

Research paper

Evidence for two types of *Aquilegia ecalcarata* and its implications for adaptation to new environments[☆]Lei Huang¹, Fang-Dong Geng¹, Jing-Jing Fan, Wei Zhai, Cheng Xue, Xiao-Hui Zhang, Yi Ren^{**}, Ju-Qing Kang^{*}

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ABSTRACT

Spurs have played an important role in the radiation of the genus *Aquilegia*, but little is known about how the spurless state arose in *A. ecalcarata*. Here we aim to characterize the genetic divergence within *A. ecalcarata* and gain insights into the origin of this species. A total of 19 populations from *A. ecalcarata* and 23 populations from three of its closest relatives (*Aquilegia kansuensis*, *Aquilegia rockii* and *Aquilegia yabeana*) were sampled in this study. We sequenced fifteen nuclear gene fragments across the genome and three chloroplast loci to conduct phylogenetic, PCoA and STRUCTURE analyses.

Our analyses indicate that *A. ecalcarata* may not be monophyletic and can be divided into two distinct lineages (*A. ecalcarata* I and *A. ecalcarata* II). *A. ecalcarata* I is genetically close to *A. kansuensis*, whereas *A. ecalcarata* II is close to *A. rockii*. Isolation-with-migration analysis suggested that historical gene flow was low between *A. ecalcarata* I and *A. rockii*, as well as between *A. ecalcarata* II and *A. kansuensis*. The two distinct lineages of *A. ecalcarata* show significant divergence in 13 floral traits and also have distinct distributions. In addition, both *A. ecalcarata* I and II are adapted to a stony environment that differs from that of their closest relatives, indicating a habitat shift may have driven new adaptations. Our findings enrich the understanding of how floral evolution contributes to species diversification.

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1. Introduction

Individuals or populations with similar phenotypes are generally classified as one species, which is the traditional taxonomic approach (Duminil and Di Michele, 2009). Populations with similar phenotypes but multiple origins would pose a challenge to this definition of a species. Nevertheless, sharp changes in the plant environment that occur over short distances (e.g., changes in soil type, moisture and temperature) may cause individual species to adapt to environmental changes by adaptive genetic

divergence (Hoekstra and Coyne, 2007). Local adaptation across an environmental gradient, either with or without gene flow, may be the first step that ultimately leads to the origin of fully reproductively isolated forms (i.e., biological species concept) (Mayr, 1942; Abbott, 2017). Therefore, investigating how certain traits originate and evolve in response to environmental changes and whether adaptive genetic divergence is impaired by gene flow would provide new insights into the delimitation of species with complicated evolutionary histories.

Recently, *Aquilegia* L. (also known as columbine) has emerged as a model system for floral evolution due to its unusual floral morphology (Kramer, 2009; Kramer and Hodges, 2010). The genus *Aquilegia* represents a recent adaptive radiation, with a Eurasia origin followed by an expansion to North America over the last 1–3 million years (Fior et al., 2013). The radiation of *Aquilegia* species was driven by diversification in both pollinators and ecological habitats (Hodges and Arnold, 1995; Hodges, 1997; Hodges et al., 2003). Different lengths of nectar spur are associated with different pollinators in North America (Whittall and Hodges, 2007).

[☆] This paper is dedicated to the memory of Prof. Yi Ren (1959–2019) who passed away shortly before the submission of this manuscript. We will always remember him for his optimism, bravery and wisdom.

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Nevertheless, there is a spurless species, *Aquilegia ecalcarata* Maxim., that has a reduced nectar spur that makes it appear spurless. The extremely reduced spur (referred as the spurless status) and the loss of nectar in *A. ecalcarata* is unique in the genus and believed to be a derived character rather than ancestral state (Hodges and Arnold, 1995; Fior et al., 2013). *A. ecalcarata* together with *Aquilegia yabeana* Kitag., *Aquilegia oxysepala* Trautv. and C.A. Mey. var. *kansuensis* Brühl (treated as *Aquilegia kansuensis* by Erst et al., 2014) and *Aquilegia rockii* Munz. are clustered as a monophyletic clade (Fior et al., 2013). Interestingly, these four closely related species are all endemic to China and differ in spur length and degree of curvature (Xiao, 1979; Fu and Robinson, 2001). *A. rockii* has long but straight or slightly incurved spurs, while *A. yabeana* and *A. kansuensis* (= *A. oxysepala* var. *kansuensis*) both have long and hooked or coiled spurs. The petal blade is the same color as the sepals in *A. yabeana* and *A. rockii*, but different in *A. kansuensis* (Fig. S1). All three species have nectar tissue at the apex of their spurs. Given that *A. ecalcarata* harbors unique phenotypes in lacking both spurs and nectar tissue, we regard *A. ecalcarata* and its relatives (*A. yabeana*, *A. kansuensis* and *A. rockii*) as a good model for investigating floral evolution, species divergence and speciation at the infrageneric level. These four species also differ in habitat (Fig. S2). The different habitat makes the origin of *A. ecalcarata* more intriguing and may provide important information about how *A. ecalcarata* adapted to a new environment.

Although Fior et al. (2013) constructed the phylogeny of the four closely related species, only one individual for each species was sampled; thus, how the spurless status originated remains unknown. Our previous research indicated that *Aquilegia ecalcarata* shows significant genetic divergence among populations (Huang et al., 2018). Morphological studies have suggested that the floral traits (such as the length of sepals, spurs and carpels) differ significantly among 19 sampled populations of *A. ecalcarata* (Xue et al., 2020). Therefore, we propose that spur loss in *Aquilegia*, as well as in *A. ecalcarata*, had independent origins. However, researchers have shown that gene flow has been a common feature throughout *Aquilegia* (Filiault et al., 2018). Therefore, the possibility of hybridization between *A. ecalcarata* and its closest relatives may not be excluded. Further analyses, such as gene flow estimation, is required to further clarify how these evolutionary forces may have impacted the origin of *A. ecalcarata*. Moreover, continuous variations in both vegetative and floral traits have been observed in some populations among *A. yabeana*, *A. kansuensis* and *A. rockii* in the field. Whether introgression occurred in sympatric areas among the three species deserves more investigation. Here we use both nuclear and chloroplast gene fragments to determine whether hybridization affected the evolutionary origin of *A. ecalcarata*, and to characterize the genetic differentiation and gene flow among *A. ecalcarata* and its relatives. This study will enhance our understanding of how floral evolution, especially the diversity of nectar spurs, contributes to parallel speciation in closely related species.

2. Materials and methods

2.1. Taxon sampling and loci

In this study, we sampled a total of 634 individuals (Fig. 1 and Table 1). We sampled nineteen populations of *Aquilegia ecalcarata*, nine of *A. kansuensis*, six of *A. rockii* and eight of *A. yabeana*. Fifteen and five individuals were sampled from each population for nuclear gene fragment and chloroplast locus sequencing, respectively. For outgroups, we sampled four individuals each from *Aquilegia canadensis* L., *A. chrysantha* A. Gray, *A. atrovinosa* Popov ex Gamojun. and *A. oxysepala*.

We used three chloroplast loci (*rpl32-trnL*, *trnK-rps 16*, *rps16-trnQ*), following previous research (Fior et al., 2013). For nuclear gene fragments, we developed markers based on the genome of *Aquilegia coerulea* E. James in the Phytozome database (<https://phytozome.jgi.doe.gov/pz/portal>). In addition to six markers used in our previous work (Huang et al., 2018), we selected an additional nine gene fragments; these fifteen loci cover seven chromosomes. We screened genes of lengths from 2 kb to 5 kb that contained both exons and introns. Then we blasted the candidate genes against the database to ensure they only mapped to one genome position. Details are shown in Fig. S3 and Table S1. Total DNA was extracted from 1 g of silica gel dried leaf material using a CTAB protocol (Zhu et al., 2007).

2.2. Amplification, sequencing and sequence analysis

PCR amplification followed a protocol described in our previous study (Zhang and Ge, 2007). Sequencing was done on an ABI3730XL automatic sequencer (Applied Biosystems Corp.). If dual peaks were found, PCR fragments were cloned into pGEM T-easy vectors (Promega Corp.) with a Pharmacia purification kit (Amersham Pharmacia Biotech) and three cloned DNA fragments were sequenced for each individual. To correct for errors in cloned fragments, we identified individuals in the alignments that contained singletons and then re-sequenced at least four clones after a second round of PCR. All sequences have been deposited in GenBank, with the accession numbers KY582911–KY582935, MG710812–MG711310, MH238358–MH238411, MH720548–MH720938, MH766660–MH766870, and MH788640–MH788921. Sequences were assembled with ContigExpress (Informax Inc., North Bethesda, MD), aligned with Clustal X1.83 (Thompson et al., 1997) and further refined with BioEdit 7.0.9.0 (Hall, 1999).

2.3. Genetic diversity and neutrality test

For each locus, we calculated the number of segregating sites (S), number of haplotypes (h), pairwise differences π (Nei, 1987) and Watterson's θ (Watterson, 1975). We also estimated the minimum number of recombination events (R_m) with the four-gamete test (Hudson and Kaplan, 1985). Tajima's D (Tajima, 1989) and D^* and F^* (Fu and Li, 1993) were calculated to test the neutrality of each locus. We obtained the associated one-tailed test p -value for Tajima's D and Fu and Li's D^* and F^* by computing 1000 coalescent simulations, accounting for estimates of the recombination per gene. The multilocus HKA tests (Hudson et al., 1987) across loci were conducted at the species level using *Aquilegia canadensis*, *A. chrysantha*, *A. atrovinosa* and *A. oxysepala* as outgroups. All analyses were performed in DnaSP 5.1 (Librado and Rozas, 2009).

2.4. Phylogenetic analysis

The genealogical trees of all alleles were constructed by Mega 7 (Kumar et al., 2016), using the Neighbor-Joining (NJ) method with Kimura's 2-parameter distances (Kimura, 1980). Maximum Parsimony (MP) and Maximum Likelihood (ML) methods were also performed and a heuristic search with tree bisection–reconnection, ACCTRAN and 1000 random taxon-addition replicates was implemented in these analyses. The optimal model of sequence evolution for each data set was determined using Akaike's information criterion (AIC) implemented in MODEST 3.7 (Posada and Crandall, 2001). We conducted bootstrap analysis to assess the confidence of internal nodes with 1000 replicates. Bayesian posterior probability was calculated by MrBayes 3.2 (Huelsenbeck and Ronquist, 2001). The incongruence length difference (ILD) test in PAUP 4.0

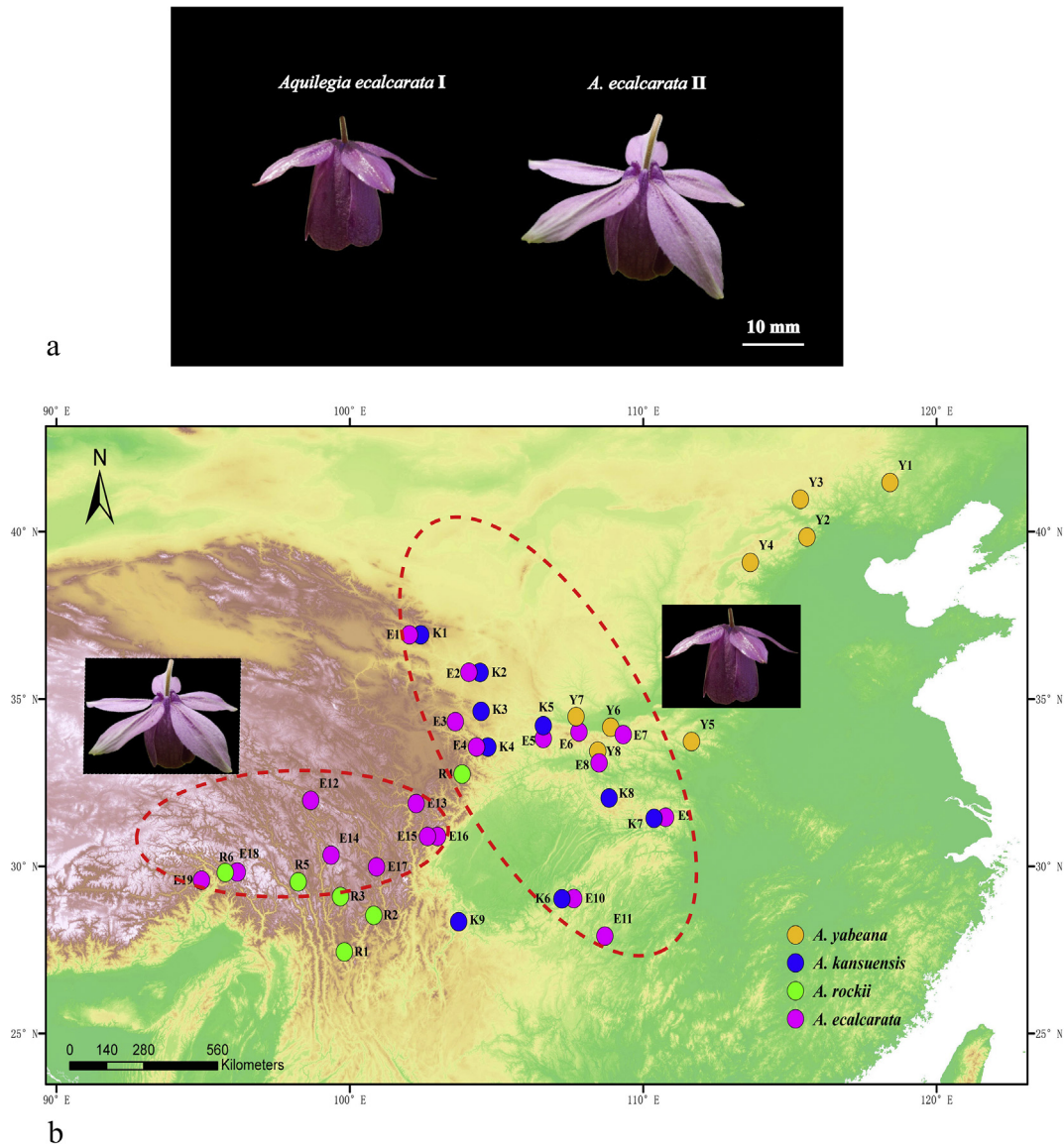


Fig. 1. The phenotypic comparison between *Aquilegia ecalcarata* I and II (a) and the geographical distribution of 42 sampled populations (b), with red dash circles for *A. ecalcarata* I and II.

was used to evaluate whether it is appropriate to combine chloroplast and nuclear data to construct phylogenetic trees (Farris et al., 1994).

2.5. Divergence time estimation

BEAST 2.3.2 was used to estimate the divergence time under a strict molecular clock model (Bouckaert et al., 2014). We set the nucleotide substitution model as GTR + I + G, and the chain-length of Markov chain Monte Carlo (MCMC) and sampling frequency to 2,000,000 and 2000, respectively. Tree prior was specified as a Yule process. Previous work indicated that *Aquilegia* diverged from other clades of Ranunculaceae 4.76 million years ago (Ma); the outgroups used in this study diverged from genus *Aquilegia* 3.88 Ma; and the four closely related species (*A. ecalcarata*, *A. kansuensis*, *A. rockii* and *A. yabeana*) diverged from the outgroups 2.79 Ma (Fior et al., 2013). These three time-scales were set as time constraints. The convergence was assessed by effective sample sizes (ESS) using Tracer v.1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>). Finally, the divergence times were estimated by TreeAnnotator v.1.6.1 with half of the trees

treated as burn-in and the divergence times visualized using Fig-Tree v.1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

2.6. Population genetic structure

The overall distribution of nucleotide diversity among populations was investigated using an Analysis of Molecular Variance (AMOVA) in Arlequin 3.01. Sequence variation was hierarchically partitioned between two species, between populations, within species, and within populations. The significance of all estimated fixation indices was tested using 10,000 permutations as described in Excoffier et al. (1992). Pairwise F_{ST} was used to measure population differentiation within and between species. We performed a Bayesian clustering analysis in STRUCTURE 2.2.3 (Pritchard et al., 2000) and principal coordinate analysis (PCoA) using DARwin (Perrier and Jacquemond-Collet, 2006). For the former analysis, the program was run with SNP markers for K-values from 2 to 10, with 50,000 burn-in iterations followed by 300,000 MCMC iterations for accurate parameter estimates. To verify the consistency of the results, we performed 10 independent runs for each k using an

Table 1
Geographic information of 42 populations used in this study.

Taxon	Population name	Sample size	Locality	Latitude (N)	Longitude (E)	Altitude (m)
<i>Aquilegia yabeana</i>	Y1	15	Ningcheng, Inner Mongolia	41°27'	118°24'	1128
	Y2	15	Mentougou, Beijing	39°55'	115°26'	1550
	Y3	15	Chongli, Hebei	40°58'	115°21'	1720
	Y4	15	Fanshi, Shanxi	39°05'	113°38'	2125
	Y5	15	Luanchuan, Henan	33°43'	111°38'	1789
	Y6	15	Huxian, Shaanxi	34°09'	108°53'	1800
	Y7	15	Meixian, Shaanxi	34°05'	107°42'	1182
	Y8	15	Ningshan, Shaanxi	33°26'	108°26'	1582
<i>A. kansuensis</i>	K1	15	Huzhu, Qinghai	36°55'	102°24'	2517
	K2	15	Yuzhong, Gansu	35°47'	104°03'	2561
	K3	15	Zhangxian, Gansu	34°38'	104°28'	2451
	K4	15	Zhouqu, Gansu	33°33'	104°19'	2705
	K5	15	Fengxian, Shaanxi	34°11'	106°35'	1578
	K6	15	Nanchuan, Chongqing	29°01'	107°13'	1953
	K7	15	Xingshan, Hubei	31°26'	110°21'	1608
	K8	15	Chengkou, Chongqing	32°02'	108°50'	2245
	K9	15	Leibo, Sichuan	28°20'	103°42'	1822
<i>A. rockii</i>	R1	15	Shangarila, Yunan	27°26'	099°48'	2934
	R2	15	Muli, Sichuan	28°31'	100°48'	3263
	R3	15	Xiangcheng, Sichuan	29°05'	099°40'	3412
	R4	15	Songpan, Sichuan	32°45'	103°49'	3199
	R5	15	Mangkang, Tibet	29°32'	098°14'	3489
	R6	15	Bomi, Tibet	29°48'	095°44'	3253
<i>A. ecalcarata</i>	E1	15	Huzhu, Qinghai	36°54'	102°24'	2583
	E2	15	Yuzhong, Gansu	35°47'	104°3'	2349
	E3	15	Zhuoni, Gansu	34°19'	103°35'	2877
	E4	15	Zhouqu, Gansu	33°33'	104°18'	2770
	E5	15	Fengxian, Shaanxi	34°12'	106°35'	1632
	E6	15	Meixian, Shaanxi	34°00'	107°48'	2770
	E7	15	Huxian, Shaanxi	33°49'	108°36'	2439
	E8	15	Ningshan, Shaanxi	33°28'	108°29'	2167
	E9	15	Xingshan, Hubei	31°27'	110°16'	2580
	E10	15	Nanchuan, Chongqing	29°02'	107°11'	2121
	E11	15	Jiangkou, Guizhou	27°54'	108°41'	2426
	E12	15	Dege, Sichuan	31°57'	98°39'	3532
	E13	15	Barkam, Sichuan	31°52'	102°37'	3193
	E14	15	Batang, Sichuan	30°19'	099°21'	3470
	E15	15	Xiaojin, Sichuan	30°53'	102°38'	3268
E16	15	Wenchuan, Sichuan	30°53'	102°59'	2646	
E17	15	Yajiang, Sichuan	29°59'	100°54'	3545	
E18	15	Bomi, Tibet	29°49'	095°42'	3211	
E19	15	Milin, Tibet	29°35'	094°56'	3282	

admixture model with correlated allele frequencies. Estimation of the uppermost hierarchical level of the genetic structure was accomplished using a statistic ΔK following the procedure described in previous methods (Evanno et al., 2005).

2.7. Fitting an isolation-with-migration model

IMa 2 is based on an isolation-with-migration model and estimates effective population sizes (present and ancestral), splitting times, and population migration rates using MCMC simulations (Hey, 2010). As the isolation-with-migration model assumes no recombination within markers (Hey and Nielsen, 2004), we conducted recombination rate tests of fifteen nuclear loci using IMgc (http://hammerlab.biosci.arizona.edu/imgc_online.html) (Woerner et al., 2007). By discarding the recombination sections in each locus, the nonrecombining sections of the fifteen gene fragments (in total 8339 bp) were used for IMa2 calculation. After a burn-in of 10^7 generations and 2,000,000 steps, 20,000 genealogies were saved. The analysis was done with four independent runs with different random seeds. Convergence was assessed based on ESS >50, stable parameter trend plots, and similar parameter estimates from the first and the second half of the runs. We estimated the mutation rate of each locus based on a modified method (Tenaillon et al.,

2004). The geometric average mutation rate of the fifteen gene fragments (2×10^{-5}) was used to convert the effective population size (θ) to effective number of individuals (N_e). In “L mode” of IMa2, marginal posterior probability density estimates and LLR tests were used for assessing whether migration rates were significantly different from zero.

2.8. Re-examination of morphological data

Xue et al. (2020) reported a detailed morphological investigation (i.e., 41 vegetative and floral traits) on the 42 populations of *Aquilegia ecalcarata*. Here we re-examined these data and compared what we refer to as *A. ecalcarata* I and II to demonstrate their morphological divergence. Statistical methods followed descriptions in Xue et al. (2020).

3. Results

3.1. Genetic diversity and neutrality test

The concatenated lengths of the fifteen nuclear gene fragments were 11,083 bp and the aligned sequence lengths ranged from 620 to 870 bp (739 bp on average). A total of 1880 segregating sites were

detected (524 in *Aquilegia ecalcarata*, 297 in *A. kansuensis*, 259 in *A. rockii* and 520 in *A. yabeana*), and a total of 363 haplotypes were found across all individuals (Tables S2 and S3). For the chloroplast loci (*rpl32-trnL*, *trnK-rps 16*, *rps16-trnQ*), the concatenated lengths were 1809 bp. We detected a total of 41 segregating sites and 37 haplotypes ($H_d = 0.946$) in all 210 individuals.

We calculated π_{sil} and θ_{sil} on combined nuclear sequence data to evaluate the level of genetic diversity among the four species. At the species level, *A. yabeana* possessed the highest genetic diversity (π_{sil} 0.0114 and θ_{sil} 0.0141), whereas *A. kansuensis* had the lowest (π_{sil} 0.0070 and θ_{sil} 0.0078). *A. ecalcarata* (π_{sil} 0.0067/ θ_{sil} 0.0135) and *A. rockii* (π_{sil} 0.0074/ θ_{sil} 0.0073) had a genetic diversity at intermediate levels. Notably, *A. ecalcarata* conserved more segregating sites (θ_{sil} 0.0135) than either *A. kansuensis* (θ_{sil} 0.0078) or *A. rockii* (θ_{sil} 0.0073).

We performed three neutrality tests (Tajima's *D*, Fu and Li's *D*, Fu and Li's *F*) in each species: the majority of the results showed non-significant values in each test, except for certain species in which significant values were shown for *Lap 3* (*A. ecalcarata*), *Cpt* (*A. ecalcarata*), *Bst 10* (*A. rockii*), *Cer* (*A. ecalcarata* and *A. yabeana*). We then conducted a multilocus HKA test to ensure all loci were neutral. The χ^2 value of *A. ecalcarata*/outgroups was 0.112 ($p = 0.738$), *A. kansuensis*/outgroups was $\chi^2 = 0.169$ ($p = 0.681$), *A. rockii*/outgroups was $\chi^2 = 0.047$ ($p = 0.829$), and *A. yabeana*/outgroups was $\chi^2 = 0.005$ ($p = 0.945$). No significant values were found by the multilocus HKA test, implying that all nuclear sequence data were evolved neutrally and are appropriate for analyses.

3.2. Phylogeny with nuclear and chloroplast data

Neighbor-Joining, Maximum Parsimony, Maximum Likelihood and Bayesian methods were used to construct the genealogies of 42

populations of four species. Phylogenetic trees using these four methods with chloroplast sequence data showed similar topologies (Fig. 2a). The four outgroups were positioned as a sister clade; the ingroup populations were split into two clades, each with high bootstrap values (100) and Bayesian posterior probabilities. Interestingly, *Aquilegia ecalcarata* could be found in both clades and clustered with different species. Of the nineteen *A. ecalcarata* populations, seven (E5–E11) clustered with all *A. yabeana* populations and three *A. kansuensis* populations, whereas the other twelve clustered with all *A. rockii* populations and six *A. kansuensis* populations.

For fifteen nuclear gene fragments, we carried out an ILD test to decide whether we could combine all loci ($p = 0.07$), then we constructed phylogenetic trees with the three spurred species (*A. yabeana*, *A. kansuensis* and *A. rockii*) (Fig. S4). When we excluded *A. ecalcarata*, different methods generated phylogenetic trees with similar topologies, in which each of the three spurred species were monophyletic. Specifically, *A. yabeana*, *A. kansuensis* and *A. rockii* split into three clades with all outgroups falling out as sister. Although some individuals from population K9 (*A. kansuensis*) were positioned in the clade of *A. rockii* and other individuals from populations R2, R4 and R5 (*A. rockii*) fell into the clade of *A. kansuensis*, the majority of *A. kansuensis* and *A. rockii* populations remained monophyletic. One possible explanation for these exceptions could be introgression, as we found that some populations, such as R4, also fell into different clades in the chloroplast sequence NJ tree. Hence, we discarded these exceptions in later phylogenetic analyses.

We then analyzed the phylogeny of these population when the nineteen populations of *A. ecalcarata* were included. The phylogeny based on nuclear gene fragments differed from that based on chloroplast loci (Fig. 2b), although all populations split into three

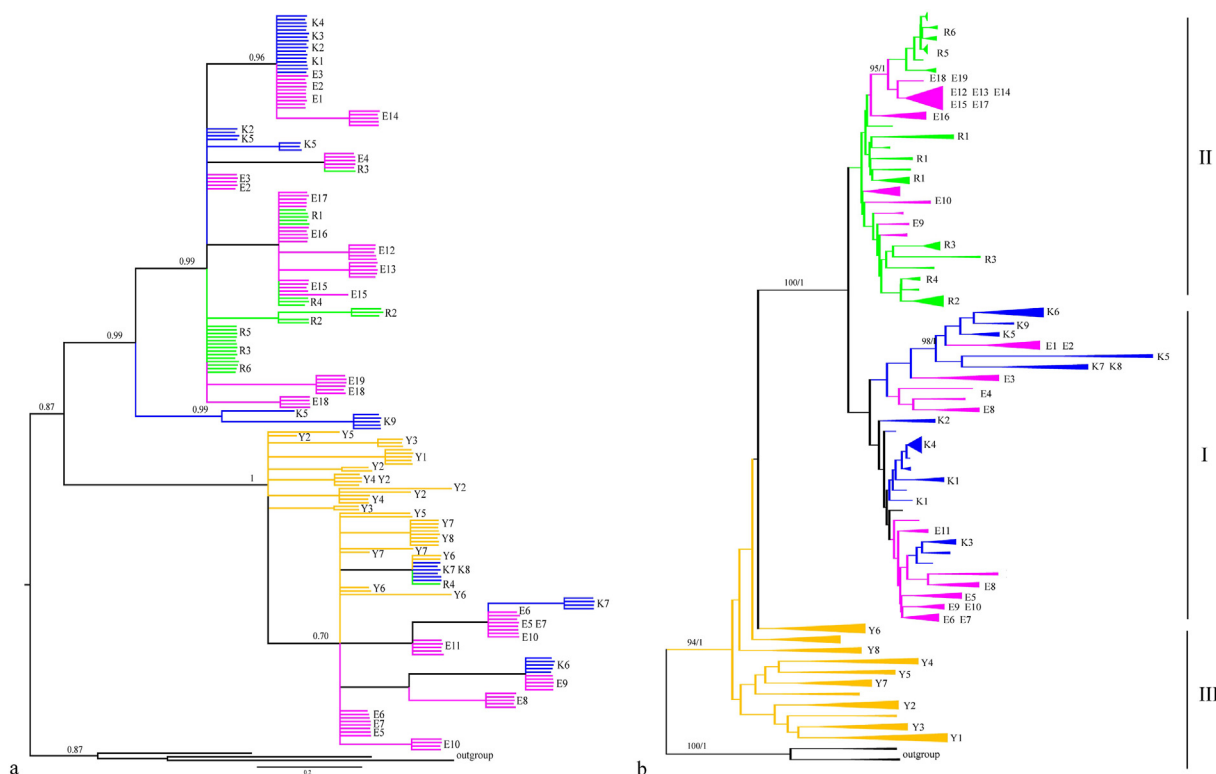


Fig. 2. Phylogenetic trees of all 42 populations based on three combined chloroplast gene fragments (a); all populations except partial individuals from K9, R2, R4, R5 based on the combined nuclear gene fragments (b); Numbers along branches represent bootstrap support (1000 replicates) and Bayesian posterior probability. *Aquilegia yabeana* (orange), *A. kansuensis* (blue), *A. rockii* (green) and *A. ecalcarata* (pink).

clades. The first diverging clade (named III, with a bootstrap value 94 and Bayesian posterior probability of 1) was composed only of *A. yabeana*, which is in contrast to our findings in the NJ tree based on chloroplast sequence data. The monophyly of *A. yabeana* suggested that *A. yabeana* is the ancestral species of the four taxa;

furthermore, phylogenetic analysis based on nuclear gene fragments indicate that *A. yabeana* may not have contributed to the origin of *A. ecalcarata*. The nineteen populations of *A. ecalcarata* fell into two groups, with eleven populations clustered with *A. kansuensis* (named clade I), while eight populations clustered

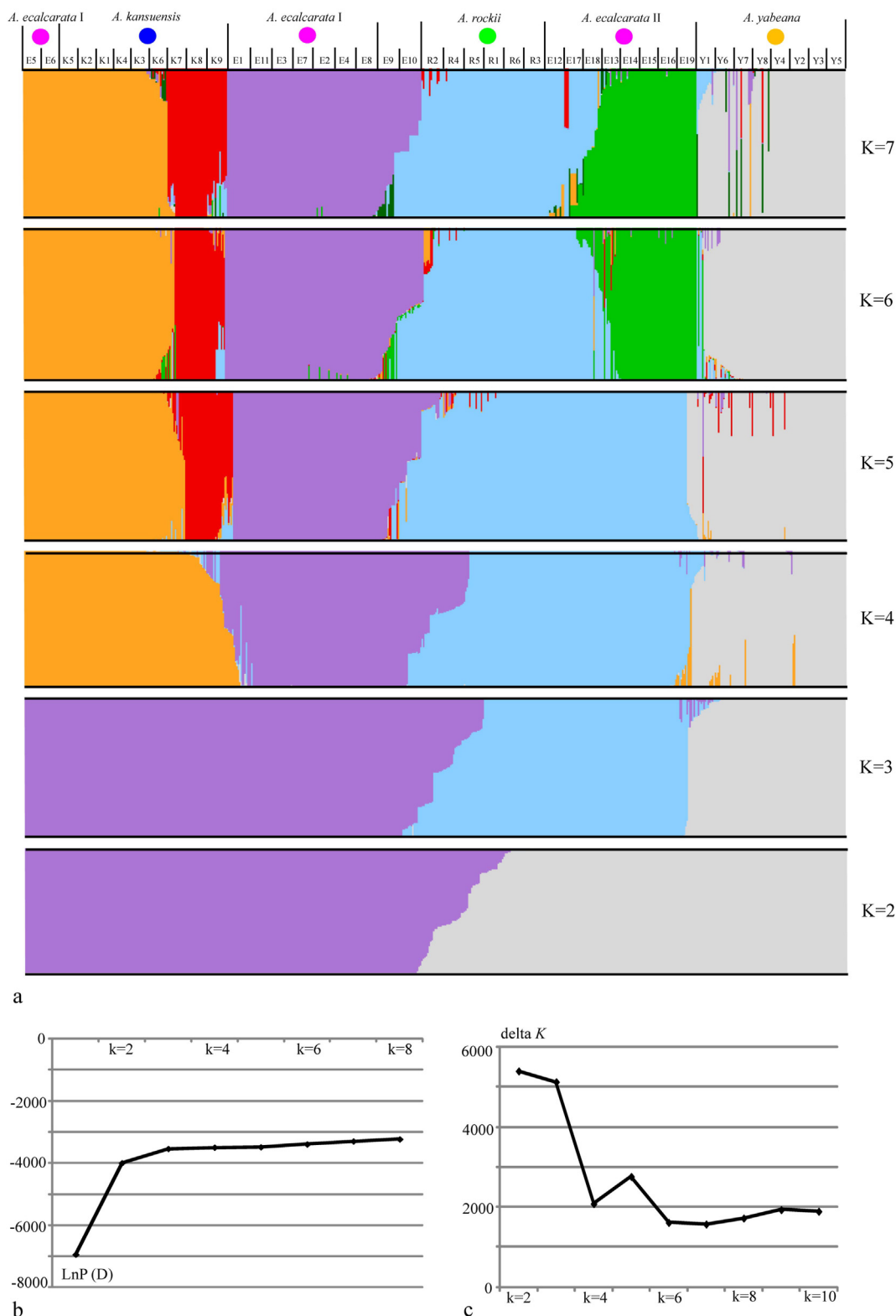


Fig. 3. Structure analysis of 42 populations with combined nuclear data. Results of structure analysis at K = 2–7, (a); the maximum LnP(D) at different K (b); delta K at different K (c).

with *A. rockii* (named clade II). The branch lengths of clade I and clade II were relatively short with bootstrap values of only 56 and 63, respectively. Notably, different individuals from two populations of *A. ecalcarata* (E9 and E10) could be found in both clade I and clade II, while those from the remaining populations clustered into only one clade.

We also constructed gene trees based on each of the fifteen nuclear gene fragments (Fig. S5). Gene trees of six gene fragments (*Hsh*, *Cta1*, *lap 3*, *Ger*, *Vap 4*, and *Cer*) had different topologies compared to the remaining nine. *A. ecalcarata* did not split into two branches on those six loci. Two populations (E9 and E10) fell into different branches among different gene trees. The unstable phylogenetic positions of E9 and E10 indicates that they could have more complicated evolutionary histories.

Furthermore, we found that the populations of *A. ecalcarata* in the two clades varied slightly from the chloroplast gene tree to the nuclear gene tree. Specifically, the majority of clade I and II (K1–K5, K9, R1–R3, R5–R6, E1–E4 and E12–E19) from nuclear gene tree belonged to the same clade as the chloroplast gene tree, whereas the remaining part of clade I and II (K6–K8, R4 and E5–E11) belonged to the other clade of the chloroplast gene tree. It was concerning that E5–E11 were clustered with *A. yabeana* in the chloroplast data. Four out of the seven of these populations (E5–E11) are sympatrically distributed with *A. yabeana*, which may have provided opportunity for gene introgression between population pairs.

We used an ILD test to decide whether we could construct phylogenetic trees with combined chloroplast and nuclear loci. The results showed the two data sets were not suitable for combination ($p = 0.01$).

The divergence time between outgroups and *Aquilegia* populations was about 2.86–3.00 million years (Myr); the divergence time of *A. yabeana* (clade III) from the other three species was about 2.57–2.64 Myr; the divergence time between clade I and II was 1.69–1.94 Myr; *A. ecalcarata* in clade I diverged from its closest relative about 0.71–0.92 Ma, whereas *A. ecalcarata* in clade II diverged from its closest relative about 0.73–0.85 Ma.

3.3. Structure and PCoA analysis

We found that nineteen populations of *Aquilegia ecalcarata* were split into different genetic groups and two populations (E9 and E10) appeared to be the products of hybridization (Fig. 3a). When $K = 2$, all individuals of *A. ecalcarata* had already been separated into different groups (in Fig. 3a, some were purple and the others were grey) with *A. kansuensis* or *A. rockii*, while *A. yabeana* and *A. rockii* still shared the same genetic component (grey), which indicated that different groups of *A. ecalcarata* diverged much earlier than *A. rockii* from *A. yabeana*. When $K = 3$, *A. rockii* and a proportion of *A. ecalcarata* were separated from *A. yabeana*, corresponding to clades I, II, III in the nuclear gene fragments tree. Notably, all *A. ecalcarata* still shared the same color with their closest relatives. When $K = 4$, the majority of *A. kansuensis* (K1–K6) and two populations of *A. ecalcarata* (E5 and E6) diverged from the others, implying that seven populations of *A. ecalcarata* (E1–E4, E7–E8, E11) were genetically differentiated from *A. kansuensis*. When $K = 5$, three populations of *A. kansuensis* (K7–K9) diverged from the

rest six populations (K1–K6), indicating a substructure within the species. When $K = 6$, five populations of *A. ecalcarata* (E13–E16, E19) were separated into distinct genetic components compared with six populations of *A. rockii* (R1–R6) and $K = 7$ simply repeated the pattern of $K = 6$. Interestingly, E5 and E6 of *A. ecalcarata* were never separated from *A. kansuensis* (K1–K6) even when $K = 7$. In addition, E9 and E10 had two main different genetic components. Notably, partial individuals from K9, R2, R4 and R5, which showed different topology on the NJ tree (Fig. S4) were shown to have different genetic components when $K = 6$ and 7. K9 might have experienced gene exchange with *A. rockii*, while R2, R4 and R5 might have experienced gene flow from *A. kansuensis*. In Fig. 3b, we observed a significant increase of $\ln P(D)$ with K from 1 to 6 and a mild increase with K from 6 to 8. In Fig. 3c, ΔK showed peaks at $K = 3$ and $K = 5$. The two peaks implied that it would be more proper to divide 42 populations into three groups and there were substructures within two groups. Overall, STRUCTURE results were in accordance with the phylogeny based on nuclear data. The geographical distribution when $K = 3$ (Fig. S6) showed that the three colors had a distinct geographical pattern.

PCoA results confirmed the species divergence revealed by STRUCTURE (Fig. S7). We observed three groups of scattered dots among 42 populations. On the PC1 and PC2 plot, the clades I and II were clustered separately, *A. ecalcarata* within each clade was surrounded by *A. kansuensis* or *A. rockii*. The three clades diverged greatly on PC1 axis and strikingly, PC1 accounts for 27.25% in total divergence. By contrast, PC2 and PC3 accounted for 11.18%, 6.84%, respectively. Those populations that showed evidence of introgression (i.e., E9, E10, K9, R2, R4 and R5) were located in the middle of clades I and II on the PCoA plot. All the above results suggest that *A. ecalcarata* had multiple origins of *A. ecalcarata* in clades I diverged from its closest relative *A. kansuensis* when $K = 4$, while *A. ecalcarata* in clades II diverged from *A. rockii* when $K = 6$. Thus we suggested that the nine populations (E1–E8, E11) from clade I were addressed as *A. ecalcarata* I and the eight populations (E12–E19) from clade II were *A. ecalcarata* II. E9 and E10 showed evidence for hybridization between *A. ecalcarata* I and *A. rockii*.

3.4. Divergence among and within species

The pairwise F_{ST} values between and within species are shown in Tables 2 and S4. The minimum F_{ST} was between *Aquilegia ecalcarata* I and *A. kansuensis* (0.080); while the maximum F_{ST} was between *A. ecalcarata* II and *A. yabeana* (0.406). The genetic differentiation between *A. ecalcarata* I and II was 0.308, higher than 0.211 (*A. kansuensis*/*A. rockii*), 0.080 (*A. ecalcarata* I/*A. kansuensis*) or 0.127 (*A. ecalcarata* II/*A. rockii*) (see Fig. S8). If *A. ecalcarata* I or II resulted from hybridization with either *A. kansuensis* or *A. rockii*, the genetic divergence between *A. ecalcarata* I and II would not be very high and vice versa. Within species, the minimum F_{ST} was *A. ecalcarata* I (0.349), the maximum was *A. rockii* (0.610); *A. yabeana* and *A. ecalcarata* II were at intermediate levels (0.403 and 0.472). In addition, AMOVA showed that variations were mostly enriched among populations within species (up to 49.24% based on the combined nuclear gene fragments) (Table S5), which implies strong population structure in these closely related species. Mantel test results are shown in Table 3; the p -values of

Table 2
Summary of F_{ST} among species with combined nuclear gene fragments.

	<i>A. ecalcarata</i> II	<i>A. kansuensis</i>	<i>A. rockii</i>	<i>A. yabeana</i>
<i>Aquilegia ecalcarata</i> I	0.308	0.080	0.239	0.398
<i>A. ecalcarata</i> II		0.284	0.127	0.406
<i>A. kansuensis</i>			0.211	0.404
<i>A. rockii</i>				0.381

Table 3
Mantel test of four species based on the combined nuclear gene fragments.

	r	p -value
<i>Aquilegia ecalcarata</i> I	0.2827	0.163
<i>A. ecalcarata</i> II	0.4224	0.020
<i>A. kansuensis</i>	0.2149	0.214
<i>A. rockii</i>	−0.6138	0.104
<i>A. yabeana</i>	0.5224	0.008

A. yabeana and *A. ecalcarata* II were significant, whereas those of *A. kansuensis* and *A. rockii* were not.

3.5. Isolation-with-migration model

To test whether *Aquilegia ecalcarata* I underwent hybridization between *A. ecalcarata* II and *A. kansuensis*, or *A. ecalcarata* II underwent hybridization between *A. ecalcarata* I and *A. rockii*, we estimated the migration rate of the above species (Fig. S9; Table S6). *A. ecalcarata* populations E9 and E10 were excluded from IMA analysis because they were not ascribed as either *A. ecalcarata* I or II. The hypothesized hybridizations between *A. ecalcarata* I and *A. rockii*, or *A. ecalcarata* II and *A. kansuensis* are not supported by our gene flow results. For instance, the $2N_1M_1$ between *A. ecalcarata* I and *A. kansuensis* was 0.177 (95% HPD 0.029–4.993) and $2N_2M_2$ was significant 0.494 (95% HPD 0.207–4.841). Because *A. kansuensis* was assumed to be the closest relative of *A. ecalcarata* I, the gene flow from *A. kansuensis* to *A. ecalcarata* I was relatively high. In contrast, gene flow was only 0.064 (95% HPD 0.018–4.783) from *A. ecalcarata* I to *A. rockii*, and in the reverse direction, 0.091 (95% HPD 0.013–4.636). Thus, no significant introgressions were detected between *A. ecalcarata* I and *A. rockii*. Similarly, the gene flow between *A. ecalcarata* II and its closest relatives *A. rockii* was $2N_2M_2$ 0.306 (95% HPD 0.102–4.762), which was also significantly higher than that between *A. ecalcarata* II and *A. kansuensis* $2N_1M_1$ 0.106 (95% HPD 0.029–4.972) and $2N_2M_2$ 0.026 (95% HPD 0.008–4.993). The gene flow between *A. ecalcarata* I and *A. ecalcarata* II was calculated at a rate of $2N_1M_1$ 0 (95% HPD 0–4.993) and $2N_2M_2$ was 0.172 (95% HPD 0.023–4.710). Interestingly, our analysis indicated that there was a low level of bidirectional gene flow between *A. kansuensis* and *A. rockii*, at a rate of $2N_1M_1$ 0.241 (0–4.552) and $2N_2M_2$ 0.192 (0–5.224). This low level of gene flow may explain the different topologies of partial individuals from populations K9, R2, R4 and R5. In addition, the gene flow between *A. ecalcarata* I and *A. ecalcarata* II for each gene fragment (Table S7) indicated that the majority of gene fragments showed very low level of gene flow, except *Hsh*, *Cta1* and *Exp*.

3.6. Re-examination of morphological data between *Aquilegia ecalcarata* I and II

We found that 13 out of 22 floral traits showed significant differentiation between *Aquilegia ecalcarata* I and II (Fig. S10). Notably, *A. ecalcarata* II had larger flower size (both the length and width of petal, sepal, carpel) and longer spurs than *A. ecalcarata* I, whereas *A. ecalcarata* I had more fertile stamens than *A. ecalcarata* II (Fig. 1a). More importantly, the geographical distribution of *A. ecalcarata* I and II also differed: *A. ecalcarata* I is mainly distributed in Gansu, Shaanxi, and Hubei provinces, whereas *A. ecalcarata* II is mostly distributed in Sichuan and Tibet provinces (Fig. 1b). In addition, we carried out Pearson correlation coefficients between the lengths of spurs and altitude in 42 populations (Fig. S11), the result was significant ($r = -0.432$, $p = 0.009$), suggesting the higher the population located, the shorter the spur length was.

4. Discussion

4.1. The distinct genetic divergence between *Aquilegia ecalcarata* I and II and the dispute over a single or multiple origins

Sample size and the number of molecular markers could affect the evaluation and interpretation of the genetic divergence or phylogenetic analysis in evolution studies. For instance, the origin of *Oryza sativa* (Asian cultivated rice) has been under debate for

many years (Londo et al., 2006; Huang et al., 2012; Wang et al., 2018). One study sampled hundreds of rice individuals and used a few nuclear gene fragments, revealing that *O. sativa* was domesticated from its ancestor independently at different areas (Londo et al., 2006), whereas another study provided solid evidence of single origin of *O. sativa* based on whole-genome resequencing of more than 1000 individuals (Huang et al., 2012). Interestingly, the most recent research, which re-sequenced 3000 accessions of cultivated rice, supports the multiple-origin hypothesis of *O. sativa* (Wang et al., 2018). The debate over single- or multiple-origins of some species has been an ongoing topic in evolutionary biology, and studies commonly support opposite hypotheses. Given that *Aquilegia* is widely known for its ability to hybridize readily and easily (Hodges and Arnold, 1995), a hypothesis of a single origin followed by hybridization hypothesis would be a plausible explanation for the different types of *A. ecalcarata*. In fact, the *Aquilegia* genome project revealed that gene flow had been a common feature throughout the genus (Filiault et al., 2018). Nevertheless, evidence from the present study does not support the single-origin hypothesis. First, IMA 2 results indicate no strong gene flow between *A. ecalcarata* I and *A. rockii*, or *A. ecalcarata* II and *A. kansuensis*. Second, STRUCTURE analysis showed that both *A. ecalcarata* I and II have very pure genetic components. If hybridization occurred, *A. ecalcarata* I or II should have mixed genetic components similar to the *A. ecalcarata* populations E9 and E10. Besides, the fact that *A. ecalcarata* I or II diverged from its closest relatives at different times, the former emerged when $K = 4$ while the latter $K = 6$, this could be at least the evidence to against the single origin hypothesis. Third, chloroplast gene fragment trees showed that all *A. ecalcarata* are separated into different clades. If the single-origin hypothesis were true, *A. ecalcarata* I and II should be in the same clade since they would share the same maternal DNA. Fourth, the nucleotide polymorphism of all nineteen *A. ecalcarata* populations was much higher (θ_{sil} 0.0135) than that of *A. ecalcarata* I (θ_{sil} 0.0100) or *A. ecalcarata* II (θ_{sil} 0.0098). This indicated that more segregating sites emerged when we mixed two types of *A. ecalcarata* for calculation. Hybridization might also reduce the F_{ST} between *A. ecalcarata* I and II, but the real F_{ST} estimated in this study was even higher than that between *A. kansuensis* and *A. rockii*. Fifth, *A. ecalcarata* I and II have quite different distributions. The former is mainly distributed in the northwestern part of the entire distribution area (Shaanxi, Gansu, Qinghai, and Hubei provinces), whereas the latter is distributed in the southern part of distribution area (Sichuan, Tibet provinces). Finally, the genetic and phenotypic divergence between *A. ecalcarata* I and II are consistent. Morphological study on the same 42 populations divided *A. ecalcarata* into clusters referred to as E and F (Xue et al., 2020), with cluster E corresponding to *A. ecalcarata* II and cluster F corresponding to *A. ecalcarata* I (except E9 and E10) in this study.

Moreover, populations E9 and E10 appear to be an exception in that they showed signs of hybridization between *A. ecalcarata* I and *A. rockii* in the results of STRUCTURE. We inferred that *A. ecalcarata* I diverged and expanded into a new area where *A. rockii* was distributed. The possibility that *A. ecalcarata* populations E9 and E10 were the outcome of introgression suggests that *A. ecalcarata* might have a more complex evolutionary history and require further study based on more populations and molecular locus samplings. In addition, the phenotypic comparison indicates that divergence within *A. kansuensis* or between *A. kansuensis* and *A. rockii* are distinct; thus, spur loss is not the only phenotypic difference that should be considered when comparing *A. ecalcarata* with its closest relatives. Flower size, the extent of spur curvature and the color of dehiscent anthers are also major transitions. Taken

together, evidence indicates that the genetic differentiation between *A. ecalcarata* I and II may be best explained by the multiple-origin hypothesis. However, to fully rule out the single-origin hypothesis and disentangle the complex evolutionary history *A. ecalcarata* and its relatives, whole-genome re-sequencing is required.

4.2. The complicated evolutionary history of an important trait and its challenges for species delimitation

Morphological markers are the main characters used for plant species delimitation, as phenotypic similarity has been the criterion used historically by taxonomists to group individuals into species (Duminil and Di Michele, 2009). However, species diversification and morphological evolution are not always correlated. Consequently, morphological markers may fail to discriminate between morphologically similar species when complicated evolutionary histories have occurred. Researchers have reported independent origins of a series of traits from *Oryza rufipogon* Griff. to *Oryza nivara* Sharma et (Cai et al., 2019), but have not ascribed different populations of *O. nivara* to different species since the phenotypes are consistent within *O. nivara*. In this study, we recommend ascribing *Aquilegia ecalcarata* I and II to different species due to genetic, morphological and biogeographical differentiation. Moreover, *A. ecalcarata* contains a variety named *A. ecalcarata* form. *semicalcarata*. The longer spur length of *A. ecalcarata* II conforms to the description of *A. ecalcarata* form. *semicalcarata* (spur length >2 mm; *Flora of China*). In addition, the type specimen of *A. ecalcarata* form. *semicalcarata* was collected in Sichuan province, where *A. ecalcarata* II is distributed. Therefore, we suggest upgrading *A. ecalcarata* form. *semicalcarata* to *Aquilegia semicalcarata* (Schipcz) Huang et Ren. Thus, *A. ecalcarata* I can be referred to *A. ecalcarata*, whereas *A. ecalcarata* II can be referred to as *A. semicalcarata*. Furthermore, traditional taxonomy usually focuses on macroscopic changes between closely related species, such as spur loss, but neglects subtle changes in quantitative traits such as flower size. If pivotal traits show similarity but other phenotypes are differentiated, these differences should be evaluated. Our findings provide a good example of how to delimitate species that have independent evolutionary histories.

4.3. Implications for the adaptation of *Aquilegia ecalcarata* to new environment

Aquilegia species diversity is assumed to be an example of ecological speciation, rather than being driven by the development of intrinsic barriers to gene flow (Filiault et al., 2018). In the present study, we note that both *A. ecalcarata* I and II grow in the stony environments along the slopes or peaks of mountains or by riversides. In contrast, *A. kansuensis* and *A. rockii* mostly grow in forest margins and riverside or under forest environments. These habitat differences suggest that *A. ecalcarata* I and II adapted to a new environment. Previous studies showed that natural selection by different pollinators, geographic isolation, and habitat shifts may all play important roles in diversification within *Aquilegia* (Hodges, 1997; Hodges et al., 2003; Whittall and Hodges, 2007; Bastida et al., 2010). Future work on pollination ecology and the investigation of ecological factors in stony habitats are required to help a better understanding of the driving forces of the adaptation in *A. ecalcarata* I and II. Because the spurless state is a derived rather than ancestral state in *Aquilegia* (Hodges and Arnold, 1995), we are still uncertain whether the loss of spurs is adaptive. Recently, Ballerini et al. (2020) identified a gene, *POPOVICH* (*POP*), crucial for nectar spur development in *Aquilegia*. *POP* plays a central role in regulating cell proliferation in the *Aquilegia* petal

during the early phase of spur development. Neutrality tests on this gene would help us understand whether it was under positive selection. *A. ecalcarata* I and II might have independently fixed an ancestral spur loss allele, or have converged on a spur loss phenotype via completely different genetic means. Whether *A. ecalcarata* I and II share the alleles controlling the spurless status is yet to be explored.

5. Conclusions

In this study, evidence indicates that the multiple-origin hypothesis might be more plausible for interpreting the genetic differentiation between *Aquilegia ecalcarata* I and II. However, whether spur loss has occurred independently within two distinct lineages of *A. ecalcarata* requires more evidence from whole genome re-sequencing data. Habitat differences relative to their closest relatives indicate that *A. ecalcarata* I and II may undergo habitat shift when they adapted to new environment. This study provides new insights into the relationship of floral diversification and species divergence.

Author contributions

Y. Ren designed the study, L. Huang and F.-D. Geng conducted the experiments and performed the analyses. J.-J. Fan, X.-Y. Zhang and C. Xue joined the sample collection, DNA extraction and phenotype measurement. J.-Q. Kang and X.-H. Zhang participated in data discussion. L. Huang and Y. Ren wrote the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pld.2021.06.006>.

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