

 Open access • Journal Article • DOI:10.1111/JBI.13664

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Published on: 01 Oct 2019 - Journal of Biogeography (John Wiley & Sons, Ltd)

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Cryptic phylogeographic history sheds light on the generation of species diversity in sky-island mountains

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Keywords: allopatry, Approximate Bayesian Computation, cryptic corridor, interglacial refugia, niche modeling, species delimitation

Running title: Cryptic sky-island phylogeography

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ABSTRACT

Biodiversity hotspots should be given high priority for conservation under the situation of global climate change. The sky islands in southwestern China are characterized by extraordinarily high species diversity and are among one of the world's top biodiversity hotspots. However, neither the actual species diversity in this region or mechanisms generating this diversity are well explored. Here, we report on the phylogeographic analysis of the long-tailed mole (*Scaptonyx fuscicaudus*), a semi-fossorial mammal that inhabits the montane cool forests across the Chinese sky islands and is considered to represent one species divided into two subspecies. Analyses using DNA sequence data from one mitochondrial and six nuclear genes revealed that populations inhabiting different mountains exhibited exceptionally strong geographic structure. The lowlands and large rivers act as “soft” and “hard” barriers to dispersal, respectively, isolating evolutionary lineages for up to 11 million years. Our results suggest that the mountain ranges act as interglacial refugia buffering populations from climate fluctuations, further facilitating allopatric diversification. Strikingly, species delimitation analyses suggests that the long-tailed mole may comprise 18 operational taxonomic units and 17 putative species. Our results suggest that for low-vagility species, the complex topography of the Chinese sky islands has shaped genetic diversity and structure and promoted exceptional diversification through a combination of eco-environmental stability as well as geographic fragmentation. The patterns observed in *S. fuscicaudus* may be representative for other cold-adapted species, reflecting the generation of mammalian faunal diversity in the sky-island mountains of southwestern China.

1 INTRODUCTION

2 As the climate continues to change, one in six species are facing an accelerating risk of extinction [1].
3 This is especially true for narrowly endemic species [2]. To reduce the impact of climate change,
4 optimizing the spatial priorities of conservation and protecting such areas from further anthropogenic
5 activities could be a practical strategy [3]. Biodiversity hotspots should have the highest priority for
6 conservation, because these areas represent only 2.3% of Earth's land surface, but harbor more than half
7 of the world's plant species as endemics, as well as nearly 43% of birds, mammals, reptiles and
8 amphibians [4].

9 Many of the biodiversity hotspots are low-latitude mountains that occur on different continents such
10 as the mountains of the Eastern Afromontane in Africa, the Madrean pine-oak woodlands in North and
11 Central America, the tropical Andes in South America, and the Himalayas as well as the mountains of
12 southwestern China in Asia [4]. The extraordinarily high species diversity in these mountains are
13 supported by various climatic and environmental conditions, resulting in altitudinal zonation and
14 biologically complex ecosystems [5]. In addition, most low-latitude mountain regions are characterized
15 by complex geographic features, composed of a series of discrete mountain archipelagos, also known as
16 "sky islands" [6]. In a sky-island complex, each mountain is a fragmented habitat, surrounded by
17 lowlands, which act as geographic barriers to montane species, leading to "speciation via isolation" in
18 allopatry or parapatry [7]. The sky islands also act as refugia. Species can find hospitable habitat by
19 moving up and down a few hundred meters and thereby survive climate fluctuations and avoid extinction.
20 Thus, a large number of ancient relict species can be found on sky islands, known as the "species museum"
21 effect [8]. Climatic changes may result in periodic distributional shifts of montane species, promoting
22 allopatric speciation. Consequently, sky islands may also act as "species pumps".

23 The mountains of southwestern China (also known as the Hengduan Mountains) and the adjacent
24 mountains are among one of the world's top global biodiversity hotspots [4]. The mountains span
25 approximately 10° of latitude (N24°-N34°) and were recently recognized as a sky-island complex [9, 10].

26 This area is similar to other sky islands on Earth in its extremely complex topography, but distinctive by
27 its well-developed river systems. The river canyons embedded between mountain ridges are comparable
28 to the largest river basin in the world [11]. The sky islands in southwestern China are relatively young
29 compared with the other sky islands in the world. The uplift started before 23 million years ago (Ma), but
30 the major surfaces uplifted after 13 Ma [12]. Nevertheless, approximately 40% of the vascular plant and
31 50% of the mammal species in China are distributed in this area, a large portion of which are endemic.
32 Many ancient and relic species are distributed in these mountains, including the giant panda (Ursidae), red
33 panda (Ailuridae), shrew-like moles (Talpidae, *Uropsilus*), and gymnures (Erinaceidae, *Neotetracus*),
34 demonstrating the buffering capacity of these mountains against climate change over geological
35 timescales.

36 Despite the recognition as a biodiversity hotspot, the actual species diversity within the Hengduan
37 Mountains or the mechanisms behind its generation is not well understood. Recent studies have shown
38 that species diversity in this area is highly underestimated [13], and recently, the area has become a
39 significant hotspot for the discovery of new species [e.g., 14, 15]. Inter- and intraspecific phylogeographic
40 studies have revealed strong geographic structuring of lineages, which were associated with the complex
41 topography of the region, geomorphologic changes (i.e., uplift of the mountains), or Pleistocene
42 glaciations [16, 17]. However, the relative degree to which they promote diversification and speciation is
43 not well known [10]. For example, previous studies showed the deep river valleys such as the Dadu, Min,
44 and Yalong Rivers in the north sub-region as well as the Mekong and Salween Rivers in the southern sub-
45 region of the sky islands (see **Fig. 1**) could act as barriers to dispersal of terrestrial small mammals
46 resulting in intraspecific diversification [17, 18], while it is not known when these barriers appeared.
47 Moreover, the montane archipelagos to the east and southeast of the Sichuan Basin including Mts. Bashan
48 and Dalou in between of Mts. Jiaozi and Qinling (Fig. 1) have received little attention [10]. Although
49 these archipelagos are quite discrete, they are inhabited by cold-adapted species, and might act as cryptic
50 refugia and corridors of dispersal for montane species [17, 19].

51 The long-tailed mole (*Scaptonyx fusicaudus*) is the sole representative of a monotypic genus within
52 the family Talpidae (moles, shrew moles, desmans, and their allies), and is divided into two subspecies
53 [20]. It is a small (weight 8-14 grams) semi-fossorial mammal mostly distributed in southwestern China.
54 It is considered a relic species because its closest relatives are distributed in Japan (*Dymecodon* and
55 *Urotrichus*) and North America (*Neurotrichus*), which diverged from the long-tailed mole approximately
56 30 million years ago [13]. It is also characterized by a strikingly deep intraspecific divergence (~16
57 million years) in our previous estimation, which is more ancient than the most recent common ancestor
58 (MRCA) of the shrew-like moles (*Uropsilus*), and that of the fossorial mole tribe Talpini [13]. We
59 consider this species as an ideal model for phylogeographic study for the following reasons: i) it is a low-
60 vagile species exclusively distributed at middle to high elevations. The populations are physically isolated
61 among discrete mountain islands, so that its evolution could be strongly shaped by endemic geographic
62 features as well as geomorphologic history; ii) it only inhabits humid environment, so that its genetic
63 diversity could be shaped by climate change and following habitat turnover; iii) it is widely distributed
64 through the sky-island mountains including those surrounding the Sichuan Basin, so it is good
65 representative to reveal potential different effect in each sub-region or along the latitude gradient; and
66 finally iv) its evolutionary time within these mountains is long enough to be comparable with the uplift
67 history of the mountains, so that its evolution could be associated with the generation of species diversity
68 in the sky islands.

69 In the present study, we analyzed mitochondrial and nuclear DNA sequences of long-tailed moles
70 collected from throughout the sky islands of southwestern China to analyze genetic structure, estimate
71 evolutionary relationships, and conduct species delimitation, approximate Bayesian computation and
72 niche modeling analyses. We aimed to recover the phylogeographic patterns and the timescale for
73 diversification, and unravel the underestimated species diversity. Furthermore, we examine to what
74 degree geographic versus climatic factors have preserved and shaped the diversity found in long-tailed

75 moles and thereby shed light on the underlying mechanisms that have driven diversification and
76 speciation in the sky islands of southwestern China.

77 **MATERIALS AND METHODS**

78 **Study area and sampling strategy**

79 Our study areas include the northern Hengduan mountains (also known as the western Sichuan
80 mountains; Nos. 22-25 in Fig. 1), the south Hengduan mountains (northwestern Yunnan; Nos. 1-7, 9-15,
81 17), the southern outliers of the Hengduan Mountains (nos. 8, 16, 19-21), montane archipelagos
82 distributed from the south to the northeast of the Sichuan Basin including Mts. Jiaozi (no. 18), Dalou (no.
83 28), Bashan (27) and Qinling (no. 26). The study areas cover nearly all known distribution range except in
84 adjacent northern Myanmar and northmost Vietnam. The sample localities were isolated by Dadu, Jinsha,
85 Mekong, Red, and Salween Rivers, all of which likely act as barriers to dispersal for small mammals. The
86 western Sichuan Mountains are less patchy than the other areas. The northwestern Yunnan is
87 characterized by the most complex topography because the mountain chains are isolated by the Jinshan,
88 Mekong and Salween Rivers, and also known as the Longitudinal Range-Gorge Region. The river
89 valleys are as low as 300 m above sea level, supporting only Savanna vegetation and are unlikely suitable
90 for the long-tailed moles. We collected 113 specimens of long-tailed moles from 1997 to 2013
91 **(Electronic supplementary material, Table S1)**, four of which were sequenced in a previous study [13].
92 Skin and skull specimens and tissue samples were deposited in the Kunming Institute of Zoology (KIZ),
93 Chinese Academy of Sciences (CAS) and the Sichuan Academy of Forestry (SAF).

94 **Sequence acquisitions**

95 We extracted total DNA using the standard phenol/chloroform protocol [21]. Sample localities
96 are shown in **Table S1**. We amplified one complete mitochondrial gene (*CYTB*) and six nuclear gene
97 segments including *ADORA3*, *APP*, *ATP7*, *BCHE*, *BRCA1*, and *RAG1* using primers provided in **Table**
98 **S2 (Electronic supplementary material)**. Sequences were assembled and edited using Lasergene v7.1
99 (DNASar, Madison, WI) and aligned using MUSCLE (Edgar, 2004). Sequences of other shrew moles

100 including *Dymecodon pilirostris* (n=1), *Neurotrichus gibbsii* (3) and *Urotrichus talpoides* (1) were
101 obtained from GenBank (**Electronic supplementary material, Table S3**), and *Condylura cristata* (1)
102 was used to root the trees. From the samples we collected, we obtained 113 *CYTB* (1140 bp), 112
103 *ADROA3* (358 bp), 111 *ATP7* (666 bp), 112 *APP* (640 bp), 103 *BCHE*, 113 *BRCA1* (969 bp), and 104
104 *RAG1* (1047 bp) sequences. Twenty-three sequences (3%) are missing in our alignments.

105 **Mitochondrial gene tree, tree-based and distance-based species delimitations**

106 We estimated the *CYTB* gene tree, which was also used for tree-based species delimitation. We
107 partitioned the *CYTB* gene by codon position and evaluated the best-fitting partitioning scheme and
108 evolutionary models (**Electronic supplementary material, Table S4**) using PartitionFinder v1.1 [22].
109 Bayesian analyses were performed using BEAST v1.8 [23]. We constrained the monophyly of
110 *Dymecodon* + *Neurotrichus* + *Scaptonyx* + *Urotrichus* based on our best knowledge regarding their
111 relationships [13]. For each analysis, we selected a lognormal relaxed-clock model, a birth–death tree
112 prior, and ran the Markov chain Monte Carlo for 50 million generations, with a sampling interval of 5,000
113 generations. The analyses were repeated four times and were performed on the CIPRES Science Gateway
114 [24]. Posterior distributions and effective sample sizes were calculated using Tracer v1.6.

115 We used two approaches including Poisson Tree Processes (PTP) and the Generalized Mixed
116 Yule Coalescent (GMYC) to conduct tree-based species delimitation. The Bayesian tree with branch
117 lengths representing substitutions/site was used for the PTP analyses and implemented in the bPTP server
118 [25]. We used the default settings for all parameters and confirmed the convergence by examining the
119 trace plot. The tree with branch lengths proportional to time was used for GMYC analyses and
120 implemented using the R package "splits" (<http://r-forge.r-project.org/projects/splits>). For the distance-
121 based species delimitation, we calculated the number of operational taxonomic units (OTUs) using
122 USEARCH v8.1 [26]. We used a minimum identity of 97% as the “radius” of an OTU.

123 **Nuclear gene trees and molecular dating**

124 We estimated the concatenated nuclear gene tree using phased alleles. We phased each of the
125 nuclear genes of the ingroup taxa using PHASE [27] and implemented in DnaSP v 5.1 [28]. The best-
126 fitting partitioning schemes and evolutionary models were estimated for each gene using PartitionFinder
127 v1.1. To minimize the effect due to missing data, we only included samples from which all six genes were
128 successfully sequenced. This dataset included 198 phased alleles representing 99 samples of *S. fuscicaudus*
129 plus six unphased outgroup samples as mentioned above.

130 We estimated the Bayesian trees as described above. The phased gene alignments were
131 concatenated, and defined by genes and codon positions, and used for estimating the best partitioning
132 scheme and evolutionary models with PartitionFinder (**Electronic supplementary material, Table S4**).
133 Two fossil-based calibrations were selected for divergence time estimation. The oldest known fossil of
134 shrew moles (*Oreotalpa*) from the strata of the latest Eocene [29] was used to calibrate the root of the tree,
135 and the oldest known *Scaptonyx* fossil specimens from the early Pleistocene strata [30] from Shennongjia
136 (Mt. Bashan), Hubei, was used to calibrate the stem group of the population. The justifications for
137 placement of these calibration priors are detailed in **Text S1 (Electronic supplementary material)**.

138 The estimated MRCA of *S. fuscicaudus* was strikingly ancient (the early Miocene, see Result
139 Section), with a high level of uncertainty (i.e., large 95% confidence intervals). To increase accuracy, we
140 compared four competing hypotheses of divergence times by constraining the root age of *S. fuscicaudus* to
141 be at i) the Oligocene/Miocene boundary (23.03 Ma; H0); ii) the Middle/Late Miocene boundary (11.61
142 Ma; H1); iii) the Miocene/Pliocene boundary (5.40 Ma; H2) and iv) the Pliocene/Pleistocene boundary
143 (2.40 Ma; H3). These four hypotheses were compared using Bayes factors estimated with the path
144 sampling (PS) and stepping-stone sampling (SS) methods [31] and implemented in BEAST v1.8. We
145 employed normal distributions for the priors of the time of the MRCA for the root age and used 0.5 as the
146 standard deviation (SD) in all four analyses. To calculate the Bayes factor the marginal likelihood of each
147 analysis was estimated with 1,000,000 steps. The two fossil-based calibrations were used as

148 “checkpoints”, constraining the times of diversifications. Similar approaches have been employed in
149 previous studies [32, 33]. We considered the Bayes Factor of ≥ 10 as very strong evidence against the
150 alternative hypothesis [34].

151 **Structure and coalescent species delimitation using nuclear genes**

152 We used the phased nuclear genes to estimate population structure for *S. fusicaudus* using the
153 correlated admixture model in STRUCTURE v2.3.4 [35]. Because the mitochondrial gene tree showed a
154 strong geographical pattern, we assigned individuals from the same mountain area to their own
155 populations. To reduce computational cost, we split the samples into three datasets, corresponding to the
156 three major clades recovered in both the mitochondrial and nuclear gene trees. The first dataset included
157 10 phased samples from Mt. Gaoligong, and we tested K values from 1 to 4. The second dataset included
158 14 phased samples from the western Sichuan mountains, and we tested K values from 1 to 4. The third
159 dataset included all the other samples (n=96), and we tested K values from 3 to 15. Each run consisted of
160 1,000,000 step Markov chain Monte Carlo (MCMC) replicates after a burn-in of 100,000 replicates. We
161 repeated the analysis 20 times for each dataset. We used a “LargeKGreedy” algorithm for aligning
162 multiple replicate analyses as implemented in CLUMPK using the default parameters [36, 37], and
163 determined the optimal number of clusters (i.e., the best K value) for each dataset using $\ln(\text{Pr}(X|K))$ values
164 (see STRUCTURE manual).

165 We conducted coalescent-based species delimitation analyses of the phased nuclear genes using BPP
166 v3.1 [38] and BEAST v2.3 [39]. Because the algorithms of BPP were not sensitive to missing data, we
167 included all ingroup samples. To avoid the potential of poor mixture of the reversible-jump MCMC
168 algorithms, we split our samples into four datasets, corresponding to clade I, clade II, subclade A, and
169 subclades B+C, according to the topology of the concatenated nuclear gene tree. Samples were assigned
170 to candidate species based on the results of STRUCTURE. We followed Yang [40] for running the BPP
171 pipelines. First, the genetic distances in each dataset were calculated using MEGA v7.0 [41], and the
172 values were used for the priors of “thetaprior.” We then estimated the priors for “tauprior” using the A00

173 model. The species tree topologies were fixed according to the mitochondrial gene tree for parameter
174 estimations (A00). To avoid false positive errors due to incorrect guide trees [42], we used the A11 model
175 to allow simultaneous species delimitation and species-tree estimation [43]. To allow variable mutation
176 rates among loci, we set the parameter α to 20 for “locusrate.” We used both algorithms 0 and 1, and
177 repeated the analyses for each dataset at least six times with combinations of different parameters and
178 priors. For each run, 5,000 samples were collected by sampling every 10 iterations after a burn-in of 5000
179 iterations. We used a conservative criterion, recognizing posterior probabilities ≥ 0.95 as strong support
180 for putative species.

181 We also conducted species delimitation and species tree estimation using the BEAST2 package
182 STACEY v1.1, which is also based on a multispecies coalescent model [44]. To reduce computational
183 costs, we assigned an HKY+G model to each of the nuclear genes and employed a strict molecular clock
184 model with a mean evolutionary rate of 0.0015 based on the results of the BEAST analyses using the
185 concatenated nuclear gene dataset. Samples were assigned to candidate species based on the results of
186 STRUCTURE. The analyses were conducted using BEAST2 v2.3.3.

187 *Approximate Bayesian Computation (ABC)*

188 DIYABC v.2.1 [45] was used for ABC-based scenario comparisons, and to examine potential gene
189 flow along the mountains to the east of the Sichuan Basin, which may serve as stepping stones among
190 isolated populations. To reduce computational cost, a subset of samples (n=40) from Mts. Ailao, Bashan,
191 Dalou, Jiaozi, Wuliang and Qinling (i.e., subclades A and C except for the single individual from Mt.
192 Wuding) were selected and were assigned to six populations according to their distributions. All six
193 nuclear genes were included in one group of the mutation model. We selected HKY as the substitution
194 model and estimated the percentage of invariant sites and the shape of the gamma distribution using
195 MEGA6 [46].

196 We performed four runs of analyses to test competing scenarios. The priors for divergence times,
197 population sizes, and mutation rates are given in **Text S2 (Electronic supplementary material)**. First,

198 we compared four diversification scenarios to test for the potential of simultaneous divergence. Second,
199 we compared the best topology with three gene-flow scenarios. Third, we compared four alternative
200 scenarios regarding the time of gene flow. Finally, we compared scenarios regarding demographic
201 changes (**Test S2**). We simulated all summary statistics within populations except for private segregating
202 sites, and all summary statistics between two populations. We simulated a minimum of 10,000 datasets
203 for each comparison. After simulations, we ran pre-evaluations to confirm that the simulated datasets
204 were not significantly different from the observed data. Then we compared scenarios based on a logistic
205 regression estimation of the posterior probabilities (PP) of each scenario. The best scenario was selected
206 based on non-overlapping 95% confidence intervals (CIs) of PP. Two scenarios with overlapping 95%
207 CIs were not considered as significantly different and the simpler scenario was then selected.

208 *Species distribution modeling (SDM)*

209 We obtained 76 geographic coordinates in our field work and adopted another two reliable
210 coordinates from previous studies [47, 48]. To reduce the effect of sample bias, we consequently
211 subsampled 63 coordinates by retaining one record per grid cell of $0.2^\circ \times 0.2^\circ$ using the R package Raster.
212 Nineteen climate variables for the present day were downloaded from the WorldClim website. We
213 extracted the 19 variables for each coordinate using DIVA-GIS v7.5. Nine variables that were not
214 strongly correlated (Pearson's $R < 0.9$; Bio 1, 2, 3, 5, 7, 12-15) were retained for further analyses. We
215 conducted principal components analyses (PCA) and discriminant factor analyses (DFA). We assigned all
216 samples into five groups based on their phylogenetic positions (clades and subclades) in the DFA. We
217 used MaxEnt v.3.3.3k to conduct niche modeling [49], calculating the potentially habitable areas in the
218 present day, during the last interglacial maximum (LIGM), and during the last glacial maximum (LGM).
219 For estimating niches in LGM and LIGM, climatic datasets were also downloaded from WorldClim. The
220 area under the receiver operating characteristic curve (AUC) was used to evaluate the accuracy of our
221 models, and AUCs higher than 0.9 were considered as an indicator of good projecting.

222 **RESULT**

223 ***Structure and diversity of the mitochondrial data***

224 Three major clades were recovered in the *CYTB* gene tree (Clades I, II and III; **Fig. 1**), showing a
225 high level of geographic structure. Five specimens from Mt. Gaoligong (the westernmost mountain in
226 Yunnan) formed a basal position of *S. fuscicaudus* (clade I). The samples from the western Sichuan
227 mountains composed the second major clade (clade II). Clade III was comprised of three subclades
228 including i) samples from Mt. Qinling (Shaanxi), Mt. Bashan (Hubei) and Mt. Dalou (Chongqing)
229 (subclade A); ii) samples approximately distributed to the west of 100.5°E longitude in Yunnan, but to the
230 east of the Mekong River (subclade B); and iii) samples from middle and eastern Yunnan, approximately
231 to the east of the 100.5°E longitude (subclade C). The mitochondrial *CYTB* gene was characterized by
232 high haplotype and nucleotide diversities ($Hd = 0.99$, $Pi = 0.09$; **Electronic supplementary material,**
233 **Table S5**).

234 ***Nuclear gene tree and ancient divergence times***

235 In the concatenated nuclear gene tree, three major clades (I, II and III) were recovered (**Fig. 2**),
236 and the monophyly of 15 mitochondrial lineages was strongly supported. Within clade III, the monophyly
237 of subclade A was highly supported ($PP=0.99$). Lineages within subclades B and C of the *CYTB* gene tree
238 showed an alternative pattern of relationships: the population from Mt. Jiaozi formed a basal clade to the
239 other populations ($PP=0.97$), the single specimen from Mt. Wuding was recovered as a distinct lineage,
240 and relationships among populations from Dali and Lijiang mountain ranges were not resolved.

241 Divergence time estimation based on nuclear genes supported the tMRCA of *S. fuscicaudus* at
242 25.04 Ma, but this was associated with a large uncertainty (95% confident interval [CI] = 37.68-15.29;
243 **Fig. S1A, Electronic supplementary material**). The age was three times older than that between the two
244 shrew mole genera in Japan (*Dymecodon* and *Urotrichus*; 6.65 [95% CI=11.12-3.38]). The Bayesian-
245 based scenario comparison (**Table 1**) rejected hypotheses H2 and H3 (ΔPS and $\Delta SS \geq 15$), but could not
246 reject H1 (ΔPS and $\Delta SS \leq 2$; **Fig. 3A**), indicating the tMRCA is no younger than 11 Ma (**Fig. S1B-E**).

247 ***Genetic structure, species delimitation and species tree***

248 When using 97% similarity as a criterion, the USEARCH analysis recovered 18 OTUs of *S.*
249 *fusicaudus* (**Table 1, Fig. 2**). Mitochondrial tree-based analyses including PTP and GMYC recognized 24
250 (confidential interval [CI] = 22 - 30) and 26 (CI=23-29) putative species, respectively (**Fig. 2**).
251 STRUCTURE, as well as BPP and STACEY delimitation analyses, supported 17 isolated populations or
252 putative species. The coalescent species tree recovered using the STACEY model was similar to that of
253 the concatenated nuclear gene tree, although relationships among the putative species recovered in
254 subclades B + C in the mitochondrial gene tree were remained unresolved (**Fig. 3B**).

255 ***Detection of gene flow using DIYABC***

256 DIYABC analyses were limited to the populations to the east (Nos. 26, 27 in **Fig. 1**), north (No.
257 28) and south (Nos. 18-21) of the Sichuan Basin to detect the possibility of long-distance dispersal among
258 a set of discrete mountains (**Fig. 3C**). These analyses suggest that after the first split somewhere between
259 the eastern (Mt. Dalou) and southern populations (Mts. Wuliang + Ailao), gene flow occurred between
260 these populations, resulting in admixture (admixture rate = 0.47 [CI=0.28-0.66]) at Mt. Jiaozi (No. 18 in
261 **Fig. 1**) in northeastern Yunnan. The dispersal and admixture was accompanied by a demographic
262 expansion ($N_2=4.36 * 10^4$ [CI=3.17-5.85] > $N_1=2.91 * 10^4$ [CI=2.35-3.57]).

263 **Species distribution modeling**

264 In the PCA plot based on nine climatic variables (**Fig. 3D**), subclade A (comprising samples to
265 the east and northeast of the Sichuan Basin) was well separated from the others along PC1. Clade I and
266 subclades B and C plot together and partly overlap with each other, suggesting all populations from
267 Yunnan inhabit similar environments, despite their distinct phylogenetic histories. A similar pattern was
268 also revealed in the DFA (**Fig. S2, Electronic supplementary material**).

269 The results of SDM showed that suitable habitats in the present day are larger than during the
270 Last Interglacial Maximum, but smaller than during the Last Glacial Maximum (**Fig. 4A-C**). The SDM
271 did not support the existence of suitable habitats during the Last Interglacial Maximum at Mt. Qinling and

272 to the east of the Sichuan Basin. On the other hand, suitable habitats were always present in northwestern
273 and northeastern Yunnan as well as in the adjacent southern Sichuan mountain areas during all three time
274 periods (**Fig. 4D**).

275 **DISCUSSION**

276 Our results indicate that *S. fusicaudus* is characterized by strikingly ancient and *in situ*
277 diversification of multiple lineages and high genetic diversity, which is unevenly distributed across the
278 sky islands in southwestern China. The patterns we have uncovered represent, to the best of our
279 knowledge, the most extreme case of geographic diversification in this area, and supports the idea that
280 geographical factors could have played a more important role than climatic changes in shaping genetic
281 diversity and geographic structure within the sky islands of southwestern China. Our results suggest that
282 the mountains have acted as a buffer against climate change, and have provided continuously suitable
283 habitats for *S. fusicaudus* since the early Late Miocene, which supports the hypothesis of the sky islands
284 as a “museum” of ancient species and lineages. Our phylogeographic results suggest that the local rivers
285 could act as “hard” barriers preventing gene flow, while the montane archipelagos act as stepping stones
286 to facilitate dispersal.

287 ***Striking diversification and underestimated diversity***

288 The most striking finding of our study are the multiple ancient intra-specific splits among lineages within
289 *S. fusicaudus*. The estimated ages on the basis of two fossil calibrations are characterized by wide
290 confidence intervals, which is probably due to the uncertainty of the priors used to calibrate the timetree.
291 Nonetheless, PS/SS-based scenario comparisons support the MRCA of *S. fusicaudus* to have split no
292 younger than the earliest late Miocene, ~11 million years ago (**Figs. 3 and S1**), twice as old as the
293 ancestor of the two shrew mole genera *Dymecodon* and *Urotrichus* (**Table 1**).

294 The ancient diversification of lineages is accompanied by a minimum of 17 and as many as 26
295 OTUs or putative species, as recovered in all mitochondrial-based and nuclear-based structure and species
296 delimitation analyses (**Fig. 1**). A recent study showed that in a sky-island system, cryptic speciation as

297 well as extreme population genetic structuring (but not speciation) could occur and the latter also result in
298 oversplitting of lineages [50]. Distinguishing the two requires further taxonomic revision, and the patterns
299 we found should be considered as hypotheses that requires validation based on morphological and
300 ecological analyses. We did not perform morphological analyses due to the small sample size of skulls; in
301 addition, most specimens have broken skulls. Nevertheless, the large number of OTUs and putative
302 species recovered have important implications for conservation. Because each of them inhabit restricted
303 mountain ranges (see below), loss of habitats could result in the extirpation of an entire distinctive genetic
304 lineage or evolutionary significant unit (Ryder, 1986).

305 *Allopatric diversification shaped by the drainage and mountain systems*

306 The second striking finding was the strong geographic structure of populations among different
307 mountain ranges of the sky islands. Because *S. fusicaudus* is a montane inhabitant, and because the
308 landscape in southwestern China is patchy, the habitats of *S. fusicaudus* are discontinuous as revealed in
309 the SDM results (**Fig. 4A**), which could have led to geographic isolation and genetic drift. As a result,
310 each of the 18 mtDNA OTUs (most of which were also recovered by the nuclear genes) is endemic to
311 specific mountain range (**Fig. 1**).

312 The deep rivers may be considered as "hard" barriers because they strengthen the barriers to
313 dispersal. For example, clades I and III diverged in the Late Miocene but geographically they are only
314 isolated by the Salween River (**Fig. 1**). PCA and DFA using climatic variables of the present day suggests
315 habitats on both sides of the Salween River were similar, at least much more similar than between
316 Yunnan and Shaanxi, which are inhabited by subclades A-C (**Figs. 3D & S2**). Therefore, the 11-million-
317 year divergence between clades I and II is due to geographic isolation instead of adaptation to a new
318 environmental habitat. The barrier effect could be due to: i) the Salween River, which acts as a physical
319 barrier to dispersal, ii) the extremely hot and dry climate due to the foehn winds in the valley (see He and
320 Jiang 2014), and iii) the very limited dispersal ability of the species. Similarly, the other large rivers

321 including the Dadu, Mekong, and Red Rivers also seem to have acted as barriers, similar to the patterns
322 revealed in previous studies [17, 18].

323 Basins and lowlands could be considered as “soft” barriers, allowing dispersal and gene flow
324 during climate fluctuations [altitudinal movements; 51]. Conflicting gene trees and species delimitation
325 patterns using mitochondrial and nuclear DNA suggest a recent mitochondrial introgression from Mt.
326 Jiaozi to Mt. Wuding (Nos. 17 and 18 in **Fig. 1; Fig. 2**). Similarly, the close genetic affinity between the
327 populations in Mt. Qinling and eastern Yunnan (clade III) was recovered in all phylogenetic analyses
328 (**Figs. 2, 3A-B**) as well as the ABC results supporting a gene flow along the mountains to the east of the
329 Sichuan Basin. The results suggest these montane archipelagos have acted as a stepping-stone corridor,
330 facilitating migration of *S. fusicaudus* between montane habitats. This result was *unexpected*, because the
331 Hengduan Mountains throughout western Sichuan have been hypothesized to play this role [52], acting as
332 the primary corridor between the Palearctic and Oriental realms [53, 54]. Compared with the Hengduan
333 Mountains, the geomorphology of mountains to the east of the Sichuan Basin is patchier and lower in
334 elevation. Importantly, the discovery of this cryptic corridor demonstrates montane species could be
335 distributed around the Sichuan Basin in a ring, which is a precondition of the existence of ring species
336 [55].

337 ***Interglacial refugia maintaining genetic diversity***

338 The geomorphology of the sky islands is likely the primary driver of diversification in *S. fusicaudus*, but
339 it does not necessarily support diversity for extended periods of time. Instead, diversity is maintained over
340 time in stable habitats during interglacial periods (namely, interglacial refugia).

341 According to the SDM results, the potentially suitable habitats of *S. fusicaudus* enlarged during
342 the LGM and reduced during the LIGM (**Fig. 4 B-C**). This is a general pattern for low-altitude montane
343 inhabitants [56] and the term of refugia should be more specifically referred as “interglacial refugia”
344 instead of “glacial refugia” [51]. Our phylogeographic and divergence time results suggest that suitable
345 habitats for *S. fusicaudus* were available during most of the Pleistocene and beyond. This pattern is not

346 unexpected because the mountains in this area have a wide altitudinal range-span, therefore montane
347 inhabitants only need to migrate a few hundred meters in altitude to avoid extirpation.

348 The SDM analyses also supported more stable habitats in the southern distribution area (**Fig. 4D**).
349 We note that our SDM analyses were conducted only for the last 130,000 years, which is much shorter
350 than the evolutionary timeframe estimated for *S. fusicaudus*. We also ignored any geomorphological
351 changes due to the uplift of the Himalaya and Hengduan mountains. Furthermore, the populations in the
352 Mts. Qinling, Bashan, and Dalou could have adapted to new climatic environments, as observed in the
353 PCA and DFA plots (**Figs. 3D & S2**, which could result in an underestimation of the potential distribution
354 in the northeastern part of the range of *S. fusicaudus*. Nevertheless, the patterns revealed in the SDM
355 coincide with the unevenly distributed genetic diversity. Specifically, thirteen out of the 18 OTUs are
356 distributed in the south, whereas Mt. Gaoligong has supported *S. fusicaudus* for up to 11 million years,
357 while only 5 are distributed in the north.

358 ***Implication for endemic species diversity of the mountains***

359 The phylogeographic pattern observed in *S. fusicaudus* is the most extreme case that we have ever
360 seen for a single species inhabiting southwestern China. Although it is taxonomically considered a
361 monotypic genus and species, the >10-million-year evolutionary timeframe and wide distribution makes
362 the long-tailed mole a good representative of endemic montane organisms with low vagility, and likely
363 reflect the generation of species diversity in the sky-island mountains. Indeed, the unevenly distributed
364 genetic diversity in *S. fusicaudus* analogue the species richness pattern of vascular plants, of which higher
365 species richness and endemism was observed in the southern sub-region [57].

366 First, the species diversity is probably underestimated. The cryptic diversity we observed in *S.*
367 *fusicaudus*, was also found in most of the mammalian genera we have examined [e.g., 58, 59], indicating
368 existence of unknown species [14]. Second, the extraordinary species diversity should thanks to the
369 highly stable environments, which buffer dramatic global cooling and desiccating since the late Miocene
370 and periodical climatic changes during the Pliocene and Pleistocene. This is especially true for Mt.

371 Gaoligong, which has possibly harbored *S. fusicaudus* for as long as 11 million years. The stabilized
372 climatic condition makes the mountains a species “museum”. Third, the complex topography of the sky-
373 island mountains has facilitated allopatric speciation even over long evolutionary timescales, resulting in
374 the appearance of narrowly endemic species. Deep rivers such as the Salween and Mekong Rivers may
375 act as strong barriers to dispersal, which could further lead to high species diversity and the unique fauna
376 found in northwestern Yunnan such as on Mts. Gaoligong and Biluo. The allopatric diversification pattern
377 is likely applicable for most cold-adapted as well as hot-adapted terrestrial organisms, although for hot-
378 adapted organisms, the mountains likely act as barriers and low lands acting as corridors, conversely [60].

379

380 **ACKNOWLEDGEMENTS**

381 We thank Mr. Chang-Zhe Pu for sample collections. This work was supported by the Key Research
382 Program of the Chinese Academy of Sciences (KJZD-EW-L07), the Yunnan Applied Basic Research
383 Projects (2014FB176) and the National Natural Science Foundation of China (31301869).

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- 552

553 DATA ACCESSIBILITY

554 DNA sequences have been deposited in GenBank nucleotide database under the accession codes
555 XXXXXX to XXXXXXXX.

556 COMPETING INTERESTS

557 We declare we have no competing interests.

558 AUTHOR CONTRIBUTIONS

559 K.H. and X.L.J conceived the idea for the study. All authors contributed to the data collection. K.H. and
560 T.W analyzed the data. K.H. and K.P.K. wrote the manuscript.

561 ELECTRONIC SUPPLEMENTARY MATERIAL

562 **Table S1.** Samples used for the molecular work in the present study.

563 **Table S2.** Primers used for amplification and sequencing.

564 **Table S3.** Species and sequences used as outgroups for divergence time analyses.

565 **Table S4.** Partitioning schemes and evolutionary models used for the phylogenetic analyses of the
566 concatenated nuclear gene dataset.

567 **Table S5.** Genetic diversity of each gene segment used in the study.

568 **Text S1.** Justifications for the two fossil-based calibration priors used in divergence time estimation
569 analyses.

570 **Text S2.** Parameters, priors, scenarios and posterior probabilities for the four runs of ABC analyses.

571 **Figure S1.** Chronograms estimated using the concatenated nuclear gene da. Divergence times were
572 estimated based on two fossil-based calibrations (A), and with additional constraints on the MRCA of *S.*
573 *fusicaudus* (B-E). Branch lengths represent time. Node bars indicate the 95% CI for the clade age. The
574 result of H1 is also presented in **Fig. 3A**.

575 **Figure S2.** Discriminant function analysis (DFA) for the habitats of clades I and II and subclades A-C.
576 Plots of scores on functions 1 and 2 were from DFA of nine climatic variables.

577

578 FIGURE LEGENDS

579 **Figure 1.** A map showing the sky islands in southwestern China, and the localities of *S. fusicaudus*
580 samples collected for the current study. The locality numbers refer to those in **Material S1**.

581 **Figure 2.** A comparison of the mitochondrial gene tree (left) and the concatenated nuclear gene tree
582 (right). The estimated OTUs, the result of clustering analysis using STRUCTURE, and the results of
583 species delimitation analyses using bPTP, split, BPP and STACEY are shown in the middle. The numbers
584 at the nodes refer to Bayesian posterior probabilities. Branch lengths represent substitutions per site (scale
585 bars shown).

586 **Figure 3.** Divergence times estimated using concatenated nuclear genes (A), and a coalescent species tree
587 estimated using the STACEY model in BEAST2 (B). Values at nodes represent posterior probabilities
588 (PPs). PPs <0.95 are not shown. Horizontal bars in (A) represent 95% confidence intervals (CIs) for
589 divergence times. The timetree shown is the one estimated using the H1 scenario for calibration priors, in
590 which the MRCA was constrained at 11.61 Ma. (C) The best-fitting scenarios selected in four runs of the
591 ABC analyses. (D) Principal components analyses (PCA) for the habitats of clades I and II and subclades
592 A-C. Plots of scores on PC1 and PC2 were from PCA of nine climatic variables.

593 **Figure 4.** Ecological niche model projections for the distribution of *S. fuscicaudus* in the present day (A),
594 during the Last Glacial Maximum (B), and the Last Interglacial Maximum (C). Potential distributions
595 with probabilities higher than 0.5 in the present day, the LGM and LIGM are extracted and shown in (D).

TABLES

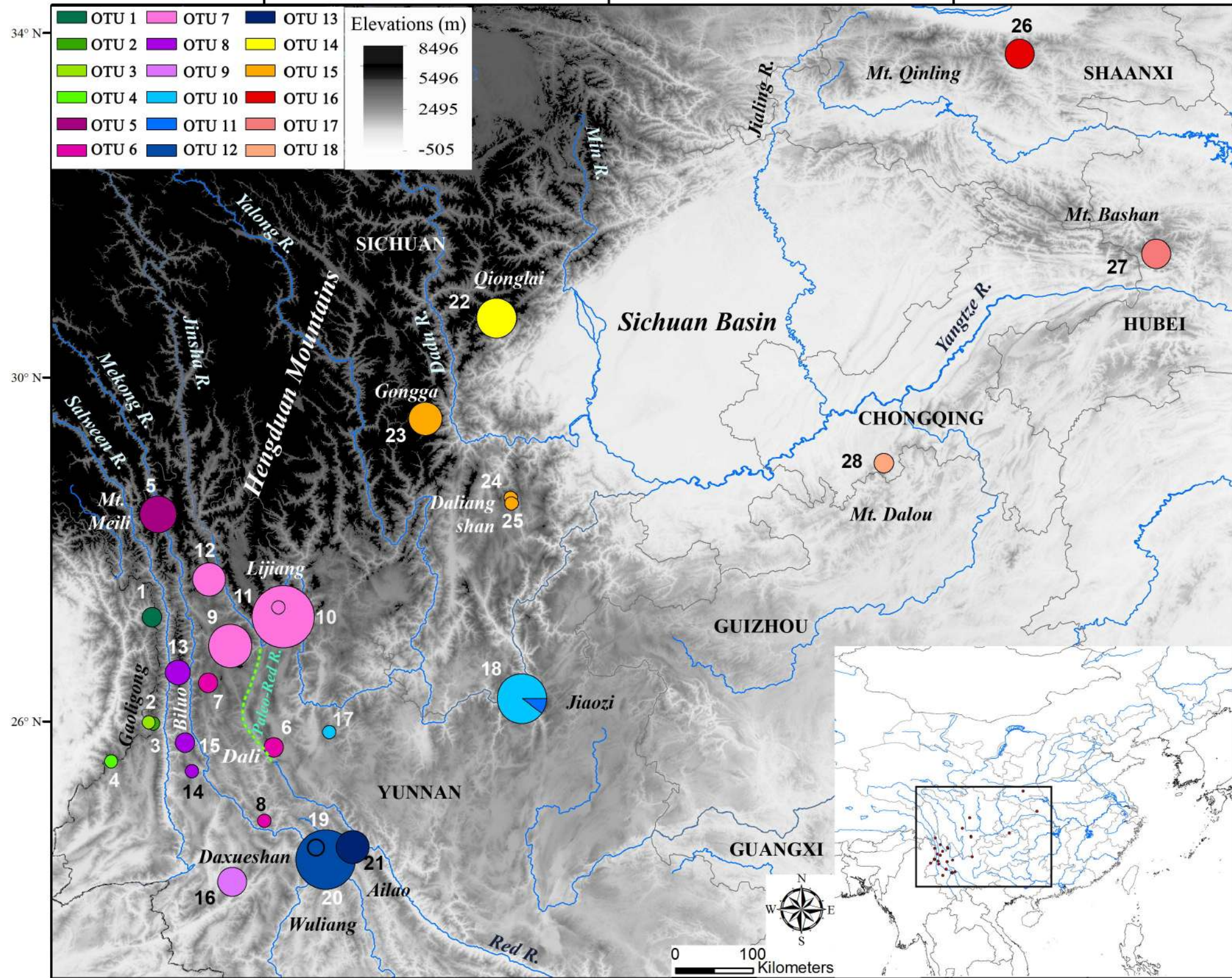
Table 1. Model comparison for diversification scenarios. Divergence times and Bayes factors estimated with the path and stepping-stone sampling (PS/SS) methods are presented.

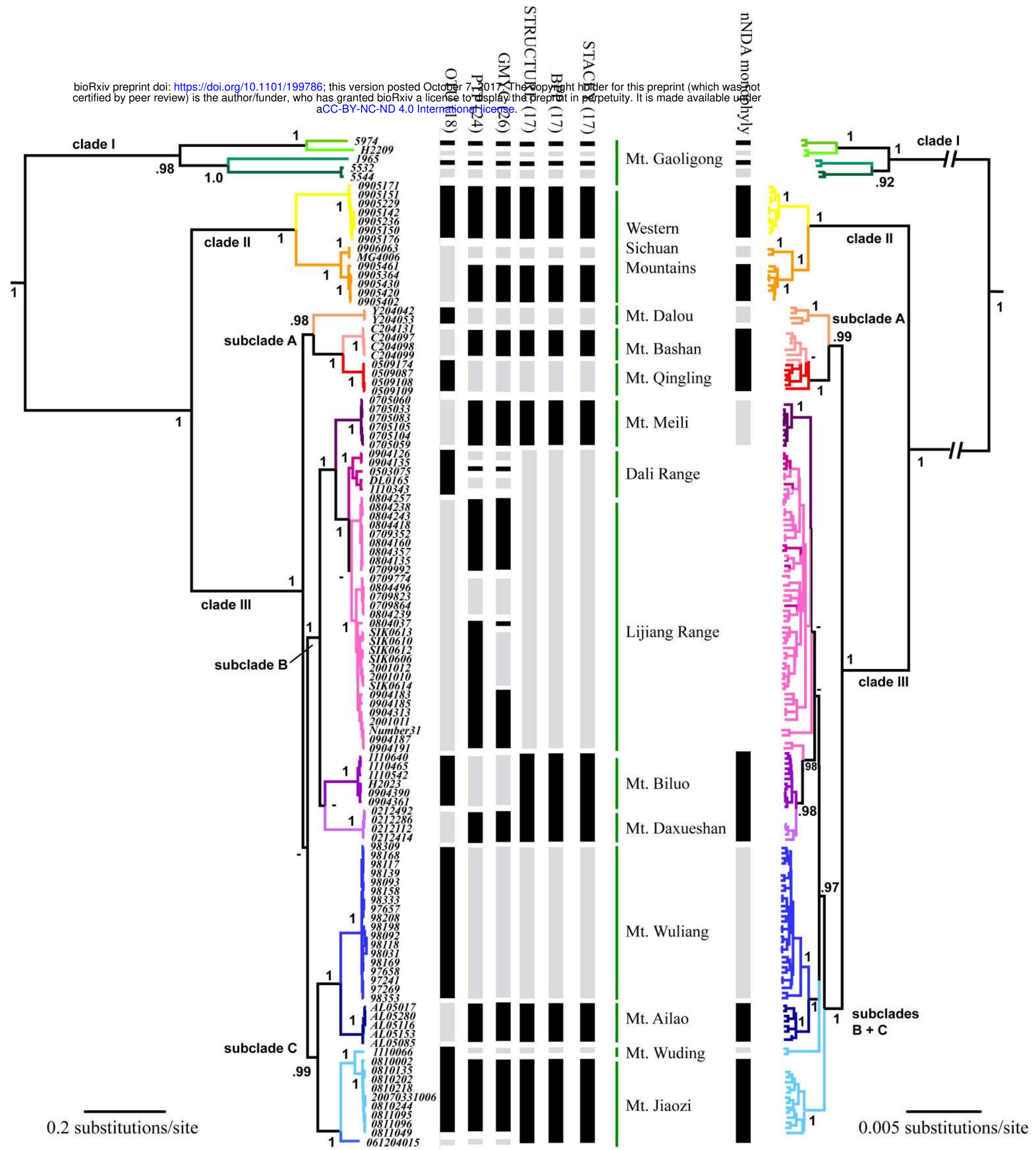
Hypothesis	TMRCAs of <i>S. fusicaudus</i>	Prior /Posterior of TMRCAs (Ma)	path sampling	Δ PS	stepping-stone	Δ SS	<i>Dymecodon / Urotrichus</i> (95%CI)
H0	Oligocene / Miocene	23.03 / 23.05	-15672	-	-15676	2	6.12 (9.03-3.48)
H1	middle / late Miocene	11.61 / 11.84	-15673	1	-15674	-	4.84 (8.70-1.91)
H2	Miocene / Pliocene	5.4 / 6.08	-15688	16	-15689	15	3.32 (7.09-1.11)
H3	Pliocene / Pleistocene	2.4 / 3.83	-15701	29	-15702	28	2.50 (5.57-0.71)

100° E

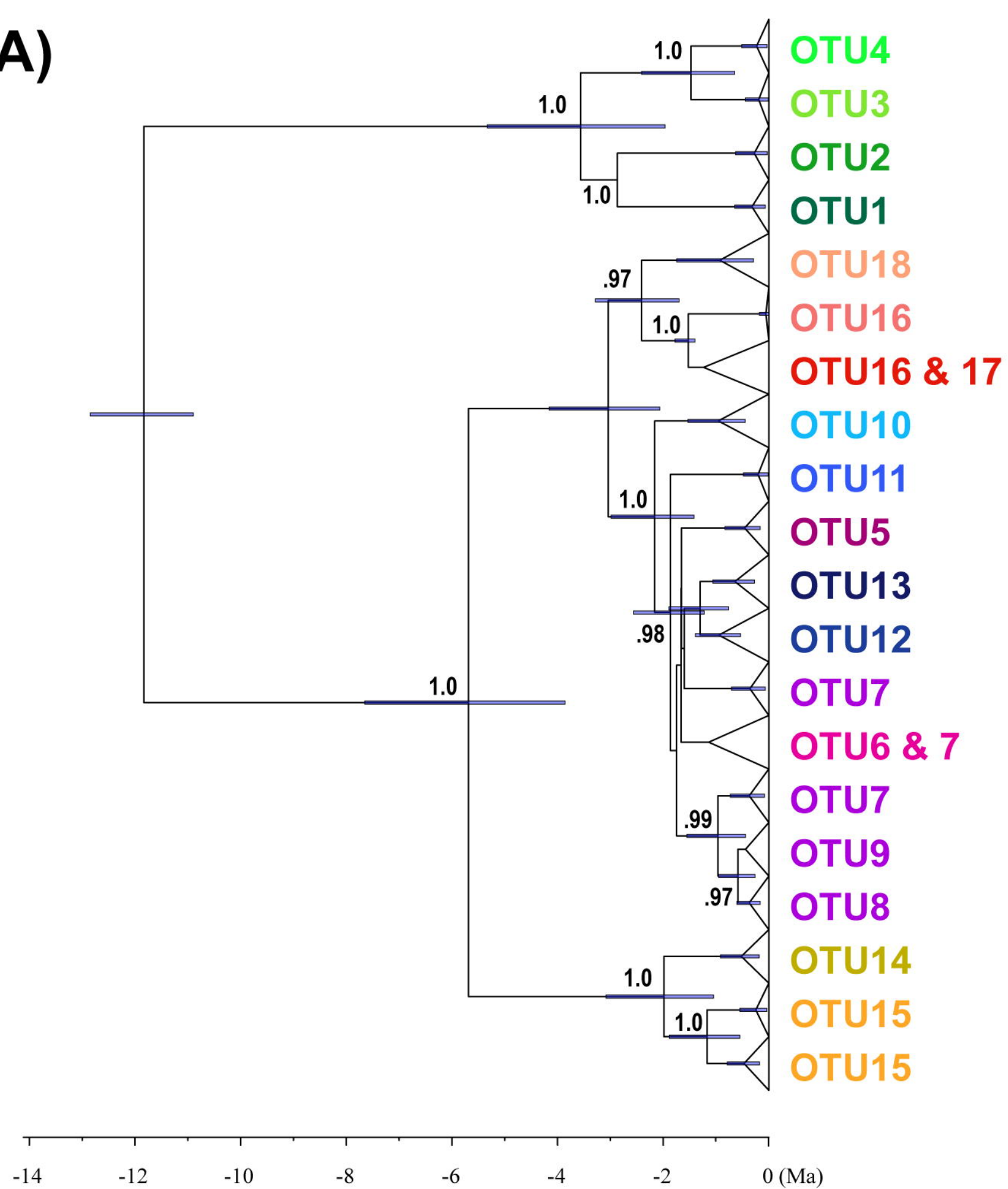
104° E

108° E

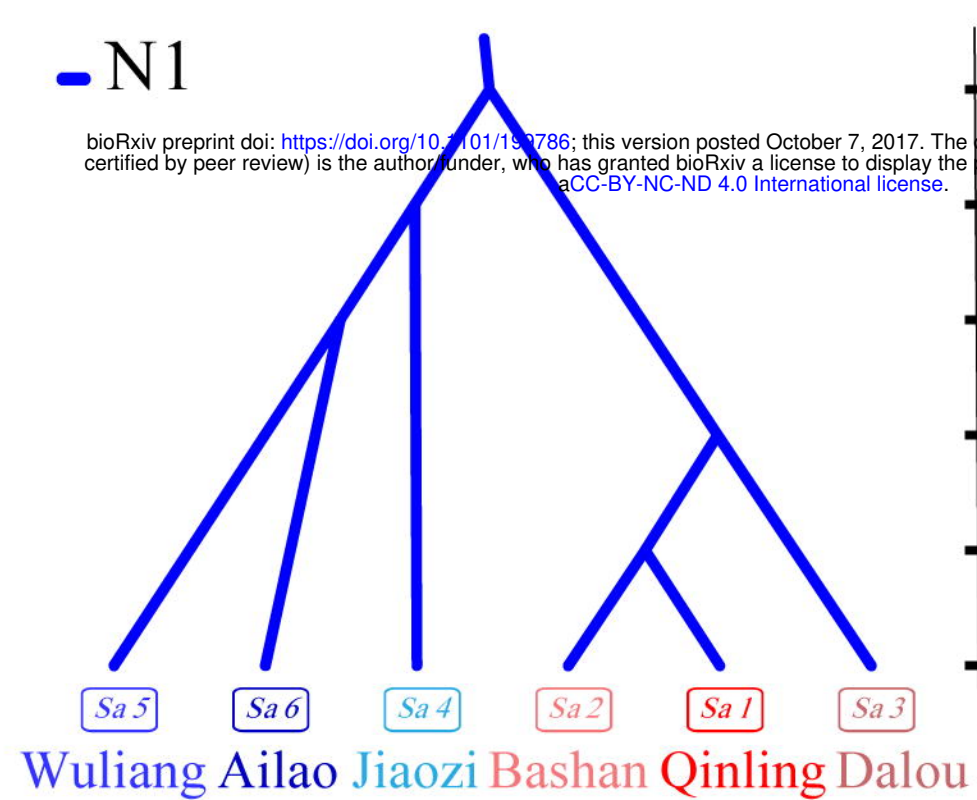
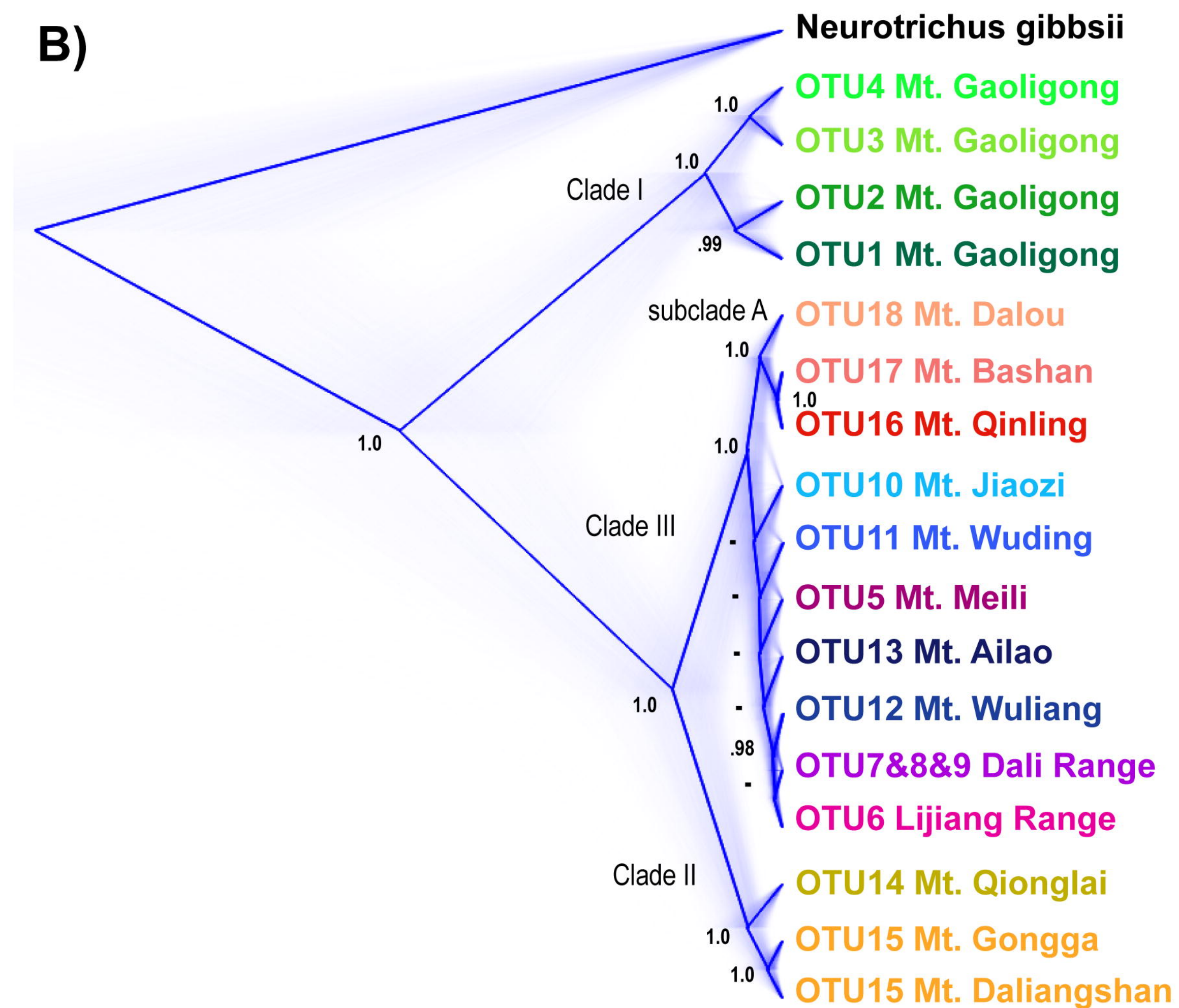




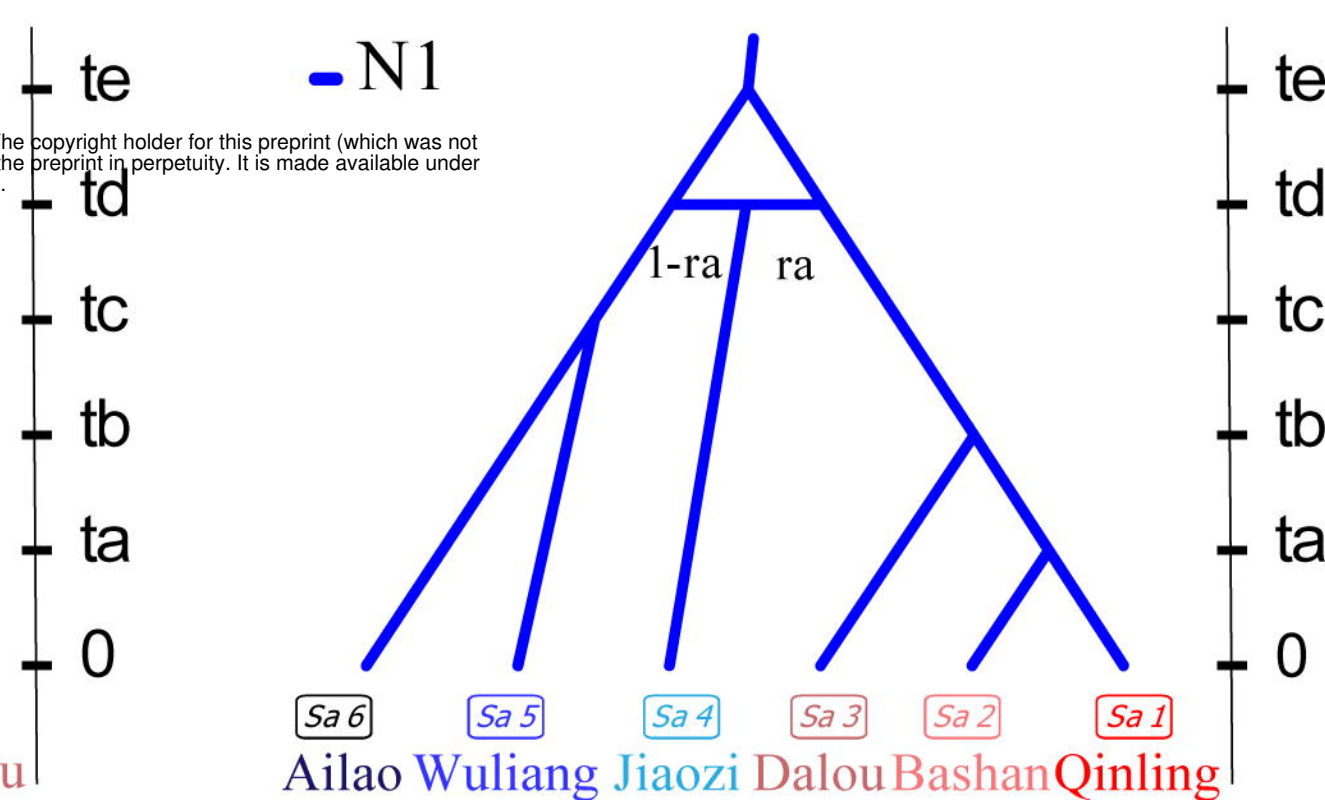
A)



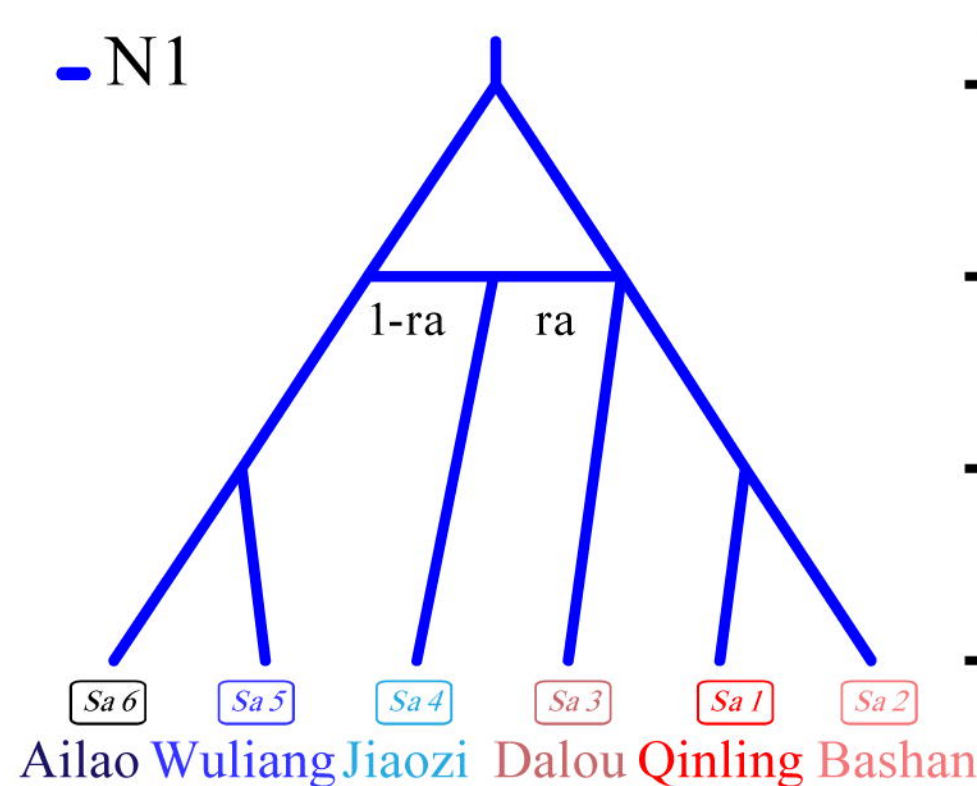
B)



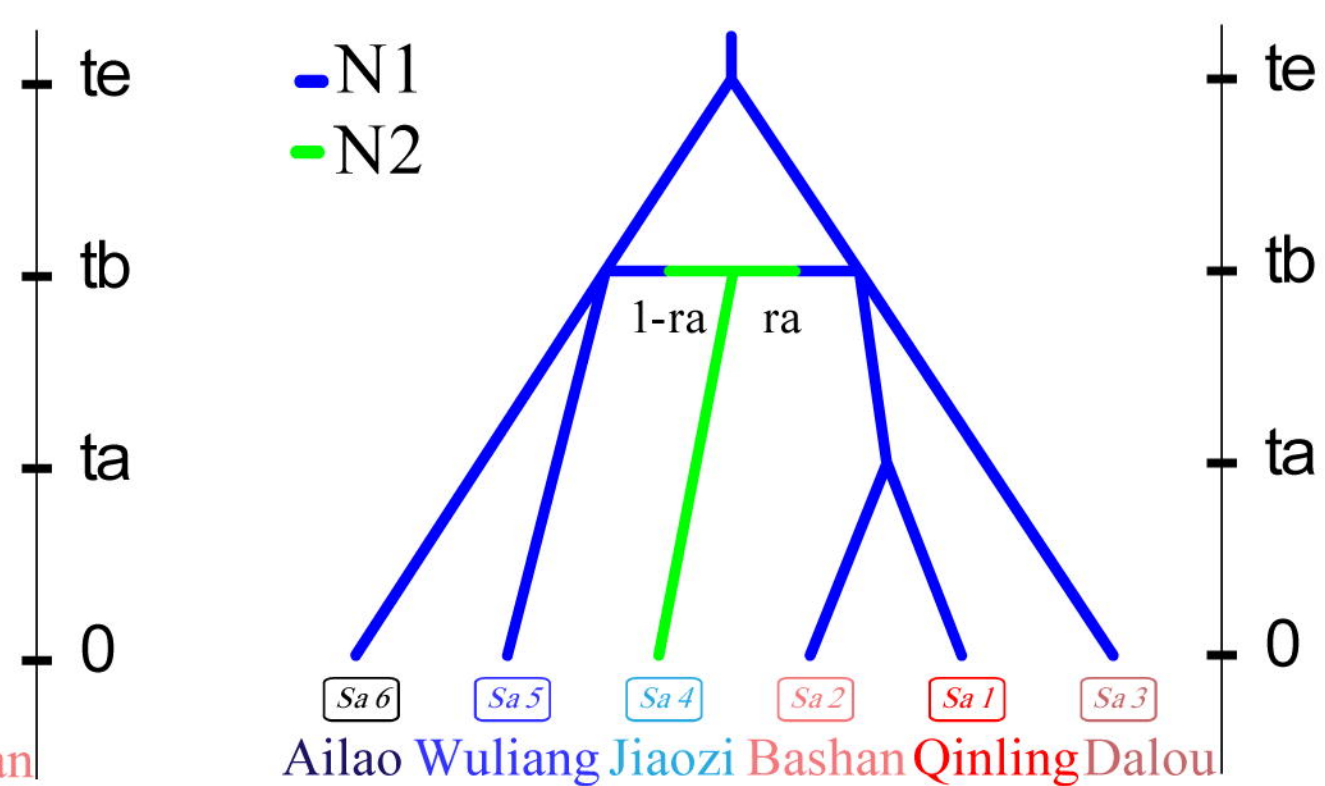
Run1: Best scenario for evolutionary relationships



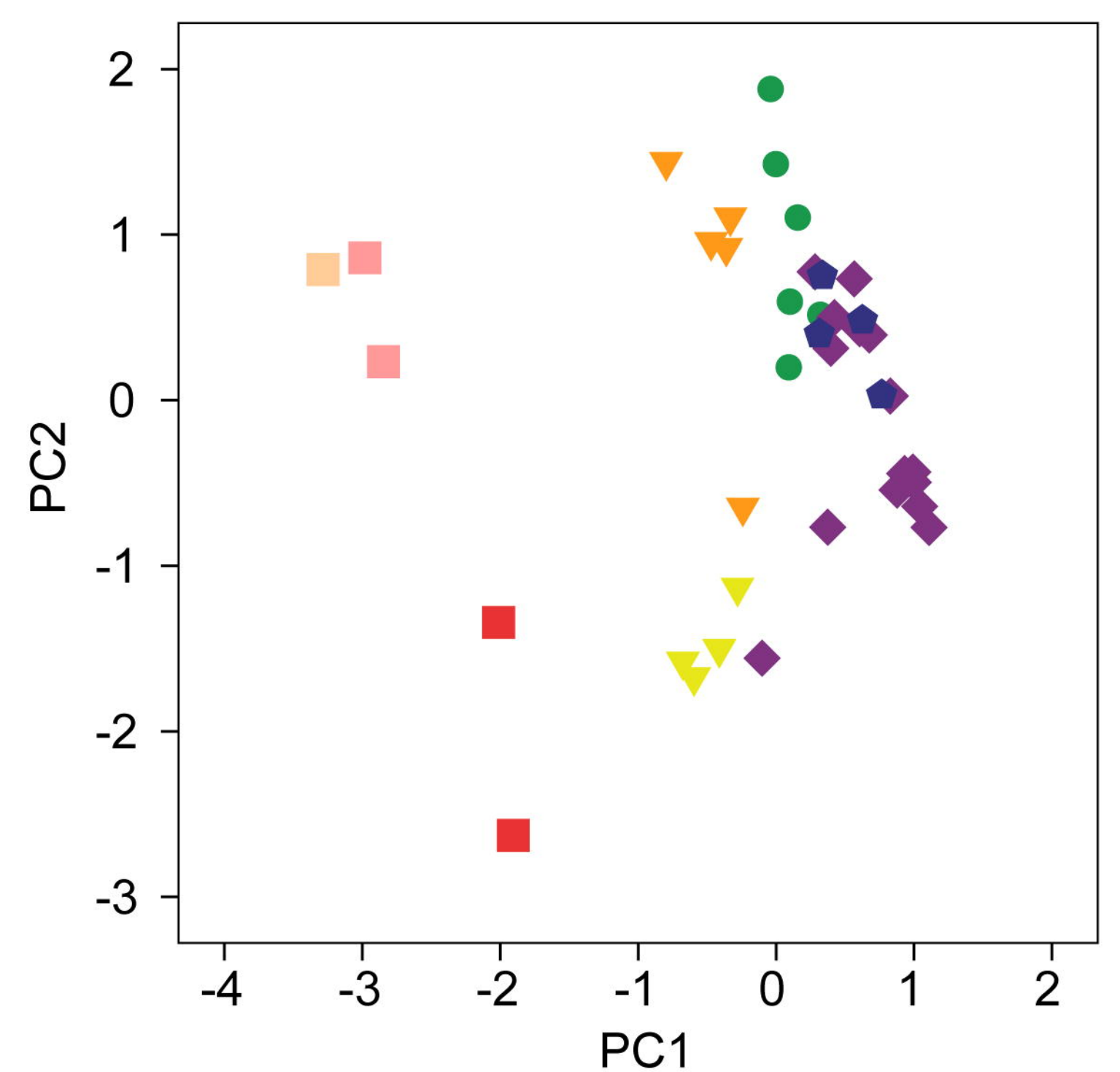
Run2: Best scenario for of the possibility of gene flow



Run3: Best scenario for the time of gene flow



Run4: Best scenario for change of population size



C)

D)

