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PHYLOGEOGRAPHIC STUDY OF *MUSELLA LASIOCARPA* (MUSACEAE): PROVIDING INSIGHT INTO THE HISTORICAL RIVER CAPTURE EVENTS

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Abstract

In the late Pliocene, the Qinghai-Tibet plateau experienced several rapid uplifts. The resulting Hengduan Mountains have since played an important role in the plant distribution pattern in China. Here, a phylogeographic study on wild populations of *Musella lasiocarpa* (Musaceae), which is endemic to southwest China, is reported. We have discovered 9 wild populations of *M. lasiocarpa* throughout its natural distribution in cliff habitats in southwest China since 2004. In order to reveal the factors causing the current distribution pattern of wild *M. lasiocarpa* populations and its spread pathways, phylogenetic analysis by sequencing 3 chloroplast DNA fragments (*psbA-trnH*, *trnL-F* and *rpl16*) were performed. Based on the results, we deduced that the origin of wild *M. lasiocarpa* populations was in the region of the Lijiang aera of the Jinsha River, which was close to Yanbian, Panzhihua. The differentiation was probably caused by the uplift of the Qinghai-Tibet Plateau, the Hengduan Mountain orogeny, and the westward retreat of the ancient Mediterranean Sea. The main factors that contributed to the fragmentation of wild *M. lasiocarpa* population habitat include diversion of the ancient Red River and stream capture of its tributaries.

Key words: Fragmentation, *Musella lasiocarpa*, Phylogeography, Population differentiation, The Qinghai-Tibet Plateau.

Introduction

Several studies suggested that plate collisions between Asia, Europe and the Indian sub-continent caused the Qinghai-Tibet Plateau in China to experience tectonic activity, including 3 uplift events and 2 planation events, among which the third uplift was the strongest (Li *et al.*, 1979; Chen *et al.*, 1999; Shi *et al.*, 1999). After the third uplift, the Qinghai-Tibet Plateau reached its current height, and the Hengduan Mountains, located in the southeast of Qinghai-Tibet Plateau west of Yunnan Province, were formed. The formation of the Hengduan Mountains caused the ancient Red River, which originally flowed from north to south, was moved cut transversely, and a number of tributaries flowing from west to east, such as the Jinsha River and Yalong River, were formed (Zhang *et al.*, 2011). Geographical isolation caused the locations of plants that grew within the original geographic range were separated. In addition, during the subsequent Quaternary glacial period, these blocked and scattered plants migrated to other suitable habitats (refuges). Thus, their distribution became more fragmented, and their number was further reduced. This has led to insularization of plant distributions in this area, and some ancient species have become endangered or even extinct.

Plant phylogeography have frequently combined the methods of cytoplasmic DNA (chloroplast DNA) sequencing and the study of geographic distribution patterns. Previous phylogeographic studies suggest that chloroplast gene fragments have many advantages, including small molecular weight, simple structure, monolepsis, richness in genetic information and exemption from genetic recombination (i.e., not subject to selective pressure), a moderate nucleotide substitution rate, independent evolution, feasibility of constructing molecular phylogenetic trees and identifying plant evolutionary history without depending on any other data, and ease of genetic analysis (Fofana *et al.*, 2001; Malm & Prentice, 2005; Jakob & Blattner, 2006; Lemes *et al.*, 2010; Gutiérrez-Rodríguez *et al.*, 2011; Hunt *et al.*, 2011; Oberlander *et al.*, 2012; Yu & Nason, 2013; Zhang *et al.*, 2013; Du & Wang, 2016; Gamar *et al.*, 2018). Plants that grow in harsh habitats or extreme environments may have experienced many historical events, such as orogeny, glacier formation and ice sheet migration (Wang *et al.*, 2011). Therefore, phylogeographic studies on species growing in harsh or extreme habitats not only are of high significance for revealing the origin and differentiation history of the species but also provide evidence for speculations on paleogeographic events.

As an ancient species endemic to southwestern China, *Musella lasiocarpa*, commonly known as Chinese dwarf banana, represents the monotypic genus *Musella* in the family Musaceae (Li, 1979; Christelová *et al.*, 2011; Hřibová *et al.*, 2011). The species is a large perennial tufted herb and is of considerable value for phylogeographic studies due to its scarcity in wild populations, narrow and specific distribution range, and harsh cliff habitat. It is widely cultivated in most parts of Yunnan Province and parts of Sichuan Province. Some scholars believe that its wild populations are difficult to find or are already extinct (Long *et al.*, 2003). Hence, there have been almost no studies on the origin and evolution of *M. lasiocarpa*. Our group, for the first time, discovered multiple wild *M. lasiocarpa* populations in cliff habitats in Yunnan and Sichuan Provinces. These populations are distributed on both sides of the Jinsha River and close to the Yalong River (Pan *et al.*, 2007), and the straight-line distances between the two most separated populations are only 140 km north-south and 130 km east-west. The habitats are broken, and the wild populations are endangered, as there are only a very few plants in most populations, even fewer than 10 plants in

some populations. However, studies have shown that although the distribution of *M. lasiocarpa* wild populations is narrow, their genetic diversity is high, and the genetic distance is essentially consistent with the geographic distance (Pan *et al.,* 2007). While this is associated with rigorous cross-pollination of *M. lasiocarpa* (Tian *et al.,* 2008), historical geological events may also be an important factor (Zhu, 2000; Soltis *et al.,* 2006; An *et al.,* 2015; Zhu *et al.,* 2016; Pascual *et al.,* 2016). In geological history, Yunnan is considered to be a convergence zone of ancient southern and northern continents; biogeographically, it is a transition zone from tropical Asian biota to East Asian subtropical-temperate biota, and it is also a global biodiversity hotspot (Wang *et al.,* 2011; Zhu *et al.,* 2016). The formation and evolution of biota in Yunnan were greatly influenced by geological events in history, including subsidence of the ancient Mediterranean Sea, uplifts of the Himalayas and Qinghai-Tibet Plateau, and formation of the East Asian monsoon climate (Chen *et al.,* 1999; Shi *et al.,* 1999; Zhang *et al.,* 2011). Based on the distribution characteristics and growth environment of wild *M. lasiocarpa* populations, we speculate that the ancient Indian continental plate movement, uplift of the Qinghai-Tibet Plateau, formation of the Hengduan Mountains, and diversion of the ancient Red River were the main causes that contributed to the current distribution of *M. lasiocarpa*. However, the question of how geological events in history affected the evolution and migration routes of this species still remains. These questions need to be addressed with phylogeographic methods.

In this study, 9 wild *M. lasiocarpa* populations were studied, and the amplification products of 3 specific fragments in the non-coding region of chloroplast DNA (*cp*DNA), i.e., *psbA-trnH*, *trnL-F* and *rpl16* intron, were sequenced. Data were analyzed and a phylogenetic tree was constructed to investigate the refuge locations of this ancient species, as well as its migration routes, genetic structure and causes that led to its current distribution pattern.

Materials and Methods

Sources of experimental materials: Materials used in this study were collected from all 9 knownwild *M. lasiocarpa* populations. From each population, 20-25 young leaves (one leaf per plant) were collected from individual plants with distances greater than 20m (for populations with fewer than 20 plants, all were sampled). A total of 208 samples (Table 1 and Fig. 3) were collected, and these were quickly dried in allochroic silica gel before being taken to the lab. A total of 127 samples from the 9 populations were actually used for experiments. In addition, *Musa basjoo* and *Enseteglaucum* in the Musaceae family were used as out-groups.

Table 1. Information on the collection sites of *Musella lasiocarpa* wild populations and the Haplotype diversity index and nucleotide diversity index.

No.	Population code	Location	Sample size	Longitude (E)	Latitude (N)	Altitude (m)	Haplotype NO.	Haplotype diversity index (Hd)	Nucleotide diversity index (Pi)
1.	YL	Yuanmou, Chuxiong	14	101.58	25.39	1600	H10(14)	0	0
2.	HW	Wenquan, Lijiang	15	101.21	26.23	1462	H5(15)	0	0
3.	BBH	Binchuan, Dali	15	100.35	25.47	1430	H4(15)	0	0
4.	YQ	Yanbian, Panzhihua	15	101.38	27.05	1200	H8(15)	0	0
5.	LJ	Jinjiang, Lijiang	15	100.23	26.37	1343	H7(15)	0	0
6.	WL	Wenle, Lijiang	15	101.31	26.6	1312	H6(15)	0	0
7.	YR	Yongsheng, Lijiang	8	100.75	26.68	2140	H1(5), H2(3)	0.5357	0
8.	WDDY	Wuding, Chuxiong	15	102.4	25.53	1910	H3(15)	0	0
9.	HHSL	Heqing, Dali	15	100.18	26.57	2196	H9(15)	0	0
Total			**127**					**0.878**	**0.00271**

Total DNA extraction and PCR amplification: An improved CTAB method was adopted to extract total DNA from leaves dried with allochroic silica gel (Doyle, 1987). PCR amplifications of the *psbA-trnH*, *trnL-F* and *rpl16* intron fragments of *cp*DNA were performed, and universal primers were used (Taberlet *et al.,* 1991; Sang *et al.,* 1997; Tate & Simpson, 2003; Shaw *et al.,* 2005). The PCR system was 50 µl in volume, including 25 µL of 2×Power *Taq* PCR MasterMix (Bioteke Corporation Beijing), 2 µL of DNA template (20-50 ng), 2 µL of each forward and reverse primer (10 µmol·L^{-1}) and 20 µLof ddH_2O. PCR amplification was performed using a BIO-RAD C1000, and the parameters were as follows: 94°C for 3 min, 94°C for 45 s, 50°C for 50 s, 72°C for 1 min and 72°C for 10 min; then, the temperature was set at 12°C. The amplification product was loaded on a 1% agarose and sent out for sequencing (Shanghai Majorbio Bio-Pharm Technology Co., Ltd). Sequencing was performed using a GS FLX Titanium System (Roche 454).

Data analysis: The whole amplification products of 127 samples from 9 populations were sequenced, and the results were aligned using ClustalX 1.81 software (Thompson *et al.,* 1997) and corrected manually. Dnasp 5.10 was used to determine *cp*DNA haplotypes in the populations. Haplotype frequency (H_d) was calculated by dividing the total number of individuals by the number of individuals with each haplotype. Using the parameters 1) haplotype composition of each population, 2) number of individuals of each haplotype in different populations and 3) variable sites in each haplotype sequence, Permut 2.0 was used to calculate genetic diversities within and among populations (H_S and H_T) and genetic differentiations (G_{ST} and N_{ST}). For permutation tests, the number of permutations was set at 1000 times (Pons & Petit, 1996).

Calculations of average estimated gene flow among populations (N_m), the haplotype diversity index (H_d), nucleotide diversity index (*Pi*) (Nei, 1987), neutrality

tests using Tajima's D (Tajima, 1989), Fu and Li's D* (Fu & Li, 1993), two models with infinite number of variable sites and mismatch distribution analysis (Rogers & Harpending, 1992) were performed with Dnasp 5.10.

Levels of genetic variation within and among wild *M. lasiocarpa* populations were analyzed using the AMOVA program in the Arlequin 3.5 software package, and F_{ST} of the haplotype distribution pattern (1000 permutations) was evaluated to further analyze the degree of population differentiation (Weir & Cockerham, 1984; Excoffier *et al.,* 2005).

The maximum likelihood (ML) method in PAUP* 4.0b10 software (Swofford, 2002)was used for phylogenetic analysis of the haplotypes. Previously obtained haplotype sequences were processed with Gap Coder software to fill in the missing gaps and to complete the genetic codes, which were then subjected to phylogenetic tree analysis using PAUP. A heuristic search was performed with 1000 random sequence additions. The reliability of the clades in the obtained phylogenetic tree was evaluated using a Bootstrap method, and clade support was tested with 1000 repetitions. The obtained tree file was viewed and edited using Treeview32 software (Page, 1996).

The haplotype output data from Dnasp 5.10 (*.rdf) were used as input data for Network 4.6 software, and a kinship diagram for haplotypes from different populations was constructed. Beast v1.7.4 and Fig. 3 v1.2.2 software were used to calculate the times of differentiation of wild *M. lasiocarpa* populations and an evolutionary rate of 1.0×10^{-9} was used as standard.

Results

Sequences and variations: The complete PCR products of *psbA-trnH*, *trnL-F* and *rpl16* intron, from 127 individual samples collected from 9 wild *M. lasiocarpa* populations (for related information, see table 1), were sequenced and subjected to alignment using ClustalX. The lengths of the *psbA-trnH*, *trnL-F* and *rpl16* intron fragments were 877 bp, 945 bp and 992 bp, respectively. The combined length of the three fragments was 2814 bp. Dnasp 5.10 analysis showed 10 haplotypes (Hap_1-Hap_10) and the variable sites of different haplotypes are shown in Table 2.

Table 2. Basic information of variable sites in 10 haplotypes of the joint fragment of 3 Musella lasiocarpa cpDNA, the dashes in the table indicating gaps.

Haplotype	Variable sites														
								1	**1**	**1**	**1**	**1**	**2**	**2**	**2**
		2	2	2	2	2	3	3	6	8	8	8	0	1	3
	7	0	3	6	6	9	5	1	2	1	5	5	8	1	7
	8	5	6	0	5	2	4	1	4	1	3	7	8	2	1
Hap_1	G	C	T	T	A	G	C	C	C	T	T	C	T	A	G
Hap_2	-	-	-	-	-	-	-	-	-	G	-	-	-	-	A
Hap_3	-	-	-	-	-	-	-	-	-	G	-	-	-	-	A
Hap_4	-	-	-	-	-	T	-	-	A	-	-	-	-	-	-
Hap_5	-	-	C	-	-	-	-	-	-	-	-	-	C	G	-
Hap_6	A	A	C	A	T	T	A	T	-	-	-	-	C	-	-
Hap_7	A	A	C	A	T	T	A	T	-	G	A	T	C	-	A
Hap_8	A	A	C	A	T	T	A	-	A	-	-	-	C	-	-
Hap_9	A	A	C	A	T	T	A	-	-	G	-	-	C	-	-
Hap_10	A	A	C	A	T	T	A	-	-	-	-	-	C	-	-

Diversity index, genetic structure and mismatch distribution analysis of haplotypes: Dnasp 5.10 was used for diversity analysis on haplotypes in the 9 wild *M. lasiocarpa* populations. The overall haplotype diversity index H_d was 0.878, the nucleotide diversity index P_i was 0.00271, and the average number of nucleotide differences *k* was 7.428. The YR population had a total of 8 individual plants and contained 2 haplotypes, among which the YR 01 population (5 individuals) occupied haplotype 1 and the YR 02 population (3 individuals) occupied haplotype 2. All other populations contained only a single haplotype and that no haplotype was found in more than 1 population. Based on haplotype data and analyses of estimated gene flow and genetic differentiation, the average estimated gene flow among wild *M. lasiocarpa* populations N_m was 0.02, suggesting infrequent estimated gene flow among the wild populations. Chloroplast DNA mismatch distribution analysis was performed on the haplotype data, and the obtained mismatch diagram is shown in Fig. 1.

The mismatch diagram was multimodal, not unimodal. Both Tajima's and Fu & Li's neutrality tests showed that Tajima's D was 2.59591, whereas Fu & Li's D* was 1.77301 and Fu and Li's F* was 2.50292. All these results were positive and the differences were significant.

AMOVA of haplotypes using Arlequin 3.5 showed that variation within wild *M. lasiocarpa* populations was 2.38%, whereas variation among populations was 97.62%, i.e., F_{ST} =0.97621 (Table 3). This suggested that the variation in wild *M. lasiocarpa* populations existed mainly between populations, and the level of genetic differentiation was rather high.

Using Permut 2.0 software, the average genetic diversity (H_S) within wild *M. lasiocarpa* populations was calculated to be 0.060 (0.0595) and total genetic diversity (H_T) was 1.000 (0.0116). Genetic differentiation coefficients among populations (G_{ST} and N_{ST}) were 0.940 (0.0595) and 0.981 (0.0183), respectively. The level of genetic differentiation among populations was rather high (G_{ST}=0.940) and $N_{ST}>>G_{ST}$.

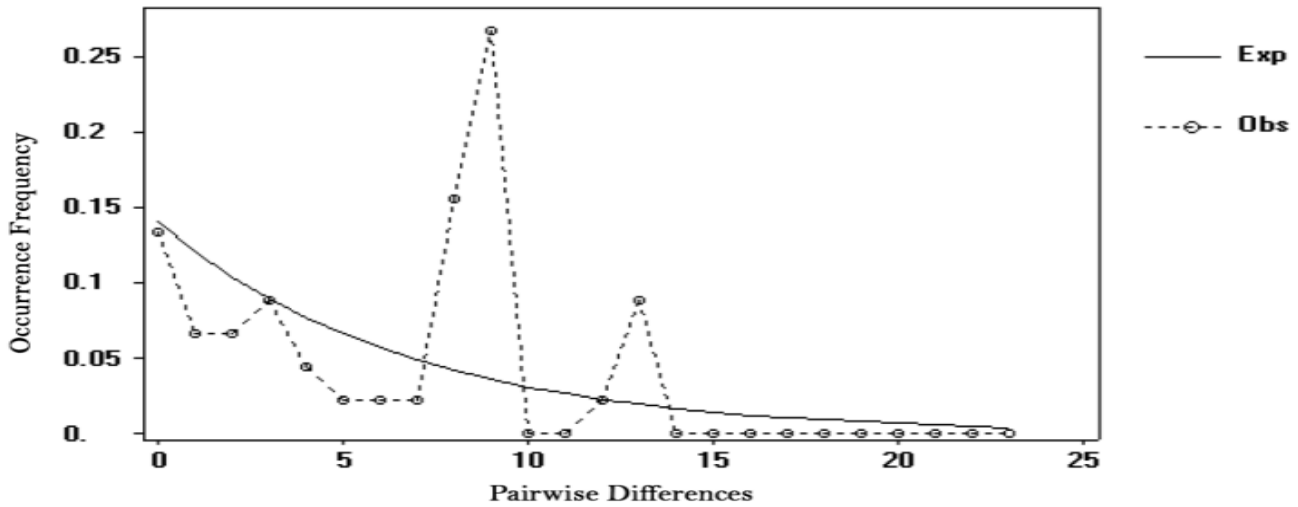

Fig. 1. Mismatch analysis chart of joint data on chloroplast-specific fragments of 127 individual plants from 9 *Musella lasiocarpa* wild populations.

Table 3. Results of AMOVA analysis on haplotypes in *Musella lasiocarpa* wild populations.

Source of variation	Degree of freedom d.f.	Sum of squares	Variation components	Variation percentage
Variation among populations	8	1691.048	14.99873	97.62
Variation within populations	118	43.125	0.36547	2.38
Total	126	1734.173	15.36419	
Fixation Index		F_{ST} = 0.97621		

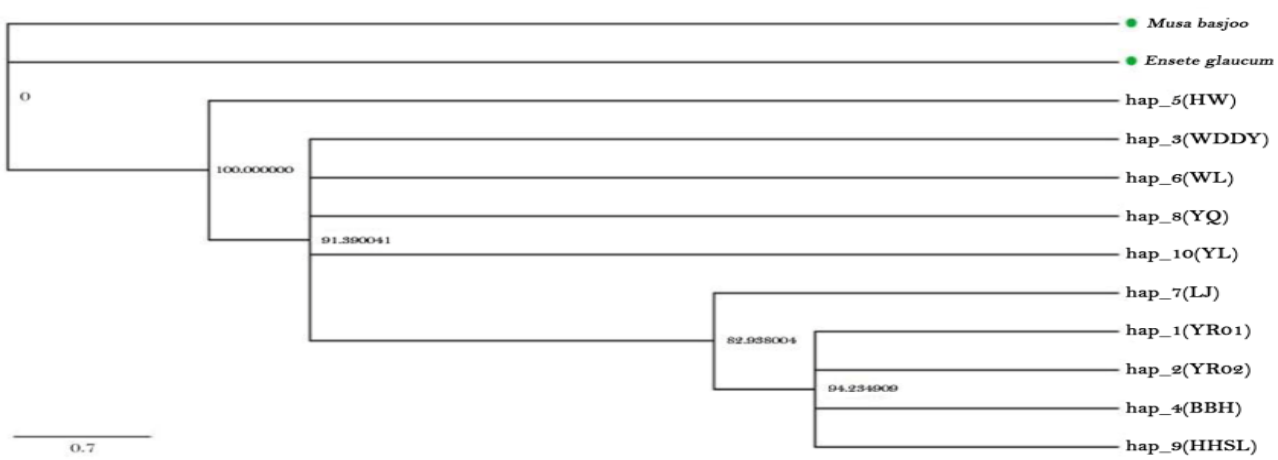

Fig. 2. Maximum likelihood tree of 10 haplotypes (Hap_1-10) from 9 *Musella lasiocarpa* wild populations constructed using a maximum likelihood (ML) method in PAUP*.

Construction of a phylogenetic tree and kinship: The 10 haplotypes in wild *M. lasiocarpa* populations were analyzed using PAUP. *Enseteglaucum* and *Musa basjoo* were selected as out-groups for phylogenetic analysis. The ML method was adopted to construct the phylogenetic tree (Fig. 2).

In the maximum likelihood tree, the 9 wild *M. lasiocarpa* populations were divided into two main clades, one comprising populations HHSL, BBH, YR 01, YR 02 and LJ and the other comprising populations HW, WDDY, WL, YQ and YL. Network software was used to plot the kinship diagram of *M. lasiocarpa* haplotypes (Fig. 3) to reveal population differentiation in a more direct way.

Calculation of time of differentiation for wild populations: Beast v1.7.4 was used to calculate the time of differentiation of the 9 wild *M. lasiocarpa* populations. Fig. 3 v1.2.2 was used to plot a differentiation time tree using the out-groups *Musa basjoo* and *E. glaucum* to root the tree (Fig. 4).

The results for differentiation times suggested that the 10 haplotypes in the 9 wild *M. lasiocarpa* populations were divided into two clades (Clade I and II). Clade I comprised the 5 populations HW, YQ, WDDY, YL and WL, whereas Clade II comprised the 5 populations LJ, YR 01, YR 02, BBH and HHSL. The structure of the differentiation time tree was similar to that of the maximum likelihood tree plotted using PAUP. The number at each node showed differentiation times (Mya) for the two populations.

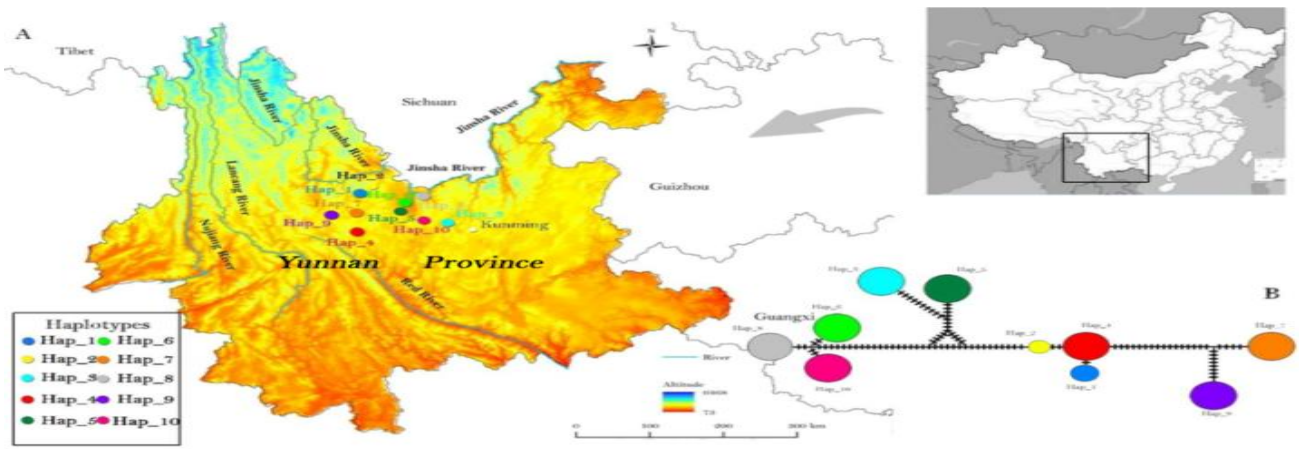

Fig. 3. Distribution diagrams of sample collection points for 9 *Musella lasiocarpa* wild populations and haplotypes, generated by the mapping software Arcmap (9.3 version). Kinship diagram of *Musella lasiocarpa* wild populations, plotted using Network. Hap_- denotes haplotypes; small black rectangular boxes denote mutation.

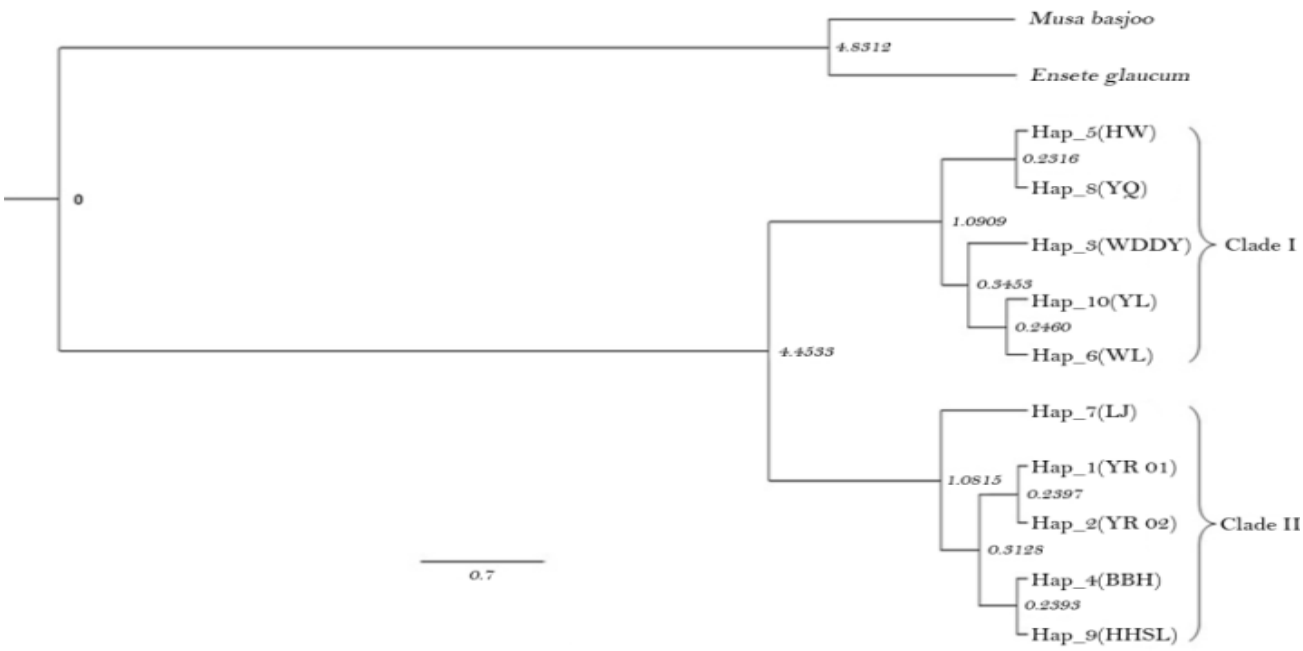

Fig. 4. Rooted differentiation time tree diagram calculated and plotted using Beast and Fig. 3.

Discussion

Based on the geographic condition and relevant phylogeographic studies (Li *et al.*, 1979; Chen *et al.*, 1999; Shi *et al.*, 1999; Zhang *et al.*, 2011; Wang *et al.*, 2011b; Yu & Nason, 2013; Zhang *et al.*, 2013; Du & Wang, 2016), we speculate that the origin of wild *M. lasiocarpa* populations is what is now the Jinsha River in the Lijiang area, specifically from Jinjiang, Lijiang to Yanbian, Panzhihua in the east-west direction, and from Huaping, Lijiang to Wenquan, Lijiang in the north-south direction. This speculation was based on the following rationale.

Evidence from phylogeographic studies: As shown in the phylogenetic tree and kinship diagram, the 9 wild *M. lasiocarpa* populations were divided into two clades. One comprised 4 populations, including HHSL, located at the west side of Dajian mountain and Pingding mountain, and the other comprised 5 populations, including HW, located at the east side of Dajian mountain and Pingding mountain. The AMOVA and genetic diversity analyses suggested that variation among the populations was far greater than that within the populations (97.62%>>2.38%). The level of genetic differentiation among the populations was rather high and was also far greater than that within the populations (0.940>>0.060). Estimated gene flow among the populations was relatively low (N_m=0.02), suggesting infrequent estimated gene flow among the populations. These results are consistent with the speculation on why *M. lasiocarpa* distribution has an island shape. The mismatch analysis diagram based on haplotypes and neutrality tests also provided evidence. The mismatch analysis diagram is multimodal, indicating that there has been no expansion or bottleneck effect occurring in the wild *M. lasiocarpa* populations in recent times. Based on the evolution model of neutrality, if in the Tajima test the number of segregating sites and the nucleotide diversity are significantly different, the null hypothesis (H_0) is rejected. In the results of the

present study, Tajima's D=2.59591, Fu & Li's D*=1.77301 and Fu & Li's F*=2.50292; these were positive values, and the differences were significant, indicating that evolution of the target sequence in *M. lasiocarpa* did not fit the neutral evolution model. That is, the main factor contributing to the current distribution patterns of *M. lasiocarpa* wild populations may not be an isolated mutation. Rather, natural selection and environmental factors both contributed to the current distribution pattern (Rogers & Harpending, 1992; Harpending *et al.,* 1998; Mayer *et al.,* 2015). This further supported the clade structure of the obtained phylogenetic tree and kinship diagram and was consistent with the current geographic distribution of *M. lasiocarpa*.

Differentiation times: Based on the calculation results for differentiation times, the 10 haplotypes in the 9 wild *M. lasiocarpa* populations were divided into Clade I and Clade II. Differentiation of Clade I from Clade II occurred during the Pliocene Epoch in the Tertiary Period (5-1.8 Mya), and the differentiation times of other branches were, in general, in the Quaternary-Pleistocene (1.8-0.011 Mya). The differentiation time between populations HW and YQ and the other three populations in Clade I was in the middle Pleistocene, and differentiation between population LJ and the other 3 populations in Clade II occurred in the late Pleistocene. The time of differentiation between Clade I and Clade II was approximately 4.5 Mya, in the early Pliocene. During the early and middle Pliocene epoch, the Western Sichuan Northern Yunnan Plateau and the Qinghai-Tibet Plateau in southwest China were relatively stable (Li *et al.,* 1979; Zhang *et al.,* 2011). Erosion and planation were prevalent, terrain height differences were reduced, and the area gradually approached peneplain (Zhang *et al.,* 2013; Du & Wang, 2016). At that time, the Qinghai-Tibet Plateau had not experienced uplift. Some scholars speculate that the altitude of Qinghai-Tibet Plateau was approximately 1000 m in the Pliocene epoch (Zheng & Li, 1990; Jiang *et al.,* 1999; Wang *et al.,* 2011a). The building of Jianchuanzu volcanic clastic rocks and large-scale eruption of trachyte indicated the end of the quiet period in the western Sichuan and northern Yunnan crustal movement, and a new period of activity began. The uplift of the western Sichuan and northern Yunnan plateaus, complete disintegration of unified plantations, and the formation of the edge of the Qinghai-Tibet Plateau and peripheral rift basins occurred at almost the same time (Li *et al.,* 1979; Clark *et al.,* 2004). This tectonic event occurred slightly earlier than 4.5 Mya, and the Hengduan Mountains might have started to experience folding and uplift at that time (Chen, 1992). This is consistent with the conclusion that the uplift of the Qinghai-Tibet Plateau caused the formation of the Hengduan Mountains. In addition, this also fits the time of differentiation between the two clades, approximately 4.5 Mya, suggesting that differentiation between the two clades may be because the Hengduan Mountain formation separated the original distribution of wild *M. lasiocarpa* populations. Studies aiming to determine the time of the formation of the Pliocene neotectonic structure by the Chinese Academy of Geological Sciences have observed that rock structures in the Panzhihua, Huaping area are more complex than those in other terrains, probably due to the high levels of tectonic movement and extensive geological changes that occurred in this area (CAGS, 1977). This is also consistent with the observations in the present study that the populations HW and YQ grouped together and diverged early from the other *M. lasiocarpa* populations.

From the early Pliocene to the middle Pleistocene, the climate in the Yunnan-Guizhou Plateau and southern Tibet was temperate to subtropical (Team of Comprehensive Science Expedition to the Qinghai-Xizang Plateau from Chinese Academy of Sciences, 1986), which was a suitable climate for *M. lasiocarpa*. Therefore, a drastic decrease in the number of *M. lasiocarpa* populations caused by climate change did not lead to the differentiation of the populations LJ, YQ and HW from other populations. The cause of this differentiation is likely to be terrain structural changes in western Sichuan and northern Yunnan during the middle Pleistocene, and tectonic movements producing new topographic forms and erosion strengthening (Zhang *et al.,* 2011). The Qinghai-Tibet Plateau experienced a substantial uplift in the Pliocene-Quaternary Pleistocene, bringing about the formation of the Hengduan Mountains, which is likely to have had a profound impact on *M. lasiocarpa* populations at that time. As a result of thermodynamic effects and dynamic action caused by the huge uplift, the inner plateau developed drought and the temperature decreased with increasing altitude. This made the climate in this region cold and dry, which was not suitable for *M. lasiocarpa*. Thus, *M. lasiocarpa* populations were only preserved in the marginal areas of the western Sichuan, northern Yunnan plateaus and the Hengduan Mountains.

The uplift of the Qinghai-Tibet Plateau also had a large impact on climate of the surrounding area. The combined changes in topography, climate and intrinsic factors of the plant accelerated plant evolution (Axelrod, 1973). Therefore, changes in the climate and terrain in areas surrounding the plateau created conditions suitable for species differentiation, and the Hengduan Mountains became a differentiation center for *M. lasiocarpa.* However, many years of field surveys by our group indicated that the adaptability of *M. lasiocarpa* populations was not significantly enhanced through evolutionary changes. This is illustrated by the fact that there are only currently approximately 10 individual plants in some populations, and some populations that existed 30 years ago have now disappeared. Recent studies on flora have shown that the Himalayas and Hengduan Mountains region are the areas that have the most endemic species, and some of these species are typical transitional types (Team of Comprehensive Science Expedition to the Qinghai-Xizang Plateau from Chinese Academy of Sciences, 1983). In geological history, the Hengduan Mountain region was located in the ancient land of Kangdian and had not been submerged by the sea since as early as the Tertiary Period (Zhong, 1988). In the subsequent period, the ancient Mediterranean gradually retreated westward, and this might have also led to the movement of *M. lasiocarpa* from the original east side to the west side. We speculate that the origin of *M. lasiocarpa* populations was close to Wenquan-Huaping, Lijiang.

Relation between the geographic distribution pattern of *M. lasiocarpa* populations and the ancient Red River: According to relevant geological records, the

current distribution pattern of *M. lasiocarpa* populations may be closely related to changes and diversions of the ancient Red River. In geological history, the Qinghai-Tibet Plateau experienced several rapid uplifts, causing drastic changes in the southeast edge of the plateau (Gregory & Gregory, 1923, 1925). These changes led to drastic changes in the location and flow direction of major river systems in this region of southwest China. Clark *et al.*, (2004) studied the pattern of the water system of the eastern part of the Qinghai-Tibet Plateau and deduced that the flow directions of the Jinsha, Yalong and Dadu rivers had not always been as they are today (Clark *et al.*, 2004). As with the upstream tributaries of the ancient Red River, many rivers, including the ancient Jinsha and Yalong rivers, had been flowing from north to south, joining the ancient Red River and eventually entering the South China Sea (Clark *et al.*, 2004). Due to the movement of the Himalayas, displacements or changes occurred in Hengduan Mountains the original ancient Red River watercourse was then cut transversely, several tributaries upstream of the ancient Red River were also broken apart, and the interrupted river flows were forced to switch direction. Under the impact of the rivers and erosion, low-lying areas favoring the direction of river flow gradually formed new watercourses (stream capture). These are the new river systems formed by the current Jinsha River, Yalong River and Dadu River (Clark *et al.*, 2004; Zhang *et al.*, 2011; Zhang & Sun, 2011). We examined the distribution of the 9 known wild *M. lasiocarpa* populations and found that all of them were closely related to the patterns of the ancient Red River and its tributaries (Fig. 5). The ancient Red River changed dramatically after stream capture. Of the 9 *M. lasiocarpa* populations, 7 were distributed on both sides of the rivers, including the Jinsha River and Yalong River. We speculate that the original growth environment of *M. lasiocarpa* populations was destroyed by geological events, such as orogeny, and with stream capture of the different tributaries of the ancient Red River, the broken wild populations were spread along the direction of river flow. Among these, 4 populations (YR, HHSL, LJ and BBH) were located in the upstream section of the ancient Red River. Three populations, i.e., YQ, WL and HW, were located near the main stream or tributaries of the ancient Yalong River. Clark *et al.*, (2004) speculated that stream capture of the Dadu River occurred at 12-5 Mya, and this time period was basically in line with the differentiation time between the two clades of the 9 *M. lasiocarpa* populations. This could also explain why most of the current *M. lasiocarpa* populations grow close to rivers. Due to the low germination rate of *M. lasiocarpa* plus seeds damages caused by humans and livestock, the current habitats of wild *M. lasiocarpa* populations are fragmented and reduced in number.

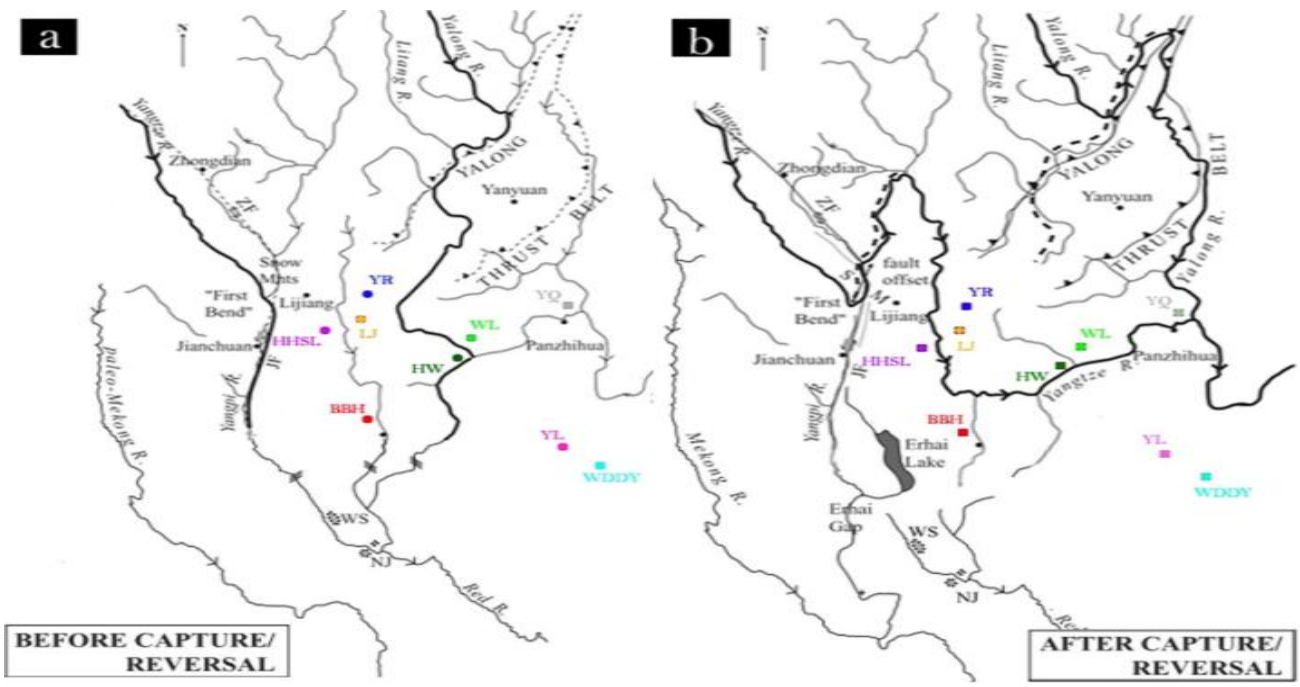

Fig. 5. Watercourses of different tributaries of the ancient Red River before and after stream capture, and their relation with the growing points of 9 *Musella lasiocarpa* wild populations (adapted from Clark, M.K. *et al*., permission conveyed through Copyright Clearance Center, Inc.).

Conclusions

M. lasiocarpa is an ancient species unique to China. Under the impact of rapid uplifts of the Qinghai-Tibet Plateau and Quaternary glacial climate, the distribution of *M. lasiocarpa* populations showed patterns of geographic isolation and fragmentation. Low levels of estimated gene flow are likely to have increased the chance of inbreeding within each population, and genetic diversity was reduced. Together with the low seed germination rate and high degree of human interference, these factors contributed to the fragmentation of *M. lasiocarpa* habitats and their reduced numbers. Therefore, the development of conservation measures for this endangered species are urgently needed, and these should include habitat protection, *ex situ* measures and artificial estimated gene flow.

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