ABSTRACT

CONTRERAS, RYAN NELSON. Azaleodendrons: Investigating Parentage, Fertility, and Effects of Polyploidy among Hybrids of Deciduous Azaleas and Evergreen Rhododendrons. (Under the direction of Dr. Thomas G. Ranney.)

Studies were conducted in order to develop a fertile form of the azaleodendron cultivar *Rhododendron* L. 'Fragrant Affinity' and determine parentage of this and two other azaleodendron cultivars. Wide hybridization can potentially lead to recombination of diverse traits and creation of unique phenotypes, but these hybrids are often sterile as is the case with the inter-subgeneric hybrid *R.* 'Fragrant Affinity'. Sterility in wide hybrids can either be genic or chromosomal; the latter may be overcome by induction of polyploidy which can restore chromosome homology and fertility. Initial cytological studies of 'Fragrant Affinity' showed bridges between bivalents in metaphase I and laggard chromosomes in anaphase I. In this study we successfully developed an allotetraploid form of *R.* 'Fragrant Affinity' using oryzalin (4-(dipropylamino)-3, 5-dinitro-benzenesulfonamide) as a mitotic inhibitor and chromosomal doubling agent. Genome sizes (2C) were determined using flow cytometry and found to be approximately 1.6 and 3.2 pg for the diploid and allotetraploid, respectively. Pollen viability, determined by staining and germination tests, was 4% and 0%, respectively for the diploid and 68% and 45%, respectively for the allotetraploid. No seeds were produced when the diploid *R.* 'Fragrant Affinity' was used as a maternal parent when crossed with pollen from viable diploid and tetraploid parents. The allotetraploid produced viable seeds and seedlings when crossed with pollen from either diploid or tetraploid parents, including self pollination, demonstrating restored male and female fertility. Additional crosses were successfully completed using the allotetraploid as part of an ongoing breeding program to develop new fragrant, cold hardy, evergreen rhododendron. Morphological

analysis has historically been used to determine parentage of unknown hybrids. This can be difficult when the purported parental taxa have very similar morphology as in the case of the three azaleodendron cultivars *R.* 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'. The three cultivars are purported hybrids of *R. catawbiense* Michx. or *R. ponticum* L. and *R. viscosum* (L.) Torr., the three cultivars are very similar morphologically as are the purported parents. In this study, morphological and AFLP analyses were conducted to determine if these are unique cultivars or clones and to elucidate the parents. The three cultivars, potential parents from 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. Morphological comparison indicated that *R. ponticum* is likely the evergreen rhododendron parent of *R.* 'Fragrant Affinity' based on intermediate petiole and leaf blade lengths and floral characteristics. Genetic similarity calculated using Jaccard's coefficient of similarity was highest between the hybrids and *R. ponticum* among the evergreen rhododendrons and *R. viscosum* among the deciduous azaleas, respectively. Genetic similarities among the three azaleodendrons indicated that they are unique cultivars and not synonyms, but likely share the same parental species. A dendrogram generated using the genetic similarity matrix grouped taxa into generally accepted taxonomic groups and nested *R. ponticum* with the hybrids suggesting it is the evergreen rhododendron parent. Additionally, a plot of the first three principle components also showed *R. ponticum* to be grouped more closely with the hybrids. Furthermore, there were 18 fragments unique to *R. ponticum* and the hybrids, however no unique bands were found for the purported deciduous azalea parent, suggesting the original parent may have been a hybrid.

AZALEODENDRONS: INVESTIGATING PARENTAGE, FERTILITY, AND EFFECTS OF POLYPLOIDY AMONG HYBRIDS OF DECIDUOUS AZALEAS AND EVERGREEN RHODODENDRONS

by
RYAN NELSON CONTRERAS

A thesis submitted to the Graduate Faculty of
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APPROVED BY:

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Dr. Thomas G. Ranney
Chair of Advisory Committee

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Dr. Dennis J. Werner Dr. G. Craig Yencho

DEDICATION

In loving memory of the greatest person

I shall ever know.

Anything I am is because of you.

I love you S.A.M.

To:

My mother
Carol English Contreras
1943-2005

Biography

Ryan N. Contreras was born in Morehead City, North Carolina on August 20, 1978. He was raised in the small farming community of Harlowe. He attended Beaufort Elementary and Middle Schools and East Carteret High School, graduating in 1996.

At this time he began his collegiate education at NC State University, majoring in Agriculture Education and Extension with a concentration in Horticultural Science. After taking his first Horticulture class in 1997 he transferred into the Department of Horticultural Science. In the summer of 1999, he conducted an internship at the Center for the Study of Early Events in Photosynthesis at Arizona State University under the direction of Dr. Kenneth J. Hoober. From 2000-2002 Ryan took a hiatus from college and worked as a landscaper. In 2002 he returned to NC State and finished his B.S. in Horticultural Science. During his last semester he conducted an independent study project with Dr. Dennis J. Werner working on the cytology of *Buddleia* ×*weyeriana*. Ryan conducted this research in the lab of Dr. Shyamalrau Tallury, Senior Researcher in Crop Science at NC State. After finishing the project Ryan stayed on with the Peanut Genetics project.

In 2004 Ryan began working toward his M.S. degree at NC State under the direction of Dr. Thomas G. Ranney at the Mountain Horticultural Crops Research and Extension Center. While conducting his thesis research Ryan spent summers in Fletcher, N.C. and semesters in Raleigh, N.C. After graduation he will begin work on his Ph.D. degree at University of Georgia under the direction of Dr. Jon Ruter. He will spend the three semesters in Athens on main campus and the last two years in Tifton conducting his dissertation research.

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Before I begin I would like to apologize to any friends or family I omit, there are so many people who have helped me along the way I can't possibly recognize you all here, but rest assured that your help and friendship were not taken for granted. I would like to begin this bittersweet process of saying goodbye to the Department of Horticultural Science at NC State by thanking my advisor, Dr. Tom Ranney. I am honored to have been one of your graduate students. I appreciate you taking a chance on me even though I had a few abysmal grades during my undergraduate career. It has been a pleasure working with you and I value you as a mentor and friend. There are few things I have enjoyed more in my entire life than sitting on the patio at the Ranney house with the rest of the research group, having a cold beverage, and talking shop. Thanks to the rest of my committee: Drs. Denny Werner, Craig Yencho, and Shyam Tallury. Denny, I credit you for giving me a challenging project to work on as a senior and introducing me to cytology and Tallury. My career path would likely be quite different without your guidance. Craig, I can always count on you to challenge my assumptions when I come to your office to discuss the project and for that I thank you. Tallury, you were a great boss and are a great friend and cytological mentor. I appreciate the opportunity to work with you and I value your friendship. To you all, it was an honor and a pleasure to work with you.

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Thank you to all graduate students that I served with. I learned as much from you as any of my classes. Brian Krug, you've been a good friend, a worthy opponent at the showdown, and I look forward to a great career from you. Carrie Judge, you were the first one that I went and had a beer with in the grad office. You have been a confidant, friend, and role model. Jim Owen, if ever there was a driven soul you are it. I measure my enthusiasm and passion for my work by you. Richard Olsen, you have been like an in-office mentor to me. Working on my M.S. would have been a lot harder and certainly a lot less fun without working along side you. You gave me something to shoot for. I thank you all for giving me an exemplary example of graduate researchers and for your friendship.

I'd like to thank the staff of the Mountain Horticultural Crops Research and Extension Center. Tom Eaker, Joel Mowrey, and Nathan Lynch were invaluable in my time at Fletcher. I've said it before and I'll say it again; you guys make us look good. I thank you for keeping my plants alive, counting those tiny rhododendron seeds and being good company.

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I would like to thank my extended Southern Style family. To Margaret: you have always been like a mother to me. Buddy, no one can tell me that I don't know what an honest day of work means. Dwain, Kay, and Rae: you've been like another brother and sisters to me.

Charlotte and Calleiope, the women in my life. They say that behind every great man there is an even greater woman. I don't think of myself as great but your support during this process has meant the world to me. I know that I was not always in the greatest spirits and I thank you for putting up with that Charlotte. Calleiope, you have been with me from the very beginning. You don't have a lot to say but what you do is profound. I could not have done it without you. I love you both.

To my father Ron: you have instilled in me the sense of honor and responsibility that all fathers hope their sons will have. We are not close physically but I try to keep in mind the morals that you hold in high regard. Christian: your intelligence is intimidating sometimes but I use it to push myself. Success is twice as sweet when I get to tell my big brother about it.

Finally, to my mother Carol: you were the embodiment of all that is good and true. You taught me to laugh, love, and cry. You taught me to have compassion for others and pride in myself. I love you.

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GENERAL INTRODUCTION

Rhododendrons and azaleas are among the most widely grown garden plants in the world. The genus *Rhododendron* L. is large and comprised of over 1,000 species separated into 8 subgenera (Chamberlain et al., 1996) within the tribe Rhodoreae DC. ex Duby of Ericaceae Sweet (Stevens, 1971). The eight subgenera are *Azaleastrum* Planch. (evergreen azaleas from China and Taiwan), *Cadidastrum* Franch. (*R. albiflorum* Hook.), *Hymenanthes* (Blume) K.Koch (elepidote or non-scaly rhododendrons), *Mumeazalea* (Sleumer) W.R.Philipson & M.N.Philipson (*R. semibarbatum* Maxim.), *Pentanthera* (G.Don) Pojarkova (deciduous azaleas), *Rhododendron* L. (lepidote or scaly leaved rhododendrons), *Therorhodion* (Maxim.) A.Gray (*R. camtschaticum* Pall. and *R. redowskianum* Maxim.), and *Tsustusi* (Sweet) Pojarkova (evegreen azaleas from Japan, China, and Taiwan). The base chromosome number is 13 and most species are diploid ($2n = 2x = 26$), though natural polyploids exist and include triploids ($2n = 3x = 39$), tetraploids ($2n = 4x = 52$), hexaploids ($2n = 6x = 78$), octaploids ($2n = 8x = 104$), and dodecaploids ($2n = 12x = 156$) (Ammal, 1950; Ammal et al., 1950).

The natural range of rhododendrons extends from the equator to above 60 ºN (Cox, 1993) and throughout the northern hemisphere. The greatest concentration of species occurs on the eastern Himalayas and southeastern Tibet, and in the mountain ranges that form the archipelago extending between mainland Asia and Australia, the latter consisting mainly of tropical species (subgenus *Rhododendron* L. section *Vireya* (Blume) Copel.f. (Cullen, 2005; Irving and Hebda, 1993). Rhododendrons are also found in northeastern Asia, northeastern Turkey, across the sub-arctic zone, and across North America (Irving and Hebda, 1993).

Within the United States there are 26 native rhododendron species in 37 states (USDA, 2006).

There is a long history of hybridization of rhododendrons, beginning in England with the work of the Waterers who developed the "ironclads hybrids", predominantly using *R. catawbiense* L. as a parent for its cold tolerance (Livingston and West, 1978). Breeding in the US began in the 1920s with Charles Dexter, Joseph Gable, and Benjamin Morrison. Dexter worked mainly in subgenus *Hymenanthes* (Blume) K.Koch section *Ponticum* G.Don using species such as *R. decorum* Franch., *R. fortunei* Lindl., and *R. auriculatum* Hemsl. in attempts to develop fragrant cultivars with novel flower color (Leonard, 1993; Livingston and West, 1978). Gable also worked in subgenus *Hymenanthes*, producing his most famous cultivar, *R.* 'Cadis'. Gable is perhaps better remembered for his early work in subgenus *Tsutsusi* (Sweet) Pojarkova, section *Tsutsusi* Sweet using *R. kaemferi* Planch. and *R. poukhanense* H.Lev. (syn. *R.* ×*transiens* Nakai) as a basis for many of his crosses. Morrison's legacy is the Glenn Dale azaleas. Morrison concentrated much of his efforts in subgenus *Tsutsusi* to develop azaleas for the mid-atlantic region that were evergreen, of dense habit, slow growing, had abundant flowers, and generally well adapted to the region (Livingston and West, 1978).

These early hybridizers concentrated their efforts on intrasectional crosses. However, intersectional and intersubgeneric crosses are possible. The first reported interspecific hybrid within the genus was the azaleodendron *R.* 'Azaleoides' (*R. periclymenoides* (Michx.) Shinners x *R. ponticum* L.) (Hillier, 2002). As of 2002, there were 108 azaleodendrons, hybrids between elepidote rhododendrons (*Hymenanthes*) and deciduous azaleas (*Pentanthera*), registered with the Royal Horticulture Society (Leslie, 2002). These cultivars

are mostly novelties sought after by rhododendron enthusiasts as opposed to superior cultivars for the average gardener. This is largely due to the fact that these individuals are a dead end in breeding. Wide hybridization (intergeneric or inter-subgeneric) often results in sterile progeny due to differences in parental chromosomes. Induced polyploidization offers the potential to restore fertility in these wide hybrids (Hadley and Openshaw, 1980).

Another issue that presents difficulty to modern rhododendron breeders is the lack of information on parentage of many of the superior cultivars in the trade. This creates difficulties for breeders trying to develop breeding strategies. Historically, morphological taxonomy has been used to elucidate parents. In rhododendrons so much hybridization has taken place between closely related (intrasectional) taxa that it is difficult to accurately determine parents of hybrids. Molecular techniques such as amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993) can distinguish between species and can be used to determine parentage (Beismann et al., 1997).

This thesis presents the results of a studies to restore fertility in the sterile azaleodendron *R*. 'Fragrant Affinity' by doubling the chromosome complement and elucidate the parentage and hybridity of three azaleodendrons; 'Fragrant Affinity', 'Fragrans', and 'Fragrans Affinity' using AFLP analysis.

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Chapter 1

Reproductive Behavior of Diploid and Allotetraploid *Rhododendron* L. 'Fragrant Affinity'

(In the format appropriate for submission to HortScience

Reproductive Behavior of Diploid and Allotetraploid *Rhododendron* L. 'Fragrant Affinity'

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Reproductive Behavior of Diploid and Allotetraploid *Rhododendron* L. 'Fragrant Affinity'

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Abstract. Wide hybridization can lead to recombination of diverse traits and creation of unique phenotypes, but the resultant hybrids are often sterile as is the case with the inter-subgeneric hybrid *Rhododendron* L. 'Fragrant Affinity'. Sterility in wide hybrids can either be genic or chromosomal; the latter may be overcome by induction of polyploidy which can restore chromosome homology and fertility. Cytological studies of 'Fragrant Affinity' showed bridges between bivalents in metaphase I, and laggard chromosomes in anaphase I. In this study we developed an allotetraploid form of *R.* 'Fragrant Affinity' using oryzalin (4-(dipropylamino)-3, 5-dinitro-benzenesulfonamide) as a mitotic inhibitor and chromosomal doubling agent. Genome sizes (2C) were determined using flow cytometry and found to be approximately 1.6 and 3.2 pg for the diploid and allotetraploid, respectively. Pollen viability, determined by staining and germination tests, was 4% and 0%, respectively for the diploid and 68% and 45%, respectively for the allotetraploid. No seeds were produced when the diploid *R.* 'Fragrant Affinity' was used as a maternal parent when crossed with pollen from viable diploid and tetraploid parents. The allotetraploid produced viable seeds and seedlings when crossed with pollen from either diploid or tetraploid parents, including self pollination, demonstrating restored male and female fertility. Additional crosses were successfully

completed using the allotetraploid as part of an ongoing breeding program to develop new fragrant, cold hardy, evergreen rhododendron.

Rhododendrons and azaleas (*Rhododendron* L.) are among the most widely grown ornamental plants in the world. There are over 1,000 species recognized in eight subgenera (Chamberlain et al., 1996). Species within subgenera readily hybridize and have resulted in thousands of cultivars (Väinölä, 2000). Although intra-subgeneric hybridization is responsible for the majority of existing cultivars, species in different subgenera are sometimes capable of hybridizing.

Azaleodendrons are hybrids between deciduous azaleas (subgenus *Pentanthera* (G.Don) Pojarkova) and non-scaly leaved rhododendrons (subgenus *Hymenanthes* (Blume) K.Koch) and constitute some of the oldest hybrids within the genus. The first interspecific hybrid rhododendron reported was 'Azaleoides', resulting from a chance cross between *Rhododendron periclymendoides* (Michx.) Shinners and *R. ponticum* L. in London circa 1820 (Hillier Nurseries, 2002). Such hybrids have the potential to combine the fragrance of the deciduous azaleas with darker flower colors, larger flower size and persistent foliage of evergreen rhododendrons. One such hybrid with breeding potential is *Rhododendron* 'Fragrant Affinity'. 'Fragrant Affinity' is an azaleodendron with semi-evergreen foliage, vigorous growth, good cold-hardiness (–26 °C), and fragrant, lavender flowers (personal observations). This inter-subgeneric hybrid, believed to be a cross between *R. ponticum* and *R. viscosum* (L.) Torrey (Contreras et al., 2006), possesses unique attributes that are desirable for breeding and development of superior, cold-hardy, fragrant azaleodendrons. Unfortunately, like many other wide hybrids, it appears to be sterile.

Hybrid sterility, referred to as chromosomal sterility or chromosomal hybrid sterility, often results from improper chromosome pairing during gametogenesis due to meiotic abnormalities such as univalents, lagging chromosomes or simply due to structural

differences in the parental chromosomes; however, other mechanisms may also be involved in hybrid sterility (Lu and Bridgen, 1997). In a study using a hybrid of *Alstroemeria aurea* Graham x *A. caryophyllaea* Jacq., Lu and Bridgen (1997) determined that sterility resulted from complex fertility/sterility-regulating mechanisms, not simply due to parental chromosome differences. In cases where sterility is caused by improper chromosome pairing, doubling the chromosome complement (polyploidization) of sterile hybrids to produce allotetraploids provides a homologue for each chromosome to pair with during meiosis and can allow for the development of fertile gametes (Hadley and Openshaw, 1980; Lu and Bridgen, 1997; Stebbins, 1950; van Tuyl and De Jeu, 1997; Zadoo et al., 1975).

Natural polyploids exist in the genus *Rhododendron* including triploids ($2n = 3x = 39$), tetraploids ($2n = 4x = 52$), hexaploids ($2n = 6x = 78$), octaploids ($2n = 8x = 104$) and dodecaploids ($2n = 12x = 156$) (Ammal, 1950; Ammal et al.,1950). Artificial polyploid rhododendrons have also been developed to increase ornamental characteristics such as flower size and texture, extending time of flowering, producing more compact plants, and to facilitate crosses not possible at the diploid level (Eiselein, 1994; Kehr, 1996a; Kehr, 1996b; Pryor and Frazier, 1968; Tolstead and Glencoe, 1991; Väinölä, 2000). Polyploid rhododendrons have been induced with various chemical doubling agents including colchicine (Kehr, 1996a) and oryzalin (Väinölä, 2000).

The objective of this study was to evaluate fertility of diploid and allotetraploid forms of *R.* 'Fragrant Affinity' and to determine if induced polyploidy is an effective method for restoring fertility in hybrids of distantly related rhododendrons.

Materials and Methods

Plant Material. A single plant of *Rhododendron* 'Fragrant Affinity' was received from Dr. August Kehr (USDA-ARS Researcher, Beltsville, Md., 1958-1978) in 2000. Semi-hardwood cuttings were taken in late summer, treated with 5,000 ppm K-IBA, set in 1 peat : 1 perlite (by volume) and placed in a chamber with intermittent mist at a rate of 10 s every 10 min. After rooting, plants were grown in pine bark medium amended with 0.59 $kg \cdot m^{-3}$ dolomitic lime and 1.0 $kg \cdot m^{-3}$ micronutrient blend (Micromax, Scotts, Marysville, Ohio) under 50% shade. Plants were fertilized using 17N–7.4P–14.1K controlled-release fertilizer (Multicote, Vicksburg Chemical Co., Vicksburg, Mo.). Plants used in controlled crosses were container grown with the same media and conditions. Plant material was maintained at the Mountain Horticultural Crops Research Station (MHCRS), Fletcher, N.C.

Development of allotetraploids. Expanding leaves were removed from 20 actively growing shoots of diploid *Rhododendron* 'Fragrant Affinity' and shoot tips were submerged in 150 μM oryzalin (4-(dipropylamino)-3, 5-dinitro-benzenesulfonamide) (Surflan® A.S., Dow AgroScience LLC, Indianapolis, Ind.) solution for 24-h. Shoot growth temporarily ceased following treatment and ploidy levels were determined on individual shoots the following spring when new growth was present.

Determining ploidy level and genome size. Relative DNA content, approximate genome size, and ploidy level of control (untreated) and treated plants were determined using flow cytometry (De Schepper et al., 2001; Doležel et al., 1998; Galbraith, et al., 1983). Approximately 1 cm^2 of newly expanded leaf tissue was finely chopped with a razor blade in a Petri dish with 500 μL of nuclei extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer, Partec, Münster, Germany). The solution was filtered using Partec CellTrics™

disposable filters with a pore size of 50 μm to remove leaf tissue. Nuclei were stained with 1.5 mL 4', 6-Diamidino-2-phenylindole (DAPI) staining buffer (CyStain UV Precise P Staining Buffer, Partec) and incubated for 1 to 2 min at approximately 24 °C. The suspension was analyzed using a flow cytometer (Partec PA-I, Partec) to determine relative DNA fluorescence. Ploidy and genome size were determined by comparing mean relative fluorescence of each sample with the 2C peak of diploids and an internal standard of known genome size. *Pisum sativum* L. 'Ctirad', with a genome size of 9.09pg (Bennett and Smith, 1976; Doležel et al., 1998) was used as an internal standard to calculate nuclear DNA content [(2C DNA content of sample = 9.09 pg × (mean fluorescence value of sample/ mean fluorescence value of standard)].

Cytological study. Flower buds were harvested between 1000 and 1200HR on warm sunny days in fall of 2005. Buds were harvested and placed in ice water until individual flower buds were removed and fixed. Flower buds were fixed in Carnoy's solution (1 glacial acetic acid : 3 chloroform : 6 100% ethanol) for 24 to 30-h. After fixing, flower buds were transferred to 70% ethanol and stored at 4 °C. Flower buds were washed in sterile distilled water and anthers removed. Anthers were squashed in 1% acetocarmine stain on glass slides, debris removed, cover glass was laid, and cells observed using a light microscope (Carl Zeiss photomicroscope, Carl Zeiss MicroImaging, Inc., Thornwood, NY 10594) under ×600 and ×1,000 magnification.

Assessing pollen fertility. Pollen fertility was assessed using staining and germination tests (Sharma and Sharma, 1980). Pollen was collected from diploid and allotetraploid plants, dried at 25 °C for 24-h, and frozen at –25 °C. Staining was performed by adding 1% acetocarmine (w/v) solution and incubating pollen for 3-h under ambient conditions (Jahier,

1996). Tetrads that were well formed and had at least one pollen grain stained were scored as viable. The germination study was conducted using Brewbaker-Kwack media (Kearns and Inouye, 1993) with 5% sucrose (w/v). Pollen was added to the solution and incubated for 8-h under ambient conditions. Tetrads with pollen tubes equal to or greater than the width of a pollen grain were scored as germinated. Pollen was observed using a light microscope (Micromaster, Fischer Scientific, Pittsburgh) under ×100 and ×400 magnification. The experimental design for the pollen viability study was a completely randomized design with two and three replicates (plants) for the diploids and allotetraploids respectively, with five subsamples of at least 100 pollen tetrads scored per subsample. Data were subjected to analysis of variance using the PROC GLM procedure and means across treatments separated by LSD ($P \leq 0.01$) using SAS 9.1 software (SAS Inst., Cary, N.C.).

In addition to in vitro testing of male fertility, self and cross-pollination tests were used to evaluate male and female fertility, self-compatibility and interploid crossability. Diploid and allotetraploid *R*. 'Fragrant Affinity' plants were pollinated with pollen collected from a fertile diploid *R. catawbiense* and allotetraploid *R*. 'Fragrant Affinity'. Diploid and allotetraploid *R*. 'Fragrant Affinity' were also pollinated with allotetraploid *R*. 'Fragrant Affinity'. A completely randomized design was used with two and three replicates (plants) for the diploids and allotetraploids, respectively, with a minimum of 50 flowers pollinated per plant with each pollen source. Average seed set per pollinated flower and average seed germination percentage was calculated for each treatment. Means across treatments were separated using a LSD, $P \leq 0.05$.

In 2004, additional crosses were made between *R*. 'Vulcan Tetraploid' (mixaploid branch sport of *R*. 'Vulcan'), *R*. 'Supernova' (tetraploid form of R. 'Nova Zembla'), and *R*.

'Briggs Red Star' (mixaploid form of *R*. 'The Honorable Jean Marie de Montague') as female parents with the allotetraploid *R*. 'Fragrant Affinity' as the male parent.

Results and Discussion

Three allotetraploid shoots were identified using flow cytometry on oryzalin treated plants. Approximate DNA content of diploid and allotetraploid *Rhododendron* 'Fragrant Affinity' was calculated as 1.6 and 3.2 pg, respectively (Fig. 1) confirming induced polyploidy. Väinölä (2000) reported 2C DNA content of a limited number of diploid *Rhododendron* taxa ranging from 1.1 to1.5 pg, which is relatively close to the 2C value observed in the current study. Allotetraploid shoots also typically had larger flowers, leaves and pollen compared to diploids.

Cytology of pollen mother cells (PMCs) showed that while there was proper bivalent pairing during metaphase I, there were bridges formed between bivalents and laggard chromosomes were present in anaphase I (Fig. 2A–C). Meiotic irregularities such as laggards support the hypothesis that sterility in *R*. 'Fragrant Affinity' is chromosomal as opposed to genic and fertility may be restored by doubling the chromosome complement.

Pollen staining and germination tests demonstrated improved pollen viability in the allotetraploids (Table 1). Diploids had well formed tetrads but stained poorly and exhibited no germination, while the allotetraploids showed improved staining and germination rates. Female fertility, self-compatibility and interploid crossability were all improved in the allotetraploids (Table 2).

No seeds were produced in crosses using diploid *R*. 'Fragrant Affinity' as a female parent (Table 2). Crosses using allotetraploid 'Fragrant Affinity' as a male or female parent

(except crosses made onto diploid 'Fragrant Affinity') resulted in viable seeds and seedlings. Additional crosses including *R*. 'Vulcan Tetraploid', 'Supernova' and 'Briggs Red Star' by the allotetraploid 'Fragrant Affinity' were also successful (data not shown). Selected F_1 progeny are currently being grown and evaluated at MHCRS.

Doubling the chromosomes of sterile cultivars resulting from wide hybridization to create fertile allotetraploids has been used in a number of crops with varying success. Zadoo et al. (1975) reported an increase of pollen staining from 0 % in diploids to a range of 91 to 98% in three induced allotetraploid *Bougainvillea* cultivars. These allotetraploids were reported to be male and female fertile when crossed with fertile diploids and other allotetraploids. Chen et al. (2003) produced a "fully fertile" interspecific hybrid (*Cucumis hystivus* Chen and Kirkbride, $2n = 4x = 38$) of *Cucumis hystrix* Chakr. ($2n = 2x = 24$) x *C. sativus* L. ($2n = 2x = 14$) by doubling an F_1 hybrid. Pollen grain germination increased from 0 to 2% in the diploid hybrid to 10 to 40% in the amphidiploid (allotetraploid). Chromosome doubling has also been used to develop fertile allotetraploid forms of the hybrids *Lilium henryi* x *L. candidum* (van Tuyl et al., 1992), *Passiflora incarnata* L. x *P. edulis* f. *flavicarpa* (Knight, 1991), and the kangaroo paw cultivar *Anigozanthos* 'Bush Ranger' (*Anigozanthos humilis* x *A. flavidus*) (Griesbach, 1990). In addition to the above examples of interspecific hybridization within a genus, doubling was used to restore fertility in ×*Chitalpa tashkentensis*, an intergeneric hybrid between *Catalpa bignoniodes* Walt. x *Chilopsis linearis* (Cav.) Sweet (Olsen, 2006). Olsen (2006) reported an increase in pollen staining from <1.0% in the diploid to nearly 99% in the allotetraploid and an increase in pollen germination from <1.0% in the diploid to nearly 66% in the allotetraploid. Female fertility of this intergeneric hybrid was also confirmed through a crossing study.

The degree of fertility restoration reported for induced allopolyploids varies considerably. For example, Chen et al. (2003) had pollen germination as low as 10% in their allotetraploid, whereas Olsen et al. (2006) reported a mean pollen germination of 66%, and in the current study nearly 45% pollen germination was observed. An explanation for this may be the degree of similarity between the genomes being combined. Genomes that are very similar behave more like autopolyploids which often have marked reduction in fertility (Stebbins, 1950) due to improper pairing between similar (homeologous) chromosomes from different genomes (Riesberg, 2001) while extreme allopolyploids (e.g., intergeneric) have a frequency of meiotic pairing of near one between homologous chromosomes and zero between homeologous chromosomes (Wu et al., 2001). Many allopolyploids, such as 'Fragrant Affinity' behave as an intermediate between these extremes. Stebbins (1950) termed these segmental allopolyploids defined as, "…a polyploid containing two pairs of genomes which possess in common a considerable number of homologous chromosomal segments or even whole chromosomes, but differ from each other in respect to a sufficiently large number of genes or chromosome segments, so that the different genomes produce sterility when present together at the diploid level." Cytological analysis showed *R.* 'Fragrant Affinity' behaves as a segmental allopolyploid. During metaphase I chromosomes exhibit 100% bivalent pairing but laggard chromosomes are observed in anaphase I indicating that chromosomes of the two genomes are similar enough to pair but different enough that proper gametogenesis cannot occur. There is a distinct advantage in utilizing taxa that exhibit this intermediate manner. In allopolyploids formed from very similar genomes the fertility is expected to be extremely low due to the formation of multivalents, making progress slow and difficult. On the other hand, in extreme allopolyploids formed

from very disparate genomes although fertility is high due to strict disomic pairing which results from 100% preferential pairing between homologous chromosomes (Wu et al., 2001), recombination between the two genomes is reduced or eliminated. Intermediate or segmental allopolyploids such as 'Fragrant Affinity' allow for some crossing over between genomes while maintaining a level of fertility high enough to make it practical in a breeding program.

There are also examples of crops where chromosome doubling is completely ineffective at restoring fertility due to a different mode of sterility. Lu and Bridgen (1997) developed allotetraploids of the hybrid *Alstroemeria aurea* x *A. caryophyllaea* which were no more fertile than the diploids. In their study they observed PMCs in the diploids and allotetraploids. They observed abnormal meiotic behavior such as no pairing, bridges and laggard chromosomes in the diploids, while the induced allotetraploids exhibited nearly normal meiotic chromosome behavior. These findings indicate that in some cases sterility is more complex than structural chromosomal differences. In light of successful restoration of fertility by chromosome doubling and the meiotic irregularities observed in 'Fragrant Affinity' it can be concluded that the mode of sterility is chromosomal, involving structural dissimilarity between parental chromosomes.

Azaleodendrons have not been utilized in breeding programs in the past due to hybrid sterility. However, the development of a fertile allotetraploid form provides new opportunities for integrating diverse traits among subgenera of *Rhododendron*. The induced allotetraploid *R*. 'Fragrant Affinity' is both male and female fertile and may serve as a valuable parent for developing new cold-hardy, evergreen rhododendrons with broad adaptability and desirable floral fragrance. Additionally, the development of triploids through interploid crosses could reduce fruit set and promote increased annual and/or

remontant flowering. This approach may be valuable for restoring fertility in other wide hybrids within the genus including other sterile azaleodendrons.

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Table 1. Pollen staining and germination of diploid (2*x*) and allotetraploid (4*x*) *Rhododendron* 'Fragrant Affinity'.

Ploidy	n^z	Staining %	Germination %
2*x*	2	3.7 ± 2.0[y] a[x]	0 a
4*x*	3	67.6 ± 19.9 b	44.9 ± 5.5 b

[z]*n* = number of replications (plants); each replication consisted of 5 sub-samples of ≥100 pollen tetrads observed.

[y]Data are presented as mean ± one standard error of the mean.

[x]Means within columns followed by different letters are significantly different based on a LSD, $P \leq 0.01$

Table 2. Fertility and interploid crossability of diploid (2*x*) and allotetraploid (4*x*) *Rhododendron* 'Fragrant Affinity' ('FA').

Female	Male	n^z	No. flowers pollinated	Average seed set[y]	Germination (%)
2*x* *R*. 'FA'	2*x* *R*. 'FA'	3	292	0.0 a[x]	---
2*x* *R*. 'FA'	4*x* *R*. 'FA'	3	403	0.0 a	---
2*x* *R*. 'FA'	*R*. *catawbiense*	2	441	0.0 a	---
4*x* *R*. 'FA'	*R*. *catawbiense*	4	436	0.1 a	25.0 a
4*x* *R*. 'FA'	4*x* *R*. 'FA'	5	409	2.0 b	12.3 a

[z]Number of replications (plants).

[y]Average number of seed set per pollinated flower.

[x]Means within columns followed by different letters are significantly different based on a LSD, $P \leq 0.05$

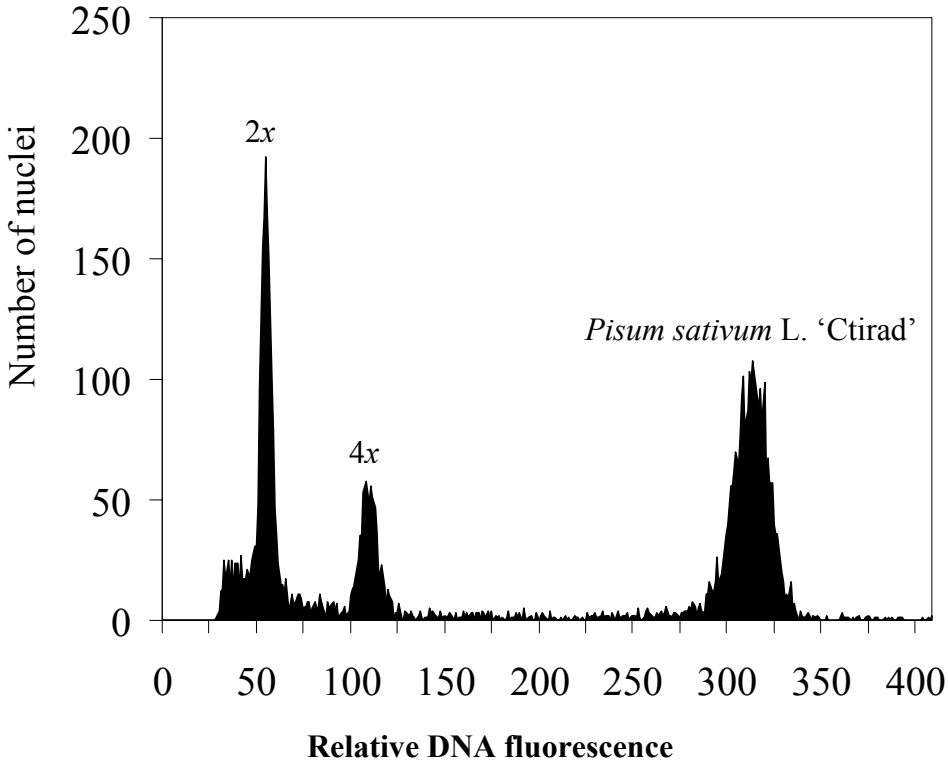

Fig. 1. Determination of ploidy level and DNA content of diploid (2*x*) and allotetraploid (4*x*) *R*. 'Fragrant Affinity' using flow cytometry with *Pisum sativum* 'Ctirad' used as an internal standard of known genome size (9.09 pg). Mean relative fluorescence was 55.42, 109.96, and 313.35 for the diploid, allotetraploid, and internal standard, respectively. Sample DNA contents were calculated using the formula: 9.09 pg * (mean fluorescence of sample/mean fluorescence of standard).

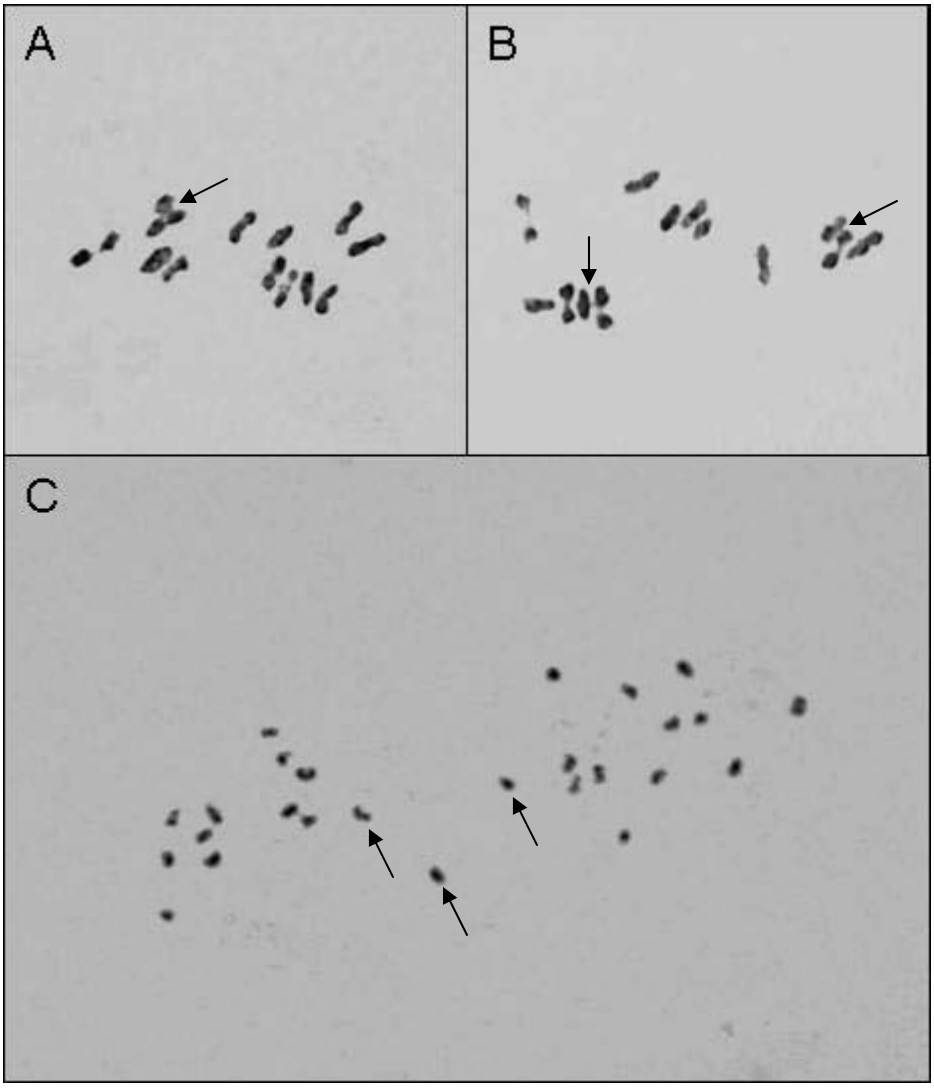

Fig. 2. Micrographs of meiotic cells from diploid ($2n = 2x = 26$) *Rhododendron* 'Fragrant Affinity'. Diploid cells in metaphase I (MI) (A–B) and anaphase I (AI) (C). Arrows indicate bridges (A–B) and laggards (C).

Chapter 2

Investigating Parentage and Hybridity of Three Azaleodendrons Using AFLP Analysis

(In the format appropriate for submission to HortScience)

Investigating Parentage and Hybridity of Three Azaleodendrons Using AFLP Analysis

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Subject Category: Breeding, Cultivars, Rootstocks, and Germplasm Resources

Investigating Parentage and Hybridity of Three Azaleodendrons Using AFLP Analysis

Additional index words. azalea, rhododendron, AFLP, 'Fragrans', 'Fragrans Affinity', 'Fragrant Affinity', plant breeding

Abstract. Morphological analysis has historically been used to determine parentage of unknown hybrids. This can be difficult when the potential parents have very similar morphology as in the case of the three azaleodendron cultivars *R*. 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity', which are purported hybrids of *R. catawbiense* Michx. or *R. ponticum* L. and *R. viscosum* (L.) Torr. Morphological and AFLP analyses were conducted to determine if 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 primer combinations. Morphological comparison suggested that *R. ponticum* is the evergreen rhododendron parent of *R*. 'Fragrant Affinity' based on intermediate petiole and leaf blade lengths and floral characteristics. Genetic similarity calculated using Jaccard's coefficient was highest between the hybrids and *R. ponticum* among the evergreen rhododendrons and *R. viscosum* among the deciduous azaleas, respectively. Genetic similarities among the three azaleodendrons indicated that they are distinct cultivars and not synonyms but likely share the same parental species. A dendrogram generated using the

genetic similarity matrix grouped taxa into generally accepted taxonomic groups. The hybrids were intermediate between the evergreen rhododendrons and deciduous azaleas but nested with subgenus *Hymenanthes* and R. ponticum in particular, suggesting it is the evergreen rhododendron parent. Additionally, a plot of the first three principle components also showed *R. ponticum* to be grouped more closely with the hybrids. Furthermore, there were 18 fragments unique to *R. ponticum* and the hybrids. However, no unique bands were found that were shared exclusively among the hybrids and the purported deciduous azalea parent, *R. visosum*, leading to the hypothesis that the original azalea parent may have been a hybrid.

The genus *Rhododendron* L. is extremely diverse with 8 subgenera and over 1,000 species (Chamberlain et al., 1996). This diversity, combined with broad crossability, has led to the development of over 28,000 cultivars registered with The Royal Horticulture Society (RHS) including 14,298 rhododendrons, 12,989 azaleas, and 108 azaleodendrons (inter subgeneric hybrids between azaleas and rhododendrons) (Leslie, 2002).

There are three azaleodendrons that exist in the trade with similar names: *R.* 'Fragrant Affinity', 'Fragrans Affinity', and 'Fragrans'. The history of *R.* 'Fragrant Affinity' is vague. We received a plant 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 RHS or the American Rhododendron Society (ARS) and the ultimate origin of this material is unknown. Although *R*. 'Fragrant Affinity' is sterile, an allotetraploid form of *R*. 'Fragrant Affinity', named *R*. 'Fragrant Affinity Tetra', was developed and exhibits restored fertility (Contreras et al., 2006). *Rhododendron* 'Fragrans Affinity' was found in a group of deciduous azalea seedlings at Greer Gardens, Eugene, Ore., in the 1950's. Harold Greer (pers. comm.) speculated that it may be a hybrid of *R. ponticum* L. and *R. viscosum* L. (Torrey) or *R. catawbiense* Michx. and *R*. *viscosum*. The plant was named 'Fragrans Affinity' due to its similarity to *R.* 'Fragrans'. *Rhododendron* 'Fragrans' is another purported hybrid of *R. catawbiense* and *R. viscosum* that was introduced by Paxton, of Chandler & Sons, Nursery, London, in 1843. *R*. 'Fragrans' is described as, "A sweet-scented azaleodendron, fast-growing and compact with trusses of small flowers, pale mauve with centers lighter to white," (Salley and Greer, 1986).

The suspected parents of these azaleodendrons are taxonomically distinct and classified in separate subgenera. *R. catawbiense* and *R. ponticum* are in the 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 the 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 el al., 1996).

Since these azaleodendrons were not the result of controlled pollinations, their parentage is difficult to determine. Furthermore, use of traditional morphological comparison to clarify parentage is complicated due to the number of species that could potentially be involved and the similarity of traits among species within each subgenus. Molecular techniques can be used to precisely assess genetic relationships among plants. Amplified fragment length polymorphism (AFLP) technique (Zabeau and Vos, 1993; Vos et al., 1995) can distinguish between species as well as cultivars of the same species (DeHaan et al., 2003; Mellsih et al., 2002; Paul et al., 1997; Perera et al., 1998; Zhang et al., 2000). Due to the use of restriction fragment analysis, AFLPs have the advantage of being highly reproducible in comparison to random amplified polymorphic DNA (RAPD) markers. Milla et al. (2005) reported reproducibility of markers used in a study of *Arachis* L. ranged from 96 to 100%. AFLP analysis also has very high resolution, requires no prior knowledge of the genome(s) being studied (Vos et al., 1995), and has the capacity to simultaneously screen for many DNA regions distributed throughout the genome producing hundreds of genetic markers (Mueller and Wolfenbarger, 1999). AFLPs have been used to confirm hybridity in a number of plants. The AFLP technique was used by Teo et al. (2002) to confirm the hybrid

status of *Mangifera odorata* Griff. and by Kiew et al. (2003) to assess hybrid status of four genera of Malesian plants. AFLP analysis was also used to differentiate between *Salix alba*, *S. fragilis*, and their hybrid *S.* ×*rubens* (Beismann et al., 1997) when morphological analysis was inconclusive.

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

Materials and Methods

Plant material. Genotypes evaluated in this study included cultivars *Rhododendron* 'Fragrant Affinity', *R.* 'Fragrans Affinity', *R.* 'Fragrans', 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 *R.* 'Marydel' (*R. atlanticum* x *R. periclymenoides*) from subgenus *Pentanthera*. *Kalmia latifolia* L. 'Sharon Rose' was used as an outgroup. Container plants were grown in pine bark medium amended with 0.59 $kg \cdot m^{-3}$ dolomitic lime and 1.0 $kg \cdot m^{-3}$ 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 (MHCRS), Fletcher, N.C. and the J.C. Raulston Arboretum (JCRA), Raleigh, N.C. (Table 1). Material maintained at JCRA was grown in display beds.

Morphological comparison. Morphological comparisons of *Rhododendron catawbiense*, *R. ponticum*, *R. viscosum*, and *R.* 'Fragrant Affinity' were conducted using 50

taxonomically relevant characters such as leaf and flower morphology in an attempt to identify the rhododendron parent of this hybrid. Flowering plants of 'Fragrans Affinity' and 'Fragrans' were not available at the time and were therefore not included. A representative sample of taxa studied was vouchered for deposit in herbaria at North Carolina State Univ., Raleigh, and the US National Arboretum, Washington D.C. They were accessioned under the voucher numbers of Dr. Paul Fantz as Fantz & Contreras 8371, 8399–8406, and 8424.

DNA extraction. Total genomic DNA was extracted using a cetyltrimethyl-ammonium bromide (CTAB) extraction method described by Stein et al. (2001), with modifications. Approximately 100-200 mg of tissue from newly opening leaves was collected in 2.0 mL conical tubes. Samples were kept on ice until submerged in 600 μL CTAB extraction buffer (0.015 g CTAB, 0.21 mL 5M NaCl, 0.03 mL 0.5M EDTA, 0.075 mL 1M Tris-HCl pH 8.0, 0.435 mL H_20, and 7.5 μL 1% β-Mercaptoethanol). Samples were ground for 35 s in a Fast Prep FP 120 (Thermo Savant, Holbrook, N.Y.) and transferred to a water bath at 65 ºC for one hour, inverting the tubes every 15 min. Proteins were precipitated by adding 500 μL of 24 chloroform : 1 isoamyl alcohol solution (by volume), shaking for 15 min and centrifuging at 12,298 g_n for 8 min. Supernatants (approximately 600 μL) were transferred to clean tubes and RNA was degraded by adding 2 μL RNAse A (1000U·ml) and incubating for 15 min at 37 °C. DNA was precipitated using 350 μL cold (–20 °C) isopropanol and incubating at –20 °C for 10 min. Samples were then centrifuged at 20,784 g_n for 8 min. Supernatants were discarded and pellets were washed with 350 μL washing solution consisting of 20μM sodium acetate and 76% ethanol. Pellets were then washed twice using 70% ethanol, allowed to air dry and resuspended in 150 μL of low TE buffer (10mM Tris-HCl pH 8.0 and 0.1 mM EDTA).

DNA quantitation and quantification. Stock DNA was diluted 1:100 using sterile distilled water and quantified using a Milton Roy Spectronic 1201 spectrophotometer (Milton Roy, Ivyland, Pa.). Absorbance at 260, 280 and 320 nm were measured and used to determine purity of DNA. The ratio of A_{260}:A_{280} was used to determine if samples were contaminated with proteins with ratios of 1.8 to 2.0 considered acceptable. A_{320} was used to determine if there was light scatter due to particulates in solution. In addition samples were run on a 0.8% agarose gel to check quality.

Digestion of DNA. Approximately 500 ng of DNA was simultaneously digested with *Eco*RI and *Mse*I at 37 °C for 1.5 hr using 12U *Eco*RI, 8U *Mse*I, and 6 µl of 5× restriction-ligation buffer (50 mM Tris-acetate, 50 mM magnesium acetate, 250 mM potassium acetate, 25 mM DTT, and 250 ng/µL BSA) in a final volume of 30 µL. DNA was run on a 0.8% agarose gel to verify complete digestion.

Adaptor ligation. Adaptor ligation was performed by adding 5 pmol *Eco*RI adapter, 50 pmol *Mse*I adapter, 10 mM ATP, 0.5U of T4 DNA ligase, and 1 µL 5× restriction-ligation buffer to 20 µL of each double-digested DNA sample (25 µL final volume) and incubating overnight at 37 °C.

Pre-amplification. A pre-amplification step was performed using primers complementary to the adaptor sequences and carrying an additional selective nucleotide (Table 2). A 1:10 dilution of the digested and adapter-ligated DNA was used as a template for this step. PCR reactions were carried out in a total volume of 20 µL containing 2 µL of 10× PCR buffer (100 mM Tris-HCl pH 8.3, 15 mM magnesium chloride, 500 mM potassium chloride), 2.5 mM of each dNTP, 30 ng primer E01-A, 30 ng primer M02-C, 1.2 U *Taq* DNA polymerase (Qiagen, Valencia, Calif.), and 5 µL of DNA template. PCR amplifications were

carried out in a PTC-100 programmable thermal controller (MJ Research Inc., Reno, Nev.) using the following temperature profile: 28 cycles of 15 s at 94 °C, 30 s at 60 °C, and 60 s + 1 s per cycle of extension at 72 °C; followed by one cycle of 2 min at 72 °C. Upon completion of amplification, 15 μL of each sample was diluted 1:20 with low TE (10 mM Tris-HCl pH 8.0 and 0.1 mM EDTA). The remaining 5 μL of each sample was checked on a 0.8% agarose gel with amplified products visible as a smear in the 100 to 1200 bp range.

Selective amplification and polyacrylamide gel electrophoresis (PAGE). For the selective amplification, primers with three selective nucleotides were used (Table 2). *Eco*RI primers were labeled with a fluorescent near-infrared group (IRD-700 or IRD-800). The PCR amplification mixture (20 μL final volume) was comprised of 2 μL of 10× PCR buffer, 2.5 mM of each dNTP, 30 ng unlabeled *Mse*I+3 primer, 5 ng labeled EcoRI+3 primer, 1.2 U *Taq* DNA polymerase, and 5 μL of diluted pre-amplification product. Selective amplification was carried out in a PTC-100 programmable thermal controller using the following temperature profile: 13 cycles of 10 s at 94 °C, 30 s at 65 °C –0.7 °C per cycle after the first cycle, and 60 s at 72 °C; followed by 25 cycles of 10 s at 94 °C, 30 s at 56 °C, and 60 s + 1 s per cycle of extension at 72 °C; followed by one cycle of 2 min at 72 °C. After amplification samples were denatured by adding 10 μL of loading dye (95% deionized formamide, 20 mM EDTA, and 0.8 $mg \cdot ml^{-1}$ bromophenol blue), heated at 94 °C for 3 min, then chilled on ice. AFLP fragments were separated by polyacrylamide gel electrophoresis (PAGE) using a LI-COR 4200 DNA Analyzer Sequencer on 25 cm gels using 8% denaturing polyacrylamide gels (7 M ultra pure Urea, 0.8× TBE, and 8% Long Ranger acrylamide (BioWhittaker Molecular Applications, Rockland, Maine)). Near-infrared labeled size

standards (LI-COR Inc., Lincoln, Nebr.) were loaded on each gel for sizing of the AFLP fragments.

Data analysis. The AFLP-Quantar 1.0 (Keygene Products B.V., Wageningen, 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 (UPGMA). 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, Inc., Richmond, Calif.) was used to create a scatter plot of the first three principle components.

Results

Morphological comparison. Out of the 50 characters evaluated, only eight are presented that helped to verify hybridity and differentiate between *R. catawbiense* and *R. ponticum* as the evergreen rhododendron parent (Table 3.). Petiole and leaf blade lengths of *R*. 'Fragrant Affinity' were intermediate between *R. ponticum* and *R. viscosum*. Furthermore, the yellow blotch on the lobes of the flowers in *R*. 'Fragrant Affinity' was very similar to that of *R. ponticum*. These characters suggested that *R. ponticum* was the evergreen rhododendron parent of *R*. 'Fragrant Affinity'.

AFLP analysis. The 31 primer combinations used generated a total of 152 scorable AFLP fragments (Table 2) ranging in size from 106.8 to 614.2 bp. Only bands that were unambiguous were scored and used for analysis (Fig. 1). The number of scored bands

generated by each primer combination ranged from one to ten with a mean of five. A total of 18 bands specific only to *R. ponticum* and the hybrids were observed (Fig. 1), however no 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 with a mean of 0.25 (Table 4). Genetic similarities among the hybrids ranged from 53 to 71%, suggesting that they are distinct cultivars and not a single clone. The degree of similarity with 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, respectively. The dendrogram generated from the similarity matrix (Fig. 2) has subgenera generally nested in accordance with recognized taxonomic groups. The upper branch contains the evergreen rhododendrons from subgenus *Hymenanthes*. All three azaleodendron cultivars nested in this clade and were particularly closely allied with *R. ponticum* suggesting that it is closely related and may be a parent. The clade below the hybrids contains the deciduous azaleas from subgenus *Pentanthera* with the exceptions of *R.* 'Marydel' and *R. arborescens*. *Kalmia latifolia* 'Sharon Rose', the outgroup, was most distantly related and formed the rooting branch.

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. A plot of the first principle component separated the taxa into two groups. In one group were the deciduous azaleas and *Kalmia latifolia* 'Sharon Rose' and the other contained the hybrids and the evergreen rhododendrons. Adding the second principle component added separation between the hybrids and the evergreen rhododendrons. The third component separates *Kalmia latifolia* 'Sharon Rose' from deciduous azaleas and

also shows that *R. arborescens* and *R.* 'Marydel' group with the other deciduous azaleas in contrast to what was observed in the dendrogram. The groups are well defined with the exception of the two accessions of *R. ponticum* (Fig. 3).

Discussion

The AFLP technique has proven effective for elucidating information about hybridity. Beismann et al. (1997) used UPGMA analysis of AFLP data to segregate the hybrid *Salix ×rubens* from one of its parents which it closely resembles morphologically and Teo et al. (2002) used cluster analysis and AFLP profiles to determine that *Mangifera odorata* is a hybrid of *M. indica* and *M. foetida*. These studies show that AFLP is a useful tool for determining hybridity as in the current study.

The results of the morphological and molecular analyses provide 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 all distinct cultivars and not a single clone. Morphological comparison showed that the leaf and flower morphology of *R. ponticum* is very similar to the hybrids. AFLP data also supports the hypothesis that *R. ponticum* is a parent of the three cultivars. The 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% with the hybrids. Cluster analysis nested the three azaleodendrons with subgenus *Hymenanthes* and they were particularly closely allied with *R. ponticum*. Principle component analysis also grouped the hybrids more closely with *R. ponticum* than any other species included. Additionally, there were 18 species specific markers unique to *R. ponticum* and the hybrids.

The data on the deciduous azalea parent is less conclusive. *Rhododendron viscosum* had the highest coefficient of similarity with the hybrids (26 to 44%) among the deciduous azaleas, but there were no markers specific to *R. viscosum* that were also common to the hybrids. The deciduous azaleas in *Pentanthera* hybridize freely in cultivation and in the wild and it is often difficult to differentiate between species and hybrids (Towe, 2004). It is possible that a hybrid azalea was a parent in these azaleodendron crosses.

The current study provides evidence that the three azaleodendron cultivars are inter-subgeneric hybrids between an evergreen rhododendron and a deciduous azalea. Morphology of 'Fragrant Affinity' was intermediate and 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 wide crosses to develop novel cultivars possessing traits from diverse taxa. Additionally, the AFLP technique proved to be a useful tool in determining parentage of cultivars of uncertain origin. It is clear that the evergreen rhododendron parent of the three azaleodendrons is *R. ponticum*, but additional AFLP analysis is necessary to accurately determine the deciduous azalea parent.

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Table 1. Taxa used in AFLP analysis to elucidate parentage and hybridity of 'Fragrans', 'Fragrans Affinity', and 'Fragrant Affinity'.

Taxa	Subgenus	Location[z]	Accession
R. '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 Plox Pink'	*Pentanthera*	JCRA	950316
R. 'Marydel'[y]	*Pentanthera*	JCRA	040705
Kalmia latifolia 'Sharon Rose'	---	JCRA	---[x]

[z]Mountain Horticultural Crops Research Station (MHCRS), Fletcher, N.C. and J.C. Raulston Arboretum (JCRA), Raleigh, N.C.

[y]Hybrid of *R. atlanticum* x *R. periclymenoides*.

[x]No accession number available, plant located in bed L14.

Table 2. 31 primer combinations used in AFLP analysis and number of polymorphic bands scored.

Primer combination	No. bands scored
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
Totals	152

Table 3. Morphological comparison of *R. catawbiense*, *R. ponticum*, *R.* 'Fragrant Affinity' and *R. viscosum*

Character	*R. catawbiense*	*R. ponticum*	*R.* 'Fragrant Affinity'	*R. viscosum*
		Leaf sizes		
Petiole	1.7 to 2.6 cm	0.8 to 1.2 cm	0.4 to 1.0 cm	2.5 to 4.0 cm
Blade length	7.0 to 9.2 cm	8.5 to 13 cm	6.5 to 11 cm	4.4 to 5.4 cm
		Leaf blade		
Margin	Entire, revolute	Entire	Entire	Entire with glandular hairs
Base	Rounded	Aequilateral to cuneate	Broadly cuneate to sub-attenuate	Cuneate
		Corolla		
Width at base	5.0 to 8.0 mm	4.0 to 6.0 mm	3.0 to 4.0 mm	2.5 to 4.0 mm
Width at throat	17 to 22 mm	25 to 30 mm	8.0 to 10 mm	4.0 to 9.0 mm
Color	Lavender to pink with whitish green blotch	Pale purple-lavender with yellow blotch	White throat with lobes flushed pale lavender to pink with yellow blotch	White; rarely pink
		Pistil		
Style length	2.5 to 3.5 cm	1.5 to 2.0 cm	3.0 to 3.5 cm	5.8 to 6.2 cm

Table 4. Genetic similarity matrix based on Jaccard's coefficient of similarity of the 14 taxa evaluated to elucidate parentage and hybridity of '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 Plox Pink'
[y]*Kalmia latifolia* 'Sharon Rose

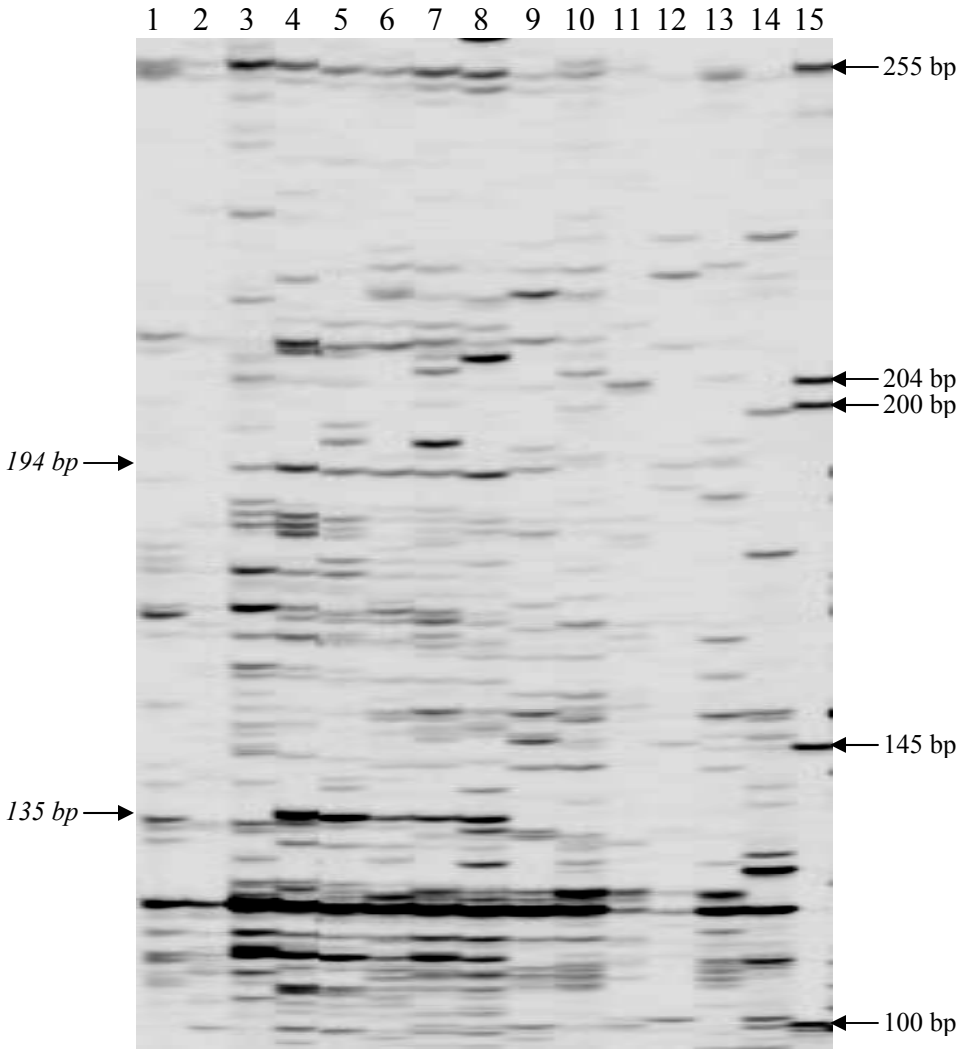

Fig. 1. AFLP profile generated by primer combination E+ATC/M+CTA. Fragment weights in italics are representative of scored fragments used in the current analysis. 135 bp fragment specific to both *R. ponticum* accessions and the hybrids. Samples are arranged from left to right in the order of (1) *R. catawbiense*, (2) *R. maximum*, (3) *R. hyperythrum*, (4) *R. ponticum* 2005-217, (5) *R. ponticum* 2004-076, (6) *R.* 'Fragrans Affinity', (7) *R.* 'Fragrant Affinity', (8) *R.* 'Fragrans', (9) *R. viscosum* 2004-116, (10) *R. viscosum* 'Roseum', (11) *R. arborescens*, (12) *R.* 'Marydel' (*R. atlanticum* x *R. periclymenoides*), (13) *R. canescens* 'Varnadoes Phlox Pink', (14) *Kalmia latifolia* 'Sharon Rose', (15) size standard.

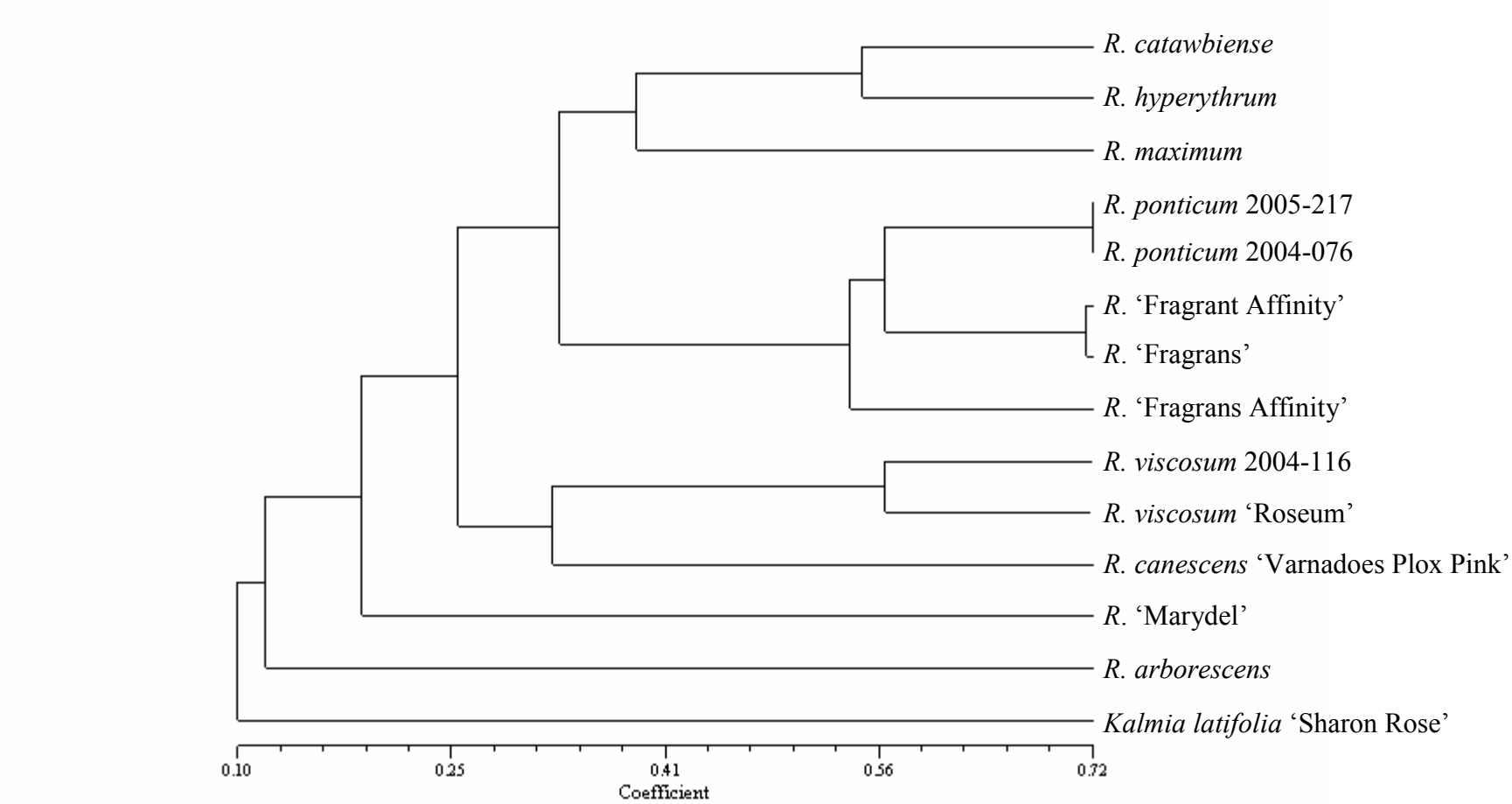

Fig. 2. Dendrogram created using the unweighted pair group method with arithmetic averages (UPGMA) based on Jaccard's coefficient of similarity of the 14 taxa included in the current study. *R.* 'Marydel' is a hybrid of *R. atlanticum* and *R. periclymenoides*.

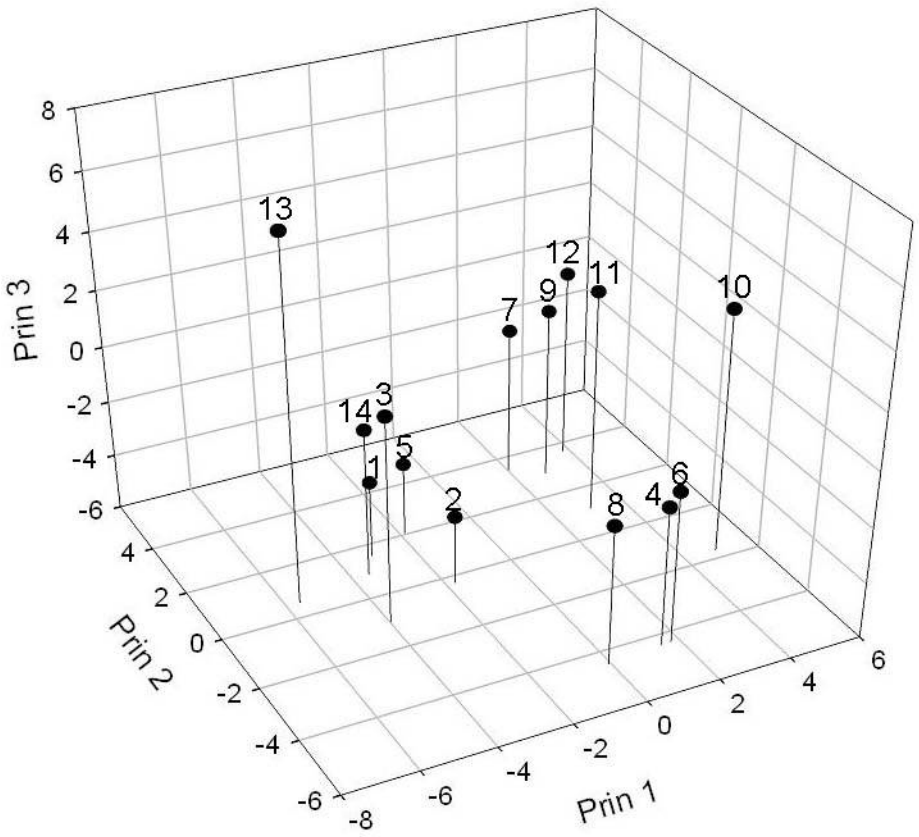

Fig. 3. Plot of first three principle components based on the variance-covariance matrix of the data using the 14 taxa evaluated. 1) *R. viscosum*, 2) *R. canescens* 'Varnadoes Plox Pink', 3) *R. arborescens*, 4) *R. hyperythrum*, 5) *R. viscosum* 'Roseum', 6) *R. catawbiense*, 7) *R.* 'Fragrans', 8) *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* x *R. periclymenoides*).