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Temporal and Spatial Pattern of *Holcoglossum* Schltr. (Orchidaceae), an East Asian Endemic Genus, Based on Nuclear and Chloroplast Genes

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East Asia is one of the plant diversity and endemism centers in the world, and the temporal and spatial patterns and processes of vascular plants attracted ecologists' and biogeographers' attention. However, the biogeographic patterns of endemic epiphytic plants in East Asia are still unclear. Here, we investigated the historical biogeography of an East Asian endemic epiphytic genus *Holcoglossum* Schltr. (Orchidaceae). Using DNA sequences of eleven chloroplast genes and one nuclear gene, we reconstructed a robust phylogenetic framework for *Holcoglossum* and used a relaxed-clock method to estimate divergent times for the genus. We inferred the ancestral range of lineages under the statistical dispersal-extinction cladogenesis (S-DEC) and statistical dispersal-vicariance analysis (S-DIVA), respectively. Biogeographical analysis suggested that the most recent common ancestor of *Holcoglossum* occurred in the Palaeotropical region in the late Miocene (6.33 Ma). Four dispersal events were inferred to explain the *Holcoglossum* expansion to Sino-Himalayan, Sino-Japanese, and Taiwan regions from the latest Miocene to Quaternary. The episodes of these events were associated with intensification of East Asian monsoon around 3.6–2.6 Ma and global cooling since the latest Pliocene. The disjunct distribution between mainland China and Taiwan was attributed by the sea-level fluctuations and climate changes during the late Pliocene. This study shed light on the biogeographic processes of endemic epiphytic plants in East Asia.

Keywords: biogeography, East Asia, *Holcoglossum*, molecular dating, phylogeny

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INTRODUCTION

East Asia is well-known for its vascular plant diversity and endemic (Qian et al., 2005). The flora of East Asia harbors ca. 258 families and more than 3000 genera, of which approximately 8.2% are endemic (Wu and Wu, 1998; Chen et al., 2018). Understanding the historical origins and processes of the plant richness of East Asia has been of major interest for ecologists and biogeographers. In the Cenozoic, East Asia underwent the uplift of the Himalaya–Tibetan Plateau (An et al., 2001) and the establishment and intensification of the Asian monsoon (Sun and Wang, 2005).

A great deal of phytogeographic research on plants in East Asia has been published. East Asia is suggested as both a "Cradle" and a "Museum" for vascular plants since the Cretaceous (e.g., Wu and Wu, 1998; Wen, 1999; Jiang et al., 2019). Xiang et al. (2019) combined molecular and fossil data and inferred that the ancestors of Hamamelidaceae occurred in tropical Asia during the mid-Cretaceous and subsequently migrated to Europe, Africa, and America during the Late Cretaceous and Early Tertiary. Luo et al. (2015) carried out phylogeographic analysis of four perennial herbs in Hengduan Mountains and showed that these species originated during the Late Pliocene or early–mid-Pleistocene and diverged by climate-induced habitat fragmentation. Further, during the glacial periods of Quaternary, East Asia acted as refugia for vascular plants' survival and evolution, especially for more recent divergent lineages (Tiffney, 1985; Ying, 2001; Qiu et al., 2011; Chen et al., 2018). However, the biogeographic patterns and processes of endemic epiphytic plants in East Asia are still poorly known.

Aeridinae is the largest and horticulturally important subtribe in Orchidaceae, which consisted of 83 genera and 1550 species (Pridgeon et al., 2014; Chase et al., 2015). The subtribe is mainly composed of epiphytes and distributed in tropical and subtropical Asia and northern Australia with some extended to northeast Asia (Hidayat et al., 2012; Chase et al., 2015). Among these, nearly 33 genera occurred across tropical Asia to temperate Asia. *Holcoglossum* Schltr. (Aeridinae, Orchidaceae) is used here as a typical case to illustrate the biogeographic processes of endemic epiphytic plants in East Asia. This genus encompasses ca. 16 currently accepted species (Xiang et al., 2012), mainly distributed in southwestern China and neighboring regions (Schlechter, 1919; Christenson, 1998; Jin and Wood, 2009; Xiang et al., 2012). These species are diverse in morphology, especially the structure of the flower (**Figure 1**). Xiang et al. (2012) employed three DNA markers (ITS, *matK*, and *trnH-psbA*) to resolve the relationship of 16 *Holcoglossum* species and found that *Holcoglossum* is monophyletic, which contains three clades, alpine clade (AC), tropical clade (TC), and the intermediate group (HC). Li et al. (2019) used the whole chloroplast genomes of 12 *Holcoglossum* species and obtained the same result of Xiang et al. (2012). Of *Holcoglossum* species, TC includes six species that occur in Southeast Asia, and AC contains five species that are mainly found in the Hengduan Mountains (**Figure 2**). Within HC, two species are restricted to Taiwan, and another two species are distributed in the mainland China (**Figure 2**). Previous studies show that the stem age of *Holcoglossum* was 7.71 Ma [95% highest posterior density (HPD): 4.12–12.04] (Xiang et al., 2016) with two *Holcoglossum* samplings. Without time estimation and biogeographic analyses, Fan et al. (2009) postulated that *Holcoglossum* originated in tropical Asia and migrated from the tropics to the temperate regions. Therefore, when and how the genus *Holcoglossum* formed current biogeographic patterns is still unclear.

In this study, we reconstruct a time-calibrated phylogenetic tree with samples of nearly all species. We also reconstruct the biogeographic history of *Holcoglossum*. Our specific objectives were to infer (1) the spatial and temporal pattern of *Holcoglossum* and (2) the possible impacts of past geological and climatic oscillations on the distribution of these species.

MATERIALS AND METHODS

Taxon Sampling

In this study, we sampled 15 species of *Holcoglossum*, representing the three clades of this genus. Based on Topik et al. (2005) and Xiang et al. (2012), the phylogenetic relationships of *Aerides* alliance is not all resolved, so ten species from *Aerides* alliance were used as outgroups (*Aerangis calligera*, *Aeranthes ramosus*, *Aerides multiflora*, *Angraecum eburneum*, *Ascocentrum ampullaceum*, *Diaphananthe odoratissima*, *Neobenthamia gracilis*, *Neofinetia falcata*, *Phalaenopsis aphrodite*, *Vanda coerulescens*). Voucher information and GenBank accession numbers are listed in **Supplementary Table 1**.

Molecular Data

Total genomic DNA was extracted from silica gel-dried leaves of a living plant using total DNA extraction kit (CWBIO, Beijing). One nuclear markers (ITS) and eleven chloroplast DNA markers (*atpH-I*, *matK*, *psbA-trnH*, *psbK-I*, *rbcL*, *rpoB*, *rpoC1*, *rpS12-rpL20*, *trnL-F*, *trnS-fM*, and *trnS-G*) were employed in this study. The primers for amplification and sequencing are listed in **Supplementary Table 2**. The PCR procedure of *atpH-I* is followed by Shaw et al. (2007); procedures of *psbK-I*, *rpoB*, and *rpoC1* are followed by CBOL Plant Working Group (2009); procedures of *rpS12-rpL20* and *trnS-G* are followed by Shaw et al. (2005); the procedure of *rbcL* is followed by Goldman et al. (2001); and the procedure of *trnS-fM* is followed by Demesure et al. (1995).

Phylogenetic Analysis

DNA sequences were aligned using the default parameters in Clustal X v.1.83 (Thompson et al., 1997) and subsequently manually adjusted with BioEdit (Hall, 1999). Topological congruence between the chloroplast and nuclear data was evaluated using the incongruence length difference (ILD) test (Farris et al., 1994).

Phylogenetic reconstruction was performed using maximum parsimony (MP), maximum likelihood (ML) in PAUP* 4.0b10 (Swofford, 2003), and Bayesian inference (BI) methods in MrBayes v.3.2 (Ronquist et al., 2012), respectively. All characters were unordered and had equal weight. Gaps were treated as missing data.

For MP analyses, heuristic searches were conducted with 1000 replicates of random addition, in combination with tree bisection–reconnection (TBR) branch-swapping, Multrees in effect, and steepest descent off. Bootstrap support values were conducted with 1000 replicates with 10 random taxon additions and heuristic search options.

Before performing ML and BI analyses, the best-fit nucleotide substitution model for each DNA region was chosen based on the Akaike information criterion (AIC) as calculated using

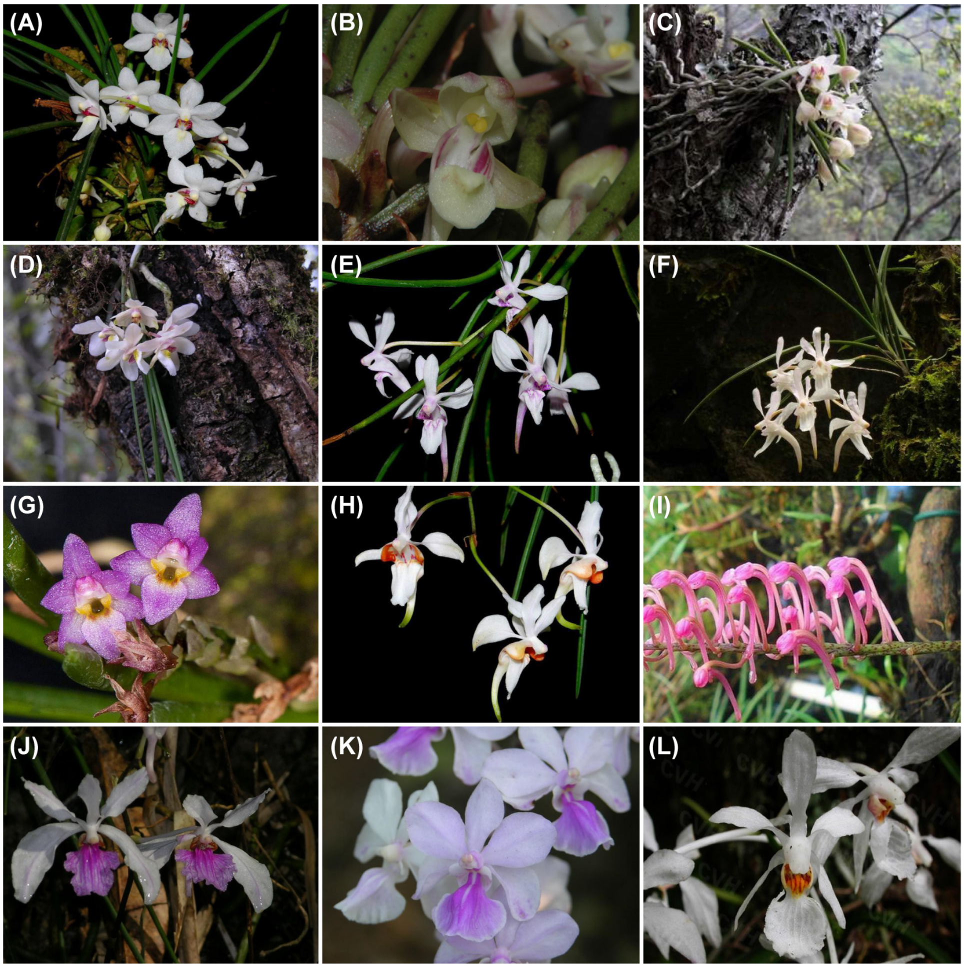

FIGURE 1 | Habitat and diversity of *Holcoglossum*. **(A)** *H. rupestre*; **(B)** *H. sinicum*; **(C)** *H. flavescens*; **(D)** *H. nujiangense*; **(E)** *H. lingulatum*; **(F)** *H. omeiense*; **(G)** *H. pumilum*; **(H)** *H. quasipinifolium*; **(I)** *H. himalaicum*; **(J)** *H. kimballianum*; **(K)** *H. amesianum*; **(L)** *H. subulifolium*. Photo by Xiaohua Jin.

Modeltest v.3.7 (Posada and Crandall, 1998). For ML analyses, we conducted a rapid bootstrap analysis (1000 replicates) and searched for the best-scoring ML tree simultaneously. Each DNA region was assigned the best-fit model, and other parameters followed the default settings.

For BI analyses, each DNA region was assigned its own model of nucleotide substitution. Four Markov-chain Monte Carlo (MCMC) were run, sampling one tree every 1000 generations for 3,000,000 generations. Runs started with a random tree. Majority-rule (>50%) consensus trees were constructed after removing the "burn-in" samples (the first 20% of the sampled trees).

Molecular Age Estimation

We first conducted a likelihood ratio test to determine whether our sequence data were evolved in a clock-like fashion. The result rejected a constant rate ($\delta = 246.55$, d.f. = 24, $P < 0.001$); we used a relaxed lognormal clock model in BEAST v.1.7.4 (Drummond and Rambaut, 2007) to generate a dated phylogeny of *Holcoglossum*. There are no fossil for *Holcoglossum* and subtribe Aeridinae, so we used the age of 33.97 Ma (95% HPD: 25.67–42.33) for the tree root prior to our analysis based on our recent broader study of Orchidaceae (Xiang et al., 2016). Following the suggestion of Ho (2007), we assigned a prior normal distribution for the calibration, with a standard deviation of 4. The YULE process was chosen as the speciation prior, and the BEAST analysis was run on the GTR + I + Γ model for each DNA region, respectively. MCMC searches were run for 100,000,000 generations, sampled every 5000 generations. Convergence was monitored using Tracer v.1.5 (Rambaut and Drummond, 2007). The effective sampling sizes (ESSs) for all parameters were more than 200. The maximum clade credibility tree was computed using TreeAnnotator v.1.7.4 (Drummond and Rambaut, 2007).

Ancestral Range Reconstruction

According to the extant distribution of *Holcoglossum* and outgroups, four main regions were categorized based on the floristic regions of Wu and Wu (1998) and Wu et al. (2003): A, Sino-Japanese region; B, Sino-Himalayan region; C,

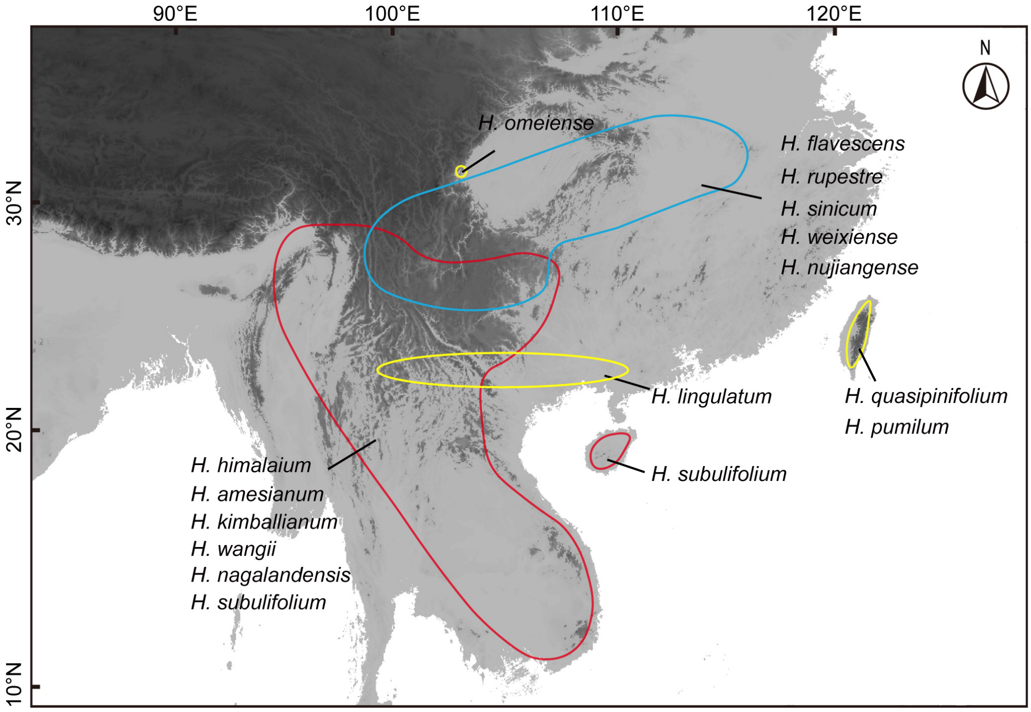

FIGURE 2 | Geographical distribution of *Holcoglossum*. The red region represents the range of tropical clade (TC), the blue region is for temperate alpine clade (AC), and the yellow region is for the group between the first two clades (HC).

Palaeotropical region; and D, Taiwan. The ancestral range reconstruction was inferred using the statistical dispersal-extinction cladogenesis (S-DEC) model and statistical dispersal-vicariance analysis (S-DIVA) as implemented in RASP (Yu et al., 2015), respectively. The maximum clade credibility tree obtained from BEAST was chosen as the input tree. The random 1000 trees from BEAST trees after burn-in were input to estimate the probabilities of the ancestral range at each node.

RESULTS

Phylogenetic Relationships Within *Holcoglossum*

A total of 103 new sequences for *atpH-I*, *rbcL*, *psbK-I*, *rpoB*, *rpoC1*, *rpS12-rpL20*, *trnS-fM*, and *trnS-G* were generated in this study (**Supplementary Table 2**). The total length of the combined chloroplast and nuclear DNA sequences was 11,176 bp, of which 1117 characters were variable, and 433 were parsimony informative (**Supplementary Table 3**). The result of ILD suggested that there is congruence between chloroplast and nuclear data (P = 1.00). The monophyly of *Holcoglossum* and the three clades previously proposed (Fan et al., 2009; Xiang et al., 2012) was strongly supported (**Figure 3**). The TC was basal to *Holcoglossum* (BI-PP: 1.00; ML-BS: 79%; MP-BS: 82%), and the AC and the group between the first two clades (HC) formed a sister group (BI-PP: 1.00; ML-BS: 100%; MP-BS: 100%). The most nodes within *Holcoglossum* were supported by moderate to high supporting values, except for interrelationships in the AC clade (**Figure 3**).

Divergent Time Estimation

The likelihood ratio test showed that our sequence data were evolving in a non-clock-like model, and a chronogram of *Holcoglossum* based on the relaxed-clock model is presented in **Figure 4**. Our time estimates suggested that *Holcoglossum* might have originated during the Late Tertiary (8.53 Ma, 95% HPD: 4.19–14.02, node 1). The crown age of *Holcoglossum* was 6.33 Ma (95% HPD: 3.3–10.6, node 2). The TC clade, containing *Holcoglossum himalaicum*, *H. nagalandensis*, *H. kimballianum*, *H. wangii*, *H. amesianum*, and *H. subulifolium*, diverged first during the Early Pliocene (5.31 Ma, 95% HPD: 2.5–8.81, node 3). Then, the HC clade and AC clade diverged during the Late Pliocene (3.63 Ma, 95% HPD: 1.64–6.24, node 4). The crown age of the HC clade, containing *Holcoglossum ligulatum*, *H. omeiense*, *H. pumilum*, and *H. quasipinifolium*, was at latest Pliocene (2.7 Ma, 95% HPD: 1.16–4.78, node 5), and then the two Taiwan species (*H. pumilum*, *H. quasipinifolium*) were divergent from their sister group. The AC clade might have started to diversify around Middle Pleistocene (1.22 Ma, 95% HPD: 0.5–2.39, node 8), and its five species (*Holcoglossum nujiangense*, *H. weixiense*, *H. rupestre*, *H. sinicum*, *H. flavescens*) rapidly differentiated since then.

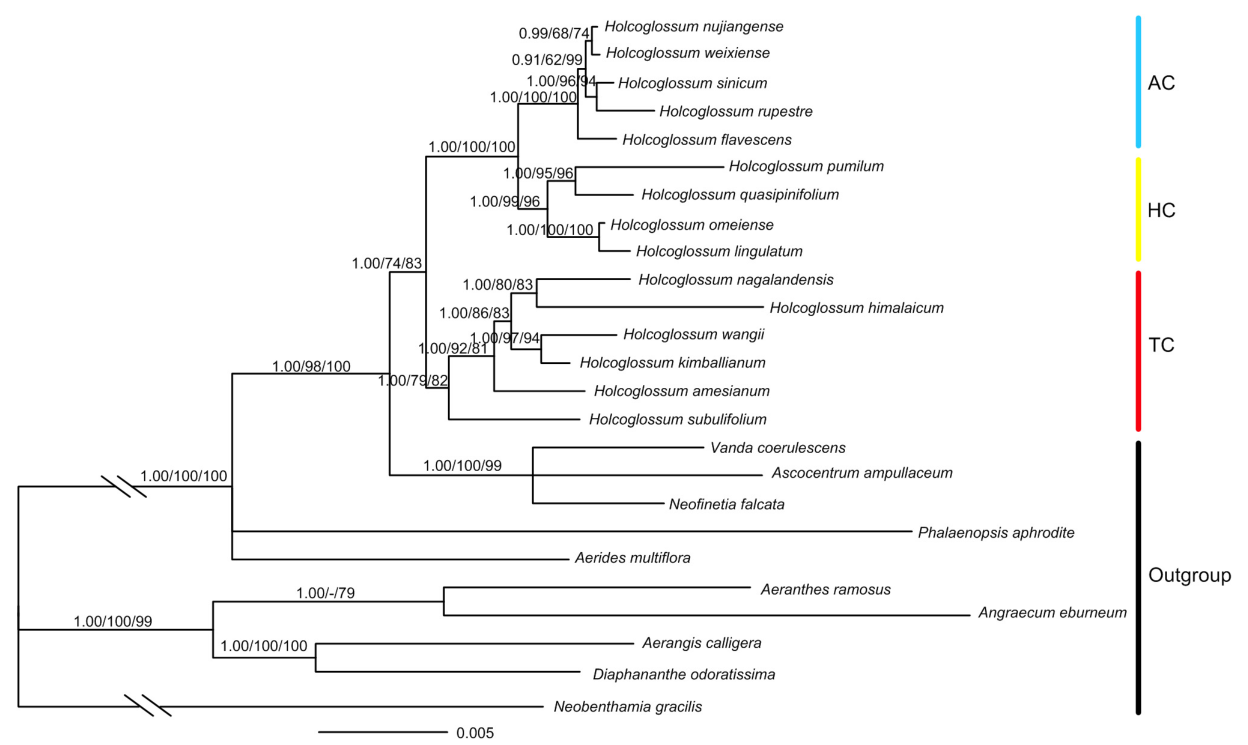

FIGURE 3 | Chronogram obtained from Bayesian inference analysis based on combined nrDNA and cpDNA data. Numbers on the branches are supporting values of Bayesian inference, maximum likelihood, and maximum parsimony, respectively. Dash indicates that the node was not supported.

Ancestral Range Reconstruction

The ancestral range reconstruction of *Holcoglossum* based on S-DEC and S-DIVA is shown in **Figure 4**. Both S-DEC and S-DIVA inferred that the most recent common ancestor (MRCA) of *Holcoglossum* was probably distributed in the Palaeotropical region (node 2). The MRCA of TC was also in the Palaeotropical region (node 3). The MRCA of HC distributed in the Palaeotropical region and Taiwan (node 5), which were later split into mainland China and Taiwan. The MRCA of AC was inferred at the Sino-Japanese region and Sino-Himalayan region by S-DEC, and Sino-Himalayan by S-DIVA (node 8), which subsequently diversified in the Sino-Himalayan region.

DISCUSSION

Phylogenetic Relationship of *Holcoglossum*

A robust phylogenetic framework is needed for biogeographical analyses. In this study, the phylogenetic result based on combined chloroplast and nuclear data strongly supported *Holcoglossum* as monophyletic, which included three clades, i.e., TC, HC, and TC, which is consistent with Xiang et al. (2012) and Li et al. (2019). However, the phylogenetic relationships within TC and AC are slightly different. For example, *Holcoglossum naglandensis* and *H. himalaicum* among TC are sisters in this study and Xiang et al. (2012), while *H. naglandensis* is close to *H. amesianum* and *H. himalaicum* is close to *H. wangii* due to different DNA sequences. Nevertheless, these species of TC and AC are distributed in the same floristic region, respectively, and there is no influence on subsequently biogeographic analyses.

Temporal and Spatial Patterns of *Holcoglossum*

Both S-DEC and S-DIVA analyses detected that the modern distribution of *Holcoglossum* is attributed to four dispersal events (**Figure 4**). Here, we adopted the S-DEC result to explain the temporal and spatial patterns of *Holcoglossum*. The biogeographical analyses indicate that the stem and crown of *Holcoglossum* most likely occurred in the Palaeotropical region, partly in agreement with the conclusion from Fan et al. (2009) that *Holcoglossum* was dispersed from the tropical region.

Within TC, all six currently recognized species distribute in the Asian tropical region. The crown group of TC happened in the Palaeotropical region since 5.31 Ma (95% HPD: 4.19–14.02, node 3) and then six species diversified there among 5.31–1.63 Ma. The weather in Southeast Asia had no distinct seasonality, and the climate is constantly moist and warm since Pliocene (Clements et al., 2006), which is beneficial for *Holcoglossum* colonization in tropical forests. Further, a putative abrupt intensification of the East Asian monsoon system during 3.6–2.6 Ma (Zhang et al., 2012) brought abundant precipitation, which provided favorable climatic conditions for the survival of epiphytic species in East Asia.

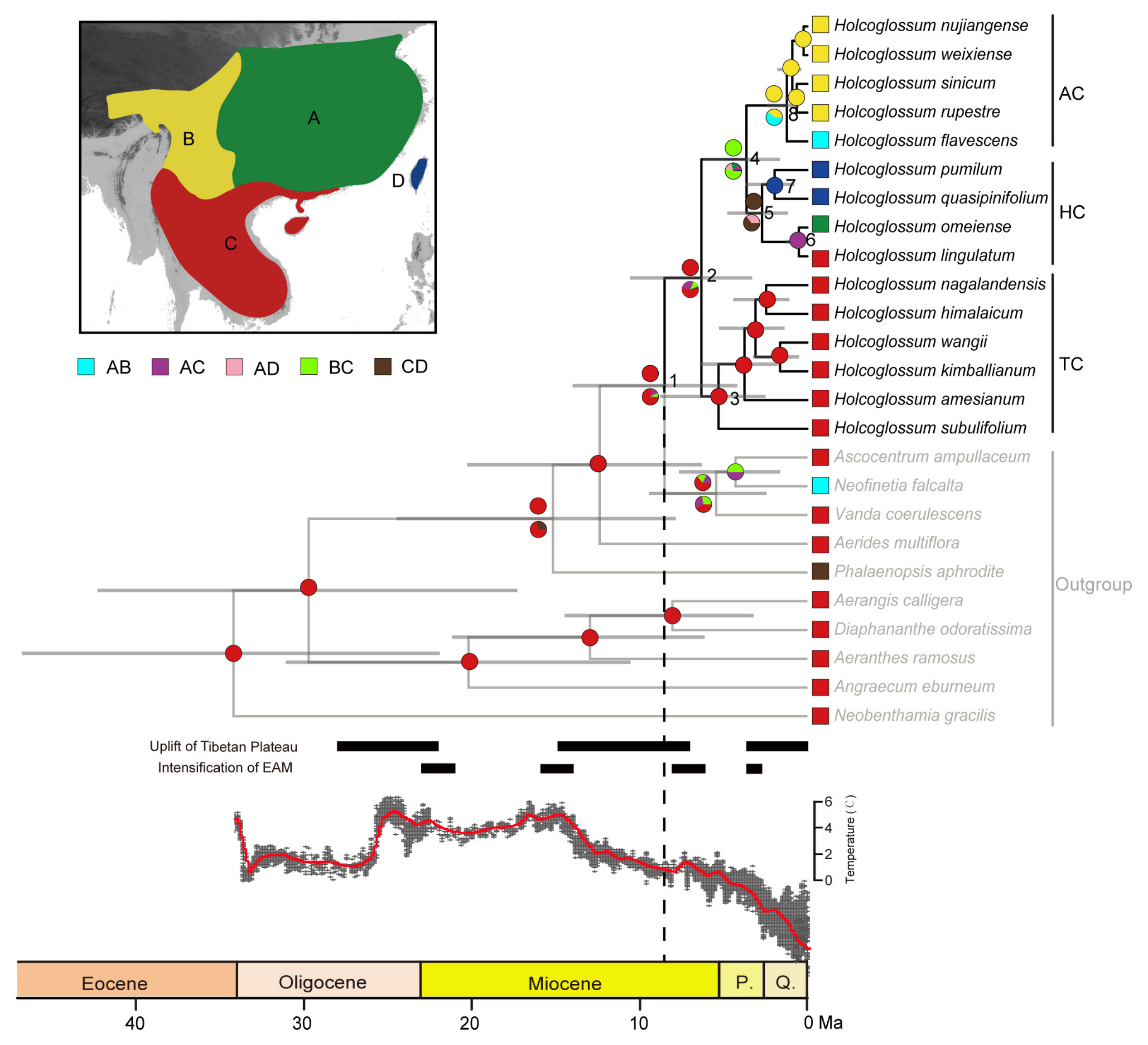

FIGURE 4 | Ancestral range reconstruction of *Holcoglossum* in RASP. The same results of S-DEC and S-DIVA are marked by one circle at the node. Colored circles at nodes above branches for S-DIVA and below branches for S-DEC. A, Sino-Japanese region; B, Sino-Himalayan region; C, Palaeotropical region; D, Taiwan. The global temperature curve is modified from Zachos et al. (2008).

After TC diverged from AC and HC, a subsequent dispersal from the Palaeotropical region to the Sino-Himalayan region occurred since the Late Miocene (3.63 Ma, 95% HPD: 1.64–6.24, node 4). Within HC, the ancestor of HC dispersed from the Palaeotropical region and Sino-Himalayan region into Taiwan (2.7 Ma, 95% HPD: 1.16–4.78, node 5). Taiwan island formed at 5–6 Ma and had been long linked to the Chinese mainland via Fujian-Taiwan land bridge (Teng, 1990; Zeng, 1993), which led to the close relationship between flora of Taiwan and mainland China (e.g., Zeng, 1993; Hsieh, 2002; Nie et al., 2007; Ying and Chen, 2011; Xiang et al., 2017). Wang (1992) proposed that plant lineages could extend from Southwest China eastward along the Nanling Corridor and along other mountain chains in Central China to East China or Taiwan. The biogeography of *Cycas taitungensis* (Huang et al., 2001), *Sassafras tzumu* (Nie et al., 2007), and *Dichocarpum basilare* (Xiang et al., 2017) showed that dispersal is attributed to the disjunct distribution between Asian mainland and Taiwan. *Holcoglossum* lineages may also have dispersed eastward along the same route to east China and then across the Fujian-Taiwan land bridge to Taiwan. Conversely, the global cooling and the subsequent rise of sea level caused the interruption of the continuous distribution of *Holcoglossum* since the latest Pliocene. The Palaeotropical *Holcoglossum lingulatum* and Sino-Japanese *H. omeiense* split at 0.56 Ma (95%: 0.11–1.17, node 6) (**Figure 4**). The global cooling and frequently glacial periods happened at Quaternary, which resulted in *H. omeiense* being restricted in Omei Mountains in China and *H. lingulatum* in Southeast Asia.

Within AC, the ancestor of AC migrated from the Palaeotropical region and Sino-Himalayan region to the Sino-Japanese region (1.22 Ma, 95% HPD: 0.5–2.36, node 8). All five currently recognized species distribute in Hengduan Mountains, except for *H. flavescens*, which extend to the Sino-Japanese region. These species are inferred to diverge rapidly during a short time interval (<1 Ma; **Figure 4**). The time is markedly later than the time of the Hengduan Mountains Region uplift (around 4–3 Ma; Chen, 1992, 1996; Shi et al., 1998). However, the uplift of the Hengduan Mountains formed a complex range of topographies, climates, and habitats (Hoorn et al., 2013), and many areas of this region are ice-free during Quaternary glaciations (Zheng et al., 1998), which can allow immigrations and survivals of plant lineages. *Holcoglossum* species of AC clade grow on the trees of evergreen broad-leaved forests, especially *Quercus* section *Heterobalanus* (Jin, 2003). The continuous uplift of Himalaya-Hengduan Mountains in the Late Miocene to Early Pliocene triggered the diversification of *Quercus* section *Heterobalanus* (Meng et al., 2017), which provided suitable habits for *Holcoglossum* in the Hengduan Mountains. Extensive biogeographical researches proved that the uplift of the Hengduan Mountains directly or indirectly plays vital roles in the species diversification in multiple lineages, such as *Rhodiola* (Crassulaceae; Zhang et al., 2014), *Rhododendron* (Eriaceae; Xing and Ree, 2017), *Saxifraga* (Saxifragaceae; Ebersbach et al., 2017), and alpine bamboo (Ye et al., 2019). The divergence of *Holcoglossum* still supported that the Hengduan Mountains Region is an adaptive plant diversification center in the Quaternary climate oscillations (Boufford, 2014; Jian et al., 2015; Xing and Ree, 2017).

CONCLUSION

In this study, we reconstructed a phylogenetic tree and estimated divergent times of *Holcoglossum*, a genus endemic to East Asia. Our biogeographic inference indicated that the MRCA of *Holcoglossum* occurred in the Palaeotropical region. Four dispersal events happened from the Palaeotropical region to the Sino-Himalayan region at ca. 3.63 Ma, from Chinese Mainland to Taiwan at ca. 2.7 Ma, from the Sino-Himalayan and Palaeotropical to Sino-Japanese regions at ca. 1.22 Ma, and from the Palaeotropical to Sino-Japanese regions at ca. 0.5 Ma. During Quaternary, the global cooling climate caused the *H. pumilum* and *H. quasipinifolium* endemic in Taiwan and the restriction *H. omeiense* in the Omei Mountains of China. The temporal and spatial patterns of *Holcoglossum* shed light on the divergence and diversification of recently evolved epiphytic plants within East Asia.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the GenBank Database under the accession numbers listed in the **Supplementary Material**.

AUTHOR CONTRIBUTIONS

XX and XJ conceived and designed the experiments. XX performed the experiments. XX, JZ, XL, PZ, and LZ analyzed the data. JZ, XX, XL, PZ, and LZ wrote the manuscript. XX, JZ, XL, PZ, LZ, and XJ revised the draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00245/full#supplementary-material

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