Molecular and Morphological Investigation of *Astilbe*

by

Brian W. Trader

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APPROVED:

Holly L. Scoggins, Chair

Joyce G. Latimer

Duncan M. Porter

Stephen E. Scheckler

Richard E. Veilleux

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Abstract

Astilbe (Saxifragaceae) is a genus of herbaceous perennials widely cultivated for their ornamental value. The genus is considered taxonomically complex because of its geographic distribution, variation within species, and the lack of adequate morphological characters to delineate taxa. To date, an inclusive investigation of the genus has not been conducted. This study was undertaken to (a) develop a well-resolved phylogeny of the genus *Astilbe* using an expanded morphological data set and sequences from the plastid gene *mat*K, (b) use single nucleotide polymorphisms to determine the lineages of cultivated varieties, and (c) successfully culture *Astilbe in vitro* and evaluate potential somaclonal variation of resulting *Astilbe* microshoots.

Phylogenetic trees generated from a morphological character matrix of 28 character states divided *Astilbe* into three distinct clades. Relationships were well resolved among the taxa, though only a few branches had greater than 50% bootstrap support. There is evidence from the phylogeny that some described species may actually represent variation within populations of species. From our analysis I propose an *Astilbe* genus with 13 to 15 species and offer a key for distinguishing species and varieties.

There was little *mat*K sequence variation among taxa of *Astilbe.* Phylogeny of *Astilbe* generated from the maximum parsimony and maximum likelihood analysis of *mat*K sequences resulted in a polytomy of seven *Astilbe* species, with relationships within the genus poorly resolved. A second phylogeny of 21 taxa of *Astilbe* was more informative, aligning cultivated varieties near species from which they were derived. The

*mat*K sequence variation for *Astilbe* taxa was aligned to reveal DNA polymorphisms. Closely related taxa retained polymorphisms at the same sites within the gene sequence. These polymorphic sites could potentially be utilized to confirm the lineage of popular cultivated *Astilbe* varieties.

Propagation of *Astilbe* seedlings in tissue culture gave rise to various numbers of microshoots from each of 15 seedlings. Multivariate and cluster analysis of morphological characters from 138 plants derived from 15 seedlings revealed potential somaclonal variants. These variants were characterized by one or more of the following traits: dwarf habit, dark green leaves (high chlorophyll content), increased flowering, or larger plant size. Somaclonal variants with desirable phenotypes may be valuable for cultivar development.

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Chapter 1. Introduction

“As your eyes move across a beautiful landscape, consider the plants that give you pause.” In the opinion of renowned garden writer Allen Lacy, *Astilbe* is one of these prized garden plants. Multidimensional, versatile, and adaptable to a variety of settings, it is one of the "steeples and spikes" in a landscape panorama. "*Astilbe* are tremendous weapons in the fight against rugs and dumplings." So stated Mr. Lacy as he cheered the use of the *Astilbe* amid one-dimensional ground covers (rugs) and mounding plants (dumplings)” (Randhava 2005).

1.1 Background of *Astilbe*

Astilbe Buch.-Ham. ex D. Don is a genus comprised of shade-loving herbaceous perennials tremendously popular in the nursery and landscape industry. Consistently ranked among the top five herbaceous perennials by the national Perennial Plant Association (PPA) in terms of wholesale value, *Astilbe* species have been extensively hybridized, selected, and released as cultivars. Unimproved species or accessions have rarely been offered in the nursery trade. Selections have been made from open-pollinated seedling populations and then given cultivar names (Armitage 1996).

The taxonomy of *Astilbe* has not been well-established and the lineages of some popular cultivars and hybrids have been lost. A search of the International Plant Name Index yielded over 60 named species of *Astilbe,* most of which are synonyms. Most authorities considered the genus to consist of 12-20 species. Recognized species of the genus *Astilbe* are endemic to eastern Asia, with the exception of *A. biternata,* which is native to the southern Appalachians of North America. Intercontinental discontinuity similar to that in *Astilbe* has been described for about 120 other genera and was first

discussed by Asa Gray (1859). Zhengy (1983) described the relationships of 117 species of plants with disjunction specific to temperate regions of eastern Asia and eastern North America.

Unlike other species within the genus, *A. biternata* has subdioecious sex expression (Olson 2001). The pattern of fruit production along with ovary position has served as a method of phylogenetic characterization and has been investigated previously in Saxifragaceae (Soltis and Hufford 2002). Other morphological character states used for inferring differences among species in *Astilbe* have included degree of pubescence, location of pubescence, leaflet ratio, leaf pinnation, floral characters, and root structure (Britton 1888; Diels 1905; Hamilton 1825; Handel-Mazzetti 1931; Hayata 1908 and 1911; Hemsley 1890; Hooker and Jackson 1895; Hutchinson 1908; Knoll 1907 and 1909; Komarov 1903; Mattfield 1931).

1.2 Saxifragaceae

Taxonomically, *Astilbe* has been placed within Saxifragaceae, which has been considered one of the most problematic families at higher taxonomic levels (family and above) to characterize morphologically and phylogenetically (Soltis et al. 1990; 1993; Soltis and Soltis 1997; Soltis et al. 2001). Engler (1930) broadly defined the family as Saxifragaceae *sensu lato*, a group with a large array of plant types and 15 subfamilies, later expanded to 17. The relationships among taxa of Saxifragaceae *sensu lato* and discrepancies among original and recent taxonomic treatments is depicted in Figure 1.1, which is taken from Soltis et al. (1990). The diverse morphological inclusion of annual, biennial, and perennial herbs, shrubs, trees, and vines within the large family has made taxonomic rank and relationships within Saxifragaceae nearly impossible to define (Soltis

et al. 1993). A narrowly defined family has been proposed (Saxifragaceae *sensu stricto*), which has better support from molecular sequence analysis and includes about 30 genera of herbaceous plants (Soltis and Soltis 1997) (Table 1.1). Though considered problematic, Saxifragaceae has served as a model for autopolyploid speciation, coevolution, geographic speciation, and gynoecial diversification and development (Soltis et al. 2001).

1.3 Morphology in *Astilbe*

The similarity of morphological character states among taxa in *Astilbe* has made it difficult to distinguish species and variants within species. Only one member of the genus has simple leaves, whereas the remaining species have varying degrees of pinnation. Other characters such as leaflet shape, pubescence, and floral traits are typically similar across the genus, but may be useful in keying out species. Though an inclusive key has not been developed for the genus, keys have been developed for Chinese species, Korean species, and species commonly found in the horticultural trade (Pan 1985; Chung et al. 1983; Hatch 2000). Pan (1985) first distinguished Chinese species of *Astilbe* by inflorescence type and petal number, then utilized sepal characteristics and petal character states to separate other members.

1.4 Gene sequences in phylogeny

In order to infer phylogenetic and taxonomic relationships among plants researchers have often considered both morphological and molecular data. In recent years advances in technology and knowledge of gene sequences have significantly impacted angiosperm phylogeny (Hilu et al. 2003). Researchers have utilized chloroplast, nuclear, and mitochondrial genes to elucidate relationships at all levels of taxonomic rank.

Molecular approaches for analyzing phylogeny have become increasingly useful, especially where morphological characters have been insufficient for distinguishing genera (Soltis and Soltis 1997). This is especially true when genera differ by only one character state. In Saxifragaceae, plant morphology has been inadequate for alignment of genera within the family because of similarities among vegetative features, with some genera distinguished by only a few pronounced differences in floral or fruit morphology (Soltis et al. 1993, 2001).

Some of the most commonly utilized genes in molecular systematics and phylogeny of plants have been *rbc*L, a chloroplast gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase (Soltis and Soltis 1990); *atp*B, a plastid gene encoding the beta subunit of ATP synthase (Savolainen et al. 2000); *mat*K, a chloroplast gene thought to be involved in splicing introns coding for a maturase (Hilu et al. 2003); and portions of the nuclear rDNA cistron unit such as 18S, 5.8S, and 26S, occurring in the nucleolar organizing region of the nucleus (Nickrent and Soltis 1995). These genes have been used in phylogeny because of their ability to be easily amplified by the polymerase chain reaction (PCR), few insertion-deletion events, and their level of evolution and conservation (White et al. 1990; Nickrent and Soltis 1995; and Hilu et al. 2003).

Genes utilized in phylogenetics and systematics have differed in their ability to provide support for relationships at different taxonomic levels. The length of a gene sequence and the number of base pairs analyzed may affect its utility in inferring phylogenetic relationships; *rbc*L, *atp*B, *mat*K and rDNA regions have been used to resolve relationships at the familial level and higher (Soltis et al. 1990; 1993; 1997; 2001;

Savolainen et al. 2000; and Hilu et al. 2003). Soltis et al. (1993) found that *rbcL* sequence data may have limited ability to resolve generic-level differences in some taxonomic groups. However, when evaluating *Heuchera*, Soltis and Kuzoff (1995) found that plastid sequences may not be useful for distinguishing phylogenetic information at lower taxonomic levels. Utilization of *mat*K sequences has been effective for inferring differences in lower taxa (genus or below) in *Saxifraga* and *Chyrsosplenium* (Saxifragaceae) and *Gilia* (Polemoniaceae) (Johnson and Soltis, 1994; 1995; Soltis et al., 1996; 2001). In a study conducted within the genus *Saxifraga,* Soltis et al. (1996) characterized the genus *Saxifragopsis* as a sister group of *Astilbe*. Nuclear DNA regions, such as 18S and 26S rDNA, have shown tremendous potential for inferring phylogenies at taxonomic levels below the genus, and have been used in many different organisms from plants to bacteria (White et al., 1990; Nickrent and Soltis, 1995; and Soltis et al., 1997; 2001).

In some instances sequences from two or more genes have been utilized to deduce phylogenetic relationships and the discrimination abilities compared to validate the use of the genes. Nickrent and Soltis (1995) used *rbc*L and nuclear 18S rDNA sequences to compare angiosperm phylogeny and determined that sequences from either were efficient to distinguish differences among flowering plants at higher taxonomic levels. In a similar study, Savolainen et al. (2000) used sequences from both *atp*B and *rbc*L to investigate phylogeny among a wide array of flowering plants, concluding that, with some discrepancies, phylogenetic trees derived from the two genes were congruent. A comprehensive study of six gene sequences was conducted by Soltis et al. (2001) to elucidate relationships within Saxifragaceae. Trees generated from nuclear sequences

agreed closely with those derived from plastid sequences and a phylogenetic classification of the family was resolved.

1.5 Molecular Techniques

Other than genetic sequence alignment, molecular marker analysis, such as amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellites or simple sequence repeats (SSR), and single nucleotide polymorphisms (SNPs), have been employed to examine differences among members of genera and subgeneric taxa. Powell et al., (1996) compared the features of many of these marker systems for plant germplasm analysis. These methods are also frequently used in plant breeding programs to link markers to genes that affect important traits. AFLPs have been used to study genetic relationships of several plant species, including some ornamentals such as daylily (*Hemerocallis* L.; Tomkins et al. 2001), ivy leaf geranium (*Pelargonium peltatum* Soland.; Barcaccia et al. 1999), and ornamental *Liriopogon* Raf. (McHaro et al. 2003). Avacado (*Persea americana* Mill) has been evaluated using RAPD, RFLP, and microsatellite markers (Fiedler et al. 1998; Sharon et al. 1997; and Schnell et al. 2003). New technology increasingly favors the use of SNPs over other molecular markers due to ease of sequencing, low cost, and prevalence within genomes. Evans et al. (2004) compared SNP markers to SSR and recommended that previous linkage studies that engendered sparse microsatellite maps could benefit substantially by use of a denser map of markers.

1.6 Single Nucleotide Polymorphisms

SNPs are DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genetic code corresponding to a gene, part of a gene, or even a stretch of DNA, that includes more than a single gene or intergenic sequence, has been altered. For a variation to be considered a SNP, it must occur in at least 1% of the population. SNPs are the most common form of DNA sequence variation and have been used in the study of animal and human genetics, including mapping of the human genome and identification of haplotypes associated with human diseases in medical research (Wang et al. 1998). In comparison with other genetic markers, SNPs are more prevalent and conserved within the genome and are used because of their occurrence, more than one per 1,000 base pairs (Osman et al. 2003).

With the advent of SNP research, numerous methods of SNP discovery have been proposed, utilized, and are still being developed. Many methods have been adapted for high-thoroughput sequencing. Some of these platforms for detecting SNP include pyrosequencing (Fakhrai-Rad et al. 2002), polymorphism ratio sequencing (Blazej et al. 2003), degenerate oligonucleotide primer PCR (Jordan et al. 2002), ecotilling (Comai et al. 2004), and SNPHunter (Wang et al. 2005). Due to the influx of SNP data, the application of SNP information, and the varied techniques for obtaining SNPs, bioinformatic techniques have been developed to facilitate the discovery and analysis of SNPs. Internet-accessible tools for data access and display have been developed to help researchers to retrieve data about SNPs based on genes of interest, genetic or physical map locations, or expression pattern (Clifford et al. 2004).

Within the last 10 years, SNPs have been extensively studied in medical research and the mapping of SNP markers within the human genome has led to the discovery of many loci associated with predisposition to various diseases (Chen and Sullivan 2003). SNPs have also been used to study population parameters and to estimate divergence within structured human populations (Nicholson et al. 2002). Researchers have investigated the ability of SNPs to estimate population parameters and found that the method of SNP determination may affect the accuracy of predicting genetic occurrences within populations (Kuhner et al. 2000). Application of SNPs has varied from studying populations of humans and cattle to bacteria and plants. Utilizing bovine SNPs, Heaton et al. (2005) tracked beef products from donor animals to consumers with incidence of a coincidental genotype match between two animals being 1 in 23 million. In a study to differentiate and identify animal fiber, Subramanian et al. (2005) identified SNP markers confirmed by RFLP that distinguished wool fibers derived from goats from those derived from sheep.

Rafalski (2002) reviewed the application of SNPs in crop genetics, discussing linkage disequilibrium, use of expressed sequence tags (ESTs), and outlining discovery procedures, assays, SNPs as markers, and SNP mapping. Studies have been conducted in both major crop plants and in specialized crops, with SNP research in plants accelerating. SNP frequency and haplotype variation were determined in an extensive study involving 25 genotypes of soybean with germplasm originating from North America and Asia (Zhu et al. 2003). Presence of conserved SNPs among genotypes confirmed that there was relatively limited genetic variability within cultivated soybean. Somers et al. (2003) utilized ESTs to mine SNPs from 12 genotypes of wheat and determined that for every

540 bps of EST, one SNP occurred, and that the SNPs were applicable to conventional genetic studies as molecular markers. In a similar study Grivet et al. (2003) used ESTs to discover SNPs in sugarcane, concluding that the polymorphisms could serve as potential markers for sugarcane breeding. Genetic diversity within populations of *Eurycoma longifolia* was determined using SNPs, with occurrence of SNPs reflecting geographic origin of individual plants and different natural populations (Osman et al. 2003). SNPs have yet to be utilized in plants to infer phylogenetic relationships. Development of SNP-based markers within a taxon would be beneficial in conducting phylogenetic analysis.

1.7 Project Summary

This study was undertaken to develop a well-resolved phylogeny of the genus *Astilbe*. Our aim was to investigate variation of *Astilbe* at various levels, from variation within a seedling population to variation among species within the genus. We incorporated an expanded morphological data set and used gene sequences of *mat*K to develop the first molecular investigation into the genus. Unlike previous investigations into the genus, we attempted to incorporate all recognized species in our study to develop a comprehensive understanding of the genus generated by molecular and morphological support.

1.8 Literature Cited

Armitage A.M. 1996. Herbaceous perennial plants: a treatise on their identification, culture, and garden attributes. 2 edn. Stipes Pub. Champaign, p.167-176.

Barcaccia G., E.Albertini and M.Falcinelli. 1999. AFLP fingerprinting in *Pelargonium peltatum*: Its development and potential in cultivar identification. J. Hort. Sci. Biotech. 74: 243-250.

Blazej R.G., B.M. Paegel and R.A. Mathies. 2003. Polymorphism ratio sequencing: A new approach for single nucleotide polymorphism discovery and genotyping. Genome Res. 13: 287-293.

Britton N. 1888. *Astilbe biternata*. Bulletin of the Torrey Botanical Club 15: 98.

Chen X. and P.F. Sullivan. 2003. Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput. Pharmacogenomics J. 3: 77-96.

Chung Y.H., B.Y. Sun and Y.C. Chung. 1983. Monographic study of the endemic plants in Korea, III Taxonomy and interspecific relationships of the genus *Astilbe*. Korean J. Bot. 26: 73-90.

Clifford R.J., M.N. Edmonson, C. Nguyen, T. Scherpbier, Y. Hu and K.H. Buetow. 2004. Bioinformatics tools for single nucleotide polymorphism discovery and analysis. Appl. Bioinformatics Cancer Detection 1020: 101-109.

Comai L., K. Young, B.J. Till, S.H. Reynolds, E.A. Greene, C.A. Codomo, L.C. Enns, J.E. Johnson, C. Burtner, A.R. Odden and S. Henikoff. 2004. Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. Plant J. 37: 778-786.

Cronquist, A. 1981. An Integrated System of Classification of Flowering Plants. Columbia University Press, New York.

Diels L. 1905. Beitrage zur Flora des Tsinling Shan und andere Zusutze zur Flora von Central China. Bot. Jahrb. 36: 48-49.

Engler A. 1930. Saxifragaceae. Die naturlichen Pflanzenfamilien. Wilhelm Engelmann, Leipzig, 226 p.

Engler A. 1964. Syllabus der Pflanzenfamilien, eds. Melchior, H. and Werederman, E. Berlin, pp 137-138.

Evans D. and L. Cardon. 2004. Guidelines for genotyping in genomewide linkage studies: Single-nucleotide polymorphism maps versus microsatellite maps. Am. J. Hum. Genet. 75: 687-692

Fakhrai-Rad H., N. Pourmand and M. Ronaghi. 2002. Pyrosequencing (TM): An accurate detection platform for single nucleotide polymorphisms. Human Mutation 19: 479-485.

Fiedler J., G. Bufler and F. Bangerth. 1998. Genetic relationships of avocado (*Persea americana* Mill) using RAPD markers. Euphytica 101: 249-255.

Gray A. 1859. Observations upon the relationships of the Japanese flora to that of North America and other parts of the Northern Hemisphere. Mem. Am. Acad. Arts 6: 377-453.

Grivet L., J.C. Glaszmann, M. Vincentz, F. da Silva and P. Arruda. 2003. ESTs as a source for sequence polymorphism discovery in sugarcane: example of the Adh genes. Theor. Appl. Genet. 106: 190-197.

Hamilton F. 1825. Prodromus Florae Nepalensis. Societ. Linnean, London.

Handel-Mazzetti H. 1931. Symbolae Sinicae. 7: 414-415.

Hatch L.C. (2000) Key to cultivated species of *Astilbe*. http://members.tripod.com/~hatch_l/astimpi.html.

Hayata B. 1908. Flora Montana Formosae. J. Coll. Sci. Tokyo 25: 86-87.

Hayata H. 1911. Material for a flora of Formosa. J. Coll. Sci. Tokyo 30: 211-212.

Heaton M.P., J.E. Keen, M.L. Clawson, G.P. Harhay, N. Bauer, C. Shultz, B.T. Green, L. Durso, C.G. Chitko-McKown and W.W. Laegreid. 2005. Use of bovine single nucleotide polymorphism markers to verify sample tracking in beef processing. Javma-J. Am. Vet. Med. Ass. 226: 1311-1314.

Hemsley W. 1890. An enumeration of all the plants known from China proper, Formosa, Hainan, Corea, the Luchu Archipelago, and the island of Hongkong, together with their distribution and synonymy. J. Linnean Societ. 26: 121-236.

Henry A. 1902. The genus *Astilbe*. Gard. Chron. 32: 154-156.

Hilu K.W., T. Borsch, K. Muller, D.E. Soltis, P.S. Soltis, V. Savolainen, M.W. Chase, M.P. Powell, L.A. Alice, R. Evans, H. Sauquet, C. Neinhuis, T.A.B. Slotta, J.G. Rohwer, C.S. Campbell and L.W. Chatrou. 2003. Angiosperm phylogeny based on *mat*K sequence information. Am. J. Bot. 90: 1758-1776.

Hooker J.D. and B.D.Jackson. 1895. Index Kewensis Plantarum Phanerogamarum. Oxford University Press London.

Hutchinson J. 1908. Miscellaneous information. .Royal Botanic Gardens Kew Bulletin of Miscellaneous Information 1: 16.

Johnson L.A. and D.E. Soltis. 1994. *mat*K DNA-Sequences and phylogenetic reconstruction in Saxifragaceae S-Str. Syst. Bot. 19: 143-156.

Johnson L.A. and D.E. Soltis. 1995. Phylogenetic inference in Saxifragaceae *sensu stricto* and *Gilia* (Polemoniaceae) using *mat*K sequences. Ann. Missouri Bot. Garden 82: 149-175.

Jordan B., A. Charest, J.F. Dowd, J.P. Blumenstiel, R.F. Yeh, A. Osman, D.E. Housman and J.E. Landers. 2002. Genome complexity reduction for SNP genotyping analysis. Proc. Nat. Acad. Sci. USA 99: 2942-2947.

Knoll F. 1907. Beitrag zur Kenntnis der Astilbe-Arten Ostasiens (Als Vorlaufige Mitteilung Einer Monographischen Bearbeitung der Gattung Astilbe). Bull. Herb. Boiss. 7: 127-135.

Knoll F. 1909. Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften, Mathematisch-Naturwissenschaftlichen Classe. Abteilung 1: 73.

Komarov V.L. 1903. Flora Manshuricae. Act. Hort. Petrop. 22: 407-409.

Kuhner M.K., P. Beerli, J. Yamato and J. Felsenstein. 2000. Usefulness of single nucleotide polymorphism data for estimating population parameters. Genetics 156: 439-447.

Mattfeld J. 1931. Saxifragaceae Novae Sinenses. Natizbl. Bot. Gart. Berlin 11: 301-302.

Maximowicz C.J. 1873. Enumeratio Plantarum in Japonia Sponte Crescentium 1: 144.

McHaro M., E. Bush, D. La Bonte, C. Broussard and L. Urbatsch. 2003. Molecular and morphological investigation of ornamental liriopogons. J. Am. Soc. Hort. Sci. 128: 575-577.

Nicholson G., A.V. Smith, F. Jonsson, O. Gustafsson, K. Stefansson and P. Donnelly. 2002. Assessing population differentiation and isolation from single-nucleotide

polymorphism data. J.Royal Stat. Soc. Series B-Statistical Methodology 64: 695-715.

Nickrent D.L. and D.E. Soltis. 1995. A comparison of angiosperm phylogenies from nuclear 18s rDNA and *rbc*L sequences. Ann. Missouri Bot. Gard. 82: 208-234.

Olson M.S. 2001. Patterns of fruit production in the subdioecious plant *Astilbe biternata* (Saxifragaceae). J. Ecology 89: 600-607.

Osman A., B. Jordan, P.A. Lessard, N. Muhammad, M.R. Haron, N.M. Riffin, A.J. Sinskey, C. Rha and D.E. Housman. 2003. Genetic diversity of *Eurycoma longifolia* inferred from single nucleotide polymorphisms. Plant Phys. 131: 1294-1301.

Pan J. 1985. A study on the genus *Astilbe* Buch.-Ham. ex D. Don from China. Acta Phytotax. Sinica 23: 432-443.

Powell W., M. Morgante, C. Andre, M. Hanafey, J. Vogel, S. Tingey and A. Rafalski. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. 2: 225-238.

Rafalski A. 2002. Applications of single nucleotide polymorphisms in crop genetics. Current Opinion Plant Biol. 5: 94-100.

Randhava L. (2005) Plant Profile: *Astilbe*. In: Chicago Botanical Garden Online Index. p www.chicagobotanic.org/plantinfo/pp/PPAstilbe.html.

Savolainen V., M.W. Chase, S.B. Hoot, C.M. Morton, D.E. Soltis, C. Bayer, M.F. Fay, A.Y. De Bruijn, S. Sullivan and Y.L. Qiu. 2000. Phylogenetics of flowering plants based on combined analysis of plastid *atp*B and *rbc*L gene sequences. Syst. Biol. 49: 306-362.

Schnell R.J., J.S. Brown, C.T. Olano, E.J. Power, C.A. Krol, D.N. Kuhn and J.C. Motamayor. 2003. Evaluation of avocado germplasm using microsatellite markers. J. Am. Soc. Hort. Sci. 128: 881-889.

Sharon D., P.B. Cregan, S. Mhameed, M. Kusharska, J. Hillel, E. Lahav, and U. Lavi. 1997. An integrated genetic linkage map of avocado. Theor. Appl. Genet. 95: 911-921.

Soltis D.E. and L. Hufford. 2002. Ovary position diversity in Saxifragaceae: Clarifying the homology of epigyny. Intl. J. Plant Sci. 163: 277-293.

Soltis D.E. and R.K. Kuzoff. 1995. Discordance between nuclear and chloroplast phylogenies in the *Heuchera* group (Saxifragaceae). Evolution 49: 727-742.

Soltis D.E., R.K. Kuzoff, E. Conti, R. Gornall and K. Ferguson. 1996. *mat*K and *rbc*L gene sequence data indicate that *Saxifraga* (Saxifragaceae) is polyphyletic. Am. J. Bot. 83: 371-382.

Soltis D.E., R.K. Kuzoff, M.E. Mort, M. Zanis, M. Fishbein, L. Hufford, J. Koontz and M.K. Arroyo. 2001. Elucidating deep-level phylogenetic relationships in Saxifragaceae using sequences for six chloroplastic and nuclear DNA regions. Ann. Missouri Bot. Garden 88: 669-693.

Soltis D.E., D.R. Morgan, A. Grable, P.S. Soltis and R. Kuzoff. 1993. Molecular systematics of Saxifragaceae-*sensu-stricto*. Am. J. Bot. 80: 1056-1081.

Soltis D.E. and P.S. Soltis. 1997. Phylogenetic relationships in Saxifragaceae *sensu lato*: a comparison of topologies based on 18S rDNA and *rbc*L sequences. Am. J. Bot. 84: 504-522.

Soltis D.E., P.S. Soltis, M.T. Clegg and M. Durbin. 1990. *rbc*L sequence divergence and phylogenetic-relationships in Saxifragaceae *sensu-lato*. Proc. Natl. Acad. Sci. U.S. 87: 4640-4644.

Soltis D.E., P.S. Soltis, D.L. Nickrent, L.A. Johnson, W.J. Hahn, S.B. Hoot, J.A. Sweere, R.K. Kuzoff, K.A. Kron, M.W. Chase, S.M. Swensen, E.A. Zimmer, S.M. Chaw, L.J. Gillespie, W.J. Kress and K.J. Sytsma. 1997. Angiosperm phylogeny inferred from 18S ribosomal DNA sequences. Ann. Missouri Bot. Garden 84: 1-49.

Soltis D.E., M. Tago-Nakazawa, Q.Y. Xiang, S. Kawano, J. Murata, M. Wakabayashi and C. Hibsch-Jetter. 2001. Phylogenetic relationships and evolution in *Chrysosplenium* (Saxifragaceae) based on *mat*K sequence data. Am. J. Bot. 88: 883-893.

Somers D.J., R. Kirkpatrick, M. Moniwa and A. Walsh. 2003. Mining single-nucleotide polymorphisms from hexaploid wheat ESTs. Genome 46: 431-437.

Subramanian S., T. Karthik and N.N. Vijayaraaghavan. 2005. Single nucleotide polymorphism for animal fibre identification. J. Biotechnol. 116: 153-158.

Takhtajan, A. 1987. System of Magnoliophyta. Academy of Sciences U.S.S.R. Leningrad.

Tomkins J.P., T.C. Wood, L.S. Barnes, A. Westman and R.A. Wing. 2001. Evaluation of genetic variation in the daylily (*Hemerocallis* spp.) using AFLP markers. Theor. Appl. Genet. 102: 489-496.

Wang D.G., J.B. Fan, C.J. Siao, A. Berno, P. Young, R. Sapolsky, G. Ghandour, N. Perkins, E. Winchester, J. Spencer, L. Kruglyak, L. Stein, L. Hsie, T. Topaloglou, E. Hubbell, E. Robinson, M. Mittmann, M.S. Morris, N.P. Shen, D. Kilburn, J. Rioux, C. Nusbaum, S. Rozen, T.J. Hudson, R. Lipshutz, M. Chee and E.S. Lander. 1998. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science 280: 1077-1082.

White T., T. Bruns, S.Lee and J.Taylor (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M., Gelfand D., J.Sninsky, T.White, eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp. 315-322.

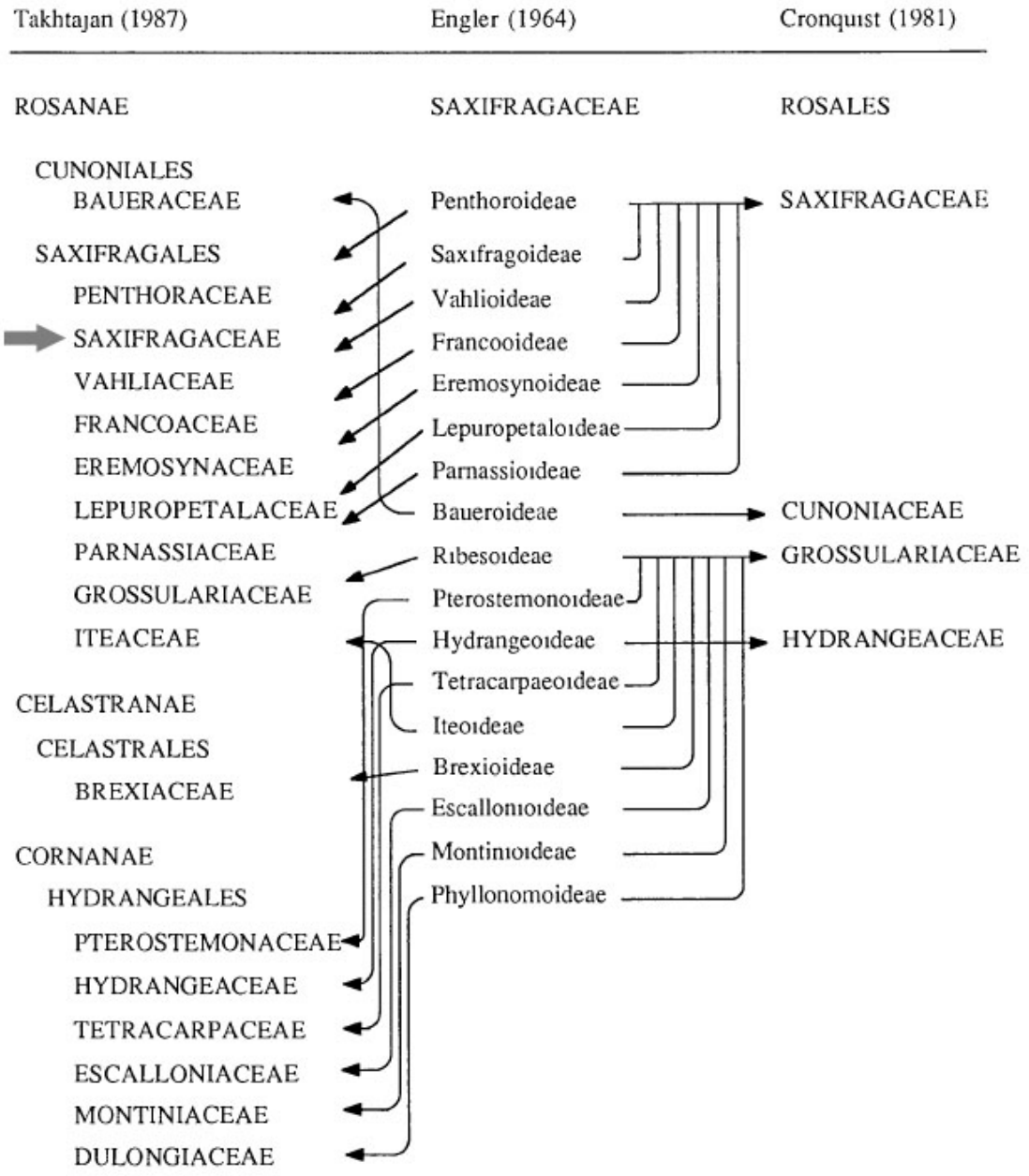
Zhengyi W. 1983. On the significance of Pacific intercontinental discontinuity. Ann. Missouri Bot. Gard. 70: 577-590.

Zhu Y.L., Q.J. Song, D.L. Hyten, C.P. Van Tassell, L.K. Matukumalli, D.R. Grimm, S.M. Hyatt, E.W. Fickus, N.D. Young and P.B. Cregan. 2003. Single-nucleotide polymorphisms in soybean. Genetics 163: 1123-1134.

Table 1.1 Current list of genera within Saxifragaceae *sensu stricto* (Soltis and Soltis 1997).

Genus
Astilbe Buch.-Ham. ex D.Don
Astilboides (Hemsl.) Engl.
Bensoniella Morton
Bergenia Moench
Bolandra A. Gray
Boykinia Nutt.
Chyrsosplenium Tourn. ex L.
Conimitella Rydb.
Darmera A. Voss
Elmera Rydb.
Francoa Cav.
Heuchera L.
Jepsonia Small
Leptarrhena R.Br
Lithophragma Torr. & Gray
Mitella Tourn. ex L.
Mukdenia Koidz.
Peltoboykinia (Engl.) Hara
Rodgersia A. Gray
Saxifraga L.
Saxifragopsis Small
Saxifragella Engl.
Suksdorfia A. Gray
Sullivantia Torr. & Gray
Tanakaea Franch. & Sav.
Telesonix Raf.
Tellima R.Br.
Tetracaraea Hook.f.
Tiarella L.
Tolmiea Torr. & Gray

Figure 1.1 Relationships among taxa of Saxifragaceae sensu lato illustrating discrepancies between original and recent taxonomic treatments. The current treatment of the family, Saxifragaceae *sensu stricto* is highlighted by the large arrow (Soltis et al. 1990).



Chapter 2. Morphological Investigation of *Astilbe*

Abstract

Astilbe (Saxifragaceae) is a genus of herbaceous plants consistently ranked among the top ten landscape perennials. The genus is disjunct with all members native to Asia with the exception of *Astilbe biternata*, which is endemic to North America. Species relationships within the genus are highly problematic. In some treatments of the genus, variants within populations have been considered as separate species, resulting in disagreement on the number of species within the genus. To establish order within the genus, we investigated 28 morphological character states from 21 taxa of *Astilbe.* *Saxifragopsis fragarioides* was chosen as the outgroup taxon for maximum parsimony and neighbor joining analysis. Resulting phylogenetic trees divided the genus into three distinct clades, with the third clade represented by only two taxa. The relationships were well resolved though only a few branches had greater than 50% bootstrap support. There is evidence from the phylogeny that some described species may actually represent variation within populations of species. From our analysis I propose that the genus has 13 to 15 species and present a key to distinguish the species.

2.1 Introduction

The genus *Astilbe* (Saxifragaceae) is comprised of herbaceous perennials valued by the horticultural industry for their distinctive fern-like foliage, attractive flowers, and low maintenance. Because of their immense popularity, *Astilbe* have been extensively hybridized, selected, and marketed to a point where the lineages of most garden varieties has been lost. In addition, the genus is complex due to intercontinental discontinuity, differences in sexual expression, and similar morphological traits. Development of character states, which would delineate the species and infer phylogenetic relationships among them, may provide clarity to the genus.

To date, there has been limited investigation into *Astilbe* other than how the genus aligns within Saxifragaceae (Johnson and Soltis 1994; 1995; Soltis et al. 1996; 2001). Within the family, *Astilbe* was found to be sister to the monotypic genus *Saxifragopsis* and aligns within the Heucheroid clade of the Saxifragaceae (Soltis et al. 2001). The number of species within the genus varies from 13 to 22 depending on the citation, and there is little agreement on the presence of subspecies and varieties within these natural populations (Griffiths 1994; Stevens 2005). An initial search on the International Plant Name Index (IPNI) yields approximately 60 different described species of *Astilbe*, many of which are synonyms of one another or have been transferred to another genus. Some of these described species likely represent variation within a population and would be better classified as a subspecies or variety.

In addition, there has been extensive breeding of *Astilbe* for larger flowers, flower color, and unique foliage. Many of the selections and cultivars currently utilized in horticulture today were derived from the breeding efforts of a famous German hybridizer,

George Arends (1862-1952). Mr. Arends has been credited with introducing over 74 cultivars of *Astilbe* in 50 years (Randhava 2005). Most of the selections were derived from only four species, noted for having dense flowers along the panicle coupled with longer petals: *A. chinensis*, *A. japonica*, *A. simplicifolia*, and *A. thunbergii* (Randhava 2005). Because so many cultivars and selections are available, the true lineage of many selections is not well documented and unimproved species are rarely utilized in gardens.

The last comprehensive investigation of *Astilbe* was conducted by Pan (1985) and concentrated primarily on Chinese species with a focus on morphology and geographical distribution. Pan broadly described the genus with 18 species, including a species (*A. crenatilobata* (Britt.) Small) thought to be extinct from North America (Tables 2.1 and 2.2). Two years prior to Pan's study, Chung et al. (1983) published a study of the relationships among eight endemic taxa of Korean *Astilbe* (Table 2.3). In their study, Chung et al. addressed three different species not mentioned by Pan, and included varieties of *A. chinensis* based upon morphology and geographical distribution. In many of the original descriptions of *Astilbe*, reference to varieties within the natural populations is made (Britton 1888; Chung et al. 1983; Diels 1905; Hamilton 1825; Handel-Mazzetti 1931; Hayata 1908,1911; Hemsley 1890; Hooker and Jackson 1895; Hutchinson 1908; Knoll 1907,1909; Komarov 1903; Mattfield 1931; Pan 1985).

Because there is debate as to whether some recognized *Astilbe* are varieties or actual species, it is important to address what in fact distinguishes the two. According to Barton (2001), speciation describes different phenotypes, use of resources, and coexistence. Methods of speciation have been examined by Endler (1977) and exemplified by Levin (2004a, 2004b, 2005). Allopatric, parapatric, and sympatric modes

of speciation proposed by Endler (1977) describe differentiation by geographic isolation, reproductive character traits, or ecological preferences, respectively. Levin addresses the idea of speciation based upon changes in niche environments, changes in environments due to levels of disturbance, and to changes in plant genotype through selection or hybridization.

Levels of speciation tend to be greater in regions where disturbances such as glacial movement, isolation, or volcanic activity are known to have occurred. Archipelagos such as Hawaii, The Galapagos, and Indonesia are ideal regions representing increased levels of speciation due to niches created from variations in climate and geographic separation (Levin 2004a). Researchers including Charles Darwin have described the diversity of species among these regions. Species evolving from common ancestors in these regions tend to have subtle differences in both genotype and phenotype. Rieseberg et al. (1997; 1999) found that species that are perennial, outcrossing, and asexually propagated are more likely to hybridize. Thus, Rieseberg noted that habitats occupied by hybrid entities are often ecologically different than those inhabited by the parents. Applying these ideals of speciation to *Astilbe* and looking at the distribution of the genus may help discern the origin and evolution of species.

Recognized species of the genus *Astilbe* are endemic to eastern Asia with, the exception of *A. biternata,* which is native to the southern Appalachians of North America (Figure 2.1). This intercontinental discontinuity observed in *Astilbe* is thought to occur in about 120 other genera and was first discussed by Asa Gray (1859). Because most *Astilbe* are endemic to Japan, Indonesia, Vietnam, Korea, the Philippines, and China, all of which have significant geographic and climatic niches, it may be hypothesized that

progenitors of the modern genus could have rapidly evolved. The similarity among character states within the genus, taxa differing only in flower color, leaf arrangement, and floral morphology, may explain how some *Astilbe* originally evolved. Hybrids of some of the species could have become established in niche environments and developed characters that favored successful inhabitation of an area. Such characters may have become fixed genetically in progeny due to natural selection. Levin (2004b) discusses how variations in gene expression may have the capacity to change flower color in some plants, erectness of flowers in others, and how a series of gene mutations could potentially affect sex expression.

Unlike other species within the genus, *A. biternata* has subdioecious sex expression (Olson 2001) and is tetraploid. This species is also the only member of the genus native to North America, except for the now extinct *A. crenatoloba* (Mellichamp 1982). The method of sex expression may be viewed as a fitness factor for ensuring variation within the species due to a lack of potential out-breeding with closely related species. The polyploid nature of the species may also be viewed as a fitness factor facilitating homeostasis for various habitats and access to resources off limits to their progenitors (Levin 2004a).

The similarity of morphological character states among taxa of *Astilbe* has made it difficult to distinguish species and variants within species. Only one member of the genus has simple leaves, while the remaining species have varying degrees of pinnation. Other characters such as leaflet shape, pubescence, and floral traits are typically similar across the genus, but may be useful for keying out only one or more species. Though an inclusive key has not been developed for the genus, separate keys have been developed

for the Chinese species, Korean species, and species commonly found in the horticultural trade. In Pan's (1985) key to Chinese species (Table 2.4), members of the genus are divided first by inflorescence type and petal number. The next step in the key relies on sepal characteristics and petal character states to delineate individual species. Chung et al. (1983) focused on different species and varieties in their treatment of *Astilbe*. Their key focused on plant size and floral characteristics such as stamen length and inflorescence branching to distinguish taxa (Table 2.5). Similarly, the key to cultivated species of *Astilbe* utilizes inflorescence type, petal, and leaflet characteristics to distinguish species and popular cultivars (Hatch 2000; Table 2.6). A character list from original species descriptions and dichotomous keys could be used to investigate the taxa of *Astilbe*.

This study was undertaken to develop a well-resolved phylogeny of *Astilbe* using an expanded morphological data set. My aim was to determine whether some described species within the genus are actual variations within a species and to determine the relationship of North American *A. biternata* to Asian species. A final goal of the research was to develop a more inclusive key to the genus based on the morphological data set.

2.2 Materials and Methods

Analysis of morphological variation in *Astilbe* was based on measurements of 101 herbarium specimens. The morphological characters in this study were selected from species descriptions (Britton 1888; Diels 1905; Hamilton 1825; Handel-Mazzetti 1931; Hayata 1908; 1911; Hemsley 1890; Hooker and Jackson 1895; Hutchinson 1908; Knoll 1907; 1909; Komarov 1903; Mattfield 1931, studies conducted by Pan (1985) and Chung et al. (1983), and from personal observations of material. For compiling the morphological data matrix, I borrowed specimens from six national herbaria (Table 2.7).

Variation of 28 vegetative and floral characters with two to five states was analyzed for 21 species, subspecies, and varieties of *Astilbe* (Table 2.8). Not all characters could be scored from each herbarium specimen. The outgroup, *Saxifragopsis fragarioides,* was selected based on studies conducted by Soltis et al. (1996), who found the monotypic genus to be the sister to *Astilbe*.

All data were scored as qualitative so there was no need for standardization of the data matrix. The Kruskal-Wallis test was implemented as the Wilcoxon npar 1-way test (SAS Institute Inc. 2005) to assess significance among characters. Characters with significant variation ($P<0.005$) were then used in multivariate cluster analysis. The multivariate cluster analysis was implemented using the FASTCLUS procedure (SAS Institute Inc. 2005), which allows for clustering of large datasets with the ability to specify the number of clusters desired. We increased the number of clusters until the cubic clustering criterion was optimized. Because some of the taxa did not fall within a single cluster, these taxa were analyzed further for character state means. Some of the herbarium sheets were incorrectly identified and the clustering permitted for detection of such sheets. From this analysis, the morphological data matrix was derived for use in phylogenetic determination (Table 2.9).

Maximum parsimony analysis was performed for the morphological data set using PAUP version 4.0b10 (Swofford 2003). All characters were weighted equally. A heuristic search strategy was implemented with 20 replicates using random taxon addition sequence, TBR (tree bisection and reconnection) branch swapping, and a maximum of 1,000 trees per replicate. Bootstrap support (Felsenstein 1985) was determined with 200 replicates using heuristic search options and TBR branch swapping, with the maxtree

option set at 1,000. Neighbor joining trees were also generated for *Astilbe* taxa using PAUP.

2.3 Results

All 28 characters were statistically informative according to the Kruskal-Wallis test implemented as the Wilcoxon npar 1-way test and, therefore, were included in further cluster and phylogenetic analysis (Table 2.10). Cluster analysis of data collected from herbarium sheets was optimized into 14 clusters according to a cubic clustering criterion of 99.02. Taxa of *Astilbe* arranged within single clusters. *Astilbe grandis*, *A. macroflora*, *A. microphylla*, *A. myriantha*, and *A. rivularis* were the exceptions, with some specimens occurring in each of two or more clusters (Table 2.11 and Figure 2.2). Even when the number of clusters was reduced, these taxa consistently were divided among one or more clusters. Because some characters were variable among herbarium sheets for those *Astilbe* species that separated into multiple clusters, characters were selected based on the clusters by which the majority of the taxa were arranged. For example, *A. microphylla* was represented by eight herbarium specimens, with seven of those specimens arranged into cluster 4 and the last in cluster 6. Data from the herbarium specimen arranged in cluster 6 were not utilized in the morphological data matrix. In the case of *A. macroflora*, half of the samples arranged in cluster 6 and the other half in cluster 9. With this taxon, three of the herbarium sheets were inconsistent with original descriptions of the species, and these three diverged into a separate cluster. Therefore, characters representing these sheets were not utilized in further analysis.

The analysis of 28 morphological characters with two to five states representing 21 taxa of *Astilbe,* using *Saxifragopsis fragarioides* as the outgroup, resulted in 76 most

parsimonious trees with a tree length of 174 steps and rescaled consistency index RC = 0.1755. Reweighting of characters yielded the single stable most parsimonious tree with length 44.2 steps and an RC of 0.2915, retention index (RI) of 0.6339, and a homoplasy index (HI) of 0.5795 (Figure 2.3).

Of the 28 characters, 25 were considered parsimony-informative and divided the genus into two distinct clades with *A. japonica* and *A. glaberrima* forming a third separate clade (Figure 2.3). The first clade is composed of taxa with linear petal shape, containing mainly Chinese taxa, the putative primitive species *A. simplicifolia*, and the North American species *A. biternata*. *Astilbe* species that typically lack petals, or that have petals spatulate in shape, comprised the second major clade. The third clade, with *A. japonica* and *A. glaberrima*, was distinguished by taxa having deeply serrated leaflet margins. Analysis removing this single character state did not affect relationships within the tree, and the two taxa still formed a separate clade. Of importance, varieties commonly disputed as species aligned closely in the tree with the species with which they are most commonly associated (e.g., *A. glaberrima* with *A. japonica*). Branches of the tree have low levels of bootstrap support, with only relationships in the second clade attaining support higher than 50%. The neighbor-joining tree (Figure 2.4), utilizing the same data matrix, correlated closely with the most parsimonious tree and differed only by the splitting of *A. simplicifolia* from *A. rubra* and *A. microphylla*, the joining of *A. koreana* to be sister with *A. austrosinensis*, and the joining of *A. myriantha* to be sister to *A. macrocarpa*.

A second analysis was conducted using 15 taxa, of *Astilbe* for which molecular data were acquired. A heuristic search of the 15 taxa using *Saxifragopsis fragarioides* as

the outgroup, resulted in two parsimonious trees with a tree length of 129 steps (Figure 2.5). Of the 28 characters, 23 were parsimony-informative and resulted in trees with an RC of 0.2267, CI of 0.408, HI of 0.529, and RI of 0.536. Two clades were generated, one comprised of *A. japonica* and *A. glaberrima* and another large clade with the remaining taxa. Branches within the tree had low levels of bootstrap support, with only four branches having support above 50%. Again, varieties commonly disputed as species aligned closely in the tree with the species with which they have been most commonly associated. The neighbor joining tree (Figure 2.6) closely correlated with the parsimonious tree, with the exception of *A. grandis* forming a sister to *A. koreana* and the alignment changing between *A. simplicifolia*, *A. rubra*, and *A. microphylla*.

2.4 Discussion

This is the first inclusive study using morphological characters to investigate relationships among members of the genus *Astilbe*. The morphological character matrix provided a well-resolved tree for both groupings of *Astilbe* taxa (Figures 2.3 and 2.5). Though bootstrap support was low for most branches in both trees, relationships formed within the trees were consistent with previous treatments of the genus, broadly grouping the Chinese species with linear petals in one group and the species lacking petals or having spatulate petals into a second (Pan 1985).

Relationships between the Chinese species *A. austrosinensis*, *A. chinensis*, *A. davidii*, *A. grandis*, and *A. koreana* have historically been problematic due to the apparent homology of morphological characters among them (Handel-Mazetti 1931; Chung et al. 1983; Pan 1985; Nakanishi 1998). Most taxonomic treatments have agreed that *A. davidii* is most appropriately recognized as a variety of *A. chinensis*. This relationship was

supported by my phylogenetic trees, where the two formed a sister group (Figures 2.3-2.6). Both *A. austrosinensis* and *A. koreana* have been described as either synonyms or varieties of *A. grandis* (Pan 1985). However, Chung et al. (1983) treated *A. koreana* as a separate species. Phylogenetic analysis (Figures 2.3 and 2.4) showed that the three taxa are closely related and that, when *A. austrosinensis* was deleted from the analysis, *A. koreana* formed a sister group with *A. grandis* (Figure 2.6).

A. rubra and *A. longicarpa* have sometimes been described as varieties of *A. chinensis* (Hayata 1908; Handel-Mazetti 1931; Pan 1985), however, our analysis supports the description of these taxa as separate species (Figures 2.3-2.6). *Astilbe biternata*, the single North American species, separated from the Chinese taxa within the first clade in all phylogenetic analyses.

Another problematic group, representing taxa of *Astilbe* with few to no petals, is presented in a subclade of the second clade: *A. angustifolia*, *A. macrocarpa*, *A. myriantha*, and *A. rivularis*. These taxa have been defined historically as either species or varieties (Diels 1905; Hara and Williams 1979; and Pan 1985). Phylogenetic analysis of these taxa using morphology revealed close relationships among the four taxa, with modest 78% support (Figure 2.3). *Astilbe angustifolia* formed a sister to *A. rivularis* with 74% support; however, when *A. angustifolia* and *A. macrocarpa* were deleted from the analysis, *A. myriantha* formed a sister group with *A. rivularis* with 71 % support. These findings support Pan's (1985) treatment of the taxa, which determined that *A. angustifolia* and *A. myriantha* are varieties of *A. rivularis*.

Astilbe formosa, *A. fujisanensis*, and *A. thunbergii* are another series of taxa that have historically been described as either species or varieties (Nakai 1922). In my

analysis, *A. formosa* and *A. fujisanensis* formed a sister group adjoined by *A. thunbergii* (Figures 2.3 and 2.4). Treatment of *A. formosa* and *A. fujisanensis* as varieties of *A. thunbergii* is supported by this analysis.

Astilbe philippinensis and *A. macroflora* formed a sister group within the second clade with bootstrap support of 61%. These taxa are both typified by dense pubescence on leaflet surfaces, stems, and peduncles. These taxa also have wide flower petals, with *A. macroflora* typically having 4-6 veins and *A. philippinensis* having up to three veins in the petal. The two taxa differ by distinct leaflet shapes.

The single clade comprised of the sister group *A. glaberrima* and *A. japonica* had high levels of bootstrap support, 88 % and 76 % (Figures 2.3 and 2.5). *Astilbe glaberrima* has often been treated as a variety of *A. japonica* and differs by a only a few characters, including a deeply serrated leaflet margin and denser flower panicles (Nakai 1922).

From the phylogenetic analysis and previous descriptions, I have developed a key to determine members of *Astilbe* (Table 2.12). The genus has been broadly defined to consider, in many instances, variances among populations to be species. Based upon my inclusive analysis of 21 taxa of *Astilbe* using 28 morphological characters, it is apparent that many described species are truly variants. From this research, I propose that the genus consist of 13 to 15 species (Figure 2.7). This treatment differs from Pan's (1985) investigation of the genus, which includes 18 species, because we did not include the now extinct *A. crenatilobata* (herbarium material was not available), *A. indica* (now *Cunonia indica*), *A. khasiana* (based on the same description as *A. rubra*), or the rare *A. platyphylla* and *A. apoensis* (herbarium samples and original descriptions were unattainable).

Future research comparing nuclear and plastid gene regions among *Astilbe* species would help validate our phylogenetic analysis of morphological character states. Integrating a geographic component into the analysis might further strengthen the proposed evaluation of the genus with 13 to 15 species.

2.5 Literature Cited

Barton N.H. 2001. Speciation. Trends Ecol. Evol. 16: 325-325.

Britton N. 1888. *Astilbe biternata*. Bull. Torrey Bot. Club 15: 98.

Chung Y.H., B.Y. Sun and Y.C. Chung. 1983. Monographic study of the endemic plants in Korea, III Taxonomy and interspecific relationships of the genus *Astilbe*. Korean J. Bot. 26: 73-90.

Diels L. 1905. Beitrage zur Flora des Tsinling Shan und andere Zusutze zur Flora von Central China. Bot Jahrb 36: 48-49.

Endler J.A. 1977. Geographic Variation, Speciation, and Clines. Princeton University Press, Trenton.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evol. 30: 783-791.

Gray A. 1859. Observations upon the relationships of the Japanese flora to that of North America and other parts of the Northern Hemisphere. Mem. Am. Acad. Arts 6: 377-453.

Griffiths M. 1994. Index of Garden Plants. Timber Press, Portland.

Hamilton F. 1825. Prodromus Florae Nepalensis. Societ. Linnean, London.

Hara H. and L. H. J. Williams eds. 1979. An Enumeration of the Flowering Plants of Nepal. Trustees of British Museum, London, 149.

Hatch L.C. (2000) Key to cultivated species of *Astilbe*. http://members.tripod.com/~hatch_l/astimpi.html.

Hayata B. 1908. Flora Montana Formosae. J. Coll. Sci. Tokyo 25: 86-87.

Hayata H. 1911. Material for a flora of Formosa. J. Coll. Sci. Tokyo 30: 211-212.

Hemsley W. 1890. An enumeration of all the plants known from China proper, Formosa, Hainan, Corea, the Luchu Archipelago, and the island of Hongkong, together with their distribution and synonymy. J. Linnean Soc. 26: 121-236.

Hooker J.D. and B.D.Jackson. 1895. Index Kewensis Plantarum Phanerogamarum. Oxford University Press, London.

Hutchinson J. 1908. Miscellaneous information. .Royal Botanic Gardens Kew Bulletin of Miscellaneous Information 1: 16.

Johnson L.A. and D.E. Soltis. 1994. *mat*K DNA-sequences and phylogenetic reconstruction in Saxifragaceae S-Str. Syst. Bot. 19: 143-156.

Johnson L.A. and D.E. Soltis. 1995. Phylogenetic inference in Saxifragaceae *sensu stricto* and *Gilia* (Polemoniaceae) using *mat*K sequences. Ann. Missouri Bot. Gard. 82: 149-175.

Knoll F. 1907. Beitrag zur Kenntnis der Astilbe-Arten Ostasiens (Als Vorlaufige Mitteilung Einer Monographischen Bearbeitung der Gattung Astilbe). Bull. Herb. Boiss. 7: 127-135.

Knoll F. 1909. Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften, Mathematisch-Naturwissenschaftlichen Classe. Abteilung 1: 73.

Komarov V.L. 1903. Flora Manshuricae. Act. Hort. Petrop. 22: 407-409.

Levin D.A. 2004a. Ecological speciation: Crossing the divide. Syst. Bot. 29: 807-816.

Levin D.A. 2004b. Ecological speciation: The role of disturbance. Syst. Bot. 29: 225-233.

Levin D.A. 2005. Niche shifts: The primary driver of novelty within Angiosperm genera. Syst. Bot. 30: 9-15.

Mattfeld J. 1931. Saxifragaceae Novae Sinenses. Natizbl. Bot. Gart. Berlin 11: 301-302.

Mellichamp T.L. 1982. *Astilbe crenatiloba*: extinct or non-existent? . Assoc. South. Biologists Bull. 29: 72.

Nakai T. 1922. Notulae ad planas Japoniae et Koreae 28. Bot. Mag. Tokyo 36: 120-124.

Nakanishi H. 1998. Distribution and ecology of *Astilbe chinensis* (Maxim.) Franch. in Tsuuchima, Japan. Hikobia 12: 313-316.

Olson M.S. 2001. Patterns of fruit production in the subdioecious plant *Astilbe biternata* (Saxifragaceae). J. Ecol. 89: 600-607.

Pan J. 1985. A study on the genus *Astilbe* Buch.-Ham. ex D. Don from China. Acta Phytotax. Sinica 23: 432-443.

Randhava L. (2005) Plant Profile: *Astilbe*. In: Chicago Botanical Garden Online Index. p www.chicagobotanic.org/plantinfo/pp/PPAstilbe.html.

Rieseberg L.H. 1997. Hybrid origins of plant species. Ann. Rev. Ecol. Syst. 28: 359-389.

Rieseberg L.H., M.A. Archer and R.K. Wayne. 1999. Transgressive segregation, adaptation and speciation. Heredity 83: 363-372.

SAS Institute. 2005. SAS Guide for Personal Computers, version 9.1. SAS Inst, Cary, N.C.

Soltis D.E., R.K. Kuzoff, E. Conti, R. Gornall and K. Ferguson. 1996. *mat*K and *rbc*L gene sequence data indicate that *Saxifraga* (Saxifragaceae) is polyphyletic. Am. J. Bot. 83: 371-382.

Soltis D.E., R.K. Kuzoff, M.E. Mort, M. Zanis, M. Fishbein, L. Hufford, J. Koontz and M.K. Arroyo. 2001. Elucidating deep-level phylogenetic relationships in Saxifragaceae using sequences for six chloroplastic and nuclear DNA regions.

Stevens, P. (2001 onwards). Angiosperm Phylogeny Website. Version 6, May 2005 [but more or less continuously updated since] http://www.mobot.org/MOBOT/research/APweb/.html

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Table 2.1 Geographical distribution of *Astilbe* Buch.-Ham. ex D. Don in Chinese provinces (Pan 1985).

	A. chinensis	*A. grandis*	*A. longicarpa*	*A. macroflora*	*A. rubra*	*A. macrocarpa*	*A. rivularis*
HeiLongJiang	X	X					
JiLin	X	X					
LiaoNing	X	X					
HeBei	X						
ShanXi	X	X					
ShanXi	X						X
GanSu	X						
QinHai	X						
ShanDong	X	X					
AnHui		X				X	
ZheJiang	X	X				X	
JiangXi	X	X					
FuJian		X				X	
TaiWan			X	X			
HeNan	X						X
HuBei	X	X					
HuNan	X	X				X	
GuangDong		X					
GuangXi		X					
SiChuan	X	X					X
GuiZhou		X					
YunNan	X				X		X
Tibet					X		X

Table 2.2 Geographical distribution of *Astilbe* Buch.-Ham. ex D. Don excluding Chinese occurrence (Pan 1985).

	A. simplicifolia	*A. chinensis*	*A. grandis*	*A. japonica*	*A. thunbergii*	*A. microphylla*	*A. philippinensis*	*A. rubra*	*A. biternata*	*A. platyphylla*	*A. rivularis*	*A. indica*	*A. crenatilobata*	*A. apoensis*	*A. khasiana*
Russia		X													
Japan	X	X		X	X	X				X					
Koreana		X	X												
Philippines							X							X	
Indonesia												X			
Thailand											X				
India								X			X				X
Bhutan											X				
Nepal											X				
Kashimir											X				
United States									X				X		

Table 2.3 Eight endemic *Astilbe* taxa in Korea investigated by Chung et al. (1983).

Genus *Astilbe* Buch. Hamilton
- Series Simplicifoliae Engler
  - *Astilbe simplicifolia* Makino
- Series Compositae Engler
  - *Astilbe taquetti* (Leveille) Koidzumi
  - *Astilbe microphylla* Knoll
  - *Astilbe chinensis* Mazimowicz ex Franchet et Savatier
    - var. *chinensis* Franchet
    - var. *paniculata* Nakai
    - var. *davidii* Franchet
  - *Astilbe divaricata* Nakai
  - *Astilbe koreana* (Komarov) Nakai

Table 2.4 Key to Chinese species of *Astilbe* described by Pan (1985).

1. Petals 5, ordinary; inflorescences densely flowered.
  2. Petals linear or spatulate linear, sepals glandular ciliate.
    3. Sepals glabrous outside.
      4. Peduncles densely covered with long, curved brown hairs; leaflets usually short-acuminate to acute at apex……..1. *A. chinensis* (Maxim.) Franch. et Savat.
      4. Peduncles covered with glandular hairs; leaflets usually short-acuminate to acuminate at apex…….. 2. *A. grandis* Stapf. ex Wils.
    3. Sepals glandular-hairy outside……... 3. *A. rubra* Hook. f. et Thoms.
  2. Petals spatulate, sepals without glandular hairs at margin.
    5. Petals retuse at apex, uninerviate; sepals obtuse at apex, subentire; plant medium-sized, 0.4-1.4 m high…….4. *A. longicarpa* (Hayata) Hayata
    5. Petals acute at apex, 4-6 nerved; sepals acute and sparsely dentate at apex; plant small, 15-30 cm high……5. *A. macroflora* Hayata
1. Petals 1-5, obsolete or absent; inflorescences sparsely flowered.
  6. Sepals 5, subcoriaceous, with glandular hairs outside; petals 2-3-5, obsolete or absent……... 6. *A. macrocarpa* Knoll
  6. Sepals 4-5, nearly membranous, glabrous outside; petals 1-(2-3-5), obsolete or absent.
    7. Petals 1-(2-3-5), obsolete or absent.
      8. Leaflets lanceolate, narrowly ovate or narrowly rhombicovate; petals usually absent or sometimes with only obsolete one……. ……. *A. rivularis* var. *angustata* C.Y. Wu ex J. T. Pan
      8. Leaflets usually ovate, broadly ovate to broadly elliptic; petals 1-(2-3-5), obsolete or absent…….. *A. rivularis* var. *myriantha* (Diels) J. T. Pan
    7. Petals absent……..7. *A. rivularis* Buch.-Ham. ex D. Don

Table 2.5 Key to endemic *Astilbe* of Korea derived by Chung et al. (1983).

1. Simple leaf …….*A. simplicifolia*
1. Compound leaf
    2. Plant size less than 20 cm; small petal length = < 1mm ……. *A. taquetti*
    2. Plant size greater than 20 cm; petal length > 1 mm.
        3. Branches of inflorescence are descending …….. *A. koreana*
        3. Braches of inflorescence are not descending
            4. Leaf length 2-4 cm; flowers sparse; leaf adaxial hairy ……..*A. microphylla*
            4. Leaf length > 4 cm; flowers dense; leaf adaxial hairy
                5. Bristle trichomes on underside of leaf; petal and stamen length equal ……. *A. divaricata*
                5. Bristle like trichomes along veins; stamen shorter than petals.
                    6. Floral axis dense with downy hairs ……*A. chinensis* var. *davidii*
                    6. Floral axis glandular hairy
                        7. Stamen length 5-6 mm and margin acute ..*A. chinensis* var. *paniculata*
                        7. Stamen length 3-4 mm and margin rounded.*A. chinensis* var. *chinensis*

Table 2.6 Key to cultivated *Astilbe* developed by Hatch (2000).

1a. Leaves simple……...….*A. simplicifolia*
1b Leaves compound
2a. Petals absent; 5 stamens …….*A. rivularis*
2b. Petals present; 10 stamens.
3a. Petals pink to purple and red; inflorescence with long curled hairs.
4a. Leaf base cuneate; inflorescence open.
5a. Plants large to 1m; hairs long and curled ……...*A. rubra*
5b. Plants dwarf 20-40 cm; some hairs short and glandular..*A. japonica* var. *glaberrima*
4b. Leaf base rounded or cordate; inflorescence open ……*A. ×arendsii*
4c. Leaf base rounded or cordate; inflorescence dense.
6a. Plants bloom late summer-fall; stems and leaves often bronze to purple……..*A. chinensis* var *taquetii*
6b. Plants bloom in summer; stems and leaves green ……*A. chinensis* and hybrids
3b. Petals pink; inflorescence with short, glandular hairs ……*A. japonica* var. *glaberrima*
3c. Petals white; inflorescence with long, curled hairs.
7a. Inflorescence open, branchlets spreading to horizontal ……*A. koreana*
7b. Inflorescence dense, branchlets erect ……*A. ×arendsii*
3d. Petals white; inflorescence with short glandular hairs.
8a. Leaf base cuneate or wedge-shaped.
9a. Plants 20-40 cm tall; calyx pinkish; leaves glossy ……..*A. japonica* var. *glaberrima*
9b. Plants 40-90 cm tall; calyx greenish; leaves dull or glossy ……..*A. japonica*
8b. Leaf base rounded to cordate.
10a. Leaflets doubly serrate; stamens shorter than petals; plants 50-80 cm tall.
11a. Leaflet apex mostly acute; petals 5-7mm wide ……*A. thunbergii* var. *formosa*
11b. Leaflet apex long acumniate; petals 3-4mm wide …….*A. thunbergii*
10b. Leaflets singly serrate; stamens longer than petals; plants 70-150 cm tall ……*A. grandis*

Table 2.7 Source of herbarium material for morphological analysis.

Herbarium	Number of sheets	Number of taxa
Gray Herbarium	20	13
BRIT Herbarium	10	6
New York Botanical Garden	30	11
Missouri Botanical Garden	33	13
Massey Herbarium	4	1
Humboldt State Univ. Herbarium	1	1

Table 2.8 Morphological characters and states utilized in phylogenetic analysis.

Vegetative Characters

1. Leaf form: (0 = simple; 1 = ternate or biternate, no more than two pairs pinnae; 2 = pinnate with 3-5 pairs pinnae; 3 = bipinnately or tripinnately compound)
2. Leaflet shape: (0 = ovate; 1 = cordate; 2 = elliptic, 3 = rhombic, 4 = lanceolate, 5 = quadrate)
3. Leaflet base: (0 = cordate, 1 = oblique, 2 = rounded, 3 = cunneate, 4 = attenuate)
4. Leaflet apex: (0 = acute; 1 = acuminate)
5. Leaflet margin: (0 = crenate, 1 = biserrate)
6. Density of leaflet pubescence abaxial: (0 = sparse, 1 = dense-surface covered)
7. Abaxial trichome type: (0 = short hispid, 1 = long strigose)
8. Density of leaflet pubescence adaxial: (0= sparse, 1 = dense-surface covered)
9. Adaxial trichome type: (0 = short hispid, 1 = long strigose)
10. Adaxial postulate: (0 = yes, 1 = no)
11. Leaflet margin pubescence: (0 = trichome present within serration, 1 = many trichomes present within serrations)
12. Stem pubescence: (0 = uniformly pubescent, 1 = hairs present mainly at nodes, 2 = mainly glabrous)
13. Leaflet ratio: (0 = <1.5, 1 = 1.6-2, 2 = 2.1-2.5, 3 = >2.6)

Sexual Characters

14. Sexual separation: (0 = monoecious; 1 = dioecious)
15. Inflorescence density per 2 cm: (0 = 10 or less; 1 = 11-20; 2 = 21-30; 3 = 30+)
16. Peduncle pubescence: (0 = long straight glandular trichomes, 1 = long curly glandular trichomes, 2 = short straight glandular trichomes)
17. Sepal #: (0= 5; 1 = >5)
18. Sepal shape: (0= ovate; 1 = elliptic)
19. Sepal apex: (0= acute; 1 = rounded; 2 = dentate; 3 = notched; 4 = fringed)
20. Sepal margin: (0= entire; 1 = hairs; 2 = membranous; 3 = both membranous and hairs)
21. Sepal abaxial: (0= hairs; 1 = glabrous)
22. Sepal adaxial: (0= hairs; 1 = glabrous)
23. Petal #: (0= 5; 1 = <5)
24. Petal shape: (0= linear; 1 = oblanceolate; 2 = spatulate; 3 = no petals)
25. Petal length mm: (0= <.9; 1 = 1-1.9; 2 = 2-2.9; 3 = 3-3.9; 4 = > 4; 5 = no petals)
26. Petal veins: (0= 1; 1 = 3; 2 = > 4; 3 = no petals)
27. Stamen #: (0= 10; 1= < 10; 2 = >10)
28. Carpel coalescence: (0= close; 1 = apart).

Table 2.9 Morphological data matrix of 28 characters utilized for phylogenetic analysis of *Astilbe*.

Saxifragopsis fragarioides	0	5	3	0	1	0	0	0	0	0	0	1	1	0	1	0	0	0	1	2	1	0	0	1	2.		0	0
Astilbe austrosinensis	2	0	3	1	1	0	0	0	0	0	0	2	1	0	3	1	0	0	1	2	0	1	0	0	4	0	0	0
Astilbe augustifolia	2	2	0	1	1	1	1	1	0	1	0	2	1	0	2	0	0	1	1	2	1	1	1	3	5	3	1	0
Astilbe biternata	1	3	1	1	0	0	0	0	0	1	0	1	1	1	1	0	0	1	1	2	2	0	0	0	0	0	0	1
Astilbe chinensis	2	0	2	0	1	0	0	0	0	0	0	2	0	0	3	0	0	0	1	3	0	0	0	0	4	0	0	0
Astilbe davidii	2	2	2	0	1	0	0	0	0	0	0	2	1	0	2	1	0	0	1	3	0	0	0	0	4	0	0	0
Astilbe formosa	3	4	3	1	1	0	0	0	0	1	0	1	1	0	2	2	0	2	1	0	0	0	0	2	4	0	0	0
Astilbe fujisanensis	2	4	3	1	1	0	0	0	0	0	0	2	1	0	2	2	0	1	1	0	0	0	0	2	3	0	0	1
Astilbe glaberrima	1	4	2	0	2	0	0	0	0	0	0	2	2	0	1	0	0	0	1	2	1	4	0	1	3	0	0	1
Astilbe grandis	2	0	2	1	1	1	0	0	0	0	0	2	1	0	1	1	0	1	1	2	1	0	0	0	3	0	0	0
Astilbe japonica	1	4	4	0	2	0	0	0	0	0	0	1	3	0	1	2	0	0	1	2	1	4	0	1	2	0	0	0
Astilbe koreana	3	0	2	1	1	1	0	0	0	0	0	2	1	0	3	2	0	0	1	2	0	0	0	0	3	0	0	0
Astilbe longicarpa	3	4	3	1	1	0	0	0	0	1	0	1	3	0	3	2	0	0	1	0	1	1	0	1	2	0	0	1
Astilbe macrocarpa	2	3	0	1	1	0	0	0	0	1	0	2	1	0	0	2	0	0	1	1	0	0	1	1	5	3	0	1
Astilbe macroflora	1	1	0	0	1	1	1	1	1	1	0	2	0	0	1	1	0	1	1	0	1	2	0	2	3	2	0	0
Astilbe microphylla	3	0	3	0	1	1	1	0	0	0	0	0	0	0	2	2	0	0	0	2	0	3	0	0	3	0	0	0
Astilbe myriantha	2	4	3	1	1	0	0	0	0	1	0	0	2	0	2	2	1	1	1	0	1	1	1	3	5	3	0	0
Astilbe philippinensis	2	4	0	1	1	1	1	1	1	1	1	2	3	0	1	0	0	0	1	2	1	1	0	2	2	1	0	1
Astilbe rivularis	2	0	3	1	1	0	0	0	0	1	0	2	1	0	2	1	1	1	1	2	1	1	1	3	5	3	1	0
Astilbe rubra	3	1	2	0	1	1	1	1	0	1	0	2	0	0	1	1	0	0	1	1	0	4	0	0	4	0	0	1
Astilbe simplicifolia	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	2	0	0	1	0	1	3	0	0	1	0	0	1
Astilbe thunbergii	2	0	3	1	1	0	0	0	0	1	0	1	2	0	2	2	0	0	1	0	1	0	0	2	3	0	0	0

Table 2.10 Statistically informative morphological charcacters according to the Kruskal-Wallis test implemented as the Wilcoxon npar 1-way test.

Character (20 degrees of freedom)	Chi-Square	Pr > Chi-Square
Leaf form	62.92	<0.0001
Leaflet shape	52.49	<0.0001
Leaflet base	52.11	<0.0001
Leaflet apex	64.28	<0.0001
Leaflet margin	72.86	<0.0001
Density leaflet pubescence abaxial	65.18	<0.0001
Abaxial trichome type	65.18	<0.0001
Density leaflet pubescence adaxial	73.19	<0.0001
Adaxial trichome type	79.5	<0.0001
Adaxial postulate	91.00	<0.0001
Leaflet margin pubescence	91.00	<0.0001
Stem pubescence	71.98	<0.0001
Leaflet ratio	61.60	<0.0001
Sexual separation	29.54	0.0523
Inflorescence density	61.08	<0.0001
Peduncle pubescence	56.28	<0.0001
Sepal #	28.67	0.0710
Sepal shape	57.31	<0.0001
Sepal apex	66.75	<0.0001
Sepal margin	50.16	0.0001
Sepal abaxial	73.75	<0.0001
Sepal adaxial	63.42	<0.0001
Petal #	69.44	<0.0001
Petal Shape	60.02	<0.0001
Petal length	55.29	<0.0001
Petal veins	38.36	0.0013
Stamen #	51.20	<0.0001
Carpel coalescence	53.95	<0.0001

Table 2.11 Arrangement of 21 *Astilbe* taxa within 14 clusters generated from 28 morphological character states measured from herbarium samples using FASTCLUS procedure in SAS.

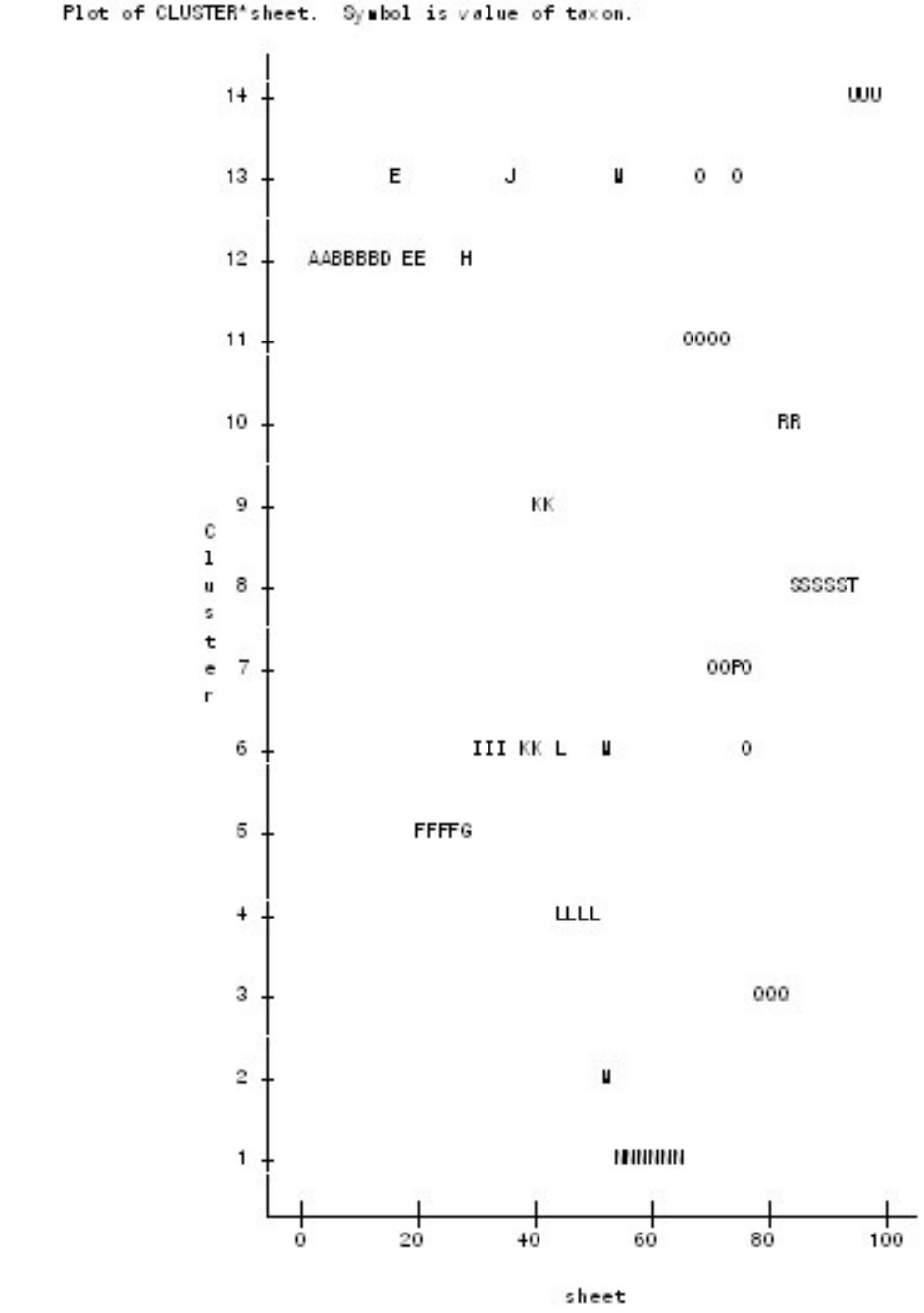
	Taxa	# of sheets	Clus.1	Clus. 2	Clus. 3	Clus. 4	Clus. 5	Clus. 6	Clus.7	Clus. 8	Clus. 9	Clus. 10	Clus. 11	Clus. 12	Clus. 13	Clus. 14
A	*A. austrosinensis*	3												3		
P	*A. augustifolia*	1							1							
U	*A. biternata*	4														4
B	*A. chinensis*	8												8		
C	*A. davidii*	1												1		
T	*A. formosa*	1								1						
D	*A. fujisanensis*	1												1		
G	*A. glaberrima*	1					1									
E	*A. grandis*	6												4	2	
F	*A. japonica*	7					7									
H	*A. koreana*	1												1		
I	*A. longicarpa*	6						6								
J	*A. macrocarpa*	2													2	
K	*A. macroflora*	6						3			3					
L	*A. microphylla*	8				7		1								
M	*A. myriantha*	3		1				1							1	
N	*A. philippinensis*	11	11													
O	*A. rivularis*	11						1	3				5		2	
Q	*A. rubra*	5			5											
R	*A. simplicifolia*	2										2				
S	*A. thunbergii*	9								9						

Table 2.12 New key based on the analysis of 28 morphological character states of 21 *Astilbe* taxa.

1a. Leaves simple ……*A. simplicifolia* Makino
1b. Leaves compound.
2a. Plants dioecious ……*A. biternata* Britton
2b. Plants monoecious.
3a. Petals 1-5, obsolete or absent; inflorescences sparsely flowered.
4a. Sepals 5, subcoriaceous, glandular hairy outside; petals 1-5 or absent.*A. macrocarpa* Knoll
4b. Sepals 4-5, nearly membranous, glabrous outside; petals 1-(2-3-5), obsolete or absent.
5a. Petals 1-(2-3-5), obsolete or absent.
6a. Leaflets lanceolate, narrowly ovate or narrowly rhombicovate; petals usually absent or sometimes with only one……..*A. rivularis* var. *angustifolia* C.Y. Wu ex J. T. Pan
6b. Leaflets usually ovate, broadly ovate to broadly elliptic; petals 1-(2-3-5), obsolete or absent…… *A. rivularis* var. *myriantha* (Diels) Pan
5b. Petals absent…… *A. rivularis* Buch.-Ham. ex D. Don
3b. Petals 5, ordinary.
7a. Inflorescences densely flowered.
8a. Petals linear or spatulate linear, sepals glandular ciliate.
9a. Sepals glabrous outside.
10a. Leaflets usually short-acuminate to acute at apex
11a. Peduncles with long, curved brown hairs...*A. chinensis* (Maxim.) Franch.
11b. Peduncle extremely downy hair …… *A. davidii* Henry
10b. Leaflets usually acuminate at apex ……..….*A .grandis* Stapf.ex Wils.
12a. Inflorescence branches mainly descending ….…… *A. koreana* Nakai
12b. Leaflets large broadly ovate to rhombic ..*A. austrosinensis* Handel-Mazz
9b. Sepals glandular hairy.
13a. Hairy on outside only…….*A. rubra* Hook. f. et Thoms.
13b. Hairy both sides…….……...*A. microphylla* Hayata
8b. Petals spatulate
14a. Sepals without glandular hairs at margin.
15a. Petals retuse at apex, single vein; sepals obtuse at apex, subentire; plant medium-sized, 0.4-1.4 m high…*A. longicarpa* (Hayata) Hayata
15b. Petals acute at apex, 4-6 nerved; sepals acute and sparsely dentate at apex; plant small, 15-30 cm high……...*A. macroflora* Hayata
14b. Sepals with hairs, membranous at margin.
16a. Leaflets lanceolate and dense with strigose hairs, leaflet margin dense with hairs……...……...……...*A. philippinensis* Henry
16b. Leaflets lanceolate sparse hispid hairs
17a. Leaflet margin doubly serrate…….…….*A. japonica* A.Gray
17b. Leaflet margins deeply incised …….…….*A. glaberrima* Nakai
7b. Inflorescences densely flowered, but sparsely branched.
18a. Petals spatulate, sepal margin entire, peduncle dense with short glandular hairs, leaflets ovate …….*A. thunbergii* (Siebold et Zucc.) Miq
18b. Leaflets lanceolate
19a. Petals longer than 3mm ……..…… *A. fujisanensis* Nakai
19b. Petals shorter than 3mm …….. *A. formosa* Nakai

Figure 2.1 Geographic distribution of the genus *Astilbe*.

Figure 2.2 Plot of *Astilbe* herbarium sheets by taxa and cluster. Taxa letters correspond to list of taxa presented in Table 2.10.



NOTE: 34 obs hidden.

Figure 2.3 Single stable most parsimonious tree derived from a morphological data matrix of 28 characters with 21 taxa of *Astilbe* using *Saxifragopsis fragarioides* as the outgroup, with length 44.2, HI of 0.5795, and an RC of 0.2915. Bootstrap support greater than 50% is presented above branches.

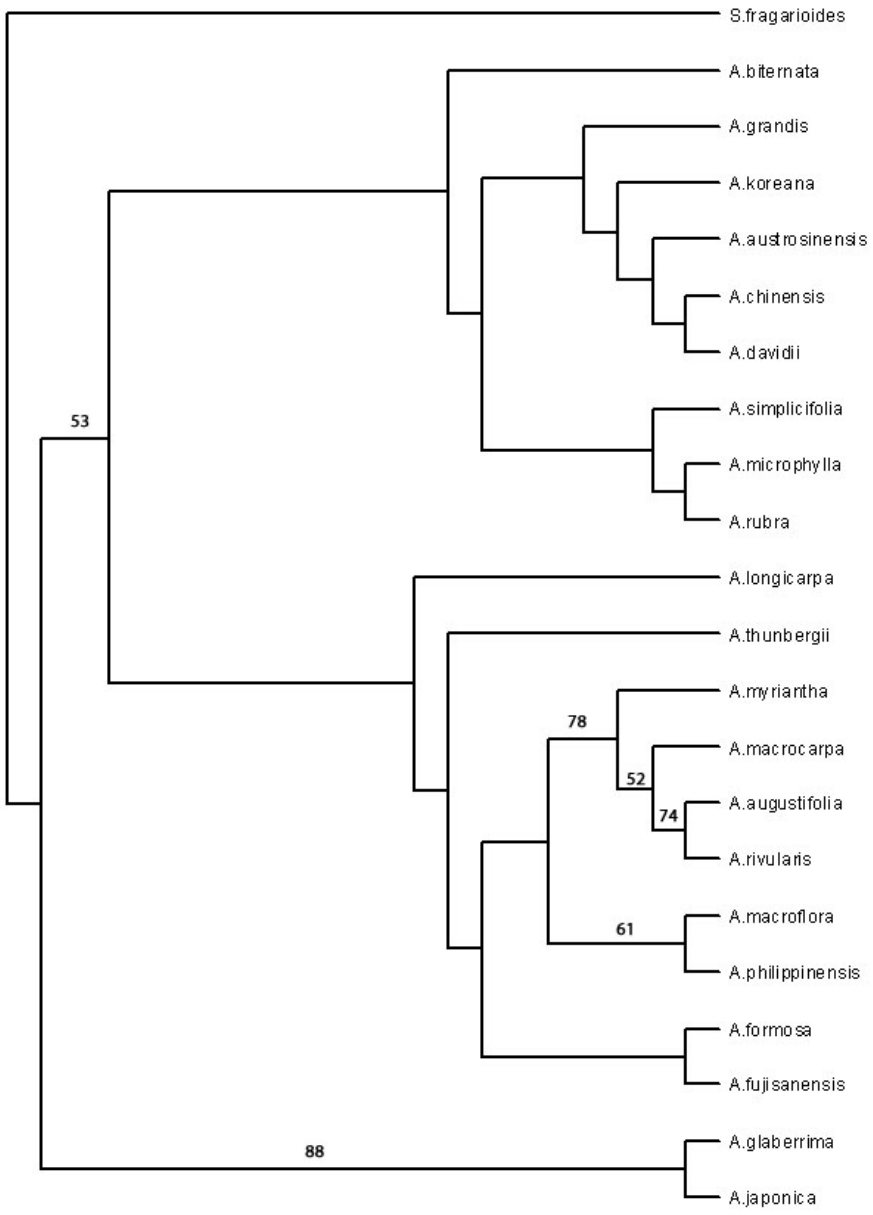


Figure 2.4 Neighbor joining tree of 28 morphological characters from 21 taxa of *Astilbe* using *Saxifragopsis fragarioides* as the outgroup taxon.

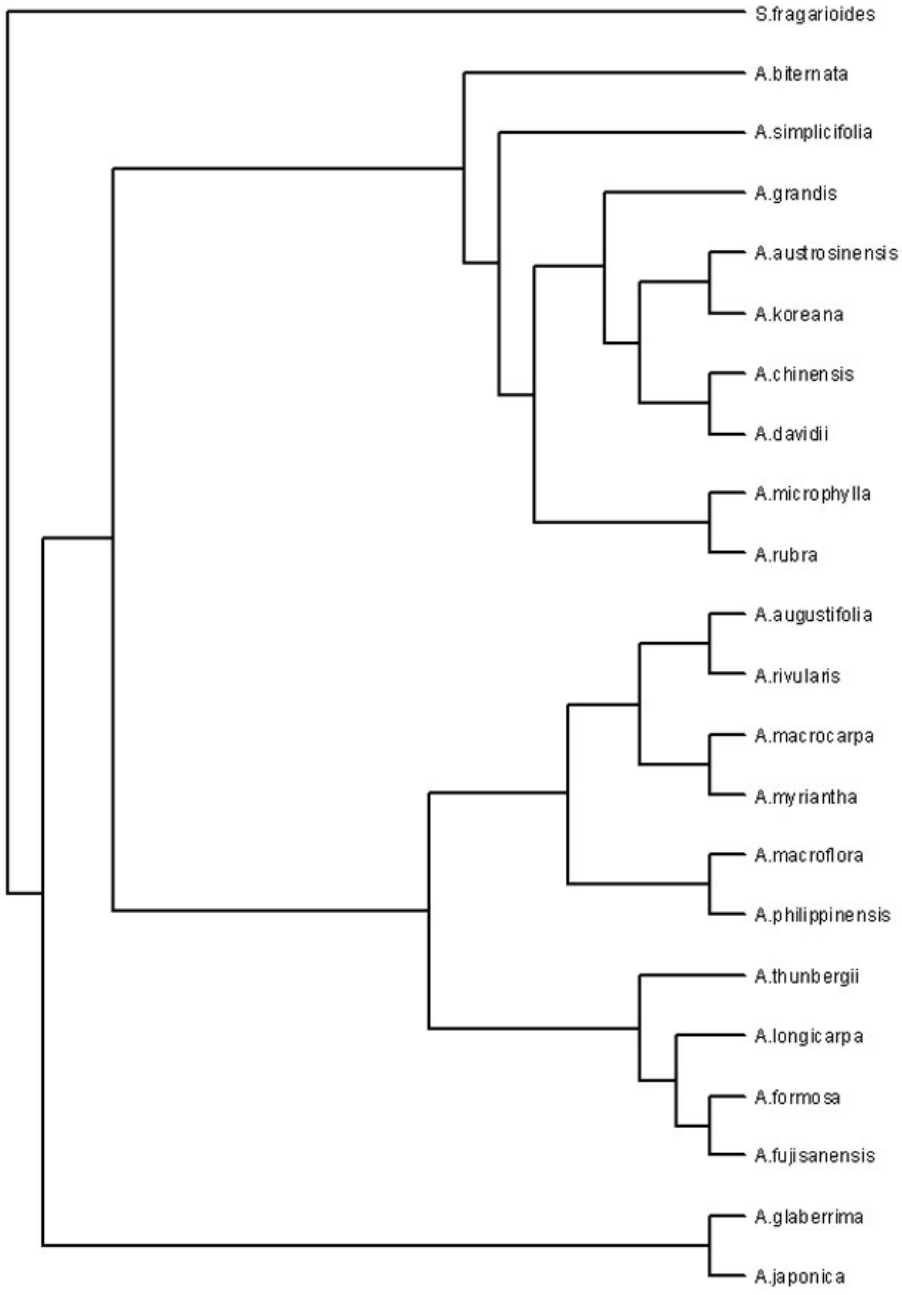


Figure 2.5 One of two most parsimonious trees derived from a morphological data matrix of 28 characters with 15 taxa of *Astilbe* using *Saxifragopsis fragarioides* as the outgroup, with length 129, HI of 0.5920, and an RC of 0.2267. Bootstrap support greater than 50% is presented above branches.

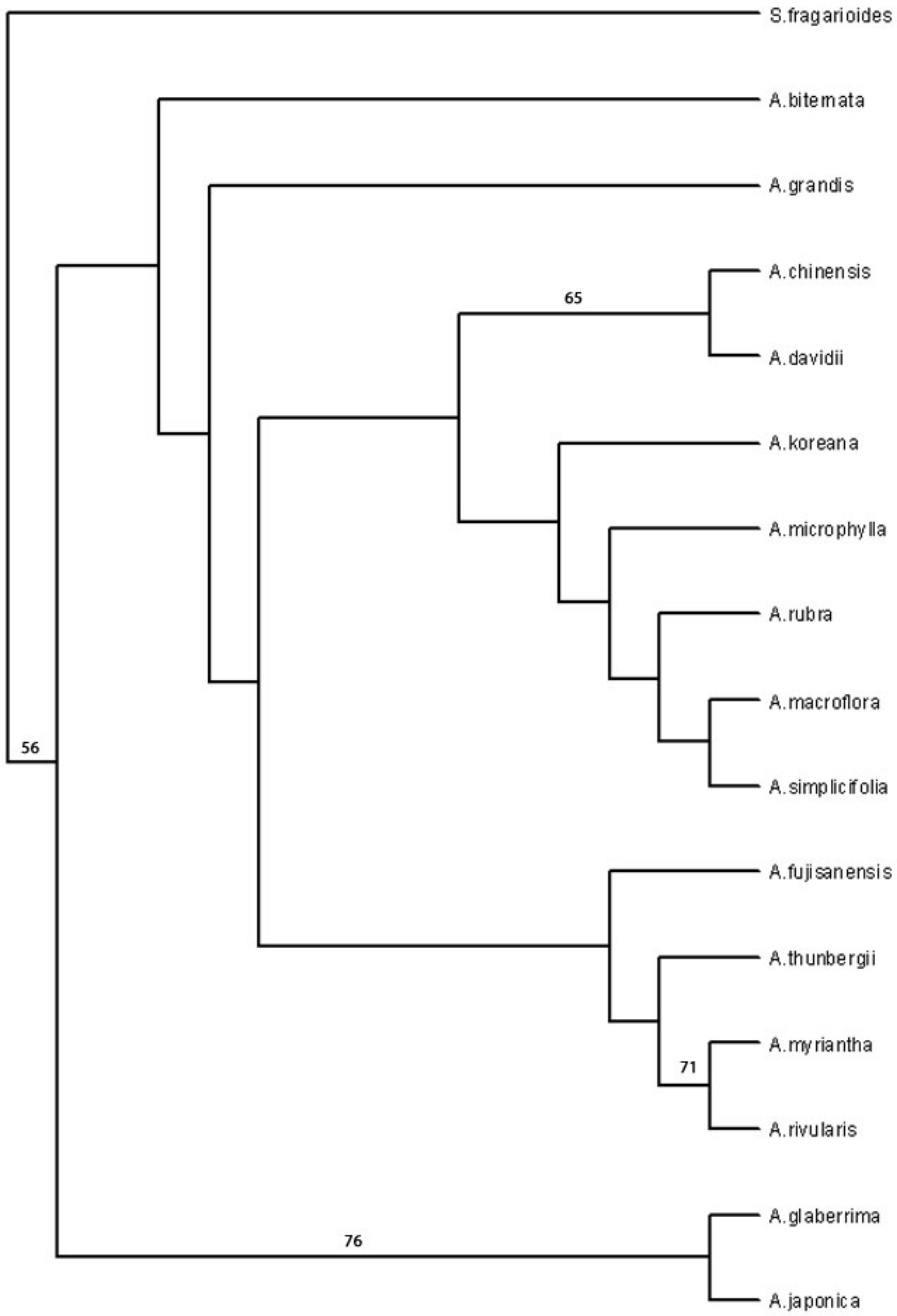


Figure 2.6 Neighbor joining tree of 28 morphological characters from 15 taxa of *Astilbe* using *Saxifragopsis fragarioides* as the outgroup taxon.

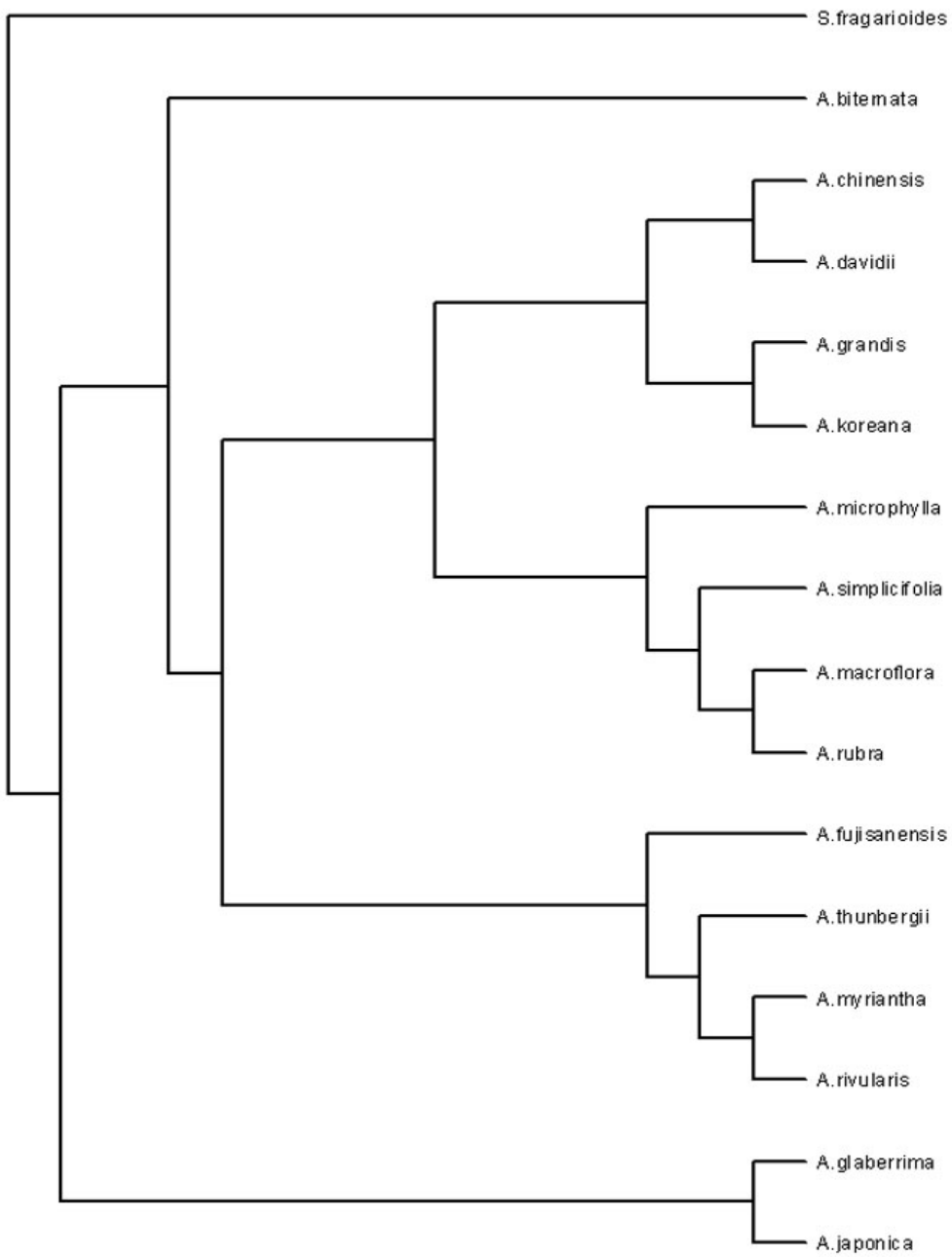
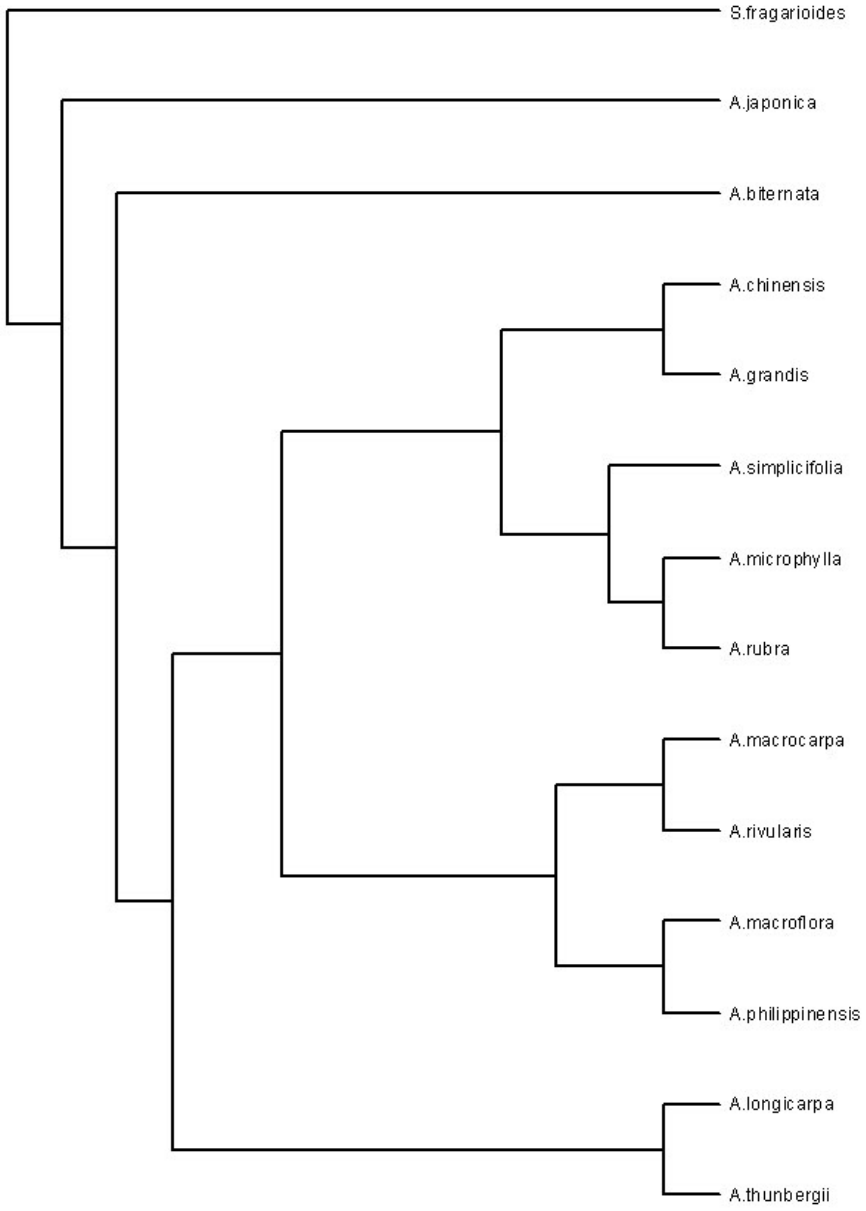


Figure 2.7 Neighbor joining tree of 28 morphological characters from 13 species of *Astilbe* using *Saxifragopsis fragarioides* as the outgroup taxon.



Chapter 3. Molecular Investigation of *Astilbe* using plastid gene *mat*K

Abstract

Astilbe (Saxifragaceae) is a genus of popular garden perennials ditributed primarily in the Northern Hemisphere, with species occurring in eastern Asia and one species in eastern North America. Though the genus is small, less than 20 species, relationships in *Astilbe* are problematic due to the similarity among morphological characters. I conducted phylogenetic analysis of DNA sequences of the chloroplast gene *mat*K for taxa of *Astilbe* to elucidate relationships, distinguish misnomered species, and confirm the lineages of popular cultivated varieties. Maximum parsimony, neighbor joining, and maximum likelihood analysis were conducted on 15 and then 21 taxa of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup. Parsimonious and likelihood analysis of 15 taxa resulted in a polytomy of seven *Astilbe* species, with relationships within the genus poorly resolved. Phylogeny of 21 hybrids, cultivars, and species of *Astilbe* was more informative, aligning cultivated varieties near species from which they were derived. The *mat*K sequence variation was aligned for *Astilbe* taxa to reveal polymorphic sites among members. Closely related taxa retained the same polymorphisms within the gene sequence. These polymorphic sites could potentially be utilized to confirm the lineages of popular cultivated *Astilbe* varieties.

3.1 Introduction

Astilbe (Saxifragaceae) is a small genus of herbaceous perennials important to the nursery and landscape industry. The genus is primarily restricted to the Northern Hemisphere with species occurring in eastern Asia and one species in eastern North America. The North American species, *Astilbe biternata* Britt., is subdioecious and tetraploid, unlike the remaining monoecious and diploid members (Olson 2001). The intercontinental disjunction involving eastern Asian and eastern North American flora has been previously described for numerous plant families (Gray 1859; Zhengy 1983) and has been observed in other genera of Saxifragaceae, such as *Chrysosplenium* (Soltis et al. 2001). Pan (1985) proposed that the center of origin of *Astilbe* was the forested parts of Japan through east and southwest China.

The genus is broadly divided into two sections: *Simplicifoliae* and *Astilbe* Engler *Simplicifoliae* is considered the more primitive of the two due to members bearing five ordinary petals (Pan 1985). *Astilbe simplicifolia*, distinguished by simple leaves, is considered the most primitive member of the genus (Pan 1985). Despite the small size of the genus, with only 13 to 22 species, relationships within *Astilbe* are problematic. Because *Astilbe* species are similar morphologically and have large ranges of distribution (contributing to natural variation within species), it is difficult to delineate members within the genus. Breeding *Astilbe* for horticultural value has further complicated the ability to distinguish species, subspecies, and varieties.

Relationships within *Astilbe* have been investigated previously by focusing on geographic distribution and morphology (Chung et al. 1983; Pan 1985). These studies

focused primarily on regional species within China and Korea, excluding other members of the genus. Chung et al. (1983) and Pan (1985) described varieties within species and discussed whether these varieties should be considered separately. Phylogenetic studies using gene sequences to support previous morphological findings have not been conducted.

In order to infer phylogenetic relationships and relationships among taxonomic ranks, it is important to consider both morphological and molecular data. In recent years advances in technology and knowledge of gene sequences have significantly impacted angiosperm phylogeny (Hilu et al. 2003). Molecular approaches for analyzing phylogeny have become increasingly useful where morphological characters are limited in distinguishing genera. The use of nuclear and plastid genomes has allowed researchers versatility in their molecular phylogenetic analyses. Because the nuclear genome is complex with many repetitive genes and high rates of evolution, variation in the chloroplast genome is frequently preferred for taxonomic separation. Chloroplast genes are useful in phylogenetic analysis because they can be amplified from total genomic DNA, are present in single copies, and have conserved rates of nucleotide substitution.

Of the chloroplast genes, *mat*K has shown potential for providing insight into evolutionary and systematic problems at various levels (Hilu and Liang 1997). The *mat*K gene is located within the intron of the chloroplast gene *trn*K and is approximately 1500 base pairs (bp) (Figure 3.1). The gene has been proposed to play a role as a maturase, splicing group II introns in the chloroplast (Sugita et al. 1985; Neuhaus and Link 1987; Mohr et al. 1993). Hilu and Liang (1997) looked at sequence variation of *mat*K among different taxonomic hierarchies (Figure 3.2) and found that even small portions of the

gene were phylogenetically informative. Using 1200 bp of *mat*K sequence could provide more taxonomic resolution than sequences of 11 other plastid genes combined (Hilu personal communication).

Phylogenetic studies of Saxifragaceae that included sequences of the chloroplast gene *mat*K for species of *Astilbe* suggested that *mat*K sequence data could be useful for resolving relationships within *Astilbe* (Johnson and Soltis 1994, 1995; Soltis et al. 2001a). The *mat*K gene sequences have been used to examine relationships of species among several genera within Saxifragaceae, including *Chrysosplenium* (Soltis et al. 2001b) and *Saxifraga* (Soltis et al. 1996). In each of these studies, *mat*K sequence analysis was able to provide resolution of relationships among species within the genera consistent with previous cpDNA restriction site data and other plastid sequences such as *rbc*L.

This study was undertaken to develop a well-resolved phylogeny of *Astilbe* using sequences of *mat*K amplified from living plants of its species. Our aim was to determine whether some described species within the genus are actual variations within a species and to elucidate the relationship of North American *A. biternata* to the Asian species. A final goal of the research was to incorporate popular cultivated varieties of *Astilbe* and see if *mat*K sequences would accurately align these with species from which they are purported to be derived.

3.2 Materials and Methods

Taxon Sampling. Fifteen species (Table 3.1) and nine popular hybrids and cultivars (Table 3.2) of *Astilbe* were included in this study. Our sampling did not include *A. austrosinensis*, *A. formosa, A. macroflora*, *A. longicarpa*, or *A. philippinensis* because

live samples of these taxa were unattainable. Voucher specimens have been placed in the Massey Herbarium and in the Hahn Horticultural Garden both located at Virginia Tech, Blacksburg, VA. *Saxifragopsis fragaroides* was chosen as the outgroup based on studies conducted by Soltis et al. (1996), who found the monotypic genus to be a sister group to *Astilbe*.

DNA Extraction, Amplification, and Sequencing. Total genomic DNA was extracted from fresh leaf material with the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA)) or using the 2X CTAB method modified from Doyle and Doyle (1987). Amplification of *mat*K was accomplished using primers *trn*K-3914F and *trn*K-2R (Johnson and Soltis 1994). These primers were used to amplify the entire *trn*K intron, which is approximately 2500 bp (Figure 3.1). PCR reactions were set up using PureTaq Ready-to-Go PCR beads (GE Biosciences, Piscataway NJ) in 25 µl reactions. The reaction consisted of 2.5 µl each primer, 6.5 µl DNA template, and 13.5 µl sterile distilled water. The following program was used for PCR amplification: 95°C (2 min), 30 cycles of 95°C (1 min), 55°C (1 min), 72°C (2 min); and 72°C (5 min). Double stranded PCR products were purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions, and the purified products were eluted in 35 µl sterile water. Purified PCR products were sequenced directly using modified dideoxy cycle sequencing with dye terminators according to the manufacturer's protocol (Applied Biosystems, ABI, Warrington, Cheshire, UK). In addition to the primers used for amplification, sequencing primers *trn*K-710F, *mat*K-1470R, *mat*K-1412F, *mat*K-2200R, and *mat*K-1235R were used to ensure complete sequencing of the gene region on both strands (Johnson and Soltis 1995) (Figure 3.1). Sequencing reactions were run on an ABI automated sequencer according to

the manufacturer's protocols at the Virginia Bioinformatics Institute in Blacksburg, VA. Sequences were assembled, aligned, and edited using Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Michigan, USA).

Phylogenetic Analysis. Consensus sequences were exported from Sequencher in nexus format and loaded into PAUP version 4.0b10 (Swofford 2003) for maximum parsimony and maximum likelihood analysis. Maximum parsimony was implemented weighing all characters equally and using a heuristic search strategy with 200 replicates using random taxon addition sequence, TBR (tree bisection and reconnection) branch swapping, and a maximum of 3000 trees per replicate. Bootstrap support (Felsenstein 1985) was determined with 100 replicates using heuristic search options and TBR branch swapping, with the maxtree option set at 20,000. Neighbor joining trees were also derived using PAUP.

3.3 Results

Sequence Variation. The *mat*K sequences were aligned in Sequencher using the Genbank sequence of *Saxifragopsis fragarioides* (Soltis et al. 2001) as a reference. Multiple sequence fragments from each pair of sequencing primers were aligned for *Astilbe* taxa to ensure both strands of DNA were present for editing. The 2500 bp of *mat*K including the *trn*K intron were trimmed to fit the sequence of *S. fragarioides* for phylogenetic analysis, resulting in about 1100 bp of edited sequence. Sequences from the *mat*K gene region showed relatively low levels of nucleotide variation. Some potential single nucleotide polymorphisms are presented in Table 3.3. Most of the variation observed is from the outgroup taxon *Saxifragopsis fragarioides* and not among the *Astilbe* taxa (Table 3.3). Some of the polymorphisms divide *Astilbe* taxa into two distinct groups (highlighted in

yellow, Table 3.3). Of particular interest is a series of nucleotide variability unique to *A. myriantha* and *A. rivularis*, closely correlating to the sequence of *S. fragarioides* (Table 3.4). Other sequence variability, a 10 bp length of sequence distinguishing the outgroup taxon from *Astilbe*, is presented in Table 3.5.

Small Data Set. Of the 1059 characters included in the matrix, 952 were constant, 92 were variable, and the remaining 1.5% were parsimony informative. Parsimony analysis of the *mat*K sequence matrix, using *Saxifragopsis fragarioides* as the outgroup, found 1629 most-parsimonious trees with 113 steps (CI = 0.80; RI = 0.88; RC = 0.84; and HI 0.20). The strict consensus parsimonious tree derived from *mat*K sequences from 15 taxa of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup, is presented in Figure 3.4. The strict consensus tree resulted in a polytomy of seven taxa, with *A. chinensis* and *A. davidii* forming a sister group within. An interesting clade that separated out from most taxa and aligned closest to the outgroup was the grouping of *A. rivularis* with *A. grandis*. *Astilbe grandis* has often been mistaken for other taxa of *Astilbe* such as *A. chinensis* and *A. davidii*, so it is unlikely to see it as sister to *A. rivularis*. Adjoining that group was *A. myriantha,* historically considered a variety of *A. rivularis*. The neighbor joining tree generated from the same data set resulted in a similar tree with a distinct separation of taxa (Figure 3.5). Maximum likelihood analysis of the dataset produced a single best tree (-ln L = 2,030.85) after 1,458 rearrangements (Figure 3.6). The tree was similar to the strict consensus tree generated from parsimony analysis, yet bootstrap support of branches was different.

Expanded Data Set. Of the 1164 characters included in the matrix, 531 were constant, 43 were variable, and the remaining 49 % were parsimony informative. Parsimony analysis

of the *mat*K sequence matrix, using *Saxifragopsis fragarioides* as the outgroup, found 2698 most-parsimonious trees with 795 steps (CI = 0.93; RI = 0.98; RC = 0.91; and HI 0.07). The strict consensus parsimonious tree derived from *mat*K sequences from 21 taxa of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup, is presented in Figure 3.7. In general, hybrids and cultivars aligned within the tree close to the taxa from which they were derived. For example, most cultivated *Astilbe* have been derived from only four species, and most of the cultivated varieties analyzed in this study aligned near two of them, *A. chinensis* and *A. japonica* (Figure 3.7). In the expanded data set, *A. myriantha* was sister to *A. rivularis* and *A. grandis* occured further in the tree. Adjoining the outgroup with 100% bootstrap support was a clade of *A. simplicifolia* and *A. microphylla*. The neighbor joining tree generated from the same data set resulted in a similar tree with a distinct separation of taxa (Figure 3.8). Maximum likelihood analysis of the same dataset produced a single best tree (-ln L = 4,731.04) after 13,920 rearrangements (Figure 3.9). The likelihood tree was similar to the consensus parsimony tree, with the exception of the relationships between *A. fujisanensis* and *A. rubra*, and *A. chinensis* and *A. davidii* (Figure 3.7 and 3.9).

3.4 Discussion

Our research initiates the first investigation into *Astilbe* using nucleotide sequences. Previous sequencing of *Astilbe* focused on alignment of this genus within Saxifragaceae (Johnson and Soltis 1994, 1995; Soltis et al. 2001a). The chloroplast gene *mat*K utilized in our study was chosen because of its ability to resolve relationships among many genera of plants. Specifically, it has been used to provide understanding of other Saxifragaceae genera: *Saxifraga*, *Heuchera*, and *Chrysosplenium* (Soltis et al. 1996;

Soltis and Kuzoff 1995; Soltis et al. 2001b). Unlike *Astilbe*, these genera are characterized by many species, up to 300 in *Saxifraga*, and are morphologically diverse. Though *mat*K has provided adequate information to resolve species relationships in some taxa, it offers less resolution at lower taxonomic levels (Shaw et al. 2005). In our study we investigated the use of *mat*K sequence variation as a tool to identify specific taxa via nucleotide polymorphisms and to understand relationships within the genus *Astilbe*.

Sequence variation among taxa of *Astilbe*, excluding cultivated varieties, was generally low. Of close to 1100 bp, less than 20 bp were considered parsimony informative. Of these informative characters, nucleotide numbers 40 and 963 divided the genus into two groups (Table 3.3 highlighted in yellow). Other informative polymorphisms distinguishing taxa were 29, 140, 500, 520, and 639 (Table 3.3 highlighted in red). In their investigation of the genus *Panax*, Komatsu et al. (2001) found that of over 1500 bp, five polymorphic sites were useful in distinguishing closely related taxa and determined these sites useful as markers. In a similar study, Yang et al. (2004) utilized *mat*K sequence polymorphisms to develop markers for the pharmaceutically important genus *Rheum*. Inter- and intraspecies variation among *mat*K sequences was found within *Rheum*, with populations growing close to one another having the same sequence due to the maternally inherited plastid genome. These studies help validate that only a few polymorphisms can distinguish taxa.

Phylogenetic analysis of *mat*K sequences from *Astilbe* resulted in poorly-resolved trees. Phylogenies of some taxa (*A. davidii* with *A. chinensis*, *A. glaberrima* with *A. japonica*, and *A. myriantha* with *A. rivularis*) were consistent with previous morphological treatments of the genus (Pan 1985; Chung et al. 1983); however, other

relationships were not as clear (Figures 3.4-3.9). In the initial analysis of 15 taxa, *A. rivularis* formed a sister group with *A. grandis*, an unexpected alignment of the two taxa. One explanation of this arrangement could be long-branch attraction. Long-branch attraction leads to the placement of unrelated taxa artificially close to one another due to the analysis of many characters with few divergent taxa (Stefanovic et al. 2004). The North American species, *A. biternata*, was aligned in a polytomy with six taxa in initial analysis (Figure 3.6) and in the expanded data set formed a polytomy with popular cultivated varieties and hybrids (Figure 3.9). This lack of resolution has been problematic in other studies such as in Valerinaceae, whereby *mat*K sequences resulted in poorly resolved American and Eurasian clades (Hidalgo et al. 2004). This lack of resolution may result from insufficient sequence variation due to rapid radiation or recent speciation (Hidalgo et al. 2004). The concept of rapid radiation has previously been addressed for Saxifragales and family members by Fishbein et al. (2001). Relationships among *Astilbe* taxa may have been similarly affected.

In a study conducted by Samuel et al. (2003) on phylogenetic relationships among species of *Hypochaeris*, *mat*K sequences were less variable and consistent in phylogeny assessment than nuclear sequences. In a similar study, Jarvinen et al. (2004) found that ITS sequences were more variable and provided higher resolution of relationships within *Betula* than did the maternally inherited *mat*K gene region. Inclusion of another gene region in our analysis, particularly a nuclear region may help resolve relationships *mat*K could not.

In our second analysis with 21 species, cultivars, and hybrids of *Astilbe*, cultivated varieties aligned close to the species from which they were derived (*A.*

'Dunkellachs' and *A.* 'Deutschland' closely aligned with *A. japonica* and hybrids; *A.*× *crispa*, *A.* 'Fanal', and *A.* 'Tamarix' closely aligned with *A. chinensis*). When determining a phylogeny of hybrid taxa with plastid gene sequences, the maternal parent must be considered. Alignment of hybrid taxa within the phylogeny will be closest to the maternal parent because of the maternally inherited plastid genome. Utilization of a nuclear gene would produce a phylogeny most representative of the parental lineages of hybrid taxa. In a study conducted by Little (2004), *mat*K demonstrated species-specific polymorphism used to confirm hybridization of *Cupressus macnabiana* and *C. sargentii*. Hybrids were likely the result of multiple hybridization events, with both species acting as the paternal parent.

In this study we have presented the first phylogeny of *Astilbe* based upon molecular data. A *mat*K sequence analysis using maximum parsimony and likelihood methods resulted in trees with somewhat low resolution, however, consistent with previous treatments of the genus in placement of varieties with species. The low levels of variation within *mat*K sequences likely contributed to the low resolution of the phylogeny. Sequencing of nuclear gene regions and additional chloroplast genes may help confirm the current proposed relationships. Sequence polymorphism in *mat*K sequences may potentially be used as molecular markers within the taxa. Investigation into the conservation of these polymorphisms among species is necessary.

3.5 Literature Cited

Chung Y.H., B.Y. Sun and Y.C. Chung. 1983. Monographic study of the endemic plants in Korea, III Taxonomy and interspecific relationships of the genus *Astilbe*. Korean J. Bot. 26: 73-90.

Doyle J.J. and J.L. Doyle 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bull. 19: 11-15.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evol. 30: 783-791.

Fishbein M., C. Hibsch-Jetter, D.E. Soltis and L. Hufford. 2001. Phylogeny of Saxifragales (Angiosperms, eudicots): Analysis of a rapid, ancient radiation. Syst. Biol. 50: 817-847.

Gray A. 1859. Observations upon the relationships of the Japanese flora to that of North America and other parts of the Northern Hemisphere. Mem. Am. Acad. Arts 6: 377-453.

Hidalgo O., T. Garnatje, A. Susanna and J. Mathez. 2004. Phylogeny of Valerianaceae based on *mat*K and ITS markers, with reference to *mat*K individual polymorphism Ann. Bot. 93: 283-293.

Hilu K.W., T. Borsch, K. Muller, D.E. Soltis, P.S. Soltis, V. Savolainen, M.W. Chase, M.P. Powell, L.A. Alice, R. Evans, H. Sauquet, C. Neinhuis, T.A.B. Slotta, J.G. Rohwer, C.S. Campbell and L.W. Chatrou. 2003. Angiosperm phylogeny based on *mat*K sequence information. Am. J. Bot. 90: 1758-1776.

Hilu K.W. and H.P. Liang. 1997. The *mat*K gene: Sequence variation and application in plant systematics. Am. J. Bot. 84: 830-839.

Jarvinen P., A. Palme, L.O. Morales, M. Lannenpaa, M. Keinanen, T. Sopanen and M. Lascoux. 2004. Phylogenetic relationships of *Betula* species (Betulaceae) based on nuclear ADH and chloroplast *mat*K sequences. Am. J. Bot. 91: 1834-1845.

Johnson L.A. and D.E. Soltis. 1994. *mat*K DNA-sequences and phylogenetic reconstruction in Saxifragaceae S-Str. Syst. Bot. 19: 143-156.

Johnson L.A. and D.E. Soltis. 1995. Phylogenetic inference in Saxifragaceae *sensu stricto* and *Gilia* (Polemoniaceae) using *mat*K sequences. Ann. Missouri Bot. Gard. 82: 149-175.

Komatsu K., S. Zhu, H. Fushimi, T.K. Qui, S.Q. Cai and S. Kadota. 2001. Phylogenetic analysis based on 18S rRNA gene and *mat*K gene sequences of *Panax vietnamensis* and five related species. Planta Medica 67: 461-465.

Little D.P. 2004. Documentation of hybridization between Californian cypresses: *Cupressus macnabiana* x *sargentii*. Syst. Bot. 29: 825-833.

Mohr G., P.S. Perlman and A.M. Lambowitz. 1993. Evolutionary relationships among group-II Intron-encoded proteins and identification of a conserved domain that may be related to maturase function. Nucleic Acids Res. 21: 4991-4997.

Neuhaus, H. and G. Link. 1987. The chloroplast $tRNA^{lys}$(UUU) gene from mustard (*Sinapis alba*) contains a class II intron potentially coding for a maturase-related polypeptide. Current Genet. 11: 251-257.

Olson M.S. 2001. Patterns of fruit production in the subdioecious plant *Astilbe biternata* (Saxifragaceae). J. Ecol. 89: 600-607.

Pan J. 1985. A study on the genus *Astilbe* Buch.-Ham. ex D. Don from China. Acta Phytotax. Sinica 23: 432-443.

Samuel R., T.F. Stuessy, K. Tremetsberger, C.M. Baeza and S. Siljak-Yakovlev. 2003. Phylogenetic relationships among species of *Hypochaeris* (Asteraceae, Cichorieae) based on ITS, plastid *trn*L intron, *trn*L-F spacer, and *mat*K sequences. Am. J. Bot. 90: 496-507.

Shaw J., E.B. Lickey, J.T. Beck, S.B. Farmer, W.S. Liu, J. Miller, K.C. Siripun, C.T. Winder, E.E. Schilling and R.L. Small. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot. 92: 142-166.

Soltis D.E. and R.K. Kuzoff. 1995. Discordance between nuclear and chloroplast phylogenies in the *Heuchera* group (Saxifragaceae). Evolution 49: 727-742.

Soltis D.E., R.K. Kuzoff, E. Conti, R. Gornall and K. Ferguson. 1996. *mat*K and *rbc*L gene sequence data indicate that *Saxifraga* (Saxifragaceae) is polyphyletic. Am. J. Bot. 83: 371-382.

Soltis D.E.(a), R.K. Kuzoff, M.E. Mort, M. Zanis, M. Fishbein, L. Hufford, J. Koontz and M.K. Arroyo. 2001. Elucidating deep-level phylogenetic relationships in Saxifragaceae using sequences for six chloroplastic and nuclear DNA regions. Ann. Missouri Bot. Gard. 88: 669-693.

Soltis D.E.(b), M. Tago-Nakazawa, Q.Y. Xiang, S. Kawano, J. Murata, M. Wakabayashi and C. Hibsch-Jetter. 2001. Phylogenetic relationships and evolution in *Chrysosplenium* (Saxifragaceae) based on *mat*K sequence data. Am. J. Bot. 88: 883-893.

Stefanovic, S., D. Rice, and J. Palmer. 2004. Long branch attraction, taxon sampling, and the earliest angiosperms: *Amborella* or monocots? BMC Evol. Biol. 4: 35

Sugita, M. K. Shinozaki, and M. Sugiura. 1985. Tobacco chloroplast $tRNA^{lys}$(UUU) gene contains a 2.5-kilobase-pair intron: an open reading frame and a conserved boundary sequence in the intron. Proc. Natl. Acad. Sci. USA 82: 3557-3561.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Yang D.Y., H. Fushimi, S.Q. Cai and K. Komatsu. 2004. Molecular analysis of *Rheum* species used as *Rhei Rhizoma* based on the chloroplast *mat*K gene sequence and its application for identification. Biol. Pharm. Bull. 27: 375-383.

Zhengyi W. 1983. On the significance of Pacific intercontinental discontinuity. Ann. Missouri Bot. Gard. 70: 577-590.

Table 3.1 List of *Astilbe* taxa, authority, and sources of plants utilized for DNA extraction and *mat*K sequencing.

Taxa	Authority	Plant Source
Astilbe biternata	Britton	Robyn's Nest Nursery, Vancouver, WA
Astilbe chinensis	Franch. & Sav.	National Arboretum
Astilbe davidii	Henry	National Arboretum
Astilbe fujisanensis	Nakai	United Kingdom National Collection of *Astilbe*
Astilbe glaberrima	Nakai	Blanchette Gardens, Carlisle, MA
Astilbe grandis	Stapf. Ex Wilson	Cornell University
Astilbe japonica	A. Gray	Blanchette Gardens, Carlisle, MA
Astilbe koreana	Nakai	Cornell University
Astilbe macroflora	Knoll	United Kingdom National Collection of *Astilbe*
Astilbe microphylla	Hayata	Blanchette Gardens, Carlisle, MA
Astilbe myriantha	Diels	United Kingdom National Collection of *Astilbe*
Astilbe rivularis	Buch.-Ham.	United Kingdom National Collection of *Astilbe*
Astilbe rubra	Hook.f .& Thomson	United Kingdom National Collection of *Astilbe*
Astilbe simplicifolia	Makino	Blanchette Gardens, Carlisle, MA
Astilbe thunbergii	(Siebold et Zucc.) Miq.	Cornell University
Saxifragopsis fragarioides	Small	Soltis et al. 2001a

Table 3.2 List of cultivated *Astilbe* and source for plants utilized for DNA extraction and *mat*K sequencing.

Plant	Source
Astilbe ×*arendsii* 'Fanal'	Dennis Garden Center, Wattsville, VA
Astilbe ×*arendsii* 'Maggie Daley'	Riverbend Nursery, Riner, VA
Astilbe ×*arendsii* 'Tamrix'	Cornell University, Cornell, NY
Astilbe ×*crispa*	Riverbend Nursery, Riner, VA
Astilbe 'Dunkellachs'	Cornell University, Cornell, NY
Astilbe japonica 'Deutschland'	Riverbend Nursery, Riner, VA

Table 3.3 The *mat*K data matrix for 15 taxa of *Astilbe* and outgroup, *Saxifragopsis fragarioides*, consisting of 89 potentially informative polymorphisms. Characters are numbered consecutively along the sequence with character one referenced to nucleotide 1, *Saxifragopsis* (Soltis et al. 2001a). A dot indicates that the same nucleotide given for *Saxifragopsis* is present and a dash represents a deleted base. Nucleotides numbers highlighted separate genus members into two groups. Nucleotide numbers highlighted in red represent polymorphisms specific to only two taxa.

Nucleotide position

Taxa	17 24 29 37 40 45 46 52 65 81	91 130 140 146 156 170 188 189 202 222	229 235 236 237 238 259 263 273 282 284	285 291 297 323 337 385 386 391 397 400	421 423 440 441 446 500 520 529 558 570	602 623 626 628 639 644 668 688 717 718	726 734 746 765 782 792 808 811 825 826	845 872 905 940 963 1012 1015
Saxifragopsis fragarioides	T G A A G T C G A C	G G A T C G C C - T	T A A C G A T C C T	G T T T A A - G A C	G A G C C C G C C T	T A G G A C A T C G	G T G T T G C A G G	A T A G G A T
Astilbe biternata	C A . G A C T . G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C . . A G G	T G T T . A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G A G .
Astilbe chinensis	C A . G A C T . G G	T . . G T C T T . A	. . . . . - C A G C	T C G G C . . A G G	T G T T . A A T . G	. . T A C T G A T T	A C . C G T . C T .	C C G G A G .
Astilbe davidii	C A . G A C T . G G	T . . G T C T T G A	. . . . . . C A G C	T C G G C . . A G G	T G T T . A A T . G	. . T A C T G A T T	A C . C G T . C T .	C C G G A G .
Astilbe fujisanensis	C A . G A C T . G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C . . A G G	T G T T . A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G A G .
Astilbe glaberrima	C A C G . C T . G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C . . A G G	T G T T . A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G . G .
Astilbe grandis	C A . G . C T . G G	T . . G T C T T . A	G . . . . . C A G C	T C G G C . . A G G	T G T T . . . T T G	. . T A . T G A T T	A C . C G T A C T .	T C G G . G C
Astilbe japonica	C A C G A C T . G G	C . . G T C T T . A	. . . . . . C A G C	T C G G C . . A G G	T G T T . A A T . G	. C T A . T G A T T	A C . C G T . C T .	C C G G . G .
Astilbe koreana	C A . G . C T . G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C . T A G G	T G T T . A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G A G .
Astilbe macroflora	C A . G . C T . G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C . . A G G	T G T T . A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G . G .
Astilbe microphylla	C A . G . C T A G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C . . A G G	T G T T A A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G A G .
Astilbe myriantha	C A . G . C T . G G	T . G G T C T T - A	. T T A . . C A G C	T C G G C . . A G G	T G T T . . . T . G	C . T A . T G A T T	A C T C G T . G T .	C C G G . G .
Astilbe rivularis	C A . G . C T . G G	T T G G T C T T . A	. . . . A . C A G C	T C G G C . . A . G	T G T T . . . T T G	. . T A . T G A T T	A C . C G T A C T A	T C G A A G C
Astilbe rubra	C A . G A C T . G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C T . A G G	T G T T . A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G . G .
Astilbe simplicifolia	C A . G A C T . G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C . A A G G	T G T T . A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G A G .
Astilbe thunbergii	C A G A C T . G G	T . . G T C T T . A	. . . . . . C A G C	T C G G C . . A G G	T G T T . A A T . G	. . T A . T G A T T	A C . C G T . C T .	C C G G A G .

Table 3.4 Variability in *mat*K sequence unique to *A. myriantha* and *A. rivularis*, closely correlating to the sequence of *S. fragarioides*. Characters are numbered consecutively with character one referenced to nucleotide 1, *Saxifragopsis* (Soltis et al. 2001a). A dot indicates that the same nucleotide given for *Saxifragopsis* is present and a dash represents a deleted base.

	Nucleotide position						
	702	703	704	705	706	707	708
Taxa							
Saxifragopsis fragarioides	T	-	T	C	-	-	C
Astilbe biternata	.	T	.	.	T	T	.
Astilbe chinensis	.	T	.	.	T	T	.
Astilbe davidii	.	T	.	.	T	T	.
Astilbe fujisanensis	.	T	.	.	T	T	.
Astilbe glaberrima	.	T	.	.	T	T	.
Astilbe grandis	.	T	.	.	T	T	.
Astilbe japonica	.	T	.	.	T	T	.
Astilbe koreana	.	T	.	.	T	T	.
Astilbe macroflora	.	T	.	.	T	T	.
Astilbe microphylla	.	T	.	.	T	T	.
Astilbe myriantha	.	-	-	-	T	T	.
Astilbe rubra	.	T	.	.	T	T	.
Astilbe rivularis	.	T	.	.	.	.	T
Astilbe simplicifolia	.	T	.	.	T	T	.
Astilbe thunbergii	.	T	.	.	T	T	.

Table 3.5 10 bp *mat*K sequence variation distinguishing *S. fragarioides* from *Astilbe* taxa. Characters are numbered consecutively with character one referenced to nucleotide 1, *Saxifragopsis* (Soltis et al. 2001a). A dot indicates that the same nucleotide given for *Saxifragopsis* is present and a dash represents a deleted base.

	Nucleotide position
	4 4 4 4 4 4 4 4 4 4 4 4 4
	6 6 6 6 6 6 6 6 6 6 6 7 7
Taxa	0 1 2 3 4 5 6 7 8 9 0 1
Saxifragopsis fragarioides	- G - - - T - - A - - -
Astilbe biternata	G . A A A . G G . T T T
Astilbe chinensis	G . A A A . G G . T T T
Astilbe davidii	G . A A A . G G . T T T
Astilbe fujisanensis	G . A A A . G G . T T T
Astilbe glaberrima	G . A A A . G G . T T T
Astilbe grandis	G . A A A . G G . T T T
Astilbe japonica	G . A A A . G G . T T T
Astilbe koreana	G . A A A . G G . T T T
Astilbe macroflora	G . A A A . G G . T T T
Astilbe microphylla	G . A A A . G G . T T T
Astilbe myriantha	G . A A A . G G . T T T
Astilbe rubra	G . A A A . G G . T T T
Astilbe rivularis	G . A A A . G G . T T T
Astilbe simplicifolia	G . A A A . G G . T T T
Astilbe thunbergii	G . A A A . G G . T T T

Figure 3.1 The *mat*K gene and *trn*K intron regions with primers utilized for amplification and for sequencing (Johnson and Soltis 1994, 1995).

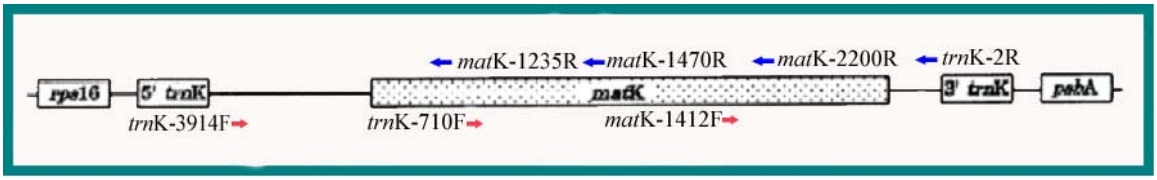


Figure 3.2 Sequence variation among taxa representing various taxonomic hierarchies using sequences of the *mat*K coding region. The x-axis represents the 5' to 3' coding region divided into 31 sectors of 50 bp; the y-axis represents number of nucleotide substitutions per sector. (Hilu and Liang 1997).

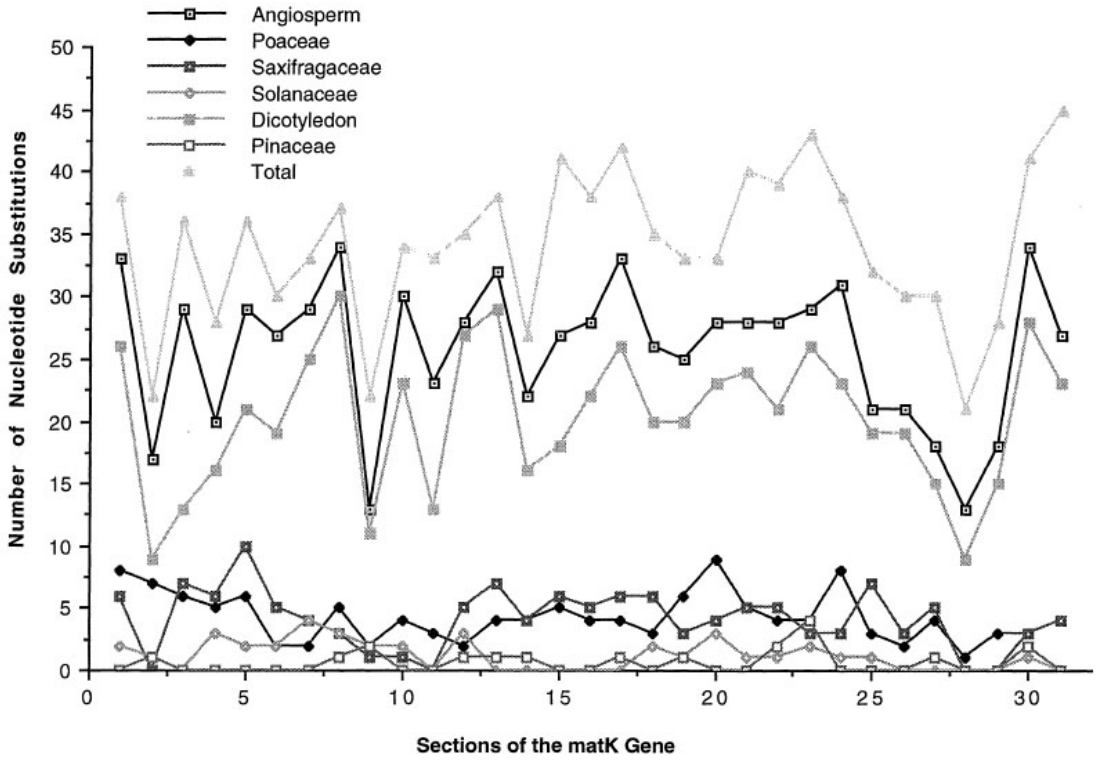


Figure 3.3 Amplification of 2500 bp of *mat*K and portions of the *trn*K intron region using primers *trn*K-3914F and *trn*K-2R (Johnson and Soltis 1994).

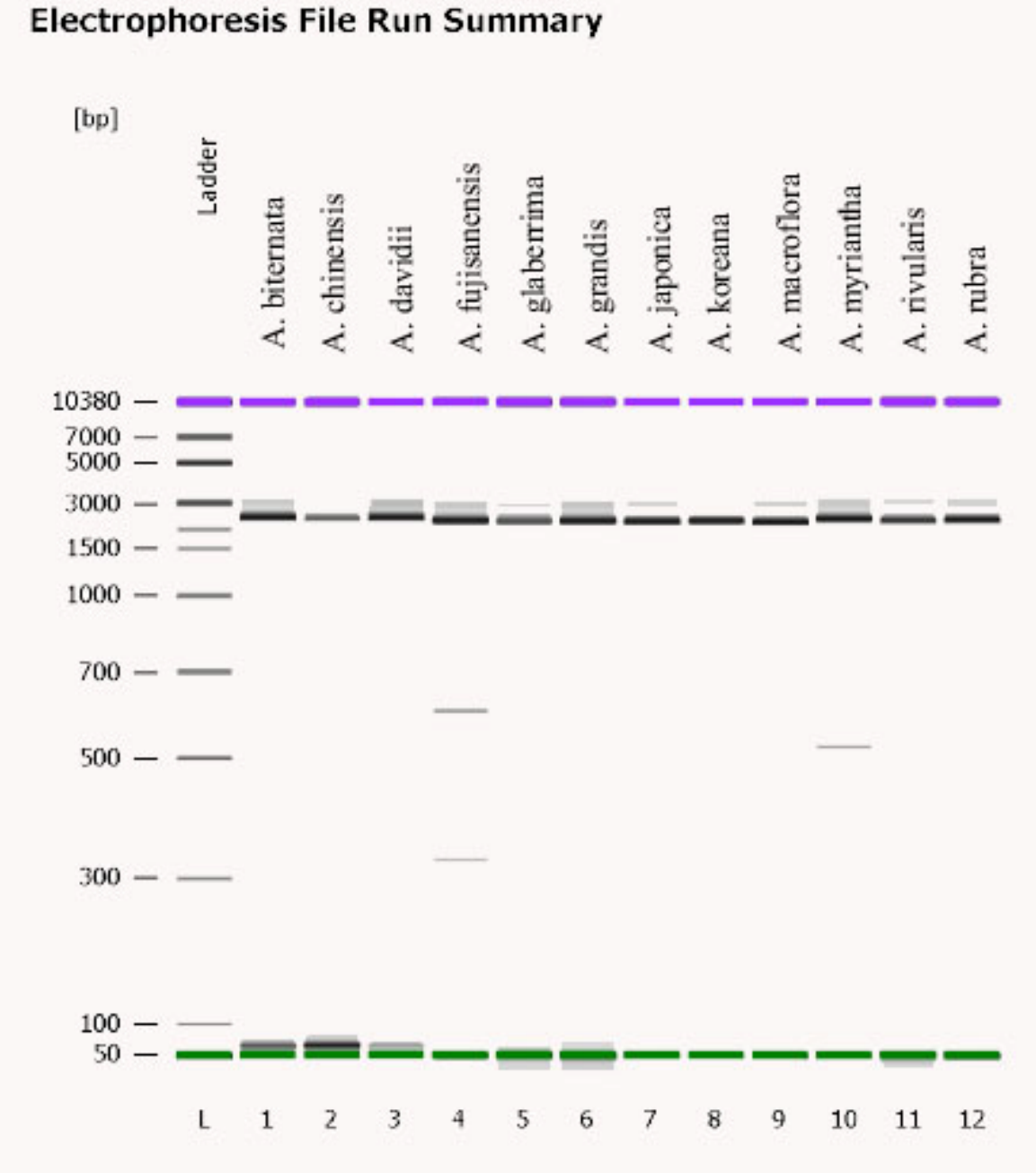


Figure 3.4 Strict consensus parsimonious tree derived from *mat*K sequences from15 taxa of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup, with length 113, HI of 0.20 and a RC of 0.84. Bootstrap support greater than 50% is presented above branches.

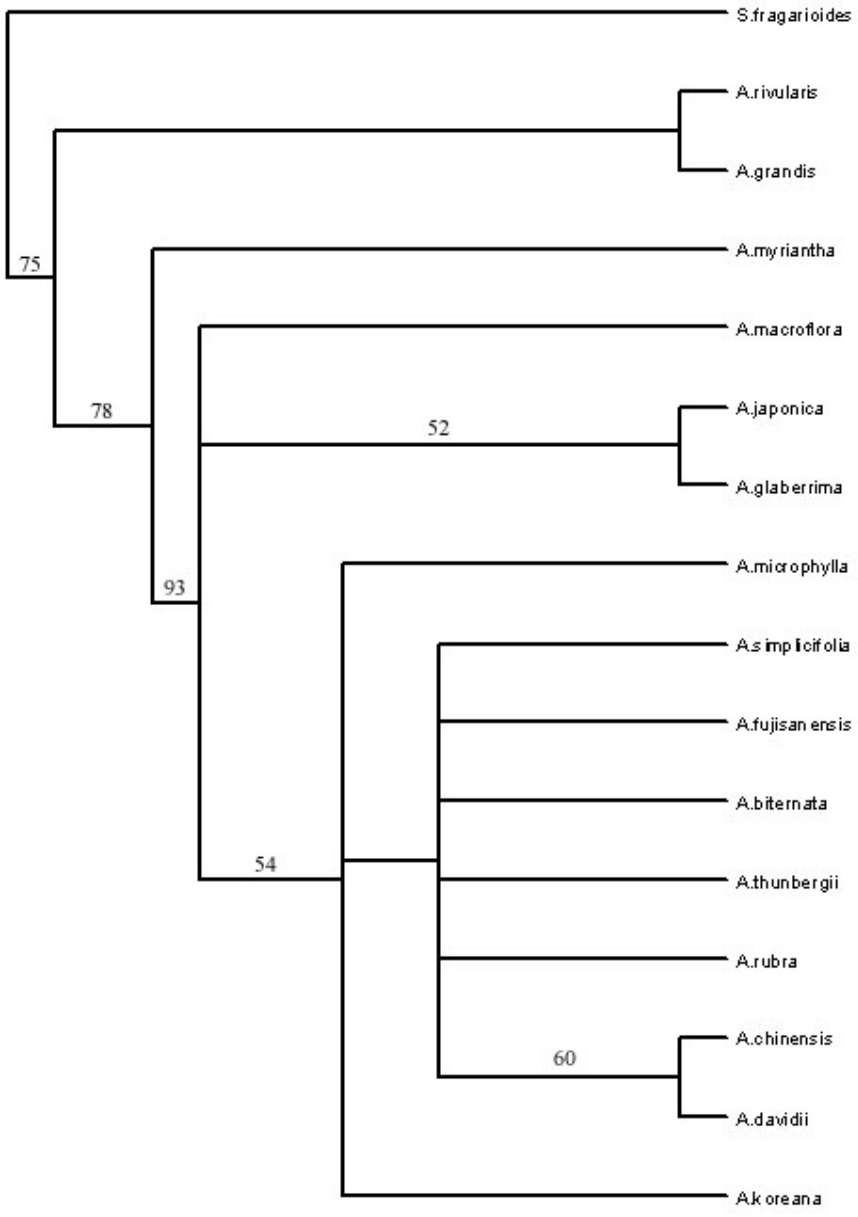


Figure 3.5 Neighbor joining tree of *mat*K sequences from 15 taxa of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup taxon.

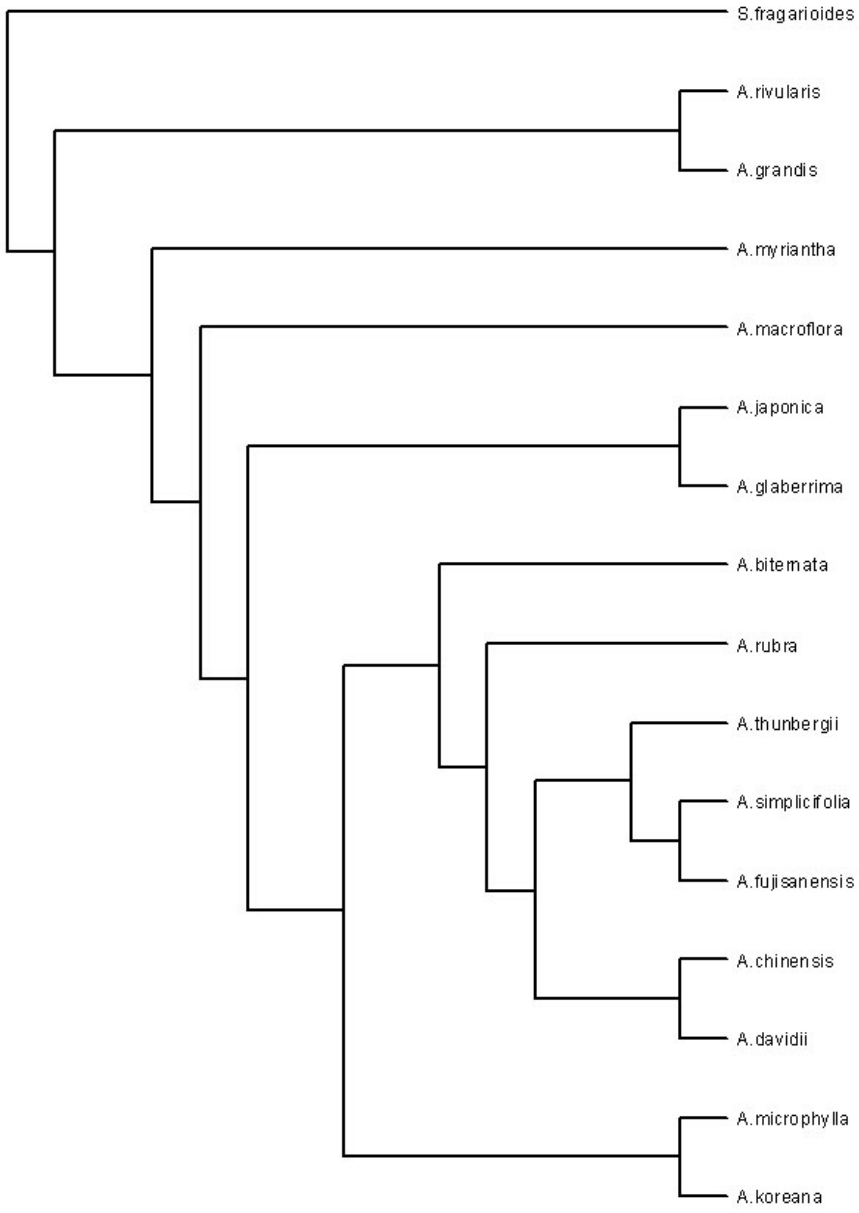


Figure 3.6 Single best tree (-ln *L* = 2,030.85) generated from maximum likelihood analysis of *mat*K sequences of 15 taxa of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup taxon. Bootstrap values greater than 50% are presented above branches.

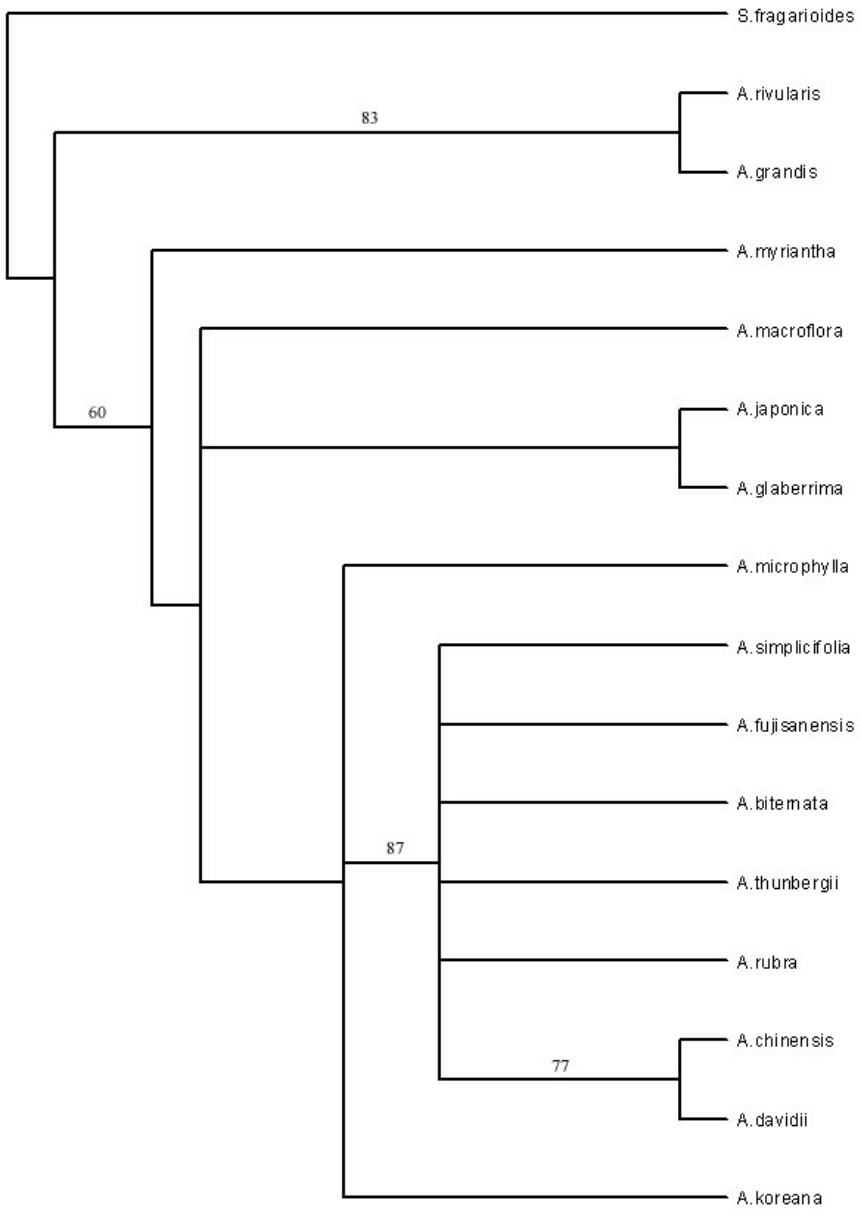


Figure 3.7 Single consensus parsimonious tree derived from *mat*K sequences from 21 species, cultivars, and hybrids of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup taxon, with length 795, HI of 0.07, and a RC of 0.91. Bootstrap support greater than 50% is presented above branches**.**

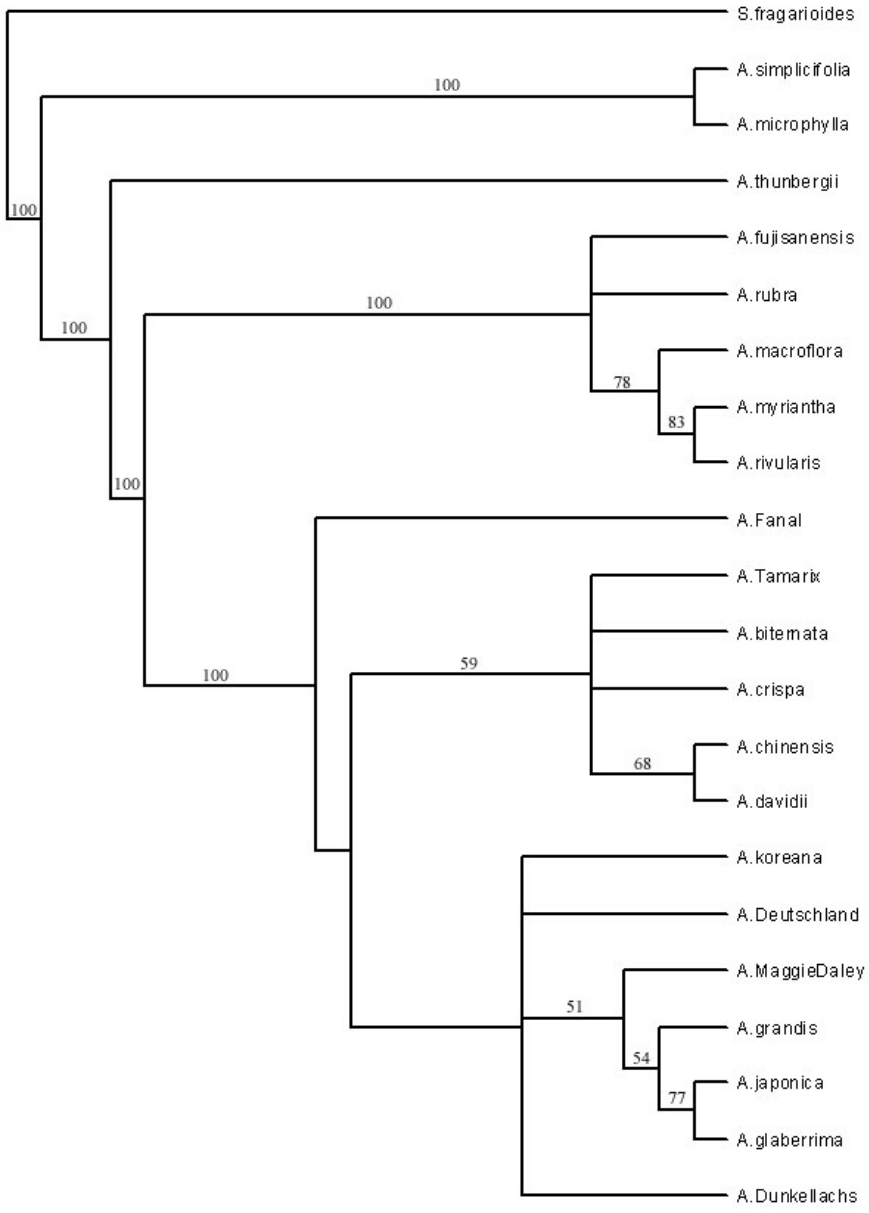


Figure 3.8 Neighbor joining tree of *mat*K sequences from 21 species, cultivars, and hybrids of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup taxon.

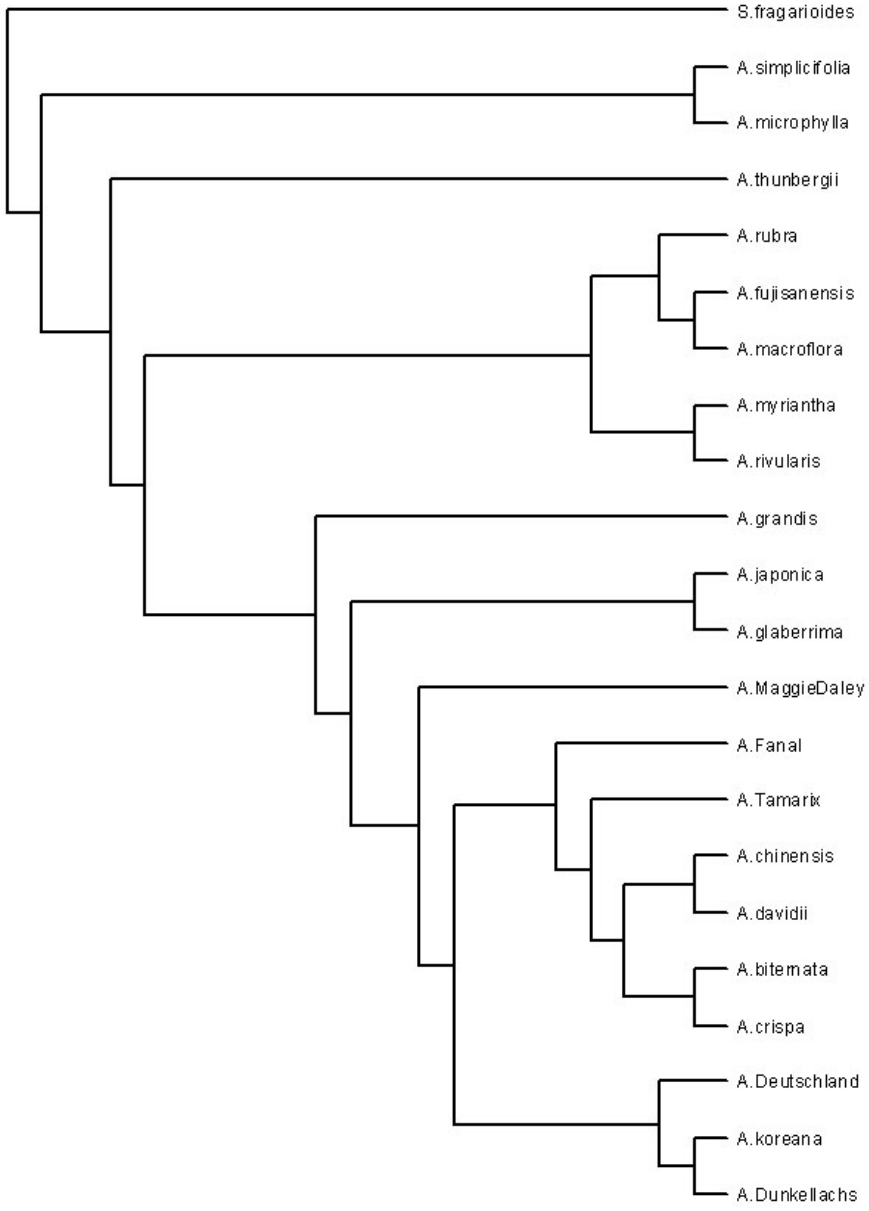
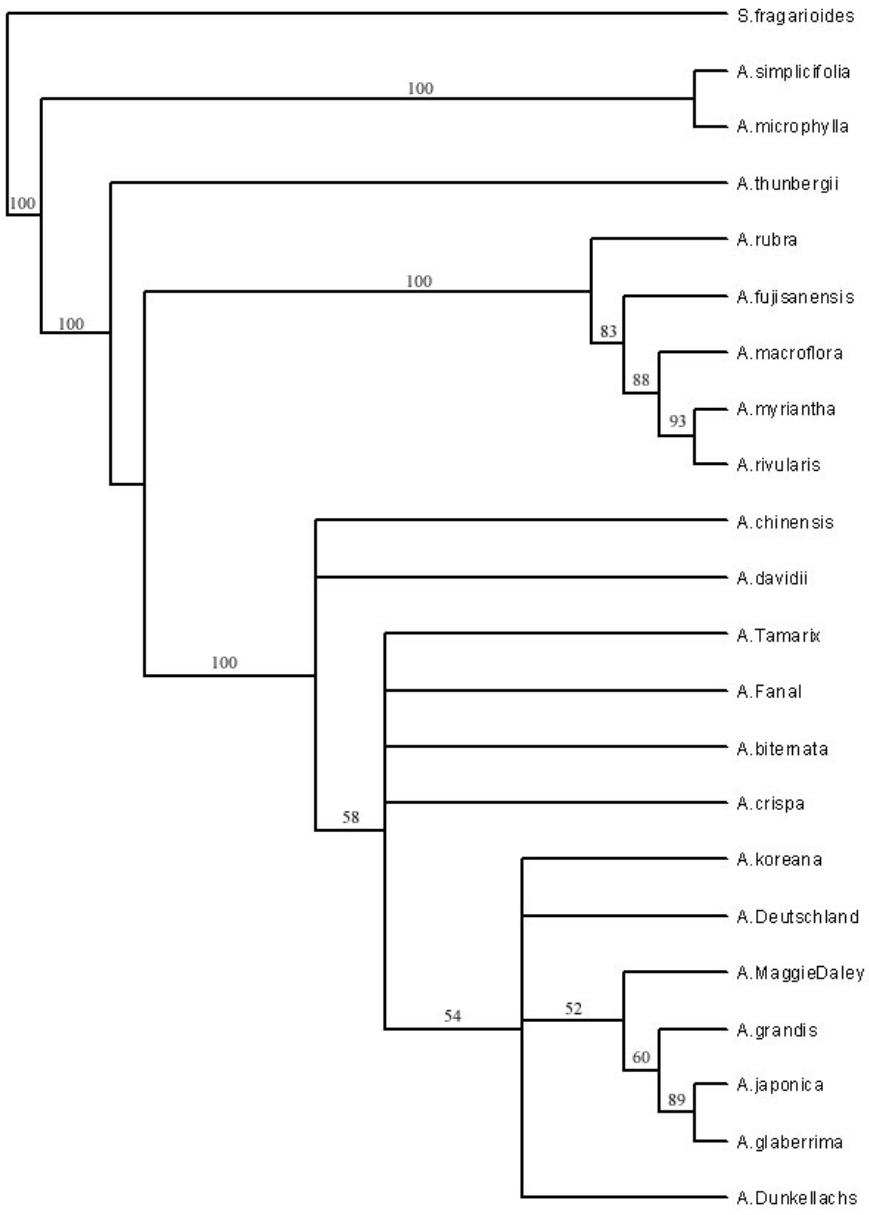


Figure 3.9 Single best tree (-ln *L* = 4,731.044) generated from maximum likelihood analysis of *mat*K sequences of 21 species, cultivars, and hybrids of *Astilbe*, using *Saxifragopsis fragarioides* as the outgroup taxon. Bootstrap values greater than 50% are presented above branches.



Chapter 4. Somaclonal Variation of *Astilbe* Microshoots

Abstract

Astilbe is a genus comprised of shade-loving herbaceous perennials tremendously popular in the nursery and landscape industry. Consistently ranked among the top five herbaceous perennials by the national Perennial Plant Association (PPA) in terms of wholesale value, *Astilbe* species have been extensively hybridized, selected, and released as cultivars. Little information exists on the regeneration of *Astilbe* through plant tissue culture. We cultured seedlings of *A.*× *arendsii in vitro* on woody plant medium (WPM) until multiple shoot regeneration occurred. Fifteen of 35 seedlings regenerated freely and produced 5 or more shoots, while others were generally recalcitrant. Shoots were acclimatized to the greenhouse from 15 seedlings, designated alphabetically A - O. About 139 plants were established and vernalized in a cold-frame before transfer back into the greenhouse and placed in a completely randomized design. Data were collected from *Astilbe* microshoots on plant emergence, plant height, average plant width, flower number, mean petiole length, leaflet ratio, and chlorophyll content. Multivariate and cluster analysis of measured characters revealed potential somaclonal variation among microshoots from seedling families. The potential somaclonal variants found among microshoots of seedling families were characterized by dwarf habit, dark green leaves (high chlorophyll content), increased flowering, or larger plant size. Somaclonal variants with desirable phenotypes will be evaluated further for cultivar development.

4.1 Introduction

The genus *Astilbe* of the family Saxifragaceae is composed of herbaceous perennials increasingly popular in the nursery and landscape industry. *Astilbe* is consistently ranked among the top five perennials by the Perennial Plant Association (PPA). Primarily hybrids and cultivars are available in the nursery trade and it is rare that unimproved species are offered; cultivated selections and hybrids of *A. chinensis*, *A. japonica*, *A. simplicifolia*, and *A. thunbergii* are most common.

Astilbe is propagated mainly by seed or vegetative crown division, with selections being made from open-pollinated seedling populations and then given hybrid names (Armitage, 1996). Many of the selections and cultivars currently utilized in horticulture are derived from the breeding efforts of a famous German hybridizer, George Arends (1862-1952). Mr. Arends has been credited with introducing over 74 cultivars of *Astilbe* in 50 years (Randhava 2005). Little information exists on genetic manipulation of the genus for desirable plant characteristics (flowering, hardiness, plant height). Introduction of new variability into difficult-to-propagate species such as the native *Astilbe biternata* and other rare species may be beneficial to the horticulture trade.

In recent years somaclonal variation has been used as a method of introducing variation into ornamental plants. Somaclonal variation has been defined as the genetic change of a plant when regenerated through a tissue culture procedure. Mechanisms, molecular analysis, and utilization of somaclonal variation have been reviewed by Veilleux and Johnson (1998). The use of somaclonal variation for crop improvement has also been investigated and summarized by Karp (1995) and Jain (2001). The benefits of

somaclonal variation in improvement of sunflower (*Helianthus annuus* L.) (Encheva et al. 2003), chili pepper (*Capsicum annuum* L.) (Houssain et al. 2003), and rose-scented geranium (*Pelargonium graveolens* (L.) Herit.) (Ravindra et al. 2004) have been reported. In these studies selected somaclones were found to be horticulturally more desirable than the explant source. Riseman and Chennareddy (2004) investigated the effect of growth regulator regimen on *Exacum* L. in tissue culture and found that different regimes caused genotypic variation in the form of varied ploidy levels. Cammareri et al. (2002) detected somaclonal variation (capitulum and disk diameter, ligulate flower number) in *Aster cordifolius* L. 'White Elegans' regenerated from leaf explants. The objectives of this study were to culture *Astilbe* seedlings *in vitro* and evaluate potential somaclonal variation of resulting *Astilbe* microshoots.

4.2 Materials and Methods

Tissue Culture. In November of 2003, seeds obtained from Stokes Seed Co. (Buffalo, N.Y.) of *A.*×*arendsii* were germinated in a growth chamber (16 h photoperiod, 22-28 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 25°C) on sterile germination paper (Figure 4.1). Germinated seedlings were dipped in 50% Plant Preservative Mixture (PPM) (Plant Cell Technology, Washington, D.C.) for 30 sec and transferred to woody plant medium (WPM) supplemented with 30 $g \cdot l^{-1}$ sucrose, 100 $mg \cdot l^{-1}$ myo-inositol, 7 $g \cdot l^{-1}$ phytagar, 0.186 $mg \cdot l^{-1}$ naphthaleneacetic acid (NAA), 2.125 $mg \cdot l^{-1}$ kinetin, and the antibiotic cefotaxime at 250 $mg \cdot l^{-1}$. Seedlings with multiple shoot proliferation were divided under sterile conditions and subcultured onto fresh medium of similar composition every 3 weeks and returned to the growth chamber (Figure 4.2).

Greenhouse Methods. In February 2004, plants derived from 15 seedlings were selected for vigor and resulting somaclones (5 to 14 representing each seedling) were acclimated to the greenhouse under 50% shade for 4 wks (Figure 4.3). Once acclimated, somaclones were transferred to 15.2 cm square plastic pots and grown without shade in the greenhouse in a completely randomized design. From October 2004 until March 2005, plants were over wintered in a cold frame for vernalization, then transferred back to greenhouse for observation.

Data were collected from *Astilbe* somaclones on plant emergence (0 = not emerged, 3 = fully emerged), plant height, average plant width, leaflet ratio, petiole length, chlorophyll fluorescence, and flower number. Degree of emergence was rated 2 wks after transfer back into the greenhouse from the cold frame. In late April 2005, leaf ratio, petiole length, and chlorophyll content were averaged from three different terminal leaflets per somaclone. Flower spike number, plant height, and average plant width were rated at this time.

Statistical Analysis. ANOVA was used to determine differences among seedling families and means were determined using the Ryan-Einot-Gabriel-Welsch Multiple Range Test (SAS Institute Inc 2005). In addition, multivariate analysis and cluster analysis by the FASTCLUS procedure of SAS (2005) were used to detect potential somaclones within seedling families.

4.3 Results

Approximately 35 seedlings survived the stringent sterilization procedures, with 22 of those seedlings readily producing multiple shoots. Of the regenerating seedlings, 20 were selected for division and sub-culturing. After acclimation to the greenhouse, only 15

seedling families yielded five or more somaclones. These were used for investigation of variability among the microshoots.

Variability of vegetative and floral morphology among the fifteen seedling families was expected and could be detected visibly. One seedling family was characterized by red pigment in the mid-veins and petioles (Figure 4.4), whereas another seedling family consistently had dark green, thick leaves (Figure 4.5). These dark green leaves, often thickened and contorted are suggestive of plants with high levels of ploidy (Briggs and Knowles 1967). Other visible differences among seedling families were flower color, leaf structure, and overall plant size. ANOVA revealed significant differences among the seedling families for each character measured (Table 4.1). Means of each character are presented by seedling family in Table 4.2. Variation present in the table is not expected to be somaclonal, but shows genetic variation among seedlings within the seedlot.

Using multivariate and cluster analysis, we revealed potential somaclones within seedling families by their departure from most family members detected on plots of canonical variables representing several traits (Figure 4.6). The cubic clustering criterion was optimized for the cluster analysis with three clusters (20.596). Standardized means of these traits by cluster are illustrated in Figure 4.7. Generally, cluster 1 consisted of the largest plants (height, width, petioles, and leaves) with the least amount of chlorophyll. Cluster 2 is composed primarily of the smallest plants (height, width, petioles, and leaves) with the latest emergence, fewest flowers, and most chlorophyll. Plants in cluster 3 were generally the first to emerge with the most flowers and had characters intermediate with respect to plant and leaf size and chlorophyll content. Seedling families

B, E ,F, and H arranged within single clusters and the remaining seedling families were dispersed in multiple clusters (Table 4.3). The potential somaclonal variants found within the fifteen seedling families (Figure 4.6 and Table 4.3) include one member of seedling family D (smaller with more chlorophyll), one member of seedling family G (more flowers and earlier to emerge), one member of seedling family L (larger with less chlorophyll), and potentially three other members of seedling family L (smaller with more chlorophyll).

4.4 Discussion

In our investigation of 15 seedling families of *Astilbe*, derived from micropropagation, we revealed potential somaclonal variation. Of the 35 surviving seedlings 22 regenerated readily. Because we utilized a hybrid seed lot as our seedling source, the genotypes and phenotypes of the seedling families were expected to be quite diverse. Evans and Sharp (1986) stated that genotype is an important variable when investigating the frequency of regeneration and somaclonal variation in tissue culture.

We utilized multivariate cluster analysis to observe outliers and potential variants in our unreplicated study. In this micropropagation system, single variant plants can be observed, but a mutation that arose during the early culture phase could have been micropropagated and resulted in severalsimilarly aberrant regenerants. This may explain why some seedling families occurred in more than a single phenotypically disctinct clusters (e.g. Family L, Table 4.3). Multivariate analysis has been used previously to evaluate aluminum tolerance of somaclonal variants of cultured *Malus* sp. rootstocks (Dantas et al. 2001). As a result of their cluster analysis, somaclonal rootstocks were classified on the basis of their tolerance to aluminum into three groups: tolerant,

somewhat tolerant and susceptible. Charlton et al. (2004) used multivariate analysis to to determine whether biochemical changes, so-called 'unintended effects', beyond those intended by incorporation of a transgene, were detectable in somaclones of *Pisum sativum*. The phenotypic stability of morphometric traits in *Lycopersicon* spp. was measured by multivariate analysis from non-regenerated plants and the progeny of regenerated plants by Pratta et al. (2000). Though no variation among the traits was found, they determined the analysis an appropriate methodology for measuring stability of traits.

Potential *Astilbe* somaclones that differed from most of the individuals within their respective seedling families varied for characteristics such as plant size, flowering, and pigmentation. Somaclonal variants of *Chrysanthemum* L. described by Chang et al. (2000) also differed from source plants for similar traits, including plant height, stem internode length, and number of disc and ray flowers. The tendency of somaclonal variants to differ with respect to height, plant form, and number of plant floral or stem structures has been discussed with regard to two species of *Picea* Link. (Tremblay et al.1999).

To further investigate the occurence of somaclonal variation among seedling families, we would need to examine chromosome stability either directly by root tip squashes or indirectly through DNA content estimation by flow cytometry. Field evaluation of potential somaclones after vegetative propagation would confirm continuation of variation through a vegetative cycle. Selection and evaluation of potential somaclonal variants could lead to new cultivar development.

4.5 Literature Cited

Armitage A.M. 1996. Herbaceous perennial plants: a treatise on their identification, culture, and garden attributes. Stipes Pub., Champaign, Illinois pp.167-176.

Briggs F.N. and P.F. Knowles. 1967. Introduction to Plant Breeding. Reinhold Pub., New York pp. 268.

Cammareri M., A. Errico, E. Filippone, S. Esposito and C. Conicella. 2002. Induction of variability in chimeric *Aster cordifolius* 'White Elegans' through somaclonal variation. Euphytica 128: 19-25.

Chang S., M. Huang, S. Chang and M. Huang. 2000. The variation in tissue culture-derived plants of *Chrysanthemum*. J. Chin. Soc. Hort. Sci. 46: 21-34.

Charlton A., T. Allnutt, S. Holmes, J. Chisholm, S. Bean, N. Ellis, P. Mullineaux and S. Oehlschlager. 2004. NMR profiling of transgenic peas. Plant Biotechnol. J. 2: 27-35.

Dantas A.C.D., G.R.D. Fortes, J.B. da Silva, A.N. Nezi and A.C. Rodrigues. 2001. Tolerance to aluminium in apple rootstocks somaclones in nutrient solution. Pesquisa Agropecuaria Brasileira 36: 615-623.

Encheva J., H. Kohler, W. Friedt, F. Tsvetkova, P. Ivanov, V. Encheva and P. Shindrova. 2003. Field evaluation of somaclonal variation in sunflower (*Helianthus annuus* L.) and its application for crop improvement. Euphytica 130: 167-175.

Evans D.A. and W.R. Sharp. 1986. Somaclonal and gametoclonal variation. Macmillan Pub., New York, pp. 97-132.

Hossain M.A., K. Konisho, M. Minami and K. Nemoto. 2003. Somaclonal variation of regenerated plants in chili pepper (*Capsicum annuum* L.). Euphytica 130: 233-239.

Jain S.M. 2001. Tissue culture-derived variation in crop improvement. Euphytica 118: 153-166.

Karp A. 1995. Somaclonal variation as a tool for crop improvement. Euphytica 85: 295-302.

Pratta G., R. Zorzoli and L.A. Picardi. 2000. Multivariate analysis as a tool for measuring the stability of morphometric traits in Lycopersicon plants from in vitro culture. Genet. Molec. Biol. 23: 479-483.

Randhava L. (2005) Plant Profile: *Astilbe*. In: Chicago Botanical Garden Online Index. p www.chicagobotanic.org/plantinfo/pp/PPAstilbe.html.

Ravindra N.S., R.N. Kulkarni, M.C. Gayathri and S. Ramesh. 2004. Somaclonal variation for some morphological traits, herb yield, essential oil content and essential oil composition in an Indian cultivar of rose-scented geranium. Plant Breed. 123: 84-86.

Riseman A. and S. Chennareddy. 2004. Genotypic variation in the micropropagation of Sri Lankan *Exacum* hybrids. J. Am. Soc. Hort. Sci 129: 698-703.

SAS Institute. 2005. SAS Guide for Personal Computers, version 9.1. SAS Inst, Cary, N.C.

Tremblay L., C. Levasseur and F.M. Tremblay. 1999. Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability. Am. J. Bot. 86: 1373-1381.

Veilleux R.E. and A.T. Johnson. 1998. Somaclonal variation: molecular analysis, transformation interaction, and utilization. Plant Breed. Rev. 16: 229-268.

Table 4.1 ANOVA table of seven characterictics among 15 seedling families of *Astilbe* ×*arendsii*.

		Emergence	Plant height	Plant width	Leaf ratio	Petiole length	Flower number	Chlorophyll
Source	DF	MS	MS	MS	MS	MS	MS	MS
Seedling family	14	2.4*	53.3*	91.4*	155.6*	29.5*	32.9*	71.1*
Error	123	0.1	10.4	11.5	11.4	3.9	3.0	11.0

* denotes significance at $\alpha<0.05$

Table 4.2 Means of fifteen seedling families for emergence, plant height, plant width, leaf ratio, petiole length, flower number, and chlorophyll content. Means separated by Ryan-Einot-Gabriel-Welsch Multiple Range Test.

		Emergence	Plant Height	Plant Width	Leaf Ratio	Petiole Length	Flower Number	Chlorophyll
Seedling	n	0 - 3	cm	cm		cm	#	
A	9	2.1 AB	16.9 BC	29.4 BC	16.6 AB	11.7 ABC	0.2 BC	31.5 D
B	8	2.0 B	16.7 BC	26.1 C	16.3 AB	8.5 CD	4.6 A	36.7 ABDC
C	8	2.0 B	16.4 BC	29.2 BC	16.3 AB	10.1 BCD	2.9 AB	31.8 D
D	12	2.7 A	15.9 BC	27.2 BC	12.7 BCDE	8.5 CD	5.3 A	36.3 ABCD
E	5	2.0 B	19.4 AB	36.8 A	16.7 AB	13.4 A	1.0 BC	32.3 D
F	10	2.2 AB	22.1 A	36.3 A	15.2 ABC	13.9 A	0.8 BC	33.1 CD
G	11	2.1 B	20.5 AB	32.7 AB	18.2 A	12.1 AB	1.3 BC	33.2 CD
H	10	2.4 AB	13.9 C	26.3 C	7.2 F	8.3 D	0.0 C	39.8A
I	9	1.0 C	15.4 BC	29.7 BC	10.7 CDEF	10.1 BCD	0.7 BC	33.6 CD
J	12	1.0 C	16.8 BC	29.6 BC	8.4 DEF	9.9 BCD	1.2 BC	34.4 BCD
K	10	2.0 B	15.8 BC	30.4 BC	7.8 EF	10.1 BCD	0.3 BC	33.3 CD
L	14	2.6 AB	15.9 BC	27.5 BC	13.1 ABCD	9.4 BCD	4.6 A	36.2 ABCD
M	9	2.2 AB	13.3 C	26.9 C	6.8 F	8.4 CD	2.8 ABC	39.6 AB
N	4	2.2 AB	13.5 C	26.6 C	15.8 ABC	7.6 D	4.3 A	40.2 A
O	7	2.1 AB	16.3 BC	27.9 BC	7.7 EF	9.8 BCD	2.9 AB	37.9 ABC

Table 4.3 Frequency of individual plants in 15 seedling families occurring among the three clusters formed by multivariate analysis using the FASTCLUS procedure in SAS.

Seedling	# of somaclones	Cluster 1	Cluster 2	Cluster 3
A	9	6	3	
B	8			8
C	8	3	2	3
D	12		1	11
E	5	5		
F	10	10		
G	11	10		1
H	10		10	
I	9	3	6	
J	12	3	9	
K	10	3	7	
L	14	1	3	10
M	9		7	2
N	4		2	2
O	7		4	3

Figure 4.1 Seedlings of *Astilbe* ×*arendsii* germinating on sterile germination paper.

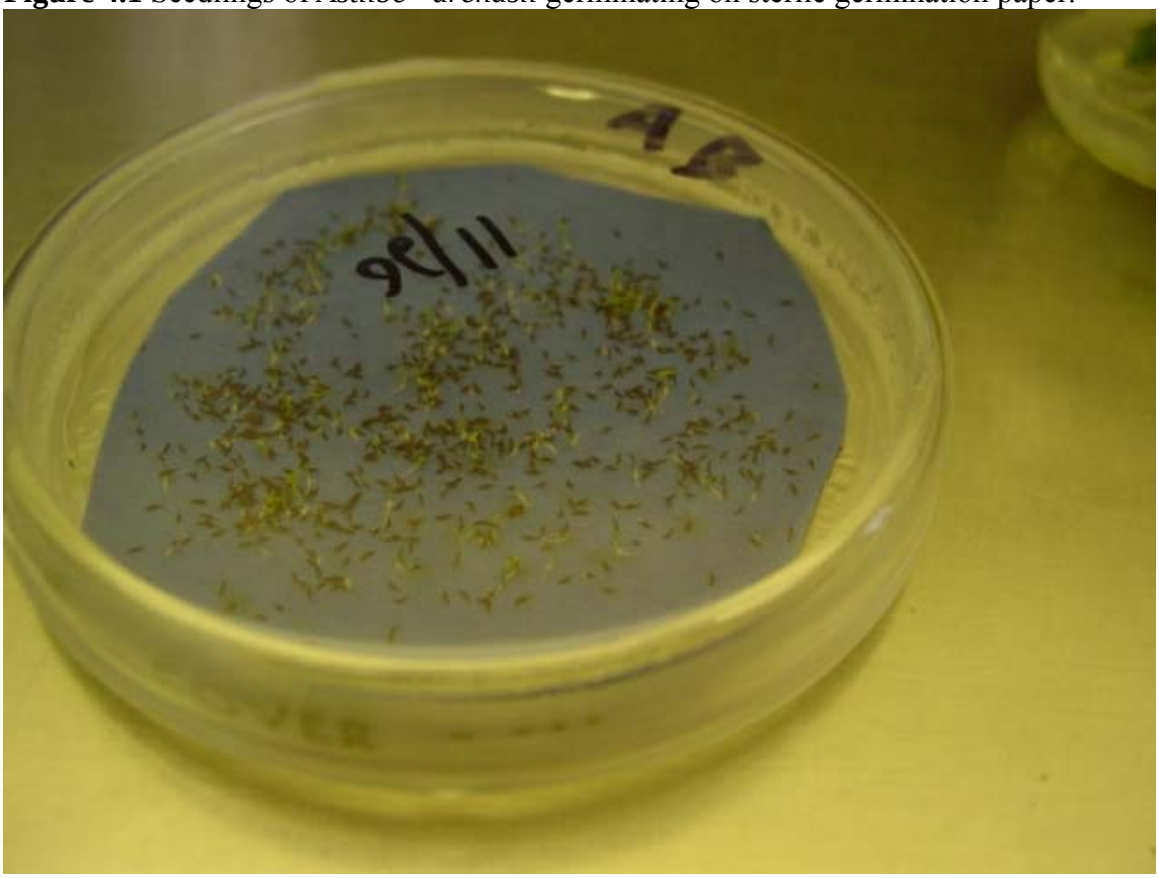


Figure 4.2 Multiple shoot proliferation developing from a single seedling of *Astilbe* ×*arendsii* before subculture *in vitro*.

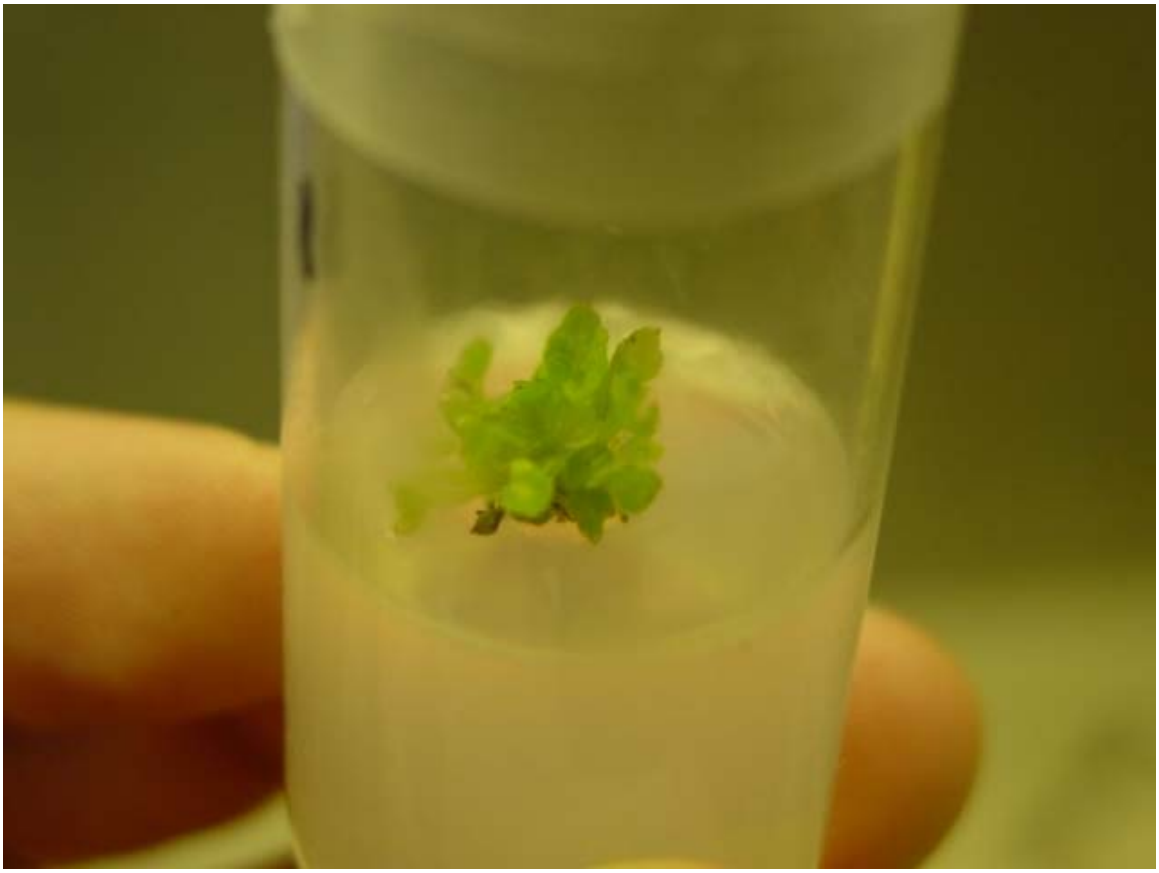
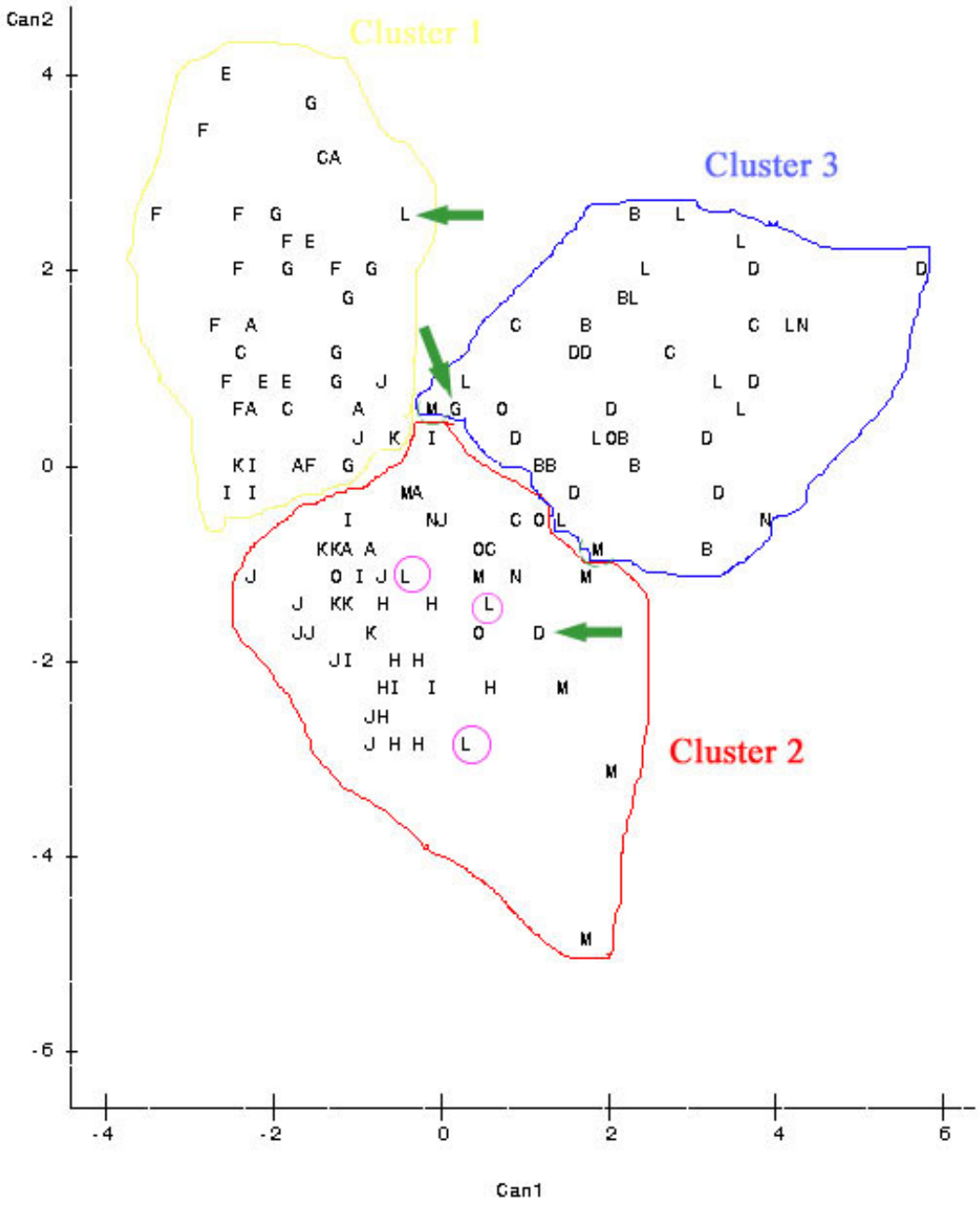


Figure 4.3 Acclimation of somaclones of *Astilbe* ×*arendsii* seedlings in the greenhouse.

Figure 4.4 Red pigment of petioles typical of somaclones belonging to seedling family G.

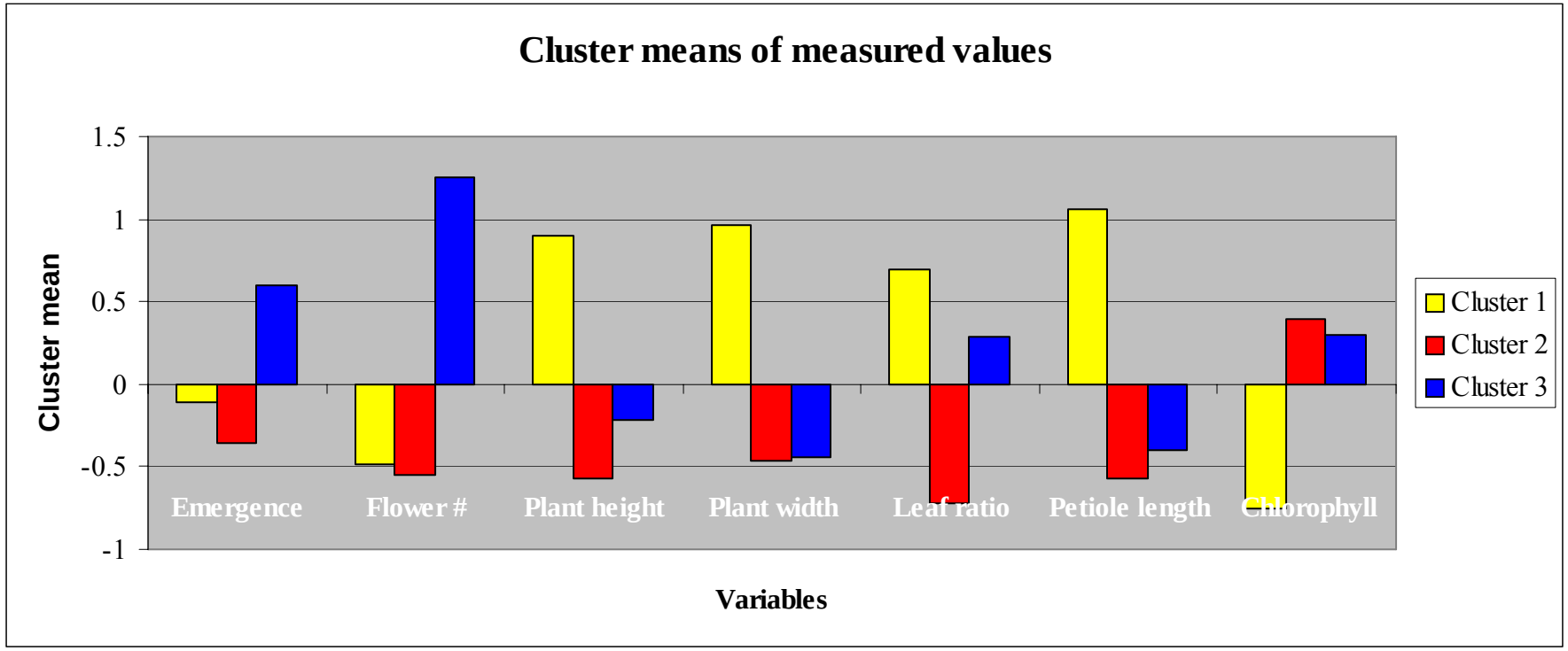
Figure 4.5 Dark green, thickened leaves typical of somaclones of seedling family H.

Figure 4.6 Plot of 138 somaclones in 15 seedling families using the first two canonical variables from multivariate analysis of characters showing their arrangement within three clusters generated by the FASTCLUS procedure in SAS. Contours enclose individuals occurring in each of three clusters. Symbols A-O are individual plants in each of the 15 families. Plants that occur in a different cluster from most of those in a family are indicated by green arrows.



ad missing values. 10 obs hidden.

Figure 4.7 Cluster means for three clusters generated using the FASTCLUS procedure of SAS based on seven characters (emergence, flower number, plant height, plant width, leaf ratio, petiole length, and chlorophyll content) and 138 plants occurring in 15 seedling families.



Chapter 5. Conclusions

The broad objective of this study was to develop a well-resolved phylogeny of *Astilbe*. We investigated variation of *Astilbe* at various levels, from variation within seedling populations to variation among the species. We incorporated an expanded morphological data set and used gene sequences of *mat*K to develop the first molecular investigation into the genus. We also looked at sequence variation within *mat*K to see if polymorphisms unique to species may be tracked to popular cultivated varieties, thus helping track the lineages of plants used in the industry. Unlike previous investigations into the genus, we tried to incorporate every recognized species in our study to develop a comprehensive understanding of the genus. Based on our results we offer a new alignment of 13 species within the genus, and a key for distinguishing them (Table 5.1).

Morphological investigation of 21 taxa of *Astilbe* using a morphological matrix of 28 character states revealed parsimonious trees giving resolution to relationships within the genus. Though bootstrap support was not high for most branches along the tree, taxa that have traditionally been hard to distinguish due to morphological homology aligned. Taxa treated as botanical varieties by some authorities consistently aligned with associated species (Table 5.2).

Molecular investigation of 15 taxa of *Astilbe* using maximum parsimony and maximum likelihood analysis of 1059 bp of *mat*K sequence resulted in 1629 trees with a score of 113 steps. Trees were poorly resolved with seven taxa forming a polytomy and bootstrap support for branches was generally low. A second analysis of 21 cultivated varieties and species of *Astilbe* resulted in 2698 trees with a score of 795 steps. Though relationships were better resolved within this tree and bootstrap support was higher,

relationships within *Astilbe* remained unclear. Investigation using a nuclear gene region and additional plastid sequences is necessary to clearly define relationships within the genus.

Single nucleotide polymorphisms found in *mat*K sequences of *Astilbe* may potentially be used as markers for distinguishing taxa. Specific polymorphic sites could determine the lineages of popular cultivated *Astilbe* taxa and identify a plant to species. To ensure the reliability of these markers, multiple samples of each taxon could be sequenced to determine if the sequence variation is persistent.

When compared, the morphological and molecular analyses of *Astilbe* were similar. Many relationships within the genus were consistent from both data sets. In both analyses proposed, varieties paired as sister taxa to their respective species, including *A. davidii* with *A. chinensis*, *A. glaberrima* with *A. japonica*, *A. koreana* with *A. grandis*, and *A. myriantha* with *A. rivularis*. These results show that distinct variation exists within species populations, and though this variation can be characterized, it may not constitute speciation.

In the final component of this research we investigated variability of micropropagated seedlings of *Astilbe* ×*arendsii*. These seedlings produced microshoots varying in number from five to fourteen and differed in time of emergence, plant size, and flowering. The clones of these seedling families were scored for various characters to identify potential occurrence of somaclonal variation. Using multivariate and cluster analysis, potential somaclones were selected based upon their separation from seedling families within a cluster. Potential somaclones were characterized by dwarf habit, dark green leaves (high chlorophyll content), increased flowering, or larger plant size.

In closing, further anlyses of the relationships within *Astilbe* need to be conducted. We plan to incorporate nuclear gene sequences for molecular analysis to help resolve the phylogeny currently presented by *mat*K sequences. We plan to include the *trn*K intron-flanking region with the current *mat*K data matrix to provide additional variation for resolution of the present phylogeny. Based upon our results, we can develop molecular markers based upon *mat*K sequence polymorphisms for determination of plant origin and lineage.

Next we would like to integrate a geographic component to our molecular and morphological analysis to further strengthen the proposed evaluation of the genus with 13 to 15 species. With regard to our potential somaclonal variants, we would like to confirm the ploidy level of the somaclones and evaluate the plants in the field through a vegetative reproductive cycle.

Table 5.1 Key to *Astilbe* species.

1a. Leaves simple ……..….*A. simplicifolia* Makino
1b. Leaves compound.
2a. Plants dioecious ……...….*A. biternata* Britton
2b. Plants monoecious.
3a. Petals 1-5, obsolete or absent; inflorescences sparsely flowered.
4a. Sepals 5, subcoriaceous, with glandular hairy outside; petals 2-3-5 or absent…….……*A. macrocarpa* Knoll
4b. Sepals 4-5, nearly membranous, glabrous outside; petals 1-(2-3-5), obsolete or absent……...……..*A. rivularis* Buch.-Ham. ex D. Don
3b. Petals 5, ordinary
5a. Inflorescences densely flowered.
6a. Petals linear, sepals glandular ciliate.
7a. Sepals glabrous outside.
8a. Peduncles covered with long, curved brown hairs; leaflets usually short-acuminate to acute at apex ……. *A. chinensis* (Maxim.) Franch.
8b. Peduncles covered with glandular hairs; leaflets usually short-acuminate to acuminate at apex…….. *A. grandis* Stapf. ex Wils.
7b. Sepals glandular-hairy
9a. Hairy only on the outside……..……*A. rubra* Hook. f. et Thoms.
9b. Sepals hairy both sides ……..……...*A. microphylla* Hayata
6b. Petals spatulate.
10a. Sepals without glandular hairs at margin.
11a. Petals retuse at apex, single vein; sepals obtuse at apex, subentire; plant medium-sized, 0.4-1.4 m high…..……*A. longicarpa* (Hayata) Hayata
11b. Petals acute at apex, 4-6 nerved; sepals acute and sparsely dentate at apex; plant small, 15-30 cm high……..……...*A. macroflora* Hayata
10b. Sepals with hairs, membranous at margin.
12a. Leaflets lanceolate and dense with strigose hairs, leaflet margin dense with hairs…..……...……...*A. philippinensis* Henry
12b. Leaflets lanceolate sparse hispid hairs and glossy ...…...*A. japonica* A.Gray
5b. Inflorescences densely flowered, but sparsely branched, petals spatulate, sepal margin entire, leaflets ovate ……...…….*A. thunbergii* (Siebold et Zucc.) Miq

Table 5.2 Table of varieties and their respectives species.

Variety	Species
A. grandis var. *austrosinensis*	*A. grandis*
A. rivularis var. *angustifolia*	*A. rivularis*
A.chinensis var. *davidii*	*A. chinensis*
A. thunbergii var. *formosa*	*A. thunbergii*
A. thunbergii var. *fujisanensis*	*A. thunbergii*
A. japonica var. *glaberrima*	*A. japonica*
A. grandis var. *koreana*	*A. grandis*
A. rivularis var. *myriantha*	*A. rivularis*

Vitae

Brian Wayne Trader

EDUCATION
Ph.D., Horticulture: **July 1, 2006**
Virginia Tech, Blacksburg, VA
Dissertation: Morphological and molecular investigation of *Astilbe.*
Advisor: Dr. Holly Scoggins
M.S. in Life Sciences: Plant Pathology, Physiology, and Weed Science, August 2002
Virginia Tech, Blacksburg, VA.
***Thesis*:** Weed control in cucumber, pumpkin, and summer squash with halosulfuron.
Advisor: Dr. Henry Wilson
B.S., Horticulture: Horticulture Crops, December 2000
Virginia Tech, Blacksburg, VA.

HONORS/
AFFILIATIONS

Future Professorate Graduate Teaching Certificate, Virginia Tech, 2005
Warren S. Barham Award, Southern Region American. Society for Horticultural Science 2004
Horticulture Graduate Student Organization, President 2004 – present
Plant Pathology, Physiology, and Weed Science Graduate Organization, President 2001-2002
Pi Alpha Xi, 1998 - present
Delta Kappa Epsilon, 1997-present

RESEARCH
INTERESTS

DNA sequence analysis for phylogenetics
Plant breeding, cultivar development
Plant tissue culture
Plant pathology
Plant growth regulation
Weed control in nursery, greenhouse, and horticultural field crops

TEACHING
EXPERIENCE

Teaching Assistant, Department of Horticulture, Virginia Tech, Blacksburg, VA
August 2002 - present
Teach the identification of woody landscape plants: instruct laboratories, administer and grade quizzes, advise students, and give help sessions

Taught undergraduate Floriculture Production, prepared and instructed laboratories
Teaching Assistant, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech, Blacksburg, VA
August 2001 - December 2001
Assisted the instruction for undergraduate Pest and Stress Management of Trees laboratory and lecture

RESEARCH EXPERIENCE

Dissertation, Department of Horticulture, Virginia Tech, Blacksburg, VA
August 2002 - present
DNA extraction, cloning, and sequencing
Phylogenetic analysis using molecular and morphological techniques
Somaclonal variation for cultivar development/plant improvement
Plant growth regulation
Research Assistant, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech, Painter, VA
January 2000 - August 2001, December 2001 - August 2002
Investigated the efficacy of various herbicide formulations in the production of several field crops
Examined herbicide-resistant weed populations in greenhouse experiments
Evaluated differential cultivar response to herbicide treatments

INDUSTRY EXPERIENCE

Retail Nusery Employee/Manager, Dennis Garden Center, Wattsville, VA
Summer Seasons June 1997 - August 2001
Managed nursery during weekends
Involved in all aspects of buying, growing, and selling of nursery plants

PRESENTATIONS

Management of weeds in the greenhouse. 2004. Southeastern Greenhouse Conference, Greenville, SC
Regional conference with trade show
Evaluated grower track session with pesticide re-certification credits offered for attendance

Ten most wanted weeds: Virginia Tech Perennials Program. 2004. Green and Growin', Greensboro, NC
Regional conference with trade show
Grower track session for pesticide re-certification credits

Weed management in perennial containers and landscapes. 2003. Virginia Flower Growers Assoc., Blacksburg, VA

Grower association meeting
Part of educational series

PUBLICATIONS

Trader, B. W., H. L. Scoggins, and R. E. Veilleux. Investigation of *Astilbe* using single nucleotide polymorphisms and morphology (in preparation)

Trader, B. W., H. A. Gruszewski, H. L. Scoggins, and R. E. Veilleux. 2006. Somaclonal variation of *Coreopsis* regenerated from leaf explants. Hort Science 41: (in press).

Trader, B. W., H. P. Wilson, and T. E. Hines**.** Weed control in Cucumber (*Cucumis sativus*) and Pumpkin (*Cucurbita maxima*) with Halosulfuron (in preparation)

Trader, B. W., H. P. Wilson, and T. E. Hines**.** Weed Control in Summer Squash (*Cucurbita pepo*) with Halosulfuron (in preparation)

Trader, B. W. 2003. Managing weeds and other pests in the greenhouse In Greenhouse Operators Training Manual 3rd ed., Virginia Flower Growers Association. Joyce Latimer and Holly Scoggins eds.

ABSTRACTS

Primary Author

Trader, B. W., H. L. Scoggins, and R. E. Veilleux. 2005. Somaclonal variation of *Astilbe* microshoots. Southern Nurs. Assoc. Res. Conf. 50: .

Trader, B. W., H. L. Scoggins, and R. E. Veilleux. 2005. Single nucleotide polymorphisms in *mat*K of *Astilbe*. Hort Science 40 (4): 1081.

Trader, B. W., H. L. Scoggins, and R. E. Veilleux. 2004. Potential micropropagation techniques for *Astilbe***.** Southern Nurs. Assoc. Res. Conf. 49: 359-361.

Trader, B. W., H. A Gruszewski, N. I. Barclift, R. E. Veilleux, and H. L. Scoggins. 2004. Somaclonal variation of *Coreopsis grandiflora* 'Domino' regenerated from leaf sections. Hort Science 39 (4): 808.

Trader, B. W., R. E. Veilleux, and H. L. Scoggins. 2004. Development of a micropropagation protocol for *Astilbe*. Hort Science 39: 646.

Trader, B. W., C. M. Whaley, H. P. Wilson, and T. E. Hines. 2002. Smooth pigweed control in cucumber with halosulfuron. Proc. Northeast. Weed Sci. Soc. 56: 138.

Trader, B. W., H. P. Wilson, T. E. Hines, and H. E. Hohlt. 2001. Weed management with halosulfuron in summer squash. Proc. Northeast. Weed Sci Soc. 55: 1.

Trader, B. W., C. M. Whaley, H. P. Wilson, and T. E. Hines. 2000. Weed control in glufosinate tolerant corn. Proc. Northeast Weed Sci. Soc. 54: 125.

Contributing Author

Armel, G. R., B. W. Trader, R. J. Richardson, and T. E. Hines. 2002. ZA-1296 combinations in herbicide-resistant corn. Proc. Northeast. Weed Sci. Soc. 56: 1.

Whaley, C. M., W. A. Bailey, B. W. Trader, H. P. Wilson, and T. E. Hines. 2002. Strategies for horseweed control in full-season no-till soybean. Proc. Northeast. Weed Sci. Soc. 56: 4.

Whaley, C. M., B. W. Trader, H. P. Wilson, and T. E. Hines. 2001. Weed control with chloroacetamide herbicides in corn. Proc. Northeast. Weed Sci. Soc. 55: 1.