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Craseonycteris thonglongyai (Chiroptera: Craseonycteridae) is a rhinolophoid: molecular evidence from cytochrome *b*

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Craseonycteris thonglongyai (Chiroptera: Craseonycteridae), an enigmatic taxon which shares morphological traits with both Rhinopomatidae and Emballonuridae was for the first time investigated with the aid of molecular phylogenetic techniques. Three methods of phylogenetic inference, parsimony, maximum-likelihood, and Bayesian phylogenetics were used. Based on 402 bp of DNA sequence from the mitochondrial cytochrome *b* gene, placement of Craseonycteridae within the superfamily Rhinolophoidea was demonstrated. Our results also suggest close proximity of Craseonycteridae to Hipposideridae rather than to Rhinopomatidae, close relationships between Megadermatidae and Rhinolophidae, sister group position of Pteropodidae to Rhinolophoidea, and closer affiliation of Nycteridae with the infraorder Yangochiroptera. Spectral analysis was in agreement with all these outcomes except for closer relationships of *Craseonycteris* with Rhinopomatidae.

Key words: *Craseonycteris*, Craseonycteridae, cytochrome *b*, phylogeny, Rhinolophoidea

INTRODUCTION

Craseonycteris thonglongyai Hill, 1974 is the only species in the genus *Craseonycteris* Hill, 1974 and the family Craseonycteridae Hill, 1974. This taxon was discovered in 1974 at Sai Yoke, Kanchanaburi Province, western Thailand (Hill, 1974; Lekagul and McNeely, 1977) and only recently it has been also recorded in SE Myanmar (Bates *et al*., 2001). The species lives in small colonies and preys on insects and spiders near the tops of bamboo trees using multiharmonic CF echolocation calls with high source level and high repetition rate (Surlykke *et al*., 1993). With a body mass of about 2 g, head and body length of 29–33 mm, and forearm length of 22–26 mm, it is considered, together with *Suncus etruscus*, to be the smallest mammal in the world. Further distinctive characters include the lack of a tail (though there are two caudal vertebrae) and calcar, the presence of a large interfemoral membrane, large-sized auricles with swollen tragi and a prominent glandular swelling at the base of the throat in males. Nostrils are slit-like and vertical, resembling that of the hog (Nowak, 1997). Wings are long and broad, similar in their phalangeal pattern to those in Nycteridae, Megadermatidae, Rhinolophidae and Hipposideridae (Hill and Smith, 1981). The humerus is characterized, similar to some hipposiderids, by extremely tapered trochiter suggesting a perfect scapulo-humeral lock, and by broad distal epiphysis with a distinct processus spinosus. There are well marked fusions in thoracic and lumbar

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vertebrae, and the particularly delicate pelvis has no pubic prominences. The skull is inflated, with prominent sagittal crest and enlarged bullae, but lacking lambdoidal crests, postorbital processes, and supraoccipital ridges. A unique character of *Craseonycteris* is the shape of premaxillae, which are not fused with the maxillae but forms a ring-like structure along the narial aperture. The dental formula is 1/2:1/1:1/2:3/3 = 28. Maxillar molars are broad with large talons resembling the state found in rhinopomatids. Mandibular molars are nyctalodont with slender walls and spacious talonids.

Craseonycteris thonglongyai is an enigmatic bat not only for its rarity but also for uncertainties concerning its past history (no fossils are available) and phylogenetic relationships. Hill (1974) and Corbet and Hill (1992) stressed that it exhibits 'some features tending towards the Rhinopomatidae and the Emballonuridae but does not appear to be intermediate between these families, but rather to derive from a dichotomy of the rhinopomatid-emballonurid stock, sharing some features of each family but considerably derived in others'. With Rhinopomatidae it shares the same dental formula, general design of skeletal, laryngeal, and skull morphology (except for specific arrangement of premaxillae) but differs in the shape of nostrils, large mutually separated auricles not joined anteriorly, in the tragus design (widest in the middle), by lacking a tail, more inflated braincase and relatively larger incisors. Characters shared with Emballonuridae include some traits of skeletal morphology and the arrangement of premaxillae that are not fused to maxillae but form a complete ring around the nasal opening. There are also differences in several other cranial, dental and external characters, including the lack of tail (see Hill and Smith, 1981 for details).

Koopman (1993, 1994) placed *Craseonycteris* in the infraorder Yinochiroptera Koopman, 1975 and superfamily Emballonuroidea Gervais, 1855 together with Rhinopomatidae and Emballonuridae. Simmons (1998) and Simmons and Geisler (1998) who scored *Craseonycteris* for 104 characters (i.e., just half those they investigated) classified it as a member of the superfamily Rhinolophoidea and a sister taxon to Rhinopomatidae, a view also adopted by Jones *et al*. (2002). On the other hand, *Craseonycteris* was omitted in the recent revision of superfamilial classification of bats (e.g., Teeling *et al*., 2000, 2002) because of the lack of any molecular information. As the first step to fill this gap we report the results of DNA sequence analysis from 402 bp of the 5' end of the cytochrome *b* gene, and these data were used to evaluate alternative hypotheses regarding the phylogenetic position of Craseonycteridae.

Materials and Methods

Tissue samples of *Craseonycteris* (pectoral muscle and chiropatagium) were obtained from a male specimen found dead at the type locality, Sai Yok Cave, Thailand in March 2002 by Mr. V. Káňa and J. Bajer. Additional material was obtained from three other bat species: *Rhinopoma hardwickei* (Jordan, coll. P. Benda), *Megaderma spasma* (Borobudur, central Java, coll. I. Horáček), and *Emballonura monticola* (Gon-Solok, E-Kalimantan, coll. I. Horáček). Tissue samples and a voucher specimen are preserved in ethanol and deposited in the collections of the Department of Zoology, Charles University, Prague, and the National Museum, Prague, respectively. Genomic DNA was extracted according to the method of Hoelzel and Green (1992). PCR was performed with 20 µl volumes containing 1x Taq buffer (Promega), 1mM $MgCl_2$, 200 µM each dNTP, 0.5 µM primers (MVZ04 and MVZ05, amplified 402 bp of the 5' part of cytochrome *b* from a wide range of taxa — Smith and Patton, 1991), 1u of Taq polymerase (Promega) and 100 ng of genomic DNA. Conditions for amplification were as described in Kennedy *et al*. (1999) with an annealing temperature of 45°C. Amplified fragments were isolated from agarose gel using

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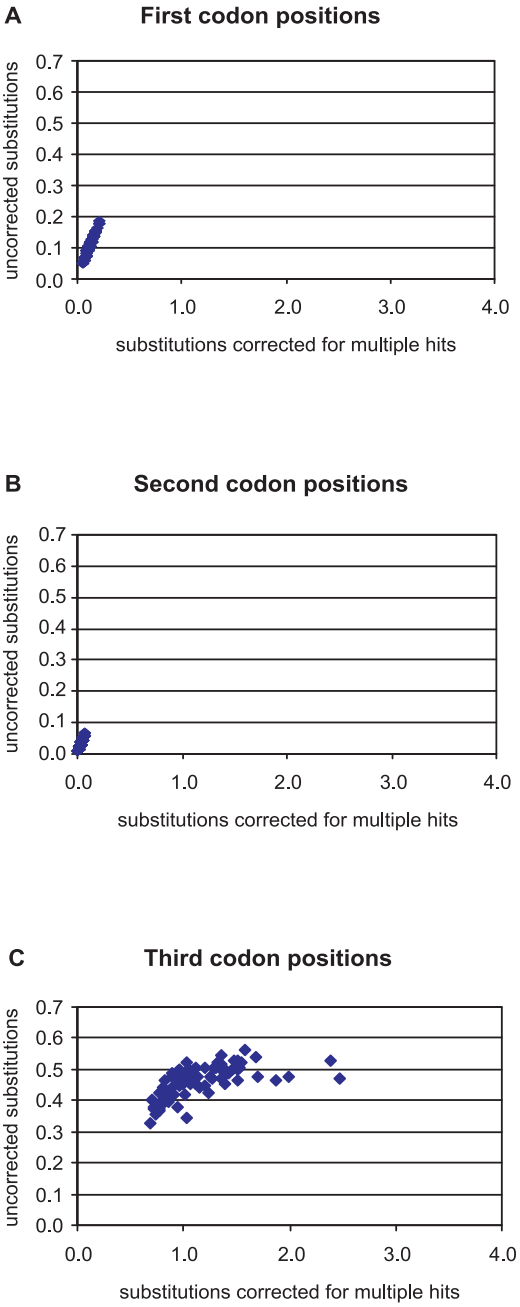


FIG. 1. Graphical plots of pairwise substitutions without correction for multiple hits (*p*-distances) against pairwise substitution corrected for multiple hits (HKY85) for first (A), second (B) and third (C) codon positions

QIAquick Gel Extraction Kit and sequenced using the ABI PRISM Terminator kit (with the same primers as PCR) and automated DNA sequencer (PE310). If possible, genomic DNA of at least two individuals of each species was used to reduce the possibility of contamination and both strands were sequenced in each sample to verify accuracy. Chromatograms were edited with Chromas (McCarthy, 1996) and contig assembly, if necessary, was performed using CAP (Huang, 1992). Sequences were submitted to GenBank (NCBI) and the following accession numbers were obtained (in parentheses): *Craseonycteris thonglongyai* (AF512008), *Rhinopoma hardwickei* (AY056462), *Megaderma spasma* (AY057942), *Emballonura monticola* (AY057946). The following accession numbers of sequences were retrieved from GenBank: *Saccopteryx bilineata* (AF044664), *Nycteris thebaica* (AF044653), *Hipposideros bicolor* (AF358131), *Rhinolophus hipposideros* (AF044660), *Molossus molossus* (L19724), *Myotis myotis* (AF246241), *Eptesicus serotinus* (AF376837), *Rousettus leschenaulti* (AF044662), *Epomophorus wahlbergi* (AF044642), *Macroglossus minimus* (AF044646), *Sorex ornatus* (AF238035).

Sequence Analysis

Sequences were aligned by the program Clustal W version 1.8 (Thompson *et al*., 1994), checked in MEGA version 2.1 (Kumar *et al*., 2001), and phylogenetic analyses were performed with PAUP* 4.0b10 (Swofford, 1993). We used three different approaches to evaluate whether the molecular sequences contained phylogenetic information (Xia *et al*., in litt.): PTP-test (1,000 replicates — Faith, 1991; Faith and Cranston, 1991), standard g_1 statistics for measuring the skewness of tree lengths of alternative trees (Hillis and Huelsenbeck, 1992) and plots of pairwise substitutions uncorrected for multiple substitutions versus corrected values. Saturation tests were plotted for the first, second and third codon position with *p*-distance and HKY85 model of sequence evolution (Fig. 1). Other distance correction models, including GTR gave similar results. Because choosing the outgroup for Chiroptera is still a subject of controversy, we provisionally accepted Eulipotyphla as possibly the nearest recent relative to bats (Murphy *et al*., 2001*a*, 2001*b*; Nikaido *et al*., 2001), and selected the genus *Sorex* for that purpose. Preliminary outgroup sensitivity analyses were also performed with *Echinosorex* and *Erinaceus*, and without the outgroup. We performed distance analyses to obtain preliminary information regarding our data by computing uncorrected (*p*-values) and corrected (HKY85) distances among all analyzed taxa (Table 1). The initial phylogenetic hypothesis was constructed using maximum parsimony (MP) – heuristic search, both with

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TABLE 1. Pairwise comparisons of sequence divergence among all taxa (uncorrected *p*-values – lower triangular matrix, corrected HKY85 distances – upper triangular matrix)

Species	Species														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 *Rhinopoma hardwickei*		0.2432	0.3074	0.1825	0.2290	0.2064	0.1916	0.1717	0.2124	0.2520	0.2326	0.2151	0.2194	0.2051	0.3087
2 *Saccopteryx bilineata*	0.2065		0.2451	0.2431	0.2450	0.2402	0.2342	0.2423	0.2324	0.2638	0.2638	0.2644	0.2553	0.2699	0.3123
3 *Emballonura monticola*	0.2488	0.2040		0.2824	0.2806	0.3018	0.2816	0.2624	0.2742	0.2761	0.2510	0.2890	0.2550	0.2884	0.3178
4 *Craseonycteris thonglongyai*	0.1592	0.2065	0.2313		0.2445	0.2073	0.2032	0.1752	0.2035	0.2377	0.2347	0.2300	0.2208	0.2341	0.3115
5 *Nycteris thebaica*	0.1940	0.2065	0.2313	0.2065		0.2536	0.2527	0.2169	0.2340	0.2753	0.2395	0.2395	0.2636	0.2418	0.2824
6 *Megaderma spasma*	0.1766	0.2015	0.2438	0.1791	0.2114		0.1654	0.1815	0.2366	0.2766	0.2458	0.2271	0.2175	0.2526	0.3305
7 *Rhinolophus hipposideros*	0.1667	0.1990	0.2313	0.1766	0.2114	0.1468		0.1791	0.2188	0.2917	0.2317	0.2328	0.2601	0.2702	0.2875
8 *Hipposideros bicolor*	0.1493	0.2040	0.2189	0.1542	0.1866	0.1592	0.1567		0.1880	0.2465	0.2474	0.2065	0.2010	0.2079	0.2684
9 *Aselliscus stoliczkanus*	0.1816	0.1990	0.2264	0.1766	0.1990	0.1990	0.1866	0.1617		0.2484	0.2492	0.2412	0.2272	0.2499	0.3267
10 *Molossus molossus*	0.2114	0.2189	0.2264	0.2015	0.2264	0.2264	0.2363	0.2065	0.2090		0.2213	0.2767	0.2515	0.2576	0.3336
11 *Myotis myotis*	0.1965	0.2189	0.2114	0.1990	0.2015	0.2065	0.1965	0.2065	0.2090	0.1891		0.2547	0.2604	0.2576	0.2826
12 *Rousettus leschenaulti*	0.1841	0.2214	0.2363	0.1965	0.2015	0.1940	0.1990	0.1791	0.2040	0.2289	0.2139		0.1520	0.2298	0.2885
13 *Epomophorus wahlbergi*	0.1866	0.2139	0.2139	0.1891	0.2189	0.1866	0.2164	0.1741	0.1940	0.2114	0.2164	0.1343		0.1829	0.2924
14 *Macroglossus minimus*	0.1766	0.2239	0.2363	0.1990	0.2040	0.2114	0.2239	0.1791	0.2090	0.2139	0.2139	0.1940	0.1592		0.3018
15 *Sorex*	0.2488	0.2512	0.2562	0.2512	0.2313	0.2637	0.2363	0.2239	0.2612	0.2637	0.2313	0.2363	0.2388	0.2438	

unweighted and weighted codon positions, 100 random addition sequences and tree bisection reconnection (TBR) branch swapping algorithm (Fig. 2). Weighting factors of 4:14:1 were computed as the inverse rate of the total number of differences in the mutation rate observed in each position in a pairwise comparison of all taxa standardized against the third position (Sudman *et al*., 1994; Kennedy *et al*., 1999). Transversions were weighted 2:1 over transitions (as suggested by exploratory maximum likelihood analyses). Support for the resultant phylogenetic hypothesis was tested via bootstrap (1,000 iterations). Maximum likelihood (ML) tree was also calculated with GTR + Γ model of sequence evolution (Fig. 3). The model and its parameters (R-matrix, base frequencies, and shape parameter of γ-distribution) were estimated using Modeltest 3.06 (Posada, 1998). The MrBayes 2.01 program (Huelsenbeck and Ronquist, 2001) served to build a tree following the rules of Bayesian phylogenetics and to compute posterior probabilities for each clade (Fig. 4). Spectral analysis (Hendy and Penny, 1993) was performed with the aid of Spectrum (Charleston, 1998). The spectrum was calculated from the matrix of Hamming distances among sampled sequences. The advantage of spectral analysis is that it emphasizes the importance of exploring the data and can help uncover patterns that might otherwise be missed (Page and Holmes, 2000). The best nontrivial splits show common characters, indicating possible phylogenetic relationships and offering good way to test alternative hypotheses. The Kishino-Hasegawa test (Kishino and Hasegawa, 1989) was omitted from this analysis because it is not useful for trees that are specified a priori (Goldman *et al*., 2000).

Alternative positions of Craseonycteridae were tested by evaluating support values of grouping with other taxa. Relative rates tests (2-dimensional method of Tajima, 1993) were performed with *Sorex* as an outgroup for all taxa. A molecular clock (Margoliash, 1963) was applied to estimate the time of divergence events, with the calibration based on HKY85 distances among taxa and on the fossil record surveyed below. Average distance between clades was divided by the time of divergence known from the fossil record in million years (Myr.). Half this value was used as the rate of evolutionary change per Myr. per lineage. Three calibration records were alternatively applied: (1) divergence of *Myotis* and *Eptesicus*: ca. 32 Myr. based on fossil record (Horáček, 2001), (2) Emballonuridae/Molossidae (41 Myr.) and (3) Hipposideridae/Rhinolophidae (43 Myr.) based on FADs of the respective genera (i.e., *Vespertiliavus, Cuvieromops, Hipposideros,* and *Rhinolophus*) in the earliest zones of Phosphorites du Quercy (Rémy *et al*., 1987).

Results

Of the 402 nucleotide positions in the 15 taxa data set, 181 were variable and 154 were parsimony informative. All variable positions involved base substitutions. Both PTP-test (1,000 replicates, $P = 0.001$) and significantly skewed tree length distribution ($g_1 = -0.306$) show our data contain phylogenetic signal. The relationship between uncorrected and corrected substitutions on the first and second codon positions is almost linear (Fig. 1), indicating that these positions have not yet reached mutational saturation. In contrast, the deflection from linearity apparent for third codon positions suggests that multiple substitutions at these sites are increasing more rapidly. In short, testing for content of the phylogenetic information confirmed that the sequenced fragment of cytochrome *b* is acceptable as a source of information on phylogenetic events up to at least about 60 Myr., i.e., the expected time range for radiation in most of the chiropteran clades.

Pairwise sequence divergences, as derived from *p*-distances, among the studied chiropteran taxa ranged from 13.4% (*Epomophorus–Rousettus*) to 24.9% (*Rhinopoma–Emballonura*) (Table 1). For *Craseonycteris* they suggest the closest relationship with Rhinolophoidea.

Maximum parsimony analyses were performed with both unweighted (Fig. 2A) and weighted models (Fig. 2B) of nucleotide substitution. The later model, with the weighting factors of 4:14:1, was applied to reduce the effect of homoplasies. Bootstrap analysis revealed relatively low values for deep branches of each tree, particularly for unweighted data. Nevertheless, obtained topologies were almost identical except for the sister taxon of *Craseonycteris*. On the other hand, the tree topology resulted from the weighted maximum parsimony analysis (Fig. 2B) had

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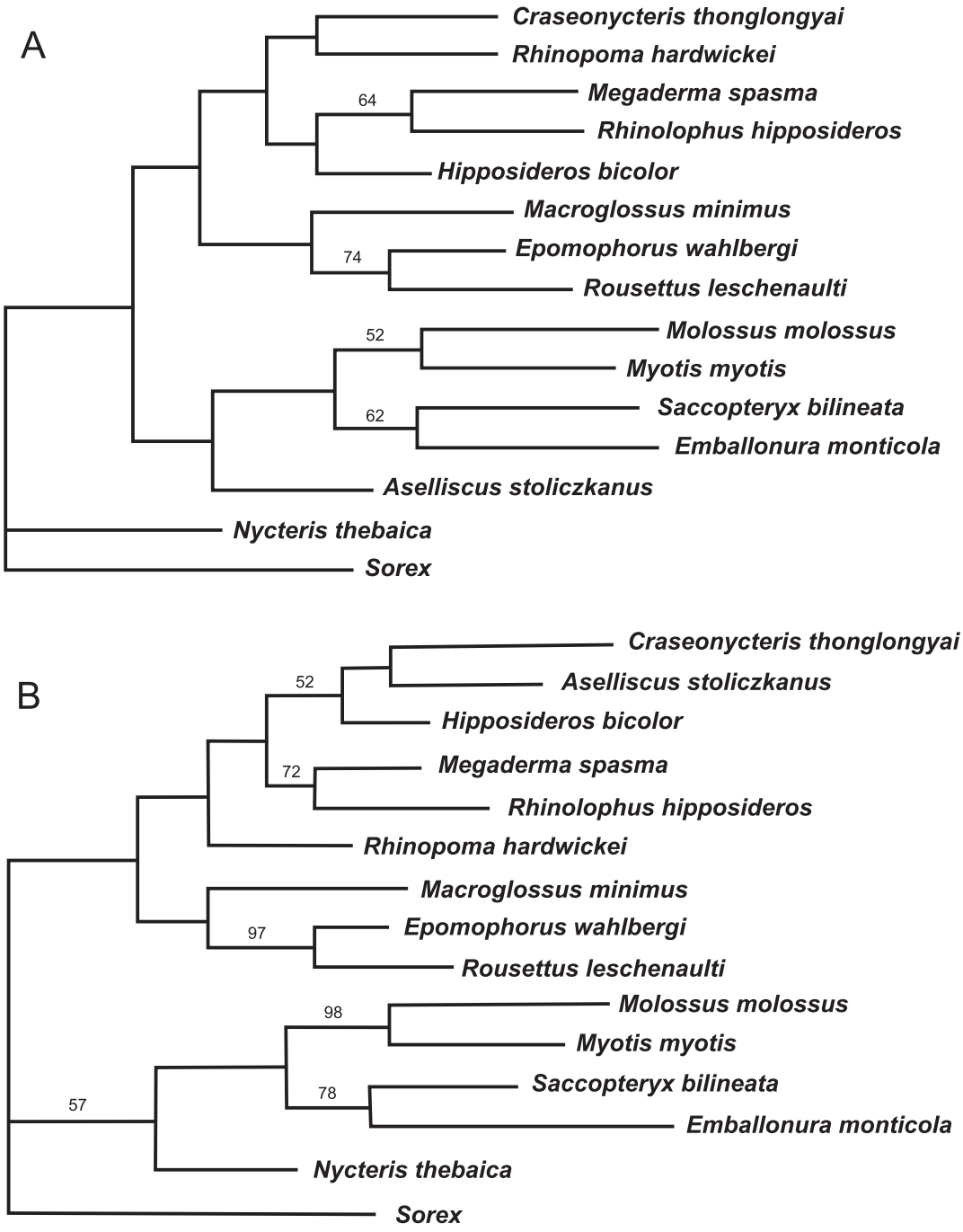


FIG. 2. Phylogenetic relationships of the family Craseonycteridae based on the cytochrome *b* sequence data. The shortest tree found by (A) unweighted maximum parsimony (length = 697 steps, CI = 0.418, RI = 0.312) and (B) with codon positions weighted 4:14:1 and transversions weighted 2:1 over transitions (length = 2,001 steps, CI = 0.456, RI = 0.392). Bootstrap values (only those > 50 are shown) were calculated with 1,000 repetitions and weights treated as repeat counts. The branch lengths represent the number of changes along each branch

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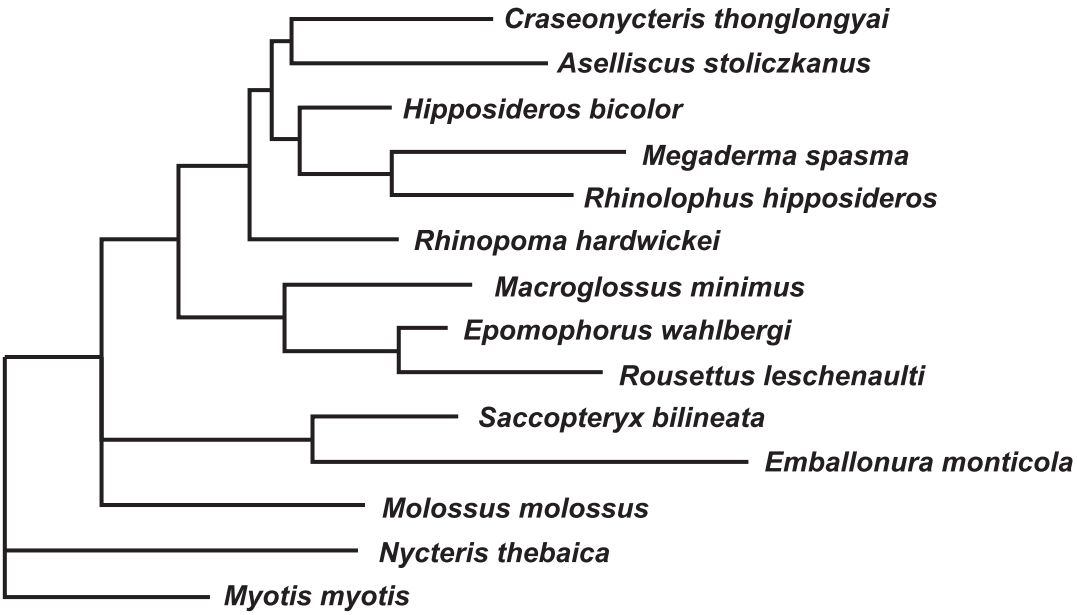


FIG. 3. The maximum likelihood (ML) tree calculated with GTR + Γ model of sequence evolution with gamma distributed rates. The parameters for ML model were estimated using the program Modeltest (R-matrix = 5.8247, 13.0624, 11.0835, 0.3571, 347.7651, 1.0000; base frequencies = 0.3280, 0.3442, 0.1383, 0.1896; shape parameter of γ-distribution = 0.143; and -ln likelihood = 1484.2)

considerably higher bootstrap support, and was very similar to trees based on the maximum likelihood algorithm (Fig. 3) and Bayesian inference (Fig. 4). In these three cases, *Craseonycteris* was closely aligned with representatives of the superfamily Rhinolophoidea. The topology of this group was as follows ((((*Craseonycteris*+*Aselliscus*) (*Megaderma*+*Rhinolophus*))*Rhinopoma*)(*Macroglossus*(*Epomophorus*+*Rousettus*))). *Hipposideros* was the only taxon with the variable position, being sister either to (*Rhinolophus*+*Megaderma*) or to (*Aselliscus*+*Craseonycteris)*. In the unweighted MP, *Aselliscus*, that in other instances is a sister taxon of *Craseonycteris*, appears in a rather improbable position close to Yangochiroptera. In all topologies, the three families, Emballonuridae, Molossidae and Vespertilionidae, were grouped together and surprisingly, Nycteridae, the group traditionally arranged within Rhinolophoidea, appeared just in this context (as a deepest branch of that clade in the weighted MP).

Results of spectral analysis (Fig. 5) are in good agreement with those derived from tree building methods except for the position of Rhinopomatidae as the closest relative of Craseonycteridae. The best nontrivial splits, such as No. 6144 (*Rousettus*+*Epomophorus*; support 0.0248, conflict 0.0117), No. 14336 (*Rousettus, Epomophorus, Macroglossus*; support 0.0120, conflict 0.0056) and No. 12288 (*Epomophorus*+*Macroglossus*; support 0.0102, conflict 0.0289), implied monophyly of the family Pteropodidae (see Fig. 5 for spectrum). Split No. 96 (*Megaderma*+*Rhinolophus*; support 0.0206, conflict 0.0013) suggested Megadermatidae and Rhinolophidae form a monophyletic clade — a relationship not supported by morphological data and calling for detailed reexamination (but see Figs. 1–3). Grouping *Molossus* and *Myotis* (split No. 1536; support 0.0144, conflict 0.0113)

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was in good agreement with traditional concepts. Less trivial is the close relationship of Emballonuridae to the core clade of Yangochiroptera (split No. 6; support 0.0096, conflict 0.0187) and quite a strong support for proximity of Emballonuridae and infraorder Yangochiroptera (split No. 1542; support 0.0043, conflict 0.0245).

The results of the spectral analysis suggest that *Craseonycteris* belongs to the superfamily Rhinolophoidea (split No. 489 — *Craseonycteris, Rhinopoma, Megaderma, Rhinolophus, Hipposideros, Aselliscus*; support 0,0039, conflict 0.0130). This position of *Craseonycteris* was also supported by splits Nos. 233 (*Craseonycteris, Rhinopoma, Megaderma, Rhinolophus, Hipposideros*; support 0.0036, conflict 0.0082) and 9 (*Craseonycteris+Rhinopoma*; support 0.0023, conflict 0.0081). Split No. 9 also indicated close relations between Rhinopomatidae and Craseonycteridae, contradicting the results of tree building analyses, in which Craseonycteridae are closely related to Hipposideridae. In short, actual relationships among particular rhinolophoid clades remains an open question. Placing Nycteridae near Emballonuridae (split No. 14825 — *Saccopteryx, Emballonura, Nycteris, Molossus, Myotis,* and *Sorex*; support 0.0038, conflict 0.0136) was another interesting outcome of the spectral analysis, which well supported the corresponding results of maximum parsimony and maximum likelihood analyses.

What were the results of alternative splits evaluation tests? Grouping *Craseonycteris* with Emballonuridae (split No. 14, not in the graph) has low support (0.0006) and high conflict (0.0149) with 28 conflicting splits (compare with support/conflict

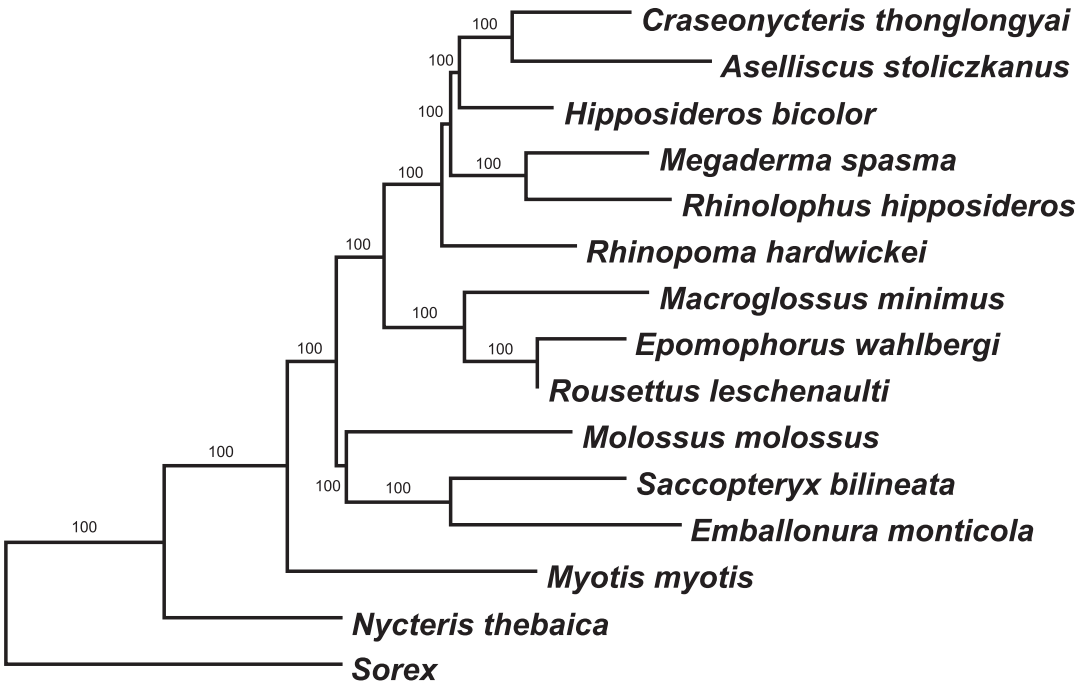


FIG. 4. Result of Bayesian phylogenetic analysis. Four chains were run in MCMC analysis with 1,000,000 replicates and GTR + I + Γ model of sequence evolution. Base frequencies and among-site rate variation were estimated according to the data, burnin was set at 10,000 according to the convergence on stable likelihood values. Posterior probabilities are shown at the nodes

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values) and only seven conflicting splits for split No. 489 placing Craseonycteridae into superfamily Rhinolophoidea. Support of Rhinolophidae incl. Rhinolophinae and Hipposiderinae (split No. 448, support 0.0003, conflict 0.0449, 11 conflicting splits) is also low compared with split number 96 (Rhinolophidae+Megadermatidae, 1 conflicting split).

Results of relative rates tests suggest that the phylogeny of selected taxa was clock-like (with no sequence under 5% significance level) and thus it was appropriate to date divergence times. The respective values for the divergence time between Craseonycteridae and related families Rhinolophoidea (omitting Nycteridae) ranged between 25 and 30 Myr. and divergence time between Craseonycteridae and Emballonuridae was 39–55 Myr.

Discussion

The major goal of this paper is to develop hypotheses for evaluating molecular relationships of Crasonycteridae (cf. Hill, 1974; Hill and Smith, 1981; Koopman, 1994; Simmons and Geisler, 1998) but the obtained results cannot be in any case considered definitive. Although cytochrome *b* and/or its fragments have been widely used to estimate phylogenetic relationships within and among bat species and it is believed to be a relevant marker for such purposes (e.g., Sudman *et al.*, 1994; Juste *et al.*, 1999; Kennedy *et al.*, 1999). Nevertheless, resolving phylogenetic relationships is generally a very difficult task, and for interfamilial relations of bats it holds even more strongly. The rapid radiation of this order, which produced most of major clades

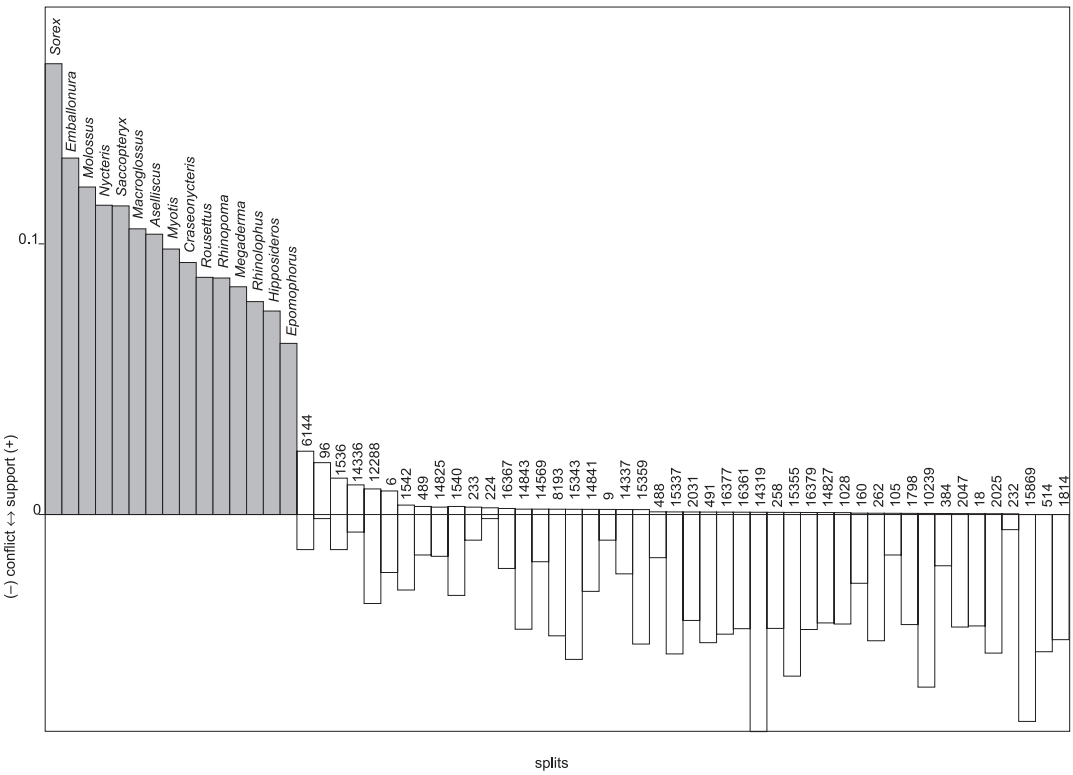


Fig. 5. Spectrum from distances calculated using Hamming distance measure plotted according to support values

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already at the beginning of its evolutionary history was followed by considerable bradytely of once established groups, which might result in various taxon-specific constrains on gradual divergence for many traits (Simmons and Geisler, 1998). Under such conditions, any phylogenetic reconstruction can be very sensitive to any ad hoc estimates and/or to precorrections of primary data and should be confronted with results of alternative approaches.

For that reason, we applied several different methods of phylogenetic inference in this study and their relevance should be discussed. The maximum parsimony method is relatively free of various assumptions (compared to maximum likelihood), and since any mathematical model currently used is a crude approximation of reality, this method may provide reliable trees (e.g., Nei and Kumar, 2000). Nevertheless, nonparametric bootstrap analysis resulted in relatively low support values for deep branches. Low bootstrap values may also be affected by the divergence pattern in chiropteran families, with its explosive radiation followed by a long period of anagenesis or stasigenesis by which cladogenetic information, although present (see random tree length distribution, PTP-test, and saturation tests), may be eroded (Kennedy *et al*., 1999; Teeling *et al*., 2000). It is especially pertinent to analyses (like this one) where individual families are represented with one or few species only. Of course, a small length of the sequenced fragment may also bias the bootstrap values, which are sensitive to the amount of characters studied (e.g., Teeling *et al*., 2000). It has to be remembered, however, that nonparametric bootstrap assesses the precision of estimate but not the proximity of a given tree to reality (Page and Holmes, 2000) and that connection between bootstrap support and statistical significance is a subject of general discussion rather than of general agreement (Hillis and Bull, 1993; Efron *et al*., 1996).

Due to this shortcoming, we applied the Bayesian inference method, a powerful tool for resolving complex questions in evolutionary biology, particularly efficient in crossing deep valleys in a landscape of phylogenetic trees (Huelsenbeck *et al*., 2001), and computed posterior probabilities on the tree we obtained. Such probabilities are considered to be more straightforward interpretation of results (Huelsenbeck *et al*., 2001; Murphy *et al*., 2001*b*). High support obtained for the respective clade composition (Fig. 3) is hence worth mentioning. The other methods we applied, i.e., maximum likelihood and spectral analysis may also help to circumvent the above problems. Maximum likelihood (Fig. 2) enables the setting of an explicit model of sequence evolution and to reduce the effect of homoplasy and long-branch attraction (Felsenstein, 1981). Unrooted tree was chosen because of incorrect position of outgroup (as a terminal taxon) in rooted variant, which is generally a problem with bats for which any possible outgroup is separated with quite a large genetic distance.

In any case, almost all applied methods provided surprisingly similar results. *Craseonycteris* clearly emerged as a member of Rhinolophoidea, a group that includes Rhinolophidae, Megadermatidae, Hipposideridae, and Rhinopomatidae. This result received, despite all possible biases, very robust support from each analysis we performed. There are other phylogenetic implications of topologies we obtained, which also should be commented upon. First, there is strong support from all tree building methods for sister relationship of Pteropodidae (megabats) and superfamily Rhinolophoidea. This outcome, quite unexpected in the light of traditional systematics, is in good concordance with our recent understanding of relationships between these

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clades, as first indicated by Kirsch and Hutcheon (1997) and Kirsch and Pettigrew (1998) (although they thought this may be an artifact of AT-GC bias), followed by Teeling *et al*. (2000), Springer *et al*. (2001), and Murphy *et al*. (2001*a*, 2001*b*). Another unexpected, but well supported result is the fact that Nycteridae are not closely aligned with Rhinolophoidea sensu Koopman (1994) but their position is in the infraorder Yangochiroptera. Teeling *et al*. (2002) also discovered that the superfamily Rhinolophoidea sensu Koopman (1994) is polyphyletic and the Nycteridae belong within Yangochiroptera along with vespertilionoids, noctilionoids, and emballonuroids.

Generic relationships of *Craseonycteris* and structure of the superfamily seem to be less unambiguous. Except for unweighted maximum parsimony, all tree building methods suggest that *Craseonycteris* is sister to *Aselliscus* (Hipposideridae), and that Rhinopomatidae form a basal clade of the superfamily Rhinolophoidea. Such possibility could be eventually accepted, if morphological traits shared by Craseonycteridae and Rhinopomatidae were symplesiomorphies. This may concern: (1) release of structural constraint on premaxilla from the maxillary developmental context, (2) lateral swelling of nasal region combined with (3) a deep interorbital constriction, (4) high sagittal crest, (5) toe phalanges formula (2-3-3-3-3), (6) elongated P_3, (7) lack of postprotocrista, and (8) continuous fossa–talon surface in M^1. The fact that remaining groups of Rhinolophoidea exhibit apparently derived states of at least some of these features (1, 2, 4, 5, 6) or share the states corresponding to those in Craseonycteridae and Rhinopomatidae (3, 7), would provide indirect support to treat respective characters as plesiomorphic. In any case, polarization of particular states should be carefully reexamined with special focus to individual rhinolophoid clades. This also holds the derived characters, including fusion of lumbar vertebrae and reduction in the number of phalanges in wing digit 2, which *Craseonycteris* shares with hipposiderids.

The results of all tree building methods as well as spectral analyses indicate that Megadermatidae and Rhinolophidae form a monophyletic group. As such, the family Rhinolophidae sensu Koopman (1994), Simmons (1998), or Simmons and Geisler (1998), i.e., including Rhinolophinae and Hipposiderinae, may represent a paraphyletic taxon. This hypothesis had been proposed at the beginning of the 20th century on the basis of morphological evidence (Miller, 1907). Another unexpected result is strong support for the close relationship between *Craseonycteris* and the hipposiderid *Aselliscus,* while nominative *Hipposideros* is distantly related to this clade. This could be interpreted as indicating that Hipposideridae, as presently arranged is not monophyletic but a collective taxon containing several clades of moderately advanced rhinolophoids. Taking into account large morphological differences between particular hipposiderid genera (in contrast to conspicuous morphological uniformity observed within the remaining rhinolophoid families) and the lack of clear autapomorphies shared by all hipposiderid genera, such a possibility is at least worth a detailed reexamination. Though two recent phylogenetic studies on Hipposideridae (Bogdanowicz and Owen, 1998; Hand and Kirsch, 1998) are largely incongruent in details, they both demonstrated a separated position of *Aselliscus* as sister to the remainder of the family.

The spectral analysis which is considered to be much less biased by possible methodological shortcomings of the tree building methods and promises to open a view to the patterns not revealed by the

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other methods (Page and Holmes, 2000) resulted in support for: (a) monophyly of Pteropodidae, (b) close relations between Megadermatidae and Rhinolophidae, (c) close affinities between core clades of Yangochiroptera and Emballonuridae, and (d) affinities of Nycteridae to Emballonuridae rather than to Rhinolophoidea (cf. also our MP and ML analyses as well as the results based on other genetic markers — Teeling *et al*., 2002). The spectral analysis demonstrated that *Craseonycteris* belongs to Rhinolophoidea though – in contrast to results of tree building methods – it suggested that Rhinopomatidae are most closely related to Craseonycteridae. This indicates that phylogenetic relationships among particular rhinolophoid clades are still far from being clearly resolved and remain a topical challenge to further study.

In conclusion, all methods used strongly support the position of *Craseonycteris thonglongyai* in the superfamily Rhinolophoidea. Within rhinolophoids, the families Hipposideridae and Rhinopomatidae are most probably the closest relatives of Craseonycteridae. Nevertheless, relationships among these clades call for additional study. Our data also suggest that Hipposiderinae sensu Koopman (1994) may not be monophyletic. Additional research with increased taxonomic and character sampling is needed to resolve this question.

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