Geographic variation of Rhinolophus affinis (Chiroptera: Rhinolophidae) in the Sundaic Subregion of Southeast Asia, including the Malay Peninsula, Borneo and Sumatra

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Recommended Citation
Ith S, Bumrungersri S, Thomas NM, Bates PJJ, Willette DA, Khan FAA, Wonglapsuwan M, Soisook P, Maryanto I, Huang JCC, Furey NM. (2016) Geographic variation of Rhinolophus affinis (Chiroptera: Rhinolophidae) in the Sundaic Subregion of Southeast Asia, including the Malay Peninsula, Borneo and Sumatra. Acta Chiropterologica, 18: 141-161.
Geographical variation of *Rhinolophus affinis* (Chiroptera: Rhinolophidae) in the Sundaic subregion of Southeast Asia, including the Malay Peninsula, Borneo and Sumatra

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*Rhinolophus affinis* sensu lato is a widespread bat species in South and Southeast Asia which shows considerable geographical variation in its morphology, echolocation call frequencies and genetics. The taxonomic status of the taxon in the Sundaic subregion remains uncertain however as the limited studies to date have been largely based on morphology. The aim of the present study was to determine the taxonomic status of subspecific forms recognized in the subregion and to evaluate phylogeographic distinctiveness between those occurring in Borneo and the Malay Peninsula using genetic, morphological and acoustic datasets. Two forms were confirmed: *R. a. nesites* from Borneo and *R. a. superans* from the peninsula. The previous recognition of a population from southernmost Sumatra as *R. a. superans* was not supported, however, as this form is likely *R. a. affinis*. Genetic divergence between these three forms is rather deep and is estimated to have occurred during the arid climatic period of the Pleistocene when suitable habitats were reduced to isolated pockets. Our results support the phylogeographic distinctiveness hypothesis as *R. affinis* sensu lato shows discrete affinities between Borneo and the Malay Peninsula. Discovery of new forms of *R. affinis* is likely with greater sampling effort throughout the region. Our study also demonstrates the importance of employing multiple datasets in taxonomic evaluations, as the use of morphological and/or acoustic datasets alone could lead to erroneous conclusions.

**Key words**: echolocation, genetics, morphology, *Rhinolophus affinis*, subspecies, Sundaic subregion

**INTRODUCTION**

The intermediate horseshoe bat, *Rhinolophus affinis* Horsfield, 1823 is a medium-sized rhinolophid (forearm length: 45–56 mm) distributed widely in South and Southeast Asia, ranging from northern India (including Andaman Islands), Nepal to southern China, mainland Southeast Asia, Borneo, and Java (Simmons, 2005; Francis, 2008). The taxon exhibits considerable morphological and acoustic variation across its range (Andersen, 1905; Csorba et al., 2003; Kingsada et al., 2011; Ith et al., 2015). Nine subspecies are traditionally recognized: *R. 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, Kayah State, Myanmar), *R. a. superans* Andersen (type locality Pahang, Peninsular Malaysia), *R. a. nesites* Andersen (type locality Bunguran Island, North Natunas, Indonesia), *R. a. princeps* Andersen (type locality Lombok, Lesser Sunda Island, Indonesia) and *R. a. hainanus* Allen (type locality Pouten, Hainan Island, China) (Csorba et al., 2003; Simmons, 2005).

The status of two subspecies, *R. a. macrurus* and *R. a. superans*, has recently been confirmed in continental Southeast Asia (Ith et al., 2015). The geographical boundary between these two forms lies in north Peninsular Thailand and accords with biogeographical boundary between these two forms lies in continental Southeast Asia (Ith et al., 2015). In contrast, the taxonomic status of other taxa such as *R. macrotis* and *R. siamensis* as these show very shallow genetic differences (Francis et al., 2010). Similar cases include *Miniopterus schreibersii* (Furman et al., 2010), *Eptesicus serotinus*, *E. nilssonii* (Mayer and von Helversen, 2001) and *Myotis annamiticus* (Kruskop and Tsytsulina, 2001; Francis et al., 2010).

*Rhinolophus a. superans* may have similar morphological and genetic variation to that found in the Indochinese form of *R. affinis*: *R. a. macrurus* (Ith et al., 2015). Francis et al. (2010) have shown that widespread taxa often have substantial geographic variation in their barcode sequences and that populations from Peninsular Malaysia and Borneo are often genetically distinct (e.g., Khan et al., 2008, 2010). As a consequence, the aim of the current study was to determine the taxonomic status of *R. a. superans* and *R. a. nesites* and evaluate the phylogeographic distinctiveness of *R. affinis* from Borneo and the Malay Peninsula using a combination of genetic, morphological and acoustic datasets.

**Materials and Methods**

**Study Specimens and Sampling Sites**

Seventy-six specimens were available for morphological study, including five from south-western Sumatra, seven from Sarawak, north-western Borneo and 64 from the Malay Peninsula. Two specimens from Central Java, Indonesia and two specimens from Mussoorie, 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 (PSU collection); Harrison Institute, UK (HZM collection); Museum Zoologicum Bogoriense, Research Center for Biology-Indonesian Institute of Science, Indonesia (MIZB collection); Museum of Texas Tech University, USA (TTU collection); and Zoological Museum of Universiti Malaysia Sarawak, Malaysia (UNIMAS collection).

Specimens from the Malay Peninsula were collected by Saveng Ith and the Small Mammals and Birds Research Unit Team of PSU between August 2011 and May 2012. Bats were surveyed in the field using a combination of harp traps, mist nets and hand nets and were captured and handled in accordance with guidelines approved by the American Society of
Mammalologists (Gannon et al., 2007). 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. 1 and collection information is given below.

**Borneo**

Sarawak: [Sa1] Mount Penrissen (01°7.88’N, 110°13.12’E) — two adult males and five females collected by Robert Baker and Peter Larsen from August 2006 to May 2010.

**Indonesia**

Sumatra: [S1] Bukit Barisan Selatan Landscape (approx. 05°37.78’N, 104°22.20’E), Lampung Province — three adult males and two females collected by Bahri Syaiful, Hesti, Karolina and Joe Chun-Chia Huang from July 2007 to May 2012.

**Peninsular Malaysia**

Kedah State: [M1] Langkawi Island (approx. 06°23.20’N, 99°47.83’E) — adult male collected by Mohd Isham Mohd Azhar. Penang State: [M2] (05°15.795’N, 100°29.076’E) — nulliparous female collected by Faisal Ali Anwarali Khan in August 1988. Kelantan State: [M3] Gua Madu, Gua Musang Division (approx. 05°10.462’N, 101°54.191’E) — parous female captured by Faisal Ali Anwarali Khan. Pahang State: [M4] Nature Study of Kuala Atok, Taman Negara National Park (04°16.281’N, 102°22.316’E) — adult male and nulliparous female collected by Faisal Ali Anwarali Khan in May 2008.

**Thailand**

Chumphon Province: [T1] Khao Kram Cave, Patiew District (10°55.13’N, 99°22.43’E); [T2] Huay Wang Cave, Tambon Khao Talu, Sawi District (10°10.00’N, 98°55.18’E); and [T3] Kao Plu Cave, Lamae District (09°43.60’N, 99°06.50’E) — five adult males and three nulliparous females collected by Sara Bumrungsri from October 2006 to January 2007.

Phang Nga Province: [T4] Koh Surin (approx. 08°46.20’N, 98°18.60’E) — two adult males collected by Sara Bumrungsri in February 2006.

Surat Thani Province: [T5] Ratchabrapha Dam and Khlong Saeng Wildlife Sanctuary (08°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 Plu Cave, Khao Ro Commune, Ron Piboon District (08°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 (07°55.517’N, 99°15.790’E) — adult male collected by Pipat Soisook in 4 May 2012.

Phatlalung Province: [T8] Khao Ban Tad Wildlife Sanctuary (approx. 07°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 (07°18.080’N, 99°41.988’E) — adult male and two nulliparous females collected by Pipat Soisook in January 2011.

Songkhla Province: [T10] Kuan Khao Wang Park, Rattaphum District (07°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. 06°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 (06°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 (06°30.878’N, 99°19.040’E) and Rawee Island (06°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.

**Morphological Measurements**

Thirty-three external and craniodental characters of each specimen were measured following Bates and Harrison (1997), Thomas (1997), Csorba et al. (2003) and Furey et al. (2009). External characters were measured using a pair of dial calipers to the nearest 0.1 mm and craniodental characters were measured to the nearest 0.01 mm using a digital caliper under a stereo microscope. Definitions for external measurements are 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; IP3D, 2P3D, IP4D, 2P4D, IP5D, 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 horsehoe to the tip of the lanct, 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: condylo-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 zygomatic arches; GBW: greatest braincase width — width of the braincase at the posterior roots of the zygomatic arches; BOW: braincase width — 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; M3M3W: posterior palatal width — taken across the widest part of the outer borders of the third upper molar; C1C4W: anterior palatal length —
Fig. 1. Sample localities and echolocation frequencies of *R. affinis* in the Sundaic subregion. M = Peninsular Malaysia, Sa = Sarawak, S = Sumatra, and T = Thailand. Abbreviations for localities are given in the methods and materials. The grey shading indicates the Sundaic biogeographic subregion following Woodruff (2010), green (zone A) and orange (B) shadings are the echolocation zones recognized in the Malay Peninsula. Dashed arrows indicate type localities and subspecies names, solid arrows indicate the transition zone of biota within the Malay Peninsula, dashed lines indicate the echolocation frequencies (min–max), and the two-headed arrows indicate the echolocation frequencies (min–max) as a whole from each echolocation zone. Note: the northern boundary of the Sundaic subregion is sometimes placed at the Isthmus of Kra (e.g., Lekagul and McNeely, 1988 and Corbet and Hill, 1992).
taken across the widest part of the outer border of the upper canine; CM^1L: upper toothrow length — from the front of the upper canine to the back of the crown of the third upper molar; CM^2L: 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; CMH: 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.

Echolocation Call Measurements

Values for the frequency of maximum energy (FMAXE) for *R. affinis* in this study were obtained from field work. To avoid pseudo-replication, one echolocation call per bat was used in analysis. In total, 72 calls (from 72 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 southwestern 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 (Pettersson Elektronik, AB). The Pettersson D-240X detector was set in ×10 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 768 kHz. A time expansion factor of ×10 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 using power spectra and the mean value was used in analysis.

Morphological and Acoustic Analyses

Statistical analyses were carried out using SPSS 16.0 (SPSS Inc., Chicago, USA) and PC-ORD 5.10 for Windows (MJM Software, Oregon, USA). Descriptive statistics (minimum, maximum, mean and standard deviation) were calculated for echolocation, 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 in size. 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.

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 pairs segment of 17 sequences of cytochrome c oxidase I (COI) was analyzed at the Canadian Center for DNA Barcoding (CCDB) using the barcoding protocols, methods of analyses were detailed in Ivanova et al. (2012). A 832 base pairs segment of 19 sequences of cytochrome b (Cytb) gene was generated and analyzed in collaboration with the Coral Triangle Partnerships in International Research and Education Project (https://www.odu.edu/imp/atree.html). Genomic DNA was isolated from bat tissue samples using the Qiagen DNeasy mini kit (Qiagen, Valencia, CA) following manufacturer’s instructions and Cytb sequences were generated, aligned and proofread as described in Willette and Padin (2014) using the primers Cytb 07 (5’-ATAGGGGTATCATTCGGGT-3’) and Cytb 09 (5’-GTGACTTGGAAACCACGGTT-3’). The full lengths (1,140 base pairs) of 13 Cytb sequences and 413 base pairs segment of five Cytb 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’-GAAAAACACCGTGTGT-3’), LGL766 (5’-GTGTTAATTAGATYTYAGCTTG-3’) with an annealing temperature of 50°C.

In total, 37 Cytb sequences and 17 sequences of COI were available. Sequences from GenBank and Barcode of Life Data Systems (BOLD) were also accessed, and eight sequences of Cytb gene (accession number: EF108156-EF108160, EU521607, JN106274 and JN106280) from Borneo and Peninsular Malaysia were included for comparison. Twenty-one sequences of COI gene were included, 11 sequences were from Peninsular Malaysia (accession no: HM541330-HM541332, HM541407-HM541414) and 10 sequences from Peninsular Thailand.

Phylogenetic relationships among sequences were reconstructed using maximum-likelihood in the MEGA 5.2.2 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, the Hasegawa-Kishino-Yano substitution model (HKY) was the best-fit model selected for COI. While General Time Reversible models (GTR) with gamma distribution (G) were the best-fit model selected for Cytb. We also performed Bayesian Analysis using MrBayes 3.2.2 (Huelsenbeck and 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 (Ramaut and Drummond, 2007). 12,000 trees of initial phase of the Markov chain were discarded as 25% burn-in. A congeneric *Rhinolophus stheno* gene was analyzed among the most recent common ancestor among the observed clades, Cytb gene was analyzed in BEAST 1.8 (Drummond and Rambaut, 2007). GTR + G was selected as the best substitution model based on jModelTest and a relaxed-clock model with an uncorrelated lognormal distribution was selected for the substitution rate. We performed two
RESULTS

Morphology

To explore sexual dimorphism, localities where male and female specimens were both available were selected; numbers of each were adjusted to balance sample sizes and so 22 males and 22 females were compared. No significant differences were found in 33 external and cranial characters between the sexes. A total of 12 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 12 characters for 74 specimens from the Sundaic subregion generated four relatively isolated groups including Borneo, Sumatra, southern Malay Peninsula and northern Malay Peninsula groups (Fig. 2). Specimens from Borneo exhibited a higher degree of isolation among the groups.
Table 1. External and craniodental measurements of *R. affinis* forms within the Sundaic subregion. Values are given as min–max, mean ± SD (in mm). Acronyms and definitions for measurements are given in the text. Sample sizes differing from those reported under *n* are given in parentheses.

|        | *n* | Sex  | FA     | TL     | EL     | TIB    | HF     | 2MT    | 3MT    | 4MT    | 5MT    | IP3D   | 2P3D   | 1P4D   |
|--------|-----|------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 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 | 25.0–26.4 | 10.0–10.3 |
| 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 | 22.8–24.8 | 8.2–10.3 |
|        |     |      | 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 | 23.9±0.7 | 9.5±0.7 |
| 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 | 23.7–26.0 | 9.7–10.0 |
|        |     |      | 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 | 24.7±0.9 | 9.8±0.2 |
| 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 | 23.8–27.0 | 9.2–10.9 |
|        |     |      | 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 | 25.3±0.8 | 10.2±0.5 |
| 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 | 24.2–27.7 | 9.5–11.5 |
|        |     |      | 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 | 25.8±0.8 | 10.3±0.5 |

Table 1. Extended

|        | *n* | Sex  | 2P4D   | IP5D   | 2P5D   | GHN    | GWN    | SL     | CCL    | ZYW    | MAW    | BW     | GBW    |
|--------|-----|------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Java   | 2   | ♀♀   | 14.4–15.2 | 12.0–12.1 | 13.5–13.5 | 10.7–11.5 | 8.2–8.4 | 22.18–22.47 | 19.50–19.69 | 10.95–11.71 | 10.16–10.71 | 9.85–10.17 | 9.31–9.48 |
| Borneo | 6   | ♀♂   | 14.0–15.8 | 9.9–12.1 | 11.8–12.4 | 11.5–14.0 | 8.5–10.3 | 21.36–22.31 | 19.06–19.75 | 10.83–11.20 | 10.22–10.58 | 9.81–10.26 | 9.38–9.82 |
|        |     |      | 14.6±0.7 | 11.0±0.7 | 12.1±0.2 | 12.9±1.1(4) | 9.1±0.6 | 21.82±0.40 | 19.36±0.26 | 11.04±0.15 | 10.38±0.15 | 10.03±0.16 | 9.57±0.18 |
| Sumatra| 5   | ♀♂   | 13.7–15.1 | 11.3–11.8 | 9.8–13.1 | 14.0–16.1 | 8.9–10.6 | 22.14–22.64 | 19.70–20.02 | 11.03–11.60 | 10.45–10.78 | 9.96–10.34 | 9.15–9.79 |
|        |     |      | 14.6±0.7 | 11.5±0.2 | 12.2±1.4 | 15.0±0.8 | 9.9±0.7 | 22.48±0.20 | 19.88±0.12 | 11.33±0.23 | 10.57±0.14 | 10.17±0.14 | 9.56±0.24 |
| Southern Malay Peninsula | 15 | ♀♂   | 11.2–15.2 | 10.6–11.9 | 12.4–13.7 | 12.8–14.9 | 8.9–10.2 | 21.59–22.62 | 19.08–19.92 | 11.07–11.49 | 10.39–10.89 | 9.80–10.44 | 9.34–10.01 |
|        |     |      | 14.4±1.0 | 11.4±0.4 | 13.0±0.4 | 13.8±0.6(14) | 9.6±0.4(14) | 22.21±0.36 | 19.51±0.27 | 11.26±0.15 | 10.70±0.15 | 10.22±0.19 | 9.74±0.19 |
| Northern Malay Peninsula | 50 | ♀♂   | 13.8–16.3 | 10.6–12.6 | 9.6–14.6 | 10.4–15.9 | 9.6–11.0 | 21.97–23.27 | 19.36–20.78 | 10.84–11.91 | 10.41–11.16 | 9.84–10.67 | 9.27–10.14 |
|        |     |      | 15.0±0.6 | 11.4±0.5 | 13.0±0.8 | 13.9±1.1 | 10.3±0.4 | 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 |
GHN (Fig. 4D and Table 1). The skulls of Sumatran specimens also have significantly smaller MAW, GBW, ALSW, AMSW, IOW, CM3L and CM3L (all $P < 0.05$ — Figs. 3, 6, 7, and Table 1). Compared with specimens from the southern Malay 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 C1C1W — $P < 0.05$). Sumatran specimens were found to have a more developed sagittal crest (Fig. 3B) however, which is well built and visible from the supraorbital ridges to the lambda.

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 of 9.9 mm; GHN is highest in the Sumatran population, averaging 15.0 mm. The median emargination of the horseshoe is as wide as specimens from Central Java and Malay Peninsula and differs from specimens from Sarawak and India (Fig. 4D). The rudimentary secondary noseleaf is visible in dorsal view, with fewer hairs compared to Sarawak and Central Java specimens (Fig. 4D). The sella is large, tall and rounded off on the top, and the lateral margin is only slightly constricted in the middle (Fig. 5D). The internarial cup is moderate in size and the margin is less developed compared to specimens from Sarawak (Fig. 4D versus Fig. 4C). 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 1). The rostral chambers are large (Fig. 6D) and ALSW and AMSW are broad, averaging 6.15 mm and 4.26 mm, respectively. The anterior median swellings are inflated (Fig. 3D) and rounded in the dorsal view (Fig. 6D). The frontal depression (Fig. 3D) and supraorbital ridges (Fig. 6D) are elongated and the palatal bridge is long, with CM3L, ML and CM3L also large (Fig. 7D). 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 the horseshoe (Fig. 4E, 4F). The sella is very broad and lacks an obvious middle constriction as the lateral margins gradually constrict (Fig. 5E, 5F). The tip of the sella is always rounded off. The internarial cup is broad with well-defined but not especially developed lateral margins.
Variation of *R. affinis* in the Sundaic subregion (Fig. 4E, 4F). 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 in some individuals.

**Baculum**

The bacula of Sumatran specimens (*n* = 2) are similar to those from the Malay Peninsula, although some differences are apparent. Overall, the bacula of Sumatran specimens are slightly shorter and the
basal portion is more inflated and rounded (Fig. 8B versus 8C). In the lateral view, the bacula of Sumatran specimens have 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 the specimen from Sarawak is similar to those of Sumatran specimens, just slightly more slender overall with a less inflated basal portion (Fig. 8A versus Fig. 8B). In the lateral view, the tip portion of the shaft is also swollen in character but is elongated and less prominent (Fig. 8A) compared to Sumatran specimens (Fig. 8B). In the dorsal view, the basal emargination is deeper and narrower.

**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: Central Java
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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 2 and Fig. 1). 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 [T17] is 65.1 kHz compared to 71.2 kHz from the central area of the peninsula. Similarly, bats from Taman Negara Pulau Pinang [M2] emit mean frequencies of 72.8 kHz compared to 77.8 kHz in Peninsular Malaysia.

**Genetics**

Results from both maximum likelihood (ML) and Bayesian analysis (BA) showed similar topologies in phylogenetic trees. Three clades were recovered based on Cytb genes (Fig. 9). Clade A and C lineages were supported by high bootstrap values (BT = 90–99%) and posterior probabilities (PP = 100%) while clade B was supported by lower BT = 60% but rather high PP = 94%. The recovery of the three lineages was very consistent in the analyses; however the recovery of basal lineage was inconsistent. The two possible basal lineage relationships through our analyses (A and B, or B and C — Fig. 9) were poorly supported (e.g., BT = 30%, PP = 75%). Clade A comprised sequences from Borneo, whereas clade B comprised sequences from Borneo, Central Java and Sumatra, and clade C comprised sequences from the Malay Peninsula (Fig. 10). Pairwise 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 Central Java-Sumatra was lower (clade A versus B:

Fig. 5. Sella variation of *R. affinis* in the Sundaic Subregion (specimen from northern India included for comparison). Kabumen, Central Java (A — MZB34475, ♀); Mussoorie, northern India (B — HZM.4.28148, ♂); Sarawak, northwest Borneo (C — TK152216, ♀); Lampung, southwestern Sumatra (D — MZB34965, ♀); Narathiwat, southern Malay Peninsula (E — IS120122.1, ♂); Songkhla, southern Malay Peninsula (F — IS110823.10, ♂). Scale = 5 mm
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 Central Java (clade C versus B: 3.6%, 3.0–4.4). Based on the mean genetic distance, the Central 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.

Results from both ML and BA illustrated similar topologies, with two clades recovered for COI gene (Fig. 11). 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. 12). 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 (clades B and C — Fig. 9), 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 Central Java and Malay Peninsula clades (A versus B, C) was 1.7 million years before present (Myr BP) (95% CI 1.09–2.35) (Fig. 9), 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).

Variation within the Malay Peninsula

Intraspecific variation was also found within the Malay Peninsula. Specimens from the high call frequency zone (green shading A: 77.3–79.3 kHz;

Table 2. Frequencies of maximum energy (FMAXE) for *R. affinis* from the Sundaic subregion. Values are given as mean ± SD, min–max

| Locality                        | No. bats | Frequency (kHz) | Source                  |
|---------------------------------|----------|-----------------|-------------------------|
|                                 |          | × ± SD          | min–max                 |
| Central Java                    | 6        | 81.8 ± 0.4      | 81.2–82.3               | This study               |
| Borneo                          | 1        | 68.9            | –                       | This study               |
| Sumatra                         | 5        | 74.2 ± 0.5      | 73.2–74.6               | This study               |
| Southern Malay Peninsula        | 16       | 77.8 ± 1.3      | 75.4–79.3               | This study and Ith *et al.* (2015) |
| Taman Negara Pulae Pinang (island) | 2      | 72.6–73.1      | –                       | This study               |
| Northern Malay Peninsula        | 31       | 70.8 ± 0.7      | 69.5–72.6               | Ith *et al.* (2015)      |
| Tarutao islands                 | 10       | 65.1 ± 1.3      | 63.6–66.6               | This study               |
| Koh Surin, Phang Nga            | 1        | 62.3            | –                       | This study               |
have significantly smaller horseshoes, SL, CCL, ALSW, PB, C\textsuperscript{1}C\textsuperscript{1}W, M\textsuperscript{3}M\textsuperscript{3}W, CM\textsubscript{1}L, CM\textsubscript{3}L and ML ($P < 0.05$) (Fig. 4E–F and Table 1). Moreover, zone A specimens have slightly smaller teeth overall (Fig. 7D–E). However, both populations have similar bacula morphology. A PCA using nine external and cranial characters of all specimens from Malay Peninsula illustrated two relatively isolated groups (Fig. 13).

**DISCUSSION**

On the basis of morphology, bacula and genetic evidence, three geographical forms of *R. affinis* are recognized in the Sundaic subregion of Southeast Asia. Two of these are referred to their existing names (*R. a. nesites* from Borneo and *R. a. superans* from the Malay Peninsula), while the population from Sumatra is provisionally referred to *R. cf. affinis* due to its morphological and genetic differences from *R. a. superans* in the Malay Peninsula. Although sampling sizes for this regionally widespread species are limited, each genetic clade identified here corresponds to a unique morphology that reflects the phylogeographic distinctiveness of different locations. Similar divergence patterns have been found in other bat species from Borneo and the peninsula (Francis *et al.*., 2010; Khan *et al.*., 2010), and also in murine rodents (Gorog *et al.*., 2004). Call frequencies in the region are not congruent with this pattern however and disparities between acoustic and other datasets have also been observed in *R. affinis* from the Indochinese subregion (Ith *et al.*., 2015), as well as *R. malayanus* (Soi sook *et al.*., 2008) and *Hipposideros larvatus* (Thahab *et al.*., 2006).

*Rhinolophus a. nesites* was described as occurring from Sarawak (Bau, Kuap) to West and South Borneo (Medway, 1977) and this form was recognized by Koopman (1994) and Csorba *et al.* (2003). The holotype is deposited in the American Museum of Natural History (AMNH.104753♀) and is badly damaged, with only the teeth and lower jaw remaining in good condition. Based on this, Andersen (1905) described *R. a. nesites* as comparable to *R. a. superans*, but with a shorter TIB and smaller MT. Specimens subsequently collected from Sarawak agree with the holotype description in having a short TIB and MT (Table 1). However, they differ in having relatively smaller EL and GWN. This is probably because the holotype was described from Bunguran Island, and our data indicate that specimens from islands (e.g., Adang, Rawee and Koh...
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Surin) tend to have larger ears and noseleaf characters (e.g., horseshoe, sella and connecting process) and emit lower call frequencies (Table 2). Our comparisons also show that *R. a. nesites* is significantly smaller on average (*P* < 0.05, table of comparisons not included) than *R. a. superans* and *R. cf. affinis* (from southwestern Sumatra) in other external characters and many cranial characters. *Rhinolophus a. nesites* also differs in noseleaf and baculum characters and genetic data support this divergence as none of the sequences from Borneo (clade A) nested with Malay Peninsula sequences (clade C) or vice versa (Figs. 9 and 10).

Andersen (1905) included Sumatra in the distribution of *R. a. superans* based on a specimen from Sirambas, central Sumatra, the only specific locality record from the region (Andersen, 1907). This was accepted by Csorba *et al.* (2003). In our study, *R. cf. affinis* from southwestern Sumatra differed in many skull and baculum characters from Malay Peninsula specimens and also genetically (COI and Cytb — Figs. 9 and 11). Sumatran specimens are more similar to Central Javan specimens craniodentally (Table 1) and genetically (Fig. 9). We therefore distinguish the southwestern Sumatran population from peninsular populations. However, since our sample was limited to the southern tip of Sumatra, the possibility that specimens from central and northern parts of the island could be allied with peninsular populations cannot be excluded as morphological and genetic affinities between adjacent areas of different islands have been found for *R. affinis* in Wallacea (Maharadatun Kamsi et al., 2000).

The presence of two Cytb sequences (GenBank) in clade B (Fig. 9) from an unspecified locality or localities in Borneo requires comment. If these...
Fig. 10. Distribution of Cytb clades of *R. affinis* within the Sundaic subregion. The shape of the symbols corresponds to clades defined in Fig. 9. Black symbols are sequences from the current study whereas grey symbols are sequences from 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.
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sequences are genuinely derived from Borneo, then their true localities are most likely to be from regions near to Java or Sumatra and result from dispersal during the Pleistocene. Another possibility is that *R. a. affinis* and *R. a. nesites* are sympatric in Borneo which would challenge their subspecies-level status. Alternatively, the location of Borneo given for these sequences could be due to incorrect labeling, and they are from Java or Sumatra but represent a genetically distinct group.

*Rhinolophus a. superans* was described as similar to the Indochinese form *R. a. macrurus*, but with a shorter TL, ALSW, AMSW, CM\(^3\)L, CM\(_{j}\)L and BW (Andersen, 1905). Ith et al. (2015) found that *R. a. superans* is significantly smaller in many external measurements, larger in many skull measurements and distinguishable genetically (D-loop, COI). Our study indicates that morphological variation of *R. a. superans* in the Malay Peninsula aligns closely with the Kangar-Pattani Line (van Steenis, 1950; Whitmore, 1984) and climatic zones (Hughes et al., 2011), although this was not supported genetically. Morphological differences were also observed between specimens from the mainland and adjacent islands (Adang, Rawee and Kho Surin) and as noted above, island specimens have larger noseleaves, rostral chambers and other skull measurements and also emit lower call frequencies. These differences reflect known relationships between call frequencies and horseshoe bat skull characters (Heller and von Helversen, 1989; Francis and Habersetzer, 1998; Barclay et al., 1999; Guillén et al., 2000), but were not supported genetically as all sequences of each gene nested together in one clade (clade C, Fig. 9 and clade I, Fig. 11).

The extensive geographical variation in echolocation call frequencies emitted by *R. affinis* sensu lato does not accord with morphological and genetic variation. Similar variation in call frequencies has also observed in *R. a. macrurus* (Ith et al., 2015) and *R. malayanus* (Soisook et al., 2008). In the Malay Peninsula, call frequencies for *R. affinis* from islands (loc. T4, T17 and M2) are lower than adjacent localities on the mainland. Two clear frequency patterns occur that correspond with morphological variation (Fig. 1). Higher frequency calls occur

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**Fig. 11.** Maximum likelihood tree based on COI. 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. Specimens are labeled by specimen codes (IS, HM, HZM and MZB) and collecting localities. The symbols of clades correspond to the genetic distribution map, Fig. 12
Fig. 12. Distribution of COI clades of *R. affinis* in the Sundaic subregion. The shape of the symbols corresponds to clades defined in Fig. 11. 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, Peninsular 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.
Variation of *R. affinis* in the Sundaic subregion south of the Kangar-Pattani Line in tropical evergreen rain forest and lower frequency calls occur north of the line in semi-evergreen rain forest. These differences are reflected in some external and craniodental characters (Fig. 13), but not by Cytb and COI genes (Figs. 9 and 11). This suggests that these differences are not a result of selection for clear social communication (which would lead to phylogenetic distinctiveness: Heller and von Helversen, 1989; Kingston *et al.*, 2000; Kingston and Rossiter, 2004), but may have recently evolved in association with climatic conditions, foraging habitats and/or prey availability. The current forest and climatic conditions of the region began as recently as the end of the last glacial maxima (ca. 9,500 years ago, after the breakup of Sunda Shelf land-bridge — Voris, 2000; Inger and Voris, 2001), which may not have been long enough for significant genetic differences to evolve.

Our results suggest that *R. a. nesites*, *R. a. superans* and *R. cf. affinis* diverged in the early Pleistocene epoch (1.7–1.3 Myr BP). This may have been caused by refugial isolation prior to the coldest Pleistocene period and accords with estimated divergence times for other taxa in the region e.g. gymnures (Ruedi and Fumagalli, 1996), murine rodents (Gorog *et al.*, 2004), bats (Khan *et al.*, 2010; Lin *et al.*, 2014), herpetofauna (Inger and Voris, 2001) and termites (Gathorne-Hardy *et al.*, 2002). The formation of *R. affinis* lineages on the Sunda shelf may be partly explained by its ecology. *Rhinolophus affinis* is a cave-dwelling bat species which forages in the understorey of forest, including mature lowland rainforest, dry forest and disturbed areas (Francis, 2008). As such, the historical transition from a relatively stable tropical environment and perhumid climate during the Miocene (Gorog *et al.*, 2004) to more arid and cool climatic conditions in the Plio-Pleistocene when suitable habitats in Southeast Asia were reduced to isolated pockets (Heaney, 1991; Morley, 1998, 2000; van der Kaars *et al.*, 2001) may explain the current biogeography of *R. affinis*.

In conclusion, our study demonstrates the importance of employing multiple datasets in taxonomic evaluations, as use of morphological and/or acoustic datasets alone could lead to erroneous conclusions. The discovery of additional population structures (e.g., *R. cf. affinis* from Sumatra) is also predicted in Southeast Asia with greater sampling effort throughout the region.

**ACKNOWLEDGEMENTS**

We are grateful to David Harrison and Malcolm Pearch for providing us access to specimens in Harrison Institute, UK. We thank Tigga Kingston from Texas Tech University (TTU) and Rosichon Ubaiddillah from Museum Zoologicum Bogoriense, Indonesian Institute of Sciences (LIPI-MZB) for facilitating specimen loans, Beatrix Lanzinger for providing access to literature from the Harrison Institute library and the directors and staff of wildlife sanctuaries and national parks in Thailand for permission and support for the field surveys. We are indebted to Chutamas Satasook and staff from the Princess Maha Chakri Sirindhorn Natural History Museum, PSU. We thank the Zoological Museum at Universiti Malaysia Sarawak, Malaysia whose research was supported by Ministry of Higher Education in Malaysia through Research Acculturation Collaborative Effort grantRACE/G(2) 1107/2013 [15] to F.A.A.K.; Coral Triangle Partnerships in International Research and Education Project (NSF OSE-0730256 to Kent E. Carpenter and Paul H. Barber). We also thank NSRL at Museum of TTU and LIPI-MZB. Finally, for their steadfast support we are also deeply grateful to the Graduate School of PSU, the Centre for Biodiversity Conservation, Fauna & Flora International (Cambodia program), Royal University of Phnom Penh, and to the Darwin Initiative (Project nos.: 18002, EIDPO028), CEPF (Critical Ecosystem Partnership Fund 2009–2011), the John D. and Catherine D. MacArthur Foundation (09-92411-000-GSS), and the Zoological Parks and Gardens Board of Victoria, American Society of Mammalogists, Bat Conservation International, and Rufford Small Grant Foundation.

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Received 28 August 2015, accepted 07 March 2016