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Genetic diversity of *Aquilegia* (Ranunculaceae) species and cultivars assessed by AFLPs

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ABSTRACT. Species of the genus *Aquilegia* are exceptionally diverse in their floral morphology and color, commonly known as columbine. They are widely planted ornamentals and are highly attractive for hummingbirds. However, little is known about their genetic diversity. We examined the genetic diversity of the species and cultivars using amplified fragment length polymorphism (AFLP) markers. Sixteen *Eco*RI/*Mse*I AFLP primer combinations produced 327 informative polymorphic bands, with a mean of 20.4 bands scored per primer. Jaccard's coefficient of similarity varied from 0.61 to 0.93, indicative of high levels of genetic variation. Cluster analysis using the unweighted pair group method with arithmetic mean algorithm placed the 64 accessions into two main clusters, each divided into two sub-clusters. The AFLP variability was significantly associated with the geographic origins, as the Asian species and the North American species grouped into two distinct clusters. The genetic diversity found among *Aquilegia* demonstrated the potential value of Chinese germplasm for cultivar improvement and for widening the genetic basis of breeding programs and breeding material selection. We concluded that

AFLPs are informative and can provide significant insights for genetic diversity research in columbine species.

Key word: Genetic diversity; AFLP; *Aquilegia*; Columbine

INTRODUCTION

Aquilegia (Ranunculaceae), commonly known as columbine, has been used for ecological and evolutionary genetics for over 50 years due to its phenotypic diversity, habitat range, and its experimental tractability (Hodge et al., 2002). The genus is distributed across temperate areas of North America, Europe, and Asia, being mostly native to northern temperate and alpine regions; numerous hybrids exist and several ornamental varieties are sold commercially. In addition, *Aquilegia* species occupy a wide range of habitats, from mesic riparian zones and desert springs to dry rocky outcrops, and from low to high elevation environments (Yang, 2008). *Aquilegia* spp, or columbines, are among the most familiar and most valued flowers to grace gardens in spring and early summer; they have been cultivated for hundreds of years as they are exceptionally diverse in their floral morphology and color. They are extremely easy to grow and reproduce by scattering their own seeds. Plants produce showy flowers in many shades of purple, blue, lavender, red, pink, yellow, or white. Most also have nectar spurs, which can be up to six inches in length in some species (Robert, 2003).

The first and foremost step in a breeding program is the choice of appropriate parents with good performance and a wide genetic base. Measures of genetic diversity, before making any cross, can help breeders to concentrate their efforts on the most promising combinations (Saxena et al., 2010). Also, genetic diversity, relatedness and structure of parental germplasm are important for breeders to design strategies in breeding program and make decisions regarding selection of parental combinations that will maximize gain from selection and maintain genetic diversity (Matus and Hayes, 2002; Hesham and Yan, 2010).

Although evolutionary relationships in the genus *Aquilegia* have been analyzed (Grant, 1992; Hodges and Arnold, 1994; Hodges et al., 2002; Whittall and Hodges, 2007; Hodges and Derieg, 2009) and a significant amount of information of floral traits influencing reproductive isolation is available (Brunet and Eckert, 1998; Medrano et al., 2006; Canto et al., 2007; Tang et al., 2007), little information is available on the extent of genetic variation in commercially cultivated columbine and the wild species (Bolaric et al., 2005). Information on genetic variation in *Aquilegia* germplasm could also be used to assist genetic mapping, quantitative trait locus (QTL) studies and the design of linkage disequilibrium mapping strategies for marker-trait association (George et al., 2006).

Diversity at marker loci is currently the most feasible strategy for characterizing diversity in wild and cultivated germplasm. The amplified fragment length polymorphism (AFLP) technique is a robust, highly effective method of DNA fingerprinting and has been successfully applied to detect genetic variation in many species, including *Brassica*, *Lupinus*, and *Swertia* (Boersma et al., 2007; Misra et al., 2010; Yildirim et al., 2010). Our objectives were to survey AFLP marker variation among selected genotypes of cultivated and wild species of columbine and describe the relationship among them based on genetic distances. We intended to assess the genetic diversity of *Aquilegia* with the hope of providing information for facilitating the efficient use of germplasm to produce new cultivars.

MATERIAL AND METHODS

Sampling of species and varieties

In this study there were 64 genotypes including 9 species from North America, 10 species from China and 45 important commercial cultivars.

The 9 species of North America were obtained from Scott Hodges, Professor of the Ecology, Evolution & Marine Biology Department of the University of California, in April 2010, and the 10 Chinese species were collected from several provinces between 2006 and 2009 (Table 1). All of these species were grown at the China National Engineering Research Center for Floriculture.

Table 1. List of *Aquilegia* (Nos. 45-64) included in the study.

No.	Name	Origin
45	*A. barnebyi*	North America
46	*A. chrysantha*	North America
47	*A. coerulea*	North America
48	*A. eximia*	North America
49	*A. flavescens*	North America
50	*A. formosa*	North America
51	*A. flabellata 'Nana Alba'*	Asia
52	*A. micrantha*	North America
53	*A. pinetorum*	North America
54	*A. pubescens*	North America
55	*A. atrovinosa*	Dongbei, China
56	*A. yabena*	Beijing, China
57	*A. oxysepala*	Dongbei, China
58	Anonymous	Dingbei, China
59	*A. parviflora*	Beijing, China
60	*A. ecalcarata*	Shanxi, China
61	Anonymous	Gansu, China
62	Anonymous	Shanxi, China
63	*A. viridiflora*	Beijing, China
64	Anonymous	Beijing, China

Cultivars No. 1-44, consisting of the most important commercial cultivars, were obtained from Pan American Seed and Dalian Century Seed from 2006 to 2009 and were grown in Beijing Dongsheng Seed Industry company fields. Photos of the plants are shown in Figure 1.

DNA extraction

Genomic DNA was extracted from silica-gel-dried leaf tissue using the FastDNA Kit, according to manufacturer instructions. The concentration of DNA was estimated with smart ladder (TianGen) on a 1% (w/v) agarose gel containing Golden View at 0.1 μg/mL in 1X TAE buffer and visualized under UV light. The DNA was stored at -20°C.

AFLP analysis

AFLP analyses were performed according to the method of Vos et al. (1995), with some modifications. Briefly, restriction enzyme digestion and adaptor ligation were done using 100 ng DNA, 5 U *Eco*RI (NEB), 5 U *Mse*I, 10 mM $MgCl_2$, 1X T4 DNA ligase buffer (NEB), 5 pmol *Eco*RI-adaptors, 50 pmol *Mse*I-adaptors, and 4 U T4 DNA ligase (NEB) made up to a

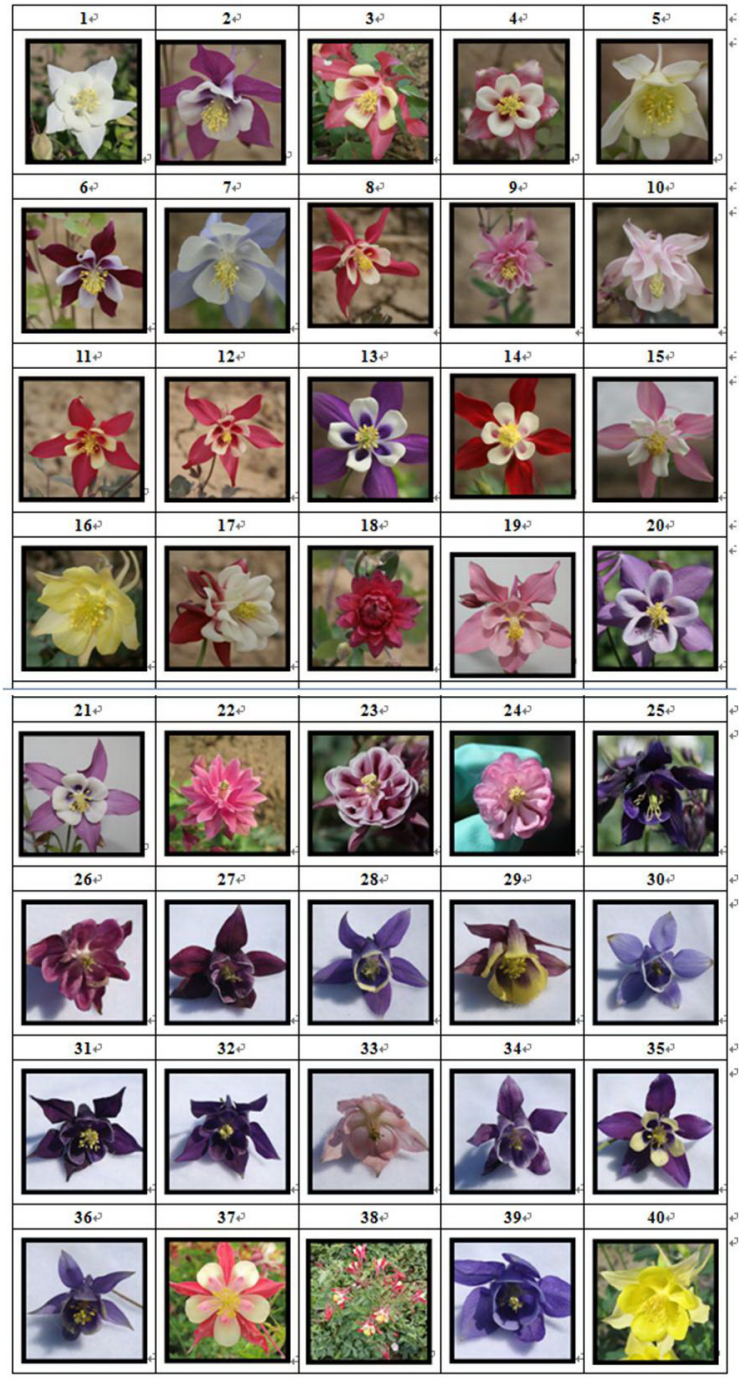

Figure 1. Photos of cultivars No. 1-40, which were obtained from Pan American Seed and Dalian Century Seed from 2006 to 2009.

final volume of 20 µL with double-distilled H_2O. This reaction was carried out at 37°C for 10 h.

The preselective polymerase chain reaction (PCR) was performed with 5 µL restriction/ligation mixture, 30 ng primer *Eco*RI-00 and 30 ng *Mse*I-00 (Table 2), 5X PCR buffer, 1.5 mM $MgCl_2$, 0.6 U Taq DNA polymerase (Promega), and 0.1 mM of each dNTP in a total volume of 20 µL. The profile of the pre-amplification was 30 cycles of 30 s at 94°C, annealing step for 30 s at 56°C and 72°C for 1 min, and a final extension step of 10 min at 72°C.

Table 2. Oligonucleotide adaptor and primers used for AFLP analysis.

	Restriction enzyme	Sequence
Adaptor	*Eco*RI	5'-GACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
	*Mse*I	5'-GACGTAGAGTCCTGAG-3' 3'-TGCTACTCAGGACTCAT-5'
Primer	*Eco*RI-00	5'-AGACTGCGTACCAATT-3'
	*Mse*I-00	5'-GACGATGAGTCCTGAGTAA-3'
	*Eco*RI+***	5'-AGACTGCGTACCAATT+***-3'
	*Mse*I+***	5'-GACGATGAGTCCTGAGTAA+***-3'

After preamplification, the samples were diluted 10 times with TE buffer. For selective amplification, 5 µL of this dilution was used in a 20-µL volume mixture containing 30 ng *Eco*RI primer and 30 ng *Mse*I primer, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP and 0.6 U Taq DNA polymerase (Promega). Amplification was performed for 42 cycles using the following profile: 94°C for 30 s, annealing step for 30 s and 72°C for 2 min. The annealing temperature in the first cycle was 65°C, which was subsequently reduced in each cycle by 0.7°C for the next 11 cycles, and continued at 56°C for the remaining 30 cycles, and finally 1 min at 72°C.

After selective amplification with the different primer combinations, 8 µL of the formamide loading dye (98% formamide, 10 mM EDTA, 1 mg/mL xylene cyanol and 1 mg/mL bromophenol blue) was added to each PCR product and all products were denatured at 95°C for 10 min. After the denaturation, the samples were immediately placed on ice to minimize renaturation before gel loading; 7-8 µL of each sample was loaded onto a 6% denaturing polyacrylamide gel, which was pre-equilibrated with preheated 1X TBE buffer. The gel was allowed to run for about 150 min, after which the plates were separated and silver-stained. The plates were then dried overnight before the polymorphic fragments were scored on a light box.

Data analysis

Polymorphic loci were identified as bands of equal molecular weight that were well-resolved and were present in some samples and absent in others. The size of every polymorphic fragment scored was estimated by comparing it with 100 bp ladders run on either side of the plate. Fragments were scored by visual inspection for presence (1) or absence (0) in the output traces, and fragments smaller than 50 and larger than 500 nucleotides were excluded from the analysis.

The similarity among the AFLP patterns was calculated with Jaccard's correlation coefficient for each primer combination. A cluster analysis was performed by the unweighted pair-group method of arithmetic averages (UPGMA) of the NTSYS-pc software. The dendrogram was created with the TREE option of NTSYS (Rohlf, 1992).

RESULTS

Levels of polymorphism within columbine

Sixty-four accessions of columbine were examined by AFLP analysis using various pairwise combinations of *Eco*RI (E) and *Mse*I (M) selective primers with two (+2) or three (+3) nucleotide additions after the cleavage site. Primer combinations of E+2 and M+2, generated profiles in which the amplification products overlapped or were too dense to allow reliable scoring and therefore not useful for analysis. In contrast, combinations involving E+3/M+3 generated too few amplification products, of which only a small proportion were polymorphic. Therefore, these combinations did not provide sufficient coverage of the whole genome to be informative.

Of the 256 primer combinations tested, the most productive and reproducible combinations that would be useful in the analysis of the *Aquilegia* genome were determined to be E+3/M+2 or E+2/M+3.

We scored 327 bands from the amplifications with 16 primers of DNA from 64 genotypes, with a mean of 20.4 bands scored per primer, demonstrating that, in spite of the low number of primers used, the AFLPs were sufficiently polymorphic and informative.

The percentage of polymorphism ranged from 52.7 to 94.5%. There was a wide variation in the number of bands, ranging from 4 to 57 bands for primers E-ACC/M-AG and four bands for primer E-AAC/M-GT. The details of the names and nucleotide sequences of primers used and summary of the number of polymorphic loci and percentages of polymorphism are shown in Table 3.

Table 3. Nucleotide sequences of primers, number of polymorphic bands and percentages of polymorphism.

Marker	Allele size range	Number of polymorphic loci	Percentage of polymorphic bands (%)
E-ACG/M-CAG	167-437	13	86.7
E-AG/M-AT	121-466	19	52.7
E-AG/M-CAC	180-360	6	66.7
E-AG/M-CAG	224-395	9	60.0
E-AT/M-CTG	236-292	7	70.0
E-GA/M-CAG	60-241	27	84.4
E-GA/M-CTA	126-458	18	75.0
E-ACC/M-AG	73-500	57	86.3
E-ACG/M-AG	66-375	32	82.0
E-ACT/M-AG	230-457	9	60.0
E-ACG/M-AT	97-400	35	92.1
E-ACT/M-GA	125-288	13	76.5
E-CGA/M-AG	190-450	18	64.3
E-TGA/M-AG	50-480	52	94.5
E-AAC/M-GT	60-280	4	80.0
E-ACT/M-GT	124-225	8	80.0
Total	50-500	327	78.9

Genetic diversity and cluster analysis

Jaccard's genetic similarity coefficient varied from 0.61 to 0.85. UPGMA cluster analysis of the Jaccard's similarity coefficient generated a dendrogram that illustrated the overall genetic relationship among the accessions and showed two main clusters split at 0.61 (Figure 2).

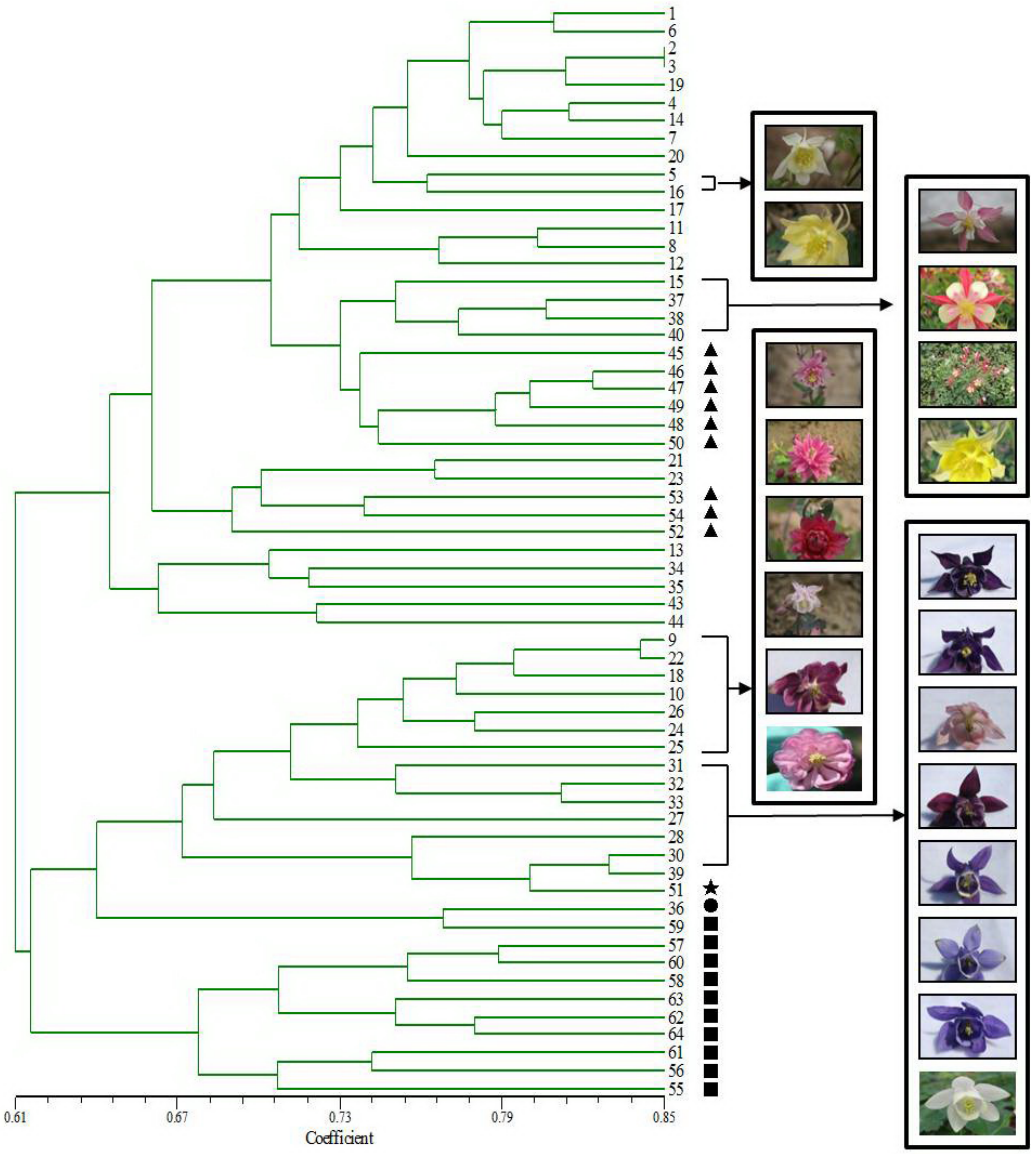

Figure 2. Cluster analysis of 64 accessions of *Aquilegia*, based on the matrix of genetic similarity calculated based on AFLP markers. The UPGMA method was the grouping criterion. *Triangles* represent the North American species, *squares* represent the species from China, the *star* represents the cultivar *A. flabellata* 'Nana Alba' from Asia, the *circle* represents the hybrid between No. 51 and 59 in our field, and the remaining genotypes with no identification were cultivars collected from Pan American Seed and Dalian Century Seed.

The dendrogram separated the 64 genotypes into two major clusters A and B, in accordance with the geographic distributions of wild accessions and traditional classification of

cultivated forms of columbine. The A cluster contained wild accessions collected from North America in addition to most cultivars in this study, while the B cluster contained wild accessions collected from China and some cultivars from other parts of Asia. Genotypes 41 and 42 were excluded from this cluster analysis.

Cluster A was further divided into two subgroups: A-I and A-II. All species from North America and most of cultivars fell into this cluster. Furthermore, six of nine wild species from North America were grouped into subcluster A-I-I, with No. 15, 37, 38, and 40 cultivars, while the remaining three species *A. micrantha*, *A. pinetorum*, and *A. pubescens* were grouped with No. 21 and 23 genotypes within subcluster A-I-II, indicating a closer relationship of North American species with most of the cultivars selected in this study. Cluster B mainly consist of all the species from China.

The 64 accessions showed different band profiles, which means that the AFLP primer combinations selected had good discriminatory power, and that the sample in this study was highly variable. The dendrogram indicates that cultivars from the same geographic origins tend to group together.

One interesting exception is No. 59 (*A. parviflora*) collected from Beijing Botanical Garden in 2007, which was not grouped into one subcluster B-II with the other nine species from China. According to the AFLP profiles and morphological characteristics, genotype No. 36 is hybrid of 59 (*A. parviflora*) and 51 (*A. flabellata* var. *nana alba*), which have been grown in our field for over two years, indicating that open cross-pollination occurs among columbines.

Genetic diversity among cultivars

This is the first study using genetic markers for cultivar discrimination in *Aquilegia*. The A cluster comprised varieties of considerable morphological variation belonging to different breeding companies.

As shown in Figure 2, genotypes No. 5 and 16 both have yellow flower group together within subcluster A-I-I; No. 15, 37, 38, and 40 all grouped into this one subcluster, which belong to the same accession. Similarily, subcluster A-II consists of many cultivars, seven of which (9, 22, 18, 10, 26, 24, and 25) are double flowering, while eight (27, 28, 30, 31, 32, 33, 39, and 51) have the same pattern (flower shape), indicating that AFLPs can distinguish the cultivars as well as morphological characteristics. In case of very high similarities between different cultivars, such as No. 2 and 3, the genotypes could be hybrids between species or share a common ancestor. Our results indicate that considerable gene flow has taken place, probably due to the work of breeders. The resulting dendrogram showed common ancestral origin for many accessions. Most of the cultivars were closely related to the wild accessions from North America. The species groupings are consistent with traditional taxonomic species delimitation.

The results indicate that enough diversity exists to broaden the genetic base of *Aquilegia* cultivars.

DISCUSSION

We used AFLP analysis to gain insight into the degree of intra- and interspecific variation in this genus, as well as to examine relationships between some of its members. Our studies show that, with appropriately defined reaction conditions and specific combinations of selec-

tive primers, it is possible to yield a sufficient number of polymorphic bands to allow objective comparisons between cultivated columbine accession lines and between species. In some cases, a single primer combination (such as E-TGA/M-AG and E-ACC/M-AG) was found to be capable of providing a sufficient number of data points (>50 polymorphic fragments) to distinguish between highly related individuals. AFLP markers were shown to reveal a high degree of genetic diversity and are capable of distinguishing between different accessions in different locations. Approximately 80% of the amplified fragments were polymorphic and provided sufficient resolution to distinguish among closely related cultivars and allow seven distinct subgroups to be recognized; the genotypes from different geographic locations tend to cluster based on their AFLP profiles. The study showed that AFLP markers for columbine can be efficiently used for cultivar discrimination. These markers are useful for genetic mapping of important traits and may become a useful tool for hybrid purity tests and marker-assisted selection.

The genus was of particular interest because it included many species with large differences in morphology, yet it was also known for having minimal sterility between species (Grant, 1992). It is worth noting that, compared with the large differences in morphology among accession lines, the measured extent of polymorphism is relatively small. Grant divided the genus into five groups of species by their floral morphology (*A. ecalcarata*, the Vulgaris group, the alpina group, the Canadensis group, and the Coerulea group) and suggested that *Aquilegia* evolved in Eurasia or Asia and subsequently spread to North America during the mid-Pliocene (approximately 3.5 million years ago). Based on our results, we also concluded that the origin of *Aquilegia* is from Asia just as Grant suggested before. We were not able to make conclusions about the frequency of gene exchange between cultivated and wild gene pools. However, species from North America were found to be genetically similar to cultivated varieties.

Our results are in good agreement with those obtained by Hodges and Arnold (1994), which indicate that within *Aquilegia* there are two distinct clades that predominately group the European and Asian species apart from the North American species. The AFLP analyses used in these two studies gave very similar phylogenetic groupings, and these groupings were consistent with the proposed clustering of species based on traditional analyses of morphological characteristics.

Numerous historical genetic studies on species of *Aquilegia* suggest that some characters separating species are controlled, at least in part, by genes of large effect, while other characters are controlled by many genes of small effect (Hodges et al., 2003). However, with the exception of inheritance studies of flower orientation, flower color and spur length (Hodges et al., 2002), no data are available that show a direct association between quantitative traits and molecular markers. In this regard, our AFLP data may provide information for more detailed analysis of the underlying genetics basis for these characteristics, and even some genetic mapping or QTL analysis.

Columbine accessions vary in phenotypic and horticultural characteristics, such as flower color, flower diameter, flower orientation, spur length, length of flower period and flowering time, plant height, resistance to disease, and so on. Columbine flower can also display a wide range of variability in size and color. The most valuable hybrids are crosses between two of the most beautiful species, *A. coerulea* and *A. chrysantha*. Some authors have suggested that *A. formosa* was thrown into the mix to add red coloration. If there is a possibility of several crosses, two parents with the greatest genetic distance between them should be crossed in order to determine QTLs involved in traits that differ. On this basis, the cross of a Chinese wild genotype and a cultivar or North America genotype is suggested as the most suitable cross for QTL analysis studies as they have low genetic similarity and are also grouped in distinct

clusters. Fewer studies have been conducted about the resources of China.

In conclusion, results from this research will be helpful for breeders to choose parents for crossing and to use marker-assisted selection in *Aquilegia* breeding. Understanding phylogenetic relationships and genetic variability in *Aquilegia* is critical for determining genetic relationships, characterizing germplasm, controlling genetic erosion, designing sampling strategies and core collections, establishing breeding programs, and the registration of new cultivars.

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REFERENCES

Boersma JG, Buirchell BJ, Sivasithamparam K and Yang H (2007). Development of a PCR marker tightly linked to molis, the gene that controls seed dormancy in *Lupinus angustifolius* L. *Plant Breed.* 126: 612-616.

Bolaric S, Barth S, Melchinger AE and Posselt UK (2005). Genetic diversity in European perennial ryegrass cultivars investigated with RAPD markers. *Plant Breed.* 124: 161-166.

Brunet J and Eckert CG (1998). Effects of floral morphology and display on outcrossing in blue columbine, *Aquilegia caerulea* (Ranunculaceae). *Funct. Ecol.* 12: 596-606.

Canto A, Perez R, Medrano M, Castellanos MC, et al. (2007). Intra-plant variation in nectar sugar composition in two *Aquilegia* species (Ranunculaceae): contrasting patterns under field and glasshouse conditions. *Ann. Bot.* 99: 653-660.

George J, Dobrowolski MP, van Zijll de JE, Cogan NO, et al. (2006). Assessment of genetic diversity in cultivars of white clover (*Trifolium repens* L.) detected by SSR polymorphisms. *Genome* 49: 919-930.

Grant V (1992). Floral isolation between ornithophilous and sphingophilous species of *Ipomopsis* and *Aquilegia*. *Proc. Natl. Acad. Sci. U. S. A.* 89: 11828-11831.

Hesham AA and Yan WG (2010). Genetic diversity and relatedness of rice cultivars resistant to straighthead disorder. *Plant Breed.* 129: 304-312.

Hodges SA and Arnold ML (1994). Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proc. Natl. Acad. Sci. U. S. A.* 91: 2493-2496.

Hodges SA and Derieg NJ (2009). Adaptive radiations: from field to genomic studies. *Proc. Natl. Acad. Sci. U. S. A.* 106 (Suppl 1): 9947-9954.

Hodges SA, Whittall JB, Fulton M and Yang JY (2002). Genetics of floral traits influencing reproductive isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Am. Nat.* 159 (Suppl 3): S51-S60.

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

Matus IA and Hayes PM (2002). Genetic diversity in three groups of barley germplasm assessed by simple sequence repeats. *Genome* 45: 1095-1106.

Medrano M, Castellanos MC and Herrera CM (2006). Comparative and vegetative differentiation between two European *Aquilegia* taxa along a narrow contact zone. *Plant Syst. Evol.* 262: 209-224.

Misra A, Shasany AK, Shukla AK, Darokar MP, et al. (2010). AFLP markers for identification of *Swertia* species (Gentianaceae). *Genet. Mol. Res.* 9: 1535-1544.

Robert N (2003). Columbines: *Aquilegia*, *Paraaquilegia*, and *Semiaquilegia*. Timber Press, Portland, 90-91.

Rohlf FJ (1992). NTSYS-pc Numerical Taxonomy and Multivariate Analysis System, Version 2.0. Exeter Software, New York.

Saxena RK, Saxena KB, Kumar RV, Hoisington DA, et al. (2010). Simple sequence repeat-based diversity in elite pigeonpea genotypes for developing mapping populations to map resistance to *Fusarium* wilt and sterility mosaic disease. *Plant Breed.* 129: 135-141.

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

Vos P, Hodgers R, Bleeker M, Reijans M, et al. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407-4414.

Whittall JB and Hodges SA (2007). Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* 447: 706-709.

Yang JY (2008). Mating patterns and reproductive isolation in *Aquilegia formosa* and *Aquilegia pubescens* (Ranunculaceae). Doctoral thesis, University of California, Santa Barbara.

Yildirim E, Yildirim N, Ercisli S and Agar G (2010). Genetic relationships among turnip (*Brassica rapa* var. *rapa*) genotypes. *Genet. Mol. Res.* 9: 987-993.

