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Systematics of Malaysian woolly bats (Vespertilionidae: *Kerivoula*) inferred from mitochondrial, nuclear, karyotypic, and morphological data

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Examining species boundaries using data from multiple independent sources is an appropriate and robust method to identify genetically isolated evolutionary units. We used 5 data sets—cytochrome *b* (*Cytb*), cytochrome *c* oxidase (COI), amplified fragment length polymorphisms (AFLPs), karyotypes, and morphology—to estimate phylogenetic relationships and species limits within woolly bats, genus *Kerivoula*, from Southeast Asia. We genetically analyzed 54 specimens of *Kerivoula* from Malaysia, assigned to 6 of the 10 species currently reported from the country. Phylogenetic analyses of nuclear AFLPs (33 specimens) and mitochondrial DNA sequences from *Cytb* (51 specimens) and COI (48 specimens) resulted in similar statistically supported species-level clades with minimal change in branching order. Using comparisons of cranial and dental morphology and original species descriptions, we assigned the resulting phylogenetic clades to *K. hardwickii*, *K. intermedia*, *K. lenis*, *K. minuta*, *K. papillosa*, *K pellucida*, and 1 unidentified species. Karyotypes further documented variability among the 6 clades. Five different karyotypes were identified, with 2 species having indistinguishable karyotypes. We compared our COI gene sequence data to 110 specimens of *Kerivoula* from Southeast Asia made available by researchers of the Barcode of Life Database. Our *Cytb* and AFLP species identifications were congruent with those in the COI database. Intraspecific geographic variation of about 5–7% sequence divergence was observed in *Cytb* and COI genes within both *K. hardwickii* and *K. minuta*. Relaxed molecular clock analyses indicated a late Oligocene to early Miocene origin of the Kerivoulinae with intraspecific diversification events coinciding with the late Pliocene and Pleistocene epochs in at least 6 species. Specimens from Sabah (northeastern Borneo) showed relatively high genetic divergence compared to those between Peninsular Malaysia and Sarawak (southwestern Borneo), indicating Pleistocene or Pliocene refugia in Borneo. We conclude that at least 1 distinct lineage of *Kerivoula* is yet to be described from Borneo and that the intraspecific geographic variation in some species agrees with previous studies on the diversification of flora and fauna in Borneo. DOI: 10.1644/09-MAMM-A-361.1.

Key words: amplified fragment length polymorphism, Borneo, cytochrome *b*, cytochrome *c* oxidase, karyotypes, Kerivoulinae, Malaysia, morphology, taxonomy

The chiropteran family Vespertilionidae is a critical component of tropical bat assemblages (Kingston et al. 2003; Struebig et al. 2006) and represents more than one-third of total bat diversity (Simmons 2005). Yet, taxonomic studies of this family face major challenges using only genetic or morphological data. This is due to mainly cryptic morphology (Kiefer et al. 2002; Mayer and von Helversen 2001; von Helversen et al. 2001) and limited voucher specimens available for comparisons to identify specimens. Previous genetic studies in the family Vespertilionidae did not support monophyly of some putative taxa in gene trees (*Kerivoula* species [Francis et al. 2007] and *Myotis* subgenera [Ruedi and Mayer 2001]). Such complexity in gene trees can be explained by convergent morphological evolution during

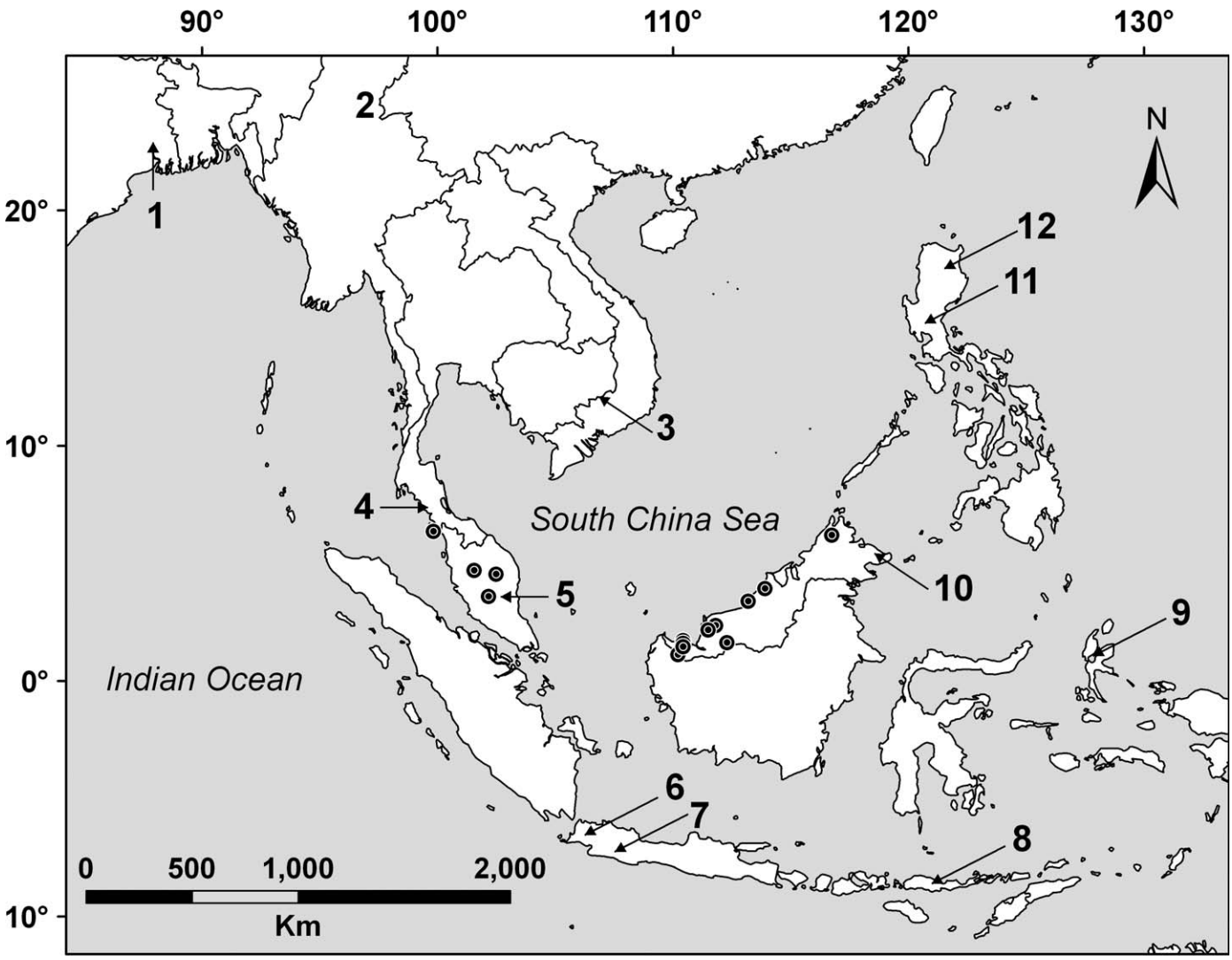

Fig. 1.—Map of Southeast Asia showing collecting localities for specimens examined herein (see Appendix I for details) and type localities for 12 recognized species within Southeast Asia. ◎ = collecting localities. Type localities beginning at the upper left corner, counterclockwise, are 1 = *Kerivoula lenis*, 2 = *K. kachinensis*, 3 = *K. titania*, 4 = *K. minuta*, 5 = *K. krauensis*, 6 = *K. papillosa*, 7 = *K. hardwickii*, 8 = *K. flora*, 9 = *K. picta*, 10 = *K. intermedia*, 11 = *K. pellucida*, and 12 = *K. whiteheadi*.

intrageneric diversification, resulting in similar phenotypes among members of nonrelated lineages (Ruedi and Mayer 2001). Increasing levels of phenotypic convergence ultimately can lead to an underestimation of species-level diversity. Therefore, systematic studies using singular data sets (e.g., morphology, karyotypes, mitochondrial DNA [mtDNA], or nuclear DNA sequences) may estimate diversity inaccurately. However, combining mitochondrial, nuclear, karyotypic, and morphological data sets is a powerful test of congruence of species boundaries defined with singular data sets. We used a multifaceted approach to estimate phylogenetic and species relationships within woolly bats, genus *Kerivoula*, from Malaysia.

The genus *Kerivoula* includes small, delicate woolly bats from Africa, Asia, and Australia (Simmons 2005). Hill (1965) revised the genus and addressed a number of taxonomic issues, including the recognition of *Phoniscus* Miller as a separate genus. His emphasis was on the 9 species that he recognized within the Asiatic range. Corbet and Hill (1992) reviewed and updated the taxonomy of the Indomalayan (South Asia subcontinent and Southeast Asia) taxa, recognizing 9 species. Simmons (2005) recognized 9 Indomalayan species with minor revisions from Corbet and Hill (1992), including the recognition of *K. lenis*, but questioned the presence of *K. myrella* in the Lesser Sunda Islands (Fig. 1). Three species have been described recently: *K. kachinensis* from Myanmar (Bates et al. 2004), *K. titania* from Cambodia (Bates et al. 2007), and *K. krauensis* from Peninsular Malaysia (Francis et al. 2007).

In Malaysia *Kerivoula* is represented by at least 10 of 12 currently recognized species from the Indomalayan region. These include *K. flora*, *K. hardwickii*, *K. intermedia*, *K. krauensis*, *K. lenis*, *K. minuta*, *K. papillosa*, *K. pellucida*, *K. picta*, and *K. whiteheadi*. Although most of the Indomalayan *Kerivoula* occur in Malaysia, the amount of information available for these species varies, depending on their

abundance in nature and availability in museums. Commonly, most of the recent faunal surveys or field guides in Malaysia document *K. hardwickii*, *K. intermedia*, *K. minuta*, *K. papillosa*, and *K. pellucida* (Anwarali et al. 2008; Francis 2008; Jayaraj et al. 2006; Kingston et al. 2006; Payne and Francis 1985). *K. picta*, which has a unique orange and black pelage, was recorded from Penang Island in Peninsular Malaysia (Medway 1983); however, its occurrence in Borneo is questioned by Corbet and Hill (1992). Similarly, although *K. flora* has been reported from Sabah (Hill and Rozendaal 1989), no subsequent verification has been reported for this species in Borneo, suggesting that this species could be rare or of limited range in Borneo, or the specimen might actually represent a morphologically similar species (*K. hardwickii*—Corbet and Hill 1992). Three subspecies are recognized within *K. whiteheadi*: *K. w. bicolor* (southern Thailand, Biserat), *K. w. pusilla* (Borneo, Sarawak), and *K. w. whiteheadi* (Philippines, Luzon). Although the holotype of *K. w. bicolor* was collected from the southern tip of Thailand and is known from central Peninsular Malaysia, none of the recent surveys have documented the occurrence of any of these subspecies in Malaysia (Anwarali et al. 2008; Kingston et al. 2006).

Recently, *K. lenis* was elevated from subspecific rank to specific rank (Vanitharani et al. 2003), and *K. krauensis* was described from Peninsular Malaysia (Francis et al. 2007). Francis et al. (2007) conducted the 1st systematic analysis of *Kerivoula* using genetic data through the DNA Barcoding initiative (Hebert et al. 2003) and demonstrated large genetic divergence in the cytochrome *c* oxidase (COI) gene among recognized species and moderate intraspecific divergence between mainland Southeast Asian and Bornean populations. Details of this intraspecific genetic divergence were not provided in their paper because the gene tree was used to delimit the new species (*K. krauensis*). Although such species delineation is the objective of the DNA Barcoding initiative, the accuracy of this method is limited to the availability of similar sequences for comparison and possibly limited by the representation of a particular species across its geographic range (Moritz and Cicero 2004; Will and Rubinoff 2004).

The gene tree in Francis et al. (2007) indicated the possibility that >1 taxonomic unit exists within each of *K. hardwickii*, *K. lenis*, and *K. papillosa*. Given potential divergence within these taxa, it is important to document the geographic extent of inter- and intraspecific genetic variation along with associated morphological characters. Although genetic data can provide statistically supported monophyletic resolution for *Kerivoula* clades, these clades are evaluated best in a context of broad taxonomic and geographic sampling. Morphological characters of these genetically recognized species could be used to delimit morphological variation within each defined clade (Baker and Bradley 2006). Furthermore, it is possible to use the morphology of genetically identified specimens for comparison with existing descriptions of holotypes to determine accurate application of Linnaean species-level names.

In light of the minimal number of systematic studies and preliminary understanding of the genus *Kerivoula*, we evaluated the diversity within 6 currently recognized Malaysian *Kerivoula*: *K. hardwickii*, *K. intermedia*, *K. lenis*, *K. minuta*, *K. papillosa*, and *K. pellucida*. We phylogenetically analyzed data from mitochondrial cytochrome-*b* (*Cytb*) and COI genes, and nuclear amplified fragment length polymorphisms (AFLPs), and compared data on karyotypes and external, cranial, and dental morphology, to better understand the evolutionary history of this genus. Furthermore, we performed relaxed molecular clock analyses to investigate the potential timescale of inter- and intraspecific diversification events within the genus. We discuss our results with respect to the mode of diversification of this group in reference to vicariant events in Southeast Asia as previously suggested for such other taxa as gymnures (Ruedi and Fumagalli 1996), rodents (Gorog et al. 2004), shrews (Ruedi 1996), and snakes and frogs (Inger and Voris 2001).

Materials and Methods

Taxonomic sampling for molecular analyses.—During a series of field trips from 2006 through 2008, 45 individuals of *Kerivoula* were collected in Peninsular Malaysia, Sabah, and Sarawak (Fig. 1; Appendix I). Additionally, 9 tissue samples were acquired through a tissue loan from the Universiti Malaysia Sarawak (UNIMAS) and the Department of Wildlife and National Parks, Malaysia (DWNP). Specimens from Borneo were divided into 3 major geographic regions: southwestern Borneo (located in southwestern Sarawak), central Borneo (located in northeastern Sarawak), and eastern Borneo (located in Sabah) for inferring phylogeographic hypotheses (similar to the geographic grouping in Gorog et al. [2004]). Specimens were collected from natural populations (Anwarali et al. 2008) using 4-bank harp traps (Francis 1989) and mist nets. Voucher specimens were prepared either as skin and skeleton or as fluid-preserved specimens. Liver and muscle tissues were preserved in both lysis buffer and 95% ethanol, and blood samples were collected using Nobuto Blood Filter Strips (Advantec MFS, Inc., Dublin, California). These materials were deposited at the Natural Science Research Laboratory, Museum of Texas Tech University (NSRL-TTU), and the Natural History Museum of Universiti Malaysia Sarawak. Museum vouchers or tissues and GenBank accession numbers for all specimens examined are listed in Appendix I. All animals were handled following the guidelines for animal care and use established by Texas Tech University (Animal Care and Use Committee; permit 02217-02) and the American Society of Mammalogists (Gannon et al. 2007).

Mitochondrial DNA sequencing.—Total genomic DNA was extracted from muscle or liver tissues following standard phenol methods (Longmire et al. 1997). The entire *Cytb* gene and 657 base pairs (bp) of the COI gene were amplified using standard polymerase chain reaction procedures (Saiki et al. 1988). Thermal cycle amplifications (polymerase chain

reactions) were performed using the flanking primers L14724 and H15915R (*Cytb*—Irwin et al. 1991) and VF1 and VR1 (COI—Ivanova et al. 2006) in a 50-µl reaction that included approximately 400 ng of DNA, 0.24 µM of each primer, 3.0 mM of $MgCl_2$, 0.024 mM of deoxynucleoside triphosphates, 1X reaction buffer, 0.64 mg/ml of bovine serum albumin, and 1.25 U of Taq DNA polymerase (Promega Corporation, Madison, Wisconsin). The thermal profile used was 94°C for 2 min, then amplification for 34 cycles of denaturation at 94°C for 45 s, annealing at 40.2°C (50°C for COI amplification) for 1 min, extension at 72°C for 1 min 15 s, followed by 72°C for 10 min. When necessary, further optimization was performed with alternate DNA template quantities. Double-stranded polymerase chain reaction amplicons were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Chatsworth, California) and sequenced using ABI Big Dye chemistry chain terminators version 3.1, on an ABI 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, California). We sequenced the entire *Cytb* gene, 1,140 bp, by using the internal primers MVZ04 and MVZ16 (Smith and Patton 1993) and 3 newly designed primers: Myotis16 (5′-TAR-AAA-GTA-TCA-YTC-TGG-TT-3′), Ker5L (5′-CCC-HGA-YAA-YTA-YAY-ACC-AGC-3′), and Ker1L (5′-THG-TAG-AAT-GAA-TCT-GAG-G-3′). A 618-bp segment of the COI gene was sequenced with the same primers used for amplification.

Amplified fragment length polymorphisms.—Our AFLP protocol followed those of Vos et al. (1995), Phillips et al. (2007), and McDonough et al. (2008) with slight modifications. Specifically, our AseI adapter sequences were as follows: 5′-GAC-GAT-GAG-TCC-TGA-G-3′ and 5′-TAC-TCA-GGA-CTC-AT-3′. A labeled selective EcoRI primer and 7 other selective primers (McDonough et al. 2008) were used to produce unambiguous and distinct AFLPs from 33 individuals of *Kerivoula*. Genotyping was performed with an ABI 3100-*Avant* Genetic Analyzer. AFLP profiles were double read anonymously, and only fragments (50–400 bp in length) with intensity $>$ 100 relative fluorescence units were selected using GeneMapper version 4.0 software (Applied Biosystems). A binary data matrix was created using GENALEX version 6 software (Peakall and Smouse 2006). Error rates (technical error rate and observer error rate) were calculated following Bonin et al. (2004) using 40 replicated samples (approximately 17% of the overall sample size).

Phylogenetic analyses.—Complete *Cytb* sequences and AFLP fragments were used independently in phylogenetic analyses. *Cytb* sequences were verified, checked for correct open-reading frame, aligned, and coded following Hoofer and Van Den Bussche (2003). Two GenBank sequences from Stadelmann et al. (2004) from Laos that are referable to *K.* cf. *lenis* were deposited as AJ841969 and AJ841970, but the voucher specimen numbers for these specimens were reversed. After further investigation (C. M. Francis, Canadian Wildlife Service, pers. comm.), we found that these GenBank sequences should be referred to *K. kachinensis* (AJ841969, ROM 118279—field number CMF980326-61) and *K.* cf. *lenis* (AJ841970, ROM 110520—field number CMF970503-17). These sequences were included in our analyses. A single base missing at position 19 in GenBank sequence AJ841969 was identified as ''N'' and treated as missing data in all analyses. Sequences of *Myotis muricola* (AJ841957), *Harpiocephalus mordax* (AJ841971), and *Murina* cf. *cyclotis* (AJ841973) were retrieved from GenBank and used as outgroups in all *Cytb* analyses (Hoofer and Van Den Bussche 2003), whereas AFLP bands generated from *Myotis muricola* (TK 152162, TK 152224) and *Murina rozendaali* (TK 152086—Anwarali et al. 2008) were included and used as outgroups in all AFLP analyses.

Phylogenetic analyses were performed using PAUP* software version 4.0b10 (Swofford 2002), PhyML (online version—Guindon and Gascuel 2003), and MrBayes software, version 3.1.2 (Huelsenbeck and Ronquist 2001). For the *Cytb* data set, minimum-evolution, maximum-likelihood, maximum-parsimony (unweighted), and Bayesian analyses were used to infer phylogenies. Of 56 models of evolution, Modeltest version 3.7 (Posada and Crandall 1998) showed that the general time reversible (GTR) model of substitution, with allowance for gamma distribution (Γ) of rate variation and for proportion of invariant sites (I), best fit the data. This model was used in minimum-evolution and maximum-likelihood analyses with full heuristic searches using neighbor-joining and BioNJ (Gascuel 1997) starting trees, respectively, for *Cytb* sequences. Maximum-parsimony analysis was performed using heuristic searches, 25 replicates of the random taxon addition option, each with random starting trees, and tree-bisection-reconnection branch-swapping for both the *Cytb* and AFLP data sets. AFLP characters were defined as unweighted, unordered characters. For bootstrap support values, 1,000 replicates were conducted using the heuristic search criterion.

Of 24 models of evolution, MrModeltest version 2.3 (Nylander 2004) showed that the GTR + I + Γ best fit the data and was implemented in Bayesian analyses for the *Cytb* data set. The binary AFLP data set was analyzed using the binary evolutionary model as implemented in MrBayes software. For both *Cytb* and AFLP data sets posterior probabilities were generated with 4 Monte Carlo chains for 2 million generations. Three separate Bayesian analyses were performed with different outgroup positions in the data set. Trees were sampled every 100 generations with a burn-in value of 1,000 trees. Phylogenetic signal present in *Cytb*, COI, and AFLP data sets were examined in PAUP* software through evaluation of 100,000 random trees using the $g1$ statistic (Hillis and Huelsenbeck 1992). The *Cytb* genetic distance matrix was generated using the Kimura 2-parameter model in MEGA version 4.0 software (Kumar et al. 2004), whereas Nei genetic distances were generated from the AFLP binary data set using GENALEX version 6 software (Peakall and Smouse 2006). We regarded nodes with $\geq$70% bootstrap values and $\geq$0.95 Bayesian posterior probability as having significant statistical support.

Partial gene sequences from the COI gene (618 bp) were used to verify and compare with 110 COI sequences used by

Francis et al. (2007) and were deposited in the DNA Barcode of Life Database (http://www.boldsystems.org/views/login.php). Species identifications were verified through clade similarities in neighbor-joining trees as identified in Francis et al. (2007). We compared our results with those of Francis et al. (2007) and estimated the geographic range for each species of *Kerivoula* identified in our analyses. The DAMBE version 4.5.65 software package (Xia and Xie 2001) was used to estimate transition and transversion saturation in *Cytb* and COI data sets as a test for phylogenetic resolution.

Divergence time estimates.—We performed a likelihood ratio test on our *Cytb* data set to test for the presence of a molecular clock. BEAST version 1.5 software (Drummond and Rambaut 2007) was used to examine the timescale of diversification for Southeast Asian species of *Kerivoula* using paleontological constraints from the fossil record. Two fossil priors were used as secondary calibration points with exponential distribution priors (Ho 2007) in all analyses: time to most recent common ancestor for Kerivoulinae, Murininae, and Vespertilionidae ~30 million years ago (mya); and time to most recent common ancestor for *Myotis* ~20 mya (Horáček 2001). Time of origin and diversification for *Kerivoula* was examined by performing relaxed molecular clock analyses on *Cytb* gene DNA sequence data from 6 species. Sequence data from *Myotis latirostris* (Stadelmann et al. 2004), *M. alcathoe*, *M. scotti*, *Harpiocephalus mordax*, and *Murina* cf. *cyclotis* were used as outgroups. Node dates were examined using a Yule species prior and random selection of single representatives of each species with preliminary analyses consisting of 5 runs at 10,000,000 generations (10% burn-in). Final analysis consisted of the combination of 2 runs at 20,000,000 generations (10% burn-in). Tracer version 1.3 (Rambaut and Drummond 2003) was used to examine convergence, effective sample sizes, and 95% highest posterior density (95% HPD) for all mean divergence dates estimated.

Morphological variation and species nomenclature.—Information about species' morphological characters was compiled by reviewing the literature and identification of diagnostic traits used in original descriptions or taxonomic revisions (Hill 1965; Hill and Francis 1984; Tate 1941; Tomes 1858; Vanitharani et al. 2003). We evaluated variation in external, cranial, and dental characters of voucher specimens for representatives of each genetically defined clade. First, specimens were grouped by forearm (FA) length and upper toothrow length (C-M3) into 3 size morphotypes: small (FA = 27–30 mm, C-M3 < 5.2 mm), medium (FA = 29–34 mm, C-M3 = 5.2–6.9 mm), and large (FA > 38 mm, C-M3 > 6.9 mm). Only adult specimens were used for morphological analysis. Standard measurements were taken following Corbet and Hill (1992) and Bates and Harrison (2000).

We used our most highly supported molecular phylogenies as a hypothesis of species boundaries and systematic relationships. We included available species-group names and type localities of each taxon name in Southeast Asia (Fig. 1) and other verified geographic records (Corbet and Hill 1992; Hill 1965; Tate 1941) to provide a geographic background to our species identifications (Solari et al. 2009). We examined morphological characters of the voucher specimens corresponding to the monophyletic clades within our molecular phylogeny to verify the morphological features diagnosing each taxon. Then, we compared our specimens with the original species descriptions or taxonomic accounts (Corbet and Hill 1992; Francis et al. 2007; Hill 1965; Hill and Francis 1984). Finally, we cross-checked our genetic and morphological identifications with those identified in the gene tree of Francis et al. (2007). Thus, every clade of the molecular tree can be assigned Linnaean names based on morphology and distribution of the corresponding voucher specimens (Baker and Bradley 2006).

Karyotypes.—Karyotypes were prepared in the field the morning after capture using the in vivo bone marrow method (Baker et al. 2003). Bone marrow cell suspensions were preserved in a mixture of acetic acid and methanol (1:3) and stored at −20°C. Slides were made by flame drying and stained with Giemsa. Metaphase spreads were examined using an Olympus epi-fluorescence microscope BX51 (Olympus Imaging America Inc., Center Valley, Pennsylvania) and photographed using an Applied Imaging camera (Applied Imaging Systems, San Jose, California). Images were captured and karyotypes were arranged using the Genus System 3.1 (Applied Imaging Systems). Karyotypes were described by diploid number (2n) and fundamental number (FN). Centromere placement on respective chromosomes were defined as: metacentric = biarmed chromosome with arm of equal length or the shortest arm to be ≥ 90% of the longer arm; submetacentric = long arm ≥ 90% of the length of short arm or < 2 times the length of short arm; subtelocentric = long arm > 2 times the length of short arm; acrocentric = telomere placement terminal, but a minute short arm may be present.

Results

Phylogenetic analyses.—The entire *Cytb* gene (GenBank EU188768–188791 and GU585640–GU585664) was sequenced for 49 specimens, and partial fragments of 404 bp (GenBank GU585665 and GU585666) were sequenced for 2 specimens (Appendix I). Partial COI gene sequences for 48 specimens (618 bp, GenBank GU585592–GU585639) were used for estimating genetic distances (Table 1) and for comparison with clades identified in *Cytb* gene sequence and AFLP data sets. Alignment of complete *Cytb* sequences, plus 2 from GenBank, was unequivocal and without internal stop codons, resulting in 42 unique haplotypes. Of 1,140 bp, 673 were invariant, 53 were autapomorphic, and 414 were parsimony informative. Including outgroups, 79 informative characters were at 1st codon positions, 21 at 2nd positions, and 314 at 3rd positions. Parsimony analyses generated a single most-parsimonious tree of 1,629 steps (consistency index = 0.3966, retention index = 0.8161). Maximum-likelihood analyses resulted in a single optimal tree (−lnL = 7,846.79; Fig. 2), and the minimum-evolution, least-evolved tree had a

Table 1.—Average percentage of Kimura 2-parameter distance values within (boldface type along diagonal for cytochrome-*b* [*Cytb*] followed by cytochrome *c* oxidase [COI] value) and among species of *Kerivoula* from different clades based on entire *Cytb* (below diagonal) and partial COI (above diagonal) gene sequences. *n* = sample sizes of each species for *Cytb* and COI sequences, respectively. NA = not available.

Taxon	Clade	1	2	3	4	5	6	7	8	9
1) *K. minuta* (*n* = 14, 15)	A	**3.12/3.98**	17.46	15.01	17.61	18.59	18.84	21.55	NA	NA
2) *K. intermedia* (*n* = 7, 7)	B	15.94	**0.41/0.33**	16.39	20.14	16.1	17.53	20.32	NA	NA
3) *K. hardwickii* (*n* = 10, 6)	C	14.43	16.33	**3.68/4.35**	16.51	17.69	17.62	19.82	NA	NA
4) *K. pellucida* (*n* = 7, 7)	D	17.81	19.34	18.31	**1.34/1.11**	21.21	18.93	21.94	NA	NA
5) *K. papillosa* (*n* = 8, 8)	E	14.67	15.78	13.23	16.81	**1.85/1.78**	12.47	15.5	NA	NA
6) *K. lenis* (*n* = 2, 2)	F	16.13	17.45	14.53	19.24	10.2	**3.90/5.62**	15.65	NA	NA
7) *Kerivoula* sp. (*n* = 3, 3)	G	15.95	17.67	15.99	18.56	11.08	15.52	**0.00/0.00**	NA	NA
8) *K. kachinensis* AJ841969 Laos		14.01	14.28	10.08	15.97	13.62	13.8	14.47	**NA**	NA
9) *K.* cf. *lenis* AJ841970 Laos		16.64	14.71	14.54	16.42	7.1	11.1	12.23	10.18	**NA**

score of 1.26. Bayesian analyses, regardless of outgroup choice (3 different outgroup arrangements), resulted in statistically supported clades with identical content of individuals (Fig. 2). No statistically supported conflicts were found between results from different analyses (Fig. 2). The *g*1 statistic was −0.62 for the *Cytb* data set and −0.47 for COI, indicating significant phylogenetic signal ($P < 0.01$) in both data sets (Hillis and Huelsenbeck 1992). However, we found an indication of transitional saturation above 19.5% and 19.0% of Kimura 2-parameter model distances in both *Cytb* and COI genes, respectively (Xia and Xie 2001). All phylogenetic topologies revealed 7 strongly supported monophyletic clades (A–G; Fig. 2) with ≥10% (*Cytb*) and ≥12% (COI) divergence from each other (Table 1).

Phylogenetic analyses of AFLP fragments resulted in 481 scored bands with an average 69 bands scored per primer pair. An error rate of 3.7% (18 bands of 481) was calculated, with most discrepancies originating from poor amplification. Seven

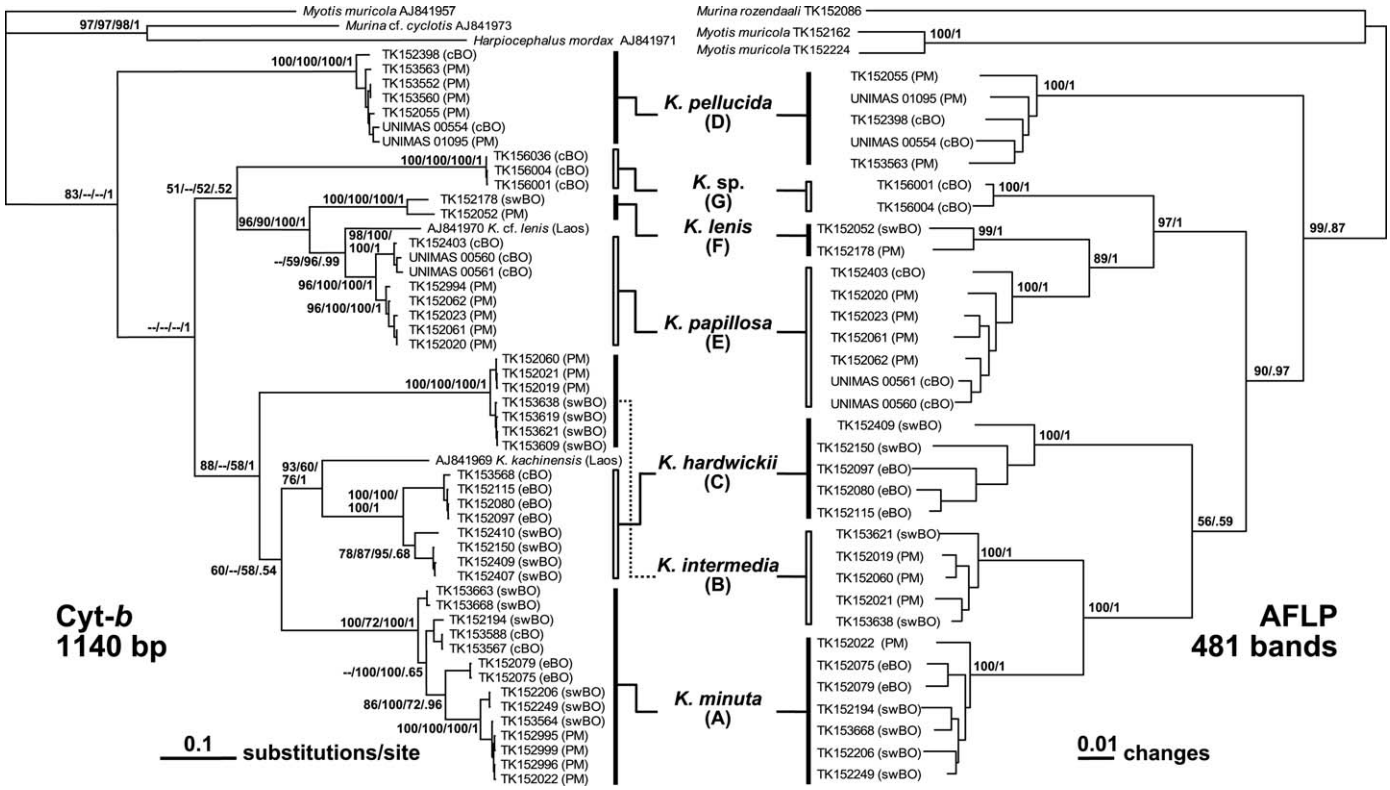

Fig. 2.—Bayesian phylogram of left) cytochrome-*b* (*Cytb*) sequences and right) neighbor-joining phylogram of amplified fragment length polymorphisms (AFLPs). Scores on the *Cytb* branches refer to bootstrap support values (1,000 iterations) from maximum likelihood (1st score), maximum parsimony (2nd score), minimum evolution (3rd score), and Bayesian posterior probabilities (4th score); -- = no support value. Maximum parsimony (1st score) and Bayesian posterior probabilities (2nd score) are shown on the AFLP phylogram. Clades (A, B, C, D, E, F, and G) are identified according to species identification. Some internal branches and similar haplotype support values were omitted from the figure. Specimen labeled by tissue number (TK, UNIMAS) and collecting region: swBO = southwestern Borneo, cBO = central Borneo, eBO = eastern Borneo, and PM = Peninsular Malaysia. Refer to ''Materials and Methods'' for suggested changes in voucher specimen numbers in Stadelmann et al. (2004).

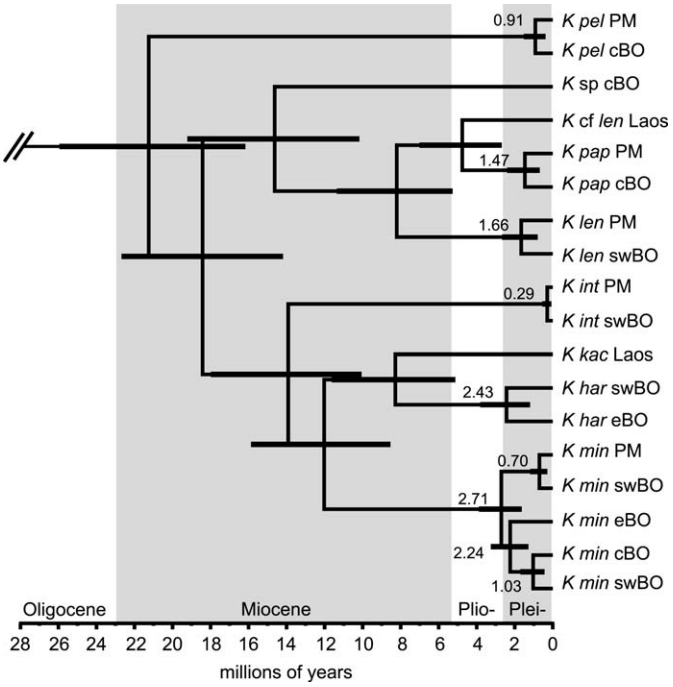

Fig. 3.—Chronogram of Southeast Asian *Kerivoula* studied here. Values at each node represent mean time to most recent common ancestor estimates from dating analyses performed in BEAST version 1.5. Gray bars represent the 95% highest posterior density intervals for the divergence estimates. Specimens: *K har* = *K. hardwickii*, *K int* = *K. intermedia*, *K kac* = *K. kachinensis* (AJ841969), *K len* = *K. lenis*, *K min* = *K. minuta*, *K pap* = *K. papillosa*, *K pel* = *K pellucida*, *K* cf *len* = *K.* cf. *lenis* (AJ841970), and *K* sp = potential undescribed species (see ''Discussion''; TK 156001). Collecting regions: swBO = southwestern Borneo, cBO = central Borneo, eBO = eastern Borneo, and PM = Peninsular Malaysia. Geological time: Plio = Pliocene and Plei = Pleistocene. Outgroup not shown.

statistically supported clusters corresponding to the 7 clades identified in *Cytb* and COI gene trees were present (Fig. 2). The average number of private bands per species was 17 (from 6 within *K. lenis* to 29 within *K. pellucida*). AFLP genetic distance values ranged from 13% (*K. lenis* versus *K. papillosa*) to 31% (*K. pellucida* versus *K. papillosa*). Phylogenetic trees recovered from neighbor-joining, parsimony, and Bayesian analyses were identical in topology (Fig. 2). The $g1$ statistic for the AFLP data set was -0.57, indicating significant phylogenetic signal ($P < 0.01$—Hillis and Huelsenbeck 1992).

Relaxed molecular clock analyses.—The assumption of a strict molecular clock was significantly rejected by the likelihood ratio test ($2\Delta L = 52.64$; $P < 0.01$). Our results indicate that the diversification of Southeast Asian *Kerivoula* began during the early to middle Miocene (Fig. 3). Interspecific diversification dates presented herein should be viewed with caution because to date only 7 of at least 26 *Kerivoula* species (Simmons 2005) have available *Cytb* sequence data. The mean rate of evolution was 0.017 substitutions per site per million years (95% HPD = 0.013–0.022), and the estimated Yule birthrate was 0.088 (95% HPD = 0.054–0.125). Diversification events within species ranged from 2.71 mya (3.87–1.65 mya) within *K. minuta* to 0.29 mya (0.54–0.08 mya) within *K. intermedia* (Fig. 3).

Morphological variation and species nomenclature.—External, cranial, and dental characters of genetically identified specimens (Table 2; Fig. 4) were studied to assign the available species names. The smallest morphotypes (Fig. 4I: FA = 27–30 mm, C-M3 < 5.2 mm; clades A and B) were compared to morphological descriptions of *K. intermedia*, *K. krauensis*, *K. minuta*, and *K. whiteheadi* (Francis et al. 2007; Hill 1965; Hill and Francis 1984), which occur within the geographic range of our specimens (Chasen 1940; Medway 1969). Clades A and B were assigned to *K. minuta* Miller and *K. intermedia* Hill and Francis, respectively. The medium-sized morphotypes (Fig. 4II: FA = 29–34 mm, C-M3 > 5.2 mm; clades C and D) were compared to morphological descriptions of *K. hardwickii* and *K. pellucida*, the closest in external morphology (Corbet and Hill 1992; Hill 1965; Vanitharani et al. 2003). Clades C and D were assigned to *K. hardwickii* Horsefield and *K. pellucida* (Waterhouse), respectively. The large-sized specimens of *Kerivoula* with domed skulls (Fig. 4III: FA > 38 mm, C-M3 > 6.9 mm; clades E, F, and G) were compared with the descriptions and measurements by Vanitharani et al. (2003) for *K. lenis* and *K. papillosa*. With respect to size, specimens in clade F corresponded to *K. lenis* Thomas, whereas the available names for clades E and G would be *K. p. papillosa* and *K. p. malayana*. Specimens from clade G had the largest size, similar to *K. p. malayana* (Chasen); however, none were collected from within the known distribution of *K. p. malayana* (Peninsular Malaysia). Therefore, we recognized clade E as *K. papillosa*, but no available Linnaean name corresponded to specimens in clade G.

Karyotypes.—We have karyotypic data for 11 specimens representing 5 of the 6 genetically defined clades (excluding *K. pellucida*). Karyotypic data are described in Table 3. Karyotype(s) for each species are presented in Figs. 5 and 6.

Discussion

Phylogenetic analyses of *Cytb* and AFLP data sets resolved 7 monophyletic clades with reciprocal monophyly meriting examination for possible species status (Genetic Species Concept [Baker and Bradley 2006] and Phylogenetic Species Concept [Mishler and Theriot 2000]). Linnaean names have been described primarily based on morphology; however, due to limited resources we were not able to travel to museums to compare our voucher specimens to holotypes, and no genetic data are available for any of those holotypes. Two papers (Francis et al. 2007; Soisook et al. 2007) have used genetic data to examine species diversity within *Kerivoula* based on COI data sets. These authors have access to most of the holotypes and have identified their genetic clades to Linnaean names. We included the COI sequence data from Francis et al. (2007) in our analyses, thus permitting us to verify the interpretation of specific epithets for our clades with specimens of *Kerivoula* from Malaysia to the rest of species

Table 2.—External and cranial and dental measurements of the species of *Kerivoula* identified by our phylogenetic analyses. Number of specimens (by sex; F = female, M = male) are listed beside each species. Superscripts indicate number of individuals actually measured for a particular character if less than the total number of specimens indicated beside each species. All measurements were taken in millimeters (mm), weight in grams (g). Abbreviations follow Vanitharani et al. (2003).

Taxon	Clade	External						Cranial and dental						
		Forearm	Ear	Tragus	Hind foot	Tail	Total	Weight	GTL	CBL	CCL	ZB	C–M3	M3–M3	C–M3	MDL
K. minuta (5F, 8M)	A	26.0–29.8[12]	10–12	7–9[11]	5–7[11]	28–41	63–78[12]	2.0–3.0[12]	10.8–11.7	10.3–11.1	10.1–10.8	7.1–7.9	4.4–4.8	4.5–4.8	4.6–4.9	8.0–8.8
K. intermedia (5F, 2M)	B	28.6–30.0	11–14	6–9[6]	6–7	34–42	68–78	3.1–3.5	12.3–12.7	11.7	11.2–11.3	7.7–8.0	4.8–5.1	5.1–5.2	5.0–5.1	9.0–9.2
K. hardwickii (3F, 6M)	C	29.8–36.0	11–15	8–9[5]	5.7–8.0	39–48	78–89[5]	2.9–3.8[5]	13.1–13.8	12.5–13.4	12.0–13.0	8.2–8.6[3]	5.3–5.6	5.0–5.4	5.4–5.8	9.4–10.5
K. pellucida (3F, 3M)	D	29.0–32.0	14–17	9–11[4]	6–7[4]	45–50	55–90	4.0–5.0	13.5	12.8	12.3	8	5.5	5	5.7	9.8
K. papillosa (1F, 5M)	E	39.5–43.0	12–19	8–12[4]	10[4]	47–53	91–106	7.9–9.2	16.9–17.3	16.2–16.4	15.8–16.1	10.8–11.1	7.1–7.5	6.6–6.9	4.5–7.8	13.2–13.4
K. lenis (2F)	F	38.7–38.9	16	11	9–10	47–53	96–101	6.7–6.9	15.7–16.2	15.2–15.3	14.7–14.8	10.1–10.8	6.9	6.0–6.4	7.3–7.4	12.4–12.7
Kerivoula sp. (3M)	G	45–46.5	15–18	8–10	9.5–10	50[1]	104–111	9–10	17.7–18	16.6–16.9	16.1–16.4	11.2–11.4	7.2–7.5	6.5–6.9	7.8–8.3	12.9–13.3

Fig. 4.—Ventral and lateral view of the skulls arranged according to morphotypes and clades in the phylogenetic analyses. Top row (clade A versus B): I) small morphotypes: *Kerivoula minuta* (TK 152075—A) and *K. intermedia* (TK 152021—B); middle row (clade C versus D): II) medium morphotypes: *K. hardwickii* (TK 152150—C) and *K. pellucida* (TK 152055—D); bottom row (clade E versus F): III) large morphotypes: *K. papillosa* (TK 152061—E) and *K. lenis* (TK 152052—F). Scale bar = 10 mm. Skull of clade G is not shown.

of *Kerivoula* from Southeast Asia. All 7 of our clades were present in their gene tree. Therefore, we identify these clades to the following genetically and morphologically defined species: *K. hardwickii* (clade C), *K. intermedia* (clade B), *K. lenis* (clade F), *K. minuta* (clade A), *K. papillosa* (clade E), *K. pellucida* (clade D), and a potentially undescribed species of *Kerivoula* from Borneo (clade G). Below we discuss the implications of genetic data with respect to morphologically similar species.

Kerivoula intermedia and K. minuta.—Clades A and B were identified as *K. minuta* and *K. intermedia*, respectively (Fig. 2). Unfortunately, no detailed description of *K. minuta* is available for comparison (Hill and Francis 1984; Miller 1898), and the primary character separating *K. intermedia* and *K. minuta* is size. Based on the presence of small ears, dorsal hairs with dark brown bases for almost one-third of their length with light brown tips, short forearm (FA < 30 mm), dark wing membranes and uropatagium, and proximal dorsal half of uropatagium with sparse, long golden brown hairs (Hill and Francis 1984), clade A was assigned to *K. minuta* Miller. Specimens in clade B (*K. intermedia*) exhibited similar

Table 3.—Karyotypic data for the species of *Kerivoula* identified in our study. BO = Borneo; PM = Peninsular Malaysia; 2n = diploid number, FN = fundamental number (number of arms of autosomal complements). Number of specimens karyotyped (by sex; F = female, M = male) are listed beside each species. Definitions for centromere placements used in this table: M = metacentric; SM = submetacentric; A = acrocentric, follows descriptions in ''Karyotypes'' section in ''Materials and Methods''; D = dot-sized or minute size, indistinguishable as either acrocentric or biarmed. Biarm elements include metacentric, submetacentric, and subtelocentric chromosomes. Sex chromosomes are identified as X and Y. — = not available; * = previous karyotype by McBee et al. (1986). Clades A–F are identified in Fig. 2. *K. pellucida* was not karyotyped.

			Chromosome characteristics					
Taxon	Clade	Locality	Pairs of biarms	Pairs of acrocentrics	2n	FN	X	Y
K. minuta (2F)	A	BO	13	0	28	52	M	—
K. intermedia (2F)	B	PM	13	0	28	52	M	—
K. hardwickii (1F)	C	BO	12	0	26	48	M	—
K. hardwickii (1M)	C	BO	11	1	26	46	M	A
K. papillosa (1F, 2M)	E	PM	9	9	38	54	M	D
K. papillosa (1F)	*	Thailand	6	12	38	48	SM	—
K. lenis (1F)	F	BO	8	10	38	52	M	—
K. lenis (1F)	F	PM	9	9	38	54	M	—

external and cranial attributes to those of *K. minuta*, with slightly larger size, wider ears, and lacking the conspicuous banding (dark bases) of dorsal fur. Comparison with *K. krauensis* shows that for almost 90% of its length this species has a conspicuous dark band of hairs with golden bronze tips on the dorsal pelage (Francis et al. 2007). These features were absent in both clade A and B specimens. Additionally, the posterior projection of the palate was longer than wide, with a

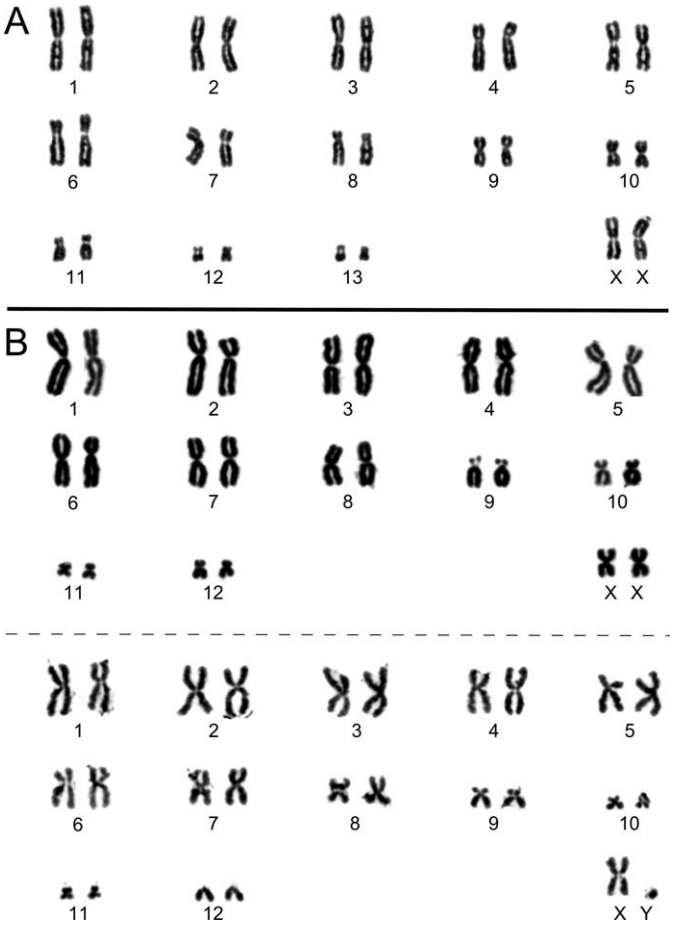

Fig. 5.—Karyotypes of 2 species of *Kerivoula*. A) Karyotype of *K. intermedia* (TK 152019; female; 2n = 28, FN = 52). This karyotype was identical to that of *K. minuta*. B) Karyotypes of *K. hardwickii* (top: TK 152097; female; 2n = 26, FN = 48; and bottom: TK 152115; male; 2n = 26, FN = 46).

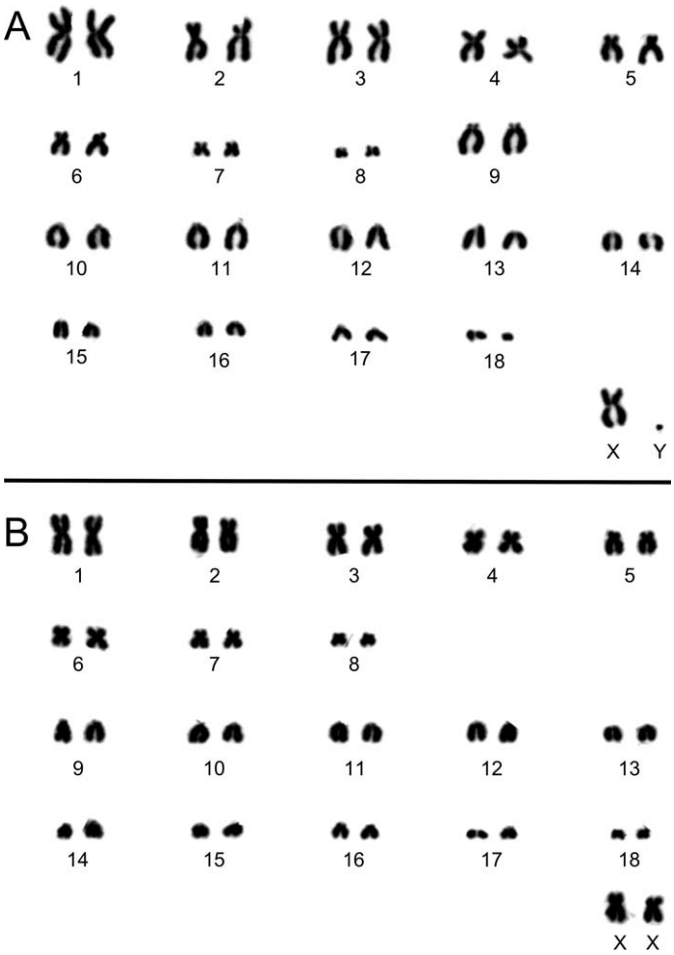

Fig. 6.—Karyotypes of 2 species of *Kerivoula*. A) *K. papillosa* (TK 152061; male; 2n = 38, FN = 54). This karyotype was identical to that of *K. lenis* of Peninsular Malaysia. B) *K. lenis* from Borneo (TK 152178; female; 2n = 38, FN = 52).

distal projection in *K. intermedia* (Fig. 4IB) that was lacking in our specimens of *K. minuta*. The utility of these characters to identify these species in the field and in natural history collections merits exploration. Initially, we assigned specimens from clade B as *K. whiteheadi* (following Corbet and Hill 1992) based on the presence of an elongate P2, which is not present in *K. minuta* (Fig. 4I, clade A). However, comparison with the skull morphology of the holotype indicated that *K. whiteheadi* has a unique elongated muzzle, with premolars that are oval in cross section and nearly twice as long as they are wide, which was not obvious in our specimens (C. M. Francis, Canadian Wildlife Service, pers. comm.).

Previous studies have indicated that *K. intermedia* is distributed widely throughout Borneo (Payne and Francis 1985) and that *K. minuta* is rare (Jayaraj et al. 2006). However, these conclusions need to be verified using genetic sequence data because these species overlap in morphological size and are indistinguishable in their karyotypes (Fig. 5A: 2n = 28, FN = 52). Genetically identified *K. intermedia* and *K. minuta* have been collected sympatrically from both Borneo and Peninsular Malaysia (Lanjak Entimau Wildlife Sanctuary and Krau Wildlife Reserve—Kingston et al. 2006; Appendix I), indicating that these taxa have a widely sympatric distribution. Thus, *K. minuta* seems to have a greater distribution than previously identified, an observation that is likely attributed to misidentification as *K. intermedia*. In studies of *K. intermedia* and *K. minuta* (Kingston et al. 1999), differences in echolocation calls were identified, and we hypothesize that these differences may reduce competition between the 2.

Kerivoula hardwickii and K. pellucida.—Clades C and D were identified as *K. hardwickii* and *K. pellucida*, respectively (Fig. 2). Characters separating these clades included a more globular braincase rising abruptly from the rostrum, a narrow extension of the posterior palate in specimens of clade D (as opposed to a less globular and lower braincase, with a wider posterior palate extension in specimens of clade C; Fig. 4II), and a glandular swelling in the terminal third portion of the tail (Hill 1965; Waterhouse 1845). In addition, a wider bulla (2.8 versus 2.5 mm) and a larger basal area of P2 in *K. hardwickii* were observed when compared to *K. pellucida*. The most obvious characters discriminating these 2 species was pelage color of pale orange to orange-brown with pale bases in *K. pellucida*, and gray or gray-brown with dark bases in *K. hardwickii*, and ear shape and length (longer and narrower ears in *K. pellucida*) as defined in Payne and Francis (1985). External characters separating these species may be applicable in the field.

We also compared our specimens of *K. hardwickii* to the original description of *K. depressa* Miller (listed as a synonym of *K. hardwickii* by Hill [1965]), which may be a valid species (Bates et al. 2007). Analysis of the braincase height (BH) allowed for separation of *K. hardwickii* from *K. depressa*, because our specimen has a larger BH (5.75–5.90 mm) relative to that present in the holotype of *K. depressa* (BH = 5.5 mm). Difficulties have arisen in the specific classification of specimens of *K. pellucida* and *K. hardwickii* throughout the geographic distributions of the 2. For example, Bornean representatives of *K. pellucida* (referred to as *K. bombifrons* Lyon—Lyon 1911), seem almost indistinguishable from typical *K. pellucida* in size and morphology (Hill 1965), and multiple forms of *K. hardwickii* and possible polyphyly were described by Miller (1906), Hill (1965), and Francis et al. (2007). However, in our study, we documented only a single form of *K. hardwickii*, and this genotype also occurs in Thailand, Laos, and Vietnam. This observation was based on comparison with the COI gene tree (deposited by researchers in the Barcode of Life Database) of Francis et al. (2007) and was further reinforced in our chromosomal analysis. Specimens of *K. hardwickii* (TK 152097, TK 152115) from the same locality in Borneo have a diploid number of 26 but have different fundamental numbers, 48 and 46 (Fig. 5B), respectively. In the karyotype TK 152097 (Fig. 5B) chromosome pair 12 is distinctly biarmed, whereas in TK 152115 (Fig. 5B) this pair is acrocentric with a small 2nd arm. Individuals with different karyotypes had similar mtDNA haplotypes, thus indicating a chromosomal polymorphism but probably not cryptic species.

Kerivoula lenis and K. papillosa.—Clades E and F were identified as *K. papillosa* and *K. lenis*, respectively (Fig. 2). As identified by *Cytb*, COI, and AFLPs, both *K. papillosa* and *K. lenis* are present in Peninsular Malaysia and Borneo. *K. lenis* and *K. papillosa* were sister to each other in both the *Cytb* and AFLP trees. Size (Table 2) discriminated *K. lenis* and *K. papillosa*, with *K. lenis* being the smaller form (Corbet and Hill 1992; Vanitharani et al. 2003). Therefore, we recognized clade F as *K. lenis*. The remaining clades (E and G) include specimens that are similar to the body-size and skull profile of *K. papillosa papillosa* (type locality Borneo) and *K. papillosa malayana* (type locality on the Selangor–Pahang boundary), respectively. According to a review by Chasen (1940), *K. p. malayana* ([GTL] = 18.3–18.4 mm) is larger than *K. p. papillosa* (GTL $\leq$ 17 mm). Therefore we recognize clade E (FA = 38–43 mm, GTL = 16.9–17.3 mm) as *K. p. papillosa*; however, the taxonomic status of clade G (composed of individuals with the largest phenotype in Malaysia) is problematic. This is because members of clade G might represent an undescribed species or be *K. p. malayana* (due to overlap in GTL; Table 2). However, all specimens from clade G were collected from outside of the known distribution of *K. p. malayana*. Thus we provisionally identify members within clade G as representing an undescribed species pending genetic data from *K. p. malayana*.

The karyotypic variation in *K. papillosa* and *K. lenis* is more complex. Mainland Malaysian populations of *K. papillosa* and *K. lenis* have indistinguishable karyotypes (Fig. 6A; 2n = 38, FN = 54), and our specimens of *K. papillosa* from Borneo also have this same karyotype. However, when compared to our mainland specimens (Fig. 6A), our Bornean specimen of *K. lenis* (Fig. 6B; 2n = 38, FN = 52) has a unique karyotype resulting from chromosome pair 9 being acrocentric.

McBee et al. (1986) reported another karyotype from a specimen of *K. papillosa* collected from Thailand (2n = 38, FN = 48). McBee et al. (1986) included the X chromosome in the fundamental number, whereas we removed the score from the sex chromosome for a consistent calculation of the fundamental number. After comparisons of measurements of *K. lenis* and *K. papillosa*, the specimen of McBee et al. (1986—CM 88164, FA = 43.4 mm) is more closely related to *K. lenis* in terms of size and skull morphology. If the specimen of McBee et al. (1986) is *K. lenis*, the karyotype would be new for *K. lenis*.

In our gene tree 2 monophyletic clades provide critical data to understanding the biodiversity of Southeast Asian *Kerivoula*. First, what is identified as *K. lenis* in Malaysia (clade F; Fig. 2) is different from what is called *K. lenis* in Laos (Fig. 2, *K.* cf. *lenis* AJ841970). Second, the status of specimens from clade G needs to be verified relative to *K. p. malayana*. Clearly C- and G-band studies and chromosomal painting would be valuable in understanding karyotypic evolution in this complex. It is not possible to relate our genetic data to the review of call parameters reported by Kingston et al. (1999), but the variation in echolocation calls they detected could be beneficial in understanding the evolutionary history of this group.

Phylogenetic relationships and differences between nuclear and mitochondrial data.—The genetic divergence values among congeneric lineages in *Kerivoula* identified in both *Cytb* and COI sequence data are greater than is typical of congeneric species in other bats. This observation was interpreted by Francis et al. (2007) and Soisook et al. (2007) as evidence that the COI gene was evolving faster in *Kerivoula* than in most other bats. Because of saturation and convergence, especially at the 3rd position of codons, fast-evolving genes might not provide accurate resolution to determine phylogenetic branching orders in this genus. Another explanation of large divergence values is that the lineages could have separated from each other at an earlier geologic time than is typical for extant vespertilionid congeneric species. In either case, we agree that saturation is a problem. Comparing *Cytb* and COI genetic divergence values (Table 1) suggests that higher genetic divergence values with slightly faster transitional saturation (19.0%) are present in COI sequences when compared to those in *Cytb* sequences, meaning that the COI gene will reach saturation faster than the *Cytb* gene for similar taxon pairwise comparisons. Nonetheless, our species-level identifications based on both mitochondrial genes were congruent. Furthermore the topology of the *Cytb* tree is similar to the nuclear AFLP tree, thus increasing the probability that our gene tree is concordant with the species tree (Edwards 2009).

Consistencies in nuclear and mtDNA phylogenetic trees include *K. pellucida* being basal to the branching order of the remaining *Kerivoula* in our sample; and within the large-sized *Kerivoula* (clades E, F, and G), the potentially undescribed species being basal to the sister species *K. lenis* and *K. papillosa*. Inconsistencies involve clades A, B, and C, because *K. intermedia* is basal in the *Cytb* gene tree but is sister to *K. minuta* in the AFLP tree. *K. hardwickii* is basal in the AFLP tree but sister to *K. minuta* in the *Cytb* gene tree. However, this inconsistency is not supported statistically in both trees, and therefore no conflict exists.

Geographic structure in genetic data.—Phylogroups (described as genetically defined clades with geographic structure) have been documented in many vertebrate species (Avise and Walker 1999), including mammals (Baker and Bradley 2006; da Silva and Patton 1998). In our samples of Malaysian *Kerivoula* 6 of the 7 species were present in both Borneo and Peninsular Malaysia. The South China Sea could be a geographic barrier that resulted in allopatric populations (Francis et al. 2007) distinguishable by levels of genetic divergence between Peninsular Malaysia and Borneo. Phylogroups within *K. intermedia*, *K. minuta*, and *K. papillosa* reflect this separation (Fig. 2). However, an absence of phylogeographic structuring within our sample of *K. pellucida* (Fig. 2) indicates a recent common ancestor of Peninsular Malaysian and Bornean populations of this species. Within our sample of *K. hardwickii* are phylogroups separated by a genetic distance value of 5.7% in *Cytb* gene sequence variation, indicating that a possible speciation event has occurred in this species (Baker and Bradley 2006). However, these distinct phylogroups are found within Borneo, indicating potential (perhaps ancient) population fragmentation within Borneo itself.

The results of this study have 2 broad implications for understanding the paleoenvironments across the Sunda shelf. First, the presence of statistically supported, monophyletic lineages associated with geographic regions within our sample of *Kerivoula* refutes the hypothesis that Pleistocene land bridges would have allowed periodic migrations between Peninsular Malaysia and Borneo until ~9,500 years ago (Inger and Voris 2001; Voris 2000). Relaxed molecular clock analyses indicated that intraspecific diversification dates of several species of *Kerivoula* predate the most recent land bridge connection between Peninsular Malaysia and Borneo (Fig. 3). Mean diversification dates for *K. intermedia*, *K. lenis*, *K. minuta*, *K. papillosa*, and *K. pellucida* were all within the late Pliocene to early Pleistocene epochs (2.7–0.3 mya). Concordance among multiple data sets (i.e., mtDNA and nuclear AFLPs) lessens concerns of incomplete lineage sorting, thus the intraspecific patterns identified herein are consistent with ancient vicariant events. This hypothesis is reinforced by similar patterns within multiple taxonomic groups studied throughout this region, including mammals (e.g., gymnures [Ruedi and Fumagalli 1996] and rodents [Gorog et al. 2004]) and other taxa (e.g., snakes and frogs [Inger and Voris 2001] and termites [Gathorne-Hardy et al. 2002]). Furthermore, the data presented herein may provide a greater understanding of pre-Pleistocene speciation events across Southeast Asia, specifically as a result of isolation during inundations of the Isthmus of Kra (the region connecting mainland Southeast Asia and the Malaysian peninsula, ~10° latitude in Fig. 1). Our estimations (within the associated 95% HPD) for the time of origin for 2 species

of *Kerivoula* collected in Laos (*K.* cf. *lenis* ~4.76 mya and *K. kachinensis* ~8.28 mya; Fig. 3) coincide with the flooding of the Isthmus of Kra during the late Miocene and early Pliocene (Woodruff 2003) and suggest periods of isolation that could have facilitated speciation events in this region.

Second, species of *Kerivoula* are interior forest-dwelling bats that forage in cluttered environments (Kingston et al. 2003). Knowing this, the forest expansion and contraction events across the Sunda shelf during the last 3 million years might have fragmented northeastern Bornean (Sabah) populations before Peninsular Malaysian and southwestern Bornean populations (Heaney 1991; Verstappen 1997; Whitmore 1981). The diversification events within Bornean populations of *K. minuta* and *K. hardwickii* are consistent with this hypothesis in that they predate divergences between Peninsular Malaysian and Bornean populations. Examination of the data indicates the presence of ancient, isolated refugia within eastern Borneo approximately 2.5 mya (Fig. 3). This hypothesis is consistent with other faunal studies (Gathorne-Hardy et al. 2002; Gorog et al. 2004), which suggest that speciation events for multiple taxonomic groups within Sundaland might have resulted from climate and vegetational change during both the Pliocene and Pleistocene epochs (Meijaard 2003; Verstappen 1997; Whitmore 1981).

Knowledge of the diversification of Southeast Asian species of *Kerivoula* would be greatly enhanced with additional data (karyotypic, morphological, and DNA sequence) from specimens throughout the Indochinese region, the Philippines (e.g., Luzon, Mindanao, and Palawan), and Indonesia (e.g., Bali, Java, Sulawesi, and Sumatra). Such data will be critical to test hypotheses of ancient forest connections throughout this region as described in Inger and Voris (2001). We posit that the genus *Kerivoula* is an appropriate model system to examine the processes underlying speciation events in Southeast Asia.

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Appendix I

Taxa, geographic localities, tissue numbers, and GenBank accession numbers of cytochrome-*b* (*Cytb*) and cytochrome *c* oxidase (COI) data used for phylogenetic analysis. TK = tissue numbers at Natural Science Research Laboratory, Museum of Texas Tech University; UNIMAS = tissue numbers at Zoology Museum, Universiti Malaysia Sarawak; and DWNP = specimen numbers at Department of Wildlife and National Parks, Malaysia. NA = not available.

Taxa	Locality	Tissue no.	GenBank no.	
			Cytb	COI
K. hardwickii[a,b]	Monggis Sub Station, Sabah, Malaysian Borneo	TK 152080	EU188768	GU585631
K. hardwickii[a,b,c]	Monggis Sub Station, Sabah, Malaysian Borneo	TK 152097	EU188769	GU585629
K. hardwickii[a,b,c,d]	Monggis Sub Station, Sabah, Malaysian Borneo	TK 152115	EU188770	GU585630
K. hardwickii[a,b]	Bako National Park, Sarawak, Malaysian Borneo	TK 152150	EU188771	NA
K. hardwickii[e]	UNIMAS, Samarahan, Sarawak, Malaysian Borneo	TK 152407	GU585657	NA
K. hardwickii[a,d,e]	UNIMAS, Samarahan, Sarawak, Malaysian Borneo	TK 152409	GU585656	NA
K. hardwickii[e]	UNIMAS, Samarahan, Sarawak, Malaysian Borneo	TK 152410	GU585655	NA
K. hardwickii[e]	Bukit Aub, Sibu, Sarawak, Malaysia Borneo	TK 153568	GU585640	GU585635
K. hardwickii[e,f]	Langkawi Island, Kedah, Peninsular Malaysia	DWNP 1399	GU585665	GU585636
K. hardwickii[e]	Langkawi Island, Kedah, Peninsular Malaysia	DWNP 1409	NA	GU585637
K. hardwickii[e,f]	Lembar Krau, Krau, Pahang. Peninsular Malaysia	DWNP 3966	GU585666	NA
K. intermedia[a,c,e]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152019	EU188789	GU585621
K. intermedia[a,b]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152021	EU188790	GU585599
K. intermedia[a,b,c,d]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152060	EU188791	GU585600
K. intermedia[d,e]	Lanjak Entimau Wildlife Sanctuary, Sarawak, Malaysian Borneo	TK 153609	GU585658	GU585598
K. intermedia[e]	Lanjak Entimau Wildlife Sanctuary, Sarawak, Malaysian Borneo	TK 153619	GU585653	GU585597
K. intermedia[a,e]	Lanjak Entimau Wildlife Sanctuary, Sarawak, Malaysian Borneo	TK 153621	GU585649	GU585601
K. intermedia[a,e]	Lanjak Entimau Wildlife Sanctuary, Sarawak, Malaysian Borneo	TK 153638	GU585652	GU585602
K. lenis[a,b,c,d]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152052	EU188772	GU585595
K. lenis[a,b,c,d]	Kubah National Park, Sarawak, Malaysian Borneo	TK 152178	EU188773	GU585596
K. minuta[a,b]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152022	EU188774	GU585607
K. minuta[a,b,c]	Monggis Sub Station, Sabah, Malaysian Borneo	TK 152075	EU188775	GU585593
K. minuta[a,b,d]	Monggis Sub Station, Sabah, Malaysian Borneo	TK 152079	EU188776	GU585594
K. minuta[a,b]	Kubah National Park, Sarawak, Malaysian Borneo	TK 152194	EU188777	GU585605
K. minuta[a,b,c,d]	Mount Penrisen, Sarawak, Malaysian Borneo	TK 152206	EU188778	GU585603
K. minuta[a,b]	Mount Penrisen, Sarawak, Malaysian Borneo	TK 152249	EU188779	GU585604
K. minuta[e]	Kuala Atok, Taman Negara, Pahang, Peninsular Malaysia	TK 152995	GU585641	GU585612
K. minuta[d,e]	Kuala Atok, Taman Negara, Pahang, Peninsular Malaysia	TK 152996	GU585646	GU585608
K. minuta[e]	Kuala Atok, Taman Negara, Pahang, Peninsular Malaysia	TK 152999	GU585645	GU585613
K. minuta[e]	Kuala Atok, Taman Negara, Pahang, Peninsular Malaysia	TK 153564	GU585643	GU585606
K. minuta[d,e]	Bukit Aub, Sibu, Sarawak, Malaysia Borneo	TK 153567	GU585644	GU585610
K. minuta[e]	Bukit Aub, Sibu, Sarawak, Malaysia Borneo	TK 153588	GU585642	GU585611
K. minuta[d,e]	Lanjak Entimau Wildlife Sanctuary, Sarawak, Malaysian Borneo	TK 153663	GU585659	GU585609
K. minuta[a,e]	Lanjak Entimau Wildlife Sanctuary, Sarawak, Malaysian Borneo	TK 153668	GU585660	GU585592
K. minuta[e]	Taman Negara, Pahang, Peninsular Malaysia	DWNP 2132	NA	GU585638
K. papillosa[a,c,d,e]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152020	EU188782	GU585632
K. papillosa[a,b,c]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152061	EU188784	GU585618
K. papillosa[a,b,c]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152062	EU188785	GU585622
K. papillosa[a,b]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152023	EU188783	GU585619
K. papillosa[a,d,e]	Similajau National Park, Sarawak, Malaysian Borneo	UNIMAS 00561	EU188780	GU585624
K. papillosa[a,e]	Similajau National Park, Sarawak, Malaysian Borneo	UNIMAS 00560	EU188781	GU585623
K. papillosa[a,e]	Niah National Park, Sarawak, Malaysian Borneo	TK 152403	GU585663	GU585625
K. papillosa[e]	Kuala Atok, Taman Negara, Pahang, Peninsular Malaysia	TK 152994	GU585664	GU585620
K. pellucida[a,b]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	TK 152055	EU188788	GU585633
K. pellucida[a,d,e]	Similajau National Park, Sarawak, Malaysian Borneo	UNIMAS 00554	EU188786	GU585617
K. pellucida[a,e]	Pahang National Park, Pahang, Peninsular Malaysia	UNIMAS 01095	EU188787	NA
K. pellucida[a,d]	Niah National Park, Sarawak, Malaysian Borneo	TK 152398	GU585661	GU585616
K. pellucida[e]	Kuala Atok, Taman Negara, Pahang, Peninsular Malaysia	TK 153552	GU585647	GU585614
K. pellucida[e]	Kuala Atok, Taman Negara, Pahang, Peninsular Malaysia	TK 153560	GU585648	GU585615
K. pellucida[a,e]	Kuala Atok, Taman Negara, Pahang, Peninsular Malaysia	TK 153563	GU585662	GU585634
K. pellucida[e]	Krau Wildlife Reserve, Pahang, Peninsular Malaysia	DWNP 3912	NA	GU585639
Kerivoula sp.[a,b,d]	Niah National Park, Sarawak, Malaysian Borneo	TK 156001	GU585651	GU585627
Kerivoula sp.[a,b]	Niah National Park, Sarawak, Malaysian Borneo	TK 156004	GU585650	GU585628
Kerivoula sp.[b]	Niah National Park, Sarawak, Malaysian Borneo	TK 156036	GU585654	GU585626

[a] Amplified fragment length polymorphism genotyped.
[b] External and cranial measurements taken.
[c] Karyotyped.
[d] Used in BEAST analysis.
[e] External measurements taken.
[f] Partial *Cytb* sequence of 428 bp.