Phylogeographical analysis on the alpine endemic plant Meconopsis punicea (Papaveraceae) based on chloroplast and nuclear genes

Zhi Ou
Southwest Forestry University
Jian Xiong
Southwest Forestry University
Yuanqi Jiang
Southwest Forestry University
Yongdong Dai
Guizhou University of Traditional Chinese Medicine
Yan Qu (quyan@swfu.edu.cn)
Southwest Forestry University

Research Article

Keywords: 4-locus phylogeny, genetic structure, molecular clock, divergence time, Origin, suitable distribution prediction

Posted Date: July 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1844011/v1

Abstract

Meconopsis punicea (Papaveraceae) is only distributed in the alpine areas of the Qinghai Plateau (QHP) and Hengduan Mountains (HDM) in China. As an important ornamental flower and traditional Tibetan medicine, in addition to habitat loss, its higher economic value drives over-exploitation of this resource, which has accelerated the reduction of its distribution. How to make its protection strategy is still unfounded as unclear genetic diversity characteristics of *M. punicea*. In this study, we analyzed the genetic diversity and phylogeographic structures of *M. punicea* representing 11 populations 106 individuals using 4-locus dataset (*matK*, *ndhF*, *rbcL* and *rpb2*, a total of 2576 bp). A total of 76 sequence types were identified (Hd = 0.987, Pi = 0.00447). Six phylogenetic clades were recognized, and 3 clades were restricted to unique geographic distribution (Clade **A** corresponded to population SD, **B** to LT, and **C** to BL). The zoige area was referred as the differentiation center and a glacial refuge of *M. punicea*. *M. punicea* most likely originated from the southern HDM in the early Miocene (~ 22 Myr). Two dispersal routes were suggested and the ecological and geographical difference were revealed as the main driving forces for species differentiation of *M. punicea.* The suitable distribution area was predicted about 158,000 km^2 mainly in Sichuan, Gansu and Qinghai. Our current results provide much clear and detailed understanding for the diversity and geographical spatial distribution of the endemic alpine plant *M. punicea*. It will also be able to provide theoretical guidance for formulating the urgently needed protection strategies of *M. punicea*.

Introduction

Meconopsis punicea, a perennial herb in the family Papaveraceae, is one of the most distinctive members of the genus with its sumptuous satiny red pendent flower (Wu and Chuang, 1980; Ying et al. 2006). It has very high ornamental value, and is also a traditional Tibetan medicine, which has kinds of pharmacological effects such as heat-clearing, analgesic, hypotensive, and antibacterial (Kala 2003; Liu et al. 2012; Shang et al. 2015).

M. punicea has a rather narrow distribution which mainly distribute on plateau hillsides or meadows at an altitude of about 3000–4500 m in northwestern Sichuan, southeastern Qinghai, and southwestern Gansu (Chuang 1981; Zhang and Grey-Wilson 2008). Thus, as driven by the benefits of the industrialized production of medicine, the limited wild resources of *M. punicea* are drastically reduced. And it has been listed as wild plant under State protection (category II) (China Plant Red Data Book 1992). Phylogeography could explore the entire geographical distribution pattern, and discuss the historical factors and evolutionary process of the speciation (Avise 2000). Phylogeography of two *Meconopsis* species (i.e. *Meconopsis cambrica, Meconopsis integrifolia) explain the* genetic differentiation pattern and the potential refugia, it is of great significance to deeply understand the speciation and evolution of species (Valtueña 2012; Yang 2012). In our current study, we conducted a range-wide sampling across the distribution of *M. punicea* and collected 11 populations with a total of 106 samples. Three chloroplast DNA sequences, i.e., *matK* (maturase K), *ndhF* (NADH dehydrogenase subunit F), *rbcL* (ribulose-15-bisphosphate carboxylase/oxygenase large subunit) and one nuclear gene *rpb2* (the second largest subunit of RNA polymerase II), were sequenced and analyzed for all samples. The purpose of this study was attempted to characterize the genetic diversity, genetic differentiation, and the genetic structure of *M. punicea*. Furthermore, we also aimed to evaluate the spatial pattern and the historical process of population demography, and explore the correlations between species differentiation and geological history of the Qinghai-Tibetan Plateau.

Materials And Methods

Sampling

M. punicea is mainly distributed in the Qinghai Plateau (QHP) and Hengduan Mountains (HDM). In terms of administrative divisions, it contains Qinghai, Sichuan and Gansu provinces in China. Therefore, the samples used in this study were collected from the three provinces and cover the distribution range of *M. punicea*. A total of 11 populations were collected: five populations in Qinghai, four populations in Sichuan, and two populations in Gansu (Fig.1, Supplementary table 1). The samplings were distributed ranging from 30.97° N -35.58° N to 95.87° E -103.74° E, and the altitudes ranged from 3000 m to 4500 m.

Ten individuals were collected from each population, with a total of 110 samples. The distance between sample of individuals in each population was at least 50 m apart. To ensure the quality of experimental materials, fresh leave s collected in the field were

dried with silica gel desiccant and stored in -20 °C.

DNA extraction, gene sequence amplification and sequencing

Genomic DNA was extracted from silica-dried leaf materials using TIANGEN Fast Plant Genome Kit. The extracted DNA were tested on a 1.2% agarose gel for gel electrophoresis, and the purity and concentration were measured using the Nano Drop 2000 ultraviolet spectrophotometer.

Candidate molecular markers were screened based on the success rate of amplification and sequencing. Three chloroplast gene fragments *matK* (maturase K), *ndhF* (NADH dehydrogenase subunit F), *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit), and one nuclear gene *rpb2* (the second largest subunit sequences of RNA polymerase II) were selected to conduct the further phylogeographic analysis of *M. punicea*.

Partial chloroplast *matK* gene of *M. punicea* was amplified using the primers matK-F (5'-ACTGTATCGCACTATGTATCA-3') and matK-R (5'- GAACTAGTC GGATGGAGTAG -3'). The *ndhF* gene sequences were amplified using the primers ndhF-F (5'-CTGTCTATTCAGCAAATAAAT-3') and ndhF-R (5'-CGATTATAGG ACCAATCATATA-3'). The *rbcL* gene was amplified using the primers rbcL-F (5'-ATGTCACCACAAACAGARACTAAAGC-3') and rbcL-R (5'-CTTTTAGTAAAA GATTGGGCCGAG-3'). The *rpb2* gene was amplified with the primer pair *Rpb2*-105F (5'-GATAGTATGTGGGACCA AGG-3') and *Rpb2*-1164R (5'-CCCTCTCGA ATGACTAT TGG-3') (Xiao 2013). The polymerase chain reaction (PCR) mixture was performed following the manufacturer's instructions (Tsingke Biotech Co. Ltd.) and the PCR procedure was performed according to our previous screening results (Xiong 2016; Jiang 2018; Qu et al. 2018).

The PCR products were separated by electrophoresis in 1.5% agarose gels. The clear bands were cut and removed from the gels for purification using a Gel Band Purification Kit (BioTeke Co. Ltd, Beijing, China) and directly sequenced with an automatic sequence analyzer (ABI 7500, Sangon Biotech Co. Ltd).

The sequences were assembled with SeqMan 7.1. Each locus was aligned using MAFFT 3.7 with the default settings (Katoh et al. 2002) and manual adjustments were made in MEGA 5 (Tamura et al. 2011).

Population genetic diversity and genetic structure analyses

Nei's nucleotide diversity (***Pi***) and haplotype diversity (**Hd**) of the *matK*, *ndhF*, *rbcL*, and *rpb2* DNA sequences were estimated by using DnaSP 6.12.01 (Librado & Rozas, 2009). To assess the genetic differentiation in *M. punicea*, we performed an analysis of molecular variance (AMOVA) on the populations of *M. punicae* using Arlequin 3.1 (Excoffier et al. 2007). Gene flow was estimated according to Wright's principles Nm = (1-Fst)/4Fst. Geographic mapping was illustrated in Google Earth 7.1. Mantel tests were carried out to test the correlation between genetic distance and geographic distance using TFPGA 1.3 (Miller 1997).

Mismatch distribution, neutrality test and historical demography

A mismatch distribution for *M. punicea* was investigated to using DnaSP 6.12.01. A unimodal distribution indicates that populations have experienced recent demographic expansions, while multimodal distributions are consistent with stability (Fu 1997).

In addition, historical demographic changes were examined with neutrality tests for the species and individual populations, including *Tajima's* D, *Fu and Li's* D*, *Fu and Li's* F* (Excoffier 2004). *Tajima's* D test detects the relationship between the number of separation sites and nucleotide diversity (Pi). *Fu-Li's D* & *F* test measures the difference between total polymorphic sites and single-base mutations. The paraments of sum-of-squared deviations (SSD) and the Harpending's Raggedness index (Rag) were calculated with Arlequin 3.1. These parameters provide information about demographic histories, with significant negative values indicating evidence of demographic expansions, whereas the positive values indicating evidence of population bottlenecks.

Phylogenetic relationships and geographic structure analysis

The phylogenetic relationships of haplotypes of *M. punicea* were constructed by using the single and combined 3 chloroplast genes. Meanwhile, the relationship of alleles was conducted by using the nuclear gene *rpb2*. Furthermore, DNA sequences of

chloroplast genes and nuclear gene were combined to retrieve the evolutionary history of *M. punicea. M. horridula* and *M. betonicifolia* chosen as outgroups as their suitable phylogenetic relationship (Liu et al. 2014). The best model was selected in each dataset by using Modeltest 3.7 (Posada and Crandall 2001). Phylogenetic tree of the haplotypes and alleles of *M. punicae* were analyzed using the Maximum Likelihood (ML) method in Raxml 7.2 (Stamatakis 2014).

Divergence time estimation

The divergence time of *M. punicea* and its relatives was estimated with combined the 4 genes using a Bayesian method in BEAST 1.8.4 (Drummond and Rambaut, 2007). A total of 156 sequences were sampled within the subf. Papaveroideae to estimate the divergence time in *M. punicea* (Jork and Kadereit 1995; Yuan 2002; Xiao 2013; Liu et al. 2014) (Supplementary table 2).. *Nandinadomestica* and *Hydrastis canadensis* were chosen as the outgroups.

The oldest known fossil of Papaveroideae, *Palaeoaster* sp. is assigned to the Latest Cretaceous (74.5–64.5 million years ago, Myr) (Smith et al. 2001). The calibration 74.5 Myr was settled according to the previous research (Valtueña et al. 2012). Accordingly, subf. Papaveroideae was defined as monophyletic, and its root was set with a normally distributed prior with a mean of 70 Myr and a standard deviation of 2 Myr. The uncorrelated relaxed clock was used as the clock model, and a birth death prior was set for branch lengths. Other priors were made in default settings, and the Markov Chain Monte Carlo (MCMC) was initiated on a random starting tree. Two MCMC analyses were run for 100 million generations with parameters sampled every 1000 generations. The first runs were used to examine the MCMC performance, and operators were adjusted as suggested by the output analysis. Finally, two BEAST runs were performed. Convergence of the runs was assessed using Tracer 1.6 (Rambaut and Drummond 2009), which indicated that most parameter values had effective sample sizes well above 150, then the two separate runs dataset were combined using LogCombiner 2.1.2 (Bouckaert et al. 2014). TreeAnnotator 2.1.2 was used to calculate node ages and the upper and lower bounds of the 95% highest posterior density interval (HPD) for divergence time from the combined tree file with the burn-in of 20% aged trees (Bouckaert et al. 2014). The chronogram was visualized using Figtree 1.6.2 (Drummond and Rambaut 2007).

Prediction of suitable distribution area of *M. punicea*

Species occurrence data was collected from ongoing field studies and published research papers. Bioclim variables were downloaded from the CliMond Archive (https://www.climond.org/) (Kriticos et al., 2012)

A total of20 species occurrence data were used (Supplementary table 3). And a total of 35 typical climate variables at a grid resolution of 10' were obtained from CliMond the CRU CL2.0 dataset. (https://www.climond.org/). These factors contained the core set of 19 variables (temperature and precipitation), and an extended set of 16 additional variables (solar radiation and soil moisture) at a global extent (Supplementary table 4). All climate variables data collection sources were from 1961 - 1990 (30 years centered on 1975).

Species distribution modelling were constructed based on species redundance with MaxEnt v3.4.1 (Phillips et al., 2006). randomly 25% of the data points were extracted as the test data, and “do jackknife to measure variable importance” was selected. The output grid format was set as “cloglog” The output result “.asc” was visualized with Global mapper17.

Results

The genetic diversity analysis

A total of 106 samples of *M. punicea* were successfully amplified and sequenced. The aligned length of *matK* was 660 bp, containing 41 polymorphic sites (variation rates: 6.24%), with values of **Hd** (0.931) and ***Pi*** (0.00788), respectively. The length of *ndhF* was 691 bp, containing 21 polymorphic sites (variation rates: 3.04%), with lower values of **Hd** (0.438) and ***Pi*** (0.00135). The length of *rbcL* was 683 bp, containing 6 polymorphic sites (variation rates: 0.88%), with the significant low **Hd** (0.110) and ***Pi*** (0.00027). The length of the nuclear gene *rpb2* was 542 bp, containing 69 polymorphic sites (variation rates: 12.78%), and its **Hd** and ***Pi*** were 0.987, 0.00447, respectively.

The combinatorial dataset was contained 2576 bp after combined the 3 chloroplast DNA fragments and the nuclear gene *rpb2*, and total polymorphic sites were 136, with mutation rate 5.28%. Sixty-nine parsimony informative sites were recognized, accounting for 2.65% of the total sequence. The *matK* and *rpb2* gene sequences have relatively more information sites among the 4 genes. A total of 76 sequence types were identified from the concatenated sequences of 4 genes (Table 1). All 76 sequence types were submitted to GenBank (*matK*: OM640468-OM640543; *ndhF*: OM640544-OM640619; *rbcL*:OM654956-OM655031; *rpb2*:OM655032- OM655107).

Table 1 Genetic diversity of *M. punicea* based on the 4 genes datasets

Paraments	*matK*	*ndhF*	*rbcL*	3 chloroplast genes	*rpb2*	**4 genes**
Length	660	691	683	2034	542	**2576**
Total number of mutations, Eta	45	21	6	72	83	**155**
Number of polymorphic sites	41	21	6	68	69	**136**
Parsimony informative sites	32	11	2	45	25	**69**
Sequence types	36	15	5	46	38	**76**
Hd	0.931	0.438	0.110	0.949	0.844	**0.987**
sd of Hd	0.011	0.06	0.042	0.010	0.031	**0.005**
Pi	0.00788	0.00135	0.00027	0.00310	0.00963	**0.00447**
sd of Pi	0.00065	0.00034	0.00011	0.00028	0.00154	**0.00041**
Theta (per site) from Eta	0.01308	0.0058	0.00168	0.00677	0.02936	**0.01151**

A high level of haplotype diversity (**Hd**= 0.987) but a relatively low level of nucleotide diversity (***Pi*** = 0.00447) is detected among the populations of *M. punicea*, (Table 2). The populations GD and BM have the highest haplotype diversity (**Hd**= 1.00). Among the 76 sequence types, types 2, 40, 44, 46 and 50 are distributed in two populations, the others were unique to only one population.

Table 2 Genetic diversity of the 11 populations of *M. punicea*

Population	**Samples num.**	**Total Seq. types**	**Seq. types num.**	**Hd**	***Pi***	**Shared hap.**	**Fst**	***Nm***
REG	10	7	1-7	0.867	0.00400	2	0.43318	0.32713
SD	10	7	8-14	0.911	0.00128		0.49481	0.25524
HL	10	6	2, 15-19	0.778	0.00398	2	0.43375	0.32637
DLJ	10	7	20-26	0.911	0.00104		0.50012	0.24988
BL	6	4	27-30	0.800	0.00171		0.48811	0.26218
LT	10	8	31-38	0.933	0.00212		0.47452	0.27685
CD	10	8	39-46	0.956	0.00162	40,44,46	0.48722	0.26312
GD	10	10	47-56	1.000	0.00309	50	0.45498	0.29947
BM	10	10	57-66	1.000	0.00386		0.43811	0.32063
MQ	10	8	40,44,46,50, 67-70	0.956	0.00346	40,44,46,50	0.44683	0.30950
JZ	10	6	71-76	0.844	0.00106		0.49955	0.25045

Genetic structure and historical dynamics of *M. punicea*

Mismatch analysis and neutrality tests were calculated based on the 4-locus sequences of the 11 populations. Mismatch analysis of *M. punicea* were calculated with two curves (expected and observed curves) to detect historical demographic changes. Neutrality tests for the whole and different populations were carried out using *Tajima's* D, *Fu and Li's* D*, and *Fu and Li's* F* methods (Excoffier 2004), respectively.

The results of neutrality tests show that, except population MQ, all the populations do not experience a significant expansion as their negative parameters of *Tajima's* D, *Fu and Li's D** and *Fu and Li's* F*. Although the *Tajima's* D, *Fu and Li's* D*, *Fu and Li's* F* of MQ are significant, SSD and Raggedness are not significant, and the mismatch curve shows obvious multimodal characteristics, the non-significant expansion with demographic equilibrium is also suggested. (Table 3, Fig. 2).

Table 3 Neutrality tests of each population of *M. punicea*

Population	***Fu and Li's* D***	***Fu and Li's* F***	***Tajima's* D**	SSD	Raggedness
REG	-1.75684	-1.95124	-1.64125	0.04110	0.06025
SD	-1.78608	-1.94152	-1.53620	0.03983	0.07605
HL	-1.32553	-1.54795	-1.50721	0.07663	0.09877
DLJ	-1.00080	-1.04388	-0.70843	0.03977	0.14519
BL	-0.96650	-1.05215	-0.99024	0.49859**	0.13778
LT	-1.51599	-1.67817	-1.40434	0.04364	0.06765
CD	-0.41245	-0.54857	-0.71356	0.23406*	0.04938
GD	-0.18999	-0.29439	-0.47492	0.01944	0.05531
BM	-1.65303	-1.78240	-1.35554	0.04351	0.48000
MQ	-2.08729*	-2.27426*	-1.79512*	0.03376	0.09580
JZ	-0.76777	-0.92940	-1.00504	0.04421	0.11309
Whole	-4.53570**	-4.13466**	-2.02875*	0.00280	0.00241

Phylogenetic relationships and phylogeographic patterns

To clarify the phylogenetic relationships among *M. punicea*, the combined 3 chloroplast genes and the single *rpb2* gene dataset were conducted and analyzed, respectively. Meanwhile, the combinatorial dataset of 4 genes were also analyzed as more informative sites obtained.

Three chloroplast genes dataset acquired a total of 46 haplotypes, which could divide into 8 clades (Clade A-H) (Table 4, Fig. 3A, B), with each clade including 2 to 19 haplotypes. Among them, Clade D and E are distributed in QHP. Clade E contains 19 haplotypes of 5 populations. The haplotypes of Clade D are derived from 4 populations. In contrast, clade A, G and H are all from single population—LT, SD, and BL, respectively, which distribute in the HDM. In terms of population genetic differentiation, REG contains 4 phylogenetic clades, while SD, BL and MQ had only one phylogenetic clade. It reveals that there are significant differences in the genetic diversity of different populations.

Rpb2 acquired a total of 38 alleles derived from 106 sequences data, and it forms 7 clades: Clade□-□ (Table 4, Fig. 3C, D). The numbers of alleles in each clade were between 1 and 11. Among them, Clade □ and □ have very abundant alleles, containing 11

and 10 respectively. Clade □ contained 10 populations (only without SD), and SD was belonged to Clade □.

When combined the 4 genes to form a multilocus dataset, 76 sequence types were obtained and 6 main clades could be divided (Clade **A-F**) (Table4, Fig. 3E, F). The numbers of sequence types in each branch were between 4 and 27. Among them, clade **F** had the most 27 sequence types. The spatial genetic structure of 11 populations presented certain valuable phylogenetic structures. And their phylogeographic structures were described as followed.

Clade **A** contained 8 sequence types, which were mainly from population SD. Clade **B** contained 7sequence types, which were only distributed in the LT population. There were 4 sequence types in Clade **C**, which are only distributed in the BL population. These 3 clades all had narrow distribution, suggesting that genetic isolation was formed in different geographic environments. Clade **D** had 18 sequence types, which were mainly from the northern HDM (populations of HL, REG and DLJ). Clade **E** contained 12 sequence types, which were distributed in 4 populations: JZ, GD, MQ and CD, all located in the QHP. Clade **F** contained 27 sequence types, which were from JZ, GD, MQ, BM, and CD, all in QHP. The wide distributions of Clade **D-F** indicated that these 3 lineages spread out around QHP and HDM, exhibiting wide distribution characteristics with low genetic isolation. These results also demonstrated that the genetic differentiation happened between QHP and HDM.

Table 4 Phylogenetic structure of *M. punicea* based on 3 different datasets

Pop.	Sample	**3 chloroplast genes**								**nuclear gene *rpb2***							**4 genes**					
		A	B	C	D	E	F	G	H	□	□	□	□	□	□	□	**A**	**B**	**C**	**D**	**E**	**F**
REG	10		2	5		1	2			2					7	1				9		1
SD	10							10		10							10					
HL	10		2	8						1					9					10		
DLJ	10		10												9	1				10		
BL	6								6						4	2			6			
LT	10	9					1			1	1				4	4	1	9				
CD	10				5	5								1	7	2					5	5
GD	10				4	6								3	5	2					3	7
BM	10					10						4	1	1	4							10
MQ	10				10					1		1			5	3					9	1
JZ	10				7	3									10						7	3

Mantel test and AMOVA analyses

The mantel test was used to test the correlations between the genetic distance and geographic distance of *M. punicea* with the combined 4 genes. The results reveal that the correlations were not significant (y=0.0103x+0.3764, r=0.075, p=0.29). It does not show obvious isolation-by-distance characteristics.

The AMOVA analyses uncover 46.75% variation among populations, and 53.25% genetic variation within populations. Genetic differentiation is not significant among all populations. While based on the haplotypes geographic mapping, the genetic difference was significantly observed between HDM and QHP, indicating that the difference of geographical environment between HDM and QHP had big influence on the species differentiation of *M. punicea.*

The prediction of the suitable distribution area of *M. punicea*

The prediction result of the suitable distribution area was obtained of ***M. punicea*** with the species distribution modeling method. The main suitable distribution areas appear in the north edge of Hengduan mountains and Qinghai plateau, containing Sichuan, Gansu and Qinghai, the total suitable distribution area was 158,000 km^2 (suitable index>0.7) (Fig. 5). this result revealed that the urgently needed protection strategies should be carried out as its quite small distribution area.

Divergence time estimation and evolutionary routes revealing

The divergence time were estimated with the combined 4 genes of main taxa of Papaveroideae, with emphasis on *M. punicea* by using fossil calibration, and the differentiation time of each node was obtained for *M. punicea* (Fig. 6A). The divergence of the crown group of *M. punicea* occurred approximately 22.24 Myr (95% HPD=13.34-29.63). The main clades of *M. punicea* differentiated from 12.10-18.26 Myr (Fig. 6B). This differentiation time just coincided with the later period of third geological uplift event of the QTP (Harrison 1995; Li and Fang 1998). The huge geological uplift changes have increased the species differentiation of *M. punicea.* While the stable stage after the third uplift of the QTP just provided opportunities for the migration and spread of species and gene exchange, which made *M. punicea* expand on the plateau and its ecological niche further expanded.

The divergence time estimation indicates that the most recent common ancestor of *M. punicea* and its close relatives was dated to the early Miocene (18.26 Myr) (95% HPD=12.67-25.03) in the southern HDM (Fig. 6C). Two possible routes are suggested from south to north and northwest (Fig. 6C). It spread westward to SD and northward to HDM (HL, REG, LT and DLJ), two important secondary endemic centers are suggested: SD and LT populations. According to the presence of Clade E in the REG population, it is speculated that the distribution around QHP (MQ, GD, JZ and BM) resulted from the diffusion of REG. And then scattered to westward on the plateau (to CD population).

Discussion

In this study, the phylogeography of 106 samplings representing 11 populations of *M. punicea* were analyzed. The conjoint analysis with four genes (*matK*, *ndhF* and *rbcL* and *rpb2*) uncovers the high genetic diversity and genetic differentiation characteristics of *M. punicea*. There are at least 6 main phylogenetic clades, three of which are endemic to local geographic populations. The geographical difference may play the important role in the genetic differentiation of this species.

We clearly identified the ecogeographical variation patterns based on the 4 gene phylogeney. REG contains the most 4 phylogenetic clades, it is suggested that the population REG should be a glacial refuge, and might be an important genetic diversity center.

According to molecular clock dating, *M. punicea* most likely originated from the southern HDM (BL population) in the early Miocene. The gene flow spread north to the high-altitude areas of the HHM, and then northward to HDM (HL, REG, LT and DLJ) along two dispersal routes. The differentiation time of main nodes of *M. punicea* (12.10-18.26 Myr) coincided with the later period of the third geological uplift event of the QTP (Qiu et al. 2011). It was suggested that ecological and geographical environment changes were the main driving forces for the species differentiation of *M. punicea*.

The suitable distribution area was predicted about 158,000 km^2. The small distribution range shows that this species has high requirement on the ecological environment. which revealed that the urgently needed protection strategies should be carried out in order to keep the population stable. Meanwhile, the sampling point CD is far from the predicted suitable distribution area, it implies that the possibility of diversity loss at this point, so protection efforts should be increased.

Declarations

Funding

This project was supported by the National Natural Science Foundation of China (Grant No. 32160404, and No. 31460218), and supported by Scientific Research Fund of Yunnan Provincial Department of Education project (2020J0408), and the youth talent support program of Yunnan Ten Thousand Talent Program (YNWR-QNBJ-2019-211).

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

All authors contributed to the study conception and design. Zhi Ou collected samples, analyzed the genetic diversity and phylogeographic structures. Jian Xiong collected samples, amplified and sequenced 4 genes (matK, ndhF and rbcL). Yuanqi Jiang collected samples, amplified and sequenced rpb2 genes. Yongdong Dai estimated the divergence time and predicted the suitable distribution area. Yan Qu wrote, and reviewed this paper. *all authors commented on this version of the manuscript. All authors read and approved the final manuscript.*

Data Availability

DNA sequences and other metadata have been submitted to GenBank. (matK: OM640468-OM640543; ndhF: OM640544-OM640619; rbcL:OM654956-OM655031; rpb2:OM655032- OM655107) [http://www.ncbi.nlm.nih.gov/]

References

1. Avise JC (2000) Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge, Massachusetts
2. Bouckaert R, Heled J, Kühnert D et al (2014) Beast 2: a software platform for Bayesian evolutionary analysis. PLoS Comput Biol 10:e1003537. https://doi.org/10.1371/journal.pcbi.1003537
3. Chuang H (1981) The systematic evolution and the geographical distribution of *Meconopsis* vig. Acta Bot Yunnanica 3:139–146
4. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Bio 7:214. https://doi.org/10.1186/1471-2148-7-214
5. Excoffier L (2004) Patterns of DNA sequence diversity and genetic structure after a range expansion:lessons from the infinite-island model. Mol Ecol 13:853–864. https://doi.org/10.1046/j.1365-294X.2003.02004.x
6. Excoffier L, Laval G, Schneider S (2007) Arlequin version 3.0, An integrated software package for population genetics data analysis. Evol Bioinform Online 1:47. doi:10.1177/117693430500100003
7. Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925. https://doi.org/10.3389/fmicb.2017.01179
8. Harrison TM, Copeland P, Kidd WSF, Yin AN (1992) Raising Tibet. Science 255:1663–1670
9. Jiang YQ (2018) Genetic diversity of *Meconopsis punicea* based on ISSR molecular marker and nuclear gene fragment RPB2. A Master Thesis of Southwest Forestry University
10. Jork KB, Kadereit JW (1995) Molecular phylogeny of the old world representatives of Papaveraceae subf. Papaveroideae with special emphasis on the genus *Meconopsis* Vig. In: Jensen U, Kadereit JW, editors. Systematics and evolution of the Ranunculiflorae. Plant Syst Evol 9 (Suppl.) 9: 171–180
11. Kala CP (2003) Medicinal plants of Indian trans-Himalaya: focus on Tibetan use of medicinal resources. Bishen Singh Mahendra Pal Singh
12. Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT, a novel method for rapid multiple sequence alignment based on fast fourier transform. Nucleic Acids Res 30(14):3059–3066. https://doi.org/10.1093/nar/gkf436
13. Li JJ, Fang XM (1999) Uplift of the Tibetan Plateau and environmental changes. Chin Sci Bull 44:2117–2125
14. Librado P, Rozas J (2009) DnaSP v5, a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452. https://doi.org/10.1093/bioinformatics/btp187
15. Liu YC, Liu YN, Yang FS, Wang XQ (2014) Molecular phylogeny of Asian *Meconopsis* based on nuclear ribosomal and chloroplast DNA sequence sata. PLoS ONE 9(8):e104823. https://doi.org/10.1371/journal.pone.0104823

16. Liu YS, Gao LY, Wang B, Bai W (2012) The research advance of *Meconopsis punicea*. Mod Hortic 6:14–15
17. Miller MP (1997) Tools for Population Genetic Analyses (TFPGA) 1.3: A Windows Program for the Analysis of Allozyme and Molecular Population Genetic Data. Northern Arizona University
18. Phillips SJ, Dudík M, Schapire RE (2006) Maxent Software for Modeling Species Niches and Distributions. Available online at: http://biodiversityinformatics.amnh.org/open_source/maxent
19. Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144:1237–1245
20. Posada D, Crandall KA (2001) Selecting the best-fit model of nucleotide substitution. Syst Biol 50:580–601. https://doi.org/10.1080/10635150118469
21. Qiu YX, Fu CX, Comes HP (2011) Plant molecular phylogeography in China and adjacent regions: Tracing the genetic imprints of Quaternary climate and environmental change in the world's most diverse temperate flora. Mol Phylogenet Evol 59:225–244
22. Qu Y, Zhao WY, Ou Z, Leng QS, Xiong J (2018) Analysis of chloroplast gene *ndhF* and *rbcL* sequences of Tibetan medicine plants of *Meconopsis*. J Cent South Univ Forestry Technol 38(6):84–89
23. Rambaut A, Drummond A (2009) Tracer: MCMC Trace Analysis Tool, Version 1.5.0. Available at: http://tree.bio.ed.ac.uk/software/tracer/University of Oxford
24. Ronquist F, Teslenko M, Mark PVD, Ayres DL, Darling A, Hohna S (2012) Mrbayes 3.2, efficient bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61:539–542. https://doi.org/10.1093/sysbio/sys029
25. Shang XF, Wang DS, Miao XL, Wang Yu, Zhang JJ, Wang XZ, Zhang Y, Pan H (2015) Antinociceptive and anti-tussive activities of the ethanol extract of the flowers of *Meconopsis punicea* maxim. BMC Complem Altern M 15:154. https://doi.org/10.1186/s12906-015-0671-y
26. Smith UR (2001) Revision of the Cretaceous fossil genus Palaeoaster (Papaveraceae) and clarification of pertinent species of Eriocaulon, Palaeoaster, and Sterculiocarpus. Novon 11:258–260
27. Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313. https://doi.org/10.1093/bioinformatics/btu033
28. Valtueña FJ, Preston CD, Kadereit JW (2012) Phylogeography of a Tertiary relict plant, *Meconopsis cambrica* (Papaveraceae), implies the existence of northern refugia for a temperate herb. Mol Ecol 21(6):1423–1437. https://doi.org/10.1111/j.1365-294X.2012.05473.x
29. Wu C, Chuang H (1980) A study on the taxonomic system of the genus *Meconopsis*. Acta Bot Yunnanica 2:371–381
30. Xiao W (2013) Molecular systematics of Meconopsis Vig. (Papaveraceae): taxonomy, polyploidy evolution, and historical biogeography from a phylogenetic insight. The University of Texas at Austin
31. Xiong J (2016) Genetic diversity and pedigree geography of the endangered plant *Meconopsis punicea*. A Master Thesis of Southwest Forestry University
32. Yang FS, Qin AL, Li YF, Wang XQ (2012) Great genetic differentiation among populations of *Meconopsis integrifolia* and its implication for plant speciation in the Qinghai-Tibetan plateau. PLoS ONE 7:e37196
33. Ying M, Xie H, Nie Z, Gu Z, Yang Y (2006) A karyomorphological study on four species of *Meconopsis* vig. (Papaveraceae) from the Hengduan Mountains, SW China. Caryologia 59:1
34. Yuan CC (2002) The phylogeny, systematics and biogeography of *Meconopsis* vig. (Papaveraceae) and *Craigia* W.W. Sm. & W.E. Evans (Tiliaceae). Dissertation (Zhong Shan University)
35. Zhang ML, Grey-Wilson C (2008) *Meconopsis* Viguier. In: Wu ZY, Raven PH (eds) Flora of China, vol 7. Science Press/Missouri Botanical Garden Press, Beijing/St Louis, pp 262–278

Figures

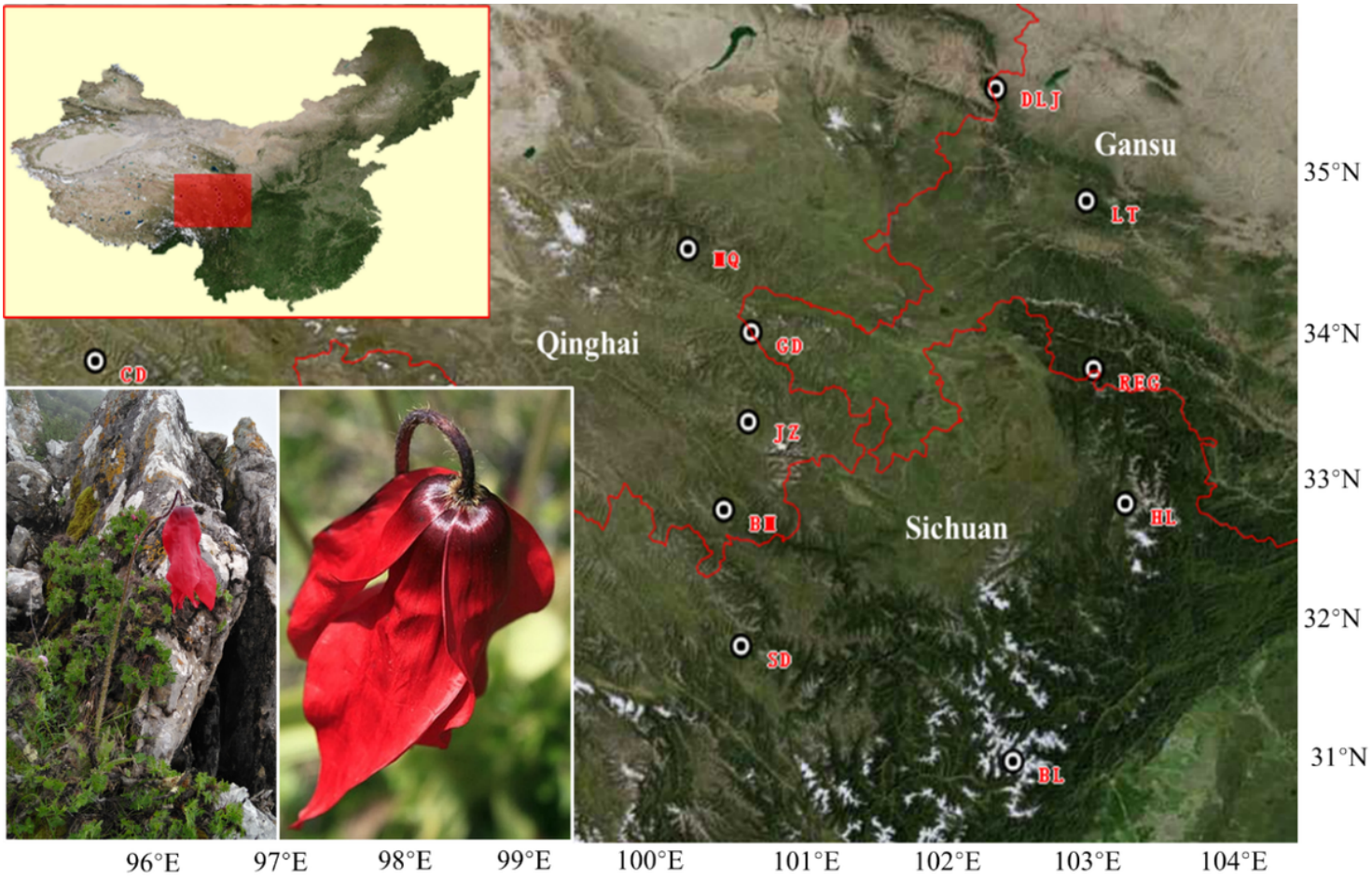

Figure 1

Geographical distribution of 11 populations of *Meconopsis punicea*

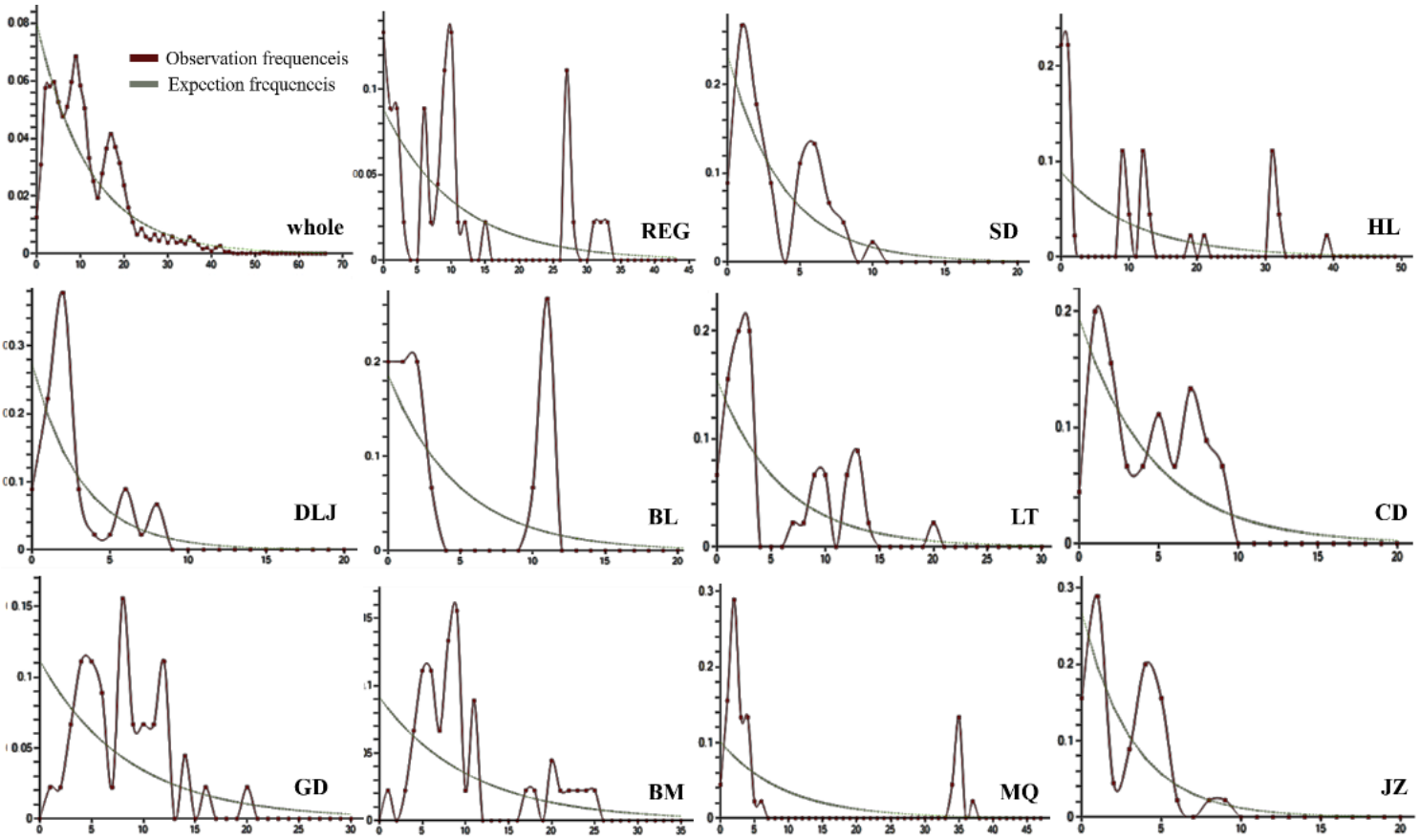

Figure 2

Mismatch distribution analyses of each population of *M. punicea*

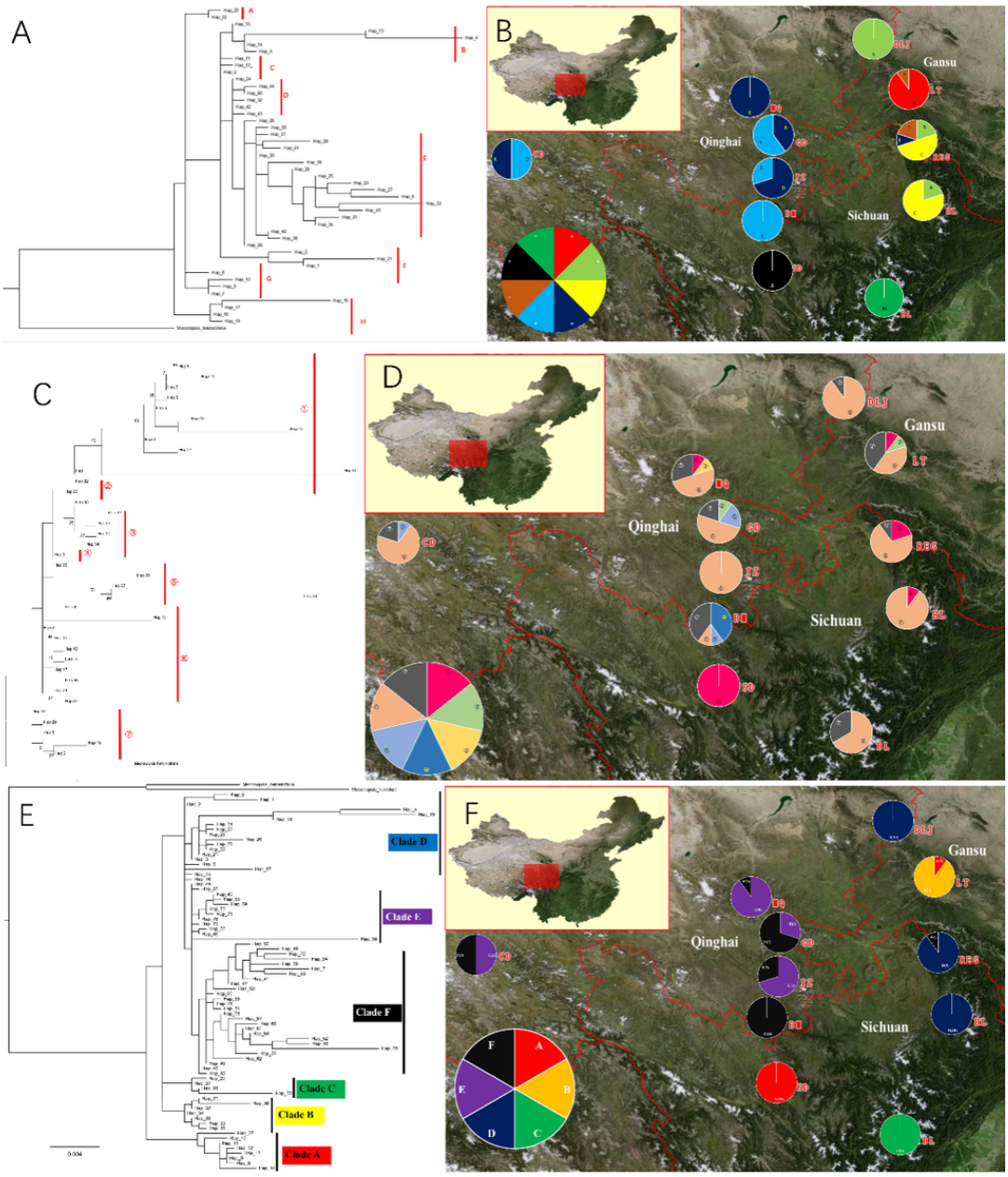

Figure 3

Phylogeographic structure and geographic mapping of *M. punicea*

A,B the phylogenetic tree and phylogeographic mapping based on three chloroplast genes;

C,D the phylogenetic tree and phylogeographic mapping based on *rpb2* gene;

E,F the phylogenetic tree and phylogeographic mapping based on 4 genes combined three chloroplast genes and nucleic *rpb2* gene

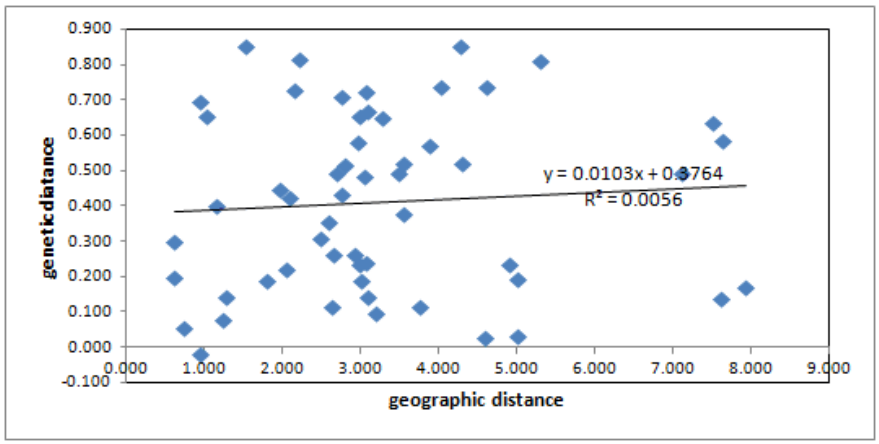

Figure 4

Correlation analysis between geographic distance and genetic distance of *M. punicea* by mantel test

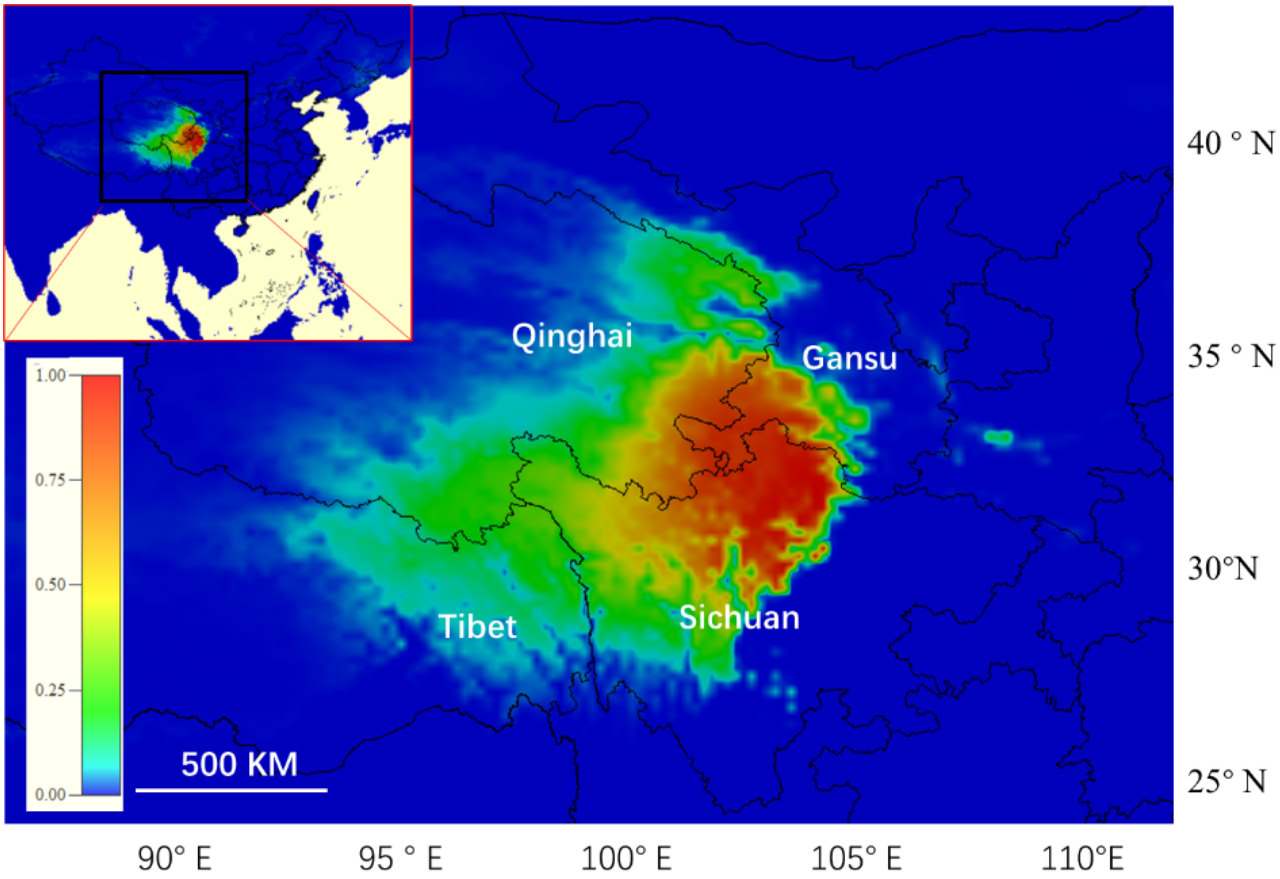

Figure 5

The prediction of the suitable distribution area of *M. punicea*

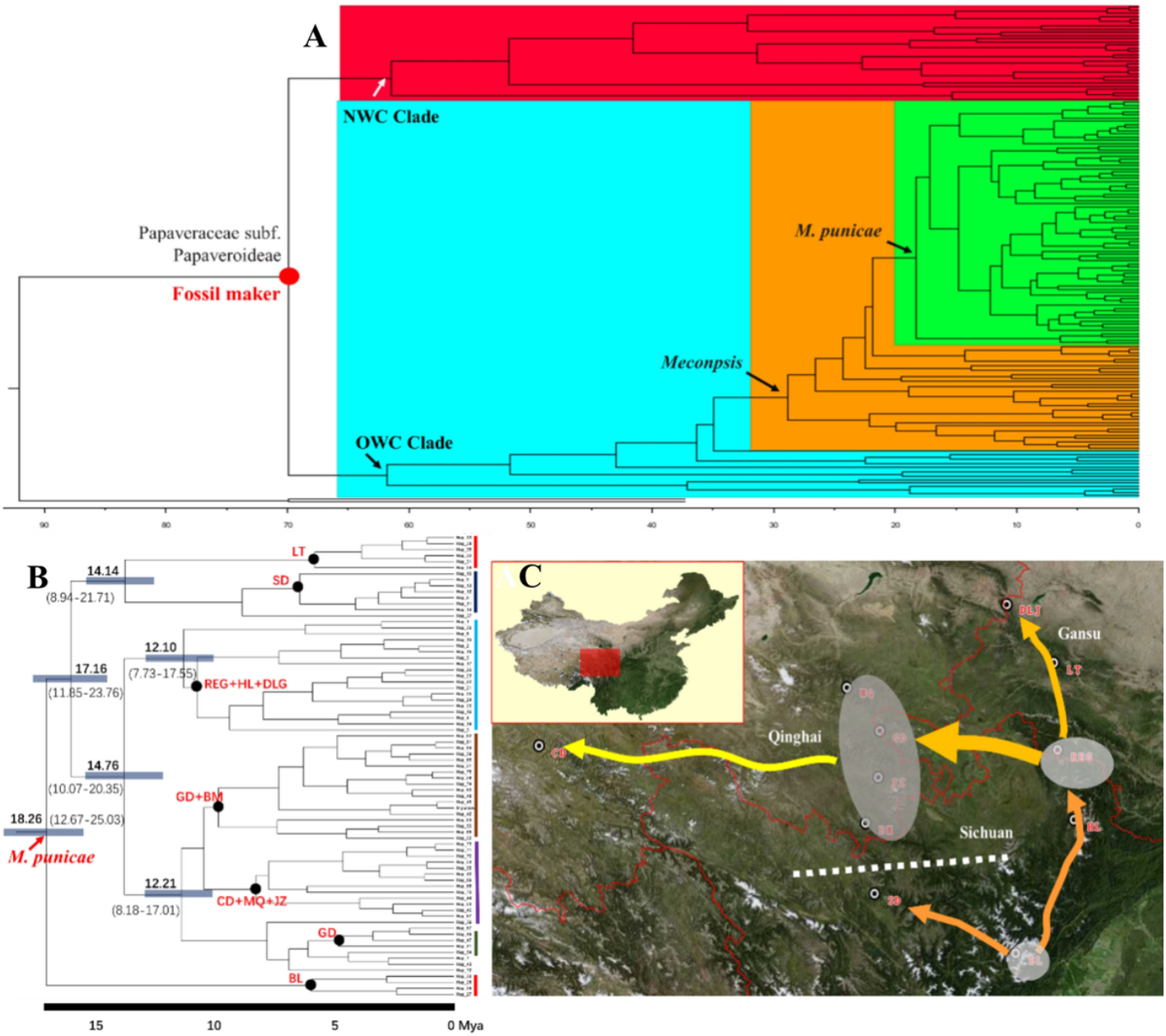

Figure 6

The divergence time estimation and the possible dispersal routes of *M. punicea*

A. The divergence time estimation of Papaveroideae; B. The divergence time estimation of *M. punicea*; C. the possible dispersal routes of *M. punicea*

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplymentarytable1.xlsx