Phylogenetic relationships and evolutionary history of the greater horseshoe bat, *Rhinolophus ferrumequinum*, in Northeast Asia

Tong Liu [1] , Keping Sun [Corresp., 1] , Yung Chul Park [2] , Jiang Feng [1]

[1] Jilin Key Laboratory of Animal Resource Conservation and Utilization, Northeast Normal University, Changchun, China

[2] Department of Forest Environment Protection, College of Forest and Environmental Science, Kangwon National University, Chuncheon, South Korea

Corresponding Author: Keping Sun
Email address: sunkp129@nenu.edu.cn

The greater horseshoe bat, *Rhinolophus ferrumequinum*, is an important model organism for studies on chiropteran phylogeographic patterns. Previous studies revealed the population history of *R. ferrumequinum* from Europe and most Asian regions, but there have still been arguments about their evolutionary process in Northeast Asia. In this study, we obtained mitochondrial DNA cyt *b* and D-loop data of *R. ferrumequinum* from Northeast China, South Korea and Japan, to clarify their phylogenetic relationships and evolutionary process. Our results indicated a highly supported monophyletic group of Northeast Asian greater horseshoe bats, in which Japanese populations formed a single clade, and clustered into the mixed branches of South Korean and Northeast Chinese populations. We inferred that *R. ferrumequinum* in Northeast Asia could be originated in Northeast China and South Korea during a cold glacial period, then some ancestors might arrive in Japan by flying or land bridge and subsequently adapt to local environment. During the warm Eemian interglaciation, the Korea Strait became a barrier to Japanese populations and inland populations, while Changbai Mountains didn't play the role as geographical barriers between populations from South Korea and Northeast China.

1 **Phylogenetic relationships and evolutionary history of the greater horseshoe**

2 **bat, *Rhinolophus ferrumequinum*, in Northeast Asia**

3 Tong Liu[1], Keping Sun[1]*, Yung Chul Park[2], Jiang Feng[1]*

4 *[1]Jilin Provincial Key Laboratory of Animal Resource Conservation and Utilization,*

5 *Northeast Normal University, Changchun, China*

6 *[2]Department of Forest Environment Protection, College of Forest and*

7 *Environmental Science, Kangwon National University, Chuncheon 200-701, South*

8 *Korea.*

9 **Abstract** The greater horseshoe bat, *Rhinolophus ferrumequinum*, is an important

10 model organism for studies on chiropteran phylogeographic patterns. Previous

11 studies revealed the population history of *R. ferrumequinum* from Europe and most

12 Asian regions, but there have still been arguments about their evolutionary process

13 in Northeast Asia. In this study, we obtained mitochondrial DNA cyt *b* and D-loop

14 data of *R. ferrumequinum* from Northeast China, South Korea and Japan, to clarify

15 their phylogenetic relationships and evolutionary process. Our results indicated a

16 highly supported monophyletic group of Northeast Asian greater horseshoe bats, in

17 which Japanese populations formed a single clade, and clustered into the mixed

18 branches of South Korean and Northeast Chinese populations. We inferred that *R.*

19 *ferrumequinum* in Northeast Asia could be originated in Northeast China and South

20 Korea during a cold glacial period, then some ancestors might arrive in Japan by

21 flying or land bridge and subsequently adapt to local environment. During the

22 warm Eemian interglaciation, the Korea Strait became a barrier to Japanese

23 populations and inland populations, while Changbai Mountains didn't play the role

24 as geographical barriers between populations from South Korea and Northeast

25 China.

26 **Introduction**

27 During the past 2.5 million years, earth has been in Quaternary glacial and

28 interglacial periods (Capinera, 2011). Climatic change and the existence of refugia

29 have influenced effective population size and demographic history of organisms,

30 and left genetic signatures in current populations (Avise, 2000; Hewitt, 2000; Qu et

31 al., 2014). The effective population size of organisms may decrease during the

32 glacial period or remain stable or grow due to intermittent gene flow between

33 refugia during warming periods (Li et al., 2009; Qu et al., 2014). The large volume

34 of accumulated ice during Quaternary glaciation period caused a worldwide sea

35 level drop by 120-140 m below the present sea level (Lambeck et al., 2002). Land

36 bridges appeared in several parts of the world, inevitably led to range changes for

37 most living organisms (Hewitt, 2000). Then, organisms adapted to different

38 environments and new neighbors, which would cause their genetic variation, both

39 through selection and drift, and ultimately led to speciation (Harrison, 1993;

40 Hewitt, 2000).

41 The greater horseshoe bat, *Rhinolophus ferrumequinum* (Rhinolophidae,

42 *Rhinolophus*), is widely distributed in northern Africa, southern Europe, and Asia

43 (Csorba et al., 2003). In China, it ranges from northeastern to southwestern regions

44 (Wang, 2003). Previous studies (Rossiter et al., 2000; Rossiter et al., 2007; Bilgin et

45 al., 2009; Flanders et al., 2009; Flanders et al., 2011) have revealed the impact of

46 glaciations on their geographic patterns in Europe and most Asian regions, but little

47 is known about this species from Northeast Asia. Flanders et al. (2009, 2011)

48 showed that *R. ferrumequinum* from Jilin Province was affiliated with those from

49 Japan unexpectedly based on mtDNA *ND2* gene, which suggested the greater

50 horseshoe bats might move between east China and Japan by using South Korea as

51 a stepping-stone (Flanders et al., 2009), or by Korean Peninsula-Japanese land

52 bridge during glacial period (Flanders et al., 2011). However, no Korean sample

53 and only one sample from Jilin Province were analyzed in Flanders et al. (2009,

54 2011). Their results indicated that Jilin's sample was located in East clade of China,

55 whereas Sun et al. (2013) showed that all samples from Jilin Province were divided

56 into another Northeast clade based on mtDNA D-loop region. Therefore, it's very

57 necessary to combine more samples from Northeast China, South Korea and Japan,

58 to investigate the population evolutionary process of *R. ferrumequinum* in

59 Northeast Asia.

60 Natural landscape features, such as mountains and rivers, can function as genetic

61 boundaries and shape the population structure of animals because they can hinder

62 dispersal and gene flow (Funk et al., 2008; Bilgin et al., 2009; Fünfstück et al.,

63 2014). For *R. ferrumequinum* in Northeast Asia, Yalu River and Changbai

64   Mountains separated the populations from Northeast China and Korean Peninsula.

65   The Korea Strait separated the populations in Korea from those in Japan. Koh et al.

66   (2014) considered that the Yalu River and Changbai Mountains did not play the

67   role as physical barriers for Korean populations and adjacent Northeast Chinese

68   populations in *R. ferrumequinum* based on mtDNA cyt *b* gene. However, only one

69   sample from Northeast China was included in their analyses.

70   In this study, we collected and sequenced mtDNA cyt *b* and D-loop sequences of

71   more *R. ferrumequinum* samples from Northeast China and South Korea, and

72   analyzed them with all of the previously published mtDNA sequences from China,

73   Japan and South Korea. Our aims are (i) to clarify the phylogenetic relationships of

74   *R. ferrumequinum* in Northeast Asia, (ii) to infer their evolutionary process in

75   Northeast Asia, and (iii) to detect whether the Changbai Mountains and Korea

76   Strait act as geographical barriers for *R. ferrumequinum*.

77   **Materials and methods**

78   Twenty-two and 49 individuals of *R. ferrumequinum* from Northeast China and

79   South Korea were sampled and used to sequence mtDNA cyt *b* and D-loop region,

80   respectively. Total 76 sequences (63 cyt *b* sequences and 13 D-loop sequences) of

81   *R. ferrumequinum* were collected from Japan, South Korea and China (Fig.1; Table

82   S1, supporting information). For the D-loop region, our study did not include the

83   sequences from Japan because no D-loop sequence of Japanese *R. ferrumequinum*

84 was deposited in GenBank. All field studies were approved by National Animal

85 Research Authority in Northeast Normal University, China (approval number:

86 NENU-20080416).

87 Previous studies and this study both showed South Korean, Japanese and Northeast

88 Chinese *R. ferrumequinum* have very low genetic divergence (Sakai et al., 2003;

89 Koh et al., 2014). Therefore, we regarded the bats from South Korea, Japan or

90 Northeast China as a single geographic population.

91 **DNA extraction and amplification**

92 Bats' wing membrane tissues were taken and stored at 95% ethanol solution until

93 genome extraction by the UNIQ-10 Column Animal Genomic DNA Isolation Kit

94 (SK1205) (Sangon, China). Mitochondrial DNA cyt *b* and D-loop region were

95 amplified by polymerase chain reaction (PCR) using universal primers L14724 and

96 H15915 (Irwin et al., 1991) and P and E (Wilkinson & Chapman, 1991),

97 respectively. Amplified products were purified and sequenced by Shanghai Sangon

98 Biotechnology Co., Ltd. Sequences were edited and aligned using Geneious v8.0.2

99 (Kearse et al., 2012), and then were assembled by eye. Sequence data were

100 deposited in GenBank (accession number: KX237527-KX237538 and KX237546).

101 **Genetic diversity**

102 Haplotype diversity, nucleotide diversity and polymorphic sites of each population

103 were calculated based on cyt *b* and D-loop sequences, respectively. The gene flow

104 was calculated using cyt *b* gene sequences based on an equation: $F_{st} = 1 / (1 +$

105   4Nm). All calculations were carried out using DnaSP v4.0 (Rozas et al., 2003).

106   Pairwise distances between populations were obtained using Kimura-2-Parameter

107   (K2P) distance model (Kimura, 1980), with 1,000 bootstrap replications, using

108   MEGA v5.0 (Tamura et al., 2013).

109   **Phylogenetic analysis**

110   Phylogenetic trees of cyt *b* and D-loop were reconstructed using maximum

111   likelihood (ML) methods in PhyML v3.1 (Guindon et al., 2010) and Bayesian

112   Inference (BI) in MrBayes v3.2.2 (Ronquist & Huelsenbeck, 2001; Ronquist &

113   Huelsenbeck, 2003). PhyML starts with a BioNJ tree by default (Gascuel, 1997),

114   and statistical support for branching patterns was estimated by bootstrap with 1,000

115   replicates. BI was run with four Markov Chains Monte Carlo (MCMC), each of

116   1×10^7 generations, sampled every 100 generations. Two congeneric species from

117   the Afro-Paleartic clade, *Rhinolophus euryale* (GenBank nos. EU436671 and

118   KF031268) and *R. fumigatus* (GenBank nos. EU436678 and KU531336) were used

119   as outgroups.

120   ML and BI methods are sensitive to nucleotide substitution models, which can be

121   estimated by jModelTest v0.1 (Posada, 2008). According to the Akaike information

122   criterion (AIC) (Posada & Buckley, 2004), the HKY + G (transition/transversion =

123   12.9820; gamma shape = 0.1510) and HKY + G (transition/transversion = 4.5832;

124   gamma shape = 0.1840) were selected for cyt *b* and D-loop, respectively.

125   The program NETWORK v4.6 (Bandelt et al., 1999) was used to build a maximum

126 parsimony network using median-joining method which simplified same-possible

127 trees and removed redundant nodes and connections (Puizina et al., 2013). This

128 method has advantage in representing the intraspecific genetic variation (Posada &

129 Crandall, 2001).

130 In order to estimate divergent time, the time to the most recent common ancestor

131 (TMRCA) was estimated using BEAST v1.6 (Drummond & Rambaut, 2007) under

132 a strict clock and a constant-size tree prior. The chain was run for 1×10^7

133 generations, with the ESS values >200 taken as evidence for convergence. Cyt *b*

134 gene was chosen to calculate TMRCA because of its moderate evolutionary rate. A

135 mean substitution rate of 1.3% per million years (Nabholz et al., 2008) used in

136 *Hipposideros turpis* complex (Thong et al., 2012)*, Hipposideros commersoni*

137 (Rakotoarivelo et al., 2015) and *Myotis nattereri* complex (Puechmaille et al.,

138 2012) was used in this analysis.

139 **Demographic analysis**

140 The demographic expansion of Northeast Asian *R. ferrumequinum* was examined in

141 Arlequin v3.1 (Schneider et al., 2000). Tajima's *D* test (Tajima, 1989) and Fu's *Fs*

142 test (Fu, 1997) were employed to confirm neutral expectation based on 1,000

143 coalescent simulations. Significant negative Tajima's *D* and Fu's *Fs* values indicate

144 a sudden expansion, whereas significant positive values indicate processes such as

145 population subdivision or recent bottlenecks. When the values nearly zero, they

146 represent population of constant size (Liao et al., 2010). Then the raggedness index

147 (Hri; Harpending, 1994) and sum of squared deviations (SSD; Schneider &

148 Excoffier, 1999) were generated with 10,000 replicates parametric bootstrapping.

149 Hri was calculated to describe the smoothness of observed mismatch distribution.

150 The small value means a population which has experienced sudden expansion

151 event, and the higher value indicates a stationary or bottlenecked population

152 (Harpending, 1994; Liao et al., 2010). SSD value was used to describe the

153 goodness-of-fit of observed mismatch distribution to that expected under the spatial

154 expansion model. A non-significant SSD value ($P_{SSD} > 0.05$) suggests a good fitness

155 (Excoffier et al., 2005). The mismatch distribution graphs were drawn in DnaSP

156 v4.0. A smooth or unimodal mismatch distribution indicates an expanded

157 population, while a ragged or multimodal distribution indicates a more stable

158 population (Rogers & Harpending, 1992; Flanders et al., 2011).

159 If the expansion was detected, the time of expansion in generations (*t*) can be

160 estimated by an equation,τ= 2*ut*, whereτ(tau) means the time to expansion in

161 mutational units and *u* is the mutation rate per generation for the DNA sequence

162 being studied. Cyt *b* mutation rate is 1.3% per million years (Nabholz et al., 2008).

163 The generation time is 2 years (Ransome, 1995).

164 **Results**

165 **Genetic diversity and divergence**

166 A total of 85 sequences based on cyt *b* gene (1140 bp) and 62 sequences based on

167   D-loop region (465 bp) were obtained and analyzed (Table S1, supporting

168   information). For cyt *b* gene, 15 different haplotypes were identified from 79

169   sequences of Northeast Asian *R. ferrumequinum*. Japanese population had 7 unique

170   haplotypes, while Chinese and South Korean populations shared haplotype H15,

171   which was the most shared haplotype. For D-loop region, 10 unique haplotypes

172   were identified from 51 sequences of *R. ferrumequinum* in Northeast Asia. H9 was

173   shared by individuals from Ji'an and Benxi in Northeast China; whereas H10 was

174   shared by most individuals and populations (including individuals in all four

175   localities of China and some individuals in South Korea) (Table S2, supporting

176   information).

177   Within cyt *b* and D-loop haplotypes, there were 15 (1.3%) and 9 (2.0%)

178   polymorphic sites and 10 (0.87%) and 5 (1.1%) parsimonious informative sites,

179   respectively. Genetic diversity of South Korean population was highest, while that

180   of the Northeast Chinese population was lowest (Table 1).

181   The cyt *b* divergence of Northeast Asian *R. ferrumequinum* was lower than 1%.

182   The average K2P distances between populations from Northeast China and South

183   Korea (0.07%) were lower than those between Japanese and other Northeast Asian

184   populations (0.21%-0.26%). Furthermore, the gene flow between South Korea and

185   Northeast China (Nm$\geq$3) were highest, which was enough to prevent genetic

186   divergence caused by genetic drift (Slatkin, 1987; Yang et al., 2011). However, the

187   gene flow levels between Japanese and the other Northeast Asian populations were

188 low (Table 2).

189 Phylogenetic relationships and TMRCA

190 The ML and BI tree topologies based on cyt *b* gene produced highly concordant

191 phylogenetic relationships. All samples from Northeast Asia formed a highly

192 supported monophyletic clade (Fig. 2). In the tree, the relationship between

193 Northeast Chinese and South Korean haplotypes was much less resolved, probably

194 because of only a few mutations. Japanese haplotypes formed their own clade, but

195 with relatively low bootstrap values (62/79% in ML/BI, respectively) (Fig. 2A).

196 The Japanese clade clustered into the mixed branches of South Korean and

197 Northeast Chinese haplotypes. The haplotypes of Northeast Asia were sister to

198 those of the Central-East China (Fig. 2A). The haplotype network showed similar

199 relationships with the phylogenetic trees, but showing relationship between the

200 haplotypes more clearly (Fig. 2B).

201 The noncoding D-loop region generally provides sufficient variation for studies at

202 intraspecific level (Qu et al., 2009). However, in the phylogenetic tree and

203 haplotype network based on D-loop region, the Northeast Chinese haplotypes were

204 still hardly separated from South Korean haplotypes (Fig. 3).

205 The TMRCA of all examined *R. ferrumequinum* individuals could be dated to 1.87

206 Ma (95% CI 1.39-2.35 Ma). The TMRCA estimates obtained for Clade CE

207 China/Northeast Asia were 0.87 Ma (95% CI 0.60-1.16 Ma). For East Asian *R.*

208 *ferrumequinum*, the TMRCA was 0.22 Ma (95% CI 0.12-0.34 Ma), which could be

209   traced back to late Pleistocene.

210   **Historical demography**

211   Mismatch distribution analysis based on cyt *b* revealed different historical

212   demography. Japanese and South Korean populations failed to reject the model of

213   population expansion based on Hri, SSD (P_{SSD}> 0.05, P_{Hri}> 0.05) (Table 3), and

214   their smooth or unimodal mismatch distributions (Fig. S1, supporting information).

215   The significant negative Fu's *Fs* value also indicated a sudden expansion. The most

216   recent expansion times were estimated to be 0.15 Ma (95% CI 0.05-0.27 Ma) and

217   0.12 Ma (95% CI 0.03-0.20 Ma) for Japanese and South Korean populations,

218   respectively. However, the high Hri, non-significant positive Fu's *Fs* value and

219   multiple mismatch distribution of Northeast Chinese population suggested a stable

220   population history or population bottlenecks (Liao et al., 2010) (Table 3, Fig S1,

221   supporting information).

222   **Discussion**

223   *Rhinolophus ferrumequinum* from Northeast Asia diverged from other populations

224   during 0.87-0.22 Ma, corresponding to Quaternary Pleistocene (Ehlers et al., 2011).

225   Climate fluctuations of this epoch are supposed to have played important roles in

226   shaping the geographical distribution, historical demography and genetic

227   diversification of many organisms in the Palaearctic region (Qu et al., 2009).

228   In this study, the TMRCA of the greater horseshoe bats from Northeast Asia could

229 date back to 0.22 Ma (95% CI 0.12-0.34 Ma), a time at the Saale glaciation (0.13-

230 0.30 Ma) (Lisiecki & Raymo, 2005). Accompanied with temperature dropping in

231 this period, the sea level declined gradually, and reached the lowest (about 130 m

232 lower than it is today) at 0.14 Ma (Rohling et al., 1998; Molodkov &

233 Bolikhovskaya, 2002), which could be beneficial for bats to go through the sea.

234 Flanders et al. (2011) supposed that the greater horseshoe bats are most likely to

235 have originated in Shandong Province of China. Therefore, we inferred that *R.*

236 *ferrumequinum* might disperse to Northeast China and South Korea from Shandong

237 Province by the Bohai Sea, because this is the shortest way and the Bohai Sea

238 would have disappeared if the sea level dropped 120 m (Ray & Adams, 2001).

239 Furthermore, haplotype network seemed to support this scenario, where H15 is a

240 most likely ancestor haplotype occupying the center of network with lots of

241 connects (Fig. 2B).

242 In Northeast China, the population might have undergone a founder effect or a

243 bottleneck event due to its low genetic diversity. However, a specific haplotype

244 H14 was detected in Northeast China, which may be not congruent with founder

245 effect. Combining the high Hri and non-significant positive Fu's *Fs* value, we

246 inferred a bottlenecked event may have occurred in Northeast China (Liao et al.,

247 2010). However, compared with previous studies on *R. ferrumequinum* (Flanders et

248 al., 2011; Sun et al., 2013), an expanding event was firstly detected at 0.12 Ma

249 (95% CI 0.03-0.20 Ma) in the South Korean population, which corresponds to the

250 relatively warm Eemian interglaciation (0.12-0.13 Ma) in Pleistocene.

251 The Changbai Mountains are the boundary of Northeast China and Korean

252 Peninsula. Extremely low genetic divergence and high gene flow level between

253 populations from Northeast China and South Korea suggested that the Changbai

254 Mountains haven't acted as geographic barriers. In previous studies, Sun et al.

255 (2013) and Flanders et al. (2011) found the Qinling Mountains have played an

256 important role in forming different lineages of *R. ferrumequinum* bats; however,

257 Rossiter et al. (2007) considered the Pyrenees (above 2,000 meters) haven't

258 hindered gene flow of *R. ferrumequinum* and Bilgin et al. (2009) showed the

259 Taurus Mountains and eastern Anatolian Diagonal Mountain Chain haven't limited

260 western clade of *R. ferrumequinum* bats' distribution. Therefore, the isolation effect

261 of different mountains is variable. Moreover, we can't rule out other reasons, such

262 as incomplete lineage sorting and ancestral ploymorphism, which can also cause

263 low divergence between populations from Northeast China and South Korea

264 (Flanders et al., 2009).

265 In our study, Japanese population formed a single sub-clade and diverged more

266 recently than the populations from Northeast China and South Korea (Fig. 2A),

267 which is contrast with that inferred by Flanders et al. (2009, 2011). Flanders et al.

268 (2009) considered that *R. ferrumequinum* might colonize from Japan to East China.

269 However, our results showed that Japanese population colonized most recently

270 from Eurasian continent. It was known that the Korea Strait is about 130 m deep, so

271 the land bridge can only be formed during main glacial period (McKay, 2012).

272 Ohshima (1990) mentioned that the Korean Peninsula-Japanese land bridge was

273 estimated to be remained in place until 0.15 Ma (also see Watanobe, Ishiguro &

274 Nakano, 2003). Thus, we inferred that the emergence of land bridge favored some

275 *R. ferrumequinum* bats colonize in Japan from northeast China and South Korea.

276 Other mammals, such as Japanese wild boar (*Sus scrofa leucomystax*) (Watanobe et

277 al., 2003), sika deer (*Cervus nippon*) (Nagata et al., 1999) and Asian black bear

278 (*Ursus thibetanus*) (Ohnishi et al., 2009) were also found to colonize in Japan from

279 Eurasian continent via Korean Peninsula-Japanese land bridge (Flanders et al.,

280 2011).

281 However, some studies showed that Japan was not connected to Eurasian continent

282 during this period. There was a narrow seaway (about 20 km wide) in Korea Strait

283 connecting the East China Sea and the East Japan Sea (Park et al., 2000). Bats of *R.*

284 *ferrumequinum* are able to fly, typically travelling up to 30 km between the winter

285 and summer roosts, with the longest recorded movement being 180 km (Paz et al.,

286 1986). And Bilgin et al. (2009) found the Marmara Sea (70 km) does not seem to

287 limit the dispersal in *R. ferrumequinum*. Although narrow seaway would have been

288 present, *R. ferrumequinum* from Eurasian continent could go through the Korea

289 Strait and enter Japan during the glacial period. *Rhinolophus ferrumequinum* in

290 Japan might have expanded at 0.15 Ma (95% CI 0.05-0.27 Ma), which is consistent

291 with the expansion time (0.13-0.19 Ma) calculated by Flanders et al. (2011). This

292 expansion time is in the Saale glaciation, which suggested that Japan might act as a

293 refuge for mammals in Northeast Asia during glacial periods.

294 With the arrival of Eemian interglaciation (0.12-0.13 Ma), the temperature was

295 higher gradually, resulting in rising of sea level. Korea Strait became a natural

296 barrier which isolated Japanese *R. ferrumequinum* from other Eurasian continental

297 populations. Some other studies on bats also showed that gene flow can be

298 hindered by water bodies, such as the Taiwan Strait (131 km) and English Channel

299 (100 km) (Chen et al., 2006; Rossiter et al., 2007). The wider Korea Strait (180 km)

300 was inferred to play an important role in acting as a barrier to hinder the gene flow

301 between Japanese and Eurasian continental populations.

302 **Acknowledgements**

303 We would like to thank Tinglei Jiang and Guanjun Lu who worked hard with us in

304 the field to collect the samples used in this study. We also thank Ying Tang for the

305 lab work.

Reference

Avise JC. 2000. *Phylogeography: the history and formation of species.* Harvard: Harvard university press.

Bandelt HJ, Forster P, and Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Phylogenetics and Evolution* **16**:37-48.

Bilgin R, Çoraman E, Karataş A, and Morales JC. 2009. Phylogeography of the Greater Horseshoe Bat, *Rhinolophus ferrumequinum* (Chiroptera: Rhinolophidae), in Southeastern Europe and Anatolia, with a Specific Focus on Whether the Sea of Marmara is a Barrier to Gene Flow. *Acta Chiropterologica* **11**:53-60.

Capinera J. 2011. *Insects and wildlife: arthropods and their relationships with wild vertebrate animals.* John Wiley & Sons.

Chen Sf, Rossiter SJ, Faulkes CG, and Jones G. 2006. Population genetic structure and demographic history of the endemic Formosan lesser horseshoe bat (*Rhinolophus monoceros*). *Molecular Ecology* **15**:1643-1656.

Csorba G, Ujhelyi P, and Thomas N. 2003. *Horseshoe bats of the world:(Chiroptera: Rhinolophidae).* Alana Books.

Drummond AJ, and Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**:214.

Ehlers J, Gibbard PL, and Hughes PD. 2011. *Quaternary glaciations-extent and chronology: a closer look.* Elsevier.

Excoffier L, Laval G, and Schneider S. 2005. ARLEQUIN ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics* **1**:47-50.

Fünfstück T, Arandjelovic M, Morgan DB, Sanz C, Breuer T, Stokes EJ, Reed P, Olson SH, Cameron K, and Ondzie A. 2014. The genetic population structure of wild western lowland gorillas (*Gorilla gorilla gorilla*) living in continuous rain forest. *American journal of primatology* **76**:868-878.

Flanders J, Jones G, Benda P, Dietz C, Zhang S, Li G, Sharifi M, and Rossiter SJ. 2009. Phylogeography of the greater horseshoe bat, *Rhinolophus ferrumequinum*: contrasting results from mitochondrial and microsatellite data. *Molecular Ecology* **18**:306-318.

Flanders J, Wei L, Rossiter SJ, and Zhang S. 2011. Identifying the effects of the Pleistocene on the greater horseshoe bat, *Rhinolophus ferrumequinum*, in East Asia using ecological niche modelling and phylogenetic analyses. *Journal of Biogeography* **38**:439-452.

Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**:915-925.

Funk WC, Forsman ED, Mullins TD, and Haig SM. 2008. *Landscape features shape genetic structure in threatened Northern Spotted Owls.* US : Geological Survey (US).

Gascuel O. 1997. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *molecular biology and evolution* **14**:685-695.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, and Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic biology* **59**:307-321.

Harpending H. 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human biology*:591-600.

Harrison RG. 1993. *Hybrid zones and the evolutionary process.* Oxford: Oxford University Press on Demand.

Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. *NATURE* **405**:907-913.

Irwin DM, Kocher TD, and Wilson AC. 1991. Evolution of the cytochrome *b* gene of mammals. *Journal of molecular evolution* **32**:128-144.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, and Duran C.

348   2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and
349   analysis of sequence data. *Bioinformatics* **28**:1647-1649.

350   Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative
351   studies of nucleotide sequences. *Journal of molecular evolution* **16**:111-120.

352   Koh HS, Jo JE, Oh JG, Kweon GH, Ahn NH, Sin WH, and Sin DS. 2014. Little genetic divergence of the greater
353   horseshoe bat *Rhinolophus ferrumequinum* from far-eastern Asia, with a preliminary report on genetic
354   differentiation of *R. ferrumequinum* from Eurasia and northern Africa examined from cytochrome *b*
355   sequences. *RUSSIAN JOURNAL OF THERIOLOGY* **13**:97-103.

356   Lambeck K, Esat TM, and Potter E-K. 2002. Links between climate and sea levels for the past three million years.
357   *NATURE* **419**:199-206.

358   Li SH, Yeung CL, Feinstein J, Han L, Le MH, Wang CX, and Ding P. 2009. Sailing through the Late Pleistocene: unusual
359   historical demography of an East Asian endemic, the Chinese Hwamei (*Leucodioptron canorum canorum*),
360   during the last glacial period. *Molecular Ecology* **18**:622-633.

361   Liao PC, Kuo DC, Lin CC, Ho KC, Lin TP, and Hwang SY. 2010. Historical spatial range expansion and a very recent
362   bottleneck of *Cinnamomum kanehirae* Hay. (Lauraceae) in Taiwan inferred from nuclear genes. *BMC*
363   *Evolutionary Biology* **10(1)**:1.

364   Lisiecki LE, and Raymo ME. 2005. A Pliocene-Pleistocene stack of 57 globally distributed benthic δ^{18}O records.
365   *Paleoceanography* **20**:n/a-n/a.

366   McKay BD. 2012. A new timeframe for the diversification of Japan's mammals. *Journal of Biogeography* **39**:1134-
367   1143.

368   Molodkov AN, and Bolikhovskaya NS. 2002. Eustatic sea-level and climate changes over the last 600 ka as derived
369   from mollusc-based ESR-chronostratigraphy and pollen evidence in Northern Eurasia. *Sedimentary*
370   *Geology* **150**:185-201.

371   Nabholz B, Glemin S, and Galtier N. 2008. Strong Variations of Mitochondrial Mutation Rate across Mammals—the
372   Longevity Hypothesis. *molecular biology and evolution* **25**:120-130.

373   Nagata J, Masuda R, Tamate HB, Hamasaki S-i, Ochiai K, Asada M, Tatsuzawa S, Suda K, Tado H, and Yoshida MC.
374   1999. Two genetically distinct lineages of the sika deer, *Cervus nippon*, in Japanese islands: Comparison of
375   mitochondrial D-loop region sequences. *Molecular Phylogenetics and Evolution* **13**:511-519.

376   Ohnishi N, Uno R, Ishibashi Y, Tamate H, and Oi T. 2009. The influence of climatic oscillations during the Quaternary
377   Era on the genetic structure of Asian black bears in Japan. *Heredity* **102**:579-589.

378   Ohshima K. 1990. The history of straits around the Japanese Islands in the late-Quaternary. *The Quaternary*
379   *Research* **29**:193-208.

380   Park S-C, Yoo D-G, Lee C-W, and Lee E-I. 2000. Last glacial sea-level changes and paleogeography of the Korea
381   (Tsushima) Strait. *Geo-Marine Letters* **20**:64-71.

382   Paz Od, Fernandez R, and Benzal J. 1986. El anillamiento de quiropteros en el centro de la peninsula iberica durante
383   el periodo 1977–86. *Bolentin de la Estacion Central de Ecologica* **30**:113-138.

384   Posada D. 2008. jModelTest: phylogenetic model averaging. *molecular biology and evolution* **25**:1253-1256.

385   Posada D, and Buckley TR. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike
386   information criterion and Bayesian approaches over likelihood ratio tests. *Systematic biology* **53**:793-808.

387   Posada D, and Crandall KA. 2001. Selecting the best-fit model of nucleotide substitution. *Systematic biology* **50**:580-
388   601.

389   Puechmaille SJ, Allegrini B, Boston ESM, Dubourg-Savage M-J, Evin A, Knochel A, Bris YL, Lecoq V, Lemaire M, Rist D,
390   and Teeling EC. 2012. Genetic analyses reveal further cryptic lineages within the *Myotis nattereri* species

391   complex. *Mammalian Biology* **77**:224-228.

392   Puizina J, Puljas S, Fredotović Ž, Šamanić I, and Pleslić G. 2013. Phylogenetic Relationships among Populations of the

393   Vineyard Snail *Cernuella virgata* (Da Costa, 1778). *ISRN Zoology* 2013.

394   Qu J, Liu N, Bao X, and Wang X. 2009. Phylogeography of the ring-necked pheasant (*Phasianus colchicus*) in China.

395   *Molecular Phylogenetics and Evolution* **52**:125-132.

396   Qu Y, Zhao Q, Lu H, and Ji X. 2014. Population Dynamics Following the Last Glacial Maximum in Two Sympatric

397   Lizards in Northern China. *Asian Herpetological Research* **5**:213-227.

398   Rakotoarivelo AR, Willows-Munro S, Schoeman MC, Lamb JM, and Goodman SM. 2015. Cryptic diversity in

399   *Hipposideros commersoni* sensu stricto (Chiroptera: Hipposideridae) in the western portion of Madagascar.

400   *BMC Evolutionary Biology* **15(1)**:1.

401   Ransome RD. 1995. Earlier breeding shortens life in female greater horseshoe bats. *Philosophical Transactions of*

402   *the Royal Society of London B: Biological Sciences* **350**:153-161.

403   Ray N, and Adams JM. 2001. A GIS-based vegetation map of the world at the Last Glacial Maximum (25,000-15,000

404   BP). *Internet Archaeology* **11**.

405   Rogers AR, and Harpending H. 1992. Population growth makes waves in the distribution of pairwise genetic

406   differences. *Molecular Biology & Evolution* **9**:552-569.

407   Rohling EJ, Fenton M, Jorissen FJ, Bertrand P, Ganssen G, and Caulet JP. 1998. Magnitudes of sea-level lowstands of

408   the past 500,000 years. *NATURE* **394**:162-165.

409   Ronquist F, and Huelsenbeck JP. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**:754-

410   755.

411   Ronquist F, and Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models.

412   *Bioinformatics* **19**:1572-1574.

413   Rossiter SJ, Benda P, Dietz CN, Zhang S, and Jones G. 2007. Rangewide phylogeography in the greater horseshoe bat

414   inferred from microsatellites: implications for population history, taxonomy and conservation. *Molecular*

415   *Ecology* **16**:4699-4714.

416   Rossiter SJ, Jones G, Ransome RD, and Barratt EM. 2000. Genetic variation and population structure in the

417   endangered greater horseshoe bat *Rhinolophus ferrumequinum*. *Molecular Ecology* **9**:1131-1135.

418   Rozas J, Sánchez-DelBarrio JC, Messeguer X, and Rozas R. 2003. DnaSP, DNA polymorphism analyses by the

419   coalescent and other methods. *Bioinformatics* **19**:2496-2497.

420   Sakai T, Kikkawa Y, and Tsuchiya K. 2003. Molecular phylogeny of Japanese Rhinolophidae based on variations in the

421   complete sequence of the mitochondrial cytochrome b gene. *Genes & Genetic Systems* **78**:179-189.

422   Schneider S, and Excoffier L. 1999. Estimation of past demographic parameters from the distribution of pairwise

423   differences when the mutation rates vary among sites: application to human mitochondrial DNA. *Genetics*

424   **152**:1079-1089.

425   Schneider S, Roessli D, and Excoffier L. 2000. Arlequin: a software for population genetics data analysis. *User*

426   *manual ver* **2**:2496-2497.

427   Slatkin M. 1987. Gene flow and the geographic structure of natural populations. *Science* **236**:787-792.

428   Sun K, Luo L, Kimball RT, Wei X, Jin L, Jiang T, Li G, and Feng J. 2013. Geographic Variation in the Acoustic Traits of

429   Greater Horseshoe Bats: Testing the Importance of Drift and Ecological Selection in Evolutionary Processes.

430   *Plos one* **8**:e70368.

431   Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*

432   **123**:585-595.

433   Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis

434   version 6.0. *molecular biology and evolution*:mst197.

435   Thong VD, Puechmaille SJ, Denzinger A, Bates PJJ, Dietz C, Csorba G, Soisook P, Teeling EC, Matsumura S, Furey NM,

436   and Schnitzler H-U. 2012. Systematics of the *Hipposideros turpis* complex and a description of a new

437   subspecies from Vietnam. *mammal review* **42**:166-192.

438   Wang Y. 2003. *A complete checklist of mammal species and subspecies in China: a taxonomic and geographic*

439   *reference*. China: China Forestry Publishing House.

440   Watanobe T, Ishiguro N, and Nakano M. 2003. Phylogeography and Population Structure of the Japanese Wild Boar

441   *Sus scrofa leucomystax* : Mitochondrial DNA Variation. *ZOOLOGICAL SCIENCE* **20**:1477-1489.

442   Wilkinson GS, and Chapman AM. 1991. Length and sequence variation in evening bat D-loop mtDNA. *Genetics*

443   **128**:607-617.

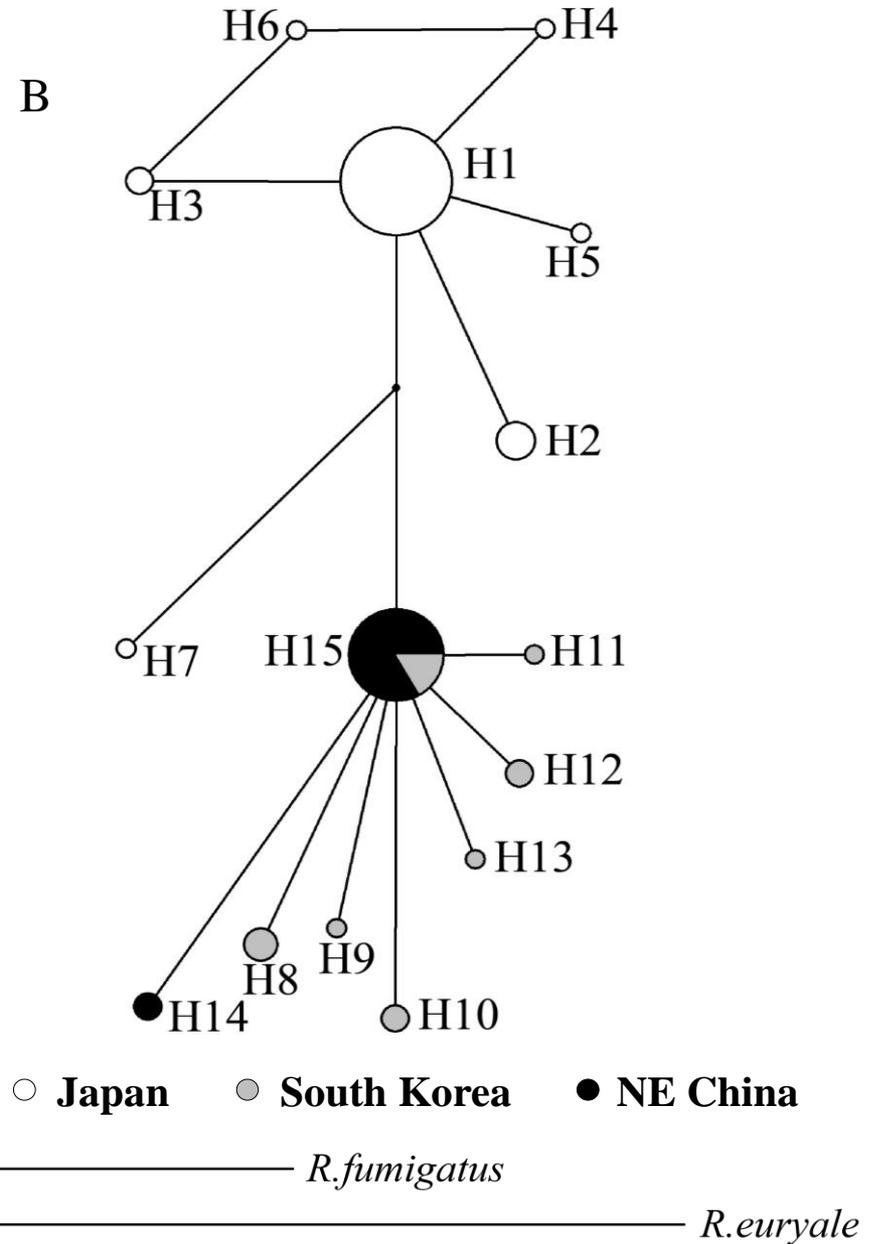
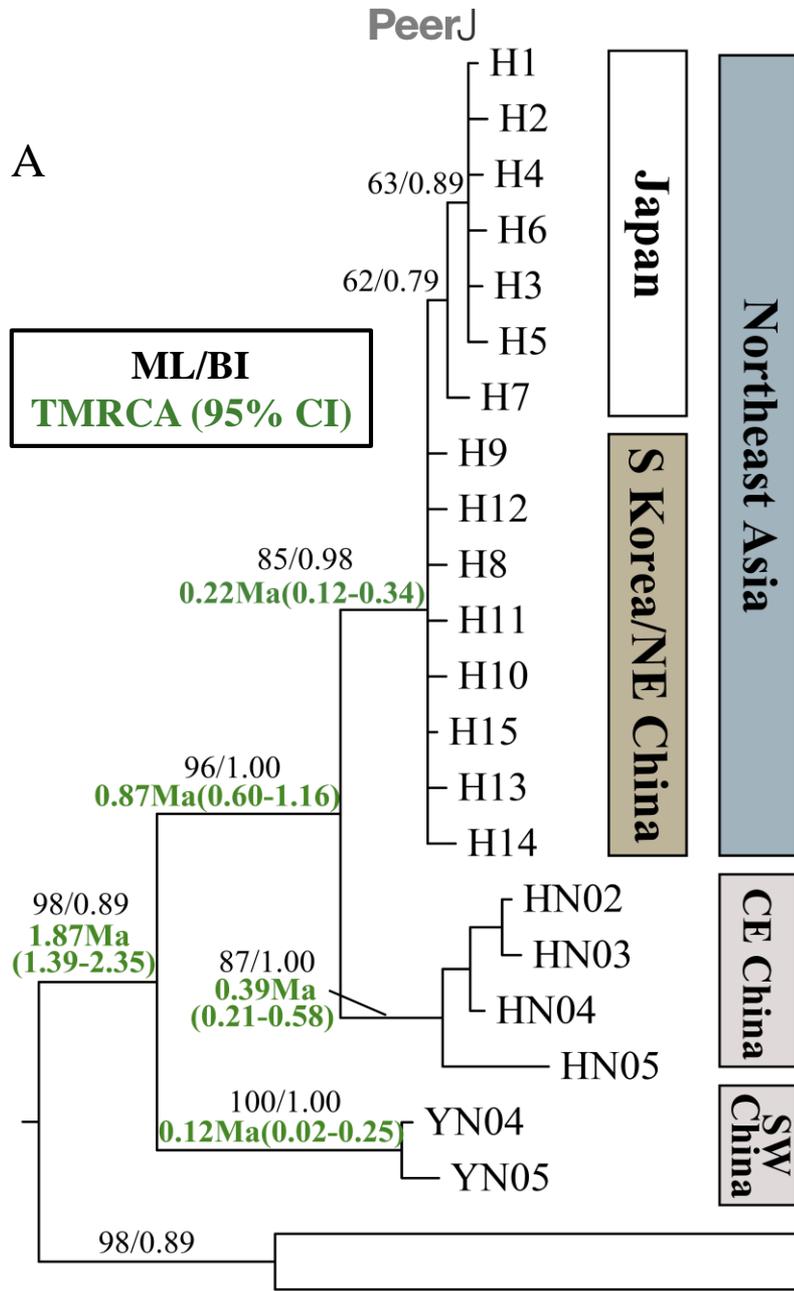
444   Yang M, Ma Y, and Wu J. 2011. Mitochondrial genetic differentiation across populations of the malaria vector

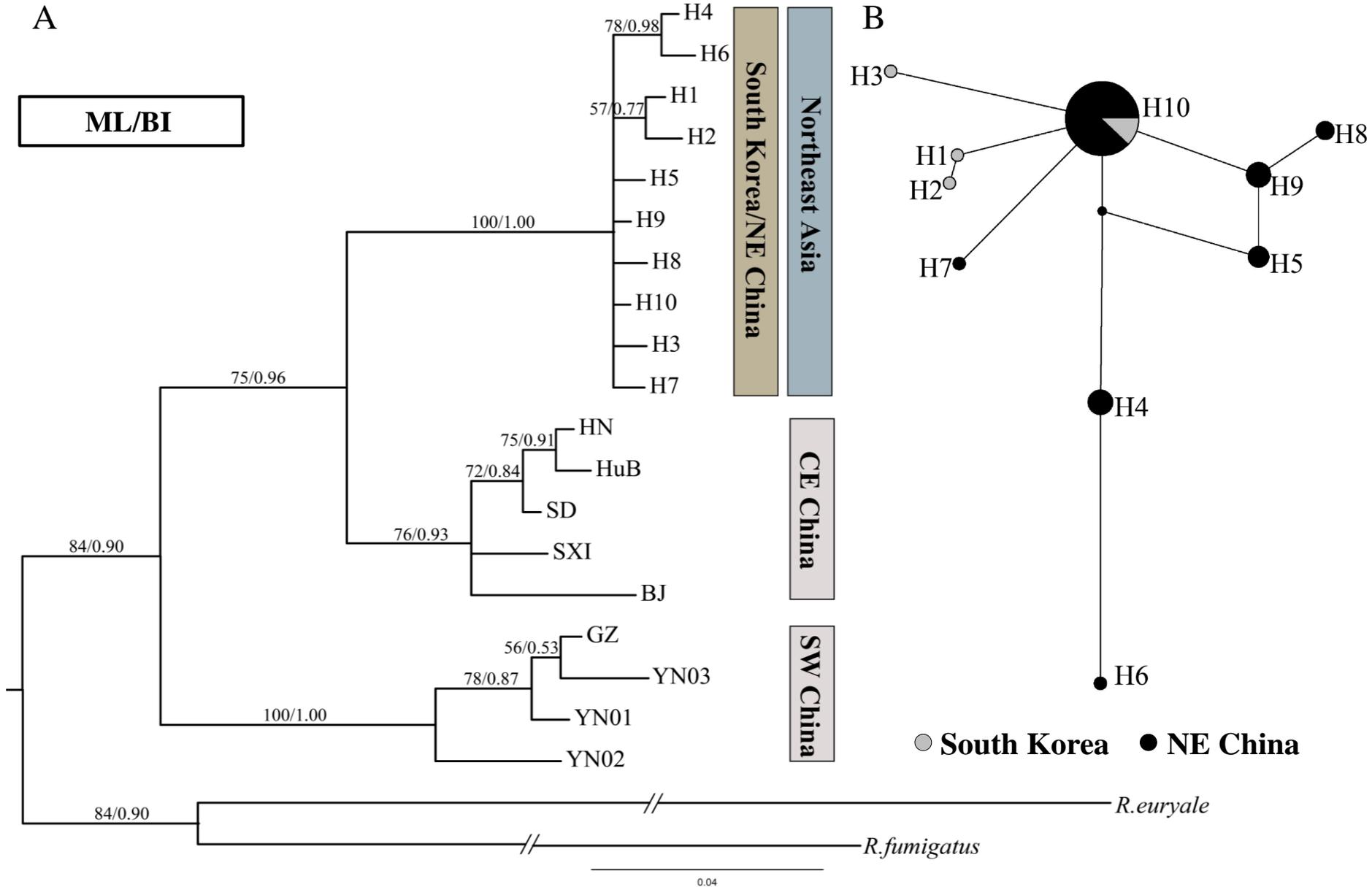
445   *Anopheles lesteri* from China (Diptera: Culicidae). *Malaria journal* **10**:216.

Sampling localities of *Rhinolophus ferrumequinum* in this study.

The colors of sampling points fit with clades identified in Fig. 2. Locality codes are identical to those in Table S1 (Supporting information). The map was freely reused and modified under the terms of the GNU Free Documentation License , Version 1.2.

Phylogenetic trees and network for *Rhinolophus ferrumequinum* populations based on cyt *b* haplotypes.

(A) Phylogenetic trees constructed by ML and BI methods. (B) Median-joining network for the East Asian *R. ferrumequinum* haplotypes. The circle size is proportional to the frequency of that haplotype. Small black dots represent missing haplotypes. Locality codes and haplotype are described in Table S1 and Table S2 (Supporting information), respectively.

Phylogenetic trees and network for *Rhinolophus ferrumequinum* populations based on D-loop haplotypes.

(A) Phylogenetic trees constructed by ML and BI methods. The D-loop data of Japanese population was unavailable. (B) Median-joining network for the South Korean and Northeast Chinese *R. ferrumequinum* haplotypes. The circle size is proportional to the frequency of that haplotype. Small black dots represent missing haplotypes. Locality codes and haplotype are described in Table S1 and Table S2 (Supporting information), respectively.

Genetic diversity of *Rhinolophus ferrumequinum* in Northeast Asia.

1   **Table 1** Genetic diversity of *Rhinolophus ferrumequinum* in Northeast Asia.

	N_s	N_h	N_{ss}	h (cyt *b*/D-loop)	π (cyt *b*/D-loop)
NE China	22/43	2/7	2/6	0.173/0.564	0.030/0.226
South Korea	14/8	7/4	6/3	0.879/0.648	0.116/0.201
Japan	43/-	7/-	6/-	0.408/-	0.047/-
Northeast Asia	79/51	15/10	15/9	0.735/0.573	0.143/0.229

2   N_s, the number of sequences; N_h, the number of haplotypes; N_{ss}, the number of segregating sites;

3   h, haplotype diversity; π, nucleotide diversity; -, missing data.

Average K2P distance (%) and gene flow of *Rhinolophus ferrumequinum* based on cyt *b* sequences.

1   **Table 2** Average K2P distance (%) and gene flow of *Rhinolophus ferrumequinum* based on cyt *b*

2   sequences.

Population	NE China	South Korea	Japan	Henan	Yunnan
NE China		3.11	0.06	0.06	0.01
South Korea	0.07		0.11	0.06	0.01
Japan	0.21	0.26		0.05	0.01
Henan	1.44	2.01	2.15		0.03
Yunnan	3.96	4.02	4.17	4.27	

3   Nm: above the diagonal; Average K2P distance (%): below the diagonal.

Results of mismatch distribution analyses and neutrality tests for *Rhinolophus ferrumequinum* based on cyt *b* sequences.

1 **Table 3** Results of mismatch distribution analyses and neutrality tests for *Rhinolophus*

2 *ferrumequinum* based on cyt *b* sequences.

	SSD	Hri	Tau (95%CI)	*t* (95%CI)	Tajima's *D*	Fu's *Fs*
NE China	0.04	0.74	3.00 (0.55-3.00)	-	-0.84	0.81
South Korea	0.07	0.35	1.71 (0.43-2.98)	0.12 Ma (0.03-0.20 Ma)	-1.73*	-5.26**
Japan	0.04	0.16	2.27 (0.75-3.94)	0.15 Ma (0.05-0.27 Ma)	-0.73	-5.58**

3 Hri, raggedness index; SSD, sum of squared deviations; NA, data deficiencies; -, no expansion

4 was detected; statistically significant results were indicated by asterisks: * $P < 0.05$, ** $P < 0.01$.