Phylogeny and biogeography of South Chinese brown frogs (Ranidae, Anura)

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Abstract

Few studies have explored the role of Cenozoic tectonic evolution in shaping the patterns and processes of extant animal distributions in and around East Asia. In this study, we selected South Chinese brown frogs as a model to examine the phylogenetic and biogeographical consequences of Miocene tectonic events within South China and its margins. We used mitochondrial and nuclear molecular data to reconstruct phylogenetic interrelationships among Chinese brown frogs using Bayesian and maximum likelihood analyses. The phylogeny results show that there are four main clades of Chinese brown frogs. Excepting the three commonly known Chinese brown frog species groups, _R. maoershanensis_ forms an independent clade nearest to the _R. japonica_ group. Phylogeny and P-distance analyses confirmed _R. maoershanensis_ as a valid species. Among South Chinese brown frogs, there are four subclades associated with four geographical areas: (I) _R. maoershanensis_; (II) _R. japonica_; (III) _R. chaochiaoensis_; and (IV) other species of the _R. longicrus_ species group.

Divergence times, estimated using mitochondrial sequences, place the vicariance events among the four subclades in the middle to late Miocene epoch. Our results suggest that (1) South Chinese brown frogs originated due to a vicariance event separating them from the _R. chensinensis_ species group at the time of the Geological movement (~18 million years ago, Ma) in southern Tibet and the Himalayan region; (2) the separation and speciation of _R. maoershanensis_ from the _R. japonica_ group occurred due to the dry climate at approximately 16 Ma; (3) South Chinese brown frogs migrated from South China to Japan at the time (~10.8 Ma) that the global sea-level fell and the East China Sea Shelf Basin was swamp facies, when a land gallery may have formed across the sea to connect the two areas; and (4) _R. chaochiaoensis_ separated from other species of the _R. longicrus_ species group during the uplift of the Tibetan Plateau at approximately 9.5 Ma.

Introduction

The taxonomy of _Rana_ (brown frogs), a genus of the family Ranidae, has been intensely debated in the last 20 years. _Rana_ is widely distributed from the Western Palearctic to Northeast Asia. South China is one of the most richly diverse regions for brown frogs, with approximately 12 species [1, 2]. Most South Chinese brown frogs are classified in the _Rana longicrus_
species group, which is one of the major species groups of Chinese brown frogs; the other two species groups are the *R. chensinensis* group and the *R. amurensis* species group [3, 4]. Additionally, the frogs of the *R. longicrus* species group were first known as the *R. japonica* group [5, 6] prior to their recognition as a new species and their classification into the *R. longicrus* species group based on morphological and nucleotide differences [4, 5].

In addition to the frogs of the *R. longicrus* species group, there are also four other commonly known species distributed in South China that share a close phylogenetic relationship with the three Chinese *Rana* species groups: *R. johnsi*, *R. shuchinae*, *R. zhengi* and *R. sauteri* [7]. Additionally, one endemic species, *R. maoershanensis* [8], is found only at the Maoershan National Nature Reserve in Guangxi Province in South China; its validity and phylogenetic relationships with other Chinese brown frogs remains unknown because of conflicting results in previous studies. *R. maoershanensis* was first reported as a new distribution record of *R. chaochiaoensis* from Guangxi Province [9] but was later described as a new species based on morphology [8]. Its status as a new species was supported by a phylogenetic analysis based on 16S ribosomal RNA [16S] showing that *R. maoershanensis* is closely related to the *R. chensinensis* species group [10]. Conversely, Yan et al. [11] considered *R. maoershanensis* as a junior synonym of *Rana hanluica* based on the mitochondrial cytochrome b [Cytb] and cytochrome oxidase subunit I [COI] genes. Therefore, substantial differences in the molecular phylogeny results of previous studies make *R. maoershanensis* a mysterious and controversial species. Therefore, a detailed estimate of the phylogenetic relationship of *R. maoershanensis* to other Chinese brown frogs from correct samples is necessary.

South Chinese brown frogs are common and widespread, from the southeast Chinese coastal hills to the Hengduan Mountains below 3,100 meters in elevation [4]. These species occur in a region of extreme Cenozoic tectonic and environmental changes [12], including the striking orogenesis of the Tibetan Plateau, which greatly altered the global climate [13–15]. Cenozoic global sea-level changes also affected the environment in East Asian coastal areas. Tectonic, environmental and sea-level changes could have affected the evolutionary history of amphibians because of their relatively low mobility, high philopatry, strict habitat specificity and physiological requirements [16]. In this study, we analyze both mitochondrial and nuclear genes to clarify the phylogeny and biogeography of frogs in South China, to reconstruct their phylogenetic relationships and to evaluate their evolutionary history.

**Materials and methods**

**Sampling and laboratory work**

We sampled 20 individuals of 15 *Rana* species, including two species of the *R. amurensis* species group, four species of the *R. chensinensis* species group and all nine ingroup species (the seven species of the *R. longicrus* species group, *R. japonica* and *R. maoershanensis*). This study included ten species that we sequenced ourselves and five species from GenBank; detailed information is shown in Table 1 and Fig 1. Within Table 1, The sequences with superscript numbers among the GenBank accession numbers are referenced as follows: 1, Zhou et al. [17]; 2, Che et al. [3]; 3, Zhou et al. [18]; 4, Yan et al. [11]; 5, Sumida et al. [19]; 6, Lin et al. [20]; 7, Ni et al. [21]. This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on Animal Care and the Ethics Committee of the College of Wildlife Resources, Northeast Forestry University (Permit Number: 100201–2015). For live frogs, we refer to Li et al. [22] only toe tip (1–2 mm²) tissues were collected and stored, and then they were released immediately after treating wounds with antiseptic. We have one week laboratory observation of *R. dybowskii* shown that the non-destructive sampling of toe
Table 1. Information about brown frogs included in this study.

| Species name          | Voucher No.      | Locality               | Longitude | Latitude | Locality No. | GenBank accession numbers |
|-----------------------|------------------|------------------------|-----------|----------|---------------|---------------------------|
| R. dybowskii*         | SYNU08100690     | Mudanjiang, Heilongjiang, China | 129°33'  | 44°35'  | 1             | KF204617 KF020592 KF020621 KF020606 KX025047 KX024980 |
| R. chensinensis*      | SYNU-hld1        | Huludao, Liaoning prov., China | 120°53'  | 40°45'  | 2             | KF204616 KF020598 KF020627 KF020612 KJ371973 KJ371956 |
| R. huanrensis*        | SYNU07040035     | Huanren, Liaoning, China   | 125°25'  | 41°17'  | 3             | KF204614 KF204642 KF204668 KX139725 KX139740 KX139734 |
| R. kukunoris          | SCUM045101WD     | Luoyang Co., Aba State, Sichuan prov., China | 102°56'  | 33°35'  | 4             | DQ289091 DQ289116 JN984231 |
| R. coreana*           | SYNU13030005     | Mt. Kunyushan, Shandong prov., China | 129°33'  | 44°35'  | 5             | KF020621 |
| R. amurensis*         | SYNU11100267     | Taiyangdao, Heilongjiang prov., China | 126°35'  | 45°47'  | 6             | KF020612 KJ371958 |
| R. huanrensis*        | SCUM0450986WD    | Zhongdian, Yunnan prov., China | 102°56'  | 33°35'  | 7             | DQ289081 DQ289106 |
| R. jemuxiensis        | KZ05263          | Jiemuxi National Nature Reserve, Hunan prov., China | 110°24'  | 28°52'  | 8             | - |
| R. culaiensis*        | SYNU07050129     | Mt. Culaishan, Shandong prov., China | 117°21'  | 36°03'  | 9             | - |
| R. zhenvaiensis*      | SYNU08040100     | Hangzhou, Zhejiang prov., China | 120°08'  | 30°13'  | 10            | KF020488 KF204620 KF204644 KX139726 KX139741 KX139735 |
| R. hanliuta*          | SYNU07100490     | Mt. Yangmingshan, Hunan prov., China | 111°54'  | 26°06'  | 11            | KF020484 KF204623 KF204646 KX139724 KX139743 KX139735 |
| R. omeinontis*        | SYNU08060317     | Mt. Emei, Sichuan prov., China | 103°20'  | 29°29'  | 12            | KF020485 KF204637 KF204646 KX139723 KX139742 KX139736 |
| R. longicrus          | long.T           | Taipe, Taiwan prov., China | -         | -       | 13            | AB058865 AB058881 JF939107 JF939067 |
| R. japonica           | jep. J*1         | Hiroshima, Japan        | -         | -       | 14            | AB058885 AB058876 JF939107 JF939067 |
| R. maoershanensis*    | SYNU08030661     | Mt. Maershans, Guangxi prov., China | 110°24'  | 25°52'  | 15            | JF939108 JF939104 |
| R. omeinontis*        | SYNU08030602     | Mt. Emei, Sichuan prov., China | 110°24'  | 25°52'  | 16            | KF049927 KF049927 KF049927 KF049927 |
| Lithobates catesbeiana|                 | Jinhua, Zhejiang, China | -         | -       | 17            | - |
| Lithobates sylvatica  |                 | Southeastern Ontario, Canada | -         | -       | 18            | - |
| Babina daunchina*     | SYNU12050567     | Hangzhou, Zhejiang, China | 120°08'  | 30°13'  | 19            | KF0204618 KF204600 KF204630 KF204615 KJ371971 KJ371955 |

Table legend: Species names marked by asterisks were sequenced in our own laboratory. Numbers in Locality No. correspond to the collection locations in Fig 1.
tips minimizes the suffering of them and does not influence their survival. Toe tips were preserved in 95% ethanol.

Genomic DNA was extracted using a standard phenol-chloroform protocol [23]. The six gene fragments that we sequenced included four partial mitochondrial DNA (mtDNA) genes, namely, the 12S ribosomal RNA gene [12S], the 16S, the Cytb, and the COI, and two partial nuclear DNA (nuDNA) genes, namely, recombination-activating protein 2 [RAG2] and ATP-dependent DNA ligase IV [LIG4]. We amplified genomic DNA via the polymerase chain reaction (PCR) in 25-μl reactions using marker-specific primers (Table 2). Thermal cycling began with a denaturation period of 5 min at 95˚C that was followed by 36 cycles of 94˚C for 1 min, primer-specific annealing at 46–55˚C for 2 min, and 72˚C for 1 min, with a final extension at 72˚C for 10 min.
Sequence analyses

Nucleotide sequences were edited using MEGA 7 [30] and aligned using the L-INS-i method in MAFFT version 5 [31] with the default parameters. Ambiguous alignments were removed using Gblocks ver. 0.91b [32] with the ‘with half’ option and the default block parameters. Mitochondrial (Mt) and nuclear genes from Babina daunchina, which we sequenced ourselves, and Mt genome sequences of Lithobates (L. catesbeiana and L. sylvatica) from GenBank [20, 21] were selected as outgroups for phylogeny and divergent time analyses. P-distances were calculated using MEGA v.7.0. B. daunchina, L. catesbeiana and L. sylvatica were selected as outgroup taxa based on the results of Frost et al. [2].

Partitioned Bayesian (BA) and maximum likelihood (ML) analyses were performed on the concatenated mtDNA and concatenated mtDNA+nuDNA datasets. For mtDNA phylogenetic analyses, an 8-partition scheme was applied: the 12S and 16S genes were treated as two separate partitions, and Cytb and COI were partitioned according to codon positions. For mtDNA+nuDNA, six additional partitions were added relative to the mtDNA phylogeny analyses, with a 12-partition scheme according to the codon positions of RAG2 and LIG4. The incongruence length difference test (ILD) [33] was used to assess the informational congruence between mtDNA and nuDNA and was performed with PAUP version 4.0b10 [34].

For BA analyses, each partition had independent models of substitution as suggested by MrModeltest v.2.3 [35] using the Akaike Information Criterion (Table 3). Markov chain Monte Carlo (MCMC) was run for 10 million generations and was implemented in MrBayes v.3.1.2 [36]. Trees were sampled every 1,000 generations. Stationarity was checked graphically

Table 2. Primers used for PCR and sequencing.

| Locus | Primer name | Primer sequence (5’-3’) | AT | PS | Cited source |
|-------|-------------|-------------------------|----|----|--------------|
| 12S   | FS01        | AACGCGAAGTATGACCCAAAAGTTCT | 54 | 390 | Sumida et al. [24] |
|       | R16         | ATAGTTGGGGATCATATCCCATGGTTGTTT | | | |
| 16S   | F51         | CCCGCTGGTTTACCAAAACA     | 55 | 510 | Sumida et al. [25] |
|       | R51         | GGTCAAGCTGAGATCCGGTA      | | | |
| Cytb  | Cytba       | AAGAAGATTTTGGCAGATGGG     | 50 | 800 | Zhou et al. [18] |
|       | Cytbs       | TAAATCTGACCCCCCTCTCAA     | | | |
|       | L14850      | TCTGACTGATGAACTTTGGCTC    | 570| | Tanaka-Ueno et al. [26] |
|       | H15502      | GGATTGGGTGTGAAATTGTCCTGGG | | | |
| CO1   | ChmF        | TTYTCNACTRYACAAAYAGATCCG | 50 | 650 | Che et al. [27] |
|       | ChmR        | ACYTCKGRTGRCRAARAACTCA   | | | |
|       | L-turtCOlc  | TACCGTGAATTACGGTTGAT      | 800| | Stuart and Parham [28] |
|       | H-turtCOlc  | TGGTGGGCTCATACAAATAGGC   | | | |
| RAG2  | RAG2_F1     | TTTCGNCAARGGGNTGCNCAA     | 46 | 800 | Shen et al. [29] |
|       | RAG2_R1     | CATRCAATGNGCRTCNGGCCCARTG| | | |
|       | RAG2_F2     | AGGGTTTTCCAGTCGACGGWGGKA A RACCCNAAXAYAYGA | | | |
|       | RAG2_R2     | AGATAACATTTTCACACCCGACCAAT TGDATCCACATC | | | |
| LIG4  | LIG4_F1     | GAYTCNACTRYACAAAYAGATCCG | 46 | 1000| Shen et al. [29] |
|       | LIG4_R1     | TCMGGYTDATYTTNARCACANCC  | | | |
|       | LIG4_F2     | AGGGTTTTCCAGTCGACGGWGGKA A TAYGGMATHAARGA | | | |
|       | LIG4_R2     | AGATAACATTTTCACACCCGACCAAT TGDATCCACATC | | | |

Abbreviations: AT, annealing temperature (˚C); PS, approximate product size (bp).

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by plotting log-likelihood scores in Tracer ver. 1.6 [37]. The first one million generations were
discarded as burn-in, and the remaining trees were used to build a consensus tree.

The ML analyses was implemented using a rapid-hill-climbing algorithm in RAxML v.7.0.4
[38]. First, the best-scoring ML tree was inferred with 200 replicates under the CTRCAT
model. Next, a nonparametric bootstrap analysis with 200 replicates was conducted under the
CTRCAT model.

**Divergence time estimates**

The estimation of divergence time was performed in BEAST ver. 1.8.3 [39]. We assumed a
relaxed lognormal clock (uncorrelated) for the rate-variation model and a Yule process for the
tree prior. An independent substitution model was assigned to each partition corresponding
to the BI models. One constraint, with a calculated age of $31.2 \pm 8.1$ million years ago (Ma)
and representing the split between *Rana* and *Lithobates* and corresponding to node 19 in the
study by Bossuyt et al. [40], was imposed on the tree to establish the divergence time. Analyses
were undertaken with 20 million generations while sampling every 1,000th tree; the first 25%
of sampled trees were treated as burn-in. Burn-in and convergence of the chains were deter-
mined with Tracer ver. 1.6 [37].

**Results**

**Sequence characteristics**

A total of ~4,200 sites were obtained, among which ~2,410 sites were from mtDNA and
~1,800 sites were from nuDNA. All sequences were deposited in GenBank; detailed informa-
tion is shown in Table 1. From mtDNA, all species except *R. jiemuiensis* had sequences for all
four mtDNA loci. For twelve species, the mtDNA sequences of each species were from the
same specimen. For the remaining three species, *R. kukunoris*, *R. longicrus* and *R. japonica*,
the mtDNA sequences were from different sources and were concatenated together based on
direct or indirect evidence to represent the genetic characteristics of one species. A detailed
description of the direct or indirect evidence for concatenating sequences from different
sources is described in S1 Text. In the nuDNA dataset, ten species had both nuDNA genes

| No. of partition | Gene | Partition | AIC model |
|------------------|------|-----------|-----------|
| 1                | 12S  |           | GTR+G     |
| 2                | 16S  |           | GTR+I+G   |
| 3                | Cytb | codon 1   | K80+I+G   |
| 4                |      | codon 2   | HKY+I     |
| 5                |      | codon 3   | GTR+G     |
| 6                | COI  | codon 1   | GTR+G     |
| 7                |      | codon 2   | HKY       |
| 8                |      | codon 3   | GTR+G     |
| 9                | RAG2 | codon 1   | F81+I     |
| 10               |      | codon 2   | F81+I     |
| 11               |      | codon 3   | K80+I     |
| 12               | LIG4 | codon 1   | F81+I     |
| 13               |      | codon 2   | HKY       |
| 14               |      | codon 3   | HKY+G     |

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(Table 1). The results of the ILD test showed no significant phylogenetic incongruence (P = 0.985) between the mtDNA and the nuDNA; therefore, we combined them into a single dataset for the phylogeny analyses.

### Phylogeny, nucleotide diversity and divergence times

BA and ML analyses of the mtDNA dataset and the mtDNA+nuDNA dataset inferred similar stable topologies, with four well-supported clades of Chinese brown frogs (Fig 2). Three clades corresponded to previously recognized species groups, namely, *R. amurensis*, *R. chensiensis*, and *R. longicrus* (or *R. japonica*), while the fourth clade represented *R. maoershanensis*. Phylogenetic analyses showed that *R. maoershanensis* had a close relationship with the *R. longicrus* species group, with strong support [ML bootstrap (ML) = 81% and Bayesian posterior probability (BPP) = 0.99 by mtDNA; ML = 70% and BP = 0.92 by mtDNA+nuDNA]; together, they displayed a sister relationship with the *R. chensiensis* species group.

There were four main conspicuous subclades within our ingroup species, three of which are South Chinese subclades, namely, *R. maoershanensis* (subclade I), *R. chaochiaoensis* (III) and other species in the *R. longicrus* species group (IV), and one of which is a Japanese subclade...
with *R. japonica* (II). Within the *R. japonica* group, subclades III and IV have the closest relationship, and they then join with subclade II, all with absolute supports.

P-distances between *R. maoershanensis* and the other Chinese brown frogs were all above 13.2% for *Cytb* gene sequences and above 12.2% for *COI* gene sequences (S1 Table). Compared to the major clades, *R. maoershanensis* differed by at least 16.1% and 12.7% in the *Cytb* and *COI* genes, respectively (S2 Table).

The timing of our favored internal nodes of Chinese brown frogs is shown in Fig 3. The crown age of Chinese brown frogs is circa 20.7 Ma (95% highest posterior density (HPD))
intervals, 15.4–26.1 Ma), which also represents the divergence time of the *R. amurensis* species group and other Chinese brown frogs. The split between the *R. chensinensis* species group and the South Chinese brown frog group occurred approximately 18 Ma (95% HPD: 13.4–23.4 Ma). The divergence time between *R. maoershanensis* and the *R. japonica* group is approximately 16 Ma (95% HPD: 11.5–20.7 Ma). The divergence time between *R. japonica* and the *R. longicrus* species group is 10.8 Ma (95% HPD: 7.4–14.5 Ma), and that between *R. chaochiaoenensis* and other species of the *R. longicrus* species group is 9.5 Ma (95% HPD: 6.6–13.1 Ma).

**Discussion**

**Phylogeny of South Chinese brown frogs**

Our study provides a well-resolved phylogeny of Chinese brown frogs, with a near-complete taxonomic sampling of the three commonly known Chinese *Rana* species groups (13 of 14 species) and of one *R. longicrus* species group with a related species, *R. japonica*, from Japan. The South Chinese brown frogs first have a sister relationship with the *R. chensinensis* species group; these groups together have a sister relationship with the *R. amurensis* species group. Within South Chinese brown frogs, there are two main clades, namely, the *R. japonica* group and a single-species clade of *R. maoershanensis*. Within the *R. japonica* group, there are four subclades associated with four geographical areas. The subclades are *R. maoershanensis* (I); *R. japonica* (II); *R. chaochiaoenensis* (III); and other brown frogs from the *R. longicrus* species group (IV).

**Validity of *R. maoershanensis***

Although previous studies have disagreed on the taxonomic status of *R. maoershanensis* [11], our phylogeny confirms the validity of this species. We performed phylogenetic analyses on independent genetic datasets (mtDNA and mtDNA+nuDNA), and all of our results recovered a monophyletic *R. maoershanensis* (Fig 2) that is distinct from the three other commonly known Chinese *Rana* species groups.

P-distances have been used as a line of evidence to delineate species among Chinese brown frogs [10, 41]. Kartavtsev et al. [42] used a large Cytb and COI dataset to compare genetic divergence at different levels of taxonomic rank and found that sibling species differed by P-distances of 5.52±1.34 (Cytb) and 4.91±0.83 (COI). Our values comparing *R. maoershanensis* with other Chinese brown frogs were above 0.13 for Cytb and above 0.12 for COI. Additionally, the P-distances between *R. maoershanensis* and other species groups were also larger than 0.16; all of these data indicate that *R. maoershanensis* differs at the species level from different genera within a family.

In recent studies sampling *Rana* species at Mount Maoershan and placing *R. maoershanensis* as a synonymous species with *R. hanluica* [11], we believe that incorrect samples were collected. Our samples were collected by the same *R. maoershanensis* finders as in Lu et al. [8]. The difficult sample collection of *R. maoershanensis* and the importance of its phylogenetic position also imply that this species needs more care and protection.

**Origin and biogeography of South Chinese brown frogs**

From our own and previously published *Rana* location data [4, 11, 18], we first conclude that the main boundary between the South Chinese brown frog and the *R. chensinensis* species group runs from the Sichuan basin to the middle and lower Yangtze River plain. *R. chaochiaoenensis* from among the South Chinese brown frogs and *R. kukunoris* from the *R. chensinensis* species group have adjacent plateau distributions around Tibetan plateau and they split by middle Hengduan Mountains and Sichuan Basin. Based on our divergence-time analyses, the
South Chinese brown frogs display a sister relationship with the *R. chensinensis* species group (Fig 2) and they diverged at 18 Ma (95% HPD: 13.4–23.4 Ma). Approximately 23–17 Ma, the global sea-level rose by over 100 m [43–45]; the sea-level rise could have caused most of the middle and lower Yangtze River plain to be flooded by seawater (Fig 1). Therefore, we argue that brown frogs could not have migrated across the lower Yangtze River plain before 17 Ma and that the origin of South Chinese brown frogs was not in these areas. During 18–8 Ma, the change in the Tibetan deformation pattern occurred when north-south contraction was replaced by coeval development of conjugate strike-slip faulting and east-west extension in Tibet [46–51], which may be the main reason that South Chinese brown frogs separated from the *R. chensinensis* species group. Our analyses suggest that the common ancestor of South Chinese brown frogs and the *R. chensinensis* species group was widely distributed throughout the Tibetan area before 18 Ma, after which a vicariance event of the two main clades, the South Chinese brown frog clade and the *R. chensinensis* species group clade, was caused by the Geological movement that occurred in southern Tibet and the Himalayan region (Fig 4A).

Currently, *R. maoershanensis* is found only at an approximately 2,000-m elevation on Mount Maoershan in Guangxi Province, China [8]. *R. maoershanensis* is distributed near the boundary area of the *R. longicrus* species group, with an overlap in distribution with *R. hainiensis* and *R. hanluica* in central South China (see Fig 1). According to our phylogeny and divergence-time analyses, *R. maoershanensis* has a sister relationship with the *R. japonica* group as a result of a vicariance event at ~16 Ma. Numerous studies suggest that an intensification of climatic aridity occurred in central Asia during the middle Miocene (16–12 Ma), based on pollen records in this area [52–55]. We suggest that the dry periods during this stage made the brown frogs’ habitat shrink and resulted in the separation and speciation of *R. maoershanensis* from the *R. japonica* group (Fig 4B).

After *R. maoershanensis* split with the *R. japonica* group, *R. japonica* separated from the *R. longicrus* species group at ~10.8 Ma, which was at the end stage of the late-Middle-Miocene sharp global sea-level decrease [44]. The amplitude of the late Middle Miocene sea-level decrease was 45–68 m [56], and the shallow sea areas of East Asian margins could have become land during the sharp sea-level decrease. The East China Sea Shelf Basin (ECSSB) entered a new developmental stage, the neotectonic movement stage, after the Miocene [57]. Previous geophysical prospecting and drilling data have confirmed that the ECSSB was swamp facies during the early to middle Miocene and was in the transition phase during the Pliocene [58, 59]. Therefore, there was once a land gallery connecting Southeast China and South Japan during the sea-level decrease and when the ECSSB was in its swamp stage. Our hypothesis suggests that the ancestor of *R. japonica* migrated from South China, for instance, from the Fujian region of China, to Japan through the land gallery at ~10.8 Ma. Separation between the *R. japonica* and *R. longicrus* species groups then occurred when the land gallery was destroyed as the sea level recovered and the swamp facies of the ECSSB subsided (Fig 4C).

Orogenesis is responsible for driving speciation in many taxa via vicariance. In the orogenesis of the Tibetan plateau, the rapid uplift and unroofing of southern Tibet began at approximately 20 Ma; the further significant increases in the altitude of the Tibetan plateau are thought to have occurred approximately 10–8 Ma [60–62]. For several species, the uplift of the Tibetan plateau has been hypothesized as the driving force of vicariant speciation (e.g., Macey et al. [63]; Che et al. [64]). *R. chaochiaensis* is the only living species among South Chinese brown frogs that lives on a plateau; these frogs are mainly distributed at the 1,150–3,500-m plateau in the south part of the Hengduan Mountains [4]. Our divergence-time analyses show that *R. chaochiaensis* separated from other species of the *R. longicrus* species group at approximately 9.5 Ma, coincident with the end period of the Tibetan Plateau uplift, suggesting that the geological event resulted in the speciation event (Fig 4D).
This study evaluated the phylogeny and evolutionary history of the frequently studied South Chinese brown frogs. We successfully recovered the phylogenetic relationships of South Chinese brown frogs and most Chinese brown frogs using mitochondrial and nuclear genes. The phylogenetic analyses also confirmed *R. maoershanensis* as a valid species composing an
independent clade that is distinct from the other three commonly known Chinese Rana spe-
cies groups. By comparing our divergence-time estimates with the ancient climatic and geological history of the distribution areas of South Chinese brown frogs, our findings suggest that the middle Miocene dry climate, the late Middle Miocene sea-level decrease, the neotectonic movement of the ECSSB and the Tibetan plateau uplift all played important roles in shaping the disjunctive distributions of South Chinese brown frogs.

Similar biogeographical processes might be found for other taxa that possess distribution ranges in the Tibetan plateau and East Asian margins and have evolutionary histories during the Miocene. Previous biogeographical studies of Tibetan amphibians [18, 63, 64] also agree that the late-Miocene uplift of the Tibetan plateau may have resulted in speciation processes. Miocene migrations from South China to Japan were also observed in phylogenetic and biogeographical analyses of giant flying squirrels [65] and salamanders [66]. We are the first authors to suggest that the late-Miocene sea-level fall and the swamp facies of the ECSSB may have built a land gallery for the migration of some organisms between South China and Japan.

Supporting information
S1 Table. P-distances among species of Chinese brown frogs based on Cytb (below the diagonal) and COI (above the diagonal).
(DOCX)

S2 Table. P-distances among four main clades of Chinese brown frogs based on Cytb (below the diagonal) and COI (above the diagonal).
(DOCX)

S1 Text. Combination of same-species sequences from different specimens.
(DOCX)

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