmanayake, E., Dinerstein, E., Loucks, C., Olson, D., Morrison, J., Lamoreux, J., *et al.* (2002). *Terrestrial ecoregions of the Indo-Pacific: a conservation assessment.* Washington DC.: Island Press.

Willette, D. A., & Padin, J. I. M. (2014). Identifying the biodiversity of marine jacks (Carangidae) in the freshwater Taal Lake, Philippines using phenotypic features and mitochondrial DNA. *Journal of Applied Ichthyology*, 1-6.

Winston, J. E. (1999). *Describing species, practical taxonomic procedure for biologist*. New York: Columbia University Press.

Woodruff, D. S. (2003). Neogene marine transgressions, palaeogeography and biogeographic trasitions on the Thai-Malay Peninsula. *Journal of Biogeography, 30*, 551-567.

Woodruff, D. S. (2010). Biogeography and conservation in Southeast Asia: how 2.7 million years of repeated environmental fluctuations affect today's patterns and the future of the remaining refugial-phase biodiversity. *Biodiversity and Conservation*, *19*, 919-941.

Woodruff, D. S., & Turner, L. M. (2009). The Indochinesd-Sundaic zoogeographic transition: a description and analysis of terrestrial mammals species distributions. *Journal of Biogeography*, 1-19.

Wu, Y., & Thong, V. D. (2011). A new species of Rhinolophus (Chiroptera: Rhinolophidae) from China. *Zoological Science*, *28*, 235-241.