

Status of the world's smallest mammal, the bumble-bee bat *Craseonycteris thonglongyai*, in Myanmar

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Abstract The bumble-bee bat *Craseonycteris thonglongyai* of the monospecific family Craseonycteridae is categorized as Endangered on the IUCN Red List. First discovered in 1973, it was until recently only known from a small population of approximately 2,300 individuals restricted to the catchment area of the River Kwai, Thailand. However, in 2001 a single craseonycterid was discovered in Mon State, Myanmar, extending its geographical range by approximately 250 km. In October and November 2002 a survey was undertaken to examine the status of *C. thonglongyai* in Myanmar and assess its geographical distribution and population size. *C. thonglongyai* calls were recorded from bats emerging from nine of 19 caves surveyed; the population size was estimated to be 1,500. The phylogenetic relationships

between the Thai and Myanmar populations were investigated using molecular, morphological and acoustic data. Morphologically, the two populations are indistinguishable. However, there is an 8–10 kHz echolocation call divergence between the populations. *Cytochrome b* data suggest that the two populations are similar and that the Myanmar population may be monophyletic. Annual surveys of the known bat roosts and *in situ* education programmes for local people are recommended together with the establishment of an integrated, transboundary Myanmar/Thai conservation strategy.

Keywords Bumble-bee bat, *Craseonycteris thonglongyai*, Chiroptera, *cytochrome b*, distribution, echolocation, genetics, Myanmar.

Introduction

The bumble-bee bat *Craseonycteris thonglongyai* (Hill, 1974) is considered to be the world's smallest mammal and is categorized as Endangered on the IUCN Red List (Hutson *et al.*, 2001; IUCN, 2006). Until recently it was thought to be restricted to the catchment area of the River Kwai in Thailand (Duangkhae, 1991) because

surveys of apparently suitable areas elsewhere in Thailand had not located this species (Hutson *et al.*, 2001).

In 2001 a single individual of *C. thonglongyai* was captured at a cave in a limestone outcrop on the flood plain of the Thanlwin River (=Salween River) in Mon State, Myanmar. This significantly extended the known range of the species (Bates *et al.*, 2001). From a conservation viewpoint, a detailed study of the status of the apparently allopatric Myanmar population and its relationship with the Thai population was of considerable interest.

The five principal objectives of the study were to locate *Craseonycteris* roosting sites in the limestone outcrops of Kayin and Mon States in south-east Myanmar, determine the size of the colonies at each cave site, characterize the population with respect to morphometry and echolocation, undertake a genetic study of the population using DNA analysis, and determine the threats to, and make recommendations for, the protection of the most important roosting sites.

Methods

Survey

Nineteen caves in limestone outcrops in the vicinities (<40 km) of the towns of Hpa-an, Kayin State and

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Mawlamyine, Mon State (Fig. 1) were surveyed during October and November 2002. During daylight hours the caves were inspected for the presence of bats. From 17.30 (before sunset) to 19.30 (post sunset), bat echolocation detectors were used to determine the presence of *Craseonycteris* among the emerging bats. Two bat detectors with time expansion technology (a Pettersson D240x; Pettersson Elektronik, Uppsala, Sweden), and a Tranquility II (David Bale, Cheltenham, UK) were used to record simultaneously the echolocation calls of bats emerging from each cave entrance. The calls were stored on a mini-disc recorder and a digital audio player (DAP) recorder (file recorded in WAV format with a sampling rate of 44.1 kHz).

Craseonycteris were caught with a mist net and harp trap at the entrance to one cave. A flip net was used to capture bats at two caves. Colony sizes were estimated by visually counting emerging bats. This methodology seems to be reliable for *Craseonycteris* because they appear a few minutes before dusk, have a distinctively small size, and a characteristic echolocation call (Duangkhae, 1991).

Handling procedures

Upon capture, weight and forearm length were recorded. To minimize stress from handling two skin biopsies of 3 mm in diameter were taken from the wings of only 11 individuals, according to the procedure of Worthington Wilmer & Barratt (1996). The samples were stored in 70% ethanol. With the exception of three individuals taken as voucher specimens for taxonomic study, all individuals were released.

Echolocation characterization

Echolocation calls were recorded after the bats were released, using a D-240x ultra sound detector and a DAP recorder. Bats were visually followed and echolocation calls were recorded in open areas so that their calls would be representative of search phase echolocation. Feeding calls were also recorded. These recordings were used as reference for comparison with others made during the cave survey. Subsequent analysis was carried out using *Bat Sound 3.31* (Pettersson Elektronik,

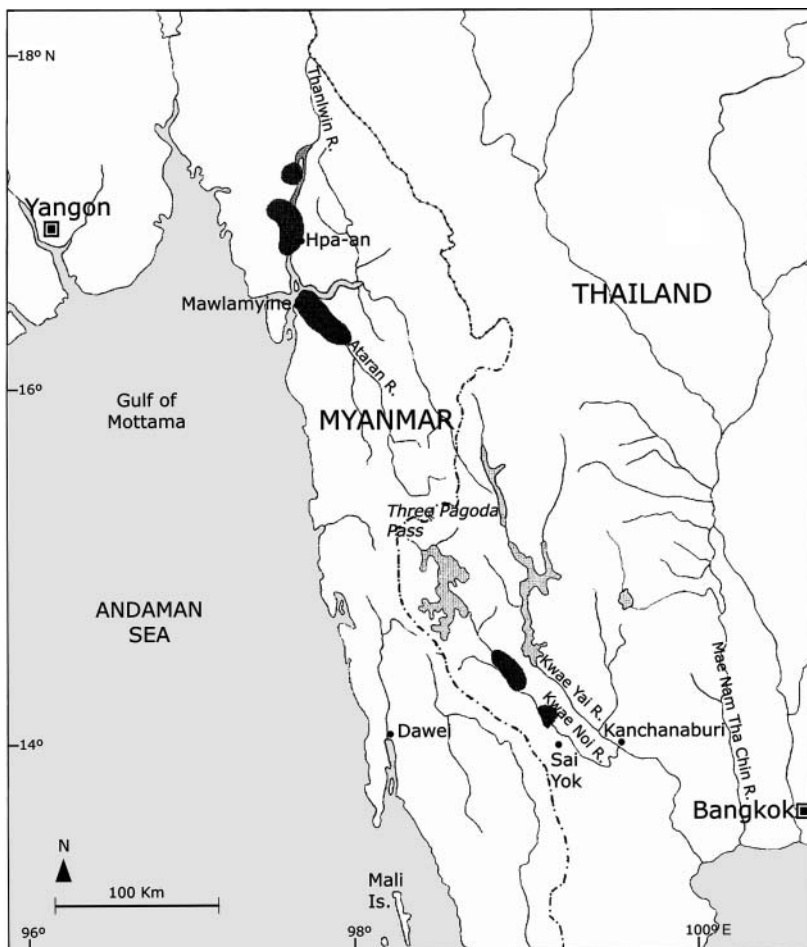


Fig. 1 Known distribution (dark shaded areas) of *C. thonglongyai* in Myanmar and Thailand.

Uppsala, Sweden) using methods described in Kalko & Schnitzler (1989).

Morphometrics and specimen collection

Four individuals were used in a detailed morphological study. One was collected in March 2001 (Bates *et al.*, 2001) and three in November 2002. All were subsequently deposited either at the University of Yangon, Myanmar, or the Harrison Institute, UK. Twenty-five external, cranial and dental measurements (Appendix) were taken from the voucher specimens using digital callipers.

Genetic analysis

Genomic DNA was extracted from the biopsy punches using the Qiagen DNesay tissue kit. Four hundred and two base pairs from the 5' end of the *cytochrome b* mitochondrial gene were amplified and sequenced for each individual using MVZ04 and MVZ05 primers (Smith & Patton, 1991) following methods detailed in Murphy *et al.* (2001). Published *cytochrome b* sequences for members of the superfamily Rhinolophoidea (*sensu* Teeling *et al.*, 2005), a Thai craseonycterid and for four pteropodid species (outgroups) were included in the analyses. The data set was aligned using ClustalX (Thompson *et al.*, 1997) and further modified in SE-AL (Rambaut, 1996). All species and GenBank accession numbers are listed in Table 1.

With the exception of Bayesian analyses all phylogenetic methods were performed using *PAUP** 4.0b10

Table 1 Genbank accession numbers.

Species	Accession number
<i>Cynopterus brachyotis</i>	AB046321
<i>Nyctimene robinsoni</i>	AF144066
<i>Pteropus rufus</i>	AB085732
<i>Rousettus amplexicaudatus</i>	AB046329
<i>Rhinopoma hardwickii</i>	AY056462
<i>Megaderma spasma</i>	AY057942
<i>Rhinolophus cornutus</i>	AY141041
<i>Rhinolophus ferrumequinum</i>	AB085731
<i>Rhinolophus hipposideros</i>	AY141040
<i>Rhinolophus perditus</i>	AY141039
<i>Aselliscus stoliczkanus</i>	AF451336
<i>Hipposideros armiger</i>	AF451332
<i>Hipposideros bicolor</i>	AF451335
<i>Hipposideros larvatus</i>	AF451333
<i>Hipposideros pratti</i>	AF451334
<i>C. thonglongyai</i> (Thailand)	AF512008
<i>C. thonglongyai</i> (Myanmar)-Type1	EF035011*
<i>C. thonglongyai</i> (Myanmar)-Type2	EF035012*

*sequences new to this study

(Swofford, 2003) and included Maximum Likelihood (ML), Minimum Evolution (ME), and Maximum Parsimony (MP). Bayesian analyses were carried out with *MrBayes 3.0b4* (Huelsenbeck & Ronquist, 2001). ML analyses incorporated the GTR+ Γ +I model of sequence evolution as suggested by ModelTest (Posada & Crandall, 1998); base frequencies = (0.3162 0.3962 0.1138 0.1738); R matrix = (6.0886 27.5234 5.6446 0.4807 67.9956); shape parameter of the gamma distribution = 1.3970; proportion of the invariant sites = 0.5581. ML analyses were also completed with third positions removed. In ML and ME bootstrap analyses the starting tree was obtained via neighbour-joining. ME analyses were performed with ML distances corrected according to the GTR+ Γ +I model of sequence evolution. Bootstrap analyses included 100 replications with ML and 500 replications with ME and MP. Heuristic searches with tree-bisection and reconnection branch-swapping for all analyses were used. Bayesian posterior probabilities were calculated using a Markov chain Monte Carlo sampling approach incorporated in *MrBayes 3.0b4*. Bayesian analyses used the general time reversible (GTR) model of sequence evolution with an allowance for a gamma distribution of rates (Γ) and a proportion of invariant sites (I). Starting trees were random, four simultaneous Markov chains were run for 1 million generations, burn-in values were set at 50,000 generations, and trees were sampled every 20 generations.

Results

Distribution and colony size

C. thonglongyai were located at nine cave sites, i.e. 47.4% of the caves surveyed (Fig. 1; details of the caves are omitted to minimize the possibility of future disturbance). At seven sites echolocation calls were recorded outside the cave entrance. Additionally, at two sites, individuals were collected within the caves. At one of these, a flip net was used, which captured three bats. Subsequent trapping at dusk inside the cave entrance, using a mist net and a harp trap, yielded many more individuals. At the other site, a single individual was captured using a flip net. However, a harp trap and mist nets set within the cave failed to catch further individuals.

The population size in this region was estimated to be *c.* 1,500 individuals. First emergence time was $17.35 \pm \text{SD } 4$ minutes ($n = 11$), with individuals of *C. thonglongyai* returning to the roost $31 \pm \text{SD } 7$ minutes ($n = 11$) after emergence, thus showing a very short dusk activity period. The use of ultrasound detectors confirmed the presence of this species at nine caves.

Morphology

Individuals of *C. thonglongyai* caught in the field weighed on average $2.4 \pm \text{SD } 0.2$ g ($n = 14$) and had a forearm length of $24.1 \pm \text{SD } 0.87$ mm ($n = 14$). Twenty-five external, cranial and dental measurements taken from the voucher specimens were compared to those from 26 specimens from Thailand (Hill, 1974). All, except ear length, were essentially similar, with little difference in mean values and an overlap in the ranges (Tables 2 & 3). A comparison of the Myanmar material with specimens from Thailand in the Natural History Museum, London, UK, showed that the apparent difference in the ear length was a result of differences in measuring techniques. On the dentition Hill (1974) wrote that the lower canine had 'no accessory cusps' and illustrated it as such. However, an examination of the 17 skulls of Thai specimens in the Natural History Museum, London, and the four skulls from Myanmar revealed that anterior and posterior cingular cusps were

always present. This confirms that there are no apparent morphological or morphometric differences between the two populations and supports the view of Bates *et al.* (2001).

Echolocation

The echolocation call of *C. thonglongyai* from the Myanmar study site (Fig. 2) is clearly distinct from that of the Thailand population (Surlykke *et al.*, 1993). Table 4 presents descriptor variables of the species' echolocation calls. The difference in the peak frequency between Myanmar and Thailand (Surlykke *et al.*, 1993) is approximately 8–10 kHz. Our data suggest that there may be differences in the bandwidth, pulse duration and interval between pulses but these results are based on small sample sizes and must be verified by future studies. *C. thonglongyai* recordings are available on request from MJRP.

Table 2 Eleven external measurements (mm) and body mass (g) of *C. thonglongyai* (for abbreviations, see Appendix). Minimum-maximum values are given where $n \geq 2$; sample sizes differing from those reported under n are given in parentheses.

	n	FA	III ^M	III ¹	III ²	IV ^M	IV ¹	IV ²	V ^M	TIBIA	HF ²	E	MASS
Myanmar (males)	3	24.6 24.5–24.7	20.7 (2) 20.7, 20.7	7.0 (2) 6.7, 7.2	20.4 (2) 20.3, 20.5	22.5 (2) 22.3, 22.7	4.0 (2) 3.7, 4.3	9.6 (2) 8.9, 10.3	22.5 (2) 22.1, 22.8	12.7 12.4–13.1	6.2 (2) 6.1, 6.2	11.0 10.5–11.4	2.3 2.0–2.5
Myanmar (female)	1	25.8	21.2	7.0	21.2	23.6	4.1	11.0	23.4	12.8	6.0	11.2	2.6
Thailand (males) ¹	17	24.6 (16) 24.1–25.2	20.7 (16) 19.8–21.6	6.7 6.4–7.0	20.3 (16) 18.7–21.5	22.4 21.7–23.2	3.9 3.5–4.2	10.2 9.7–10.7	22.4 21.7–23.0	12.3 (16) 11.9–12.9	6.3 5.9–6.8	9.5 9.0–10.0	
Thailand (females) ¹	9	24.9 22.5–25.8	21.1 19.7–21.7	6.8 6.5–7.1	20.1 19.4–21.2	23.1 21.7–23.8	3.9 3.6–4.3	10.3 9.7–10.8	23.2 21.8–23.7	12.3 11.7–12.8	6.2 5.8–6.6	9.8 9.0–10.2	

¹After Hill (1974)

²Includes claw

Table 3 Fourteen cranial and dental measurements (in mm) of *C. thonglongyai* (for abbreviations, see Appendix). Minimum-maximum values are given where $n \geq 3$; sample sizes differing from those reported under n are given in parentheses.

	n	GTL	CCL	ZB	BB	BH	PC	TBL	TBW	BW	C-M ³	M ³ -M ³	C ¹ -C ¹	C-M ₃	MDL
Myanmar (males)	3	10.8 10.4–11.0	9.7 9.4–10.1	5.8 (2) 5.7, 5.9	5.5 5.4–5.7	4.2 (2) 4.2, 4.2	2.2 (2) 2.2, 2.2	2.6 (2) 2.5, 2.7	2.0 (2) 1.9, 2.0	0.7 (2) 0.7, 0.7	3.8 3.6–3.9	4.6 (2) 4.6, 4.6	3.1 (2) 3.0, 3.1	4.3 4.2–4.5	7.2 7.0–7.3
Myanmar (female)	1	10.9	10.1		5.7	4.3	2.2	2.5	2.0	0.7	3.8	4.8	3.1	4.4	7.2
Thailand (males)*	12	11.2 (11) 10.6–11.5	10.0 9.9–10.1	5.8 5.5–6.0	5.5 5.3–5.7	4.1 4.0–4.3	2.2 2.1–2.3	2.6 2.4–2.7	1.7 1.5–1.9	0.6 0.5–0.7	3.8 3.7–3.9	4.7 4.6–4.9	3.0 2.9–3.2	4.1 4.0–4.2	7.1 7.0–7.3
Thailand (females)*	5	10.9 10.3–11.1	9.9 9.6–10.0	5.6 5.5–5.7	5.5 5.4–5.5	4.0 3.9–4.0	2.1 2.1–2.2	2.6 2.3–2.6	1.7 1.6–1.8	0.6 0.5–0.6	3.7 3.6–3.8	4.6 4.5–4.6	2.8 2.8–2.9	4.0 3.9–4.0	6.8 6.7–6.9

*After Hill (1974)

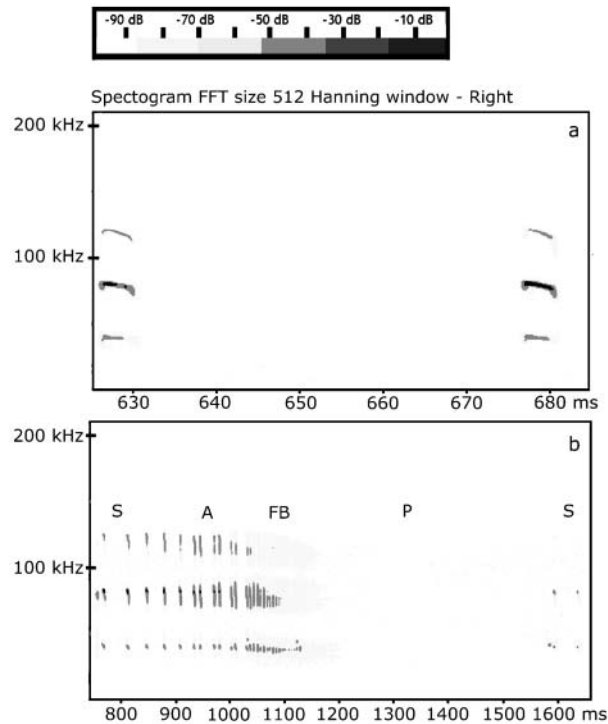


Fig. 2 Sonograms of examples of echolocation calls produced by *C. thonglongyai* from Myanmar: (a) two search phase echolocation calls, (b) a feeding buzz.

Genetics

The Craseonycteridae formed a monophyletic clade; the Thailand representative grouped with the Myanmar craseonycterids with 100% bootstrap support and a posterior probability of 1.000. In 11 Myanmar craseonycterids only two haplotypes were found. One individual differed from all others by an A-G substitution at nucleotide position 394. The Myanmar population appeared to form a monophyletic clade and received moderate bootstrap support (71–81% bootstrap support; posterior probability of 0.84), however only three substitution differences separated the Thai and Myanmar craseonycterids (T-C at position 223; G-A at position 343; C-T at position 390) and the branch length was short. The phylogenetic trees (Fig. 3) differed with and without the inclusion of third positions, which have been shown to be saturated (Hulva & Horacek, 2002).

The optimal topology resulting from analyses without third positions is more similar to the topology reported in Teeling *et al.* (2005), in which they investigated the interfamilial relationships within Chiroptera with a 13 kb nuclear gene data for representatives of all bat families.

Discussion

This study confirms the suggestion of Bates *et al.* (2001) that *C. thonglongyai* may be distributed widely amongst the limestone outcrops of the flood plains of the Thanlwin, Ataran (and Gyaing) Rivers in Kayin and Mon States, Myanmar. Originally known from one cave (Bates *et al.*, 2001), it has now been recorded from nine separate sites that extend 102 km from north to south. Unfortunately, we were not able to survey all the potential geographical range. In future, if the security situation permits, it would be of value to explore other areas adjacent to the Thanlwin and Ataran Rivers that are known to have extensive limestone outcrops (Foley, 1836; Tickell & Parish, 1859).

However, although only a small proportion of the potential caves within the study area were surveyed, the population size is two-thirds of that cited for Thailand or some 40% of the known world population of 3,800 individuals. The number of individuals in some of the caves (600–800) was also high when compared to numbers estimated in Thailand (a maximum of 560 individuals; Duangkhae, 1991).

Currently, the *C. thonglongyai* population in Myanmar does not appear to be greatly threatened. Potential future threats are the extraction of limestone for cement production, pollution, and human disturbance. At present no known locality of *C. thonglongyai* is directly affected by limestone extraction but there is pollution, in the form of smoke and dust, generated by two local cement factories within the bats' foraging area (Bates, 2001). Meanwhile, a number of the caves at which *C. thonglongyai* was recorded are Buddhist sites attracting a growing number of pilgrims. Although most pilgrims do not penetrate deep into the caves, they do have an impact on the temperature, humidity, light and noise levels of the cave environment and could provoke *C. thonglongyai* to abandon its roost. Human disturbance

Table 4 Descriptor variables (mean \pm SD) of the echolocation calls of *C. thonglongyai* from Myanmar and Thailand. Sample size is for the number of individual bats sampled.

Population	Peak frequency (kHz)	Maximum frequency (kHz)	Minimum frequency (kHz)	Band width (kHz)	Pulse duration (ms)	Interval between pulses (ms)	Sweep rate (kHz/ms)	Duty cycle (%)
Myanmar (n = 19)	81.78 \pm 0.91	85.04 \pm 1.44	70.69 \pm 1.41	14.35 \pm 3.22	3.68 \pm 0.33	54.19 \pm 7.92	3.95 \pm 0.87	6.89 \pm 0.86
Thailand (n = 4)	73.2 \pm 0.4			12 \pm 4	3.5 \pm 0.4	45.45 \pm 10.89		

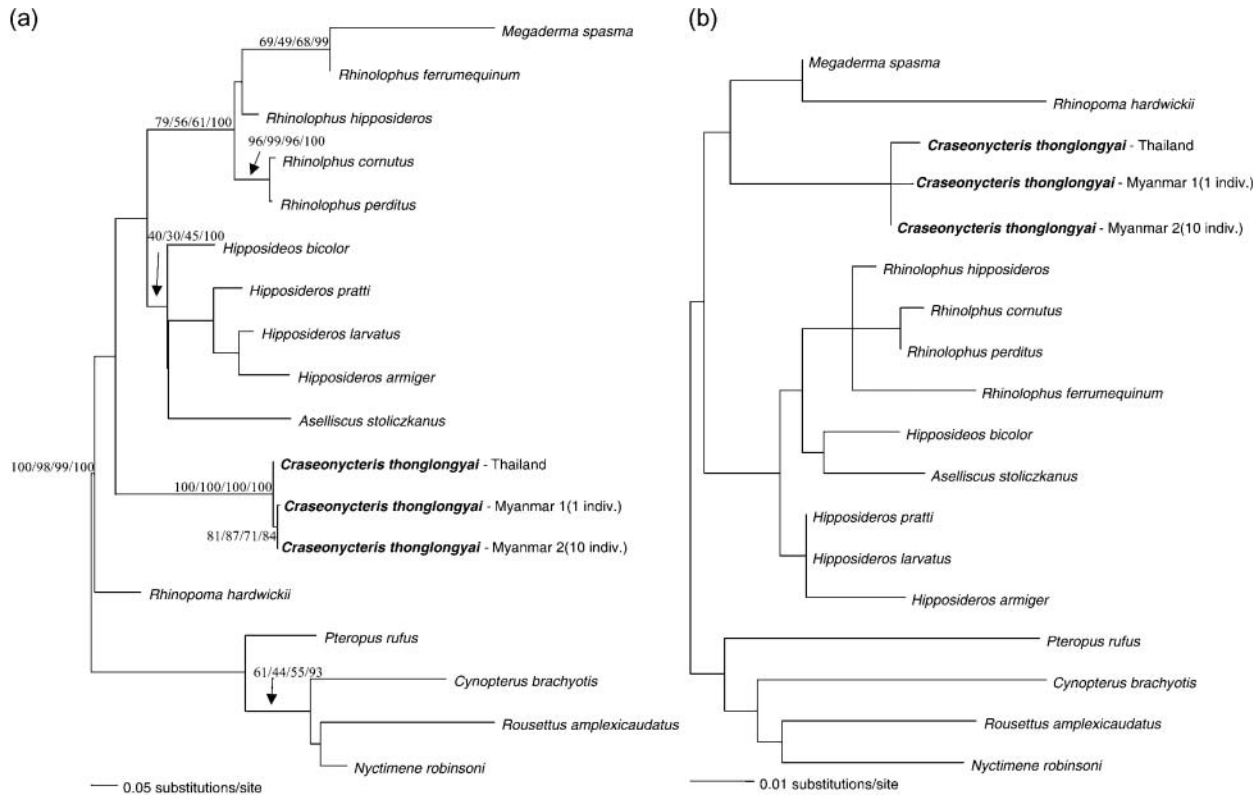


Fig. 3 Phylogenetic trees for *C. thonglongyai* in Myanmar and Thailand and related species (see text for details): (A) The unrooted maximum likelihood (ML) tree for 402 base pairs of *cytochrome b* under the GTR+ Γ +I model of sequence evolution ($-\ln$ likelihood = 2581.61). Numbers plotted along the branches indicate bootstrap values and Bayesian posterior probabilities shown as percentages in the following order: ML/ MP/ ME/ Bayesian. (B) The unrooted maximum likelihood (ML) tree for 268 bp of *cytochrome b* with third positions removed under the GTR+ Γ +I model of sequence evolution ($-\ln$ likelihood = 800.5).

has led to caves being abandoned by *C. thonglongyai* in Thailand (Hutson *et al.*, 2001). On the positive side, local monks restrict the hunting of bats within Buddhist caves.

It is still not clear whether the Myanmar and Thai populations are reproductively isolated. In terms of echolocation, the calls of the Myanmar population are clearly distinct, whereas morphologically they are similar. Currently, the taxonomic and evolutionary significance of this acoustic variation is not known. Information from other taxa suggests that geographical variation in peak frequency within species is not uncommon. Several examples of differences in excess of 10 kHz are known from the Rhinolophidae (Csorba *et al.*, 2003). In the case of *C. thonglongyai* a complete survey of the echolocation calls along a transect linking the known ranges in Myanmar and Thailand would provide information as to whether the variation is clinal or step-like, with two distinct acoustic populations.

The molecular phylogenetic analyses suggest that the Myanmar population may form a monophyletic clade. However, this needs to be confirmed through the collection of more samples. A more detailed study

involving microsatellites is also desirable because of the faster mutation rate of these genetic markers and because bi-parentally inherited nuclear markers have been shown to support an alternative phylogenetic relationship, which cannot be observed using only maternally inherited mitochondrial DNA (Roca *et al.*, 2004).

Based on this work our conservation recommendations, in accordance with the species action plan (Hutson *et al.*, 2001) and tailored to the specific needs of the Myanmar population, are:

- Conduct further studies to determine whether the Myanmar and Thai populations are monospecific.
- Conduct an annual census of the known cave sites within Kayin and Mon States, with an assessment of threats.
- Conduct further surveys to establish distribution and population levels. Possible locations include: in Kayin State, the extensive limestone hills north of Yinbaing on the Thanlwin River (information based on Foley, 1836) and a vast limestone outcrop c. 30 km to the north-west of Three

Pagoda Pass (information based on Tickell & Parish, 1859); in Tanintharyi Division, a large limestone cave 33 km from the northern border of Sai Yok National Park (information from PJJB). Unfortunately, all three areas are currently off-limits because of security considerations.

- Conduct a local education programme to reinforce ideas about the positive value of bats, including *C. thonglongyai*, within the local community, to reduce disturbance at cave roosting sites.
- Minimize the impact of limestone extraction on cave roosts through dialogue with representatives of local cement factories.
- Assess the bats' diet and describe the insect population within the foraging areas.
- Study the population structure, behaviour, and echolocation calls of both the Myanmar and Thai populations.

To address and implement these recommendations a consortium of research scientists from University College Dublin, Ireland, Harrison Institute, UK, Prince of Songkla University, Thailand, and Yangon University, Myanmar, created a multinational *Craseonycteris* team, in November 2005, dedicated to the transboundary conservation of this species. Currently the consortium is completing extensive surveys throughout the species' range, examining the acoustic variation, roost preferences, population genetics and evolutionary history of the Thai and Myanmar *craseonycterid* populations. This information will be used to create long-term conservation management plans.

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Appendix Measurements taken from voucher specimens.

FA, Forearm length, from the extremity of the elbow to the extremity of the carpus with the wings folded. *III^m*, *IV^m*, *V^m*, Metacarpal length, from the extremity of the carpus to the distal extremity of the third, fourth and fifth metacarpals respectively. *III¹*, *IV¹*, First phalanx length, from the proximal to the distal extremity of the first phalanx of the third and fourth digits respectively. *III²*, *IV²*, Second phalanx length, from the proximal to the distal extremity of the second phalanx of the third and fourth digits respectively. *TIBIA*, Tibia length, from the knee joint to the ankle. *HF*, Foot length, from the extremity of the heel behind the *os calcis* to the extremity of the longest digit, including the claws. *E*, Ear length, from the lower border of the external auditory meatus to the tip of the pinna. *MASS*, Body mass taken using a 10 g Pesola scale. *GTL*, Greatest length of skull, the greatest antero-posterior length of the skull, taken from the most projecting point at each extremity. *CCL*, Condylar length, from an exoccipital condyle to the alveolus of the anterior canine. *ZB*, Zygomatic breadth, the greatest width of the skull across the zygomatic arches. *BB*, Breadth of braincase, greatest width of the braincase at

the posterior roots of the zygomatic arches. *BH*, Height of braincase, from the midpoint of exoccipital condyle to a point on the parietal vertically above it. *PC*, Postorbital constriction, the narrowest width across the constriction posterior to the orbits. *TBL*, Tympanic bulla length, greatest oblique antero-posterior diameter from the apex of the tympanic bulla internal to the hamular process to the most posterior part of the mastoid chamber of the tympanic bulla external to the base of the paroccipital process. *TBW*, Tympanic bulla width, taken across the bulla at the widest point perpendicular to the line of the antero-posterior diameter. *BW*, Basioccipital width, taken at the narrowest point between the two cochlea. *C-M³*, Maxillary toothrow length, from the front of the upper canine to the back of the crown of the third upper molar. *M³-M³*, Posterior palatal width taken across the outer borders of the third upper molars. *C¹-C¹*, Anterior palatal width, taken across the outer borders of the upper canines. *C-M₃*, Mandibular toothrow length, from the front of the lower canine to the back of the crown of the third lower molar. *MDL*, Mandible length, from the most posterior part of the condyle to the most anterior part of the mandible.

Biographical sketches

Maria João Ramos Pereira and Hugo Rebelo are studying aspects of bat population genetics, habitat selection and roosting ecology.

Emma Teeling has a long-standing interest in bat systematics, evolution, conservation and the molecular process governing sensory perception and speciation.

Stephen J. O'Brien's principal research focus involves complex and multi-factorial disease gene discovery in infectious diseases. He has a secondary research interest in mammalian comparative genomics.

Iain Mackie, Si Si Hla Bu, Khin Maung Swe, Khin Mie Mie and Paul Bates have spent the last 5 years conducting bat research in Myanmar, with particular interest in acoustics, taxonomy and conservation.