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Species-level phylogeographical history of *Myricaria* plants in the mountain ranges of western China and the origin of *M. laxiflora* in the Three Gorges mountain region

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Abstract

***Myricaria* species in China occur mostly in the major high-altitude mountain areas in and around the Qinghai-Tibetan Plateau. The one major exception to this is *M. laxiflora* which is restricted to the Three Gorges mountain region. In this study, we investigate species-level phylogeographical patterns of *Myricaria* species in western China and the origin of *M. laxiflora*. The results show that most chloroplast haplotypes are species-specific, except for one haplotype which is shared by three widespread species. Higher haplotype diversity within the Qinghai-Tibetan Plateau region supports the hypothesis that the Himalayas are the centre of origin for *Myricaria*. The phylogeny of *Myricaria* was geographically structured, and an estimated Bayesian chronology suggested the main divergence events occurred during the Late Pliocene and Early Pleistocene (~1.46–2.30 million years ago). The overall phylogeographical pattern was characterized by vicariance events and regional demographical expansion, reflecting a major influence of geological and climatic events on the evolution of *Myricaria* species. Our data suggest that *M. laxiflora* has an ancient origin, but has experienced recent population expansion through the Three Gorges Valley. The origin of *M. laxiflora* was estimated to be during the Early Pleistocene but its demographical expansion was more recent at about 0.015 million years ago. This highlights the unique phylogeographical history of the Three Gorges mountain region, and the deep imprint of the watercourse connections of the Yangtze River Valley on the phylogeographical structure of the species in this region.**

Keywords: biogeography, chloroplast DNA, *Myricaria*, phylogeography, Qinghai-Tibetan Plateau, vicariance

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Introduction

Phylogeography examines the spatial arrangements of genetic lineages and is a powerful tool for inferring the processes that determine the genetic composition of species or species groups (Avise 2000; Hardy *et al*. 2002). Phylogeographical studies can disentangle historical changes in patterns of gene flow, isolation and secondary contact among divergent populations at various spatial and temporal scales (Schaal & Olsen 2000; Hewitt 2001). Mountain ranges are seen as particularly important drivers of plant evolutionary divergence and speciation, both in the tropics and in more temperate regions (Hewitt 2004). Topographically diverse mountain ranges can create both geologically and climatically induced species range changes (Hewitt 2004). Quaternary climatic oscillations instigated cycles of habitat contraction/expansion and latitudinal/altitudinal shifts of species' distributions, as evidenced by phylogeographical studies of many plants and animals in Europe and North America (e.g. Soltis *et al*. 1997; Taberlet *et al*. 1998; Petit *et al*. 2003). The effects of geological events result mostly in altitudinal shifts, predominantly the continuous uplift of mountain tops in alpine regions, whereas geographical barriers, resulting from orogenesis, may impel phylogeographical patterns of montane species and leave deeper phylogenetic imprints underlined by strong genetic drift and limited gene flow.

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Understanding the combined effect of geology and climate on the evolution and dispersal of species is thus important when developing generalized models of mountain taxa phylogeography (Haenel 2007).

The Qinghai-Tibetan Plateau (QTP) together with adjacent regions in western China is a complex mountain system that shows large altitudinal changes, ranging from the Himalayas-Hengduan mountains at an average altitude of about 4500 m above sea level (a.s.l.) (Shi *et al*. 1998) to the Three Gorges Mountain Region (TGMR) at an altitude of about 1000 m a.s.l. at its eastern boundary. Most parts of these mountain ranges in western China are geographically and ecologically dynamic. In the Late Tertiary and Early Pleistocene, the drastic Himalayan orogenic movement drove a rapid uplift in the QTP area and the Palaeo-Mediterranean sea regressed westwards (Zhang *et al*. 2000). Previous studies based on pollen cores, fossils and moraine have attempted to explain the possible roles of geology and multiple glaciations and climate oscillations in shaping the current geo-ecological system across these mountain ranges (Tang & Shen 1996; Tang *et al*. 1998; Li *et al*. 2004; Owen *et al*. 2005). Some core areas have been seen as either a biodiversity hotspot/refuge (e.g. the southeastern part of the QTP, Myers *et al*. 2000) or an ancient relict area through the Quaternary (e.g. the TGMR, where the living fossil *Metasequoia glyptostroboides* was found, Hu 1948). A growing body of studies have begun to elucidate the complex roles of past geological and climatic changes on the evolution of species occurring in the western mountain ranges of China (e.g. Liu *et al*. 2002; Wang *et al*. 2005; Zhang *et al*. 2005; Yang *et al*. 2006; Yuan *et al*. 2008), but the overall phylogeographical patterns are still poorly understood.

The genus *Myricaria* Desv. provides an excellent system for examining the potential influence of geological and climate effects on species divergence across the mountain ranges in western China. It belongs to the family Tamaricaceae, which comprises four genera occurring in the Palaeo-Mediterranean sea region since the Tertiary (Zhang & Zhang 1984). The genus contains 13 described species and the majority of these mainly occur in high altitudes in the mountain ranges of eastern Asia, centring on the QTP and adjacent regions but extending to central Asia and Europe. Seven endemic species and three widespread species (*M. bracteata*, *M. paniculata* and *M. squamosa*) occur in the mountain ranges of western China, whereas one dominant species *M. germanica* and two local species, *M. dahurica* and *M. longifolia*, occur in central Asia and Europe (Zhang & Zhang 1984; Wang *et al*. 2006). The vertical distribution of these species varies greatly from 1000 to 5200 m a.s.l., the only exception being *M. laxiflora* which is restricted to the TGMR along the Yangtze River Valley (TGV) at lower altitudes, 70–160 m a.s.l. (Zhang & Zhang 1984; Wang *et al*. 2003). *Myricaria* species are semi-evergreen shrubs or small trees and occur in niche habitats along mountain streams and other sandy or occasionally inundated places. Their dispersal capabilities are limited, and both pollen and seed dispersals are mainly by wind and/or water (Liu *et al*. 2006a; Wang *et al*. 2006). The genus is thought to have evolved in ancient seaside habitats and most likely speciated rapidly through the Quaternary. Past climate oscillations and the uplift of the Himalayas are believed to have driven genetic diversification, migration and local adaptation of *Myricaria* species (Zhang & Zhang 1984; Wang *et al*. 2006).

Understanding the phylogeographical history of *Myricaria* will provide insights into the evolutionary responses of plants to past geological and/or climatic changes in western China. In particular, *M. laxiflora* is notable for being the first plant to become extinct in the wild due to the Three Gorges Dam construction (Wang *et al*. 2003; Liu *et al*. 2006a). Besides a great concern for the conservation and restoration of this species, its unique geographical distribution has evoked enthusiastic interest in the Chinese conservation community to better understand its origin and historical demographic changes (Wang *et al*. 2003; Wu *et al*. 2003; Liu *et al*. 2006a). Wang *et al*. (2003) have proposed that the most recent ancestors of *M. laxiflora* were derived by gene flow from ancestral species that originated in the QTP region, during the Quaternary glacial period when its ancestor migrated eastward along the Yangtze River valley with the rapid uplift of the QTP, and survived in the palaeo-Chuan Jiang River (the upper Yangtze River) within the TGMR region (gene flow hypothesis). This hypothesis is based on the presence of the morphologically similar species *M. paniculata* in the upper Yangtze River and is thought to have contributed to the origin and evolution of *M. laxiflora*. Alternatively, Wu *et al*. (2003) have suggested that *M. laxiflora* is phylogenetically more closely related to the European species *M. germanica*, and that is more likely that one of the ancestral lineages of the *Myricaria* genus originated during the early extension of the North Pacific plate and further dispersed into the Palaeo-Mediterranean sea region, contributing to the initial development of *M. germanica* (ancient origin hypothesis). Detailed information on the phylogeographical pattern of *M. laxiflora* is needed to test these different hypotheses and to uncover its evolutionary history.

Chloroplast DNA (cpDNA) is maternally inherited in most of flowering plants and is commonly considered a single, nonrecombining unit of inheritance (Clegg *et al*. 1994). Genetic variation in the chloroplast genome is often geographically structured and it is a useful molecule for studying recent or historical isolation and dispersal in plants (Petit *et al*. 1997), although phylogeographical inferences based solely on organellar genes may not necessarily be representative of the overall genetic aspects of the organisms themselves. In this study, we survey cpDNA variation in *Myricaria* across the mountain ranges in western China to determine species-level phylogeographical patterns. Specifically, we address the following questions.

(i) What are impacts of the past geological and climatic oscillations on the evolution and dispersal of these important montane riverine plants? (ii) Which of the hypotheses discussed above is most likely to account for the origin and demographic expansion of *M. laxiflora* along the Yangtze River in the TGMR? As a model, species-level phylogeographical studies of *Myricaria* in western China will facilitate understanding the general patterns of ecogeographical effects across large mountain ranges in the recent past.

Materials and methods

Taxon and population sampling

A total of 311 samples were analysed in this study representing all 10 species and one variety of the genus *Myricaria* in China, two samples of European *M. germanica* and three outgroup samples of *Tamarix albiflonum* (Table S1, Supporting information). We sampled sites covering the complete range of *Myricaria* in western China, but to account for the origin and evolution of *M. laxiflora*, emphasis was placed on sampling from the TGMR and its adjacent montane stream areas in Sichuan and Gansu provinces. Hence, three widespread species *M. bracteata*, *M. paniculata* and *M. squamosa*, which are geographically and morphologically closely related to *M. laxiflora*, have been sampled more widely than others to test possible hypothesis of the origin of *M. laxiflora*. Species were identified using the key of Zhang & Zhang (1984). The latitude, longitude and altitude of each sampling site were recorded using a global positioning system (GPS). Leaves were sampled randomly from trees and quick-dried with silica-gel and stored frozen until DNA extraction. Voucher specimens from each sampling site have been deposited in the Herbarium of the Wuhan Botanical Garden.

Laboratory procedures

Total genomic DNA was extracted following a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987). A preliminary screen for variation in cpDNA used five available universal primer pairs [*trn*S–*trn*G by Hamilton (1999); *psb*A–*trn*H by Sang *et al*. (1997); *trn*L–*trn*F and *trn*T–*trn*F by Taberlet *et al*. (1991) and *rpL*16 intron by Small *et al*. (1998)] and 10 samples from 10 different *Myricaria* taxa in China. Two regions, *psb*A–*trn*H and *rpL*16 intron, were found to be the most variable. Thus, large-scale screening of haplotype variation was then performed on all individuals and populations. Polymerase chain reaction (PCR) amplifications were performed in 25 μL reactions consisting of 1 U *Taq* polymerase (MBI Fermentas), 1× *Taq* Buffer, 0.5 mM dNTPs, 1.5 mM $MgCl_2$, 1 μM of each primer, and 1 μL of template genomic DNA (10–100 ng). The PCR temperature profile for the *psb*A–*trn*H spacer was 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 53 °C for 2 min and 72 °C for 2 min, and finally 72 °C for 7 min, and for *rpL*16 intron was 95 °C for 5 min, followed by 25 cycles of 95 °C for 1 min, annealing temperature (50–65 °C, +0.6 °C/cycle) for 1 min and 65 °C for 4 min, and finally 65 °C for 7 min. Amplification products were purified using an ExoSAP-IT PCR Purification Kit (USB Corp.) and sequenced by a commercial laboratory (Beijing Sunbiotech Co., Ltd).

Sequence analyses

The sequences of the two regions were combined and aligned using Clustal_ X (Thompson *et al*. 1997) and then double-checked manually. Recent studies have demonstrated that length variation in DNA sequences might comprise a substantial part of the potentially useful phylogenetic information in sequence-based data sets (Simmons & Ochoterena 2000). The sequences from *Myricaria* included microsatellites, mononucleotide repeats and hypervariable indels (Table S2, Supporting information). In order to use all of this information for phylogeographical analyses, a recent method called modified complex indel coding (MCIC) (Müller 2006) was implemented in the program SeqState (Müller 2005) to code the character states of different length variants in our cpDNA sequence data. cpDNA haplotypes were thus identified from both base-pair substitutions and length variants coded as multistate characters. Sequence divergences were estimated using the Kimura 2-parameter algorithm in PAUP* 4.0B10 (Swofford 2002).

Phylogenetic analyses and estimate of divergence time

Maximum-parsimony (MP) analyses were carried out using PAUP*4.0B10 to detect the phylogenetic relationship of all cpDNA haplotypes. This allows several options to deal with gaps, such as coding gaps as multistate characters in MCIC. A heuristic search was performed for optimal MP trees with 1000 random addition sequence replicates and starting trees obtained via stepwise addition. Settings for MP analysis included tree-bisection–reconnection (TBR) swapping and steepest descent off with Multrees option on (Swofford 2002). Node confidence was assessed by 1000 bootstrap replicates with an initial random addition of taxa. A maximum-likelihood (ML) analysis treating gaps as missing data was used to assess the reliability of the MP tree (which was based on the data set coded by MCIC). The best-fit substitution model employed was the IVM + I + G (AIC: 4106. 1973) for the ML analysis as determined by ModelTest 3.7 (Posada & Crandall 1998).

Approximate times of lineage divergence were estimated using a Bayesian relaxed clock method, implemented in PAML (Yang 1997) and MultiDistribute (Thorne & Kishino

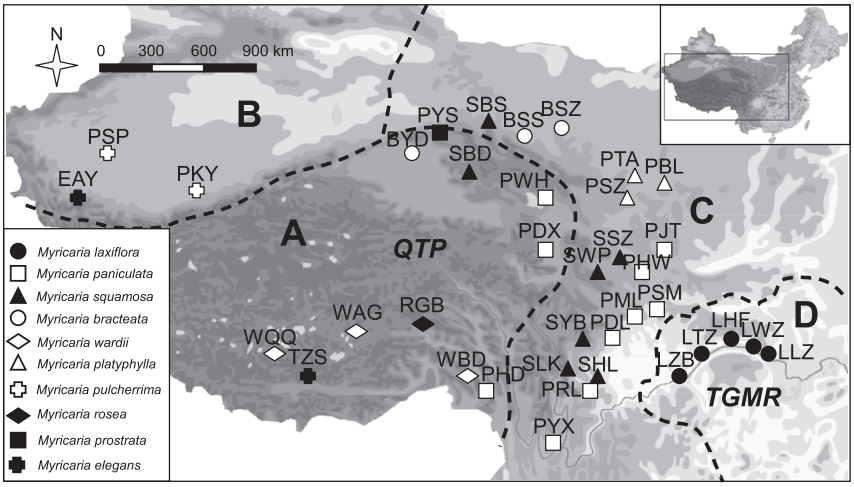


Fig. 1 Mountain ranges and sampling localities of *Myricaria* species in western China. Location details are given in Table S1 and four potential geographical regions (A–D) in western China were used for DIVA analyses.

2002). Although no fossil records of *Myricaria* are available, the upper age for the root node of this genus can be deduced from its sister genus *Tamarix*. The earliest known fossils identified as *Tamarix* sp. were from Late Oligocene deposits of the Tertiary. *Tamarix* was possibly present during the Eocene period of the Early Tertiary (Zhang *et al*. 2003). Since *Myricaria* and *Tamarix* are closely related, but *Myricaria* is found over a smaller area, *Myricaria* may have evolved more recently. Thus, the upper boundary of divergence of *Myricaria* can be constrained at the Eocene/Palaeocene boundary at about 57 million years ago (Ma; the earliest likely occurrence of *Tamarix*). One old spore-pollen record of *Myricaria* sp. (~0.15 Ma) in Xinjiang region (Zhao *et al*. 1993) was used to specify the lower age boundary of the evolutionary haplotypes found in this region. Each analysis consisted of 1×10^6 generations with sampling of Markov chains at intervals of 100 generations (100 000 generations discarded as burn-in).

Phylogeographical analyses

A haplotype network was constructed using statistical parsimony as implemented in TCS version1.18 (Clement *et al*. 2000). This parsimony network represents genealogical relationships among haplotypes even in cases of shallow genetic divergence which tree-building methods (e.g. MP and ML methods) do not always detect. We followed a two-step strategy which was developed by Bänfer *et al*. (2006) to construct the network by which the presumably different mutation rates underlying base substitutions, indels and microsatellites can be evaluated and used. The first step was to exclude all five microsatellite and mononucleotide repeat loci and one hypervariable indel region and then construct a backbone network of haplotypes. In the second step, additional haplotypes defined by the above excluded length variants were added manually at appropriate positions on the backbone. Mutational steps between each pair of haplotypes in the network were finally determined based upon the MCIC-coded data set. Criteria from several empirical predictions derived from coalescent theory have been applied to decide alternative solutions of the loops (Crandall & Templeton 1993; Pfenninger & Posada 2002).

The possible ancestral biogeographical scenarios in the phylogeny of *Myricaria* haplotypes were tested by a dispersal and vicariance (DIVA) analysis using the program DIVA version 1.1 (Ronquist 1997). Distribution data of *Myricaria* species in China was gathered from documented collections (specimens from several Chinese herbaria), reports (e.g. Zhang & Zhang 1984) and a recent field survey (Wang *et al*. 2006). Increasing the number of regions considered may lead to a greater chance of inconclusive or nonexclusive hypotheses of ancestral areas for the phylogenetic lineages of interest (Noonan & Gaucher 2006). We thus chose to be conservative in our definition of regions and designated five unit areas based on geographical contiguities and similar altitudes: the QTP region (A, including Tibet, Qinghai and Yunnan provinces), the Xinjiang region (B), the secondary region (C, including the regions around the QTP extending to the northeast such as Sichuan, Gansu, Ningxia, and others provinces), the TGMR region (D, including Chongqing and Hubei provinces) and the central Asia and Europe regions (E) (Fig. 1). As DIVA analysis requires a simple, bifurcating tree, one representative individual/haplotype for each paraphyletic group was used to construct a parsimony tree (Fig. 2). Outgroup individuals were excluded in the DIVA analysis.

Population genetic parameters including nucleotide diversity (π), haplotypic diversity (h) and mean number of haplotypes per population for each taxon (N_p) were estimated using the program Arlequin 2.0 (Schneider *et al*. 2000). Genetic variation in different taxa and geographical regions (A–E regions used in the DIVA analysis) was also partitioned by analysis of molecular variance (AMOVA),

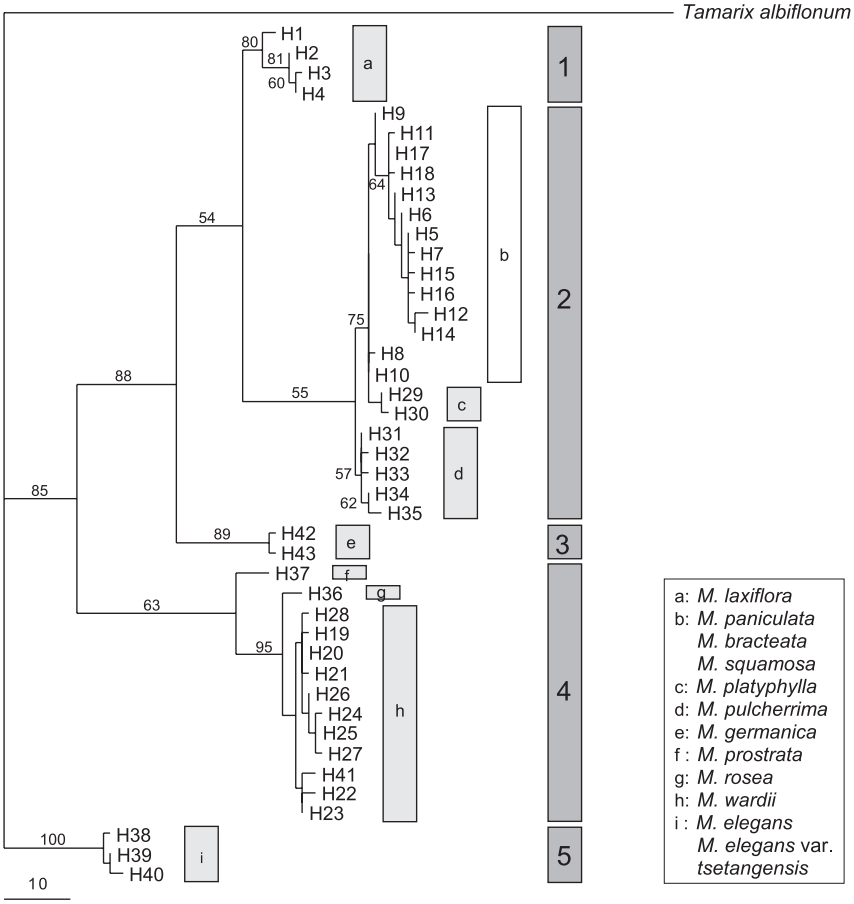


Fig. 2 Phylogram resulting from maximum-parsimony phylogenetic analysis for *Myricaria* based on the combined data set. Numbers on branches indicate bootstrap values (% > 50). Clades 1–5 denote five main genetic clades based on all identified haplotypes. The haplotype numbers refer to those in Table S1.

respectively. Phylogeographical signals in the defined regions were inferred by testing $N_{ST} > G_{ST}$ with 1000 replicates using Permut 2.0 (Pons & Petit 1996; Burban *et al*. 1999). The presence of a phylogeographical structure is indicated by N_{ST} being higher than G_{ST} (Pons & Petit 1996). A pattern of isolation by distance was tested in each region by a Mantel test with 10 000 permutations using GenePop 4.0 (Rousset 2008). The potential geographical zones associated with genetic discontinuities across the total mountain region were further investigated by the maximum difference Monmonier's algorithm of Manni *et al*. (2004) implemented in the program Barrier 2.2.

Historical demographic expansions were investigated by the *D* test of Tajima (1989) and F_S test of Fu (1997). Significant *D* values can be due to factors such as population expansion, bottlenecks and selection (Tajima 1989), and large negative F_S values generally suggest recent demographic expansion (Fu 1997). A mismatch analysis (Schneider & Excoffier 1999) was also performed to compare the demographic histories of four main clades (1, 2, 4, 5) in China according to the phylogenetic analysis. Various expansion parameters were estimated and the moment estimator of time to expansion (τ) was used in the equation $\tau = 2\mu t$ (where μ is the mutation rate for the whole sequence and t is the coalescent time in generations, this corresponds to the time of the last common ancestor) to infer a time scale for the demographic expansion. All these demographic tests were performed using the program Arlequin 2.0.

Results

Sequence variation

Sequence analyses of *Myricaria* resulted in an aligned fragment length of 351 base pairs (bp) of *psb*A-*trn*H and 516 bp of the partial *rpL*16 intron sequence (3′ end). A total of 12 aligned regions consisting of 29 different length variants (1–90 bp) was found, including six positions of simple indels (1–15 bp), one position of hypervariable indels and five positions of microsatellites (AT repeats) and mononucleotide repeats (A/T repeats) (Table S2). Combining all base substitutions and length variation character states, a total of 43 haplotypes were identified from all *Myricaria* plants analysed (Tables S1 and S2). All defined haplotype sequences have been deposited in GenBank databases

Table 1 Genetic diversity indices, phylogeographical signal test ($N_{ST} > G_{ST}$) and isolation by distance (IBD) effects of *Myricaria* plants within and across five defined geographical regions

Region	No. of populations	No. of plants	HT	π (SD)	*h* (SD)	Phylogeographical signal	IBD
A	11	93	20	0.0092 ± 0.0048	0.833 ± 0.034	0.666, *	*b* = 0.00034, *
B	3	22	7	0.0110 ± 0.0059	0.825 ± 0.041	0.513, NS	*b* = –0.00006, NS
C	18	142	11	0.0014 ± 0.0010	0.506 ± 0.048	0.845, *	*b* = 0.00036, *
D	5	49	4	0.0006 ± 0.0006	0.664 ± 0.039	0.419, NS	*b* = –0.00042, NS
E	2	2	2	0.0013 ± 0.0018	1.000 ± 0.500	—	—
Total	39	308	43	0.0076 ± 0.0041	0.802 ± 0.022	0.729, *	*b* = 0.00024, *

HT, number of haplotypes; significant level *$P < 0.05$; NS, not significant.

under Accession nos EF394254–EF394297 and EU914131–EU914132. Sequence divergence of haplotypes ranged from 0 to 6.68% among *Myricaria* species and from 0 to 17.83% when outgroups were included.

Phylogenetic relationships and divergence time

As essentially identical topologies were yielded from both the MP and ML methods, only the MP tree is presented. Parsimony analysis resulted in 288 480 equally parsimonious trees, 228 steps in length with CI = 0.854 and RI = 0.909. A maximum-parsimony phylogram showing the most basal divergence of all haplotypes is presented in Fig. 2. The phylogenetic relationships of ingroup haplotypes of *Myricaria* were well resolved except for three widespread species *M. bracteata*, *M. paniculata* and *M. squamosa* (Fig. 2). Five main haplotype clades can be recognized on the tree. Clade 1 comprised all haplotypes of *M. laxiflora* which is restricted to the TGMR area and separated from other *Myricaria* species. Clade 2 contained the haplotypes of three widespread species, *M. bracteata*, *M. paniculata* and *M. squamosa* and two local species, *M. pulcherrima* and *M. platyphylla*. These species occur around the QTP extending northward and eastward. Clade 3 included two haplotypes of European *M. germanica*. Clade 4 included the haplotypes of *M. wardii*, *M. prostrata* and *M. rosea* which are mostly distributed within the QTP. Clade 5 was the most basal group and consisted of *M. elegans* and its variety *M. elegans* var. *tsetangensis*, both of which are distributed in the QTP and the adjacent Xinjiang region. These main haplotype clades were supported by high bootstrap values ranging from 54 to 100% (Fig. 2).

Estimates of divergence times based on the MP tree suggest that the separation of clade 5 from all other clades occurred approximately 2.30 ± 1.39 Ma. Subsequently, clade 4 branched from clades 1, 2 and 3 approximately 1.99 ± 1.17 Ma, while clade 3 branched from clades 1 and 2 approximately 1.67 ± 0.98 Ma, and clade 1 separated from clade 2 approximately 1.46 ± 0.87 Ma. All these ancestral divergence events occurred between the Late Tertiary and the Early Pleistocene, which is in accordance with the period of rapid uplift of the QTP region.

Phylogeographical structure

Using the 95% criterion suggested for the statistical parsimony method implemented by TCS, a backbone network was initially generated based on 30 backbone haplotypes. The haplotypes resulting from sequence length variation were then added to this backbone network based on their phylogenetic and geographical relationships. The whole network obtained by this procedure is well resolved (Fig. 3). Haplotype arrangements in the network are generally consistent with the species' geographical distributions. In particular, 30 backbone haplotypes revealed remarkable phylogeographical patterns (Fig. 3 and Table S1). All Chinese endemic species showed unique haplotypes while most populations of three widespread species *M. paniculata*, *M. bracteata* and *M. squamosa* shared a common haplotype H5. Considering the five defined geographical regions, nearly half of the haplotypes (20) occur in region A (46.51%), while the other four regions shared the remaining haplotypes (Table 1). The number of haplotypes in region C (11) is much lower than that in region A despite its larger area and the large number of populations sampled.

The dispersal–vicariance analysis reconstructed the ancestral distributions of genetic lineages as shown in Fig. 4. Four main vicariance events and nine dispersal events were detected, revealing a general biogeographical history of *Myricaria* plants analysed. It seems that after the primary radiation of the ancestral *Myricaria* lineages in the region A of the QTP, four old vicariance events gave rise to important genetic divergence of *Myricaria* plants between regions A and BCDE, E and BCD, D and BC, B and C. Some locally endemic species in the genus, such as *M. laxiflora* and *M. pulcherrima*, are likely to have undergone speciation in response to vicariance. In contrast, dispersal events could have occurred more frequently in clade 2 of the phylogenetic

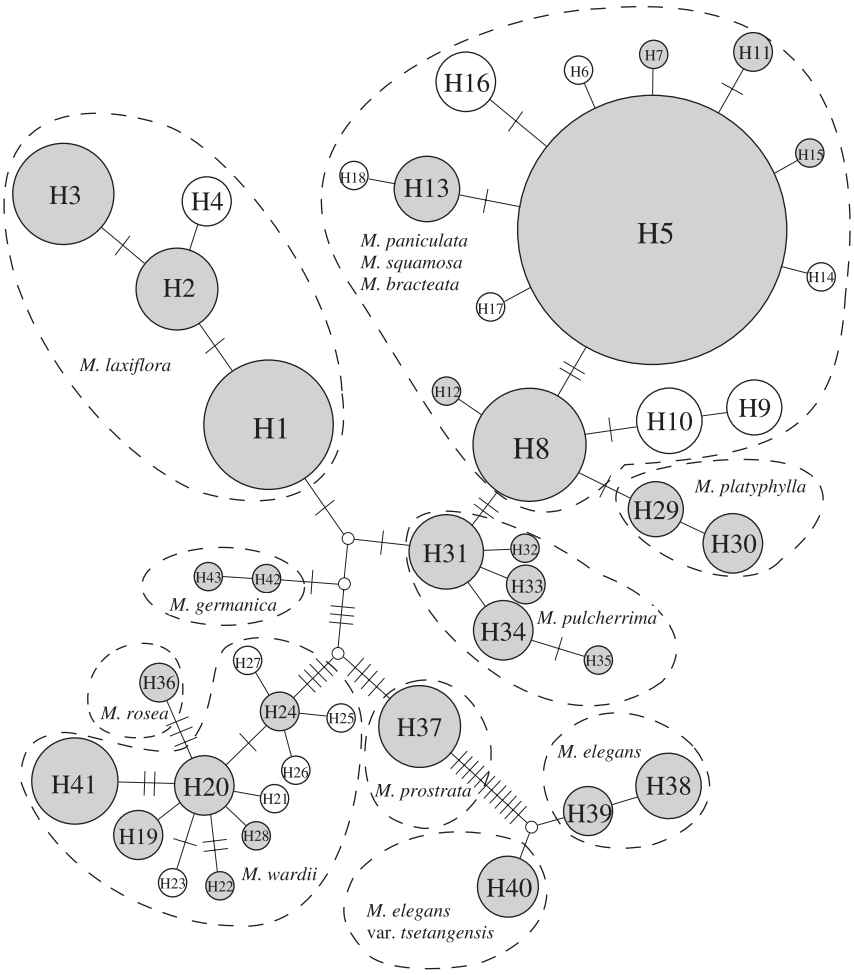


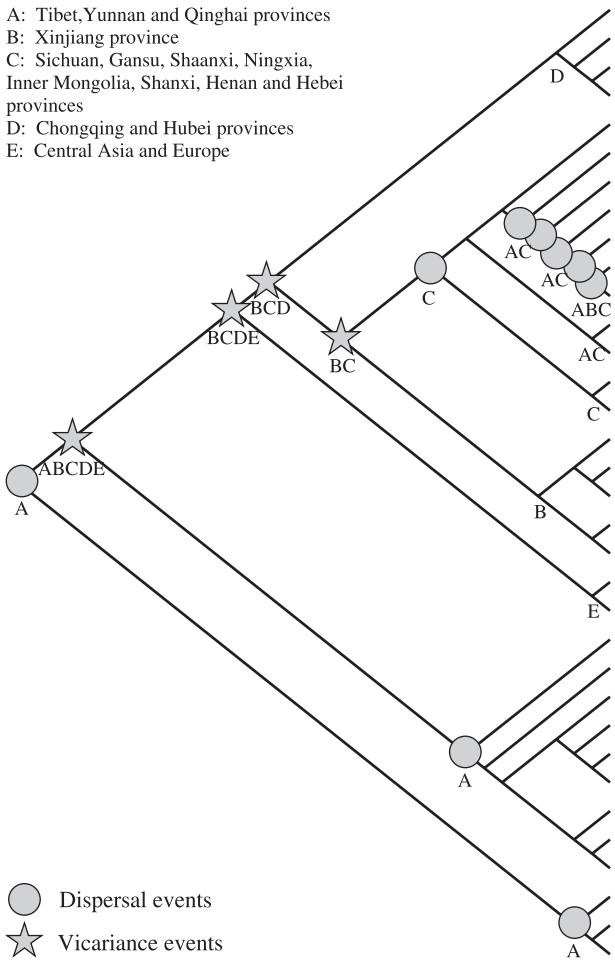
Fig. 3 Statistical parsimony network of 43 chloroplast haplotypes of *Myricaria* plants sampled in western China. Each numbered circle (H1–H43) represents a unique haplotype, with circle size reflecting the haplotype frequencies. Solid circles indicate backbone haplotypes and open numbered circles show haplotypes based on five microsatellite and mononucleotide repeat loci and one hypervariable indel region. Lines between haplotype circles indicate single mutational steps, while four small open circles indicate unsampled or extinct haplotypes. Haplotypes attributed to the same species or species group are circled by dashed lines.

tree, suggesting recent extensive range expansions of the three widespread species *M. bracteata*, *M. paniculata* and *M. squamosa* in China. Based on DIVA analysis, the optimal ancestral areas correspond to region A of the QTP region. Taking into account the geographical distributions of genetic lineages related to dispersal events, the general routes of dispersal of *Myricaria* species in China can be seen as an ancient ancestral origin from the centre of the QTP extending to outlier regions both northward and eastward.

In the five defined geographical regions, the genetic diversity in regions A and B was much higher than in the others (Table 1). At the species level, the highest mean number of haplotypes per population (N_p) was found in *M. wardii* (3.67, Table S1) which is endemic to the QTP region, despite only three populations being sampled in contrast to a total of 20 populations sampled for the three widespread species. The results of the AMOVA test are presented in Table 2. Of the total genetic variation partitioned by

Table 2 Analysis of molecular variance of *Myricaria* populations, partitioned by species and region

Partitioning	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P* value
By species	Among species	11	773.346	2.987	92.00	< 0.001
	Among populations	27	43.659	0.189	5.82	< 0.001
	Within populations	268	18.995	0.071	2.18	< 0.001
	Total	306	836.000	3.247		
By region	Among regions	4	346.873	1.555	46.33	< 0.001
	Among populations	34	470.132	1.730	51.56	< 0.001
	Within populations	268	18.995	0.071	2.11	< 0.001
	Total	306	836.000	3.356		



H1	D
H2	D
H3	D
H4	D
H9	AC
H11,H17,H18	ABCD
H13	ABCD
H6	AC
H5,H7,H15,H16	ABCD
H12	ABCD
H14	ABC
H8	AC
H10	AC
H29	C
H30	C
H31	B
H32	B
H33	B
H34	B
H35	B
H42	E
H43	E
H37	AC
H36	A
H28	A
H19,H20,H21	A
H24,H25,H27	A
H26	A
H41	A
H22	A
H23	A
H38	AB
H39	AB
H40	A

Fig. 4 Results of *Myricaria* dispersal–vicariance analyses. Haplotype numbers refer to those in Table S1. Letters below nodes indicate the inferred ancestral areas of corresponding clades.

species, 92% was attributed to the differences among species, 6% to the differences between populations, and 2% to the difference between individuals within populations. Similarly, significant genetic variation between geographical regions (46%) was detected. Phylogeographical signals ($N_{ST} > G_{ST}$) from regions A, C and the all regions combined were significant. In addition, there was evidence for weak but significant isolation-by-distance effects in these regions (Table 1). The zones associated with abrupt genetic differentiations are presented in Fig. 5. These zones are mostly independent across the mountain regions in western China, reflecting significant genetic/geographical isolations and allopatric fragmentation. For example, the genetic composition of *M. laxiflora* in TGMA is obviously different from that of others (indicated by line d in Fig. 5).

The results of demographic expansion histories tested by different analyses are shown in Table 3. Although no significant D value was detected, the large negative F_S value of clade 2 suggests a historical demographic expansion. This is consistent with the results obtained from the DIVA analysis, suggesting that most dispersal events occurred in clade 2. Estimates of time to expansion (τ) showed a more recent demographic expansion of clade 1 ($\tau = 3.168$), suggesting a distinct pattern of recent dispersal of *M. laxiflora* restricted to the TGMR along the Yangtze River Valley.

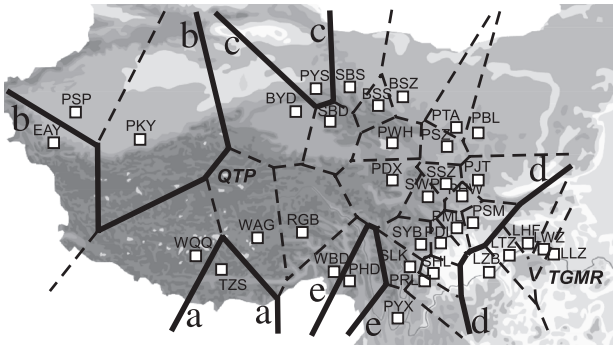


Fig. 5 Zones of genetic discontinuities as identified by Barrier 2.2. Barriers that were retained under the majority-rule criterion are identified by order of importance (a, b, c, d and e).

Table 3 Results of Tajima's *D* and Fu's F_S tests, and mismatch analyses for *Myricaria* clades

	Tajima's *D* test		Fu's F_S test		Mismatch distribution		
Groups	*D*	*P*	F_S	*P*	τ	θ_0	θ_1
Clade 1	1.712	0.975	–0.806	0.299	3.168	0.000	2.585
Clade 2	–0.719	0.258	–13.824*	0.000	6.515	0.000	1.728
Clade 3	0.000	1.000	0.000	0.243	—	—	—
Clade 4	1.052	0.876	–1.828	0.257	6.848	14.627	14.630
Clade 5	1.860	0.982	1.821	0.857	4.035	0.000	4.366

Discussion

Sequence heterogeneity

The variation in cpDNA sequences detected in this study includes base substitutions, hypervariable indels, microsatellites and mononucleotide repeats (Table S2). This variation, if neutral, is driven by different mutation rates and as a consequence could potentially be useful for inferring events that occurred at different time periods. Employing MCIC coding of length variants as multistate characters might affect the phylogenetic analysis as length variants and base substitutions are effectively treated as being equivalent (Müller 2006). However, the two-step procedure of haplotype network construction used should effectively take into account the different mutation rates of these different sequence variants (Bänfer *et al*. 2006), and in the current study, we detected a strong correspondence between genealogy and geography. The structure of the two-step network is strongly congruent with the tree topology from MP analysis (Figs 2 and 3), indicating that the data set coded by MCIC was appropriate for phylogenetic analysis. Furthermore, in comparison with the ML analysis (not shown), the MP tree provided higher resolution particularly in the relationships of tip clades on the tree topology (Fig. 2).

Genetic structure and species divergence

The results obtained clearly show significant genetic divergence and highly structured phylogeny and phylogeography of *Myricaria* populations and species (Figs 2 and 3; Table 2). Assuming maternal inheritance of cpDNA, a highly divergent genetic structure might be expected due to limited seed dispersal, as has been found in other taxa (Petit *et al*. 2003). Species of *Myricaria* are mostly riverine plants whose demography is largely dependant upon long-distance dispersal events driven by hydrochoric forces, especially by seed flow. This has been inferred from a recent amplified fragment length polymorphism analysis of *M. laxiflora* populations, which demonstrated high interpopulation genetic differentiation (F_{ST} = 0.463) attributed to limited unidirectional gene flow following watercourses (Liu *et al*. 2006a). Thus, significant phylogeographical structure is expected if associations between species richness and population differentiation are present in *Myricaria* due to the influence of strong population structure on speciation (Hughes & Hollingsworth 2008). Another explanation, probably of most importance, is the recent and extreme orogenesis movements in and around the QTP region since the Late Pliocene (Zhang & Zhang 1984). Geographical isolation and subsequent genetic drift, together with adaptation to local environments has probably led to strong genetic divergence and, hence, further speciation of *Myricaria* in China (discussed in the next section).

Myricaria wardii has the highest mean number of haplotypes per population (N_P: 3.67, Table S1). This may reflect its relatively ancient origin and long-term evolutionary history, as revealed by its basal position in the phylogeny (Fig. 2). On the other hand, unlike the clearly species-specific haplotypes of most locally endemic species in the phylogenetic tree and parsimony network, the three widespread species *M. bracteata*, *M. paniculata* and *M. squamosa* have one shared haplotype H5 and a polyphyletic mixed clade relationship with the remaining haplotypes (Fig. 2). This scenario is possibly best explained by either retained ancestral polymorphism or reticulate hybridization. The spatial distributions of the three widespread species reveal overlapping areas (Fig. 1), which could provide opportunities for hybridization. Furthermore, the close phylogenetic relationships (Fig. 2) of the three species suggest that possible genetic admixture or introgression of different evolutional lineages could have occurred upon secondary contact. However, it is not possible to establish the relative importance of hybridization and ancestral polymorphism using the data from the current study.

Phylogeography from the perspective of geological and glacial events

Based on the MP tree and haplotype network, the general structure of the phylogeny showed relatively basal and old clade positions of haplotypes representing *Myricaria* species that occur naturally within the QTP areas compared

to those in the regions around the QTP, the TGMA and Europe. Geographical structuring of clades (Figs 2 and 3) and the regional genetic diversity (Table 1) provided strong support for the previously suggested centre of origin of *Myricaria* in the Himalaya region based on species richness (Zhang & Zhang 1984). Our estimated divergence time suggests that the genetic divergence between the five main clades in the MP tree occurred about 1.46–2.30 Ma. Although caution is necessary because of the poor palaeogeographical data and fossil records, the estimated timescale for the inferred historical events fits well with known geological and climate scenarios for this geographical area. The latest and most significant uplift of QTP started from the Late Pliocene and Early Pleistocene, and extended to about 1.10–0.60 Ma (Harrison *et al*. 1992; Shi *et al*. 1998). In addition, the plateau has undergone about four or five glaciations starting from about 4.43–1.21 Ma (Zheng & Rutter 1998; Shi 2002). The largest glacier on the plateau was present during the Middle Pleistocene (~0.5 Ma) and continued until 0.17 Ma after the penultimate glaciation (0.3–0.13 Ma) (Zheng *et al*. 1998; Zhang *et al*. 2000; Shi 2002). The consistency between the estimated timeframe of genetic divergence and the geological and glacial events through the Quaternary suggest that mixed and complex 'mountain effects' forced rapid genetic divergence, local adaptation, speciation and dispersal of *Myricaria* plants across the mountain ranges of western China. This is supported by our analysis of phylogeographical signals (Table 1), genetic/geographical barriers (Fig. 5) in different regions, and by the vicariance and dispersal events identified by DIVA (Fig. 4).

In a recent study investigating the phylogeographical structure of species responding to geological events and climatic changes in southwestern USA mountain areas, Haenel (2007) suggested three main hypotheses: the geology hypothesis, the refugia hypothesis, and the fragmentation hypothesis. The general phylogeographical patterns of *Myricaria* in the mountainous regions of western China predominantly support the geology hypothesis. The results from the cpDNA analysis showed that the haplotypes were highly structured and clear species and geographical clustering patterns are indicated by the haplotype network (Fig. 3) and AMOVA test (Table 2). However, fragmentation effects cannot be ignored because they could reinforce the effects of geological events. For instance, expansion of desertification in the QTP may have restricted *Myricaria* species to habitats with sufficient water availability. Alternatively, the refugia hypothesis could be appropriate for explaining the phylogeographical patterns in localized areas or regions lacking complex mountain systems. In the present study, the haplotypes of *M. bracteata*, *M. paniculata* and *M. squamosa* grouped together in clade 2 in the MP tree. Significant negative F_S value (Table 3), short genetic distances (Fig. 5) and fewer dispersal events (Fig. 4) indicated by DIVA analysis of this clade suggest recent demographic expansion of this clade. However, phylogenetic analysis indicated that the most widely distributed haplotype H5 was not the most ancestral haplotype. In contrast, three haplotypes: H8, H9 and H10, occurring in the south of our sampling regions between Yunnan and Sichuan provinces (Fig. 1), were the most basal ones. A scenario of postglacial expansion and evolution from southern refugia by these widespread species is a better explanation for their observed phylogeographical patterns. This coincides with the proposed postglacial shifting latitude effect as a consequence of Quaternary climatic oscillations (Taberlet *et al*. 1998).

The phylogeographical history of *Myricaria* species is partly congruent with that of other taxa, including plants (Liu *et al*. 2002, 2006b; Wang *et al*. 2005) and animals (Guo *et al*. 2005; Yang *et al*. 2006) that co-occur in the QTP and adjacent regions. A phylogenetic analysis of members of the *Ligularia-Cremanthodium-Parasenecio* complex conducted by Liu *et al*. (2006b) recently showed that significant differences in geology and ecology due to the recent uplifts of the QTP triggered rapid and continuous allopatric speciation in small and isolated populations which resulted in an explosive radiation of the complex. Yang *et al*. (2006) also concluded that the phylogeographical structure of ground tit (*Pseudopodoces humilis*) coincided with important climatic and palaeogeographical changes following the uplift of the QTP. Overall, the phylogeography of mountain species in western China is primarily shaped by geological effects as a consequence of the recent uplift of the QTP, which has driven speciation and diversification. Both vicariance and dispersal theories are necessary to explain current distribution patterns at different spatial and temporal scales, as observed in this study. There are different phylogenetic histories for endemic vs. widespread species (Fig. 2) suggesting possibly divergent biogeographical scenarios including *in situ* adaptation (e.g. *M. prostrata* and *M. rosea* are both endemic species with recumbent growth forms, adapted to long-term exposure to high altitudes) and adaptive dispersal accompanied by gene flow (e.g. dispersal events identified in *M. bracteata*, *M. paniculata* and *M. squamosa*, Fig. 4).

Origin and dispersal of M. laxiflora *in the TGMR*

Molecular dating of the cpDNA data estimated a time of origin of *M. laxiflora* at about 1.46 ± 0.87 Ma (Early Pleistocene). The phylogenetic position of *M. laxiflora* in the MP tree (Fig. 2) and haplotype network (Fig. 3) suggests an origin and biogeographical history that is independent from the morphologically and geographically related species *M. paniculata* and *M. bracteata*, as depicted by an abrupt genetic discontinuity between them in Fig. 5. Moreover, it has long been recognized that drainage patterns of the old Yangtze River were remarkably different from its current

patterns (Rüber *et al*. 2004). The old upper Yangtze River (the palaeo-Chuan Jiang River) could not flow through the TGMR region and instead ran westward into the South China Sea through the palaeo-Red River (Rüber *et al*. 2004). This geological scenario and the identified phylogenetic divergence (Fig. 2) does not provide support for the gene-flow hypothesis that the most recent common ancestor of *M. laxiflora* originated from eastward migration via mountain watercourse-mediated gene flow because it would be impossible under a westward flowing palaeo-Chuan Jiang River. A more likely scenario is that the ancestor of *M. laxiflora* stems from old *Myricaria* lineages which developed from the initial demographic expansion of *Myricaria* at the most eastern shore of the palaeo-Mediterranean sea. Despite seawater retreating westward from Sichuan and the TGMR region during the Early Tertiary (Sun & Li 2003), many remnant riverine habitats within the TGMR and the neighbouring regions were available to preserve those old lineages during the Quaternary. The ancient origin hypothesis by Wu *et al*. (2003) is therefore more plausibly supported by our analyses, but the estimated time of origin of this species is later than that previously suggested. Furthermore, the TGMR is a possible secondary evolution centre and/or a climate refuge contributing to the independent evolution of mountain species such as *M. laxiflora* during climate oscillations in the Quaternary.

The observed age expansion parameter value (τ) was 3.168 for clade 1 of *M. laxiflora* (Table 3). Based on the estimated mutational rates from Bayesian relaxed clock method, the time of expansion of clade 1 occurred at ~0.015 Ma, suggesting a recent demographical expansion of *M. laxiflora* populations. The currently limited geographical distribution of *M. laxiflora* along the Yangtze River Valley from Chongqing to Yichang may reflect a time association between its demographic expansion and the geological changes that cut through the TGV region allowing the present Yangtze River to flow eastwards. Although the timescale of the watercourse connection of the TGV is still under debate, a plausible opinion suggests it occurred about 0.20–0.07 Ma (Zhao 1998; Li 2003; Zhang 2006). Thus, our estimated time of demographic expansion for *M. laxiflora* is far later than the suggested timescale of the connection of the watercourse in TGV region. Although the phylogeographical patterns of the four identified haplotypes of *M. laxiflora* in the present study do not clearly correspond to basal-derived evolutionary relationship between the upstream and downstream populations, our recent genetic analysis with a higher resolution of population collections did suggest that unidirectional demographical expansion of *M. laxiflora* from the upper palaeo-Chuan Jiang River area occurred some time after the completion of the river channel through the TGV region and the Yangtze River began to flow eastwards (Liu *et al*. 2006a).

Conclusions

Phylogeographical analysis of cpDNA has revealed an overall correspondence between the current geographical distribution of *Myricaria* and the effects of geology and climate on its evolution in mountain ranges of western China. It is notable that the main genetic divergence of *Myricaria* species occurred between the Late Tertiary and Early Quaternary and was predominantly driven by the recent rapid uplifting of mountains in the QTP region. Local survival–expansion in response to alpine glaciers in the Quaternary has also significantly contributed to genetic isolation and endemic species formation over different regional scales. The independent origin of *M. laxiflora* in the TGMR region was highlighted in the present study, and it approached a conclusion that the unique phylogeographical history of a relatively ancient origin but recent demographical expansion of *M. laxiflora* was strongly driven by the geological events that changed the topography and watercourse flow in the TGV valleys and/or climatic effects within the TGMR areas. It is our hope that this study provides a useful reference to contribute to understanding the general biogeographical history for the evolution of species in this region. Principal geological effects generally lead to periodic modifications of major biota within and around the QTP, while climatic changes alter the ecological composition of regional or local landscapes concomitantly, which in turn, impose new selective pressures and/or geographical isolation leading to genetic diversification, adaptation and evolution of mountain species in western China.

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This paper represents a portion of Yifei Liu's PhD research; he is interested in the dynamics of gene flow and adaptive evolution in plant populations, and also natural hybridization and introgression that contribute to biodiversity and speciation in wild. Dr Yong Wang is a conservation researcher involved in collecting and preserving endangered plants in the Three Gorges Region. Professor Hongwen Huang researches population and conservation genetics.

Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Geographical origins, sampling locations, number of individuals sampled from each population (*N*), assigned chloroplast haplotypes (H) and the mean number of haplotypes per population for each species (*N*p) of *Myricaria* plants used in this study.

Table S2 Haplotypes identified in all *Myricaria* samples. Chloroplast DNA sequence included the *psb*A-*trn*H region (351 bp) and the partial *rpL*16 intron sequence (3′ end, 516 bp).

Table S3 Transformation costs (steps) between two compared character states of *Myricaria* haplotype sequences after modified complex indel coding (MCIC).

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