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Evolution

Columbines: A geographically widespread species flock

(*Aquilegia*/adaptive radiation/molecular evolution/morphological evolution/Ranunculaceae)

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ABSTRACT **Species in the columbine genus, *Aquilegia*, are known for their broad variation in ecology and floral morphology. *Aquilegia* is also known for the large degree of intercompatibility among its species, which has led to the suggestion that the genus has arisen recently. However, intercompatibility does not always imply recent divergence and the widespread distribution pattern of the genus has suggested an older age. We constructed phylogenies for *Aquilegia* plus its close relatives by using nucleotide sequence data from both nuclear and chloroplast DNA. The sequence data averaged over 1250 bp per species. Among the 14 columbine species sampled from Europe, Asia, and North America only 16-bp changes and one insertion/deletion event were detected. In contrast, related genera had from 3 to 45 times this level of variation. The phylogenies derived from the chloroplast and nuclear DNA sequences were highly concordant and suggest that the columbines are the result of a recent, rapid radiation. In contrast to other examples of species flocks, *Aquilegia* has radiated on a widespread geographical scale. By comparison with their related taxa, we suggest that the evolution of the nectar spur in *Aquilegia* was a key innovation for this genus and allowed rapid speciation through specialization to specific pollinators.**

Among species of the columbine genus *Aquilegia*, there is an extensive diversity of floral morphologies and color (1–3) corresponding to different pollination syndromes (4). Columbines are also diverse in the types of habitats that they occupy, including warm-temperate forests, extremely high altitude alpine zones, and desert springs (3). Such diversity is striking within a single genus and yet columbines remain largely interfertile (1, 2). The high degree of interfertility among many columbine species suggests that, overall, species of *Aquilegia* may be genetically very similar. These observations led Clausen *et al.* (5) to state "[*Aquilegia*] possibly represents a youthful stage experienced by many other, now mature genera." The possibility that *Aquilegia* is in an early stage in the evolution of a genus is extremely interesting because this would provide an exemplar for studying the establishment of higher-order taxa.

However, Stebbins (6) and others (1, 7), relying on the distribution of columbines throughout mesophytic forests of the north-temperate region, have suggested that *Aquilegia* is not a youthful genus in terms of chronological time and may be of at least mid-Tertiary age. In addition, interfertility does not directly imply recent ancestry. For instance, there are examples of species that are known to have been separated for long periods of time, have large genetic distances, and yet remain interfertile (8). Thus, interfertility may not be indicative of recent divergence.

To assess the recency of divergence among taxa, it is necessary to compare the degree of genetic divergence within a clade with the degree of genetic divergence within its sister clade(s) (9). Recent divergence among taxa will result in lower levels of genetic variation within a clade as compared to its sister clade. A similar pattern would result if there were a slowdown in the accumulation of genetic change in the lineage of interest. However, tests of relative rates of divergence can be made by comparing the amounts of divergence since a common ancestor (10). In addition, concordance of patterns of divergence obtained from nuclear and cytoplasmic genomes will provide stronger evidence for diversification patterns than simply using one of these genomes (10).

To determine the relative recency of the diversification of *Aquilegia*, we compared the degree of DNA sequence divergence among columbine species and their close relatives. *Aquilegia* is a member of the small chromosome section of the Ranunculaceae (11). This section consists of only a few recognized genera and therefore we could sample members from each genus for our analysis. Within this small chromosome section there are two groups inferred from basal chromosome number; one of $n = 7$ and one of $n = 9$ (11). These two groups are apparently sister clades within the single monophyletic assemblage of small chromosome species (11). Confirmation of this view has been obtained based on preliminary DNA sequencing of both the *atpB* and *rbcL* coding genes for a wide range of genera within the Ranunculaceae (S. Hoot, personal communication). *Aquilegia* is a member of the $n = 7$ group. Thus, in our phylogenetic analyses we sampled extensively from the $n = 7$ group and used two $n = 9$ species for our outgroup comparison.

We sequenced both a nuclear and a chloroplast DNA (cpDNA) region from each of 27 species to compare the patterns and rates of divergence for these two distinct genomes. Because these species were suspected to be closely related, we chose to sequence two regions that have been previously shown to possess high rates of nucleotide substitution. In the nuclear genome, we chose to sequence the internal transcribed spacer (ITS) region that consists of two spacer elements and the 5.8S rRNA gene (12). This region has been shown to have high levels of sequence divergence between closely related species (13, 14). Because cpDNA evolves slowly relative to nuclear loci (15), we chose to sequence the spacer region between the *atpB* and *rbcL* coding genes; this region also appears to have increased rates of mutation (16).

MATERIALS AND METHODS

We sampled 14 species of columbines that varied in their floral morphologies, habitat associations, and biogeographic distributions (Table 1). We also sampled from the similarly widespread, related genera *Thalictrum* (from North America, *T. clavatum* and *T. fendlerii*; from Europe, *T. flavum* and *T. aquilegiifolium*) and *Isopyrum* (from North America, *I. biternatum*, *I. occiddentale*, and *I. savilei*; from Asia, *I.*

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Abbreviations: cpDNA, chloroplast DNA; ITS, internal transcribed spacer; indel, insertion/deletion.

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Table 1. Species of *Aquilegia* used in this study and their distribution (Dist) (E, Europe; A, Asia; NA, North America), pollination syndrome (Poll) (B, bee; HB, hummingbird; M, moth; F, fly), petal colors (spur, blade; if different) (Y, yellow; B, blue; R, red; W, white; P, purple; G, green), and habitat (Hab) (M, mountain; HA, high alpine; D, desert springs)

Species	Dist	Poll	Color	Hab
A. aurea (AA)	E	B	Y	M
A. caerulea (ACR)	NA	M	B, W	M
A. canadensis (ACN)	NA	HB	R, Y	M
A. chrysantha (ACH)	NA	M	Y	M
A. flavescens (AFL)	NA	B	Y	M
A. formosa (AFO)	NA	HB	R, Y	M
A. fragrans (AFR)	A	M	W	HA
A. jonesii (AJ)	NA	B	B, W	M
A. micrantha (AM)	NA	B	Y, R	M
A. pubescens (AP)	NA	M	WY	HA
A. shockleyi (ASH)	NA	HB	R, Y	D
A. skinneri (ASK)	NA	HB	R, YG	D
A. viridiflora (AVI)	A	F	G, P	M
A. vulgaris (AVU)	E	B	P	M

stoloniferum). In addition, we examined *Paraquilegia (P. grandiflora* and *P. microphylla*), *Leptopyrum fumarioides*, *Coptis trifolia*, and *Xanthorhizza simplicissima*. These species include representatives from all of the recognized genera in the small chromosome section of the Ranunculaceae (11). *C. trifolia* and *X. simplicissima* were used as outgroups. Tissue samples were obtained from plants derived from wild collected seed, which were grown to flowering for species identification or from herbarium samples.

DNA was isolated (17) and used for amplification via the PCR. The forward primer for cpDNA amplification was the ATPBE primer (5'-GTGGAAACCCCGGGACGAGAAGTAGT-3') (16) and the reverse primer was a 27-mer (5'-ACTTGCTTTAGTTTCTGTTTGTGGTGA-3')corresponding to positions 30–4 of the *rbcL* coding region of *Zea mays*. It was necessary to design a variety of internal primers to sequence this region because of sequence variation [substitutions and insertions/deletions (indels)] among the species. Primers for the ITS region were ITS5 (5'-GGAAGGAGAAGTCGTAACAAGG-3') and ITS4 (5'-TATGCTTAAACTCAGCGGGT-3'). For *T. fendlerii* we used the primer ITSL (5'-TCGTAACAAGGTTTCCGTAGGTG-3') in place of ITS5. Internal primers were ITS2 (5'-GCTACGTTCTTCATCGATGC-3') and ITS3 (5'-GCATCGATGAAGAACGTAGC-3'). For some of the species obtained from herbarium samples it was necessary to amplify subregions by using internal primers and then sequence each region separately. The PCR amplification protocol for both the ITS and cpDNA regions used 2.5 units of Promega *Thermus aquaticus (Taq)* DNA polymerase, the Promega *Taq* buffer, 1.5 mM $MgCl_2$, 25–50 pmol of each primer, and 5 μl of stock DNA. The total volume of the PCR mixtures was 50 μl. The amplifications were performed in a M.J. Research thermal cycler programmed for one cycle at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 53°C, and 1.5 min at 72°C. When internal primers were used to amplify only a portion of these regions, the same protocol was used except that the extension time was 1 min. The PCR amplification products were purified by adding an equal volume of isopropanol to the reaction mixture to precipitate the DNA followed by two washes with 70% isopropanol. Both strands of the PCR products were then directly sequenced with an automated sequencer (Applied Biosystems, model 373A). Sequences were aligned manually and the criteria for positioning indels was the same as that used by Golenberg *et al.* (16), who also sequenced the spacer region between the *atpB* and *rbcL* chloroplast coding genes for nine grass species. Indels were coded as single events; when overlapping indels occurred the overlap portion was considered a single event (16). Indels were coded only for the chloroplast region.

PAUP (18) was used to analyze the data. We performed three separate analyses to predict the phylogeny of this group: one for the nuclear DNA, one for the cpDNA, and one for the combined data. In all analyses we used the heuristic search, MULPARS option in effect, and tree bisection reconnection (TBR) branch swapping. We conducted a bootstrap analysis for each of the data sets. Tree lengths and goodness-of-fit statistics (consistency index, retention index, and rescaled consistency index) were calculated for each tree.

RESULTS

Sequences for the ITS1 region varied from 214 to 246 bp and the ITS2 region varied from 198 to 216 bp. The 5.8S rDNA region was 164 bp for all species. The spacer region between the *atpB* and *rbcL* coding genes varied from 585 to 814 bp. Seventy-two indel events were coded for this region that ranged in size from 1 to 23 bp. Most of these indels (76%) were duplicated or other transformed adjacent or proximal sequences as previously found in this region for other species (16). Heuristic searches resulted in 2, 10, and 5 equally parsimonious trees for the ITS, cpDNA, and combined data sets, respectively.

The phylogenies derived from the nuclear DNA, cpDNA, and combined data sets are completely congruent except for the relationship of *T. fendlerii* and *T. aquilegiifolium*. All phylogenetic analyses placed the *Aquilegia* species in a single monophyletic group. The nuclear and cpDNA gene trees do differ in their degree of resolution of some of the groups. The ITS data do not resolve the relationship of the Asian *Isopyrum* with the North American *Isopyrum* species, while the cpDNA data show *Isopyrum* to be paraphyletic. However, the ITS data resolve *Leptopyrum* and *Paraquilegia* as a monophyletic clade, while the cpDNA data do not. And finally, the cpDNA data resolve *Leptopyrum* and *Paraquilegia* as sister groups to *Isopyrum* and *Aquilegia*, while the ITS data do not. Bootstrap values were high for all major clades when using the combined data sets (63–100%; Fig. 1).

There was little sequence variation in either the cpDNA or the nuclear DNA region among the columbines, especially compared to other widespread, related genera (Figs. 1 and 2). In the chloroplast region, there were two base substitutions and a single indel detected among the columbines. In the ITS region, there were only 14 variable substitution sites detected. In contrast, the widespread genera *Isopyrum* (North American species only) and *Thalictrum* had from 3 to 45 times the levels of genetic variation found among the species of *Aquilegia* (Fig. 2). There was no overlap in the levels of genetic diversity among the columbines with the levels of genetic diversity among *Thalictrum* or the North American *Isopyrum* (Fig. 2).

DISCUSSION

The low levels of genetic variation among *Aquilegia* species suggest that the genus has radiated rapidly and recently. Except for their widespread distribution, this pattern is indicative of a "species flock" (19). An alternative explanation for the low level of columbine nucleotide divergence is a slowdown in the accumulation of mutations along the lineage leading to these species. However, in all phylogenetic analyses the branch leading to *Aquilegia* consists of a large number of changes (Fig. 1). Indeed, the degree of change along the lineage leading to the columbine species is similar to or larger than that found for any other genus examined in this study. For example, using the ITS data, the columbines range from 23 to 27 steps from the *Isopyrum/Aquilegia*

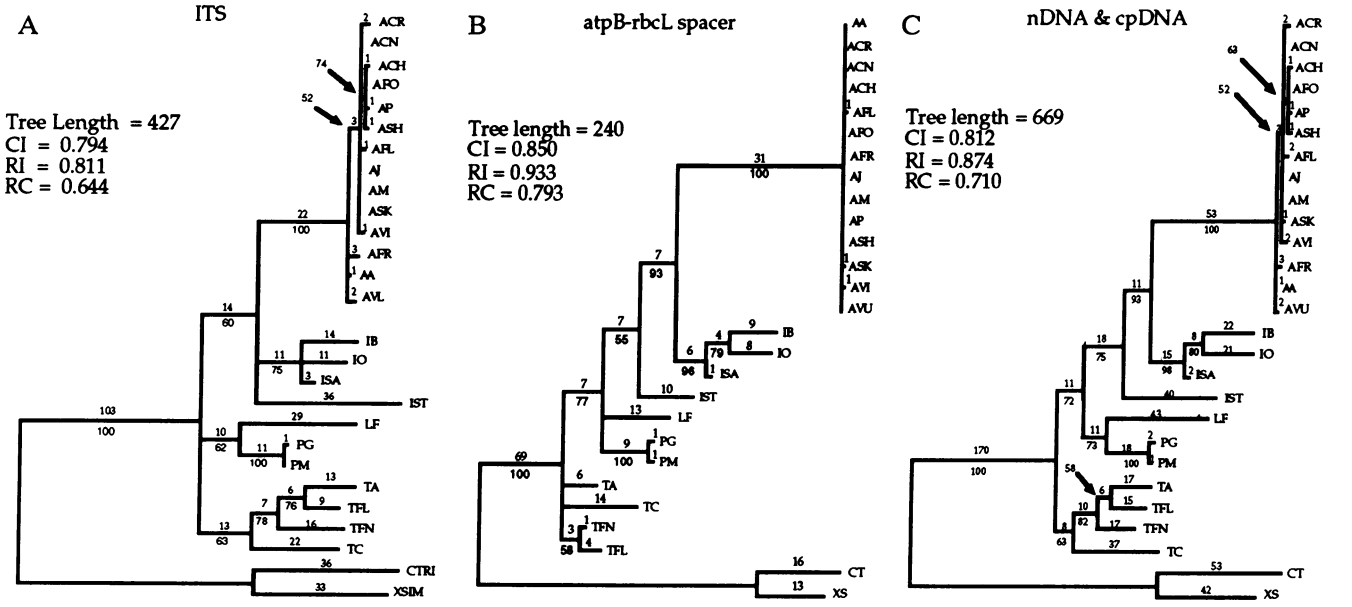

FIG. 1. Phylograms of the genus *Aquilegia* and related species. Each tree is a single most parsimonious tree resulting from a heuristic search using PAUP (18) for the ITS region of the nuclear genome (*A*), the spacer region between the *atpB* and *rbcL* genes of the chloroplast genome (*B*), and the combined data sets (nDNA, nuclear DNA) (*C*). For each analysis a single, shortest tree was chosen and truncated to show only branches that had bootstrap values over 50%. Bootstrap values are shown below the branches (or arrows) and branch lengths are shown above the branches. We sequenced 14 species of *Aquilegia* that varied in habitat associations, geographic locality, and floral pollination syndromes (see Table 1). We also sampled *I. biternatum* (IB), *I. occiddentale* (IO), *I. savilei* (ISA), *I. stoloniferum* (IST), *T. clavatum* (TC), *T. fendlerii* (TFN), *T. flavum* (TFL), *T. aquilegiifolium* (TA), *P. grandiflora* (PG), *P. microphylla* (PM), and *L. fumarioides* (LF). We used *C. trifolia* (CT) and *X. simplicissima* (XS) as outgroup species. CI, consistency index; RI, retention index; RC, rescaled consistency index.

common ancestor while the *Isopyrum* range from 14 to 36 steps. The single *Isopyrum* species with a greater number of steps than the *Aquilegia* species is resolved as ancestral to the other *Isopyrum* and *Aquilegia* in the cpDNA data, and this may explain its larger branch length. Thus, the data indicate that substitution rates have been equivalent or higher in the columbine lineage compared with sister genera. In addition, the phylogenetic patterns determined by using DNA sequences from both a nuclear DNA and cpDNA region were highly concordant (Fig. 1). This similarity in pattern of total genetic change between these phylogenies strongly supports the conclusion of recent diversification of the columbines rather than a slower rate of evolutionary change along the lineage leading to the columbines.

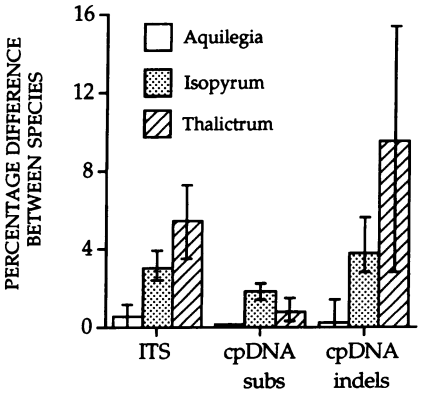

FIG. 2. Mean percentage difference between species within the widespread genera *Aquilegia*, *Isopyrum*, and *Thalictrum* in the ITS region of the nuclear genome and in the spacer region between the chloroplast *atpB* and *rbcL* genes. Lines indicate range of variation between species in a genus. Only North American species of *Isopyrum* are used here because they form a single monophyletic sister group relative to *Aquilegia* (see Fig. 1). The variation between species in the chloroplast region is dissected into the variation due to substitution events (subs) and that due to indels.

Although there was little sequence divergence among the columbine species, three groups were distinguished that suggest that the columbines originated in central Asia or Europe and subsequently spread to North America. First, both European species and one central Asian species form a basal group. Thus, it appears that the European and Asian species are ancestral to the North American species. This corresponds well with most authors' interpretation of the evolution of this genus (1–3). A single Asian species, *A. viridiflora*, groups with the North American species, suggesting that it may be ancestral to this group. It is intriguing how the columbines have rapidly spread throughout the Northern Hemisphere, given that their seed dispersal mechanism would not seem to promote long distance dispersal. The fruit is a follicle that splits open when it dries, allowing the seeds to drop to the ground (3).

Unfortunately, it is not possible to predict the absolute time of the columbine radiation. There are no fossils that could be used to calibrate nucleotide substitution rates within this group. In addition, rates of change calculated for more distantly related taxa are unlikely to be applicable to rates within this group because of variability in nucleotide substitution rates. For example, Suh *et al.* (20) calibrated substitution rates for the ITS regions in the Winteraceae but nucleotide divergence within this family was only half that found between genera or within subtribes of more recently derived families (20). Also, Bousquet *et al.* (21) found that rates of substitution of the *rbcL* gene of the chloroplast genome are quite variable among the Angiosperms, making extrapolation of cpDNA substitution rates across families questionable.

The relatively recent radiation of columbines is unlike any other example of a recent, rapid diversification because of their widespread distribution. Other examples of recent radiations include Darwin's finches (22, 23), Hawaiian honeycreepers (24), Hawaiian silverswords (25), and African cichlid fishes (26). However, each of these species groups differs from the radiation of the columbines because they are found

in restricted habitats such as island chains or lake systems. Apparently the columbines have been able to diversify and spread throughout the Northern Hemisphere in a short period of time. This unusual distribution for a recent radiation is particularly striking since both of the closely related genera *Isopyrum* and *Thalictrum* have similar distributions in the mountainous regions of the Northern Hemisphere but have apparently not radiated in a similar manner.

Explanations of the mechanisms of rapid species diversification often rely on the suggestion that low levels of competition allow taxa to radiate in species depauperate habitats (9, 27). Thus, taxa that colonize newly formed islands or relatively young lake systems have a greater opportunity to radiate into a variety of habitats. Because *Aquilegia* has radiated throughout the Northern Hemisphere, invasion of species depauperate habitats seems an unlikely mechanism for rapid radiation in this group. Again, this is particularly apparent when considering that the genera related to *Aquilegia* occupy very similar habitats, have similar distributions, but have not undergone a rapid radiation.

Taxa that undergo radiations in species depauperate habitats may also be aided by the evolution of a key innovation (9, 27). For example, it has been suggested that the radiation of cichlids in the African rift lakes has been aided by the evolution of a specific jaw apparatus. This jaw apparatus is apparently extremely flexible in that adaptations can easily be derived from it for a wide variety of feeding modes. However, it is thought that key innovations often do not promote diversification until new ecological conditions and opportunities arise (27).

The widespread diversification of the columbines suggests that they have evolved a key innovation that allowed them to immediately exploit entirely new resources and to speciate. An obvious candidate for a key innovation in *Aquilegia* would be the evolution of multiple nectar spurs, the hallmark of the genus. The evolution of multiple nectar spurs in *Aquilegia* would allow specialization to different pollinators as compared to the unspecialized flowers of their related genera. In fact, it has been hypothesized that features that change the pollinator environment may be a likely factor that would transform a neospecies into a new genus (see ref. 28). Other features of this genus such as habitat preferences and seed-dispersal biology are unlikely to be responsible for the radiation of the columbines since these features are extremely similar to those found in their close relatives that have not radiated rapidly.

Adaptation for pollination by different specific pollinators would reduce gene flow via pollen among populations and allow an increased rate of divergence among populations. For example, divergent floral morphologies have recently been shown to be associated with reduced gene flow between two columbine species (29). Similar arguments have been made to explain the diversification of the Angiosperms as a whole (30) and predominantly animal pollinated plant families in particular (31) [e.g., the families Orchidaceae (32) and Polemoniaceae (33)]. The evolution of nectar spurs in *Aquilegia* may have occurred quite suddenly as their presence appears to be controlled largely by one or a few genes (34). However, whether spurs, and thus the genus, evolved recently cannot be determined from the present phylogeny. Because of the long branch length leading to the columbines (Fig. 1), it is not possible to distinguish whether spurs evolved near the time of divergence from the *Aquilegia/Isopyrum* common ancestor or near the time of diversification of *Aquilegia* species.

The low genetic diversity combined with a high diversity in both ecology and floral morphology among the columbines suggests that *Aquilegia* represents a widespread species flock. Like other species flocks, morphological and ecological divergence has proceeded at a greater rate than genetic divergence (26). However, unlike other examples of species flocks, the columbines present the paradox of rapid speciation and rapid colonization of a large geographical area. Other examples of rapid radiations of taxa have been described from restricted localities, most likely because they are obvious in these settings. However, this study suggests that if widespread taxa are considered, examples of rapid radiations may be more common than previously thought.

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