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Investigating Parentage and Hybridity of Three Azaleodendrons Using Amplified Fragment Length Polymorphism Analysis

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Abstract. **Morphological analysis historically has been used to determine parentage of unknown hybrids. This can be difficult when potential parents have similar appearance, as in the case of three azaleodendron cultivars, *Rhododendron* L. 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'. These cultivars are similar in name and appearance, and all are purported hybrids of *R. catawbiense* Michx. or *R. ponticum* L. and *R. viscosum* (L.) Torr. Amplified fragment length polymorphism (AFLP) analysis was conducted to determine whether the cultivars are synonyms or distinct clones and to elucidate the parental species. The three cultivars, suspected to be hybrids between taxa in subgenera *Hymenanthes* (Blume) K.Koch (evergreen rhododendrons) and *Pentanthera* (G.Don) Pojarkova (deciduous azaleas), and related taxa from each subgenus were evaluated using 31 AFLP primer combinations. Genetic similarity, calculated using Jaccard's coefficient, among the hybrids ranged from 53% to 71%, indicating that they are distinct cultivars and not a single clone. Genetic similarity was highest between the hybrids and *R. ponticum* among the evergreen rhododendrons, and *R. viscosum* among the deciduous azaleas. A dendrogram generated using the genetic similarity matrix grouped taxa into their respective subgenera, with the three cultivars nested intermediately between subgenera but more closely with subgenus *Hymenanthes* and particularly *R. ponticum*, suggesting it is the evergreen rhododendron parent. Furthermore, principle components grouped *R. ponticum* more closely with the hybrids and there were 18 AFLP fragments unique to *R. ponticum* and the hybrids. However, no unique AFLP bands were shared exclusively among the hybrids and the purported deciduous azalea parent, *R. viscosum*, suggesting that the original azalea parents may have been hybrids.**

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Rhododendron L. is an extremely diverse genus with eight subgenera and more than 1000 species (Chamberlain et al., 1996). This diversity, combined with broad crossability, has led to the development of more than 28,000 cultivars registered with the Royal Horticulture Society (RHS), including 14,298 rhododendrons, 12,989 azaleas, and 108 azaleodendrons (intersubgeneric hybrids between azaleas and rhododendrons) (Leslie, 2002). Thus, this genus is among the most extensively developed and widely grown in the world. Many superior cultivars continue to be used in breeding programs, but are often of unknown parentage.

Three azaleodendron cultivars exist in the trade with similar names: 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'. 'Fragrans' is a purported hybrid of *R. catawbiense* Michx. and *R. viscosum* L. (Torr.) that was introduced by Paxton, of Chandler & Sons Nursery, London, in 1843. It is described as, "A sweet-scented azaleodendron, fast-growing and compact. Trusses of small flowers, pale mauve with centers lighter to white" (Salley and Greer, 1986, p. 110). 'Fragrans Affinity' was found in a group of deciduous azalea seedlings at Greer Gardens, Eugene, Ore., in the 1950s. Harold Greer (pers. comm., May 2004) speculated that it may be a hybrid of *R. ponticum* L. and *R. viscosum* or *R. catawbiense* and *R. viscosum*. The plant was named 'Fragrans Affinity' because of its similarity to 'Fragrans'. The history of 'Fragrant Affinity' is poorly documented. A plant was received in 2000 from the late Dr. A. Kehr, who indicated it was a hybrid between *R. viscosum* and *R. catawbiense*. The name 'Fragrant Affinity' is not registered with the RHS or the American Rhododendron Society, and the ultimate origin of this material is unknown. Although 'Fragrant Affinity' is sterile, an allotetraploid form of 'Fragrant Affinity', named 'Fragrant Affinity Tetra', was developed that exhibited restored fertility (Contreras et al., 2007). All three are semievergreen and closely resemble the description provided by Salley and Greer (1986) of 'Fragrans' (pers. obs.).

The suspected parents of these azaleodendrons are distinct taxonomically and are classified in separate subgenera. *Rhododendron catawbiense* and *R. ponticum* are in subgenus *Hymenanthes* (Blume) K.Koch, section *Ponticum* G.Don, subsection *Pontica* Sleumer. This subsection contains evergreen species from North America, Europe, and Asia, including *R. hyperythrum* L. and *R. maximum* L. *Rhododendron viscosum* is in subgenus *Pentanthera* (G.Don) Pojarkova, section *Pentanthera* G.Don. This section contains other fragrant, deciduous species from North America including *R. arborescens* (Pursh) Torrey, *R. atlanticum* (Ashe) Rehd., *R. canescens* (Michx.) Sweet, and *R. periclymenoides* (Michx.) Shinners (Chamberlain et al., 1996).

Parentage of these azaleodendrons is difficult to determine because these hybrids were not the result of controlled pollinations. Furthermore, use of traditional morphological comparison to clarify parentage is complicated because of the number of species that could potentially be involved and the similarity of traits among species within each subgenus. Molecular techniques have been used widely to assess genetic relationships among plants. The amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995; Zabeau and Vos, 1993), in particular, has been used by many scientists to distinguish between species as well as cultivars of the same species (DeHaan et al., 2003; Mellish et al., 2002; Paul et al., 1997; Perera et al., 1998; Zhang et al., 2000). Because of the inclusion of a restriction endonuclease digestion step, AFLPs have the advantage of being highly reproducible in comparison with random amplified polymorphic DNA markers. Milla et al. (2005) reported that

reproducibility of markers used in a study of *Arachis* L. germplasm ranged from 96% to 100%. Also, AFLP analysis has a very high resolution, requires no prior knowledge of the genomes being studied (Vos et al., 1995), and has the capacity to screen simultaneously for many DNA regions distributed throughout the genome, thus producing hundreds of genetic markers (Mueller and Wolfenbarger, 1999).

Amplified fragment length polymorphism markers have been used to confirm hybridity in a number of plants. Teo et al. (2002) confirmed the hybrid status of *Mangifera odorata* Griff. Kiew et al. (2003) assessed the hybrid status of four genera of Malesian plants. Beismann et al. (1997) differentiated between *Salix alba* L., *S. fragilis* L., and their hybrid *S.* ×*rubens* Schrank when morphological analysis was determined to be inconclusive.

The objectives of this study were 1) to elucidate the progenitor species of 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'; and 2) to determine whether these cultivars are all distinct clones or possibly synonyms.

Materials and Methods

Plant material. Genotypes evaluated in this study included *Rhododendron* cultivars 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'; putative parents; and related taxa. Putative parental and related taxa included *R. catawbiense*, *R. ponticum*, *R. maximum*, and *R. hyperythrum* from subgenus *Hymenanthes;* and *R. viscosum*, *R. arborescens*, *R. canescens* 'Varnadoes Phlox Pink', and 'Marydel' (*R. atlanticum* × *R. periclymenoides*) from subgenus *Pentanthera*. *Kalmia latifolia* L. 'Sharon Rose' was used as an outgroup. Plants were grown in 11.4-L containers with a pine bark medium amended with 0.59 kg·m^{-3} dolomitic lime and 1.0 kg·m^{-3} micronutrient blend (Micromax, Scotts, Marysville, Ohio) under 50% shade and fertilized using 17N–7.4P–14.1K controlled-release fertilizer (Multicote, Vicksburg Chemical Co., Vicksburg, Mo.). Plant material was maintained at the Mountain Horticultural Crops Research Station (Fletcher, N.C.) and the J.C. Raulston Arboretum (JCRA; Raleigh, N.C.; Table 1). Plants maintained at JCRA were grown in display beds.

Amplified fragment length polymorphism analysis. Total genomic DNA was extracted using a cetyltrimethyl–ammonium bromide extraction method described by Stein et al. (2001), using ≈100 to 200 mg tissue from newly opening leaves. Amplified fragment length polymorphism analysis was conducted using the protocol described by Milla et al. (2005) with 31 primer combinations (Table 2).

Data analysis. The AFLP-Quantar 1.0 (Keygene Products B.V., Wageningen, the Netherlands) software package was used to score distinct, major, unambiguous bands. Presence or absence of each AFLP fragment was scored as a binary unit character (present, 1; absent, 0). Jaccard's coefficient of similarity was calculated using the SIMQUAL function of NTSYSpc 2.1 software (Exeter Software, Setauket, N.Y.) and subsequently used to construct a dendrogram using the unweighted pair group method with arithmetic averages. Principle component analysis was based on the variance–covariance matrix of the data using the PRINCOMP function of SAS 9.1 software (SAS Institute, Cary, N.C.). SigmaPlot 9.0 (Systat Software, Richmond, Calif.) was used to create a scatter plot of the first three principle components.

Table 1. Taxa used in amplified fragment length polymorphism analysis to elucidate parentage and hybridity of *Rhododendron* 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'.

Taxa	Subgenus[z]	Location[y]	Accession
Rhododendron 'Fragrans'	—	MHCRS	2005–235
R. 'Fragrans Affinity'	—	MHCRS	2005–218
R. 'Fragrant Affinity'	—	MHCRS	2000–189
R. arborescens	*Pentanthera*	MHCRS	2004–115
R. catawbiense	*Hymenanthes*	MHCRS	2005–242
R. hyperythrum	*Hymenanthes*	MHCRS	2006–029
R. maximum	*Hymenanthes*	MHCRS	2005–243
R. ponticum	*Hymenanthes*	MHCRS	2004–076
R. ponticum	*Hymenanthes*	MHCRS	2005–217
R. viscosum 'Roseum'	*Pentanthera*	MHCRS	2004–219
R. viscosum	*Pentanthera*	MHCRS	2004–116
R. canescens 'Varnadoes Phlox Pink'	*Pentanthera*	JCRA	950316
R. 'Marydel'[x]	*Pentanthera*	JCRA	040705
Kalmia latifolia 'Sharon Rose'	—	JCRA	—[w]

[z]Species in subgenus *Pentanthera* are deciduous azaleas and species in subgenus *Hymenanthes* are evergreen rhododendrons.
[y]Mountain Horticultural Crops Research Station (MHCRS), Fletcher, N.C., and J.C. Raulston Arboretum (JCRA), Raleigh, N.C.
[x]Hybrid of *R. atlanticum* × *R. periclymenoides*.
[w]No accession number available; plant located in bed L14.

Table 2. Thirty-one primer combinations used in amplified fragment length polymorphism analysis of *Rhododendron catawbiense*, *R. ponticum*, *R. maximum*, *R. hyperythrum*, *R. viscosum*, *R. arborescens*, *R. canescens* 'Varnadoes Phlox Pink', *R.* 'Marydel' (*R. atlanticum* × *R. periclymenoides*), and *Kalmia latifolia* 'Sharon Rose', and the number of polymorphic bands scored for each combination.

Primer combination	Bands scored (n)
E+AAC/M+CTA	4
E+AAC/M+CTC	6
E+AAC/M+CTG	6
E+AAC/M+CTT	4
E+AAG/M+CTA	8
E+AAG/M+CTC	7
E+AAG/M+CTG	6
E+AAG/M+CTT	6
E+ACC/M+CGA	1
E+ACC/M+CGC	4
E+ACC/M+CGT	2
E+ACG/M+CTA	5
E+ACG/M+CTC	2
E+ACG/M+CTG	4
E+ACG/M+CTT	2
E+ACT/M+CTA	6
E+ACT/M+CTC	7
E+ACT/M+CTG	4
E+ACT/M+CTT	4
E+ATC/M+CTA	5
E+ATC/M+CTC	5
E+ATC/M+CTG	6
E+ATC/M+CTT	3
E+ATG/M+CGA	5
E+ATG/M+CGC	4
E+ATG/M+CGG	1
E+ATG/M+CGA	6
E+ATT/M+CTA	10
E+ATT/M+CTC	10
E+ATT/M+CTG	6
E+ATT/M+CTT	7
Total	152

Results

Amplified fragment length polymorphism analysis. The 31 primer combinations used generated a total of 152 useful AFLP polymorphisms ranging in size from 107 to 614 bp. Only bands that were unambiguous were scored and used for analysis. The number of scored polymorphic bands generated by each primer combination ranged from 1 to 10 (mean, 5). A total of 18 polymorphic bands specific only to *R. ponticum* and the hybrids were observed. No polymorphic bands specific only to *R. viscosum* and the hybrids were observed.

Genetic similarity matrix and cluster analysis. The pairwise genetic similarities ranged from 0.04 to 0.72 (mean, 0.25; Table 3). Genetic similarities among the hybrids ranged from 53% to 71%, indicating they are distinct cultivars and not a single clone. The degree of similarity for the hybrids was highest with *R. ponticum* (53% to 61%) among the evergreen rhododendrons, and with *R. viscosum* (26% to 48%) among the deciduous azaleas. The dendrogram generated from the similarity matrix (Fig. 1) had subgenera nested in accordance with recognized taxonomic groups (Chamberlain et al., 1996). The upper branch contained the evergreen rhododendrons from subgenus *Hymenanthes* with the exception of *R. ponticum*. *Rhododendron ponticum* was nested within the same clade as the three azaleodendron cultivars, suggesting that it was a parent. The clade below the hybrids contained the deciduous azaleas from subgenus *Pentanthera*, with the exceptions of 'Marydel' and *R. arborescens*. *Kalmia latifolia* 'Sharon Rose' was most distantly related and formed the rooting branch.

Table 3. Genetic similarity matrix based on Jaccard's coefficient of similarity of the 14 taxa evaluated to elucidate parentage and hybridity of *Rhododendron* 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'.

Species	*R. catawbiense*	*R. maximum*	*R. hyperythrum*	*R. ponticum* 2005–217	*R. ponticum* 2004–076	*R.* 'Fragrans Affinity'	*R.* 'Fragrant Affinity'	*R.* 'Fragrans'	*R. viscosum* 2004–116	*R. viscosum* 'Roseum'	*R. arborescens*	*R.* 'Marydel'	*R. canescens* 'VPP'[z]	*Kalmia latifolia* 'SR'[y]
R. catawbiense	1.00													
R. maximum	0.36	1.00												
R. hyperythrum	0.55	0.41	1.00											
R. ponticum 2005–217	0.43	0.30	0.43	1.00										
R. ponticum 2004–076	0.35	0.26	0.39	0.72	1.00									
R. 'Fragrans Affinity'	0.31	0.25	0.37	0.58	0.53	1.00								
R. 'Fragrant Affinity'	0.37	0.27	0.40	0.61	0.58	0.53	1.00							
R. 'Fragrans'	0.31	0.21	0.35	0.55	0.53	0.53	0.71	1.00						
R. viscosum 2004–116	0.16	0.12	0.16	0.14	0.20	0.26	0.33	0.38	1.00					
R. viscosum 'Roseum'	0.28	0.23	0.25	0.26	0.24	0.37	0.44	0.48	0.57	1.00				
R. arborescens	0.06	0.06	0.06	0.09	0.09	0.11	0.13	0.16	0.24	0.17	1.00			
R. 'Marydel'	0.16	0.14	0.15	0.10	0.15	0.15	0.22	0.23	0.28	0.29	0.14	1.00		
R. canescens 'VPP'[z]	0.23	0.23	0.25	0.19	0.19	0.27	0.26	0.28	0.31	0.35	0.14	0.20	1.00	
Kalmia latifolia 'SR'[y]	0.14	0.09	0.12	0.06	0.07	0.11	0.07	0.11	0.11	0.16	0.04	0.09	0.12	1.00

[z]*R. canescens* 'Varnadoes Phlox Pink'.
[y]*Kalmia latifolia* 'Sharon Rose'.

Principle component analysis. Principle components one, two, and three accounted for 22%, 16%, and 15% of the variance observed between all samples respectively, for a total of 53% of the observed variation (Fig. 2). A plot of the first principle component separated the taxa into two groups. In one group were the deciduous azaleas and *K. latifolia* 'Sharon Rose'. The second group contained the hybrids and the evergreen rhododendrons. Addition of the second principle component resulted in a separation of the hybrids and the evergreen rhododendrons. The third component separated *K. latifolia* 'Sharon Rose' from the deciduous azaleas. Also, it more closely allied the *R. arborescens* and 'Marydel' group with the other deciduous azaleas, in contrast to what was observed in the dendrogram (Fig. 1). The taxonomic groups were well defined with the exception of the two accessions of *R. ponticum* (Fig. 2).

Discussion

The current study demonstrates that the AFLP technique can be used to elucidate information about the parentage and hybridity of cultivars in the genus *Rhododendron*. Results of the molecular analysis provided strong evidence that *R. ponticum*, not *R. catawbiense*, was most likely the evergreen rhododendron parent of 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'. Genetic similarities among the three azaleodendron cultivars ranged from 53% to 71%, indicating that the three hybrids are each distinct cultivars, not a single clone.

Amplified fragment length polymorphism data supported the hypothesis that *R. ponticum* was a parent of the three cultivars. Genetic similarity with the hybrids was highest with *R. ponticum* among the evergreen rhododendrons, ranging from 53% to 61% genetically similar. The other species ranged from 21% to 37% similarity with the hybrids. Cluster analysis nested the three azaleodendrons with subgenus *Hymenanthes*. Of species in this group they were most closely allied with *R. ponticum*. Also, principle component analysis grouped the hybrids more closely with *R. ponticum* than any other species included. Furthermore, there were 18 species-specific markers unique to *R. ponticum* and the hybrids, providing very strong evidence that it is a parent.

Data on the deciduous azalea parent was less conclusive. *Rhododendron viscosum* had the highest coefficient of similarity with the hybrids (26% to 44%) among deciduous azaleas, but there were no markers specific to *R. viscosum* that were also common to the hybrids. Deciduous azaleas in *Pentanthera* hybridize freely in cultivation and in the wild, thus it often is difficult to differentiate between species and hybrids (Towe, 2004). It is probable that a hybrid azalea was a parent in these azaleodendron crosses. Considering that the hybrids have fragrant flowers of pale lavender, the potential candidates from the deciduous azaleas are somewhat

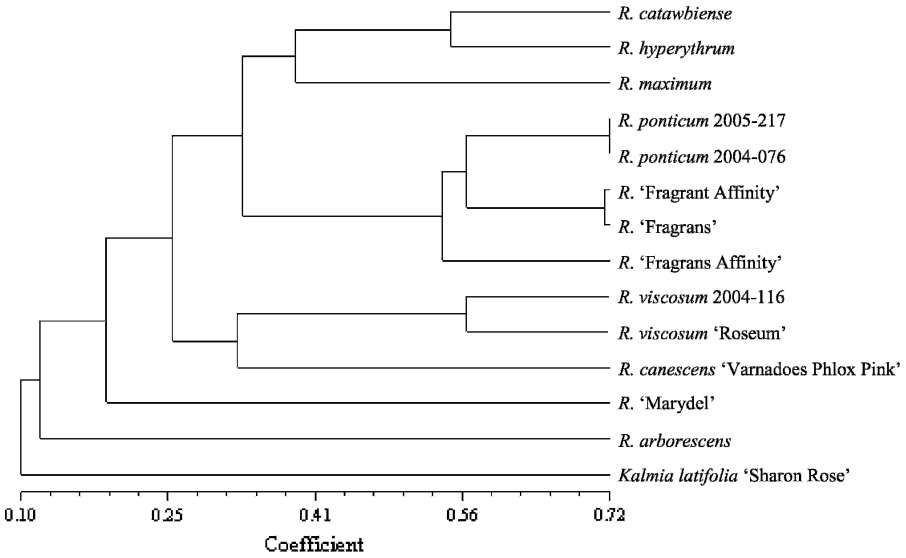

Fig. 1. Dendrogram created using the unweighted pair group method with arithmetic averages based on Jaccard's coefficient of similarity of the 14 taxa evaluated to elucidate parentage and hybridity of *Rhododendron* 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'. *R.* 'Marydel' is a hybrid of *R. atlanticum* and *R. periclymenoides*.

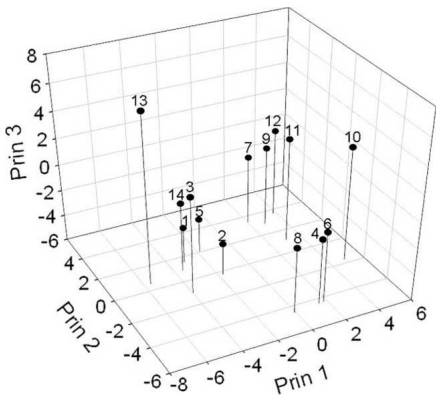

Fig. 2. Plot of first three principle components based on the variance–covariance matrix of the data using the 14 taxa evaluated to elucidate parentage and hybridity of *Rhododendron* 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'. 1, *R. viscosum;* 2, *R. canescens* 'Varnadoes Phlox Pink'; 3, *R. arborescens;* 4, *R. hyperythrum;* 5, *R. viscosum* 'Roseum'; 6, *R. catawbiense;* 7, *R.* 'Fragrans'; 8, *R. maximum;* 9, *R.* 'Fragrant Affinity'; 10, *R. ponticum* 2005–217; 11, *R. ponticum* 2004–076; 12, *R.* 'Fragrans Affinity'; 13, *Kalmia latifolia* 'Sharon Rose'; 14, *R.* 'Marydel' (*R. atlanticum* × *R. periclymenoides*).

limited. *Rhododendron luteum* (L.) Sweet and *austrinum* (Small) Rehd., for instance, are unlikely because of their brilliant yellow flower color. The limited number of species that could have contributed the morphological traits seen in the hybrids, their overlapping distribution, and their propensity for natural hybridization supports the hypothesis that the deciduous azalea parent is a hybrid of species included in the study.

The current study provided evidence that these three azaleodendron cultivars are intersubgeneric hybrids between an evergreen rhododendron and a deciduous azalea. All three azaleodendrons were nested between subgenus *Hymenanthes* and subgenus *Pentanthera* in the dendrogram. Confirmation that wide hybridization is possible may encourage more rhododendron breeders to attempt intersubgeneric hybridization to develop novel cultivars with traits from diverse taxa. The AFLP technique has proved to be a useful tool in determining parentage of rhododendron cultivars of uncertain origin. Elucidating parentage of the numerous superior cultivars of unknown parentage will allow breeders to use available germplasm more effectively. In contrast to previous literature, it is clear that *R. ponticum*, not *R. catawbiense*, was the evergreen rhododendron parent of the three azaleodendrons, but additional analysis would be necessary to determine better the deciduous azalea parents.

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