Plant Syst Evol (2010) 284:171–185
DOI 10.1007/s00606-009-0243-z

ORIGINAL ARTICLE

Extended phylogeny of *Aquilegia*: the biogeographical and ecological patterns of two simultaneous but contrasting radiations

Jesús M. Bastida · Julio M. Alcántara ·
Pedro J. Rey · Pablo Vargas · Carlos M. Herrera

Received: 29 April 2009 / Accepted: 25 October 2009 / Published online: 4 December 2009

Abstract Studies of the North American columbines (*Aquilegia*, Ranunculaceae) have supported the view that adaptive radiations in animal-pollinated plants proceed through pollinator specialisation and floral differentiation. However, although the diversity of pollinators and floral morphology is much lower in Europe and Asia than in North America, the number of columbine species is similar in the three continents. This supports the hypothesis that habitat and pollinator specialisation have contributed differently to the radiation of columbines in different continents. To establish the basic background to test this hypothesis, we expanded the molecular phylogeny of the genus to include a representative set of species from each continent. Our results suggest that the diversity of the genus is the result of two independent events of radiation, one involving Asiatic and North American species and the other involving Asiatic and European species. The ancestors of both lineages probably occupied the mountains of south-central Siberia. North American and European columbines are monophyletic within their respective lineages. The genus originated between 6.18 and 6.57 million years (Myr) ago, with the main pulses of diversification starting around 3 Myr ago both in Europe (1.25–3.96 Myr ago) and North America (1.42–5.01 Myr ago). The type of habitat occupied shifted more often in the Euroasiatic lineage, while pollination vectors shifted more often in the Asiatic-North American lineage. Moreover, while allopatric speciation predominated in the European lineage, sympatric speciation acted in the North American one. In conclusion, the radiation of columbines in Europe and North America involved similar rates of diversification and took place simultaneously and independently. However, the ecological drivers of radiation were different: geographic isolation and shifts in habitat use were more important in Europe while reproductive isolation linked to shifts in pollinator specialisation additionally acted in North America.

Keywords Adaptive radiation · Allopatric speciation · Columbines · Habitat specialisation · Historical contingency · Pollination syndromes · Sympatric speciation

Introduction

Adaptive radiations are characterised by the accumulation of substantial taxonomic, morphological and ecological diversity within a rapidly diversifying lineage (Schluter 1996, 2000). In recent years, the application of molecular techniques to the study of widespread plant groups has served to highlight that recent radiations could have played an important role in the exceptional species richness of several continental regions of the world. Examples include the genera *Ehrharta* and *Argyroderma* in South Africa (Verboom et al. 2003; Ellis et al. 2006), *Lupinus* in South

---

Electronic supplementary material The online version of this article (doi:10.1007/s00606-009-0243-z) contains supplementary material, which is available to authorized users.

J. M. Bastida · J. M. Alcántara (✉) · P. J. Rey
Departamento de Biología Animal, Biología Vegetal y Ecología,
Universidad de Jaén, 23071 Jaén, Spain
e-mail: jmalcan@ujaen.es

P. Vargas
Real Jardín Botánico, C.S.I.C., Plaza de Murillo 2,
28014 Madrid, Spain

C. M. Herrera
Estación Biológica de Doñana, C.S.I.C,
Avenida de María Luisa s/n, 41013 Sevilla, Spain

America (Hughes and Eastwood 2006), *Penstemon* in North America (Beardsly et al. 2004; Wolfe et al. 2006) and *Cistus* in the Mediterranean basin (Guzmán et al. 2009).

The canonical view of adaptive radiation in animal-pollinated plants (Grant and Grant 1965; Stebbins 1970) relies on the hypothesis that speciation proceeds through reproductive isolation resulting from pollinator specialisation and the differentiation of floral traits. Thus, sympatric speciation should be common. Much of the support for this view is due to the pioneering studies of Verne Grant on the evolution of floral isolation and pollination syndromes in columbines (genus *Aquilegia*, Ranunculaceae) (see review in Hodges et al. 2003). Studies of floral evolution based on molecular phylogenetic reconstructions of North American columbines strongly support this hypothesis (Hodges and Arnold 1994, 1995; Hodges 1997; Ro and McPheron 1997; Whittall and Hodges 2007). For example, the results of Whittall and Hodges (2007) indicate that flower morphology (particularly spur length) has diversified in North American columbines in association with changes in the type of pollinator, which shifted from bees and bumblebees to hummingbirds and finally to hawkmoths. In contrast to the canonical view, evidence is accumulating for abiotic environment-driven radiations in other lineages of flowering plants (Robichaux et al. 1990; Francisco-Ortega et al. 1996, 1997; Patterson and Givnish 2003; Givnish et al. 2004; Verboom et al. 2004; García-Maroto et al. 2009). As stated by Schluter (2000), exploring these alternative views concerns the ultimate bases of speciation, whether it results from contrasting natural selection on vegetative structure and physiology between environments or instead from divergent sexual selection on floral structures mediated by pollinators.

Available phylogenies suggest that columbines are a monophyletic group which has radiated very rapidly from an Asiatic ancestor (Ro and McPheron 1997). The genus *Aquilegia* consists of approximately 75 species (Munz 1946; Whittemore 1997; Nold 2003), with a similar number of taxa occurring in North America (22 spp.), Asia (23 spp.) and Europe (21 spp.). The species inhabit a variety of habitats, from desert springs to alpine meadows, rocky outcrops, and temperate forests, from the sea level to the slopes of the Himalayas, the Rocky Mountains or the Alps. The distribution ranges are also highly variable, from very narrowly endemic (e.g. *A. borodinii*, *A. laramiensis* and *A. barbaricina*) to almost continental-wide distributions (e.g. *A. sibirica*, *A. vulgaris*, *A. canadensis* and *A. formosa*). The main pollinators can be syrphiid flies, bumblebees, hawkmoths or hummingbirds (Whittall and Hodges 2007; Tang et al. 2007). Columbine species exhibit remarkable differentiation in floral (e.g. the shape and length of spurs, petal and sepal colour, stigma exertion, corolla size) and vegetative traits (e.g. the presence and type of pubescence in leaves and inflorescence axis, plant height, number and size of leaves, leaf morphology).

All of these features make columbines a particularly well-suited case to study the processes of adaptive radiation and speciation in relation to natural selection acting on vegetative and floral traits. However, few studies have addressed the possible role of vegetative or physiological divergence and habitat differentiation (and related issues such as geographic isolation or local adaptation) in the columbines. Chase and Raven (1975) and Hodges and Arnold (1994), concluded that barriers to gene flow between *A. formosa* and *A. pubescens* in the Sierra Nevada of California are maintained primarily through their specialisation to different types of environments and secondarily through their specialisation to different pollinators (but see Fulton and Hodges 1999). The experimental studies of LaRoche (1978, 1980) suggested that the large geographical range of *A. canadensis* might be related to its large phenotypic plasticity, both in floral and vegetative traits. What is known about European columbines also challenges the canonical view. European species are quite similar in floral traits, which seem concordant with the much lower diversity of known pollinator types (almost exclusively bumblebees) of European columbines in comparison with North American columbines. However, because the number of taxa is similar in Europe and North America, it seems possible that the process of radiation has been driven by different mechanisms in each continent. Unfortunately, studies on the evolutionary ecology of European columbines are very scarce and scattered. Medrano et al. (2007) have shown that *A. vulgaris* and *A. pyrenaica* in southern Spain differ more in vegetative than in floral traits and that populations of each species might show local adaptation for vegetative, but not floral, traits. Similarly, Gafta et al. (2006) have shown variation in vegetative traits among populations of *A. nigricans* occupying contrasting habitats in Transylvania (Romania). Lavergne et al. (2005) found selection on floral traits in the French Alps populations of *A. vulgaris*, but not in populations of *A. viscosa*, probably as a consequence of flower and seed predation.

Important aspects of the evolution of the European columbines have not been addressed thus far. The available phylogenies suggest that the genus originated in Asia and then spread to North America and Europe. However, these phylogenies are focused on the North American species. A better understanding of the phylogenetic relationships between European, Asiatic and North American columbines would open the field to formulate and test new hypotheses of trait evolution and habitat diversification. The present study is primarily intended to extend the phylogenetic analysis of *Aquilegia* to a larger set of species and to the whole geographical range of the genus. We used the obtained phylogeny to (1) explore the spatio-temporal pattern of diversification of the genus *Aquilegia* and (B) compare the patterns of diversification in relation to the pollination syndrome, habitat use and geographic isolation in different continental lineages.

Materials and methods

DNA extraction, molecular markers and sequencing

Thirty-two columbine species were included in the analysis, 9 from North America, 10 from Asia and 13 from Europe. We utilised *Semiaquilegia adoxoides* and *Urophysa henryi* as outgroups. *Semiaquilegia* has been widely recognised as sister to *Aquilegia* based on petal evolution (Tucker and Hodges 2005; Damerval and Nadot 2007) and geographical affinities (Munz 1946; Nold 2003). Recently, Wang and Chen (2007) showed that *Semiaquilegia*, *Urophysa* and *Aquilegia* form a monophyletic clade, with *Semiaquilegia* as sister to *Aquilegia*. Due to the large geographic range of the genus, the fresh plant material came largely from living collections and herbarium specimens from several European botanical gardens. Fresh material was collected from wild populations of Iberian columbines. The species used in the study and the origins of the samples are summarised in Appendix 1.

We utilised the ITS region (nrDNA) and two plastid regions not previously used in *Aquilegia* phylogenetics, the *trnK-matK* spacer and the *trnS-G* intergenic spacer. Total genomic DNA was extracted from fresh, silica-dried, and herbarium material using a DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. The DNA was PCR-amplified in a Perkin-Elmer PCR system 9700 thermal cycler (California). After a 1- to 3-min denaturation step at 94°C, the PCR conditions were 27–38 cycles of 1 min at 94°C; 0.5–1 min at 48°C (ITS), 51°C (*trnK-matK* spacer) and 54°C (*trnS-G* intergenic-spacer); and 1–1.30 min at 72°C. The amplification was accomplished using the external primers 17SE and 26SE (Sun et al. 1994) for the ITS (ITS 1, 5.8 s and ITS 2), the 3914F and 1470R primers (Johnson and Soltis 1994) for the *trnK-matK* spacer, and the *trnS*^{gcu} and *trnG*^{uuc} primers for the *trnS-trnG* intergenic spacer (Hamilton 1999). The amplified products were purified using a PCR Clean-up Kit (MoBio Laboratories, CA). The products were sequenced directly using the Big Dye Terminator v. 2.0 (Applied Biosystems, Little Chalfont, UK). The sequencing primers were the same as those used for the amplification, except for ITS, for which we used the internal primers *P1A* and *P4* (Francisco-Ortega et al. 1999). The raw sequences were assembled and edited using the software SeqEd (Applied Biosystems, CA) and Bioedit v.7.0.5 (Hall 1999).

Phylogenetic analyses

Because the number of informative characters among the three data sets was low (Table 1), we tried to get the maximum resolution by combining the ITS and cpDNA data sets. In order to test the homogeneity among the three data sets, 1,000 replicates of the incongruence length difference test (ILD, Farris et al. 1994) were implemented in PAUP*4.0b1.0 (Swofford 2003) under the heuristic search constraints. ILD test showed no significant differences between the cpDNA data sets ($P = 1.00$) and between the ITS and cpDNA data sets ($P = 0.17$). Therefore, the phylogenetic analyses were conducted separately on the ITS and cpDNA, and we then combined the evidence of the three data sets. Insertion/deletion mutations were manually coded as appended characters (Table 1), following the logic of Kelchner (2000) and Simmons and Ochotorena (2000).

The phylogenetic trees were estimated by maximum parsimony (MP) and Bayesian inference (BI) for each data set. PAUP* 4.0b10 (Swofford 2002) was used to reconstruct the phylogenetic relationships by MP with equal weighting of all of the characters and of transitions/transversions. We performed heuristic searches using tree-bisection-reconnection (TBR) branch swapping, with one tree held at each step during the stepwise addition, the MulTrees option in effect, steepest descent off, MaxTrees set to 10,000 (increase = auto) and 5,000 replicates of random addition. The internal support was estimated with

Table 1 Statistics from the analyses of the molecular datasets

Data set (no. of taxa)	TL	Ii	Vs	Is	Trees	LT	CI	RI	RC	Model	Ar	TLik
ITS (34)	606	–	25	13	40	45	0.88	0.90	0.81	HKY + Γ	16.94–72.62%	−1,306.22
Chloroplast (33)	1,880	2 (1–12 bp)	32	8	7	43	0.97	0.98	0.96	GTR + I + Γ	11.76–70.12%	−2,830.75
ITS + cpDNA (34)	2,486	2 (1–12 bp)	57	21	2	91	0.89	0.88	0.78	GTR + I + Γ	11.99–59.87%	−4,039.40

The chloroplastic dataset includes the sequences of trnK-matK + trnS-G

TL Total length of sequences, *Ii* number of informative indels (and their length *in brackets*), *Vs* number of variable sites, *Is* number of informative sites, *Trees* number of most parsimonious trees, *LT* length of the shortest trees, *CI* consistency index, *RI* rescaled index, *RC* rescaled consistency index, *Model* model of sequence evolution, *Ar* acceptance rate, *TLik* tree likelihood

2,000 bootstrap replicates (Felsenstein 1985) with 10 random additions and heuristic search options.

Bayesian inference was carried out using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). These analyses were accomplished using the best-fit models as determined by Modeltest 3.06 (Posada and Crandall 1998) (Table 1). The Bayesian inference analysis was conducted by sampling for 4,000,000 generations (four chains of the Markov Chain Monte Carlo, chain temperature = 0.2, sample frequency = 1,000, burn-in = 600). Following the completion of each analysis, we plotted the output parameter estimates through time in order to identify the point of convergence to the stationary distribution. We used the *sump* command to explore two diagnostic statistics, the acceptance rate of the proposed chain swaps between the hot and cold chains, which should be between 10 and 70%. Then, the convergence was diagnosed by the average standard deviation of the split frequencies. This number should approach 0.01 and stabilise before starting to sample trees to make up the posterior distribution. A 50% majority-rule consensus tree was calculated for each analysis from the pooled sample using the *sumt* command. Posterior probability (PP) from the 50% majority rule consensus tree was used as an estimate of robustness (Alfaro et al. 2003). The analyses were run several times to assure the convergence and mixing.

Molecular clock analyses

To estimate the ages of the main nodes in the *Aquilegia* phylogeny, a temporal dimension was added. *Aquilegia* and its closely related genera are absent from the fossil record, and because our sampling does not allow dating based on geographic history (e.g., volcanic islands), it was impossible to calibrate the *Aquilegia* tree directly. To estimate the ages of the main nodes in the *Aquilegia* phylogeny, we expanded our phylogeny to the Ranunculaceae. To this end, we extended our *trnK-matK* sampling with the *matK* region and *trnK-matK* accessions from GenBank, including 26 genera. *Cissampelos pareira*, *Cocculus trilobus* and *Pericampylus glaucus* (Menispermaceae) were used as outgroups (Appendix 2). This allowed the incorporation of several fossil calibration points, in order to minimise the bias produced by single calibration points. This data set of 70 species and 1,358 characters was analysed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), with the same settings as given above. The best-fit model was TVM + G (Rodríguez et al. 1990), determined by Modeltest 3.06 (Posada and Crandall 1998). Rate constancy in the *matK* tree (Ranunculaceae) and in the "total evidence" tree of *Aquilegia* was tested using the likelihood-ratio test (Felsenstein 1988; Sanderson 1998). The results from likelihood-ratio test strongly rejected the clock-like hypothesis in both data sets (Ranunculaceae: $\chi^2 = 586.61$, $P = 1.42 \times 10^{-83}$; and *Aquilegia*: $\chi^2 = 261.85$, $P = 3.18 \times 10^{-35}$).

In order to estimate the age of main nodes, a Bayesian relaxed-clock analysis was performed using Beast 1.4.8 (Drummond and Rambaut 2007). We applied the GTR + I + Γ model with four categories on the Ranunculaceae-*matK* data set. The uncorrelated lognormal clock model was selected (Drummond et al. 2006). Unfortunately, no pre-quaternary fossils are known for *Aquilegia* or any closely related lineages. Therefore, we chose the age of the fossil *Prototinomiscium vangerowii* (Menispermaceae). The Menispermaceae are represented in the Cretaceous of Europe by endocarps assigned to the fossil genus *Prototinomiscium*. The oldest record, from the Turonian of Central Europe, dated to 91.0 million years (Myr) ago (Knobloch and Mai 1986), sets the minimum age of the split between Menispermaceae and Ranunculaceae (Anderson et al. 2005). Additionally, we used the age interval 51–66 Myr reported by Wikström et al. (2001) for the split of *Ranunculus* (subfamily Ranunculoideae) and *Xanthorhiza* (subfamily Coptidoideae), the former genus being in the sister subfamily to Thalictroideae, which includes the genus *Aquilegia* (Ro et al. 1997). Based on this information, we set priors of 91.0 ± 7.5 and 58.0 ± 2.5 Myr respectively for each calibration point. A Yule speciation model was selected as the tree prior. This is a simple model of speciation that is more appropriate when considering sequences from different species. In Beast 1.4.8, the normal distribution was applied to reflect the credibility intervals. The distribution of all of the other priors was set to uniform. The posterior distributions of the parameters were approximated using two independent Markov chain Monte Carlo analyses of 20 million generations followed by a discarded burn-in of 2 million generations (10%). The convergence of the chains was checked using TRACER 1.4.1 (Rambaut and Drummond 2007) and the effective sampling size (ESS) parameter was found to be much greater than 500, which suggests acceptable mixing and convergence. We show the estimated mean age (excluding the values in the burn-in) with the 95% HPD interval (the shorter interval that contains 95% of the sampled values).

Secondarily, a data set from the subfamily Thalictroideae, comprising 45 species and 1,612 characters, was used to estimate the dates of the main events of speciation within *Aquilegia*. This data set includes all of the available sequences of *Aquilegia* (Appendix 1) plus sequences of the allied genera (see Appendix 2). The ages and the respective confidence intervals estimated for the origin of Thalictroideae and the stem and crown node of *Aquilegia* were used in Beast 1.4.8. The analysis was developed following the same procedure described above for the Ranunculaceae data set, except we constrained the relationships of the different allied genera of *Aquilegia* to those reported by Wang and Chen (2007).

The role of ecology and geography in the *Aquilegia* diversification

Ancestral state reconstruction

We classified each species into one of six combinations of habitat use (forest, saxicolous, forest and meadows, saxicolous and meadows, forest-saxicolous-meadows and desert seep) and six discrete states for their pollination syndrome (syrphiid flies, bumblebees/bees, moths, hummingbirds, moths and bumblebees, and hummingbirds and bumblebees). The six categories used in each case reflected the existence of specialist and generalist species. Moreover, using the same number and type of states allows a better comparison of the number of transitions in habitat and pollinator use. Habitat and pollination data were gathered from our own field observations, specimen label data and the literature (Munz 1946; Pignatti 1982; Díaz Gonzáles 1986; Whittemore 1997; Fu et al. 2001; Nold 2003; Bacchetta et al. 2004; Arrigoni 2006; Polyakova et al. 2008). Although published studies on the pollinators of Eurasian columbines are very scarce, all of the available information unequivocally points to bumblebees as the main pollinators (Müller 1883; Knuth 1906-09; Lavergne et al. 2005; Tang et al. 2007; Herrera et al. 2008). Furthermore, because the flowers of most Eurasian columbines match the bumblebee pollination syndrome as described by Whittall and Hodges (2007) for North American columbines (short spurs, blue-purple and nodding flowers), we assigned the bumblebee pollination syndrome for those Eurasian columbines for which we lacked data regarding the main pollinators (see Table 2).

We applied the Bayesian mutational mapping approach (BMM) (Nielsen 2002; Huelsenbeck et al. 2003) for mapping the characters, using the post-burn-in tree file generated in the Bayesian analysis for the three combined data sets. We used a stochastic substitution model as implemented in SIMMAP (Bollback 2006) to calculate the posterior probability of each ancestral state reconstruction (SIMMAP considers only those trees possessing the node in question). We also used SIMMAP to explore the posterior expectation of the number of transitions for the habitat and pollination syndrome and between the different biogeographic lineages (SIMMAP re-scales the branch lengths of the tree in memory such that the overall length of the tree is one).

We used different parameter values for the priors to test the effect on the results of using a low-rate or high-rate prior on the tree length: (α:1 β:1), (α:3 β:2), (α:10 β:1) with 100 samples or realisations for each tree, of the 2,000 last trees from the post-burn-in tree file. The posterior probability of the ancestral states was mapped onto the well-supported nodes ($PP > 0.97$) of the phylogeny obtained from the combined data set.

Geographical patterns of speciation

Because both habitat- and pollinator-driven evolution could be influenced by the degree of geographical isolation, we estimated the degree of sympatry (Sd, hereafter) for the North American and European lineages. For both geographical lineages, we constructed a geographical overlap matrix for all of the species (Appendix 3) based on the Atlas Florae Europaea (Jalas and Suominen 1989) and maps from the Flora of North America (Whittemore 1997). Each species pair was assigned an Sd value of 1 if it was sympatric or 0 if it was allopatric. The Sd in each lineage was estimated as the mean Sd of the species in each clade. Finally, to explore the role of geographic isolation in the process of diversification, we plotted the absolute value of the independent contrast of the Sd at each node in the phylogeny against the "height" of that node (for details see Barrclough et al. 1998). The node height (meaning relative age of the split) and independent contrasts were calculated in the ape package of R (Paradis et al. 2004) for over 100 fully resolved phylogenies. Only the species included in the phylogeny were plotted. For the North American lineage, we also performed the same analysis on the consensus tree from Whittall and Hodges (2007).

Results

Phylogenetic analyses

The results from the three analyses that were performed are summarised in Table 1. These three analyses (ITS, cpDNA and combined ITS plus cpDNA) (Figs. 1, 2) clearly indicate the monophyletic origin of *Aquilegia*. The ITS analysis did not resolve the relationships within *Aquilegia*, as the main clades found in this analysis had low support. In turn, the cpDNA analysis (Fig. 1b) showed a well-supported clade containing all of the European species plus the Asiatic *A. glandulosa, A. sibirica, A. oxysepala* and *A. olympica*. Within this clade, the European species appear as monophyletic. In addition, we found a clade composed of *A. flabellata* and *A. turczaninovii* from East Asia, and *A. coerulea* from North America.

The combined analysis suggested a basal split within *Aquilegia* into two well-defined lineages: one containing all the European and some Asiatic species (Euro-Asiatic lineage; node labelled 3 in Fig. 2), and the other containing

Table 2 Summary of floral traits related to distinct pollinator syndromes in *Aquilegia*

	Pollinator	Mean spur length (mm)	Flower colour	Flower position (respect to floral axis)	References
A. nigricans	Bumblebees[b]	14.0	Blue-purple	Nodding	1, 2, 6
A. atrata	Bumblebees	12.5	Blue-purple	Nodding	1, 2, 6, 14[a], 15[a]
A. viscosa	Bumblebees	17.5	Blue-purple	Nodding	1, 2, 5, 8[a]
A. thalictrifolia	Bumblebees[b]	9.5	Blue-purple	Nodding/suberect	1, 2, 6
A. pyrenaica	Bumblebees	13.0	Blue-purple	Nodding/suberect	1, 2, 5, 12[a]
A. ottonis	Bumblebees[b]	13.5	Blue-purple	Nodding	1, 2, 6
A. nugorensis	Bumblebees[b]	16.5	Blue-purple	Nodding	1, 2, 6, 7
A. barbaricina	Bumblebees[b]	16.0	White	Nodding	1, 2, 6, 7
A. bernardii	Bumblebees[b]	16.0	Blue-purple	Nodding/suberect	1, 2, 6
A. einseleana	Bumblebees[b]	9.0	Blue-purple	Nodding	1, 2, 6
A. aurea	Bumblebees	14.0	Yellow	Suberect	1, 2, 5, 12[a]
A. alpina	Bumblebees[b]	21.5	Blue-purple	Nodding	1, 2, 6
A. vulgaris	Bumblebees	18.5	Blue-purple	Nodding	1, 2, 5, 8[a], 13[a], 15[a], 16[a]
A. coerulea	Moths/bumblebees	50.0	Blue/white	Erect	1, 2, 3, 9[a], 11[a], 13[a], 17[a]
A. flavescens	Hummingbirds/bumblebees	14.0	Yellow	Nodding	1, 2, 3, 9[a], 11[a], 13[a]
A. skinneri	Hummingbirds	40.08	Red/yellow/green	Nodding	1, 2, 3, 9[a], 11[a], 13[a]
A. laramiensis	Bumblebees	6.5	White	Nodding	1, 2, 3, 9[a], 13[a]
A. formosa	Hummingbirds/bumblebees	17.0	Red/yellow	Nodding	1, 2, 3, 9[a], 11[a], 13[a], 17[a]
A. canadensis	Hummingbirds/bumblebees	19.0	Red/yellow	Nodding	1, 2, 3, 9[a], 11[a], 13[a], 17[a]
A. scopulorum	Moths	27.5	Blue/white/yellowish	Erect	1, 2, 3, 9[a], 13[a]
A. chrysanta	Moths	53.5	Yellow	Erect	1, 2, 3, 9[a], 11[a], 13[a], 17[a]
A. elegantula	Hummingbirds	19.5	Red/yellow	Nodding	1, 2, 3, 9[a], 13[a], 17[a]
A. ecalcarata	Syrphid flies	0.00	Blue-purple	Suberect	1, 2, 4, 10[a]
A. oxysepala	Bumblebees[b]	17.5	Blue-purple/Yellowish	Nodding	1, 2, 4
A. yabeana	Bumblebees	18.5	Blue-purple	Nodding	1, 2, 4, 10[a]
A. sibirica	Bumblebees[b]	9.0	Blue-purple	Nodding	1, 2, 4
A. glandulosa	Bumblebees[b]	9.0	Blue-purple	Nodding/suberect	1, 2, 4
A. turczaninovii	Bumblebees[b]	16.5	Blue-purple	Nodding	1, 2, 4
A. fragans	Moths	17.5	White	Nodding/suberect	1, 2, 4, 11[a]
A. flabellata	Bumblebees[b]	14.0	Blue-purple	Nodding	1, 2, 4
A. viridiflora	Flies	15.0	Greenish	Nodding	1, 2, 4, 11[a]
A. olympica	Bumblebees[b]	17.5	Blue-purple	Nodding	1, 2, 4
S. adoxoides	?	0.00	Blue-purple/white	Nodding	4
U. henryi	?	0.00	Blue-purple/white	?	4

1 Munz (1946); 2 Nold (2003); 3 Whittemore (1997); 4 Fu et al. (2001); 5 Díaz Gonzáles (1986); 6 Pignatti (1982); 7 Arrigoni (2006); 8 Lavergne et al. (2005); 9 Whittall and Hodges (2007); 10 Tang et al. (2007); 11 Hodges and Arnold (1994); 12 Herrera et al. (2008); 13 Whittall (2005); 14 Knuth (1906-09); 15 Muller (1883); 16 Chase and Raven (1975), 17 Hodges et al. (2003) and references cited therein

[a] References employed to assign pollination vectors

[b] Pollinator assignment based on floral characteristics (pollination syndromes)

all the North American and some Asiatic columbines (Asiatic-North American lineage; node labelled 2 in Fig. 2). The first split within the Euroasiatic lineage separates *A. fragrans* from the rest of species. After this split, there is a polytomy with three groups: one containing *A. flabellata*, another formed by *A. ecalcarata* and *A. yabeana* and a third group (node labelled 6 in Fig. 2) comprising Siberian, Caucasian and European species. In this last group, both the cpDNA and combined data sets showed strong support for the monophyly of the European columbines (node labelled 7 in Fig. 2). However, none of the data sets resolved which Asiatic species in the group is most closely related to the European clade. The internal support for the relationships resolved within the European columbines was low in the cpDNA and combined analyses. However, the cpDNA and combined analyses (Figs. 1b, 2)

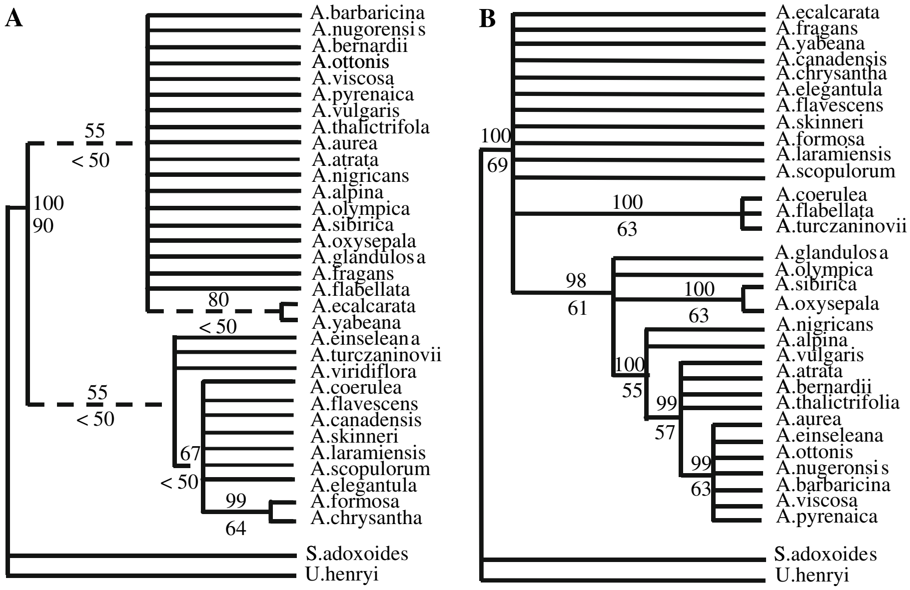


Fig. 1 Bayesian 50% majority-rule consensus trees. **a** Based on the ITS data set. **b** Based on the combined analysis of *trnK-matK* and *trnS-G* data sets. *Numbers above and below branches* are Bayesian posterior probabilities and boostrap percentages respectively. The *dashed lines* indicate the node not present in the strict consensus tree

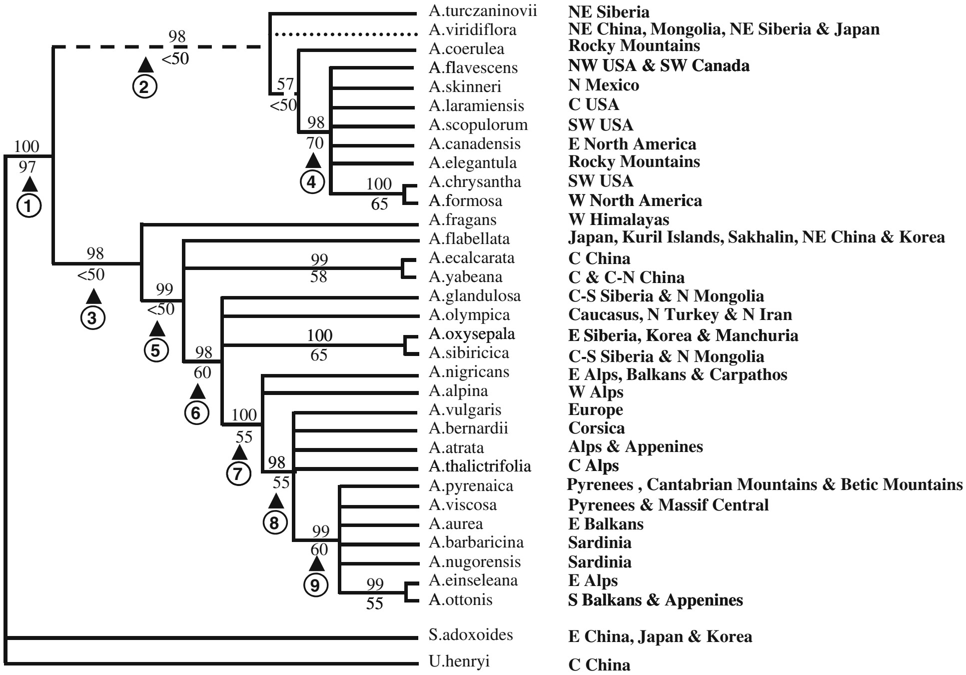


Fig. 2 Bayesian 50% majority-rule consensus tree of the combined analyses of ITS, *trnK-matK* and *trnS-G* sequences (total-evidence tree). *Numbers above and below branches* are Bayesian posterior probabilities and boostrap percentages respectively. The *dashed lines* indicate the node not present in the strict consensus tree. The *dotted line* indicates position of *A. viridiflora* based on ITS. Well-supported clades (used in the reconstruction of ancestral states) are indicated with *circled numbers*. Distribution ranges, indicated *to the right* of each species name, are based on Munz (1946), Nold (2003), Fu et al. (2001), Whittemore (1997), and Jalas and Suominen (1989)

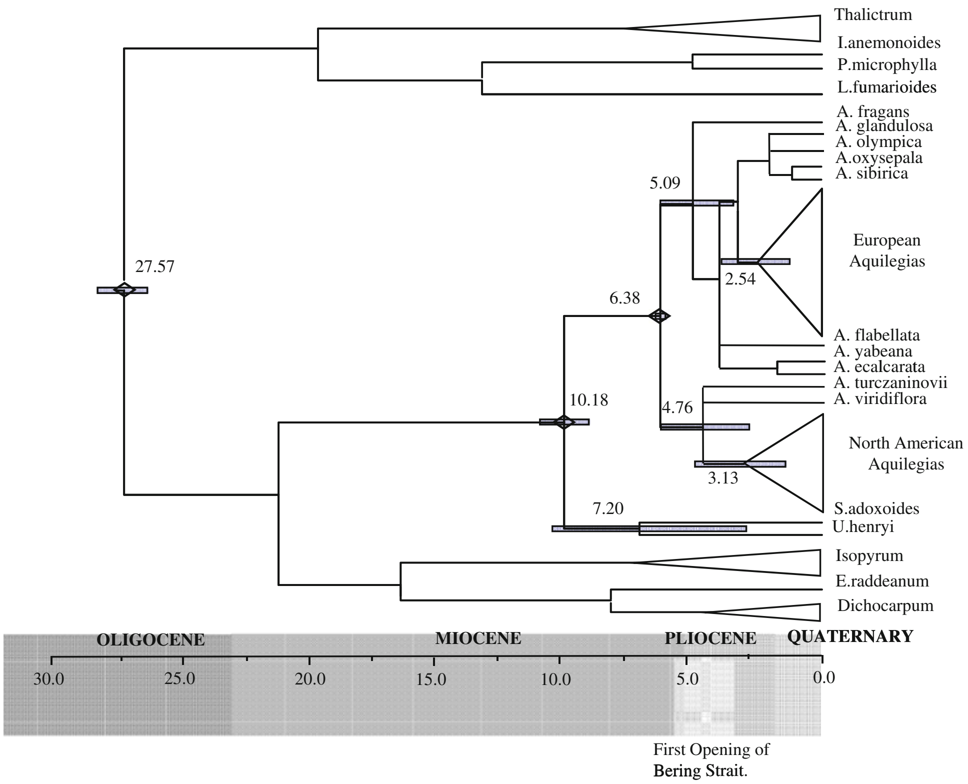
resolve *A. alpina* or *A. nigricans* as the probable sister to the rest of the species. Interestingly, the European clade contains a subclade (node labelled 9 in Fig. 2) formed by narrow endemic species from the mountains of southern Europe (*A. aurea* in the southern Balkans, *A. ottonis* in the southern Balkans and Apenines, *A. barbaricina* and *A. nugorensis* in Sardinia, *A. viscosa* in the Pyrenees, and *A. pyrenaica* in the Cantabrian, Pyrenees and Betic mountains), except for *A. einseleana,* which is widespread east of the Alps.

All of the analyses suggested a close relationship between North American *A. coerulea* and the Asiatic *A. turczaninovii* and *A. viridiflora*. The analysis based on the combined data set suggested that the rest of the North American species are a monophyletic group (node labelled 4 in Fig. 2). The intermediate position of *A. coerulea* between the Asiatic and North American species of this clade has low support.

Molecular clock analysis

The Bayesian relaxed clock of the Ranunculaceae data set dated the origin of Thalictroideae to 27.61 Myr ago (HPD interval: 26.59–28.56 Myr ago), and the stem and crown

Fig. 3 Chronogram of subfamily Thalictroideae from BEAST relaxed clock analysis. The nodes that were constrained (calibration points) are indicated with *squares*. Nodes of interest are shown with estimated age plus the node age 95% HPD interval (the shorter interval that contains 95% of the sampled values)



node of *Aquilegia* to 10.18 Myr ago (9.21–11.14 Myr ago) and 6.38 Myr ago (6.18–6.57 Myr ago) respectively. This preliminary analysis placed the origin of genus *Aquilegia* in the late Miocene. These estimates were used to calibrate the Thalictroideae data set (Fig. 3). The Bayesian relaxed clock of Thalictroideae dated a relatively early basal split within *Aquilegia,* giving rise to the Euroasiatic (mean 5.09 Myr ago, HPD interval: 3.49–6.48 Myr ago) and Asiatic-North American (mean 4.76 Myr ago, HPD interval: 2.87–6.48 Myr ago) lineages, between the late Miocene and the early Pliocene. After this initial split, some columbines entered North America in the Pliocene, where the lineage began to diversify around 3.13 Myr ago (1.42–5.01 Myr ago). Similarly, the European columbines began to diversify around 2.54 Myr ago (1.25–3.96 Myr ago).

The evolution of habitat and pollination syndrome

To obtain a general view of the evolutionary relationships among columbines and the environment in terms of the type of habitat occupied and the pollinators, we used our "total-evidence" phylogeny to reconstruct the ancestral states of these variables. Because the posterior probabilities of the ancestral states obtained using different parameter values were not different, we show only the posterior probabilities for the ancestral states for the default parameter values of SIMMAP (α: 3 and β: 2; Fig. 4).

The expected number of habitat transitions through the phylogeny ranges from 22.26 to 24.47. The Euroasiatic lineage showed slightly more habitat transitions than the Asiatic-North American lineage (Table 3). The reconstruction of the habitat occupied did not resolve the ancestral habitat states of the genus. However, the basal node leading to the Asiatic-North American lineages was probably a generalist ancestor occupying many habitats, from the forest to rocky places and meadows. In turn, the most recent common ancestor of the North American clade likely occurred mainly in forests, and the clade subsequently spread and specialised in many habitats. Although our results suggest a shift in habitat use from the direct ancestor of the North American clade to the forest habitat, this result might be biased since the species from the forested habitats are overrepresented in our sample of this clade. About 22% of our sampled species of the North American clade occur in rocky habitats (saxicolous or saxicolous and meadows), but around 57% of all North American species belong to this habitat. This bias is not as strong in the European clade because around 61% of the sampled species occur in saxicolous habitats, and this percentage is around 70% when considering all European columbines. Apparently, the opposite trend occurred in the basal node of the Euroasiatic lineages, where the most probable state was saxicolous and meadows. This ancestral state shifted to forest and meadows in the two successive nodes leading to the rest of the Asiatic species and remained as the most probable ancestral state of

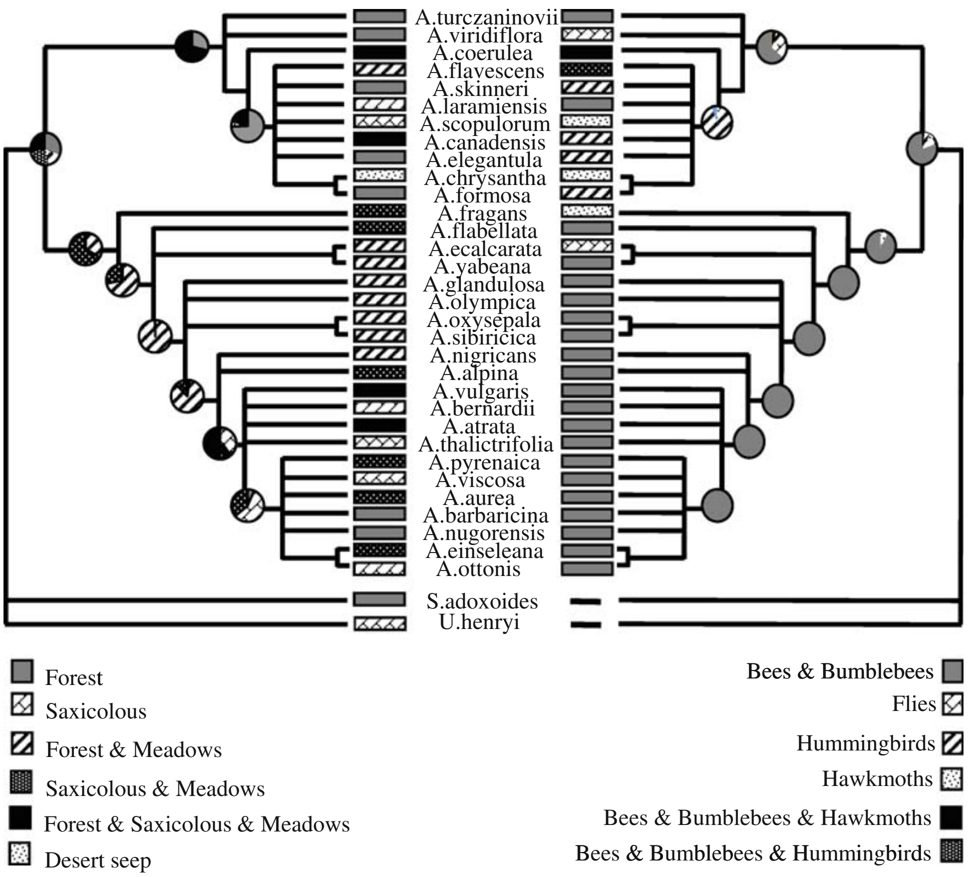


Fig. 4 Estimation of ancestral states, using the Bayesian mutational mapping approach (SIMMAP), for habitat and pollination syndrome. Each portion in the pie diagrams corresponds to mean posterior probability for each character state

the European species. Within the European species, there was a shift from the Asiatic forest and meadows ancestor to a forest, meadows and saxicolous ancestral state (a generalist ancestor). In the following node leading to the South European columbines, the ancestral state shifted to saxicolous or saxicolous and meadows.

Finally, the ancestral state reconstruction for the pollination syndrome indicates that the ancestor of the genus was pollinated by bees/bumblebees. Bees/bumblebees are the most likely pollinators of all ancestors leading to the European clade, which has retained this state. The ancestral pollination state of the Asiatic-North American lineage seems to be bee/bumblebee, although its posterior probability is relatively low. However, the most recent common ancestor of the North American clade may have been pollinated by hummingbirds. The expected number of transitions for the pollination syndrome through the phylogeny varied from 12.25 to 14.04. In contrast to the habitat transitions, the greatest number of pollination transitions was concentrated within the North American lineage (Table 3).

Table 3 Expected number of transitions for habitat and pollination syndrome in the whole sample of species and within the two main lineages identified in the phylogeny. States used for each ecological factor are indicated in Fig. 4

Ecological factors (no. of states)	Lineages	Rate priors		
		(1,1)	(3,2)	(10,1)
Habitat (6)	All species	22.26	22.14	24.47
	Eurasia	14.40	14.29	15.83
	Asia-North America	10.53	10.30	12.72
Pollination syndrome (6)	All species	13.53	12.25	14.04
	Eurasia	2.15	2.16	3.06
	Asia-North America	7.38	7.39	13.84

Three different rate priors were used to assess the influence of different choices on the estimated number of transitions

Geographical speciation patterns

Our estimation of sympatry revealed significant differences in the Sd values between the European and North American lineages (Mann–Whitney $Z_{(\text{adjusted})} = -2.16$, $P = 0.03$; mean Sd ± 1 standard error: 0.18 ± 0.037 and 0.25 ± 0.036 respectively). Interestingly, the European and American lineages showed contrasting patterns of geographical speciation when the Sd was plotted against node height (Fig. 5). The North American clade showed a negative correlation between sympatry and node height (Pearson $r = -0.92$, $P = 0.001$), which suggests that recent events of speciation have occurred in sympatry. This

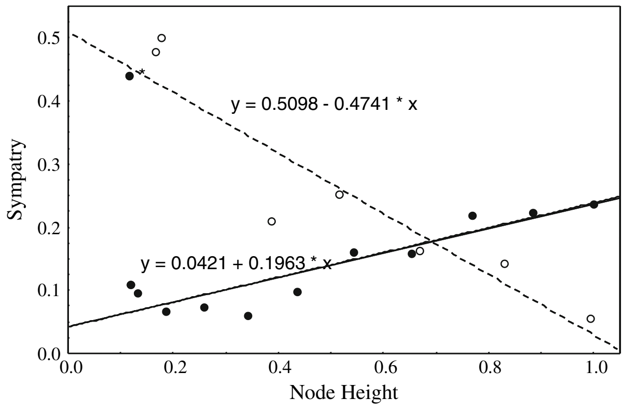


Fig. 5 Relationship between sympatry degree and node height in the European (*black points*) and North American (*white points*) lineages. A recent node with unusually high degree of sympatry in the European lineage (marked with an *asterisk*) was not included to fit the regression line. The correlations between sympatry degree and node height were $r = 0.91$ and 0.93 ($P < 0.01$) in the European and North American clade respectively

trend was confirmed for the complete North American columbine phylogeny ($r = -0.49$, $P = 0.02$). In contrast, the European clade showed a positive relationship between sympatry and node height ($r = 0.91$, $P = 0.00008$), suggesting that recent speciation events have occurred in allopatry.

Discussion

Columbine radiations in space and time

The phylogenetic analysis presented here represents the first study of the genus *Aquilegia* that includes a balanced sample of species from the three continents where it occurs. Moreover, in contrast to previous analyses of the genus, it was based on three DNA data sets, including nuclear and plastid regions: ITS, *trnS-G* and *trnK-matK*. Though none of the data sets (ITS, cpDNA, and the combined data set) had enough variation individually to fully resolve most of the inter-specific relationships in the terminal nodes, collectively they provided good resolution at most of the internal nodes.

According to our analyses, the split between *Semiaquilegia-Urophysa* and *Aquilegia* was dated 10.18 Myr ago. The calibration points that we used for the molecular clock are phylogenetically distant from *Aquilegia*, which might add error to the estimated time of origin for the genus. However, the estimated substitution rates agree with other studies. The mean substitution rate in *trnK-matK* within *Aquilegia* was 0.43×10^{-9}. Similar rates have been obtained for the genus *Paeonia* (0.4×10^{-9}, Sang et al. 1997) or within the family Leguminosae (0.21 to 2.46×10^{-9}, Lavin et al. 2005). Moreover, the mean substitution rate in ITS (2.35×10^{-9} subs/site/year) is well within the range found in other herbs (mean: 4.13×10^{-9} subs/site/year; range: $1.72–8.74 \times 10^{-9}$ subs/site/year; Kay et al. 2006). Thus, our molecular clock estimate of the time of origin of the genus seems reasonable.

The first split within *Aquilegia* gave rise to the Euro-Asiatic and Asiatic-North American clades some 6.38 Myr ago, between the late Miocene and early Pliocene. All our analyses place *A. turczaninovii* within the lineage that included all the North American species. Our analysis of ITS, like those of Hodges and Arnold (1994) and Ro and McPheron (1997), also placed *A. viridiflora* within this clade. Both *A. turczaninovii* and *A. viridiflora* occur in northeastern Asia, which suggests that columbines entered North America through Beringia. According to our molecular clock analysis, this colonisation took place during the Pliocene (Fig. 3), between 3.13 and 4.76 Myr ago, as tentatively proposed by Grant (1994). Moreover, this age of colonisation agrees with the estimated time of first opening of the Bering Strait, between 3.1 and 5.5 Myr ago (Marincovich and Gladenkov 2001, and references therein), closing the terrestrial connection between Asia and America during a time with a cool-temperate climate (Matthiessen et al. 2009). This connection (the Beringian land bridge) emerged again several times during the glacial ages, when the climate in the region was not favourable for the columbines. This may partially explain why North America has not been colonised repeatedly from Asia and why columbines have not travelled back to Asia from North America. Moreover, other features of this genus, such as the small seed size (1.5–2 mm) and lack of special appendages for seed dispersal make long-distance dispersal events highly unlikely (Hodges and Arnold 1994; Strand et al. 1996).

On the other hand, we found a Euro-Asiatic lineage in which the earliest branch corresponds to *A. fragrans* (from the Himalayas, north-western India), followed by an unresolved split involving *A. flabellata* (from the Pacific islands of northern Japan, Sakhalin and the Kurils) and the sister species *A. ecalcarata* and *A. yabeana*. This relationship was also suggested for *A. fragrans* by Ro and McPheron (1997). Interestingly, *A. ecalcarata*, pointed out by some authors as a possible ancestor of the genus (based on its spurless condition), appears as sister to *A. yabeana*. The position of *A. ecalcarata* within the Euro-Asiatic lineage is in accordance with the results of Ro and McPheron (1997), which placed it, with low support, as a sister to the European columbines. Thus, our phylogeny suggests that the spurless condition of *A. ecalcarata* may be a secondary loss, in agreement with the results of Wang and Chen (2007).

The biogeographic links between the Asiatic and European columbines seem complex according to our results. *A. ecalcarata* and *A. yabeana* overlap their distribution in central China, while *A. flabellata* occurs in north-east China, Korea and the neighbouring islands (Japan, Kurils and Sakhalin). Although the relationships between these three species and the rest of the clade have not been resolved by our analyses, their similar positions in the phylogeny and their geographic distributions suggest that they might belong to a subclade that migrated east from the Himalayas through central China, reaching the Pacific islands. On the other hand, the Asiatic species most closely related to the European columbines occur in the mountain systems of south-central Siberia (*A. sibirica*, *A. oxysepala* and *A. glandulosa*) and the Caucasus (*A. olympica*). Thus, it seems possible that the Asiatic ancestor of the European columbines lived somewhere in the central Asian mountains (Altai and Tien Shan). Actually, this is the most important centre of diversity for Asiatic columbines, because 10 out of 23 Asiatic species inhabit this area (while two species occur in the Caucasus, four in western and southern Himalaya, and seven in eastern China, Korea and Japan). These central Asian mountains have long been regarded as important source areas both for the European (Comes and Kadereit 2003) and North American (Weber 2003) mountain flora; therefore, the present phylogeny adds another piece of evidence for these biogeographical connections.

The European columbines also form a well-supported clade dated to the late Pliocene. On biogeographical grounds, a closer relationship with *A. olympica*, from the Caucasus, seems likely. Moreover, the basal European columbines are *A. nigricans* (from the Balkans, Carpathian Mountains and Apenines) or *A. alpina* (from the Alps). These results, coupled with the molecular clock analysis, suggest that some 2.54 Myr ago, a lineage from the central Asian mountains migrated to Europe via the Caucasus and Balkans, finally reaching the Alps. Similar routes have been traversed by other species. For example, populations of *Carex atrofusca* (Cyperaceae) in the Alps originated by immigration from central Asia (Altai mountains) through the Carpathian mountains in eastern Europe (Schönswetter et al. 2006). Besides the basal European columbines, a terminal clade composed of seven species is clearly identified. This clade groups species with narrow distributions in the mountains of southern Europe, from the Balkans to the Betic mountains (southern Spain). This suggests that these species may have originated from populations of a widely distributed ancestral species isolated in different glacial refugia.

Ecological and geographical patterns of diversification

One of the main objectives in the study of adaptive radiations is to establish the relationships between environmental factors and the process of diversification (Schluter 2000). We have used the reconstruction of ancestral states for several ecological factors in order to assess which ones may have been linked to the radiation of the European and North American columbines. The accuracy of the ancestral state reconstruction depends on the availability of a well-resolved phylogeny and on the inclusion of all the taxa. To minimise the effect of violating these conditions, we have considered only the ancestral states reconstructed for well-supported nodes of the phylogeny. However, if the distribution of character states of the taxa included in the phylogeny is biased compared to their distribution in the whole clade, then the reconstruction will be biased. The species that we have used in this study were chosen on the basis of their availability rather than on a particular ecological characteristic, so they most likely represent an unbiased sample of states. Still, small samples of taxa (i.e. subsamples of the terminal clades), even if chosen at random, are likely to be biased for some states. Therefore, our reconstructions of ancestral states must be taken as preliminary descriptions. On the other hand, Paradis (2008) showed that while current methods to reconstruct ancestral states might be misleading, particularly at the root of a phylogeny, they offer nearly unbiased estimates of the rates of character change. Indeed, our interest in reconstructing the ancestral character states is more centred on evaluating the frequency of changes in different ecological factors (habitat use and pollination syndrome) as a way to assess their influence in the process of radiation.

Whittall and Hodges (2007) identified at least seven independent shifts among the pollinator types within the North American clade. Our results indicate a minimum of 12.25 transitions between the types of pollinators in the whole genus (including pollination by flies, not considered by these authors). These transitions were concentrated in the Asiatic-North American clade with at least 7.38 shifts, while the Euroasiatic columbines showed only 2.15 shifts, since they have retained the ancestral bee/bumblebee pollination of the genus. This conservation of the ancestral pollinator type in Eurasia is striking because hawkmoths are relatively frequent and widespread on this continent. Thus, given the scarcity of studies on the pollinators of Eurasian columbines, we cannot discard the possibility that hawkmoths may play a role in the pollination of some European or Asiatic species. Still, combined with the current knowledge on the evolution of pollination syndromes in columbines and the palaeontological evidence on the evolution of hummingbirds, our results suggest that a historical contingency may have prevented the diversification of the pollination syndromes in the Euroasiatic columbines. Whittall and Hodges (2007) showed that the hummingbird pollination syndrome could have acted as a “stepping stone" between the bumblebee and hawkmoth pollination syndromes in North American columbines. On the other hand, hummingbirds were present in western Europe during the Oligocene (Mayr 2004; Bochenski and Bochenski 2008; Louchart et al. 2008), but they had gone extinct there before the columbines began to diversify. Thus, it is possible that the absence of hummingbirds in Eurasia could have constrained the floral evolution to a hawkmoth pollination syndrome in this continent.

Another consequence of the use of the same type of pollinators in European columbines would be a higher probability of hybridisation. Pollinator specialisation, mediated by floral traits, has been shown to act as a very effective barrier to hybridisation between sympatric columbines, such as *A. formosa* and *A. pubescens* (Fulton and Hodges 1999), *A. micrantha* and *A. elegantula* (Miller and Willard 1983), or *A. ecalcarata* and *A. incurvata* (Tang et al. 2007). However, differences in the floral phenotype are quite conspicuous between these pairs of species. For example, while the floral parts in *A. formosa* and *A. elegantula* are red and yellow, those in their sympatric counterparts can be white, pink (in *A. micrantha*), or cream, pink or yellow (in *A. pubescens*). While *A. incurvata* has spurs, *A. ecalcarata* lacks them. Accordingly, very different pollinators pollinate each member of these pairs: hummingbirds vs. hawkmoths (in *A. formosa* and *A. pubescens* respectively), hummingbirds vs. bees and bumblebees (in *A. elegantula* and *A. micrantha* respectively), and syrphiid flies vs. bumblebees (in *A. ecalcarata* and *A. incurvata* respectively). As far as we know, no studies have documented whether this type of barrier to hybridisation is as effective between pairs of sympatric species with more subtle differences in floral traits that share the same type of pollinators as would be the case for virtually every pair of locally sympatric European columbines (for example, *A. vulgaris* and *A. pyrenaica* in the Iberian Peninsula or *A. barbaricina* and *A. nugorensis* in Sardinia). In any case, sympatry among columbines is less frequent in Europe than in North America, which suggests that European columbines may share the same type of pollinators without incurring hybridisation.

The wide range of habitats occupied by the columbines and the frequency of habitat transitions in the phylogeny suggest that changes in habitat use must have been a further creative force in the diversification of the genus. This role of habitat diversification seems to have been more relevant in the Euroasiatic lineage (a minimum of 14.29 shifts) compared to the Asiatic-North American lineage (10.30 shifts). The ancestors of the European columbines likely occupied forests and meadows. However, a trend toward inhabiting more saxicolous habitats seems to have been related to the recent diversification of the group. Adaptation to these saxicolous habitats may have enhanced the probability of survival and differentiation of these species in their southern refugia during the glacial-interglacial cycles. This type of habitat, usually located at a high altitude, with a steep terrain, shallow soils, and frequent exposure to harsh weather, likely remained scarcely forested through the Holocene, providing open habitats for the persistence of alpine species after the Last Glacial Maximum (Birks and Willis 2008). It is noteworthy that this type of habitat is characteristic of the subclade of European columbines that groups the endemic species with narrow distributions in the mountain systems of southern Europe (the Pyrenees, Betic Mountains, Alps, Appenines and Balkans). This possible link between the use of rocky habitats and endemicity has been described as a general trend for the plant species of western Europe (Lavergne et al. 2004) and agrees with the allopatric mode of recent speciation we found for the clade. Thus, our results suggest that an ancestral columbine may have been widely distributed in the mountains of southern Europe, where it occupied open mountain habitats (rocky places and/or meadows) and gave rise, through allopatric speciation promoted by geographic isolation in different mountain systems, to a clade which largely retained this type of habitat.

For the North American clade, forest or generalist (forest, meadows and saxicolous) are the most likely ancestral states. In any case, the extent and density of these types of habitats available during the late Pliocene and the warm stages of the glacial cycles may have allowed a widespread distribution of the ancestral North American columbines (Strand et al. 1996), reducing the opportunity for allopatric speciation through geographic isolation. Thus, habitat and pollinator specialisation may have been the predominant forces promoting the speciation of North American columbines in sympatry.

Conclusions

These phylogenetic analyses suggest that the high diversity of species in the genus is the result of two independent events of radiation leading respectively to the North American and European clades. Both radiations show a common biogeographic pattern: (1) an origin in the south-central Siberian mountains, (2) the unidirectional, intercontinental migration of a single ancestral lineage, and (3) the subsequent colonisation and radiation in each continent starting in the Pliocene, approximately 3 Myr ago. However, the processes of diversification have been different in each continent. Our results strongly support the hypothesis that geographic isolation (the allopatric mode of speciation) coupled with habitat specialisation must have been the basic processes driving the radiation of the European columbines, while the radiation of the North American columbines must have been driven by habitat specialisation coupled with pollinator specialisation, thus promoting a sympatric mode of speciation. This difference would be largely the result of a biogeographical-historical contingency. Hummingbirds were present in North America by the time the first columbines arrived, allowing for the possibility of a radiation that was based on pollinator specialisation. However, although hummingbirds were present in Europe 30-34 Myr ago, they had left the continent by the time columbines arrived.

Acknowledgments We are grateful to the many botanical gardens (listed in Appendix 1) that provided plant material used in this study. Emilio Cano (Real Jardín Botánico de Madrid) generously offered assistance in molecular techniques. This work was supported by the Spanish Ministerio de Educación y Ciencia (projects BOS2003-03979-C02/01-02 and CGL2006-02848). During this work J.M.B. was supported with grant BES-2004-3387 of Spanish MEC. We thank Zhi Duan Chen and Wei Wang for kindly providing the molecular sequences for some of the outgroup taxa used in our study.

References

Alfaro ME, Zoller S, Lutzoni F (2003) Bayes or bootstrap: a simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and boostrapping in assessing phylogenetic confidence. Mol Biol Evol 20:255–266

Anderson CL, Bremer K, Friis EM (2005) Dating phylogenetically basal eucots using rbcl sequences and multiple fossil reference points. Am J Bot 92:1737–1748

Arrigoni PV (2006) Flora dell'Isola di Sardegna, vol 1. Carlo Delfino, Sassari

Bacchetta G, Iiriti G, Mossa L, Pontecorvo C, Serra G (2004) A phytosociological study of the *Ostrya carpinifolia* Scop. woods in Sardinia (Italy). Fitosociologia 41:67–75

Barrclough TG, Vogler AP, Harvey PH (1998) Revealing the factors that promote speciation. Phil Trans R Soc Lond B 353:241–249

Beardsly PM, Schoening CB, Whittall JB, Olmstead RG (2004) The radiation of *Mimulus* in the western North America: systematic, hybridization, chromosomal evolution, cryptic biobiversity, and patterns of rarity. Am J Bot 91:474–489

Birks HJB, Willis KJ (2008) Alpines, trees and refugia in Europe. Plant Ecol Divers 1:147–160

Bochenski Z, Bochenski ZM (2008) An old world hummingbird from the Oligocene: a new fossil from Polish Carpathians. J Ornith 149:211–216

Bollback JP (2006) SIMMAP: stochastic character mapping of discrete traits on phylogenies. Bioinformatics 7:1–17

Chase VC, Raven PH (1975) Evolutionary and ecological relationship between *Aquilegia formosa* and *Aquilegia pubescens* (Ranunculaceae): two perennial plants. Evolution 29:474–486

Comes HP, Kadereit JW (2003) Spatial and temporal patterns in the evolution of the flora of the European Alpine system. Taxon 52:451–462

Damerval C, Nadot S (2007) Evolution of perianth and stamen characteristics with respect to floral symmetry in Ranunculales. Ann Bot 100:631–640

Díaz Gonzáles TE (1986) "G. *Aquilegia*". In: Castroviejo et al. (eds) *Flora Ibérica*. Real Jardín Botánico, C.S.I.C, Madrid

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed phylogentics and dating with confidence. PLoS Biol 4:e88

Drumond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214

Ellis AG, Weis AE, Gaut BS (2006) Evolutionary radiations of "stone plants" in the genus *Argyroderma* (Aizoaceae): unravelling the effects of landscape, habitat and flowering time. Evolution 60:39–55

Farris JS, Källersjö M, Kluge AG, Bult C (1994) Testing significance of incongruence. Cladistics 10:315–319

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Felsenstein J (1988) Phylogenies from molecular sequences: inference and reliability. Annu Rev Genet 22:521–565

Francisco-Ortega J, Jansen RK, Santos-Guerra A (1996) Chloroplast DNA evidence of colonization, adaptive radiation, and hybridization in the evolution of the Macaronesian flora. Proc Natl Acad Sci USA 93:4085–4090

Francisco-Ortega J, Crawford DJ, Santos-Guerra A, Jansen RK (1997) Origin and evolution of *Argyranthemum* (Asteraceae: Anthemideae) in Macaronesia. In: Givnish TJ, Sytsma KJ (eds) Molecular evolution and adaptive radiation. Cambridge University Press, Cambridge, pp 406–431

Francisco-Ortega J, Fuertes-Aguilar J, Gómez-Campo C, Santos-Guerra A, Jansen RK (1999) ITS sequence phylogeny of *Crambe* L. (Brassicaceae): molecular data reveal two old world disjunctions. Mol Phyl Evol 11:361–380

Fu D, Li L, Bartholomew B, Brach AR, Dutton BE, Gilbert MG, Kadota Y, Robinson OR,Tamura M, Warnock MJ, Guanghua Z, Ziman SN(2001) Ranunculaceae. In: Wu Z, Raven PH, Hong D (eds) Flora of China, vol. 6.Missouri Botanical GardenPress, St. Louis, pp 133–148

Fulton M, Hodges SA (1999) Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. Proc R Soc Lond B 266:2247–2252

Gafta D, Muncaciu S, Csergö A-M (2006) Morphometric variation in a rare endemic *Aquilegia* (Ranunculaceae) in the Carpathians. Plant Biosyst 140:297–306

García-Maroto F, Mañas-Fernández A, Garrido-Cárdenas JA, López Alonso D, Guil-Guerrero JL, Guzmán B, Vargas P (2009) Δ^6-Desaturase sequence evidence for explosive Pliocene radiations within the adaptive radiation of Macaronesian *Echium* (Boraginaceae). Mol Phyl Evol 52:563–574

Givnish TJ, Montgomery RA, Goldstein G (2004) Adaptive radiation of photosynthetic physiology in the Hawaiian lobeliads: light regimes, static light responses, and whole-plant compensation points. Am J Bot 91:228–246

Grant V (1993) Origin of floral isolation between ornithophilous and sphingophilous plant species. Proc Natl Acad Sci USA 90:7729–7730

Grant V (1994) Historical development of ornithophily in the western North American flora. Proc Natl Acad Sci USA 91:10407–10411

Grant V, Grant KA (1965) Flower pollination in the phlox family. Columbia University Press, New York

Guzmán B, Lledó MD, Vargas P (2009) Adaptive radiation in Mediterranean *Cistus*. PLoS One 4:1–13

Hall TA (1999) BioEdit: a user-friendly biological sequence alignmeant edition and analysis program for Windows 95/98/NT. Nucleid Acids Symp Ser 41:95–98

Hamilton MB (1999) Four primers pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol Ecol 8:521–523

Herrera CM, García IM, Pérez R (2008) Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants. Ecology 89:2369–2376

Hodges SA (1994) Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. Proc Natl Acad Sci USA 91:2493–2496

Hodges SA (1997) Floral nectar spurs and diversification. Int J Plant Sci 158:S81–S88

Hodges SA, Arnold ML (1994) Columbines: a geographically widespread species flock. Proc Natl Acad Sci USA 91:5129–5132

Hodges SA, Arnold ML (1995) Spurring plant diversification: are floral nectar spurs a key innovation? Proc R Soc Lond B 262:343–348

Hodges SA, Fulton M, Yang JY, Whittall JB (2003) Verne Grant and evolutionary studies of *Aquilegia*. New Phytol 161:113–120

Huelsenbeck JP, Nielsen R, Bollback JP (2003) Stochastic mapping of morphological characters. Syst Biol 52:113–158

Hughes C, Eastwood R (2006) Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. Proc Natl Acad Sci USA 103:10334–10339

Jalas J, Suominen J (1989) Atlas Florae Europaeae. Distribution of vascular plants in Europe. 8. Nymphaceae to Ranunculaceae. The Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo, Helsinki

Johnson LA, Soltis DE (1994) *MatK* DNA and phylogenetic reconstruction in Saxifragaceae s. str. Syst Bot 19:143–156

Kay KM, Whittall JB, Hodges SA (2006) A survey of nuclear ribosomal internal transcribed spacer substitution rates across angiosperms: an approximate molecular clock with life history effects. BCM Evol Biol 6:36

Kelchner SA (2000) The evolution of non-coding chloroplast DNA and its applications on plant systematics. Ann Mo Bot Gard 87:482–498

Knobloch E, Mai DH (1986) Monographie der Früchte und Samen in der Kreide von Mitteleuropa. Rozpravy ústredního ústavu geologickénho Praha 47:1–219

Knuth P (1906-09) Handbook of flower pollination. Clarendon, Oxford

LaRoche G (1978) An experimental study of population differences in leaf morphology of *Aquilegia canadensis*. Am Midl Nat 100:341–349

LaRoche G (1980) The effects of restricting root growing space, decreasing nutrient supply and increasing water stress on the phenetics of *Aquilegia canadensis* L. (Ranunculaceae). Bull Torrey Bot Club 107:220–222

Lavergne S, Thompson JD, Garnier E, Debussche M (2004) The biology and ecology of narrow endemic and widespread plants: a comparative study of trait variation in 20 congeneric pairs. Oikos 107:505–518

Lavergne S, Debussche M, Thompson JD (2005) Limitations on reproductive success in endemic *Aquilegia viscosa* (Ranunculaceae) relative to its widespread congener *Aquilegia vulgaris*: the interplay of herbivory and pollination. Oecologia 142:212–220

Lavin M, Herendeen P, Wojciechowski MF (2005) Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the Tertiary. Syst Biol 54:530–549

Louchart A, Tourment N, Carrier J, Roux T, Mourer-Chauviré C (2008) Hummingbird with modern feathering: an exceptionally well-preserved Oligocene fossil from southern France. Naturwissenschaften 95:171–175

Marincovich L Jr, Gladenkov AY (2001) New evidence for the age of Bering Strait. Quaternary Sci Rev 20:329–335

Matthiessen J, Knies J, Vogt C, Stein R (2009) Pliocene palaeoceanography of the Arctic Ocean and subarctic seas. Phil Trans R Soc A 367:21–48

Mayr G (2004) Old World fossil record of modern—type hummingbirds. Science 304:810–811

Medrano M, Castellanos MC, Herrera CM (2007) Comparative floral and vegetative differentiation between two European *Aquilegia* taxa along a narrow contact zone. Plant Syst Evol 262:209–224

Miller RB, Willard CL (1983) The pollination ecology of *Aquilegia micrantha* (Ranunculaceae) in Colorado. Southw Nat 28:157–164

Müller H (1883) The fertilization of flowers. MacMillan, London

Munz PA (1946) *Aquilegia*: the cultivated and wild columbines. In: Bailey LH (eds) Gentes Herbarium 7:1-50

Nielsen R (2002) Mutations mapping on phylogenies. Syst Biol 51:729–732

Nold R (2003) Columbines. *Aquilegia*, *Paraquilegia* and *Semiaquilegia*. Timber Press, Cambridge

Paradis E (2008) Asymmetries in phylogenetic diversification and character change can be untangled. Evolution 62:241–247

Paradis E, Claude J, Strimmer K (2004) APE: analysis of phylogenetics and evolution in R language. Bioinformatics 20:289–290

Patterson TB, Givnish J (2003) Geographic cohesion, chromosomal evolution, parallel adaptive radiations, and consequent floral adaptations in *Calochortus* (Calochortaceae): evidence from a cpDNA phylogeny. New Phytol 161:253–264

Pignatti S (1982) Flora d'Italia. Edagricole, Bologna

Polyakova MA, Dits LY, Ermakov NB (2008) Studies on biological features of band pine forests in the intermontane Minusinsk depression by methods of gradient analysis. Russ J Ecol 39:238–245

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818

Rambaut A, Drummond AJ (2007) Tracer v.1.4.1. http://beast.bio.ed.ac.uk/tracer

Ro K-E, McPheron B (1997) Molecular phylogeny of the *Aquilegia* group (Ranunculaceae) based on internal transcribed spacers and 5.8S nuclear ribosomal DNA. Biochem Syst Ecol 25:445–461

Ro K-E, Keener CS, McPheron BA (1997) Molecular phylogenetic study of the Ranunculaceae: utility of the nuclear 26S ribosomal DNA in inferring intrafamilial relationships. Mol Phyl Evol 8:117–127

Robichaux RH, Carr GD, Liebman M, Pearcy RW (1990) Adaptive radiation of the Hawaiian silversword alliance (Compositae-Madiinae): ecological, morphological and physiological diversity. Ann Mo Bot Gard 77:64–72

Rodríguez F, Oliver JF, Marín A, Medina JR (1990) The general stochastic model of nucleotide substitution. J Theor Biol 142:485–501

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Sanderson MJ (1998) Estimating rate and time in molecular phylogenies: beyond the molecular clock? In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants II: DNA sequencing. Kluwer, New York, pp 242–264

Sang T, Crawford DJ, Stuessy TF (1997) Chloroplast DNA phylogeny, reticulate evolution and biogeography of *Paeonia* (Paeoniaceae). Am J Bot 84:1120–1136

Schluter D (1996) Ecological causes of adaptive radiation. Am Nat 148:S40–S64

Schluter D (2000) The ecology of adaptive radiation. Oxford Series in Ecology and Evolution. Oxford University Press, New York

Schönswetter P, Popp M, Brochmann C (2006) Central Asian origin of and strong genetic differentiation among populations of the rare and disjunct *Carex atrofusca* (Cyperaceae) in the Alps. J Biogeogr 33:948–956

Semerikov VL, Zang HQ, Sun M, Lascoux M (2003) Conflicting phylogenies of *Larix* (Pinaceae) based on cytoplasmatic and nuclear DNA. Mol Phyl Evol 27:173–184

Simmons MP, Ochotorena H (2000) Gaps as characters in sequence-based phylogenetic analyses. Syst Biol 49:369–381

Stebbins GL (1970) Adaptive radiation of reproductive characteristics in angiosperms. I. Pollination mechanisms. Annu Rev Ecol Syst 1:307–326

Strand AE, Brook GM, Pruit CS (1996) Are populations island? Analysis of chloroplast DNA variation in *Aquilegia*. Evolution 50:1822–1829

Sun Y, Skinner DZ, Liang GH, Hulbert SH (1994) Phylogenetic analysis of sorghum and related taxa using internal transcribed spacer of nuclear ribosomal DNA. Theor Appl Gen 89:26–32

Swofford DL (2002) PAUP*: phylogenetic analysis using parsimony, version 4.010b. Sinauer Associates, Sunderland

Tang L-L, Yu Q, Sun J-F, Huang S-Q (2007) Floral traits and isolation of three sympatric *Aquilegia* species in the Qinling Mountains, China. Plant Syst Evol 267:121–128

Tucker SC, Hodges SA (2005) Floral ontogeny of *Aquilegia*, *Semiaquilegia*, and *Enemion* (Ranunculaceae). Int J Plant Sci 166(4):557–574

Verboom GA, Linder HP, Stock W (2003) Phylogenetics of the grass genus *Ehrharta*: evidence for adaptive radiation in the summer-arid zone of the South African Cape. Evolution 57:1008–1021

Verboom GA, Linder HP, Stock WD (2004) Testing the adaptive nature of radiation: growth form and life history divergence in the African grass genus *Ehrharta* (Poaceae: Ehrhartoideae). Am J Bot 91:1364–1370

Wang W, Chen Z-D (2007) Generic level phylogeny of Thalictroideae (Ranunculaceae) implications for the status of *Paropyrum* and petal evolution. Taxon 56:811–821

Weber WA (2003) The Middle Asian element in the southern Rocky Mountain flora of the western United States: a critical biogeographical review. J Biogeog 30:649–685

Whittall JB (2005) Ecological speciation and convergent evolution in the North American columbine radiation (Aquilegia, Ranunculaceae). PhD Thesis, UC Santa Barbara, Santa Barbara

Whittall JB, Hodges SA (2007) Pollinator shifts drive increasingly long nectar spurs in columbine flowers. Nature 447:706–710

Whittemore AT (1997) Aquilegia. In: Morin NR (ed) Flora of North America. Oxford University Press, New York

Wikström N, Savolainen V, Chase M (2001) Evolution of angiosperms: calibrating the family tree. Proc R Soc Lond B 268:2211–2222

Wolfe AD, Randle CP, Datwyler SL, Morawetz JJ, Arguedas N, Díaz J (2006) Phylogeny, taxonomic affinities, and biogeography of *Penstemon* (Plantaginaceae) based on ITS and cpDNA sequence data. Am J Bot 93:1699–1713