Darwin Initiative Annual Report
Project reference 14-008

The Darwin Initiative Centre for Bat Conservation in China

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Project partner: Professor Shuyi Zhang, Institute of Zoology, Chinese Academy of Sciences, China

Darwin Initiative

Annual Report

1. Darwin Project Information

Project Ref. Number	14-008
Project Title	The Darwin Initiative Centre for Bat Conservation in China
Country(ies)	China
UK Contractor	University of Bristol
Partner Organisation(s)	Institute of Zoology, Chinese Academy of Sciences
Darwin Grant Value	£112,000
Start/End dates	1 July 2005 – 30 June 2008
Reporting period (1 Apr 200x to 31 Mar 200y) and annual report number (1,2,3..)	1 July 2005-30 April 2006 First annual report (covering 9 months)
Project website	
Author(s), date	

2. Project Background

The diversity of bats in China is poorly understood. With 120 species listed to date, China has one of the most speciose bat faunas worldwide. However the validity and taxonomy of many of the described species is open to question, and a more in depth study of their taxonomy is needed. Despite their rich faunal diversity, bats in China are threatened due to extreme human pressure – through habitat loss, cave disturbance and human consumption. There is a clear need for education programmes to disseminate information about the ecological importance of bats, and to increase their protection. A Red Data book of the Endemic Mammals of China listed 6 microchiropteran bats as Rare, Vulnerable or Indeterminate (Wang, 1988 – China Red data Book of Endangered Animals: Mammalia Science Press, Beijing), and in reality accurate information on population levels of any species is lacking. Our project will assist in the conservation of these Red Data species, and will provide baseline data for future surveys to detect trends in population size.

The project will be based around Beijing, and is in collaboration with the Institute of Zoology, Chinese Academy of Sciences.

3. Project Purpose and Outputs

Project purpose

The project aims to establish a unit for research and for the promotion of bat conservation in China (The Darwin Initiative Centre for Bat Conservation). We

will train 2 Chinese PhD students in the conservation biology and ecology of bats, produce a key and a web-based identification guide to Chinese bats, and promote public awareness of bat conservation by training school teachers and pupils about the importance of bats.

Regarding Year 1 milestones, we have taken on 3 staff as proposed (2 PhD students and an education officer), and have made substantial progress in writing scientific papers. The establishment of the education centre has been delayed (see below), though space for it has been purchased and development of the site is imminent.

Modifications to original proposal

Our original plan was to build the Darwin Centre in Guangzhou (Guangdong Province, southern China). Initial discussions with the Guangzhou Panyu Safari Park in July 2005 were very encouraging, but negations hit an impasse largely because of findings that bats were reservoirs of SARS-like coronoviruses in southern China (covered in high profile papers in *Science* and *Proceedings of the National Academy of Sciences, USA*). Staff at the safari park informed my Chinese collaborator, Professor Zhang, that they did not want to house bats there, and they also lost interest in hosting a conservation centre there, because of the recent findings regarding SARS.

Professor Zhang therefore suggested that we base the centre close to a building that he has established for bat research near Beijing. Professor Zhang has proposed building a museum at the new site, covering aspects of the conservation of bats, including the roles of bats in culture and history. The centre would still function as an education centre in the ways initially proposed. Professor Zhang works closely with the local government in the area, which is also a tourist region (Xiayunling district). Professor Zhang believes he can oversee the development of the site easily from the Chinese Academy of Sciences in nearby Beijing. Although the change means that the centre will be based in northern, rather than southern China, it will still be the only site devoted to bat conservation in China, and similar problems of habitat destruction and killing of bats for food apply in Beijing Province as they do elsewhere in China.

The proposed change in site was put to the Darwin Initiative by email on 17 October 2005. Approval was granted on 16 January 2006. The change in site has obviously delayed development of the education centre.

4. Progress

Summary

The project began with a visit by Professor Jones to Guangzhou in July 2005. Professors Zhang and Jones met with the Standing Deputy General manager at Guangzhou Panyu Xiangjiang Safari Park to discuss setting up the Darwin Centre there. Although the Park were initially very positive about hosting the Centre, their position changed after bats were shown to be reservoirs of SARS-like coronoviruses, and so an alternative site for the Centre had to be found. While in Guangzhou, Professor Jones met with the Consul, Science and Innovation and with the Science and Innovation Officer from the British Consulate-General, Guangzhou. The Consulate was positive about publicising the Centre. Professor Jones also met with the Head of Kadoorie Farm Conservation Centre, Hong Kong, and with the Conservation Officer of the

Agriculture, Fisheries and Conservation Department in Hong Kong, about sharing education material on bat conservation. We have taken on Li Gang and Zhang Jinshuo as PhD students on the grant, and appointed Zhang Junpeng as education officer. We have purchased one floor of a new School in Xiayunling district, near Beijing, for the Centre. We have worked on three manuscripts about the ecology and taxonomy of bats in China. One of these papers has been accepted in *Journal of Mammalogy*, another is under revision at *Acta Chiropterologica*, and the third is almost ready for submission.

Progress in relation to baseline timetable and logical framework

Progress over the last 9 months against the agreed baseline timetable and the logical framework is provided in *Annex 1*. Slippage in the development of the education centre has occurred for reasons explained above. PhD students and the employment officer have been deployed as anticipated. Material has been accumulated (echolocation call recordings, DNA sequences, photographs) for development of the website. Outputs in the form of scientific manuscripts are progressing at a rate faster than anticipated.

Achievements

Key achievements in the first 9 months of the project have been

- Purchase of site for Darwin centre in a school.
- Employment of 2 PhD students and education officer.
- Production of three manuscripts on the ecology of Chinese bats. One of these has been accepted or publication in an international, peer-reviewed journal, another is under revision following review.

Abstracts of the publications are given below (project participants highlighted in bold).

1. **Phylogenetics of small horseshoe bats from East Asia based on mitochondrial DNA sequence variation**

Gang Li, Gareth Jones, Stephen J. Rossiter, Shiang-Fan Chen, Stuart Parsons, **Shuyi Zhang**

Journal of Mammalogy, in press.

We undertook analyses of mitochondrial DNA gene sequences and echolocation calls to resolve the phylogenetic and taxonomic relationships among the related bat taxa *Rhinolophus pusillus* (sampled across China), *R. monoceros* (Taiwan), *R. cornutus* (main islands of Japan) and *R. c. pumilus* (Okinawa, Japan). Phylogenetic trees and genetic divergence analyses were constructed by combining new complete mitochondrial cytochrome *b* gene sequences and partial mitochondrial control region sequences with published sequences. Our work showed that these four taxa formed monophyletic groups in the phylogenetic tree. However, low levels of sequence divergence among the taxa, together with similarities in body size and overlapping echolocation call frequencies point to a lack of taxonomic distinctiveness. We therefore suggest that these taxa are better considered as geographical subspecies rather than distinct species, though this should not diminish the conservation importance of these island populations, which are important evolutionary significant units. Based on our findings, we suggest the

similarities in body size and echolocation call frequency in these rhinolophids result from their recent common ancestry, while similarities in body size and call frequency with *R. hipposideros* of Europe are the result of convergent evolution.

2. **Echolocation calls, wingshape, diet and phylogenetic diagnosis of the endemic Chinese bat *Myotis pequinius***

Gareth Jones, Stuart Parsons, **Shuyi Zhang**, Benoit Stadelmann, Petr Benda, and Manuel Ruedi

Under revision after review at *Acta Chiropterologica*

We describe the echolocation calls, flight morphology and diet of the endemic Chinese bat *Myotis pequinius* Thomas, 1908. Orientation calls are broadband, and reach low terminal frequencies. Diet comprised 80% beetles by volume. Wing shape and call design suggest that the bats fly in cluttered habitats, and the possession of moderately long ears and the dietary composition imply they forage at least sometimes by gleaning. *M. pequinius* resembles a larger Oriental version of the western Palaearctic species *Myotis nattereri*. Phylogenetic analysis based on sequences of the cytochrome *b* gene of mitochondrial DNA (1140 base pairs) from a range of Palaearctic *Myotis* species confirmed that *M. pequinius* is close to the *nattereri* group, but is a sister-species to the eastern Palaearctic *M. bombinus*. One bat sequenced from China could not be identified from available species descriptions. It was smaller than *M. pequinius*, and also differed from it in sequence divergence by 6.3%, suggesting the existence of additional, cryptic taxonomic diversity in this group. Our phylogenetic analysis also supports the recognition of *Myotis schaubi* as a species distinct from *M. nattereri* in Transcaucasia and south-western Asia. *M. nattereri tschuliensis is more closely related to M. schaubi* than to *M. nattereri*, and is best considered either as a subspecies of *M. schaubi*, or possibly as a distinct species.

3. **Diet, echolocation calls and phylogenetic affinities of the great evening bat *Ia io* (Vespertilionidae): another carnivorous bat**

Adora Thabah, **Gang Li**, Yinan Wang, Bing Liang, Kailiang Hu, **Shuyi Zhang** and **Gareth Jones**

MS almost ready for submission.

We studied the great evening bat *Ia io* in India (Meghalaya) and China (Guizhou), and present the first account of its feeding behaviour. Of droppings analysed from 119 bats between November-May from India, 28 included bird feathers, with most of these containing 90-100% feathers by volume. The main constituent of the diet overall was Coleoptera, although Lepidoptera and traces of Diptera, Orthoptera and Hemiptera were also found. In China bats were captured in early November, and fresh droppings were also collected from underneath the roost. Bird feathers were found to comprise 82% of the bats' diet by volume. *Ia io* emits echolocation calls that are relatively low in frequency. Such calls are well adapted for aerial hawking of large insects and we present evidence that the species also sometimes produces two-toned echolocation calls, a characteristic of species that echolocate distant targets. *Ia io* has a high wing loading (15.4 Nm^{-2}), an average aspect ratio (6.9) and a high tip shape index (1.1), features associated with fast and efficient flight. Phylogenetic analysis of a concatenation of mitochondrial ND1 and

cytochrome *b* genes showed that *Ia io* was phylogenetically close to *Scotomanes ornatus*, in a clade distinct from bats in the genera *Pipistrellus* and *Eptesicus* with which it has previously been allied. Although *Ia io* has parallels in wingshape and echolocation call design with the carnivorous, fast flying *Nyctalus lasiopterus*, its carnivorous behaviour evolved independently. It is not clear whether *Ia io* captures nesting birds, birds that occupy the same roosting site, or whether, as is proposed for *N. lasiopterus*, birds are caught at high altitude during flight.

In summary, findings from these three papers make the following contributions.

- By using data from DNA sequencing, echolocation calls and morphology we show that the current taxonomy of Chinese small horseshoe bats is flawed, and make suggestions for its revision.
- We provide the first ecological data on an endemic Chinese bat, including studies on echolocation calls and diet, and also reveal its relationship with other bats in the same genus by sequencing studies.
- We describe carnivory (specifically bird-eating) in a large, aerial feeding bat, and also reveal the evolutionary relationships of this bat with other bats in the same family by DNA sequencing. Current ideas about the closest living relatives of this bat are proven wrong.

In addition, Professor Jones attended the Darwin Initiative Workshop in London on 22 February 2006, and Professors Jones and Zhang have been in discussions with the BBC about filming at the Centre for the forthcoming series on *Wild China*.

Research: I believe that these publications are furthering the standing of bat research in China considerably on the international stage. Prior to my collaborations with Professor Zhang, no studies on Chinese bats were published in international, peer-reviewed journals.

Training: I am providing considerable input into the supervision of 2 Chinese PhD students, by giving advice about scientific methods, and by improving written English. Advice is provided especially on molecular methods, phylogenetics, and echolocation call analyses.

Planning: Planning is mainly organised through weekly emails between Professor Zhang and myself. Professor Jones will visit Beijing in July 2006 to further develop plans in depth.

Assessment: The quality of the research being conducted is being assessed by high standards of peer-review. Manuscript (1) is attached as *Appendix 1.*

Monitoring: I am in weekly contact with the PhD students by email, and will meet them in July when I will assess progress. They will visit my laboratory probably in August.

Difficulties encountered and steps taken to overcome them.

Problems regarding the positioning of the Centre have been outlined at length already. These problems have been largely overcome with a suitable site now obtained in a school building. The problem is therefore surmountable, but will delay opening of the Centre. At times the delay in getting responses from Darwin staff has been frustratingly long (e.g. 3 months for approval to move the site).

I have found managing the project difficult at times because of a lack of support staff. To maximise the chances of getting the project funded, I had to work within a relatively limited upper budget. I wanted most of the funding to benefit conservation and science in China, so the vast majority of money is being channelled directly to Chinese sources (building costs, equipment, PhD stipends). Because I already have a busy schedule as a university professor, and because I am accustomed to running research projects with support staff (e.g. postdoctoral research assistants), I have found this project especially demanding to manage at times. I have to deal with tasks such as renegotiating money transfers between the Chinese Academy of Sciences and local councils: in most of my previous experience these tasks have been dealt with by support staff. I imagine these demands will diminish as the Chinese staff become more independent over time, and once the activities of the Centre are settled into a routine.

Timetable for the next reporting period.

Our revised project implementation timetable (as agreed by Darwin Secretariat) for the current financial year was

'*Planning and development of building (January 06). Enrol 2 PhD students, begin research projects (July 05). Employ staff member to run the Centre and develop material for it (July 05). Develop education material and displays for Centre.*'

This has largely been achieved, although progress towards developing educational material is proceeding slower than anticipated. I have obtained educational material from the Bat Conservation Trust and sent it to my Chinese collaborators to give them some ideas about pamphlets and booklets that they might develop.

For April 2006-March 2007 our project implementation timetable currently states

'*Building work completed within 6 months (July-August 06). Formal opening of the Darwin Initiative Centre for Bat Conservation (August 06). Begin intensive education programmes for school children and local communities. Ongoing research onto population estimates of bats and bat taxonom*y'.

A more detailed timetable is presented below.

	GJ visits China/SZ visits Bristol	*PhD students visit Bristol*	*Development of education material*	*Class visits to centre*	*PhD Research on taxonomy and ecology*	*Website development for identification key*
May 2006						
June 2006						
July 2006						
August 2006						
September 2006						
October 2006						
November 2006						
December 2006						
January 2007						
February 2007						
March 2007						

5. Actions taken in response to previous reviews

Not relevant.

6. Partnerships

Collaboration between the UK and China has been relatively straightforward to date. A meeting was held with staff at Kadoorie Farm conservation centre in Hong Kong, who are also planning development of educational material about bats. It may be possible to collaborate in this area.

7. Impact and Sustainability

Impact: the Darwin Initiative is acknowledged in research publications that will appear in international, peer-reviewed journals. We are expecting publicity to take off when the education centre opens.

Sustainability: because the school buildings have been bought, there is the potential to keep the Centre open long-term. We will investigate sustained funding for staff with the local council once the Centre is running.

8. Outputs, Outcomes and Dissemination

At present, we see no changes to the project outputs listed in the original application. Dissemination of results is being achieved through publication of scientific papers. The Centre has already been mentioned in a letter by Professor Zhang published in the journal *Science*.

Table 1. Project Outputs (According to Standard Output Measures)

Code No.	Description	Year 1 Total	Year 2 Total	Year 3 Total	Year 4 Total	TOTAL
1A,B	*PhD students*	*2*				
8	*Time spent in China*	*2 weeks*				
11A,B	*Papers to be published in peer-reviewed journals*	*2*				

One paper is in press and one in revision.

9. Project Expenditure

Table 3: Project expenditure during the reporting period (Defra Financial Year 01 April to 31 March)

10. Monitoring, Evaluation and Lessons

Quality of publications can be assessed by their acceptance in peer-reviewed journals. One of our manuscripts has already been accepted, and has attained our benchmark of expected quality.

Annex 1 Report of progress and achievements against Logical Framework for Financial Year: 2005/2006

Project summary	***Measurable Indicators***	***Progress and Achievements April 2005-Mar 2006***	***Actions required/planned for next period***
Goal**:** *To draw on expertise relevant to biodiversity from within the United Kingdom to work with local partners in countries rich in biodiversity but poor in resources to achieve* • *The conservation of biological diversity,* • *The sustainable use of its components, and* • *The fair and equitable sharing of the benefits arising out of the utilisation of genetic resources*			
Purpose (insert original project purpose statement) *Promotion of bat conservation in southern China by establishment of a bat conservation centre in Beijing. From the centre we will increase public awareness about bat conservation, improve identification of Chinese bats, and establish baseline data to assess population changes in Chinese bat populations.*	(insert original purpose level indicators) *Completion of Darwin Initiative Centre for Bat Conservation by yr 1.* *Education dissemination strategy implemented by year 1.* *Education material disseminated by yr 3.* *Bat identification key completed by yr 3.* *Baseline data on bat populations in cave sites.*	(report impacts and achievements resulting from the project against purpose indicators – if any) *A site for the centre has been purchased, and an education officer has been employed. A change in the site of the centre will result in a delay in completion. The site is now ready for occupation, and the education dissemination strategy will be discussed when Professor Jones visits China in July. Baseline data on bat populations in cave sites is being collected by Zhang Jinshuo.*	(report any lessons learned resulting from the project & highlight key actions planning for next period) *Original plans sometimes have to be changed in the light of unforeseen events. In this case, adverse reaction to findings about bats being reservoirs to emerging diseases resulted in us having to change location of the Darwin Centre. This will put a delay in getting activities at the site running.*
Outputs			
Foundation and running of Darwin Initiative Centre for Bat	*Building completed, staffed, displays and education material*	*Building ready for occupation, education officer employed.*	

Conservation.	*developed.*		
Identification key for Chinese bats (Chinese and English versions): online version to include echolocation calls and DNA sequences.	*Key published in a peer-reviewed journal. Online version accessible. One PhD student being trained.*	*Already have one paper in press, one under revision and one ready to submit on ecology and taxonomy of Chinese bats. PhD student employed. Echolocation calls collected and DNA sequenced from ca. 30 species to date.*	*Will begin putting key online when Chinese students visit Bristol later in 2006.*
Baseline data on population sizes of cave-dwelling bats.	*Population estimates published in scientific journal. One PhD student being trained.*	*Roost counts being collected, and PhD student employed.*	
Education packages for teachers and children.	*Visits to Centre by teachers and classes. Estimated 50 school visits by yr 3, and 'pyramid' teaching by educating teachers and allowing PhD students to train undergraduates, who will then visit schools.*	*Planned for coming year.*	
Lessons learned and best practices disseminated	*CCTV documentary broadcast by yr 3. Radio broadcasts, articles (3+) in popular science magazines (e.g. National Geographic China).*	*Planned for coming 2 years.*	

Note: Please do NOT expand rows to include activities since their completion and outcomes should be reported under the column on progress and achievements at output and purpose levels.

Appendix 1: *Project reference 14-008*

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RUNNING HEAD: Phylogenetics of East Asian lesser horsehoe bats

Phylogenetics of small horseshoe bats from East Asia based on mitochondrial DNA sequence variation

GANG LI, GARETH JONES, STEPHEN J. ROSSITER, SHIANG-FAN CHEN, STUART PARSONS, * SHUYI ZHANG

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We undertook analyses of mitochondrial DNA gene sequences and echolocation calls to resolve the phylogenetic and taxonomic relationships among the related bat taxa *Rhinolophus pusillus* (sampled across China), *R. monoceros* (Taiwan), *R. cornutus* (main islands of Japan) and *R. c. pumilus* (Okinawa, Japan). Phylogenetic trees and genetic divergence analyses were constructed by combining new complete mitochondrial cytochrome *b* gene sequences and partial mitochondrial control region sequences with published sequences. Our work showed that these four taxa formed monophyletic groups in the phylogenetic tree. However, low levels of sequence divergence among the taxa, together with similarities in body size and overlapping echolocation call frequencies point to a lack of taxonomic distinctiveness. We therefore suggest that these taxa are better considered as geographical subspecies rather than distinct species, though this should not diminish the conservation importance of these island populations, which are important evolutionary significant units. Based on our findings, we suggest the similarities in body size and echolocation call frequency in these rhinolophids result from their recent common ancestry, while similarities in body size and call frequency with *R. hipposideros* of Europe are the result of convergent evolution.

Key words: Chiroptera, Rhinolophidae, *Rhinolophus*, mitochondrial DNA, phylogenetics.

The phylogenetic relationships among several species of horseshoe bat of the *pusillus* subgroup (*Rhinolophus pusillus*, *R. monoceros*, *R. cornutus* and *R. c. pumilus*,) have been discussed by numerous researchers for many years (Corbet and Hill 1992; Csorba 1997; Csorba et al. 2003; Koopman 1994; Simmons 2005; Yoshiyuki 1989, 1990).

R. pusillus (the least horseshoe bat) was described initially from Java by Temminck (1834). Temminck (1834) also first described *R. cornutus* (the little Japanese horseshoe bat) from Japan, while *R. c. pumilus* (the Okinawa least horseshoe bat) is endemic to the Ryukyu Islands of Japan, situated between Japan and Taiwan, and was first described by Andersen (1905). *R. monoceros* (the Formosan lesser horseshoe bat), was described from Taiwan (where it is endemic) by Andersen (1905).

The separate species status of *R. pusillus* and *R. cornutus* was acknowledged by Yoshiyuki (1989, 1990), but questioned by Corbet and Hill (1992) who considered that they might be conspecific, with *R. pusillus* including the types *cornutus* and *pumilus*. *R. monoceros* was speculated to be conspecific with *R. cornutus* and/or *R. pusillus* for more than a decade by different authors (Corbet and Hill 1992; Csorba et al. 2003; Koopman 1994). More recently, Guillén-Servent et al. (2003) used complete mitochondrial cytochrome *b* (*Cytb*) gene sequences to reconstruct the phylogenetic tree of the family Rhinolophidae, but did not include *R. cornutus*, *R. monoceros* and *R. c. pumilus*, and, therefore, did not solve the systematic puzzle of these small horseshoe bats of East Asia. Csorba et al. (2003) reviewed the genus *Rhinolophus* and considered *R. monoceros*, *R. pusillus* and *R. cornutus* as independent species, with *R. c. pumilus* a subspecies of the latter. In the most recent assessment, Simmons (2005) agrees with this treatment, but acknowledges that all four species might be conspecific.

Mitochondrial DNA (mtDNA) data can provide valuable information on the evolutionary histories of closely related taxa, and have been widely used to evaluate the phylogenetic relationships of bats (e.g. Baker et al. 1994; Hoofer and Van Den Bussche 2001; Kawai et al. 2002). In this study, we combined new complete *Cytb* gene sequences and partial sequences of the mtDNA control region of *R. pusillus*, *R. monoceros* and *R.*

cornutus with other species of *Rhinolophus* and *Hipposideros* to analyze the phylogenetic relationships among these species. Additionally, we included a published sequence of *R. c. pumilus*, obtained from GenBank. We also obtained recordings of echolocation calls from *R. pusillus* and *R. monoceros*, and compared these with data obtained by other authors on *R. cornutus*. By analyzing divergence in mtDNA gene sequences and echolocation call frequencies, we aimed to resolve the taxonomic positions of these taxa.

Materials and Methods

Taxonomic sampling.—R. pusillus was sampled over a large area of its range in China, which extends from Beijing to Hainan across more than 21 degrees of latitude. Samples of *R. cornutus* and *R. monoceros* were obtained or collected from Japan and Taiwan respectively (see Table 1 for locality details). Samples were either 3-mm wing membrane biopsies from live animals (which were released after being punched) or liver biopsies taken from voucher specimens. For all bats, forearm lengths were measured with dial calipers to the nearest 0.1 mm.

*DNA extraction and amplification.—*All tissue was preserved in 75% ethanol until genomic DNA was extracted using a standard phenol/chloroform protocol (Maniatis et al. 1982) and stored at 4 °C. Complete mitochondrial *Cytb* sequences and partial control region sequences were obtained from eight individuals of *R. pusillus*, four of *R. cornutus* and five of *R. monoceros*, as well as one individual from each of the following five additional *Rhinolophus* species: *R. hipposideros*, *R. affinis*, *R. pearsonii*, *R. ferrumequinum* and *R. luctus*. We also amplified the corresponding sequences in two hipposiderid species (*Hipposideros armiger* and *H. pratti*), which were used as outgroups. Published mitochondrial sequences of *R. monoceros* and *R. pumilus* were also

obtained from the GenBank database. Sampling localities and Genbank accession numbers are given in Table1.

Complete *Cytb* gene sequences were amplified via the polymerase chain reaction (PCR) from each individual DNA sample. The amplification process was conducted as follows: 94 °C (5min), 35 cycles at 94 C (50s); 50 C (40s), 72 C (50s); 72 °C (5 min). PCR mixtures were prepared in 30 µl volumes with a final concentration of 0.4 µM of each primer, 0.2 µM of each dNTP, 1.5 µM $MgCl_2$ and 1 U of *Taq* DNA polymerase. The primers L14724 (5'-GGT CTT AGG CAA AAA ATT GGT GCA ACT C-3') (Kocher et al. 1989) and H15915R (5'-TCA GCT TTG GGT GTT GAT GG-3') (Irwin et al. 1991) were used for amplification. To improve amplification performance for some species, we also designed the primer 'Bat_Cytb_1' (5'-TAG AAT ATC AGC TTT GGG TG-3'), which was also used with L14724 (Kocher et al. 1989).

For the control region, the primers DLH 16750 (5'-CCT GAA GTA GGA ACC AGA TG- 3') (Wilkinson and Chapman 1991) and THRL 16272(5'-CCC GGT CTT GTA AAC C- 3') (Stanley et al. 1996) were used with the following thermal profile: 94 C (2 min); 34 cycles of 94 C (30 s), 55 C (30 s), 72 C (30 s); 72 C (10 min). We amplified and sequenced approximately 500 control region base pairs.

Genetic analyses and phylogenetic reconstructions.—Sequence data were aligned using CLUSTALX 1.81 (Thompson et al. 1997) with the default parameters, and sequence variation and divergence were calculated using MEGA3 (Kumar et al. 2004) under the Kimura 2-parameter model.

Prior to phylogenetic analysis, the most appropriate substitution model was determined for sequences using the program MODELTEST 3.06 (Posada and Crandall 1998). We combined the *Cytb* and control region sequences to reconstruct a maximum-

likelihood phylogenetic tree using PAUP* 4.0b (Swofford 2002). These analyses were performed using heuristic searches with tree bisection and reconnection branch swapping (TBR). To assess the robustness of tree topologies obtained, bootstrapping was carried out. 2000 replicates were set for neighbor joining (evolution substitution model of distance measure is decided by MODELTEST) and maximum parsimony as well as 100 replicates for maximum likelihood. Bootstrap test were undertaken using PAUP 4.0*b, incorporating all codon positions and substitutions. In addition, maximum posterior probability tree was constructed using the program MrBayes 3.1 (Huelsenbeck and Ronquist 2001). Five million generations were used for six simultaneous Markov chains, and the trees were sampled after one million generations, when the chains approached equilibrium. In the control block of the input file, we set code type fitting to vertebrate mitochondrial sequences.

Echolocation calls analyses.—Echolocation calls were recorded using a Pettersson D980 (Pettersson Elektronik AB, Uppsala, Sweden) bat detector, and 10x time expanded calls were downloaded to either a Sony WMD6C cassette recorder (Sony, Tokyo, Japan) or a Sony TCD-D8 DAT recorder. No measurable differences in call frequency occurred depending on which recording method was used. Echolocation calls were digitized using the sound analysis software BatSound Pro, v3.0 (Pettersson Elektronik AB). The maximum intensity of the constant frequency (CF) component of the second harmonic in the power spectrum was used for measuring the resting frequency (in kilohertz, kHz) of a call. A 4096-point fast Fourier transformation (FFT) and a Hanning window were used within a 5-kHz frequency range, giving a frequency resolution of 64 Hz. Call frequencies were stable within individual bats, and so one call was chosen at random from each bat for analysis. *R. pusillus* was recorded from Yunnan, Guangdong and Huibei Provinces in China, and *R. monoceros* was recorded throughout

its range in Taiwan. All bats emitted calls typical of horseshoe bats, with a long CF portion initiated and terminated by brief frequency-modulated sweeps.

Results

Genetic analyses.—The complete mitochondrial *Cytb* gene and partial control region sequences of the bats were deposited in GenBank (Table 1). The mitochondrial *Cytb* sequences contained 1,140 base pairs, beginning with the codon ATG and ending with AGA. No gaps were found in the *Cytb* sequences among species of *Rhinolophus* and *Hipposideros* studied. The number of conserved sites of *Cytb* gene sequences was 1,045 (92%). Correspondingly, 8 percent of variable sites and 4.4 percent of parsimony informative sites were found. 494 base pairs of control region sequences were combined to carry out the phylogenetic analyses, which including 411 conserved sites (83%) and 17% variable sites. 62 sites (12.55%) yielded information for parsimony analysis. As expected, the control region was more variable than the coding gene.

Pairwise genetic distances based on complete *Cytb* and partial control region sequences shows the high homogeneity of these mitochondrial sequences (Appendix 1). Sequence divergence between *R. cornutus* and *R. c. pumilus* was found to be only 1.6% at *Cytb* and 5.2% at control region sequences. Within *R. pusillus* samples from different areas of China, sequence divergence ranged from 0.2% to 1.7% at *Cytb* sequences (mean 1.2%), and from 0.4% to 5.8% (mean 4.2%) based on the control region. Sequence divergence between *R. monoceros* and *R. cornutus* ranged from 2.1% to 2.9% (mean 2.38%) at *Cytb* and from 5.3% to 7.1% (mean 6.1%) at the control region. Between *R. monoceros* and *R. pusillus*, genetic distance based on *Cytb* sequences ranged from 1.2% to 2.2% (mean 1.66%) and control region sequences ranged from 3.1% to 7.8% (mean

5.7%). The average *Cytb* and control region sequence divergences were 3.6% to 7.7% between *R. pumilus* and *R. pusillus*.

Our results show that the genetic distances among *R. pusillus. R. monoceros*, *R. cornutus* and *R. c. pumilus* are very low values, ranging from 1.6% to 4% (mean 2.31%) for the complete mitochondrial *Cytb* gene and 5.7% to 7.8% (mean 6.2%) for the partial control region. Moreover, sequence divergence within *R. pusillus* from China is often as high as that between currently recognized species. Overlap between sequence divergence values within *R. pusillus* is therefore often as high as between species. On the other hand, much higher sequence divergence was recorded between these and other rhinolophid species. Approximate divergence values of these taxa versus the other *Rhinolophus* species studied were 11% for *Cytb* and 14% for the control region.

Phylogenetic reconstructions.—For the combined sequences, the TVM+G+I model was selected and the relative base frequencies were: A = 0.32, T = 0.24, G = 0.12 and C = 0.32. Rate matrices were: A-C = 0.57, A-G = 5.01, A-T =0.79, C-G =0.07, C-T =5.02, G-T =1.0. The proportion of invariable sites equaled 0.54 and the Gamma distribution shape parameter equaled 1.44. We used these selected models and parameters for maximum likelihood analyses and Markov chain simulation.

Based on the combined sequences, both maximum likelihood (Fig. 1.—A) and maximum posterior probability (Fig. 1.—B) trees showed that *R. pusillus*, *R. monoceros*, *R. cornutus R. c. pumilus* together formed a monophyletic group that was supported strongly by high maximum likelihood bootstrap and posterior probability values, and that all four taxa formed separate monophyletic groups. The sister relationship between *R. monoceros* and *R. pusillus* from mainland China was also strongly supported by each tree with high bootstrap values (82 for maximum likelihood, 98 for neighbor joining (distance

measure used GTR model with gamma distribution), 86 for maximum parsimony) and a posterior probability value of 1.0. These two species are more related to each other than to the taxa of the Japanese archipelago (*R. cornutus*) and Ryukyu archipelago (*R. c. pumilus*). Both the maximum likelihood and Bayesian analyses indicate with high statistical support an earlier divergence between *R. c. pumilus* and the other small horseshoe bats studied, whereas the neighbor joining and maximum parsimony bootstrap test support a sister-relationship between *R. cornutus* and *R. c. pumilus,* supporting the current taxonomic status of these taxa. Each tree rejected a close relationship between these species and the similarly sized *R. hipposideros* from Europe, indicating that similar echolocation frequency and morphological features between these bats are convergent characters.

Echolocation calls analyses.—Echolocation calls from *R. pusillus* in China contained a frequency with most energy at 106.71 $\pm$ 2.47(SD) kHz (n = 24 bats: range 102-111 kHz). Averages obtained for males and females were 105.95 $\pm$ 2.72 kHz (n = 8) and 108.48 $\pm$ 2.57 kHz (n=5), respectively. The average forearm length of males was 36.9 $\pm$ 1.4 mm (n = 17: range 35.1-40.2 mm), and of females was 37.2 $\pm$ 1.5 mm (n=8: range 34.9-39.7 mm). *R. monoceros* from Taiwan emitted calls with a frequency of most energy at 112.72 $\pm$ 2.55 kHz (n=30: range 107-118 kHz). Males called with most energy at 111.71 + 2.22 kHz (n = 15) and had an average forearm length of 37.3 $\pm$ 1.2 mm (n = 15: range 35.6-39.2 mm). Female calls contained most energy at 113.73 $\pm$ 2.51 kHz (n = 15), and their average forearm length was 37.9 $\pm$ 1.1 mm (n = 15: range 35.0-39.9 mm). *R. cornutus* from Yakushima in Japan had a mean frequency of most energy at 108 kHz (D.A. Hill; unpublished data). Wallin (1969) gives the forearm length of *R. cornutus* as 38.0-40.7 mm, that of *R. pumilus* as 37.0-40.0 mm.

All taxa therefore show considerable overlap in body size and, as far as is known, in echolocation call frequency.

Discussion

We examined the phylogenetic relationships of horseshoe bats within the *pusillus* subgroup across much of their range in East Asia. *R. pusillus* was sampled from across China. The identification of two individuals captured in a cave near Beijing, previously considered northeast to the known range of this species, was confirmed by genetic analysis. The individual from Hainan Island (currently recognized as subspecies *R. pusillus parcus*), was not found to be divergent from the mainland populations of this species, and was nested within bats captured on other Chinese localities, despite Hainan's location 29.5 kilometers off the coast of southeast China (109°49′E,19°3′N).

The Ryukyu population of *R. c. pumilus* was allocated to a subspecific level by Andersen (1905), however, based on wing color and skull and dental characters, Yoshiyuki (1989) ranked this taxon as a distinct species. Other specimens from south China (Guangdong, Guangxi and Fujian provinces) were identified as *R. pumilus*, but were referred to *R. pusillus* later (Corbet and Hill 1992). Hill and Yoshiyuki (1980) analyzed the morphology of *R. pusillus* and *R. cornutus* and suggested that differences occurred in the structure of the connecting process between *R. pusillus* and *R. cornutus*, but speculated that there was a high likelihood that these bats were conspecific. Corbet and Hill (1992) and Koopman (1994) later regarded *R. cornutus* and *R. pusillus* as two distinct species based on their allopatric distributions.

R. monoceros is currently recognized as an insular endemic species and its distribution is restricted to Taiwan. Andersen (1905) described the type specimen of *R.*

monoceros and suggested that it was a new species which could be differentiated from *R. cornutus* by the shape of the lancet in the noseleaf. However other researchers suggested that *R. monoceros* might be conspecific with *R. cornutus* and/or *R. pusillus* (Corbet and Hill 1992; Csorba 1997; Koopman 1994).

Bradley and Baker (2001) surveyed *Cytb* divergence levels across 4 genera of rodents and 7 genera of bats, and showed that genetic distances can be used to broadly evaluate the systematic status of taxa. Specifically, they suggested that genetic distance values of <2% usually indicates conspecific populations, 2-11% encompasses conspecific populations and separate species, and so require further study, and >11% usually corresponds to distinct species. Levels of divergence in bats were also generally higher than equivalent taxonomic levels in rodents (Bradley and Baker 2001). Ditchfield (2000) analyzed the mitochondrial *Cytb* sequences from 275 individual bats of 17 species, and showed low levels of sequence divergence at *Cytb* with <4% divergence (usually 1%-2.5%) within bat species, while Ruedi and Mayer (2001) analyzed complete *Cytb* sequences of the genus *Myotis*, and showed >10% sequence divergence (average 15%) among congeners. In this current study, sequence divergence based on complete mitochondrial *Cytb* was consistently low among *R. pusillus*, *R. monoceros*, *R. cornutus* and *R. c. pumilus*, not exceeding 4%, and averaging just 2.3%. This high level of sequence homogeneity indicate that these bats have diverged relatively recently in their evolutionary history. In our dataset, the pairwise genetic distances of *Cytb* gene between other *Rhinolophus* species (excluding the divergence within the *pusillus* group of East Asia) ranged from 11% to 15% and the divergence values of *Rhinolophus* and outgroup (*Hipposideros*) averaged approximately 20%, with a minimum of 17%.

It would be unwise to determine species status solely on the basis of sequence divergence at one gene locus. Indeed, some bat species that look very different in terms of their morphology differ only slightly in mtDNA sequences (e.g. *Eptesicus serotinus* and *E. nilssonii*, Mayer and von Helversen 2001). Neither genetic nor morphological data alone are sufficient to distinguish species. The body sizes (as measured by forearm lengths) and echolocation call frequencies of *R. pusillus*, *R. monoceros* and *R. cornutus* all overlap considerably, supporting their classification as one species. Echolocation call frequencies can be informative in resolving taxonomic differences among bat species. For example, many cryptic species of bats are difficult to distinguish by morphological criteria, but differ considerably in the frequency of their echolocation calls (Jones and Barlow 2004). Although *R. monoceros* calls at a slightly higher frequency and is slightly larger than *R. pusillus*, the differences are small, and overlap is extensive. Call frequency of the similar sized *R. hipposideros* in Britain varies between 109-117 kHz and is partly explained by variation in sex and age (Jones et al. 1992), so intraspecific variation in call frequency can be substantial.

The major obstacle in determining whether allopatric populations should be given species rank is that reproductive isolation cannot be proven, because the populations are spatially separated. Whether reproductive isolation occurs among these taxa could only be determined if populations established secondary contact in the future. Helbig et al. (2002) provide useful criteria for deciding whether allopatric taxa of birds should be given species status. They proposed that species status should be assigned if taxa are fully diagnosable in each of several discrete or continuously varying characters related to different functional contexts (e.g. structural features, vocalizations) or DNA sequences, and the sum of the character differences corresponds to or exceeds the level of divergence seen in related species that coexist in sympatry. These criteria do not

appear to be met in our study species. Allopatric taxa can be deemed worthy of consideration as allospecies if at least one character is fully diagnostic or if taxa are fully diagnosable by a combination of two to three characters (Helbig et al. 2002). Allospecies are also usually unambiguously phenotypically divergent, which does not appear to be the case here. Our results show that the current classification of these taxa is inconsistent: *R. c. pumilus* is considered as a subspecies of *R. cornutus*, whereas *R. monoceros* is considered as a species distinct from *R. pusillus* even though these pairs of taxa show the same average level of genetic divergence. The most parsimonious and consistent explanation to is that *R. monoceros*, *R. cornutus* and *R. c. pumilus* are all island subspecies of the more widely distributed *R. pusillus*. However, this view should not devalue the importance of the island populations from a conservation perspective. As seen by the small frequency differences between calls of *R. pusillus* and *R. monoceros*, the island populations of these bats appear to diverging from the mainland *R. pusillus* stock, so their importance as evolving populations in the process of speciation cannot be overlooked. Indeed, the monophyly of all four taxa, together with the geographical distances between these islands and China, together indicate that genetic similarity stems from recent common ancestry rather than recurrent gene flow, and thus each population represents a separate evolutionary significant unit (ESU) (Moritz 1994).

A recent phylogenetic analysis of the Rhinolophidae placed *R. pusillus*, *R. cornutus* and *R. monoceros* in the *pusillus* subgroup, and *R. hipposideros* in the *hipposideros* subgroup (Guillén-Servent et al. 2003). This study also reported deep branching between these two subgroups, suggesting divergence occurred around 15 Mya. Thus, the similarity of echolocation calls between *R. hipposideros* and the bats in the *R. pusillus* subgroup studied here is best explained by convergent evolution in

body size (forearm length 34.4-39.3 mm, after Jones et al. (1992)), and hence call frequency, among these bats.

Estimates of the accumulated mutation rate for vertebrate mitochondrial DNA range 2–5% per million years (Arbogast and Slowinski 1998; Brown et al. 1979; Shields and Wilson 1987), with a 2% per million years mutation rate for *Cytb* (Arbogast and Slowinski 1998). Based on this approximation, divergence among *R. pusillus*, *R. monoceros*, *R. cornutus* and *R. c. pumilus* probably occurred around 1 Mya. The current wide geographic distribution of *R. pusillus* includes India, Nepal, Myanmar, south China, Vietnam, Laos, Thailand, Malaysia and Indonesia, while *R. cornutus* is endemic to the Japanese archipelago, where it is widely distributed (Csorba et al. 2003). The Japanese archipelago separated from the Eurasian continent in the Neocene, but the Japanese island of Hondo became connected to the Asian continent via a land bridge during three periods between 300,000 and 1 million years ago. Land bridges also formed between the Ryukyuan islands and the Japanese archipelago in the late Pleistocene (Ujiie 1998), providing opportunities for faunal exchange. We therefore suggest that the common ancestor of *R. pusillus*, *R. cornutus* and *R. c. pumilus* reached Japan via a land bridge in the middle of the Pleistocene Epoch, and subsequently began to diverge as the Japanese archipelago became isolated from the rest of the Asian mainland and Ryukyu Islands. Similarly, although the Taiwan Strait formed around 4 Mya (Hsu 1990), a land bridge has connected Taiwan to mainland Asia around 5 times in the past 4 million years (Creer et al. 2001; Fairbanks 1989; Gascoyne et al. 1979;Yu 1995), allowing colonization of *R. pusillus* into Taiwan. An apparent lack of exchange during the most recent land bridge (see Chen et al. 2006) has allowed these populations to diverge in allopatry, forming monophyletic groups.

The weight of evidence based on sequence divergence values, morphological measurements, echolocation call data, phylogenetic analyses and information about geological time of separation, all indicate that *R. pusillus, R. monoceros*, and *R. cornutus* and *R. c. pumilus* are best considered as populations of the same species. However, these populations are almost certainly reproductively isolated by sea barriers, and represent allopatric, evolving populations that merit substantial conservation effort.

Acknowledgements

We thank K. KAWAI and F. DAI for collecting bat tissue samples from Japan. We are also grateful to Kenting National Park and Yangmingshan National Park in Taiwan for granting permission to sample R. monoceros. This study was financed by the National Natural Science Foundation of China (Grant 30270169) and the National Geographic Society (Grant 7806-05 to S.Y. Zhang), a Joint Project Grant between the Royal Society (London) and the Chinese Academy of Sciences, and a Darwin Initiative grant (Grant 14-008) to G. Jones.

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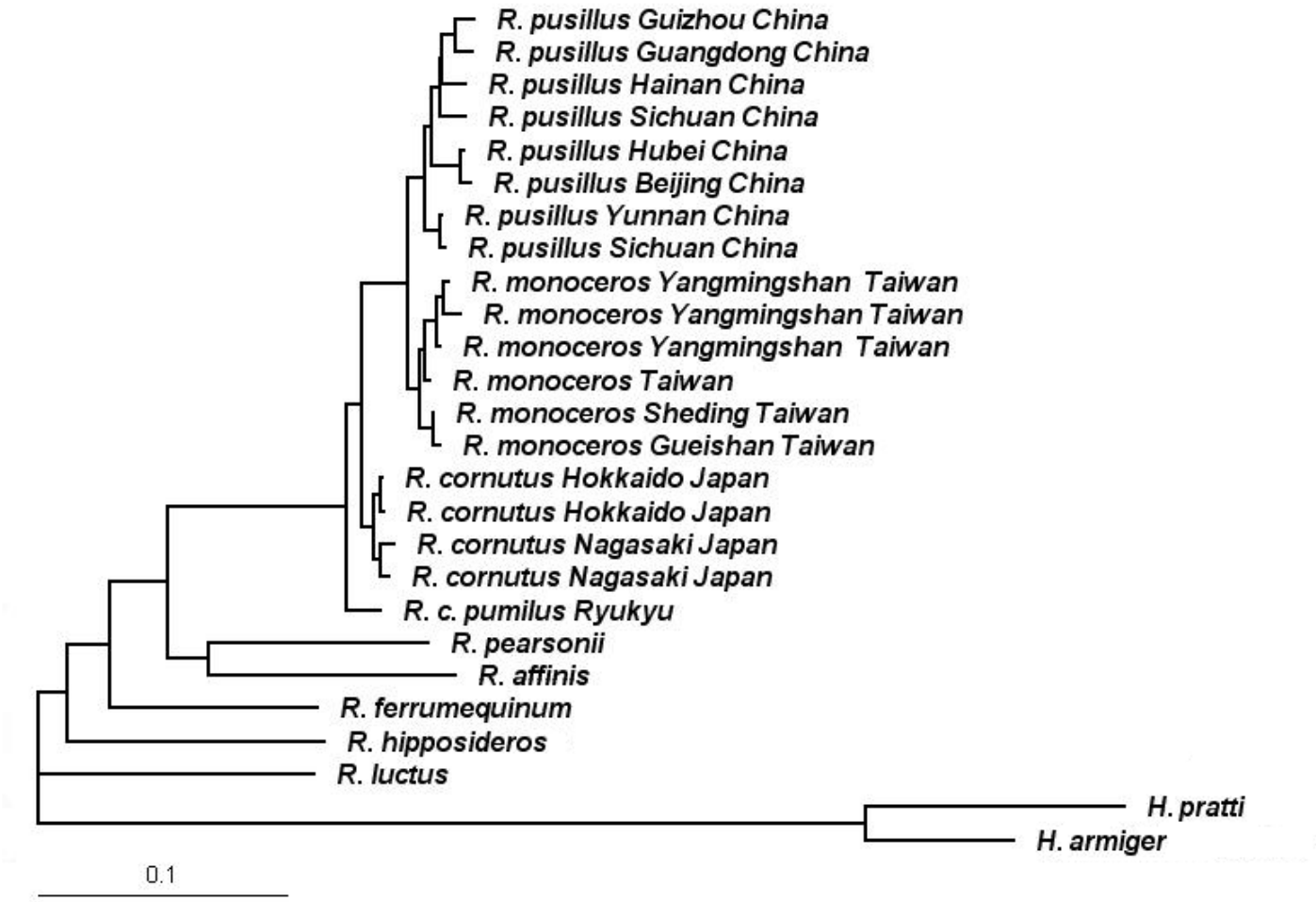
Table 1.—The collection localities of the bats analyzed, with the corresponding GenBank accession numbers.

Species	Collection locality	Accession number	
		Cytb	Control region
R. monoceros(1)	Sheding, Kenting, Taiwan	DQ297578	DQ297602
R. monoceros(2)	Gueishan, Kenting, Taiwan	DQ297579	DQ297603
*R. monoceros** (3)	Taiwan	NC_005433	NC_005433
R. monoceros(4)	Yangmingshan,Taiwan	DQ297580	DQ297604
R. monoceros(5)	Yangmingshan,Taiwan	DQ297581	DQ297605
R. monoceros(6)	Taiwan	DQ297576	DQ297600
R. pusillus(7)	Yunnan, China	DQ297574	DQ297598
R. pusillus(8)	Sichuan, China	DQ297595	DQ297618
R. pusillus(9)	Sichuan, China	DQ297589	DQ297613
R. pusillus(10)	Hainan, China	DQ297590	DQ297614
R. pusillus(11)	Guizhou, China	DQ297577	DQ297601
R. pusillus(12)	Guangdong, China	DQ297597	DQ297620
R. pusillus(13)	Hubei, China	DQ297583	DQ297607
R. pusillus(14)	Beijing, China	DQ297588	DQ297612
R. cornutus(15)	Kashi, Nagasaki, Japan	DQ297594	DQ297617
R. cornutus(16)	Kashi, Nagasaki, Japan	DQ297593	DQ297621
R. cornutus(17)	Okupirika, Hokkaido, Japan	DQ297591	DQ297615
R. cornutus(18)	Okupirika, Hokkaido, Japan	DQ297592	DQ297616
*R. c. pumilus*** (19)	Ryukyu	NC_005434	NC_005434
R. pearsonii(20)	Sichuan, China	DQ297587	DQ297611
R. affinis(21)	Guizhou, China	DQ297582	DQ297606
R. hipposideros(22)	Upper Langford, England	DQ297586	DQ297610

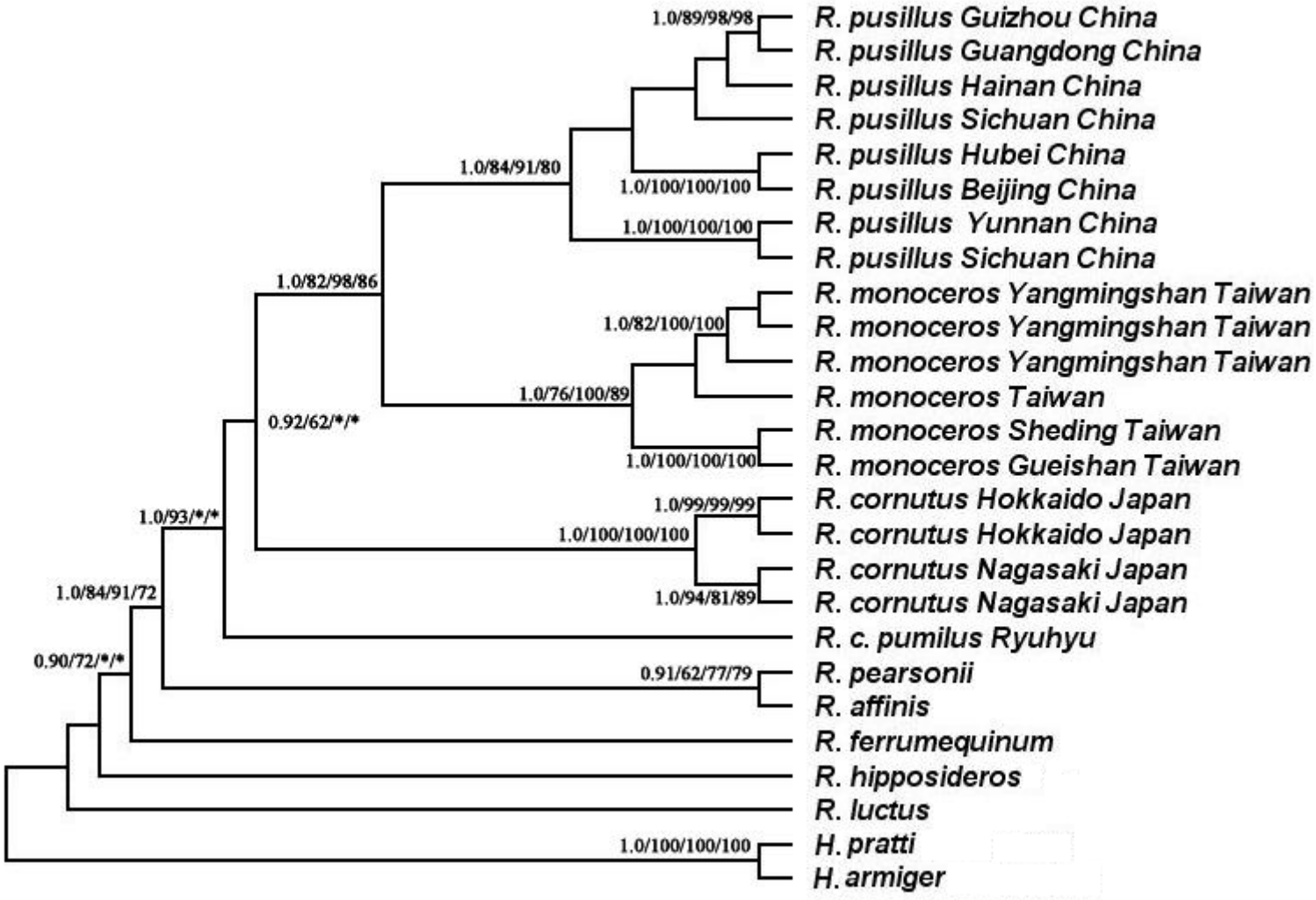
R. ferrumequinum(23)	Yunnan, China	DQ297575	DQ297599
R. luctus(24)	Huibei, China	DQ297596	DQ297619
Hipposideous armiger	Guizhou, China	DQ297585	DQ297609
H. pratti	Guangxi, China	DQ297584	DQ297608

Note: '*' (Lin et al. 2002) and '**' (Nikaido et al. 2001)indicate the sequences obtained from GenBank. The numbers in brackets correspond to the columns and rows in Appendix 1.

Fig. 1.— A: Maximum likelihood tree (lnL=-7992.58, with branch lengths) was constructed based on the combined mitochondrial complete cytochrome *b* gene and partial control region under TVM+G+I model using program PAUP*4.0b. B: Maximum posterior probability (MPP) tree (without branch lengths). *Hipposideros species* were designated as outgroups. The evolution model used in the MrBayes3.1 was also selected by MODELTEST 3.06. The numbers near the node indicate the posterior probabilities and bootstrap values of major nodes. The first number is posterior probabilities value, the 2nd, 3rd and 4th are the bootstrap values following maximum likelihood (100 replicates), neighbor joining (2000 replicates) and maximum parsimony (2000 replicates) methods respectively. The symbol '*****' indicate the relative method did not support this clade.


R. pusillus Guizhou China
R. pusillus Guangdong China
R. pusillus Hainan China
R. pusillus Sichuan China
R. pusillus Hubei China
R. pusillus Beijing China
R. pusillus Yunnan China
R. pusillus Sichuan China
R. monoceros Yangmingshan Taiwan
R. monoceros Yangmingshan Taiwan
R. monoceros Yangmingshan Taiwan
R. monoceros Taiwan
R. monoceros Sheding Taiwan
R. monoceros Gueishan Taiwan
R. cornutus Hokkaido Japan
R. cornutus Hokkaido Japan
R. cornutus Nagasaki Japan
R. cornutus Nagasaki Japan
R. c. pumilus Ryukyu
R. pearsonii
R. affinis
R. ferrumequinum
R. hipposideros
R. luctus
H. pratti
H. armiger
0.1

A:


1.0/89/98/98
R. pusillus Guizhou China
R. pusillus Guangdong China
R. pusillus Hainan China
R. pusillus Sichuan China
1.0/84/91/80
R. pusillus Hubei China
1.0/100/100/100
R. pusillus Beijing China
1.0/100/100/100
R. pusillus Yunnan China
R. pusillus Sichuan China
1.0/82/98/86
R. monoceros Yangmingshan Taiwan
1.0/82/100/100
R. monoceros Yangmingshan Taiwan
R. monoceros Yangmingshan Taiwan
1.0/76/100/89
R. monoceros Taiwan
0.92/62/*/*
R. monoceros Sheding Taiwan
1.0/100/100/100
R. monoceros Gueishan Taiwan
1.0/99/99/99
R. cornutus Hokkaido Japan
1.0/93/*/*
1.0/100/100/100
R. cornutus Hokkaido Japan
R. cornutus Nagasaki Japan
1.0/84/91/72
1.0/94/81/89
R. cornutus Nagasaki Japan
R. c. pumilus Ryuhyu
0.90/72/*/*
0.91/62/77/79
R. pearsonii
R. affinis
R. ferrumequinum
R. hipposideros
R. luctus
1.0/100/100/100
H. pratti
H. armiger

B:

APPENDIX I

Sequence divergence matrix based on complete mitochondrial *Cytb* sequences(1140 bp, above the diagonal) and partial control region region sequences(494 bp, below the diagonal).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1		**0.001**	**0.006**	**0.005**	**0.008**	**0.006**	**0.016**	**0.017**	**0.018**	**0.022**	**0.021**	**0.02**	**0.016**	**0.018**	**0.028**	**0.026**	**0.025**	**0.025**	**0.03**	0.109	0.116	0.14	0.117	0.132
2	**0.008**		**0.007**	**0.006**	**0.007**	**0.007**	**0.017**	**0.018**	**0.019**	**0.022**	**0.021**	**0.021**	**0.017**	**0.019**	**0.029**	**0.025**	**0.026**	**0.026**	**0.031**	0.11	0.117	0.141	0.118	0.133
3	**0.014**	**0.023**		**0.003**	**0.005**	**0.004**	**0.013**	**0.014**	**0.015**	**0.019**	**0.02**	**0.017**	**0.013**	**0.015**	**0.025**	**0.023**	**0.022**	**0.022**	**0.025**	0.11	0.117	0.14	0.117	0.131
4	**0.027**	**0.035**	**0.021**		**0.003**	**0.001**	**0.012**	**0.013**	**0.014**	**0.018**	**0.019**	**0.016**	**0.012**	**0.014**	**0.024**	**0.022**	**0.021**	**0.021**	**0.026**	0.109	0.116	0.139	0.117	0.132
5	**0.031**	**0.04**	**0.025**	**0.012**		**0.002**	**0.013**	**0.014**	**0.015**	**0.019**	**0.02**	**0.017**	**0.013**	**0.015**	**0.025**	**0.021**	**0.022**	**0.022**	**0.029**	0.112	0.12	0.14	0.12	0.135
6	**0.035**	**0.044**	**0.033**	**0.033**	**0.025**		**0.013**	**0.014**	**0.015**	**0.019**	**0.02**	**0.017**	**0.013**	**0.015**	**0.025**	**0.023**	**0.022**	**0.022**	**0.027**	0.11	0.117	0.137	0.118	0.133
7	**0.031**	**0.04**	**0.038**	**0.055**	**0.06**	**0.06**		**0.003**	**0.011**	**0.014**	**0.013**	**0.011**	**0.009**	**0.011**	**0.03**	**0.028**	**0.027**	**0.027**	**0.034**	0.111	0.119	0.141	0.119	0.136
8	**0.031**	**0.04**	**0.038**	**0.055**	**0.06**	**0.06**	**0.004**		**0.012**	**0.015**	**0.016**	**0.013**	**0.01**	**0.012**	**0.031**	**0.029**	**0.028**	**0.028**	**0.034**	0.112	0.12	0.142	0.123	0.137
9	**0.06**	**0.069**	**0.062**	**0.064**	**0.067**	**0.075**	**0.051**	**0.051**		**0.014**	**0.015**	**0.012**	**0.009**	**0.011**	**0.032**	**0.03**	**0.029**	**0.029**	**0.035**	0.114	0.123	0.141	0.117	0.134
10	**0.049**	**0.057**	**0.046**	**0.053**	**0.051**	**0.055**	**0.038**	**0.042**	**0.033**		**0.017**	**0.014**	**0.012**	**0.012**	**0.035**	**0.033**	**0.033**	**0.033**	**0.039**	0.114	0.117	0.136	0.12	0.132
11	**0.046**	**0.055**	**0.044**	**0.055**	**0.055**	**0.06**	**0.044**	**0.044**	**0.04**	**0.031**		**0.01**	**0.013**	**0.015**	**0.036**	**0.034**	**0.033**	**0.033**	**0.04**	0.113	0.123	0.142	0.119	0.137
12	**0.055**	**0.064**	**0.058**	**0.069**	**0.068**	**0.064**	**0.04**	**0.044**	**0.049**	**0.036**	**0.025**		**0.011**	**0.012**	**0.033**	**0.032**	**0.031**	**0.031**	**0.037**	0.112	0.12	0.14	0.116	0.134
13	**0.055**	**0.064**	**0.053**	**0.062**	**0.073**	**0.073**	**0.049**	**0.049**	**0.053**	**0.04**	**0.051**	**0.051**		**0.002**	**0.028**	**0.026**	**0.025**	**0.025**	**0.032**	0.111	0.121	0.136	0.117	0.132
14	**0.06**	**0.069**	**0.058**	**0.066**	**0.078**	**0.078**	**0.049**	**0.049**	**0.058**	**0.044**	**0.051**	**0.056**	**0.012**		**0.03**	**0.028**	**0.027**	**0.027**	**0.034**	0.111	0.121	0.134	0.115	0.13
15	**0.055**	**0.064**	**0.062**	**0.06**	**0.068**	**0.071**	**0.057**	**0.057**	**0.068**	**0.064**	**0.066**	**0.071**	**0.069**	**0.064**		**0.007**	**0.006**	**0.006**	**0.018**	0.117	0.122	0.143	0.117	0.13
16	**0.06**	**0.068**	**0.062**	**0.06**	**0.068**	**0.066**	**0.062**	**0.062**	**0.073**	**0.064**	**0.071**	**0.08**	**0.069**	**0.069**	**0.012**		**0.004**	**0.004**	**0.016**	0.116	0.12	0.144	0.115	0.131
17	**0.053**	**0.057**	**0.055**	**0.053**	**0.062**	**0.064**	**0.055**	**0.06**	**0.071**	**0.057**	**0.069**	**0.073**	**0.066**	**0.066**	**0.021**	**0.016**		**0**	**0.015**	0.114	0.115	0.139	0.112	0.128
18	**0.055**	**0.059**	**0.057**	**0.057**	**0.066**	**0.069**	**0.057**	**0.062**	**0.073**	**0.06**	**0.071**	**0.076**	**0.069**	**0.064**	**0.025**	**0.021**	**0.004**		**0.015**	0.114	0.115	0.139	0.112	0.128
19	**0.073**	**0.082**	**0.066**	**0.059**	**0.073**	**0.08**	**0.075**	**0.08**	**0.077**	**0.068**	**0.08**	**0.087**	**0.073**	**0.078**	**0.051**	**0.055**	**0.049**	**0.053**		0.114	0.122	0.136	0.112	0.127
20	0.162	0.169	0.149	0.154	0.164	0.17	0.154	0.159	0.164	0.156	0.154	0.148	0.154	0.161	0.148	0.151	0.141	0.143	0.146		0.121	0.131	0.123	0.13
21	0.167	0.174	0.159	0.159	0.169	0.172	0.177	0.183	0.182	0.167	0.177	0.164	0.158	0.169	0.166	0.172	0.166	0.172	0.164	0.147		0.14	0.121	0.135
22	0.157	0.163	0.152	0.155	0.16	0.163	0.147	0.152	0.14	0.14	0.155	0.145	0.155	0.155	0.157	0.162	0.155	0.16	0.15	0.184	0.202		0.125	0.129
23	0.168	0.175	0.17	0.168	0.173	0.173	0.168	0.173	0.152	0.142	0.158	0.16	0.165	0.171	0.157	0.16	0.15	0.155	0.155	0.166	0.187	0.138		0.126
24	0.178	0.188	0.178	0.186	0.185	0.188	0.17	0.175	0.167	0.16	0.17	0.173	0.183	0.183	0.162	0.175	0.167	0.173	0.175	0.186	0.21	0.17	0.155	

Note: 1 to 6 represent *R. monoceros*, 7 to 14 *R. pusillus*, 15 to 18 *R. cornutus*, 19 represents *R. c. pumilus*, and 20, 21, 22, 23, 24 correspond, respectively, to *R. pearsonii*, *R. affinis*, *R. hipposideros*, *R. ferrumequinum*, and *R. luctus*. The boldfaced numbers are the values of genetic distance among the little or lesser horseshoe bats of East Asia.