Introduction

*Apodemus* species are among the most common small rodents inhabiting woodlands and forests of the Palaearctic and Oriental Region [1,2,3,4]. The genus has been subdivided into four subgenera, *Apodemus*, *Sylvaeous*, *Alsonsus* and *Kastomys* [5] and comprises 20–22 extant species [2,4,6].

There are 12 extant *Apodemus* species in the Oriental Region [2], but only four forms are reported from the Eastern Trans-Himalayas [4], including *A. peninsular*, *A. latronum*, *A. chevieri* and the *A. draco* complex. The *Apodemus draco* complex includes *A. draco*, *A. ilex*, and *A. orestes* and are distributed in mountainous areas in China, Myanmar, and India [2]. These taxa have been treated as three valid species, subspecies of a single species, or synonyms of *Apodemus draco* in different taxonomic revisions [2,3,5,7,8]. The fossil records and phylogenetic analysis suggested an initial radiation of *Apodemus* in East Asia into a Japanese endemic (*A. argentatus*), a Nepalese endemic (*A. gurkha*) and the ancestral lineage of the remaining eastern Asian species (subgenus *Apodemus*) after a two-step radiation process associated with the recent tectonic movements that occurred 5–7 Mya (million years ago) and 2–5 Mya, respectively [9,10,11]. The phylogeography of *Apodemus* in the Far East of Asia showed extensive isolations within *Apodemus* species and could be linked to the presence of many biogeographic barriers such as mountains, rivers, seas, and deserts [1], similar to many other species [9,12,13,14].

The Hengduan Mountains (i.e. the mountains of Southwest China) have the most complex river systems and a profoundly complex and dynamic geological history. The uplifting of the Himalayas and the Qinghai-Tibet Plateau and the successive alternation of glaciation and interglaciation in the Pleocene-Pleistocene contributed to the formation of natural geographical barriers and habitat heterogeneity [15]. This made it not only an important center of relic survival but also decisive evolutionary localities exist. Hence it is an excellent model system for biogeographic studies [16]. Previous studies of *Apodemus* considered this region to be the Pleistocene refugium or the radiation center for the East Asian *Apodemus* species [1,8,17,18]. However, there has not been sufficient evidence from morphometric and molecular studies to describe the phylogenetic relationship...
between *A. draco* and *A. ilex*, especially the biogeography in the south of the Hengduan Mountains and the Yunnan-Guizhou Plateau.

In this study, the complete cytochrome *b* gene sequences (cyt-*b*) were determined from 203 samples of *A. draco/ilex* that were collected from southwest China, including the Hengduan Mountains and the Yunnan-Guizhou Plateau. Using phylogenetic and phylogeographical approaches, we examined the different revisions regarding the taxonomic status of *A. ilex*, the effect of the complex geological structures in the Himalayan regions on the genetic diversity of *A. ilex*, as well as the hypotheses of the biogeographic patterns and colonization history of *A. ilex*. In addition, a Bayesian method with a “relaxed” clock model [19] was applied to co-estimate the phylogenetic relationships and divergence times of *Apodemus*.

**Methods**

**Ethics Statement**

All animal samples were obtained following the regulations for the implementation of China on the protection of terrestrial wild animals (State Council Decree [1992] No. 13) and approved by Wildlife Protection Office, Yunnan and Sichuan Provincial Forestry Departments, China as well as the Ethics Committee of Kunming Institute of Zoology, Chinese Academy of Sciences, China.

**Sampling and sequencing**

A total of 203 samples of *Apodemus draco/ilex* were collected from 51 localities in the southern Hengduan-Mountains and the Yunnan-Guizhou Plateau in China (Table S1, Figure 1). Specimens were identified based on the description of Thomas [20] and Barrett-Hamilton [21].

Total genomic DNAs were extracted from muscle or liver preserved in 95% ethanol at −20°C by using the phenol/proteinase K/sodium dodecyl sulphate method [22]. Mitochondrial cyt-*b* sequences (1,140 bp) were amplified with the universal primers of L14724 and H15915 [23]. The 50 μl PCR reaction contained 5 μl of 10X PCR buffer, 2 μl of 2 mM dNTP mixture, 2 μl of bovine serum albumin (1 mg/ml), 1 μl of 10 mM of each primer, 2.5 μl of 25 mM MgCl₂, 1.25 U Taq DNA Polymerase (Takara, Dalian, China) and approximately 100 ng total genomic DNA as template, and DNase/RNase free water diluted to a final volume of 50 μl. A touchdown PCR protocol [24] was used to prevent non-specific amplification, including an initial denaturation at 94°C for 10 min, 10 cycles of denaturation at 94°C for 40 s, annealing at 52.5°C but...
Phylogenetic and molecular divergence analysis

The DNA sequences were edited with Segman and EditSeq (DNASTAR, Lasergene v.7.1) and aligned with ClustalX v.1.83 [25]. Genetic distance was calculated with MEGA v.4.0 [26] with the Kimura 2-parameter (K2P) model [27].

We apply Bayesian inference (BI) and maximum likelihood (ML) to reconstruct phylogenetic relationships. We used RAxML v.7.2.8 [28,29] for ML analyses on the CIPRES Science Gateway (ML) to reconstruct phylogenetic relationships. We used RAxML [25]. Genetic distance was calculated with MEGA v.4.0 [26], with the 95% CI. The third calibration date was 12.3–11.0 Mya, the divergence of Apodemus flavicollis, the common ancestor of Mus and Rattus was included in analyses (Table S2).

Results

Phylogeography of Apodemus ilex

Because A. draco and A. ilex are morphologically indistinguishable, the sequences of the samples were identified based on the pairwise comparison with the sequences of the specimens collected at or near their type localities. The type locality for A. draco is located at Kuatum, Fujian, China, while the type locality for A. ilex is at Salween - Mekong divide (28° 20′ N) [20]. In analysis, the sequences determined from the topotype specimen of A. draco by Liu et al. [8] (Accession number : AY389009) and the specimen from near the type locality of A. ilex from Mt. Meili, China (28° 23.8′ N) were serve as the reference sequences for A. draco and A. ilex, respectively. Of the 203 sequences generated in this study, 6 were identified as A. draco and 197 as A. ilex (Tables S1, S2). Haplotype analysis of 201 cyt-b sequences of A. ilex, including 4 sequences from GenBank, identified 134 haplotypes. The new identified haplotypes were submitted to GenBank (Accession numbers: JF503102–JF503107 (A. draco) and JF503109–JF503198, JF503200–JF503228, JF503230–JF503240 (A. ilex)).

The phylogeny estimated by RAxML and BEAST were congruent with each other and the topologies were overall highly supported. Thus, only the Bayesian trees were given and both Bayesian posterior probabilities and ML bootstrap support values (BS) were represented (Figures 2, 3). All populations of A. ilex and
A. draco formed strongly supported (BS = 94, PP = 1.0) reciprocal monophyletic groups. The sister relationship between A. draco and A. ilex was also supported (BS = 90, PP = 0.87), with 9.0% of a K2P distance. All 201 A. ilex samples were grouped into the Eastern (E) and Western (W) clades, each containing two subclades: E1/E2 and W1/W2 (Figures 2, 3). All clades and subclades were significantly supported by BI analyses (PP = 1.0) and at least moderately supported by ML analyses (BS = 62). The K2P distances between clades and subclades were: E/W = 3.1%, E1/E2 = 1.9% and W1/W2 = 1.6%.

Table 1 presents the divergence times based on the Bayesian relaxed molecular dating estimation. A. ilex and A. draco diverged from their common ancestor at about 2.25 Mya (95% CI = 1.69–2.82). The earliest split within A. draco occurred about 1.15 Mya (95% CI = 0.84–1.46), much earlier than the split of the clades E and W of A. ilex at about 0.62 Mya (95% CI = 0.44–0.84). The divergence times of subclades E1/E2 and W1/W2 were at about 0.33 Mya (95% CI = 0.23–0.45) and 0.32 Mya (95% CI = 0.22–0.45), respectively.

Genetic diversity and structure of A. ilex

Phylogenetics analysis of 201 cyt-b sequences of A. ilex detected 963 conserved sites (84.6% of all sites) and 175 variable sites (15.4% of all sites). The K2P distances between haplotypes of A. ilex ranged from 0.0% to 4.1% (average 2.1%). The overall haplotype diversity (Hd) and nucleotide diversity (Pi) were 0.993 and 0.021, respectively. The pairwise Fst estimation among populations ranged widely from 0.00 to 1.00. Most populations are strongly differentiated from each other (Fst > 0.25) indicating restricted gene flow. High levels of gene flow are more often observed within geographically close populations (e.g. population 16 and 18, Fst < 0.001; Table S3).

In clade E, the haplotypes in both E1 and E2 are widely distributed in the east and some areas of the west of the Mekong basin.
River (populations 1, 14, 34, 36, 39 containing E1 haplotypes and population 1 containing E2 haplotypes; Figure 1). In clade W, the haplotypes in W1 were mainly distributed in the west of the Salween River and two localities east of the Mekong River (populations 10 and 26); while the haplotypes in W2 were distributed mainly in the southern part of the Yunnan-Guizhou Plateau and west of the Mekong River. In addition, sympatric distribution of different maternal lineages were observed in several localities (i.e., population 1, 2, 8, 10, 11, 16, 19, 34, 36, 39; Figure 1).

Further geographical structure was examined with AMOVA using three grouping options, including (1) the populations grouped by the subclades E1, E2, W1 and W2; (2) the populations grouped by geographical distributions, namely, the individuals from the west of the Salween River as group 1, the individuals from the east of the Mekong River as group 2, and the individuals from the east of the Mekong River as group 3; and (3) the populations grouped with the same way as (2) except for populations 1 and 14 which were included in group 3 (Figure 1). The results of AMOVA showed significant genetic structures at all hierarchical levels (P<0.001) and the largest proportion of variances were always found among groups (Table 2). In size order, the variances among groups were the second grouping variances were always found among groups (Table 2).

The network analyses generated eleven most parsimony trees that were similar to the gene tree inferred from the BEAST values from 0.715, 0.737 to 0.822, respectively. W group had the third grouping option (54.12%), with the corresponding increased BS. The network analyses generated eleven most parsimony trees that were similar to the gene tree inferred from the BEAST values from 0.715, 0.737 to 0.822, respectively. W group had the third grouping option (54.12%), with the corresponding increased BS. The network analyses generated eleven most parsimony trees that were similar to the gene tree inferred from the BEAST values from 0.715, 0.737 to 0.822, respectively. W group had the third grouping option (54.12%), with the corresponding increased BS.

**Table 1.** Divergence information within and between groups of *Apodemus*

| Node | Age | 95% CI range | Divergence Event |
|------|-----|--------------|------------------|
| t1*  | 11.92 | 10.49–14.20 | Rattus/Mus |
| t2*  | 10.84 | 10.18–11.95 | Mus/Apodemus |
| t3   | 9.63  | 9.16–10.39  | Sylvaemus Group/Apodemus Group |
| t4   | 3.97  | 3.41–4.63   | sylvaemus/alpicola+flavicollis+tralenis |
| t5   | 2.87  | 2.21–3.63   | tralenis/alpicola+flavicollis |
| t6   | 2.20  | 1.55–2.85   | flavicollis/alpicola |
| t7   | 8.35  | 6.98–9.48   | Apodemus Group/mystacinus |
| t8   | 7.06  | 5.94–8.38   | agrarius subgroup/draco subgroup |
| t9   | 6.12  | 4.89–7.49   | peninsularis/(agranius+chevrieri+spectabilis) |
| t10  | 5.13  | 3.95–6.45   | speciosus/(agranius+chevrieri) |
| t11  | 1.56  | 1.12–2.08   | chevrieri/agranius |
| t12  | 4.74  | 3.64–5.99   | latrunum/(draco+ilex+semotus) |
| t13  | 2.67  | 2.02–3.34   | semotus/(draco+ilex) |
| t14  | 2.25  | 1.69–2.82   | draco/ilex |
| t15  | 1.15  | 0.84–1.46   | draco MRCA |
| t16  | 0.62  | 0.44–0.84   | igh E/W |
| t17  | 0.33  | 0.23–0.45   | igh E1/E2 |
| t18  | 0.32  | 0.22–0.45   | igh W1/W2 |

*Nodes used for calibration.

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**Discussion**

The clade E1 has the most complex structure and can be further divided into 4 subgroups (E1a, E1b, E1c, and E1d). E1a consisted of the haplotypes from Caoqian (population 14). E1b consisted of the haplotypes from Mt. Haba, Mt. Yulong, Lushi and Mt. Bangma (populations 3 and 6, 10, 24 and 36, respectively). E1c mainly included the haplotypes from the Mt. Wuliang and Mt. Ailaos in central Yunnan (populations 15–22), and E1d contained the haplotypes from the northwest of Yunnan (Table S1). Star-like structures were found in E1c. E1d, W1 and W2 (Figure 4), though the original haplotypes were not found in W1 and W2. These structures are evidence of population expansion [52].

**Population historical demography of *A. ilex***

The analysis of MDA suggested that all four groups showed multimodal distributions (Figure S1) but with small SSD and rg values (Table 3). Moreover, Fu’s and R2 test showed the large populations have significant negative Fs (i.e., E1, W1, W2) when small populations have significant small R2 (i.e., E2) (Table 3). Further analyses of the subgroups E1c and E1d revealed smooth unimodal mismatch distributions and significant negative Fu’s Fs values (Table 3, Figure S1). The insensitive values of PSSD and Prg (P>0.05) in E1c, E1d and W2 confirmed the expansions within these group/subgroups; while the significant PSSD/Prg (P<0.05) of W1 might be due to insufficient sample size [53]. Group E2 had multimodal distribution and insignificant negative Fu’s Fs value, but the significant small R2 value and the insignificant values of PSSD and Prg implied the possibility of expansion in congruent with the network topologies. Because the divergence time between group E and W was at about 0.62 Mya, the population expansions of E1c, E1d and W1 were approximately at 0.064, 0.038 and 0.039 Mya, respectively (Table 3).

**Taxonomic implication of *Apodemus ilex***

The taxonomic status of *A. draco, A. ilex* and *A. orestes* have been controversial for a long time. *Apodemus ilex* was named based on specimens collected from the Salween-Mekong divide (28°20’N), China [20] but was treated as a synonym of *A. orestes* [7] or *A. draco* [5,54]. Musser et al. [6] included *orestes* within *A. draco*, but, after comparing the cranial characteristics between *A. orestes* and *A. draco* that are distributed in Wuliang Mountain, China, Jiang and Wang considered *A. orestes* as a valid species [3]. Patterns of genetic variations observed in the complex of *A. draco* based on cyt-b genes suggested that *A. orestes* was a subspecies of *A. draco*, and *A. ilex*, which is distributed in the Yunnan-Guizhou Plateau, is a valid species [8]. However, Musser et al. [2] still considered *A. ilex* and *A. orestes* as synonyms of *A. draco*.

With inclusion of 214 samples of *A. draco/ilex* that were widespread in the southern Hengduan Mountains and Yunnan-Guizhou Plateau, two major phylogroups were identified within the *A. draco* complex, one representing *A. draco* that consists of the specimens from eastern and western China, including all specimens from the western Sichuan Plateau, and another representing *A. ilex* that contains the samples mainly from the southern Hengduan Mountains and the western Yunnan Plateau. The average genetic distance between *A. draco* and *A. ilex* was 0.09 (K2P). The molecular dating estimation suggested that the divergence between *A. draco* and *A. ilex* was at about 2.25 Mya,
earlier than the split of A. alpicola and A. flavicollis or A. agrarius and A. chevrieri at 2.20–1.56 Mya (Table 1, Figure 2). These results support the recognition of A. ilex as a valid species under genetic and phylogenetic species concepts [55,56]. The taxonomic status of A. orestes will be discussed elsewhere (Chen et al. in preparation).

Phylogeographic structure in A. ilex and topography of mountains and rivers

The Hengduan Mountains have long been recognized as a refugial area for animals [57,58]. Previous analyses either focused on the northern Hengduan Mountain [59,60,61] or treated this area as one refugium Only a few studies have addressed the effect of the extremely complex topography of the southern Hengduan Mountains and the Yunnan Plateau [62,63]. Our research revealed the significant internal genetic structure within the mountains which is relevant to the “microrefugia” [64] or “refugia within refugia” [65,66]. These concepts are usually used to explain the phylogenetic structure in the refugia such as the Iberian Peninsula or disjunctive populations surviving in isolated microhabitats. The extremely complex topography [67], climate [68] and habitats [69] in the mountains as well as the mid-high elevation distributed pattern of A. ilex could have lead to the geographically isolation of A. ilex among different mountain areas and the subsequently restricted gene flow, which are respond for the strong geographic structure and the high pairwise $F_{st}$ values [70].

The minimum-spanning network and AMOVA analyses indicated the geographic structure of A. ilex was also shaped by the Mekong and Salween river systems. When the two rivers were setup as the genetic barriers in the AMOVA analyses, the

| Groups                  | $\Phi_{ST}$ | $\Phi_{SC}$ | $\Phi_{CT}$ | %among groups | %among populations within groups | %within populations |
|-------------------------|-------------|-------------|-------------|---------------|---------------------------------|--------------------|
| Four subclades          | 0.822*      | 0.603*      | 0.551*      | 55.17         | 27.05                           | 17.78              |
| Divided by two river    | 0.715*      | 0.497*      | 0.433*      | 43.30         | 28.20                           | 28.50              |
| Third choice            | 0.737*      | 0.427*      | 0.541*      | 54.12         | 19.57                           | 26.31              |

*P < 0.05.  
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Figure 4. The median-joining network of A. ilex based on cyt-b sequences. The circle size is proportional to the haplotype frequency and the branch length is proportional to the number of mutations.  
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variances existed mainly among populations in different regions ($\Phi_{ST} = 0.715$) (Table 2). This result is congruent with the recent proposed hypothesis that deep river valleys may have acted as barriers to *Apodemus* species [1] as well as to other animals [71]. However, another montane mammal, the Yunnan hare (*Lepus conus*) has no phylogeographic pattern in the same area. Neither the area of low-elevation nor the river systems seem to be the barrier for the hares [72]. On the other hand, the paleo-drainage systems have facilitated dispersal of a frog species [62]. The discrepancy may due to the different habitats, dispersal abilities and colonization histories. The *Nanorana yunnanensis* is a semi-aquatic anuran living in cold montane streams [62]; the *L. conus* is much larger than *A. ilex* and may have colonized this area only recently [72].

**Effect of glaciation**

The biogeographic histories of the montane inhabitants are usually affected by Pleistocene glacial cycles [73,74,75]. Generally, animals respond to climate change in different ways [76]. First is by changing their distribution. The montane animals usually have larger distribution during glacial periods (but see [77,78]) when they spread to lowland [79,80], and the glacial and interglacial climate fluctuation can result in population isolation and reconnection [73,74,78,83,84]. Second is by adapting to new environments [75,76,81]. Apparently, *A. ilex* occupies the same habitats as its relatives (i.e. *A. draco* and *A. semotus*) and hasn’t adapted to a new environment, thus it had to shift distribution responding to climate change.

With a Bayesian method under a “relaxed” clock model, *A. ilex* diverged from *A. draco* at around 2.25 Mya. Therefore, the ancestor of *A. ilex* probably expanded southward from the northern Hengduan Mountains during global cooling in the period 2.7–2.5 Mya [85]. After that, divergence of *A. draco/ilex* may be attributed to the accelerated uplift of the Qinghai-Tibet Plateau and the resulting geomorphic changes of the plateau and the surrounding areas [86] as well as to turnover of vegetation and habitats [87]. The divergence between the eastern and western populations of *A. ilex* (~0.62 Mya) was within the Yulong glaciation (0.73–0.5 Mya) [88] and the simultaneous divergences of the subclades E1/E2 (0.33 Mya) and W1/W2 (0.32 Mya) were consistent with the Lijiang glaciation (0.31–0.13 Mya) [89]. Because the calibration points we used are old (4 Mya to 0.01 Mya) [90,91], our research has, however, revealed significant internal genetic structure which suggests that the “microrefugia” or “refugia within refugia” models are more relevant.

**Conclusion and Perspectives**

The Hengduan Mountains are the most important refugial region in China. Other studies have regarded the mountains as a single refugium, our research has, however, revealed significant internal genetic structure which suggests that the “microrefugia” or “refugia within refugia” models are more relevant.

Our finding suggests that both the low-elevation areas and deep river valleys are strong geographic barriers for *A. ilex*. However, for aquatic animals in this area, the drainage system is more likely to facilitate dispersal rather than prohibit it [62]. Thus, it seems the drainage system did play a role in shaping geographic patterns, but in different ways for different animals. Furthermore, the evolution of the drainage system may have led to a more complex geographic pattern.

Paleoclimatic change has also shaped genetic structure. The glacial-interglacial cycles not only resulted in inter- and intraspecific divergence, but also led to population expansion and secondary contact.

Our study has shed light on the biodiversity in this area. However, because of the complex topography of the mountains, complicated geological history of the drainage system, Pleistocene climate fluctuation and habitat turnover, it is far from clear how the high endemic biodiversity came into existence. It would be necessary to use comparative phylogeographic approaches of animals distributed in different habitats and with different dispersal abilities to examine how the topography, geographic
events and climate change have shaped the biodiversity in the mountains of Southwest China [74,93,94].

Supporting Information

Figure S1 MDA and Fu’s Fs test for four subclades of A. ilex.

Table S1 Sampling information and genetic variability of A. draco/ilex used in this study.

Table S2 Information of outgroups used in this study.

Table S3 Pairwise FST values for all pair of populations.

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