CHARACTERIZING THE GENETIC VARIATION IN SEVEN SPECIES OF DECIDUOUS NATIVE AZALEAS AND IDENTIFYING THE MECHANISM OF AZALEA LACEBUG RESISTANCE IN DECIDUOUS AZALEA

by

MATTHEW RANDOLPH CHAPPELL

(Under the direction of Dr. Carol Robacker)

ABSTRACT

Despite the ecologic and economic importance of native deciduous azaleas (*Rhododendron* spp. section *Pentanthera*), our understanding of interspecific variation of North American deciduous azalea species is limited. Furthermore, little is known concerning intraspecific or interpopulation genetic variation. The present study addresses questions of genetic diversity through the use of amplified fragment length polymorphism (AFLP) analysis. Twenty-five populations of seven species of native azalea were analyzed using three primer pairs that amplified a total of 417 bands. Based on analysis of molecular variance (AMOVA) and estimates of Nei's coefficients of gene diversity (H_S, H_T, and G_{ST}), the majority of variation in deciduous azalea occurs within populations. Both among species and among population variation was low, likely the effect of common ancestry as well as frequent introgression among members (and populations) of section *Pentanthera*. The majority of populations were grouped into species based on Nei's unbiased genetic distances viewed as a UPGMA phenogram. The significance of these results is discussed in relation to breeding in section *Pentanthera*. In addition to the lack of information concerning genetic variation in North American native azaleas, little is known concerning the insect-plant interaction between the primary azalea pest in the United States,

azalea lace bug (ALB) (*Stephanitis pyrioides* Scott), and deciduous azalea. Azaleas are largely resistant to predation by insects, with the exception of ALB. Within deciduous azalea (*Rhododendron* section Pentanthera) varying levels of resistance to ALB is observed with a continuous distribution from susceptible to highly resistant. In this study, epicuticular leaf wax from two ALB resistant [*R. canescens* Michaux and *R. periclymenoides* (Michaux) Shinners] and two ALB susceptible ('Buttercup' and 'My Mary') deciduous azalea genotypes was extracted and re-applied to fresh azalea foliage. Leaf wax extracted from ALB resistant genotypes and applied to ALB susceptible genotypes conferred a high level of resistance to ALB feeding and oviposition. Conversely, leaf wax extracted from ALB susceptible genotypes and applied to ALB resistant genotypes conferred susceptibility to ALB resistant genotypes. The results indicate that leaf wax serves as a primary mechanism of resistance of deciduous azalea to ALB.

INDEX WORDS: AFLP markers, *Rhododendron spp.*, deciduous azalea, among population diversity, within population diversity, AMOVA, phenogram, epicuticular wax, insect resistance, chloroform wax extraction

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DEDICATION

I would like to dedicate this work to two individuals.

The first is my grandfather and friend, George Randolph Chappell. I can not list the contributions that he has made to my life, as I would surely run out of space! I only hope that I am able to become half the man that he is.

Second is my mother, Mary Williams, who has given me guidance and support throughout my many years as a student. I will never forget the times we spent at the Appomattox Regional Library looking at books and deciding what direction I should take in my life's work. It is amazing to look back on where this journey started and how we have both grown over the years. One thing has been constant though, you have always been there for me. I will never forget that and am grateful to have such a wonderful mother and friend.

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Peace be with you all,
Matthew Randolph Chappell

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

PURPOSE OF STUDIES

Deciduous azalea species (Division: Magnoliophyta; Class: Magnoliopsida; Subclass Dilleniidae; Order: Ericales; Family: Ericaceae; Genus: *Rhododendron*; Section: Pentanthera) are indigenous to the eastern United States from southeastern Canada to central Florida and to east Texas. The deciduous azalea is an outstanding spring-summer blooming woody ornamental crop, and along with the evergreen azaleas (*Rhododendron* series *Azalea*), is a staple plant in southeastern U.S. landscapes (Gallee, 1987). For more than a century, researchers have been addressing genetic diversity within North American native deciduous azaleas (*Rhododendron* spp. section *Pentanthera*) through studies of chromosomes, morphology, introgression, interspecific hybridization, flavanoids, isozymes, chloroplast and ribosomal DNA, and recently molecular markers. However to date no study has utilized total genomic analysis to determine the level of genetic diversity among deciduous azalea species. Furthermore, no study has addressed among population genetic diversity or within population genetic diversity within a single species of deciduous azalea. Additionally, the insect-plant interaction between *Stephanitis pyrioides* Scott (azalea lace bug) and both deciduous and evergreen azalea has been examined via screening of cultivars for resistance to insect damage and correlations of chemical compounds of epicuticular wax with resistance in azalea genotypes. However, to date no study has conclusively determined the mechanism of azalea lace bug resistance in azalea.

The overall goal of this dissertation is to address the two aforementioned topics that are of vast importance to azalea breeders and conservationists. In the first project, amplified fragment length polymorphism (AFLP) fingerprint analysis will be used to measure genetic diversity among and within seven species of deciduous azalea indigenous to the eastern United States. Physical observations among the various species and populations within a species have

revealed morphologic differences among species, among populations within a species, and between plants of the same population. Though differences have been observed, the question remains, how much *genetic* difference is there within the aforementioned groups? In the second project, the assumption that epicuticular leaf wax mediates resistance to azalea lace bug will be examined. Epicuticular leaf wax will be extracted from resistant and susceptible genotypes and re-applied to fresh azalea foliage. Thereafter, azalea lace bugs will be introduced to the treated azalea foliage and insect behavior documented via quantifying survivability, feeding, and fecundity.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF DECIDUOUS AZALEA

Taxonomy of *Rhododendron* spp. sect. *Pentanthera.* *Rhododendron* spp. section *Pentanthera* includes sixteen species of deciduous azalea: *R. alabamense* Rehder, *R. arborescens* (Pursh) Torrey, *R. atlanticum* Ashe, *R. austrinum* (Small) Rehder, *R. calendulaceum* (Michaux) Torrey, *R. canadense* (L.) Torrey, *R. canescens* (Michaux) Sweet, *R. cumberlandense* Braun, *R. eastmanii* Kron & Creel, *R. flammeum* (Michaux) Sargent, *R. luteum* Sweet, *R. occidentale* Torrey & Gray, *R. periclymenoides* (Michaux) Shinners, *R. prinophyllum* (Small) Millais, *R. prunifolium* (Small) Millais and *R. viscosum* (L.) Torrey. All species with the exception of *R. luteum* and *R. occidentale* are native to the eastern United States, primarily east of the Mississippi River. *R. occidentale* is native to California and southwestern Oregon and *R. luteum* is native to Poland, Turkey, Slovenia, and the southern states of the former Soviet Union.

Chromosome and breeding studies. Chromosome studies by Sax (1930) and Li (1957), in conjunction with early studies on interspecific hybridization and geographic distributions, were the initial push toward determining the relatedness of species within the genus *Rhododendron*. Scharff (1911) first described the probable introduction and migration of deciduous azaleas to North America from Asia, via the Bering Strait, in the early Tertiary to Pre-Pliocene periods. The interesting and confounding issue is that over the 5+ million years since this period, little to no change has occurred in chromosome number or chromosome morphology. Therefore, it is assumed that members of *Rhododendron* spp. section *Pentanthera* are very stable. This hypothesis was corroborated first by W.H. Judd who reported in 1915 successful interspecific hybridization between the geographically isolated diploid species *R. occidentale* (California-Oregon) and the tetraploid *R. calendulaceum* (North Carolina-Georgia) and later by Wilson and Rehder (1921) who reported a successful cross between *R. luteum* (Eastern Europe) and *R. canadense* (Eastern Canada-Northeast United States) in 1938.

Sax (1930) examined chromosome stability in the genus *Rhododendron*, specifically the species *R. roseum* (now *R. prinophyllum*), *R. viscosum*, *R. calendulaceum*, and *R. arborescens*. Results indicated that, based on chromosome morphology of species and interspecific hybrids, these species were closely related. This was true even in the tetraploid species *R. calendulaceum* which freely hybridized with diploid genotypes via unreduced gametes (pollen and egg). Li (1957) assessed chromosome numbers and morphology in 15 species of *Rhododendron* spp. series Azalea and determined that while enough variability exists in chromosome morphology to differentiate between species, interspecific hybridization occurred freely. It was therefore postulated that species identity was maintained not through interspecific incompatibility but rather through geographic isolation and temporal patterns of flowering.

Early research conducted by Rehder (1921), Wherry (1943), Skinner (1961), and Galle (1967) directly addressed questions concerning the relationship of various species and cultivars found both in the U.S. and abroad. The majority of their research was conducted by assessment of morphological features of known species or by observing interspecific hybridization in cultivation and drawing conclusions of relatedness based upon morphological characteristics of the progeny. The quandary in using interspecific hybridization and morphological characteristics of progeny to determine taxonomic and/or genetic relationships lies in the fact that within *Rhododendron* spp. Sect. *Pentanthera*, species have the ability to freely interbreed and morphologic traits are often identical between species. This phenomenon is well documented, especially in the eastern United States, where interspecific hybridization often occurs where two species overlap in geographic distribution. The result is a hybrid swarm, with individuals that may morphologically resemble one parent with the exception of one or a few minor traits (Kron et al., 1993).

Bioassay and DNA studies in azalea. The first research directed at deducing relationships among deciduous azalea species using laboratory methods was conducted by King (1977) who analyzed flavanoid content of *R. canescens*, *R. austrinum*, *R. speciosum* (now *R. flammeum*), and hybrids between the three species. The goals of the study were to characterize leaf flavanoids of the three species and hybrids and to utilize flavanoid differences to differentiate between naturally occurring populations. This study was successful in that the progeny of crosses among these species had variable and distinguishable flavanoid contents, allowing for a distinction among species and evidence into parentage of hybrids.

Kron et al. (1993) successfully employed the use of chloroplast DNA markers to distinguish two species of deciduous azalea, *R. canescens* and *R. flammeum*, and also to measure

introgression in a hybrid swarm located at Stone Mountain, GA. The choice of species allowed the researchers to use morphological markers to confirm results, as the two species are distinct in traits such as flowering time, flower color, flower size, plant habit, pod size, and leaf characteristics. The use of chloroplast DNA to differentiate between the two species was critical, as it is not diluted by repeated backcrossing and hence is valuable in constructing a parental lineage in highly backcrossed progeny. Results of this study verify the value of DNA studies to distinguish between species of deciduous azaleas. In the hybrid population of *R. canescens* and *R. flammeum*, hybridization and introgression were confirmed in more than 75% of the surveyed individuals. While most of the individuals surveyed were morphologically indistinguishable from *R. flammeum*, these same individuals contained chloroplast DNA patterns of *R. canescens*, suggesting introgression of *R. canescens* into *R. flammeum*.

Randomly amplified polymorphic DNA, introduced in 1990 by Williams et al., was quickly adapted as a valuable tool for analyzing genetic similarities and/or differences among plants. RAPD analysis is useful in this regard as it has the power to identify polymorphisms, or small changes in DNA composition. These polymorphisms can be used to identify genetic distance between genes or to calculate the relatedness of two or more genotypes. Iqbal et al. (1995) used RAPD analysis to discern genetic relatedness of thirteen deciduous *Rhododendron* spp. and to test if RAPD analysis of *Rhododendron* spp. reflected their true genetic relationship. Results indicated that RAPD analysis is a valuable tool for discerning genetic relatedness in *Rhododendron*, as RAPD data plotted using cluster analysis grouped species and cultivars as predicted based upon origin and known pedigree.

Scheiber et al. (2000), using an innovative procedure, sequenced the internal transcribed spacer (ITS) region of sixteen deciduous azalea species in an effort to group species based upon

genetic similarity. The ITS region is unique in that it is transcribed yet untranslated, therefore postulated to change freely via deletion or addition without altering gene products. Hence, molecular evolution studies have embraced this genomic region as a means of both classifying closely related species and tracking evolution of a species. The results of this study were promising, as bootstrap analysis concurred with previous studies in grouping of species within *Rhododendron* section *Pentanthera*. While this was a very successful study in ascertaining genetic differences between species at the base level, little variation occurred in sequence, ranging from 0.00 to 1.67%. Additionally, only a single region of the genome was observed. Both of these factors point to the need for further examination of the entire genome to deduce genetic differences, thereby increasing the likelihood of identifying significant genetic variation.

AFLP background and technique. Studies in reproductive biology, geographic distribution, morphology, ontogeny, pedigree, breeding behavior, chromosome structure and behavior, morphological markers, and protein markers have provided valuable information on relatedness in azalea, yet none survey the total genomic DNA of a single individual or individuals within a population or species. In this study, a procedure was needed that could deduce genomic differences at the base level over the entire genome. Amplified fragment length polymorphism (AFLP) DNA fingerprint analysis is currently a premier method of discerning genetic relationships based upon genetic sequence and surveys total genomic DNA. AFLP has been used to characterize genetic diversity within many genera of plants including *Arabadopsis*, *Hordeum*, *Calycophyllum*, *Camellia*, *Manihot*, *Gossipyum*, *Physalis*, *Helianthus*, *Zea*, *Pinus*, *Solanum*, *Oryza*, *Salix*, *Glycine*, and *Triticum*. The basic theory of AFLP is the amplification of restriction digested DNA using Polymerase Chain Reaction (PCR) technology with directed primers (Vos et al., 1995).

AFLP has several advantages compared to the molecular methods of restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and microsatellites (SSR). The main advantage is the large number of polymorphisms that are generated by the AFLP method. For example, Barker et al. (1999) found 170 polymorphic bands with 20 RAPD primers in Salix, yet using 4 AFLP primers found 645 polymorphic bands. This high level of polymorphism detection is essential, especially within a population of deciduous azaleas, due to high levels of genetic homogeneity between individuals (Scheiber et al., 2000). Nearly of equal importance in this study is the ability to use AFLP to assess genetic diversity when no sequence information is available, as is the case with the species included in this study.

Yet another advantage is the speed of this technique, due to the use of PCR in amplification of DNA fragments and the high multiplex ratio, or number of different genetic loci that may be simultaneously analyzed per experiment. The high multiplex implies that the entire genome is being sampled for differences rather than a single segment, as is the case with RFLP and SSR analysis. The final advantage of the AFLP technique is a high level of reproducibility. RFLP and RAPD results are difficult to reproduce and this problem can lead to inconclusive results. For example, Winfield et al. (1998), in an AFLP analysis of Black Poplar, found that over five trials, banding patterns were 97.6% to 100% similar.

Inherent problems are associated with any technique, and AFLP is no different. High cost can be limiting when working with a large number of samples (plants). Determination of appropriate restriction enzyme and primers can also be difficult. The choice of restriction enzyme and primer will directly affect the number of polymorphisms detected. In *Hordeum* spp., Ridout and Donini, (1999) determined that the restriction enzyme combination *Pst*I/*Mse*I yielded better polymorphism detection than *Eco*RI/*Mse*II. The same has held true for peanut, *Abelia*, and

wheat (Melanie Newman, personal comm..). Qi and Lindhout (1997) discovered that at the species level, [adapter] + 2 primers amplify too many bands and [adapter]+4 primers amplify too few bands. They found that while [adapter] + 3 primers is the best choice for polymorphism detection, choice of adapters varies by genus or species. Since the plant genome is AT rich, the use of AT poor primers may reduce polymorphism detection. To alleviate this problem, we have sampled [adapter] + primer sets to find the best combinations. Several major corporations (Applied Biosystems and Qiagen), in conjunction with public institutions, have published base protocols that are in agreement. The majority of published protocols employ *Eco*RI/*Mse*I and primer +3, as this modus operandi has been effective in a majority of plant species studied.

The issue of dominance of AFLP has been debated, though researchers now concur that AFLP markers are a dominant marker. Therefore, when scoring the bands produced in an AFLP study, each band is scored as present or absent (1 or 0), with no intermediate. This raises the question of how faint bands (thought to be heterozygotes) are scored. Castiglioni et al. (1999) suggested that heterozygotes be identified based upon intensity of the bands or peaks on AFLP gels. A heterozygote would have a band half as intense as a homozygote. Vos et al. (1995) argued against this by stating that the AFLP technique is insensitive to template DNA concentration, with similar band intensities seen using a range of DNA concentrations. Three solutions for this problem have been proposed. The first is to increase sample size 2-10 fold when using a dominant marker technique. The second solution is inherently a part of the AFLP technique, as the problem of dominance can be overcome due to the high number of polymorphisms detected (Castiglioni et al. 1999). The final solution is to employ fluorescent tags and set a threshold value to use as a definition of presence or absence (ABI, 2004).

AFLP in *Rhododendron.* In Rhododendron, the first published AFLP study was conducted by Reik et al. (1999) who analyzed a collection of 75 Belgian pot azaleas for genetic conformity. Pedigree information was available for 34 cultivars, which allowed for AFLP results to be checked for accuracy. Using three primer combinations, 648 fragments were scored ranging from 70 to 450 base pairs. Primer combinations were fluorescently labeled to increase multiplex ratio and internal size standards were added to each gel lane to enable automated scoring of each fragment arising from each primer combination. Eight additional data subsets were constructed by setting differing marker selection thresholds, accomplished by adjusting the fluorescence detection levels within the automated detection device. Pairwise plant genetic similarity was attained for the nine data sets using the two most popular statistical models in use, the Simple Matching coefficient (symmetrical, including double zeros) and Jaccard coefficient (asymmetrical, excluding double zeros). These two methods were compared for accuracy and checked against known pedigree information for reliability. The use of a Simple Matching coefficient with little or no selection to signal intensity and excluding rare and abundant markers garnished the best correlation with known pedigree information.

Riek et al. (2000) expanded on the aforementioned 1999 study in which AFLP was used to ascertain genetic relatedness in azalea. In this study, plant material consisted of ten seedlings from 33 natural or semi-wild populations as well as 75 accessions from a breeder's collection (4 Hirado, 7 Krume, 55 Belgin pot azaleas, and 9 related Rhododendron species from the Tsutsusi subgenus). The goal of the study was to assess the genetic conformity between the breeder's pool of azaleas and the native Chinese populations that are postulated as being the original source of germplasm for current hybrids. Two approaches were used in answering this question: ordination analysis that includes clustering and principal co-ordinate analysis, and analysis of molecular

variance (AMOVA). Each of these methods use pairwise similarity or distance matrix as input data and were calculated on an individual plant basis using presence/absence of AFLP bands as well as on a population basis by AFLP marker frequency. The calculation of similarity or distance measures using ordination analysis allowed the researchers to directly measure the genetic relatedness of each native population and the cultivated hybrids. The use of AMOVA analysis allowed the researchers to simultaneously estimate the source of genetic variation within and between pools of plants at different levels (individuals, subpopulations, populations, or multiple populations), which added an extra dimension to the study and also served to corroborate results from the ordination analysis. The use of these two analyses was successful in determining genetic relatedness of genotypes and native populations and linking current hybrids to likely ancestors.

AZALEA LACE BUG (INSECT) - DECIDUOUS AZALEA (HOST) INTERACTION

Azalea lace bug and effects on azalea. Since its introduction from Japan in 1915, azalea lace bug (ALB) has become a significant pest on azalea throughout North America (Drake and Ruhoff, 1965; Weiss 1916). ALB damage occurs from late spring until leaf drop, with up to four generations occurring in a single growing season in the southern United States (Neal and Douglass, 1988). Adult and nymphal ALB feed on the abaxial leaf surface by inserting stylets into stomata (Ishihara and Kawai, 1981). Cell contents, principally chloroplasts, are removed from the mesophyll layer. The resulting damage is visible as a tan speckling on foliage of susceptible species and cultivars. Cast skins of nymphs and brown to black frass deposition further discolor foliage (Braman and Pendley, 1992; Buntin et al., 1996; Ishihara and Kawai, 1981; Mead, 1967).

Azalea lace bug bioassays. Bioassays performed by Braman and Pendley (1992) found the deciduous *R. canescens* and *R. prunifolium* (Small) Millais to be resistant whereas the evergreen cultivar 'Delaware Valley White', a selection of *R. indica* var. *alba*, was susceptible. Further work by Wang et al. (1998) evaluated four cultivars and eleven deciduous species selections and found *R. periclymenoides*, *R. canescens*, and *R. prunifolium* to be highly resistant and *R. serrulatum* (Small) Ahles and *R. viscosum* (L.) Torrey to be moderately resistant to ALB. Moderately susceptible to very susceptible species included *R. arborescens* (Purch) Torrey, *R. austrinum* (Small) Rehder, and *R. oblongifolium* (Small) Millais. Susceptible cultivars included 'Buttercup', a *R. austrinum* selection; 'My Mary', a complex hybrid of [(*R. atlanticum* (Ashe) Rehd. x *R. periclymenoides*) x *R. austrinum* seletion]; and 'Nacoochee', a *R. atlanticum* x *R. periclymenoides* hybrid.

Plant cuticle and epiculticular leaf wax. The principle functions of the plant cuticle are to prevent excessive water loss and protect the leaf surface from insect and pathogen damage (Hopkins, 1995). The epicuticular wax layer of the cuticle consists primarily of long-chain aliphatic compounds derived from fatty acid chains. This wax layer is deposited on the leaf surface as amorphous intracuticular wax embedded in cutin polymers, as well as wax crystalloids. Epicuticular wax components include alkanes, primary and secondary alcohols, ketones, and wax esters (Eigenbrode and Espelie, 1995; Knust and Samuels, 2003). Epicuticular wax has been recognized as a deterrent to feeding and oviposition by herbivorous insects as well as an attractant of beneficial (often predatory) insects (Eigenbrode and Espelie, 1995). Specific examples of epiculticular wax-herbivore interactions have been identified and characterized in *Allium cepa* L. (Molenaar, 1984), *Brassica rapa* L. (Bodnaryk, 1992; Srinivasachar and Malik, 1972), *Eucalyptus globulus* Labill (Brennan and Weinbaum, 2001), *Glycine max* L. (Baker et al.,

1985), *Hordeum vulgare* L. (Tsumuki et al., 1989), *Sorghum bicolor* (L.) Moench (Chapman et al., 1983; Nwanze et al., 1992; Weibel and Starks, 1986), and *Triticum aestivum* L. (Lowe et al., 1985).

Specific wax compounds linked to resistance/susceptibility. In azalea, through the use of gas chromatography-mass spectrometry, specific lipid components of epicuticular wax have been implicated in ALB resistance and susceptibility (Balsdon et al., 1995; Wang et al., 1999). Research by Balsdon et al. (1995), analyzing lipid components of four susceptible evergreen cultivars ('Delaware Valley White', 'Hino Crimson', 'President Clay', and 'Higasa') and the resistant species *R. canescens*, identified three triterpenoid components that may have an effect on ALB behavior; ursolic acid, β-amyrin and α-amyrin. However, data were not definitive as purported deterrent/stimulant compounds were found in similar concentrations in both *R. canescens* and the susceptible cultivars. Wang et al. (1999) identified lipid components correlated with ALB resistance and susceptibility in two deciduous resistant genotypes, four deciduous susceptible genotypes, and one evergreen susceptible azalea genotype. The lipid component present in the largest proportion among resistant genotypes was *n*-Henrtriacontane whereas in susceptible genotypes α- and β-amyrin were in greatest concentrations. When comparing the two studies, it is of note that α- and β-amyrin are described as a stimulant to ALB by Baldson et al. (1995) and conversely a deterrent by Wang et al. (1999). This discrepancy in findings is puzzling and while these studies suggest an association of leaf-surface lipids with ALB response, studies to quantify the actual effects of lipids on ALB behavior have not been conducted. The research described in this paper investigates the quantitative effects of leaf-surface lipids from susceptible and resistant foliage on ALB survival, feeding, and oviposition

and will address if epicuticular leaf wax is indeed a mechanism of ALB resistance/susceptibility in azalea.

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CHAPTER 2

ASSESSING THE GENETIC DIVERSITY OF SEVEN DECIDUOUS AZALEA SPECIES (*RHODODENDRON SPP. SECTION PENTANTHERA*) NATIVE TO THE EASTERN UNITED STATES [1]

[1] Chappell, M.R., C. Robacker, and T. Jenkins. Submitted to the Journal of The American Society of Horticultural Science, 04/07.

Index words: AFLP markers, *Rhododendron spp.*, among population diversity, within population diversity, AMOVA, phenogram

ABSTRACT

Despite the ecologic and economic importance of native deciduous azaleas (*Rhododendron* spp. section *Pentanthera*), our understanding of interspecific variation of North American deciduous azalea species comes principally from morphologic studies. Furthermore, little is known concerning intraspecific or interpopulation genetic variation. With ever-increasing loss and fragmentation of native azalea habitat in the eastern United States due to anthropogenic activity, it is imperative that an understanding of natural genetic variation among and within species and populations is acquired. The present study addresses questions of genetic diversity through the use of amplified fragment length polymorphism (AFLP) analysis. Twenty-five populations of seven species of native azalea were analyzed using three primer pairs that amplified a total of 417 bands. Based on analysis of molecular variance (AMOVA) and estimates of Nei's coefficients of gene diversity (H_S, H_T, and G_{ST}), the majority of variation found in deciduous azalea occurs within populations. Both among species and among population variation was low, likely the effect of common ancestry as well as frequent introgression among members (and populations) of section *Pentanthera*. The latter was evident in four populations of *R. prunifolium* and *R. canescens* that were highly related to *R. austrinum* and *R. viscosum*, respectively. Despite these outliers, the majority of populations were grouped into species based on Nei's unbiased genetic distances viewed as a UPGMA phenogram. The significance of these results is discussed in relation to breeding in section *Pentanthera*.

INTRODUCTION

Deciduous azalea species (Division: Magnoliophyta; Class: Magnoliopsida; Subclass Dilleniidae; Order: Ericales; Family: Ericaceae; Genus: *Rhododendron*; Section: *Pentanthera*) are indigenous to the eastern United States from southeastern Canada southwestward to east Texas and east to the Atlantic coast. The deciduous azalea is an outstanding spring-summer blooming woody ornamental, and along with evergreen azaleas, is a common plant in eastern U.S. landscapes (Gallee, 1987). The level of genetic diversity among species has been addressed in a previous study, yet no research has described the amount of genetic diversity among populations of a single species or within individual populations. This research was conducted to answer these questions.

Scharff (1911) postulated that the introduction and migration of deciduous azaleas to North America occurred from Asia, via the Bering Strait land bridge, in the early Tertiary to Pre-Pliocene periods. Despite the 5-65 million years since, little change was observed in chromosome number and morphology when members of sect. *Pentanthera* were compared to possible Asian founder species *Rhododendron schlippenbachii* Maximowicz from Korea, *R. molle* Blume from China and *R. japonicum* (Gray) Sur. from Japan (Sax 1930; Li, 1957). Judd (1915) successfully produced interspecific hybrids between the geographically isolated diploid *R. occidentale* (Oregon-California) and tetraploid *R. calendulaceum* (Virginia-Georgia). Rehder (1921) successfully hybridized *R. luteum* (Eastern Europe) and *R. canadense* (Eastern Canada-Northeast United States). All *Rhododendron* spp. within sect. *Pentanthera* have subsequently been hybridized (Wherry, 1943; Skinner, 1961; Galle, 1967). Coupled, the results of chromosome and breeding studies indicate a high degree of chromosome stability within *Rhododendron*

spp. sect. *Pentanthera*. They also suggest species identity is maintained through geographic isolation via habitat preference and temporal patterns of flowering rather than interspecific crossing barriers.

Kron et al. (1993a) utilized chloroplast DNA markers to distinguish two species of deciduous azalea, *R. canescens* and *R. flammeum* and also to measure introgression in a hybrid swarm located at Stone Mountain, GA. Morphological markers were utilized to confirm results and measure the level of introgression within the population. In the hybrid population of *R. canescens* and *R. flammeum*, interspecific hybridization and introgression were confirmed in more than 75% of the surveyed individuals. This study verified the value of DNA-based studies to distinguish between species of deciduous azaleas and indicated that introgression in native azaleas could hamper genome-based studies of genetic diversity. Iqbal et al. (1995) proved the usefulness of whole-genome analysis to distinguish germplasm within genus *Rhododendron*. They employed RAPD analysis to detect genetic relatedness of both evergreen and deciduous species and cultivars of *Rhododendron* and to test if RAPD analysis of *Rhododendron* spp. reflected their true genetic relationships. Using cluster analysis, species and cultivars were grouped as predicted based upon origin and known pedigree.

Scheiber et al. (2000) sequenced the entire internal transcribed spacer (ITS) region, including ITS1, ITS2, and the 5.8S subunit, of sixteen members of *Rhododendron* sect. *Pentanthera* in an effort to group species based upon genetic similarity. The ITS region is unique in that it is transcribed yet untranslated, therefore postulated to change freely via deletion or addition without altering gene products. This study was the first to focus on members of sect. *Pentanthera* and results indicated extremely little variation in

ITS sequence, ranging from 0.00 to 1.67%. Studies among species within section *Pentanthera* are needed that will sample total genomic variation and give high resolution of genomic variability. Amplified fragment length polymorphism (AFLP) has this ability as it produces a large number of polymorphisms when no prior sequence information is known (Vos et al., 1995). For example, Barker et al. (1999) found 170 polymorphic bands with 20 RAPD primers in Salix, yet using 4 AFLP primers found 645 polymorphic bands. AFLP is also highly reproducible whereas RFLP and RAPD results are difficult to reproduce. Winfield et al. (1998), in an AFLP analysis of Black Poplar, found that over five trials at five varying laboratories, banding patterns were 97.6% to 100% similar.

A naturally occurring population of native deciduous azalea is determined to be comprised of a single species if all individuals within that population resemble one species in the vast majority of phenotypically measurable traits. Results presented by Kron (1993a) suggest introgression could be a major obstacle in any genome-based analysis of native azalea species. Introgression is the infiltration of genetic material from one species into the germplasm of another through repeated backcrossing (Anderson, 1949) and can result in the selection of naturally occurring populations that resemble one species but contain genetic material from another (or several other) species(s). This phenomenon can confound results of genetic studies by creating a significant downward bias in genetic diversity estimates among species and a significant upward bias in genetic diversity within populations. Many native azalea populations contain obvious hybrid individuals as well as individuals that resemble one to several species (Galle, 1987). A far fewer number of populations contain only individuals that morphologically resemble accepted taxonomic keys. Therefore, in this study, populations were selected after

rigorous examination of morphologic characters across each population, based upon the accepted morphologic key presented by Kron (1993b). Thereafter, we utilized AFLP analysis to ascertain the level of genetic diversity among and within naturally occurring species and populations of seven species of deciduous azaleas.

MATERIALS AND METHODS

Plant Material and Collection. Seven species of deciduous *Rhododendron* spp. (sect. *Pentanthera*) native to the eastern United States were included in this study. The species included *R. austrinum* (Small) Rehder, *R. calendulaceum* (Michaux) Torrey, *R. canescens* (Michaux) Sweet, *R. flammeum* (Michaux) Sargent, *R. atlanticum* Ashe, *R. prunifolium* (Small) Millais, and *R. viscosum* (L.) Torrey. Three to six populations per species (Table 1) were selected as viable collection sites based on three parameters: isolation from other *Rhododendron* (sect. *Pentanthera*) species or hybrid swarms, size of population with a minimum of 15 individuals in the population of interest, and morphologic characteristics. An extensive morphologic key by Kron (1993b) was used to identify populations of a single species with no hybrid individuals. Traits including leaf shape and size, pubescence patterns, vein patterns, stem color, stipules, petiole length, flower color, flowering time, seed pod pubescence and size, bud scale patterns and color, and growth habit and habitat were examined at three intervals over a one year period prior to collection. Leaf samples were collected from six individuals per population approximately 7-14 days after leaf emergence yet prior to complete leaf expansion. Selection of individual plants to sample was determined by constructing a grid pattern of

each population and thereafter collecting plants evenly distributed throughout the entire population. Collected leaf tissues were individually stored at -70°C.

AFLP Procedure. The E.Z.N.A.® High Performance (HP) DNA extraction kit (Omega Bio-Tek, Inc, Doraville, GA) and corresponding protocol was utilized in total genomic DNA extraction from 100 mg of frozen leaf tissue. DNA was quantified using a spectrofluorometer and 1.5% agarose gel with Low DNA Mass Ladder™ (Invitrogen™, Carlsbad, CA) while simultaneously checking for quality (shearing). Subsequently, genomic DNA was stored at -20°C in sterile deionized water. Restriction-digestion, ligation, and pre-selective amplification (Table 2) of genomic DNA was carried out using the Li-Cor, Inc. (Lincoln, NE) IRDye™ AFLP Template Preperation Kit. All polymerase chain reactions (PCR) were carried out in a Perkin-Elmer Model 9600 Thermal Cycler® (Wellesley, MA). Thirty primer pairs (Table 2) were screened on two individuals of each species included in this study. Three primer pairs; E-ACA/M-CTG, E-ACT/M-CAC, and E-AGG/M-CAT were selected based upon number of polymorphic bands visualized on a gel and repeatability of band presence. Only *Eco*RI primers (100µM) with fluorescence at 700 nm (Li-Cor® Biosciences, Lincoln, NE) were selected as band intensity was extremely low for all 800 nm primers.

Selective amplification was carried out on all individuals with each of the three selected primer pairs. In a 1.5ml microcentrifuge tube (Fisher Scientific Company L.L.C.), based on total sample number to be selectively amplified in a single reaction, the following were combined: 2.15µl sterile deionized water, 2.0µl $MgCl^2$ (Promega Corp., Madison, WI), 0.05µl GOTaq® DNA polymerase (Promega), 2µl 5x GOTaq buffer (Promega), 0.8µl 100mM dNTP (Promega), 0.5µl *Mse*I primer, and 0.5µl *Eco*RI primer.

In each well of a Fisherbrand 96 well PCR plate (Fisher Scientific Company L.L.C.), 8µl of the aforementioned mix was combined with 4µl of template DNA from the preselective amplification stage. PCR conditions for selective amplification were set based on the Li-Cor AFLP protocol. Following completion of the selective amplification PCR program, products were immediately denatured. Three microliters of Blue Stop Solution® (Li-Cor® Biosciences) were added to each well and samples denatured at 94°C for 4min. Samples were then cooled to 4°C by placing the PCR plate on ice and/or into the refrigerator.

Gels were cast using Li-Cor 25cm glass plates with 0.25mm spacers. Twenty ml of 6.5% KB Plus acrylamide gel solution was combined with 150µl APS (Fisher Scientific Company L.L.C.) and 15µl Temed (Fisher Scientific Company L.L.C.). DNA was loaded at a volume of 0.4µl per well. Each gel included all individuals from a single species-primer pair combination. Each gel also included two standards to enable efficient and reliable gel comparison in the scoring process. The first standard employed was a Li-Cor IRDye® 50-700 size standard, placed on the outside two lanes of each gel. The second standard was a panel of each species, with one individual of each species included in all gels. Extraction and analysis was repeated in thirty-three percent of the individuals to ensure repeatability of banding patterns.

Gels were run on a Li-Cor Model 4300S DNA Analysis System using the SagaLite® software package (Li-Cor Biosciences Inc.) with laser focus adjusted on a run-by-run basis to optimize performance. Run length was set to four hours with KBplus standard electrophoresis conditions. Standard power and temperature settings were utilized with the exception of voltage that was reduced to 1000 to allow for increased low

base pair band separation. Gel images produced by SagaLite® were graphically adjusted within the program and exported to GelBuddy (Zerr and Henikoff, 2005) whereby gels were graphically aligned using monomorphic banding patterns. Image files were then exported to Adobe Photoshop® CS2 (Adobe Systems Inc., San Jose, CA) and all gel images from a single primer pair merged into a single graphics file. Individual gel images were aligned using three standards; the Li-Cor IRDye® 50-700 size standard, the panel lanes that included one sample of each species, and monomorphic bands that were shared by all species. The resulting single graphics file was utilized in the scoring of gels.

Data Analysis. Bands were manually scored in binary format as present (1) or absent (0) and values recorded in Microsoft® Excel, including monomorphic bands. Data from the three primer pair combinations required a three-fold increase in the quantity of columns available in Microsoft® Excel. Therefore, GS-Calc 7.1 (JPS Development, Palmer Lake, CO) was used to combine data sets from the three primer pairs into a single data set that was manipulated as needed for individual data analysis programs.

PopGene v.3.2 was used to calculate Nei's genetic diversity (Nei, 1987) and percentage of polymorphic loci. Settings for analysis included a significance level of $p \leq 0.05$, seven groups (one for each species), and 10,000 simulations. A matrix of Nei's unbiased genetic distances (Nei, 1978) was calculated by PopGene v.3.2 (Yeh and Boyle, 1997) using all markers, including monomorphs. Nei's unbiased genetic distance is the most accurate and reliable estimate of genetic diversity when population sizes are small (Nei, 1978). An unrooted UPGMA phenogram based on Nei's unbiased genetic distance matrix over all populations (Nei, 1978) was produced using Treeview 1.6.6 (Page, 1996).

Analyses of molecular variance (AMOVA) was calculated among species using Arlequin v.2.000 (Schneider et al., 2000) to determine the hierarchical partitioning of genetic variability among all species, populations within a single species, and within each population. Hickory v.1.0.4 (Holsinger and Lewis, 2002) was utilized to calculate panmictic heterozygosity based on mean allele frequencies (H_T), average panmictic heterozygosity within each population (H_S), and the Bayesian analog of Nei's G_{ST} (Holsinger, 1999). Data was analyzed among and within species using a 250,000 burn-in and 500,000 randomizations.

RESULTS AND DISCUSSION

Level of polymorphism. Three AFLP primer combinations (Table 2) amplified a total of 417 scorable bands. The average repeatability of AFLP fragments across two replications was 97.4% (data not shown). The primer pair *Mse*I-CTG/ *Eco*RI-ACA amplified 142 bands; the primer pair *Mse*I-CAC/ *Eco*RI-ACT amplified 134 bands; and the primer pair *Mse*I-CAT/ *Eco*RI-AGG amplified 141 bands. Unbiased genetic distance matrices were calculated from each of the three primer pairs and compared by Mantle test in three possible combinations. Results indicate that the information generated by each of the primer pairs was consistent ($r > 0.9$, $P < 0.01$) across primer combinations. The percentage of polymorphic loci across all species was 89.69% (Table 3). Within species, polymorphic band percentages ranged from 86.81-91.85%. The high degree of polymorphism is not due to a single species or population within a species, but rather polymorphic loci are spread evenly across all species and individual populations.

Diversity among species. Based on AMOVA results, the proportion of variation among species is only 14% (Table 4). Studies by Kurashige et al. (2001) and Scheiber et al. (2000) present similar findings as to the low level of genetic diversity among members of *Rhododendron* spp. sect. *Pentanthera*. In the latter study, the entire ITS region was analyzed to determine genetic relatedness of members of *Rhododendron* spp. sect. *Pentanthera*. The ITS region has been used to study interspecific genetic structure because subtle genetic differences at the base level have been observed. Therefore, the agreement of ITS data and AFLP data from this study indicate that members of *Rhododendron* spp. sect. *Pentanthera* are highly related and possibly derived from a common ancestor. Sax (1930) and Li (1957) noted considerable chromosome similarity among possible Asian founder species and North American native azalea species. These included *R. molle* from China and *R. japonicum* from Japan that are in sect. *Pentanthera*, and *Rhododendron schlippenbachii* from Korea that is in sect. *Sciadorhodion*. Further research, such as chloroplast DNA studies, is needed to conclusively determine the ancestry of U.S. native deciduous azalea species. Alone, common ancestory is unlikely to explain all of the present-day genetic similarity among species since the proposed migration of sect. *Pentanthera* into North America 5-65 million years ago. The low proportion of genetic variation among species is likely a result of common ancestry coupled with an abnormally high level of naturally occurring interspecific hybridization and introgression events such as that deduced by Kron (1993a). Breeders should be aware of the effects of introgression when using plants collected in the wild as parents, such as confounding genetic ratios and the possibility of unexpected phenotypic characters in progeny of crosses.

Diversity among populations. The proportion of species genetic diversity attributed to among population variation ($Gst = 1 - \frac{Hs}{Ht}$), whereas H_S is the average genetic diversity within populations and H_T is the genetic diversity within species, is a critical indicator of genetic diversity at the population level. The proportion of total genetic diversity that occurs among populations over all species and loci (G_{ST}) was 0.29, and within each species ranged from 0.09 in *R. flammeum* to 0.26 in *R. prunifolium* (Table 3). The relatively low overall G_{ST} indicates a low to marginal proportion of diversity (29%) is observed among populations as opposed to a high level of diversity observed within populations (71%). Low G_{ST} values also indicate a high level of gene flow among populations, which tends to homogenize a species' genetic structure. This is expected as members of *Rhododendron* spp. sect. *Pentanthera* are entomophilously pollinated (Gallee, 1987; Hamrick and Godt, 1996). Insect pollination leads to populations with a high level of genetic variation while individuals within the population share a similar complement of alleles in similar frequencies (Falk et al., 2001; Hamrick and Godt, 1996). AMOVA (Table 4) supports G_{ST} values and indicates a high level of variation is observed within populations (83%) and low proportion of variation among populations (3%; $P < 0.05$). The relatively low G_{ST} value coupled with low proportion of variation among populations from AMOVA further indicates that individuals within populations are likely to be genetically distinct; however each population contains a very similar complement of alleles in similar frequencies (Falk et al., 2001). Thus, from a breeding standpoint, the low percentage of among population variation is important in the selection of parents. Within each species, individuals obtained from geographically isolated populations will not substantially increase allelic diversity in a breeding program.

Diversity within populations. AMOVA and G_{ST} values correspond to the proportion (percentage) of genetic variation partitioned among species, among populations, and/or within populations. H_S and H_T values, conversely, are a direct measure of diversity within populations and within species, respectively (Falk et al., 2001). H_S and H_T values offer insight into the actual level, rather than proportion, of genetic variation within populations and within each species.

The average genetic diversity within populations (H_S) was low to moderate for all species, ranging from 0.29 in *R. austrinum* and *R. viscosum* to 0.35 in *R. calendulaceum* (Table 3). A likely cause for lower average diversity in *R. austrinum* and *R. viscosum* is population size. All *R. austrinum* and *R. viscosum* populations surveyed inhabited a relatively small geographic area (< 0.7 km^2). The reduced geographic size of the populations allow for pollen to be easily transported among all individuals of the population, effectively reducing within-population genetic diversity. This is unlike larger populations where the migratory range of insects may only cover a portion of the entire population and, therefore, facilitate higher within population diversity. In addition to small population size, *R. viscosum* also has a flowering time 1-2 months later than any other species with the exception of *R. prunifolium*. The offset reproductive cycle, in comparison to other members of *Rhododendron* spp. sect. *Pentanthera*, reduced the potential of introgression with adjacent species in past generations that could lead to increased within-population diversity.

The species with the highest average genetic diversity within populations (H_S), *R. calendulaceum* (0.35; Table 3), is the only tetraploid member of *Rhododendron* spp. sect. *Pentanthera* (*n*= 26, 2*n*= 52). Li (1957) concluded, based upon differing sizes of

chromosome complements, that *R. calendulaceum* is an allotetraploid, which may explain the higher within-population diversity. *R. flammeum* also has comparatively high genetic diversity within populations (0.34) and, interestingly, exhibits a high level of within-population phenotypic variability. Of all members of *Rhododendron* spp. sect. *Pentanthera*, *R. canescens* occupies the largest geographic range and inhabits the largest variety of habitats. As the habitats and bloom cycle of *R. flammeum* overlap with *R. canescens*, past introgression events, undetectable when assessing morphologic traits, may explain the observed variability. Kron (1993b) documented this in a study involving a natural population of *R. canescens*, *R. flammeum*, and hybrids of the two species. It was discovered that despite phenotypic assignment of an individual to *R. flammeum*, chloroplast DNA proved that the individual contained alleles from *R. canescens*. Because anthesis of *R. canescens* typically precedes that of *R. flammeum*, pollen of *R. canescens* is available to pollinate *R. flammeum* flowers after its flowers open yet prior to anthesis (Kron, 1993b).

Within species diversity. Genetic diversity within species (H_T) was low to moderate, ranging from 0.34 in *R. austrinum* to 0.41 in *R. prunifolium* (Table 3). The relatively low level of within species diversity of *R. austrinum* may be due to the small geographic range of the species and hence short distance between population sites (Falk et al., 2001). No two populations were greater than 34 km apart and populations from such a close proximity are more likely to be highly related than distant populations. *R. viscosum* had similar within species diversity (0.36) despite geographic distances between populations of at least 237 km. Low species diversity in *R. viscosum* is most likely a direct result of disjointed flowering time. In this study, collection of *R. viscosum* was also conducted

outside the geographic range of *R. prunifolium* to minimize the potential of contamination via introgression between these two species with parallel annual bloom cycles.

R. prunifolium exhibited the highest within species diversity (0.41) and this diversity was exhibited not only in the pooled H_T value but also in two random subsamples of three populations (H_T = 0.40, 0.41; data not shown), indicating the diversity was not due to the greater number of populations sampled. Phenotypic and habitat variation among populations explains the discrepancy between relatively low within-population genetic diversity (H_S = 0.30; Table 3) and relatively high within species genetic diversity (0.41) and is supported by a modest correlation between Nei's unbiased genetic distance (Nei, 1978) with geographic distance (r = 0.432, p = 0.02). *R. prunifolium* was the only species in the study to show a significant correlation ($p \leq 0.05$) of genetic distance and geographic distance (data not shown). Parapatric speciation may explain this result, although a much broader sampling of populations will be required to determine if this phenomenon is present. *R. prunifolium* and *R. flammeum* are the two most phenotypically variable species included in this study. *R. flammeum* principally inhabits hardwood forest ecosystems and displays increased phenotypic variation within individual populations. Conversely, *R. prunifolium* inhabits a wide variety of ecological niches with greater phenotypic diversity observed among populations, ranging from upland hardwood forests at the northern species range to stream basins at the southern range. Greater phenotypic variation observed among populations, coupled with diverse habitat preference across the species range, suggests that some level of genetic divergence is occurring along the north-south axis of *R. prunifolium*'s geographic range.

R. canescens also exhibited a relatively high level of within species variation (0.40). The level of variation among populations of *R. canescens* was unexpected as phenotypically the species is very stable. There are two possible explanations for these results. Because of the broad geographic range of *R. canescens*, the largest of any member of sect. *Pentanthera*, a small upward bias in within species diversity may be expected as species with broad ranges generally exhibit higher H_T values. The relatively higher level of variation may also be the result of past interspecific hybridization (introgression) events with a number of related species. The geographic ranges of four separate species with similar annual flowering times overlap three of the collection sites of *R. canescens*. The Meriwether Co., GA and Baldwin Co., GA populations are within the range of *R. flammeum*; the *Bullock Co., GA* population is with the *R. atlanticum* range; and the Lee Co., AL population is within the ranges of *R. alabamense* and *R. cumberlendense* (Kron 1993b). The introgression of even a single individual in past generations of these populations could be the cause of a slight upward bias in within species diversity, despite the lack of phenotypic evidence, as introgression would have introduced variable genetic material into each of the populations surveyed.

Effects of clonal structure on diversity estimates. H_S and H_T values of *R. atlanticum* were intermediate to other species in this study. However, the G_{ST} value for *R. atlanticum* is low at 0.10 (Table 3). *R. atlanticum* is the only species incorporated into this study that forms dense clonal patches. In the populations surveyed for this study, multiple genotypes formed interconnecting patches with minimal breaks. Because of dense cover, it is likely that seedling germination and/or survival is low within the bounds of the colonized area. This plant habit was possibly the result of a founder effect. Population

genesis likely occurred when one or several plants intermated and colonized the habitable area surrounding the introduction site. Such phenomena, observed in the related species, *Rhododendron ferrugineum* L. (Wolf et al., 2004), prevents the introduction of alleles from other populations or species, leading to genetically diverse yet highly related individuals that share a majority of alleles due to common ancestry.

Evidence of introgression based on genetic distance comparisons. As expected, Nei's unbiased genetic distances (Nei, 1978) among populations were lower within species compared to among species (Table 5). Additionally, the phenogram derived from Nei's unbiased genetic distances (Figure 1) provided expected grouping of species into individual branches based on the accepted morphological key generated by Kron (1993b). Only four of the 25 populations surveyed in this study showed distinct signs of introgression (Table 5; Figure 1). Genetic distances indices for *R. prunifolium* fall into two distinct groups (Table 5). The three populations collected from the southern area of the species range are highly related, with indices of 0.10-0.11. The three northern populations are also highly related, with values of 0.07-0.09. A comparison of genetic distances between populations of the northern and southern groups show less relatedness among the two sets of populations (0.21-0.24). Remarkably, these values are similar to those across species in this study. When genetic distances are visualized as a UPGMA phenogram (Figure 1), the disassociation between northern and southern populations is evident. Northern populations occupy an independent branch-point of the phenogram while southern populations share a major branch with *R. austrinum* populations. This is corroborated by genetic distance indices between southern *R. prunifolium* populations and *R. austrinum* populations that range from 0.08-0.12 (Table 5). These genetic distance

values are extremely low for between species comparisons and closely resemble values seen within a species. This was unexpected considering all populations were phenotypically verified at several growth stages and the two species exhibit distinct flowering periods, with *R. austrinum* flowering 2-3 months prior to *R. prunifolium*.

Despite present-day morphologic differences, major climate shifts possibly led to an intermingling of the two species geographic range and/or annual flowering period. Studies suggest the occurrence of such an event in woody cross pollinated species native to the eastern United States. Two examples are *Liquidambar styraciflua* and *Liriodendron tulipifera* that purportedly migrated south to the gulf coast at the period of glacial maxima of the Pleistocene period (Hoey, 1990; Parks et al., 1994). In the case of *R. prunifolium*, any migration southward of the species during this same geologic period would intermingle at least the southern populations of *R. prunifolium* with existing *R. austrinum* populations. Breeders should be aware of the genetic, morphologic, and habitat differences between southern and northern populations when breeding with *R. prunifolium*.

Within species genetic diversity of three populations of *R. canescens* was similar to that of other species in this study (0.04-0.05; Table 5), with the exception of a single population located in Bullock Co., GA (0.26-0.28). The phenogram of genetic distances (Figure 1) groups this population in a major branch shared with *R. viscosum* and a minor branch with only the Bullock Co., GA *R. viscosum* population. These two populations are located 6 km apart, an acceptable distance based on our collection parameters and considering differential bloom periods. Yet it appears that introgression has occurred at some time between the two populations, possibly the result of severe drought or

significant late freeze/cold weather pattern. During a severe drought in central Georgia during 2001-2002, the researcher witnessed no flowering in two populations of *R. viscosum* in Harris and Meriwether County, GA in the summer of 2002. The 2003 spring brought near normal rainfall and plants in each population began sporadically flowering in mid March, two months prior to normal bloom time. Such an event could align the flowering periods and may explain these results. As genetic diversity is very low within species, relatively few hybrid individuals could potentially cause an upward bias in genetic distance values.

Rehder (1921) and Skinner (1955, 1961) postulated that naturally occurring interspecific hybridization (introgression) plays an important role in creating and maintaining morphological (and genetic) diversity and Skinner went further by stating introgression was a major component in the evolution of members of sect. *Pentanthera*. We further propose that introgression within sect. *Pentanthera* serves to reduce inbreeding depression within individual populations, as AMOVA results from this study indicate an extremely low proportion of genetic diversity among populations within species (3%, Table 4), and only slightly higher genetic variation among species (14%). No studies have been conducted in native azaleas to assess the overall vigor of a species or population when introgression is minimized by geographic distance or disjointed flowering time. Yet many studies in other cross-pollinated species have noted reductions in population vigor, due to inbreeding depression, within isolated and/or small populations (Keller and Waller, 2002). Although extremely difficult to measure, the same principle should hold true in larger populations with low allelic diversity. Because members of sect. *Pentanthera* primarily outcross and contain low levels of genetic

variation among populations of a single species, it is reasonable to assume that a reduction in heterosis would occur in native azalea populations (or species) isolated for multiple generations. Therefore introgression events would serve to increase heterosis within the population, essentially 'rescuing' the population from the effects of inbreeding depression, specifically the fixation of deleterious alleles that reduce fecundity and the ability of a population to adapt to environmental stress and/or change.

AFLP markers exhibited a high level of efficiency in detecting genetic variation within *Rhododendron* section *Pentanthera*. The results of this study indicate that members of *Rhododendron* section *Pentanthera* are highly related, both among and within species, despite occupying a vast geographic area. The proportion of variation among species and among populations of each species is low, with the greatest proportion of genetic variation residing within individual populations. When assessing the actual (not proportional) variation of individual species, in addition to low among species and among population diversity, within-population variation is also relatively low.

Three points have been illuminated by this study in relation to the breeding of North American native azaleas. First, breeders should take precautions to ensure breeding material, if deemed to be a pure species, has not been compromised via introgression. Identification of populations consisting of one species without hybrid individuals, based on phenotypic keys, was difficult in the field. In several instances, multiple observations of phenotypic characters indicated a population consisted of a single species, yet genetic data suggests introgression had occurred. Second, there is a low level of genetic diversity observed among populations within species. While genetic diversity does exist among populations, the relatively low level of diversity indicates that the importance of

collecting individuals from multiple geographically isolated populations to obtain allelic diversity in parent stock is not critical. Third, results illuminate potential pitfalls in the conservation of native azaleas in the eastern United States. The low levels of genetic diversity, coupled with its entomophilous outcrossing nature, suggest that gene flow both among populations and among species (introgression) is important in maintaining the heterosis of populations and/or species. Fragmentation or isolation of populations due to anthropogenic activity could have a significant and swift negative effect on the health, vigor, and adaptability of individual populations and/or species. Studies should be conducted to identify those species that may be at risk either due to small geographic range (*R. flammeum*, *R. prunifolium*) or habitat preferences that collide with development preferences (*R. atlanticum*, *R. flammeum*).

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Table 1. Locations of populations collected, grouped by species. Each population is numbered in succession and includes six individual plant samples also listed in consecutive order.

Population Number	Species	Location	Sample Numbers
1	*Rhododendron austrinum*	Holmes Co., FL	1-6
2	*R. austrinum*	Washington Co., FL	7-12
3	*R. austrinum*	Vernon, FL	13-18
4	*R. prunifolium*	Georgetown, GA	19-24
5	*R. prunifolium*	Morris- Quitman Co., GA	25-30
6	*R. prunifolium*	Randolph Co.,GA	31-36
7	*R. prunifolium*	Randolph Co.,GA	37-42
8	*R. prunifolium*	Harris Co., GA	43-48
9	*R. prunifolium*	West Point, GA	49-54
10	*R. calendulaceum*	Amherst Co., VA	55-60
11	*R. calendulaceum*	Towns Co., GA	61-66
12	*R. calendulaceum*	Macon Co., NC	67-72
13	*R. viscosum*	Bullock Co., GA	73-78
14	*R. viscosum*	Essex Co., VA	79-84
15	*R. viscosum*	Meriwether Co., GA	85-90
16	*R. canescens*	Bullock Co., GA	91-96
17	*R. canescens*	Baldwin Co., GA	97-102
18	*R. canescens*	Lee Co., AL	103-108
19	*R. canescens*	Spalding Co., GA	109-114
20	*R. atlanticum*	Dinwiddie Co., VA	115-120
21	*R. atlanticum*	Carteret Co., NC	121-126
22	*R. atlanticum*	Florence Co., SC	127-132
23	*R. flammeum*	Milledgeville, GA	133-138
24	*R. flammeum*	Thomaston, GA	139-144
25	*R. flammeum*	Fulton Co., GA	145-150

Table 2. List of adaptors and primers screened and used in this study to characterize the amplification fragment length polymorphism (AFLP) band patterns in seven *Rhododendron* spp.

	MseI site	EcoRI site
Adaptors	5' TACTCAGGACTCAT 3' 5' GACGATGAGTCCTGAG 3'	5' CTCGTAGACTGCGTACC 3' 5' AATTGGTACGCAGTCTAC 3'
Pre-selective amplification primers	5' GATGAGTCCTGAGTAAC 3'	5' GACTGCGTACCAATTCA 3'
Selective amplification primers screened	*Mse* I-CTT *Mse* I-CAC *Mse* I-CTG *Mse* I-CAT *Mse* I-CTA	*Eco* RI-ACT 700 [z] *Eco* RI-ACA-700 *Eco* RI-AGG-700 *Eco* RI-ACC-800 *Eco* RI-AGC-800 *Eco* RI-AAC-800
Selective amplification primers sets [y]	*Mse* I-CTG *Mse* I-CAC *Mse* I-CAT	*Eco* RI-ACA-700 *Eco* RI-ACT 700 *Eco* RI-AGG-700

[z] *Eco*RI primers were labeled with one of two Li-Cor fluorescent tags with peak fluorescence at 700 or 800 nm.

[y] Three primer sets selected from 30 screened primer pairs.

Table 3. Percentage of polymorphic loci, average genetic diversity within populations (H_S), average genetic diversity within species (H_T), and proportion of species genetic diversity attributed to among population variation (G_{ST}) for seven species of *Rhododendron* section *Pentanthera*.

	Polymorphic Loci Percentage	H_S	H_T	G_{ST}
OVERALL	89.69	0.3109	0.4372	0.289
R. atlanticum	87.53	0.3287	0.3661	0.1021
R. austrinum	86.81	0.2931	0.3420	0.1431
R. calendulaceum	90.27	0.3479	0.3949	0.1190
R. canescens	91.85	0.3107	0.4023	0.2278
R. flammeum	89.69	0.3434	0.3789	0.0937
R. prunifolium	89.11	0.3011	0.4066	0.2594
R. viscosum	88.25	0.2910	0.3572	0.1853

Table 4. Analysis of molecular variation (AMOVA) for seven *Rhododendron* spp. included in this study.

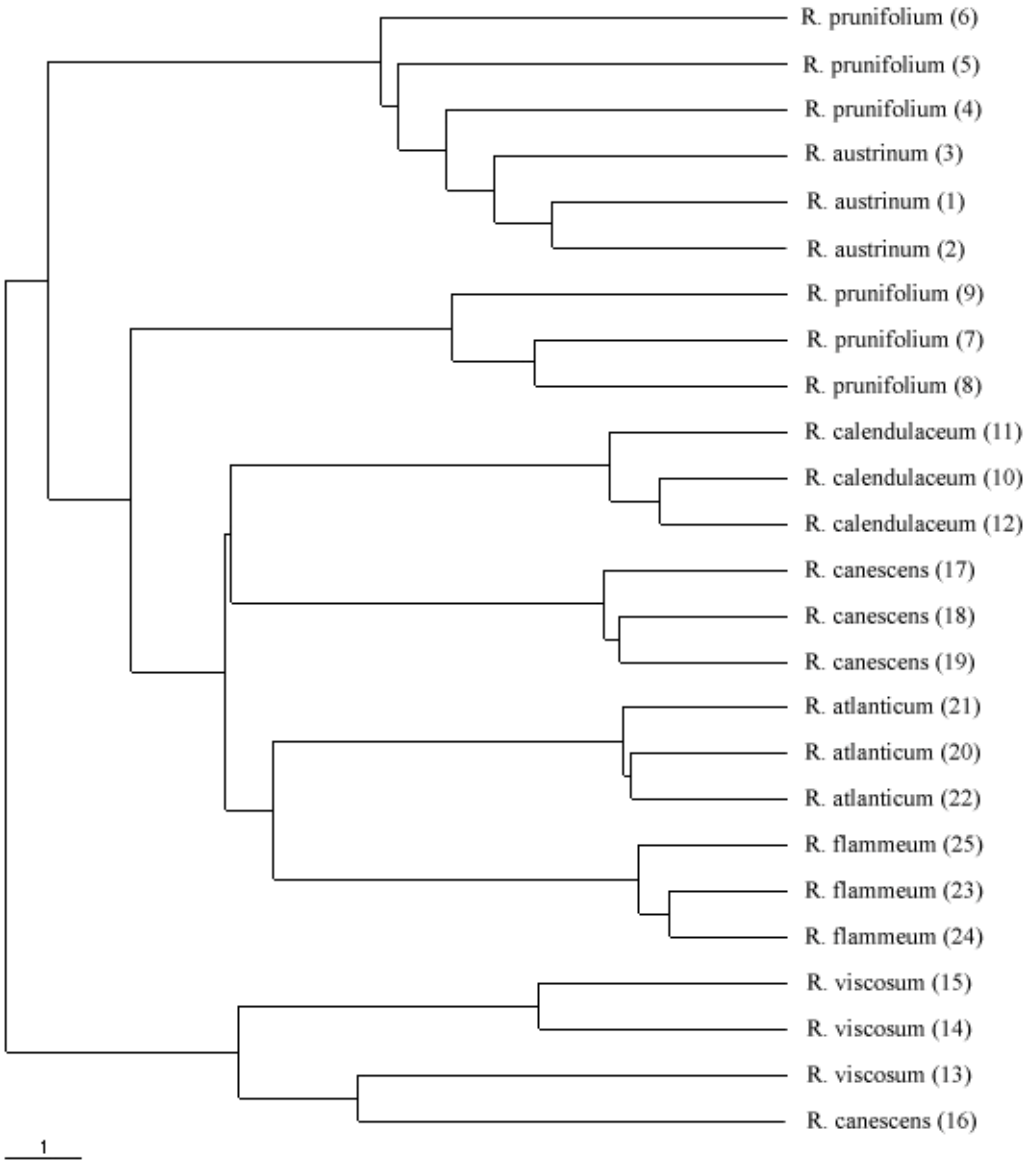
Source of variation	d.f.	Sum of squares	Variance components	Variance percentage	Probability ***P***
Among species	6	2248.74	13.30	14.14	<0.05
Among populations within species	18	1702.94	2.80	2.99	<0.05
Within populations	125	9722.33	77.80	82.88	
Total	149	13674.01	93.85		

Table 5. Nei's unbiased measures of genetic distance (Nei, 1978) below diagonal and geographic distance (km) above diagonal.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 [z]	****	14	34	151	172	151	177	254	250	987	504	549	419	1143	309	415	375	227	335	1019	958	708	364	279	362
2	0.061	****	19	155	180	174	147	254	258	990	506	546	422	1146	312	420	380	232	336	1022	963	712	362	282	364
3	0.076	0.077	****	161	164	161	177	185	259	267	998	514	554	430	1153	320	388	240	328	1032	971	721	372	290	372
4	0.083	0.099	0.083	****	26	29	16	105	108	836	351	390	311	998	156	306	232	90	180	882	839	584	219	127	209
5	0.110	0.089	0.097	0.108	****	16	29	118	122	844	364	403	308	1003	166	306	237	106	190	887	515	584	222	135	222
6	0.102	0.125	0.093	0.103	0.104	****	19	98	109	818	338	378	279	972	138	283	213	98	209	857	811	551	192	109	195
7	0.204	0.232	0.171	0.184	0.190	0.165	****	98	108	813	333	375	282	974	140	285	208	98	164	853	810	555	193	109	200
8	0.227	0.272	0.236	0.225	0.211	0.226	0.065	****	27	742	248	287	280	913	68	287	158	47	90	800	776	522	156	52	106
9	0.246	0.280	0.255	0.241	0.210	0.216	0.082	0.091	****	760	253	295	311	932	90	314	182	26	109	818	802	547	182	77	114
10	0.219	0.246	0.186	0.226	0.242	0.183	0.159	0.187	0.195	****	517	473	639	211	681	644	609	782	655	182	396	398	630	708	646
11	0.220	0.221	0.188	0.224	0.212	0.179	0.146	0.208	0.173	0.035	****	42	333	696	201	338	182	280	177	601	642	407	211	230	142
12	0.210	0.220	0.181	0.215	0.234	0.192	0.156	0.190	0.182	0.033	0.057	****	351	658	240	356	213	322	216	568	615	393	242	270	184
13	0.206	0.332	0.197	0.231	0.196	0.216	0.197	0.228	0.235	0.168	0.187	0.192	****	758	237	6	167	328	232	633	543	299	147	237	287
14	0.206	0.209	0.175	0.205	0.216	0.189	0.210	0.242	0.230	0.175	0.177	0.196	0.064	****	847	760	766	956	823	127	338	472	74	873	823
15	0.206	0.222	0.178	0.201	0.215	0.231	0.201	0.181	0.190	0.146	0.178	0.182	0.150	0.127	****	172	92	113	26	736	713	457	97	95	72
16	0.219	0.267	0.207	0.226	0.230	0.222	0.194	0.218	0.206	0.216	0.221	0.213	0.105	0.130	0.112	****	172	332	237	638	543	301	151	240	291
17	0.157	0.167	0.147	0.184	0.157	0.175	0.171	0.214	0.184	0.155	0.130	0.136	0.220	0.196	0.229	0.284	****	204	76	650	621	365	29	109	121
18	0.167	0.201	0.165	0.163	0.166	0.138	0.142	0.203	0.168	0.151	0.132	0.143	0.188	0.201	0.233	0.255	0.048	****	135	844	826	570	204	98	140
19	0.188	0.200	0.160	0.187	0.156	0.161	0.142	0.214	0.180	0.159	0.144	0.151	0.213	0.212	0.250	0.260	0.046	0.044	****	713	694	438	87	55	61
20	0.173	0.191	0.201	0.196	0.171	0.167	0.177	0.199	0.198	0.148	0.145	0.171	0.174	0.171	0.183	0.202	0.142	0.152	0.143	****	233	343	665	760	713
21	0.206	0.213	0.205	0.235	0.163	0.180	0.157	0.173	0.163	0.147	0.154	0.157	0.184	0.187	0.169	0.222	0.139	0.165	0.152	0.041	****	256	625	729	718
22	0.188	0.226	0.216	0.222	0.186	0.171	0.148	0.160	0.163	0.141	0.139	0.135	0.167	0.187	0.167	0.210	0.161	0.149	0.146	0.040	0.043	****	370	475	467
23	0.173	0.156	0.130	0.164	0.144	0.168	0.147	0.151	0.142	0.137	0.147	0.137	0.195	0.168	0.168	0.185	0.133	0.134	0.158	0.139	0.134	0.148	****	106	140
24	0.213	0.193	0.153	0.163	0.147	0.175	0.150	0.191	0.151	0.135	0.125	0.113	0.186	0.176	0.173	0.198	0.130	0.137	0.159	0.137	0.122	0.130	0.030	****	98
25	0.184	0.170	0.142	0.151	0.141	0.149	0.145	0.161	0.151	0.165	0.164	0.152	0.198	0.185	0.169	0.198	0.144	0.137	0.166	0.139	0.114	0.139	0.034	0.043	****

[z] Population numbers correspond to the following species; *Rhododendron austrinum* (1-3), *R. prunifolium* (4-9), *R. calendulaceum* (10-12), *R. viscosum* (13-15), *R. canescens* (16-19), *R. atlanticum* (20-22), and *R. flammeum* (23-25).

Figure 1. The unrooted UPGMA phenogram of Nei's unbiased genetic distance matrix (Nei, 1978) over all 25 populations surveyed. Populations are denoted in parenthesis to the right of species identification.



CHAPTER 3

IDENTIFICATION OF THE MECHANISM OF AZALEA LACEBUG RESISTANCE IN DECIDUOUS AZALEA [2]

Chappell, M. and C. Robacker. 2006. Leaf Wax Extracts of Four Deciduous Azalea Geontypes Affect Azalea Lace Bug (*Stephanitis pyrioides* Scott) Survival Rates and Behavior. J. Amer. Soc. Hort. Sci. 131(2): 225-230. [2]

Index words: *Rhododendron* spp., deciduous azalea, epicuticular wax, insect resistance, chloroform wax extraction

ABSTRACT

Azaleas (Ericales: Ericaceae: *Rhododendron* spp.) are a staple plant in many landscapes of the U.S. and are largely resistant to predation by insects, with the exception of azalea lace bug (ALB) (Heteroptera: Tingidae: *Stephanitis pyrioides* Scott). Within deciduous azalea (*Rhododendron*: sect. Pentanthera: subseries Luteum) varying levels of resistance to ALB is observed with a continuous distribution from susceptible to highly resistant. In this study, epicuticular leaf wax from two ALB resistant [*R. canescens* Michaux and *R. periclymenoides* (Michaux) Shinners] and two ALB susceptible ('Buttercup' and 'My Mary') deciduous azalea genotypes was extracted and re-applied to fresh azalea foliage. Leaf wax extracted from ALB resistant genotypes and applied to ALB susceptible genotypes conferred a high level of resistance to both ALB feeding and oviposition in the treated ALB susceptible genotypes. Conversely, leaf wax extracted from ALB susceptible genotypes and applied to ALB resistant genotypes conferred susceptibility to the treated ALB resistant genotypes. However, the effect was much less substantial than the effect of resistant wax extracts on susceptible genotypes and confined to ALB oviposition. When applied to the same genotype from which the extract was collected, leaf wax extract from ALB susceptible genotypes had no effect on susceptibility, whereas resistant wax extract had a moderate effect on ALB oviposition rate. The results indicate that leaf wax serves as a primary mechanism of resistance of deciduous azalea to ALB.

INTRODUCTION

Since its introduction from Japan in 1915, azalea lace bug (ALB) has become a significant pest on azalea throughout North America (Drake and Ruhoff, 1965; Weiss 1916). ALB damage occurs from late spring until leaf drop, as up to four generations can occur in a single growing season (Neal and Douglass, 1988). Adult and nymphal ALB feed on the abaxial leaf surface by inserting stylets into stomata (Ishihara and Kawai, 1981). Chloroplasts and other cell contents are removed from the mesophyll layer, resulting in speckling on foliage of susceptible species and cultivars. Additionally, cast skins of nymphs and brown to black frass deposition on the leaf further discolor foliage (Braman and Pendley, 1992; Buntin et al., 1996; Ishihara and Kawai, 1981; Mead, 1967).

Bioassays performed by Braman and Pendley (1992) revealed the deciduous *R. canescens* and *R. prunifolium* (Small) Millais to be resistant whereas the evergreen cultivar 'Delaware Valley White', a selection of *R. indica* var. *alba*, was susceptible. Further work by Wang et al. (1998) evaluated four cultivars and eleven deciduous species selections and found *R. periclymenoides*, *R. canescens*, and *R. prunifolium* to be highly resistant and *R. serrulatum* (Small) Ahles and *R. viscosum* (L.) Torrey to show moderate resistance to ALB. Moderately susceptible to very susceptible species included *R. arborescens* (Purch) Torrey, *R. austrinum* (Small) Rehder, and *R. oblongifolium* (Small) Millais. Susceptible cultivars included 'Buttercup', a *R. austrinum* selection; 'My Mary', a complex hybrid of (*R. atlanticum* (Ashe) Rehd. x *R. periclymenoides*) x *R. austrinum* seletion; and 'Nacoochee', a *R. atlanticum* x *R. periclymenoides* hybrid.

The principle function of the plant cuticle is to prevent excessive water loss (Hopkins, 1995). The epicuticular wax layer of the cuticle consists of long-chain aliphatic compounds

derived from fatty acid chains. This wax layer is deposited on the leaf surface as amorphous intracuticular wax embedded in cutin polymers, as well as wax crystalloids. Epicuticular wax components include alkanes, primary and secondary alcohols, ketones, and wax esters (Eigenbrode and Espelie, 1995; Knust and Samuels, 2003). Epicuticular wax has been recognized as a deterrent to feeding and oviposition by herbivorous insects as well as an attractant of beneficial (often predatory) insects (Eigenbrode and Espelie, 1995). Specific examples of epiculticular wax-herbivore interactions have been identified and characterized in *Allium cepa* L. (Molenaar, 1984), *Brassica rapa* L. (Bodnaryk, 1992; Srinivasachar and Malik, 1972), *Eucalyptus globulus* Labill (Brennan and Weinbaum, 2001), *Glycine max* L. (Baker et al., 1985), *Hordeum vulgare* L. (Tsumuki et al., 1989), *Sorghum bicolor* (L.) Moench (Chapman et al., 1983; Nwanze et al., 1992; Weibel and Starks, 1986), and *Triticum aestivum* L. (Lowe et al., 1985).

In azalea, through the use of gas chromatography-mass spectrometry, specific lipid components of epicuticular wax have been implicated in ALB resistance and susceptibility (Balsdon et al, 1995; Wang et al., 1999). Research by Balsdon et al. (1995), analyzing lipid components of four susceptible evergreen cultivars and the resistant species *R. canescens*, identified three triterpenoid components that may have an effect on ALB behavior. However, data were not definitive as purported deterrent/ stimulant compounds were found in similar concentrations in both *R. canescens* and the susceptible cultivars. Wang et al. (1999) identified lipid components correlated with ALB resistance and susceptibility in two deciduous resistant genotypes, four deciduous susceptible genotypes, and one evergreen susceptible azalea genotype. The lipid component present in the largest proportion among resistant genotypes was *n*-hentriacontane whereas in susceptible genotypes α- and β-amyrin were in greatest

concentrations. While these studies suggest an association of leaf-surface lipids with ALB response, studies to quantify the actual effects of lipids on ALB behavior have not been conducted. The research described in this paper investigates the quantitative effects of leaf-surface lipids from susceptible and resistant foliage on ALB survival, feeding, and oviposition.

MATERIALS AND METHODS

Plant Materials. Genotypes included in this study were selected based upon previous bioassays that screened azaleas for response to ALB (Braman and Pendley, 1992; Wang et al., 1998). The azalea genotypes included in this study were the resistant *R. periclymenoides* and *R. canescens* and the susceptible 'Buttercup' (*R. austrinum* selection) and 'My Mary' (Beasley hybrid). Due to a lack of sufficient numbers of adult female ALB, the experiment was undertaken in two parts. *R. periclymenoides* and 'Buttercup' were paired and tested 30 Aug. 2004 followed 1 week later by *R. canescens* and 'My Mary'. All plant material used in this research was obtained in Aug. 2004 from mature field-grown plants grown under mixed deciduous tree species. The field plots were established in Nov. 1994 and maintained through drip irrigation and annual fertilization with Osmocote Pro Controlled Release Fertilizer Plus Minors (19 N-2.2 P-7.47 K) (Scotts-Sierra Horticultural Products Company, Marysville, Ohio). No pesticides were applied.

Laboratory bioassays. Bioassays were conducted by extracting epicuticular leaf wax from two ALB resistant and two ALB susceptible deciduous azalea genotypes and re-applying to fresh azalea foliage of each of the four genotypes in a diallel design. Following leaf wax extraction and re-application, ALB were introduced to foliage in a closed and controlled environment and ALB survival, frass deposition, and oviposition were measured. To extract leaf wax, 40 azalea leaves per genotype were air-dried for 120 h, then immersed in 100 mL of chloroform for 15 s.

Chloroform was evaporated and the remaining epicuticular wax re-suspended in a 50 mL ethanol: deionized water solution (2:1) under mild heating (32.2 °C) and stirring. Upon cooling to room temperature (20 °C), the resulting solution was applied directly to the fresh foliage of a 4-5 cm cutting with two leaves. The leaf wax solution was applied by painting the solution on one side of the midrib, on both abaxial and adaxial surfaces. By applying solution to only one side of the midrib, the experiment served as a choice test, thereby allowing pairwise comparisons to be made between treated and untreated leaf surfaces. After the treatment was applied to both leaves of the cutting, the stem was inserted through a snap-on lid into a 32-mL plastic cup (4 cm ht x 4 cm diam) of water. Four female adult ALB were placed into a second 32-mL cup that was modified by replacing the bottom of the cup with organdy screen to allow ventilation. This cup was inverted and placed over the cutting, and was sealed to the water cup with Parafilm M (Fisher Scientific, Hampton, N.H.). Mature, female azalea lace bugs were collected the day of the experiment from evergreen azaleas located in the Griffin, GA area.

Experimental design. Experimental design was a full diallel, including two controls: (i) solution-only (ethanol: deionized water solution [2:1] heated and stirred, without wax extract) and (ii) non-treated leaf. The study had four treatment groups (Table 1), each consisting of a donor of epicuticular wax extract, recipient of epicuticular wax extract, solution-only control, and non-treated control. Each treatment was applied to five cuttings, with two leaves per cutting; hence five replications were employed with two sub-samples per replication. Experimental conditions were controlled by placing all entries into a growth chamber at 24 ˚C and 12 h daylength for 120 h.

Data Collection and Statistical Analysis. Data were collected 120 h after ALB were introduced to azalea foliage. Numbers of live adults, frass spots (insect excrement), and eggs were recorded.

Frass and egg data were recorded separately for treated and non-treated sides of each leaf, including solution-only and non-treated controls. Data were analyzed as a three-factor factorial in SAS using Proc GLM (SAS Institute, Cary, N.C.); the three factors were replications; donor of epicuticular leaf wax; and recipient of epicuticular wax. Mean values were compared using LSD analysis based upon the recipient of each leaf wax extract, including controls of non-treated foliage and solution-only treatment. Additionally, pairwise comparisons (dependent group t-test) were performed between frass counts on treated and untreated sides of foliage as well as egg counts on treated and untreated sides of foliage. Data subsets were constructed based on: (i) susceptible genotypes used as leaf wax donor and resistant genotypes as leaf wax recipient, (ii) resistant genotypes used as leaf wax donor and susceptible genotypes used as leaf wax recipient, (iii) susceptible genotypes used as leaf wax donor and recipient (self), (iv) resistant genotypes used as leaf wax donor and recipient (self), (v) solution-only control treatment on susceptible and resistant genotypes, respectively.

Additional Studies to Exclude Treatment Effects. To investigate the possibility that residual chloroform was contaminating the wax extract solution and thus affecting results, leaf wax samples from *R. periclymenoides*, *R. canescens*, and 'Buttercup' were screened in a Tekmar purge-and-trap (Gas Chromatography) unit (Teledyne Tekmar, Mason, Ohio, 45040) for chloroform residue. Additionally, the application of wax solution to leaf surfaces may have clogged open stomata with wax particles, preventing ALB feeding. To determine whether stomata were obstructed, foliage of *R. periclymenoides*, *R. canescens*, and 'Buttercup' was treated with leaf wax extract from each of the three genotypes and examined under a LEO 982 field emission scanning electron microscope (FE-SEM, LEO Electron Microsopy, Inc., Thornwood, N.Y.) with a Gatan Alto 2500 Cryostage and cryoprep chamber (Gatan UK,

Ferrymills 3, Osney Mead, Oxford, OX2 0ES, UK). Three leaf sections measuring 100 μm^2- per genotype, were photographed and all fully and partially open stomata scored as mechanically obstructed by accumulation of wax debris or unobstructed, with simple ratios calculated as a percentage of un-clogged stomata per 100 μm^2.

RESULTS AND DISCUSSION

Azalea lace bug survival. Variances from the two tests, performed 1 week apart, were homogeneous based on Bartlett's test for homogeneity of variance at $P > 0.05$ (data not shown). Hence, data from the two independent experiments were combined and analyzed as a single data set. The first of three parameters assessed in this study, ALB survival, directly measures the deterrent effects of epicuticular leaf wax. Results indicate that epicuticular leaf wax from ALB resistant genotypes contains a strong deterrent, as ALB survival was dramatically reduced when foliage of susceptible genotypes were treated with wax extract from resistant genotypes. When 'Buttercup' foliage was treated with *R. periclymenoides* wax extract, ALB survival was reduced from the 'Buttercup' non-treated and solution-only means of 2.9 and 3.0, respectively, to 0.6 (Table 1). This mean of 0.6 is comparable to the ALB survival on *R. periclymenoides* foliage treated with *R. periclymenoides* wax extract. Similarly, when 'My Mary' foliage was treated with *R. canescens* wax extract, ALB survival was reduced from the 'My Mary' non-treated and solution-only means of 3.0 and 2.9, respectively, to 1.0. This 1.0 ALB survival rate is comparable to that of *R. canescens* foliage treated with *R. canescens* wax extract.

Results point to an inverse effect as well, as leaf wax extracts from susceptible genotypes conferred susceptibility when applied to resistant genotypes. When 'Buttercup' wax extract was applied to *R. periclymenoides* foliage, ALB survival was increased from the *R. periclymenoides*

non-treated and solution-only means of 1.0 and 1.4, respectively, to 3.2. This 3.2 value is comparable to that of 'Buttercup' foliage treated with 'Buttercup' wax extract ($\bar{x}$=3.1). Similarly, when 'My Mary' wax extract was applied to *R. canescens* foliage, ALB survival was increased from the *R. canescens* non-treated and solution-only values of 0.8 and 0.9, respectively, to 3.2. This 3.2 value is comparable to that of 'My Mary' foliage treated with 'My Mary' wax extract ($\bar{x}$=2.9). Treatment of leaf surfaces with wax from the same genotype had no statistically significant effect on the level of resistance or susceptibility compared to non-treated or solution-only controls. *R. periclymenoides* foliage treated with *R. periclymenoides* wax extract had a mean ALB survival of 0.6, with non-treated and solution-only means statistically similar at 1.0 and 1.4, respectively. 'Buttercup' foliage treated with 'Buttercup' wax extract had a mean ALB survival of 3.1, with non-treated and solution-only means statistically similar at 2.9 and 3.0, respectively. Both *R. canescens* and 'My Mary' treatment of leaf surfaces with wax from the same genotypes followed this pattern.

Azalea lace bug frass deposition. The number of frass spots is a direct assessment of ALB feeding. Frass deposition on foliage of susceptible genotypes treated with resistant wax extract were significantly reduced compared to susceptible controls, due to both mortality of ALB in these treatment combinations and a reduction in feeding of live ALB. Mean number of frass spots from 'Buttercup' leaf surfaces treated with *R. periclymenoides* wax extract was reduced from the solution-only mean of 66.5 to 5.9 (Table 1). Mean number of frass spots from 'My Mary' leaf surfaces treated with *R. canescens* wax extract was reduced from the 'My Mary' solution-only mean of 56.3 to 16.6. Photos of feeding damage and frass deposition on 'My Mary' foliage treated with *R. canescens* wax extract is shown in Fig. 1. Conversely, when wax extracts from susceptible genotypes were applied to resistant genotypes, treated leaf surfaces of resistant

genotypes exhibited a susceptible response to ALB. Mean number of frass spots on *R. periclymenoides* leaf surfaces treated with 'Buttercup' wax extract was increased from the *R. periclymenoides* solution-only mean of 19.2 to 46.6. Similarly, mean number of frass spots on *R. canescens* leaf surfaces treated with 'My Mary' wax extract was increased from the *R. canescens* solution-only mean of 8.8 to 44.6. This treatment effect is seen in the fig. 1 photograph of a *R. canesce*ns leaf treated with 'My Mary' wax extract.

Treatment of leaf surfaces with wax extracted from the same genotype showed no significant effect on the level of resistance. Mean number of frass spots on *R. canescens* leaf surfaces treated with *R. canescens* wax extract was 11.8, comparable to that of the solution-only mean 8.8. Similarly, mean number of frass spots on *R. periclymenoides* leaf surfaces treated with *R. periclymenoides* wax extract was 2.9, comparable to that of the solution-only mean 19.2. Conversely, treatment of 'My Mary' leaf surfaces with 'My Mary' wax extract showed a significant effect on the level of susceptibility compared to the solution-only control.

Azalea lace bug oviposition. Egg deposition is an indirect measure of feeding, as eggs are typically deposited only on foliage of susceptible genotypes capable of supporting nymphal feeding and development. Oviposition rate on foliage of susceptible genotypes treated with resistant wax extract were significantly reduced compared to susceptible controls, probably due to significantly higher ALB mortality and a reduction in feeding of live ALB in these treatment combinations. Mean number of eggs on 'Buttercup' leaf surfaces treated with *R. periclymenoides* wax extract was reduced from the 'Buttercup' solution-only mean of 14.6 to 0.9 (Table 1). Similarly, mean number of eggs on 'My Mary' leaf surfaces treated with *R. canescens* wax extract was reduced from the 'My Mary' solution-only mean of 13.3 to 1.5. Conversely, oviposition was significantly increased on resistant genotypes treated with susceptible wax

extracts. Mean number of eggs on *R. periclymenoides* leaf surfaces treated with 'Buttercup' wax extract was increased from the *R. periclymenoides* solution-only mean of 2.0 to 8.5. Mean number of eggs on *R. canescens* leaf surfaces treated with 'My Mary' wax extract was increased from the solution-only mean of 0.1 to 5.2. Treatment of leaf surfaces with wax from the same genotype neither positively nor negatively affected the resistance or susceptibility of genotypes in this study. Mean number of eggs on *R. canescens* leaf surfaces treated with *R. canescens* wax extract was 0.4, comparable to the solution-only mean of 0.1. Mean number of eggs on 'Buttercup' leaf surfaces treated with 'Buttercup' wax extract was 14.8, comparable to the solution-only mean of 14.6.

Epicuticular leaf wax effects on untreated leaf surfaces. The effects of epicuticular leaf wax extracts, in addition to significantly impacting ALB behavior on treated leaf surfaces, also significantly impacted behavior on non-treated leaf surfaces. Resistant wax extract had a significant impact on susceptible cultivars in both frass deposition and oviposition, again due to significantly higher ALB mortality in treatment combinations including wax solution of resistant genotypes. When *R. periclymenoides* wax extract was applied to 'Buttercup' leaf surfaces, mean number of frass spots on non-treated leaf surfaces was reduced from the 'Buttercup' non-treated and solution-only (control) means of 112.9 and 68.7, respectively, to 6.5 (Table 1). Mean number of eggs on non-treated leaf surfaces was reduced from the 'Buttercup' non-treated and solution-only means of 25.9 and 13.8, respectively, to 0.3. When *R. canescens* wax extract was applied to 'My Mary' leaf surfaces, mean number of frass spots on non-treated leaf surfaces was reduced from the 'My Mary' non-treated and solution-only means of 119.3 and 56.6, respectively, to 13.1. Mean number of eggs on non-treated leaf surfaces was reduced from the 'My Mary' non-treated and solution-only means of 29.4 and 12.7, respectively, to 0.3.

The same effect was noted when wax extract of susceptible genotypes was applied to resistant genotypes. When 'Buttercup' wax extract was applied to *R. periclymenoides* leaf surfaces, mean number of frass spots on non-treated leaf surfaces was increased from the non-treated and solution-only means of 47.6 and 20.4, respectively, to 82.6. Mean number of eggs on non-treated leaf surfaces was also increased from the *R. periclymenoides* non-treated and solution-only means of 4.7 and 2.5, respectively, to 17.9. Similarly, when 'My Mary' wax extract was applied to *R. canescens* leaf surfaces, mean number of frass spots on non-treated leaf surfaces was increased from the *R. canescens* non-treated and solution-only means of 14.5 and 6.4, respectively, to 50.4. Mean number of eggs on non-treated leaf surfaces was also increased from the non-treated and solution-only means of 0.3 and 0.1, respectively, to 12.6.

Treatment of leaf surfaces with wax from the same genotypes mirrored controls, with no significant differences observed in frass or egg means with the exception of 'My Mary' frass means. For example, when *R. canescens* wax extract was applied to *R. canescens* leaf surfaces, mean number of frass spots on non-treated leaf surfaces was 13.2, comparable to both non-treated and solution-only values of 14.5 and 6.4, respectively. Mean number of eggs on non-treated leaf surfaces was 1.0, comparable to the non-treated and solution-only values of 0.3 and 0.1, respectively. When 'My Mary' wax was applied to 'My Mary' leaf surfaces, mean number of frass spots on non-treated leaf surfaces was 93.3, statistically different from the non-treated mean of 119.3, yet much greater than the *R. canescens* solution value of 13.1. Mean number of eggs on non-treated leaf surfaces was 18.7, comparable to the solution-only mean of 12.7.

Pairwise comparisons. To better elucidate the overall effect of wax extracts from resistant and susceptible genotypes on ALB behavior, on treated versus untreated sides of a leaf, a dependent t-test (pairwise comparison) was performed on six data subsets (Table 2). Three data subsets

yielded significant differences between both frass and/or egg counts on treated versus non-treated sides of foliage. The first subset, significant for both frass deposition and oviposition rate contained resistant genotypes as donor of leaf wax with susceptible genotypes as recipients of leaf wax. The second subset, significant only for oviposition rate, included all treatment groups containing a resistant genotype as a recipient of leaf wax with resistant genotypes as donors of leaf wax. The third subset, significant only for oviposition rate, included all treatment groups containing a susceptible genotype as a wax donor with only resistant genotypes as recipients as leaf wax extract. No significant differences in frass deposition or oviposition were observed on treated versus non-treated sides of foliage within the following data subsets: (i) susceptible genotypes as a recipient of leaf wax and susceptible genotypes as a donor of leaf wax, (ii) solution-only control as a treatment. Overall, results of the pairwise comparisons indicate a significant effect of leaf wax extracts from resistant genotypes on ALB feeding and oviposition, and united with results from mean separation (LSD) analysis, it has been determined to be a significant negative effect. Such a strong response of ALB to resistant wax extracts, as concluded from both LSD and pairwise comparisons, is likely due to an epicuticular wax component that serves as a strong deterrent to ALB.

Potential sources of experimental error. A comparison of solution-only and non-treated foliage revealed a small yet measurable negative effect on feeding and oviposition of the solution-only application, primarily on susceptible genotypes (Table 1). This solution-only effect does not overshadow or under-represent the effect of wax extracts, and with the exception of the measurement of frass on non-treated ‘My Mary’ foliage, is no greater than one standard deviation from the mean of non-treated foliage.

No chloroform (or other trace chemicals listed in the chloroform chemical MSDS as present in the chloroform stock solution) were detected in the three tested leaf wax samples of *R. periclymenoides*, *R. canescens*, and 'Buttercup' (data not shown). Therefore, it was assumed that the chloroform was evaporated prior to re-suspension of wax extracts and data was not confounded due to chloroform contamination.

Examination of foliage of *R. periclymenoides*, *R. canescens*, and 'Buttercup', using a scanning electron microscope revealed no obstructed stomata in any treatment combination (data not shown). Furthermore, photomicrographs showed no disruption of epicuticular wax on leaf surfaces treated with wax solution (Fig. 2). Apparently, leaf wax solution application provided uniform coverage and minimal disruption of the natural leaf surface texture.

The results of this study demonstrate that leaf-surface lipids from resistant deciduous azalea foliage confers a high level of resistance to ALB feeding and oviposition, as first proposed by Balsdon et al. (1995) and Wang et al. (1999). Conversely, susceptible wax extracts conferred moderate susceptibility when applied to resistant genotypes and hence may contain a stimulant to ALB, although its effect is markedly less than that of resistant wax extract. While there have been few studies in woody ornamental crops to determine the specific mechanism of insect resistance, this study conclusively demonstrates that resistance to ALB in azalea follows a pattern identified in many agronomically important crops (Bodnaryk, 1992; Brennan and Weinbaum, 2001; Molenaar, 1984). This response is most likely due to a wax component that serves as a strong deterrent to ALB. Both Balsdon et al. (1995) and Wang et al. (1999) linked several specific chemical components, present in epicuticular wax of ALB resistant genotypes, to this deterrent effect. Studies are under way to further their work and characterize whether reduced ALB survival, frass deposition (feeding), and oviposition observed on resistant

genotypes is due to a specific lipid that serves as a strong deterrent within epicuticular wax and whether increased ALB survival and oviposition in susceptible genotypes is due to a chemical stimulant within epicuticular leaf wax. The future identification of specific epicuticular leaf wax component(s) linked to ALB resistance and susceptibility has the potential to significantly change both ALB resistance breeding and management strategies employed in ALB control.

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Table 3-1. Numbers of surviving adult azalea lace bugs, frass spots, and eggs on azalea foliage treated with epicuticular wax extract from resistant (*R. periclymenoides*, *R. canescens*) or susceptible ('Buttercup', 'My Mary') azalea foliage. Numbers are means of ten replications and data are grouped based on the recipient of leaf wax. Mean separation is based upon least significant difference (LSD) at P<0.05. Means followed by the same letters for each parameter-leaf wax recipient combination are not statistically different.

	Surviving lace bugs [z]		Frass: treated surfaces [y]		Frass: non-treated surfaces [x]		Eggs: treated surfaces [w]		Eggs: non-treated surfaces [v]	
					'Buttercup' leaves (susceptible) [u]					
	'Buttercup' [t]	3.1 [s] a [r]	'Buttercup'	67.6 a	Non-treated	112.9 a	'Buttercup'	14.8 a	Non-treated	25.9 a
Wax	Solution-only	3.0 a	Solution-only	66.5 a	'Buttercup'	84.3 a	Solution-only	14.6 a	'Buttercup'	17.2 ab
Trt.	Non-treated	2.9 a	*R. periclymenoides*	5.9 b	Solution-only	68.7 a	*R. periclymenoides*	0.9 b	Solution-only	13.8 b
	R. periclymenoides	0.6 b			*R. periclymenoides*	6.5 b			*R. periclymenoides*	0.3 c
Pr<F		<.0001		0.0054		0.0055		0.0027		0.0046
					'My Mary' leaves (susceptible)					
	Non-treated	3.0 a	'My Mary'	80.1 a	Non-treated	119.3 a	'My Mary'	18.8 a	Non-treated	29.4 a
Wax	'My Mary'	2.9 a	Solution-only	56.3 b	'My Mary'	93.3 b	Solution-only	13.3 b	'My Mary'	18.7 b
Trt.	Solution-only	2.9 a	*R. canescens*	16.6 c	Solution-only	56.6 c	*R. canescens*	1.5 c	Solution-only	12.7 b
	R. canescens	1.0 b			*R. canescens*	13.1 d			*R. canescens*	0.3 c
Pr<F		0.0038		<.0001		<.0001		0.0004		<.0001
					R. periclymenoides leaves (resistant)					
	'Buttercup'	3.2 a	'Buttercup'	46.6 a	'Buttercup'	82.6 a	'Buttercup'	8.5 a	'Buttercup'	17.9 a
Wax	Solution-only	1.4 b	Solution-only	19.2 b	Non-treated	47.6 b	Solution-only	2.0 b	Non-treated	4.7 b
Trt.	Non-treated	1.0 b	*R. periclymenoides*	2.9 b	Solution-only	20.4 bc	*R. periclymenoides*	0.1 b	Solution-only	2.5 b
	R. periclymenoides	0.6 b			*R. periclymenoides*	8.1 c			*R. periclymenoides*	1.3 b
Pr<F		0.0015		0.0033		0.0038		0.0042		0.0002
					R. canescens leaves (resistant)					
	'My Mary'	3.2 a	'My Mary'	44.6 a	'My Mary'	50.4 a	'My Mary'	5.2 a	'My Mary'	12.6 a
Wax	*R. canescens*	1.0 b	*R. canescens*	11.8 b	Non-treated	14.5 b	*R. canescens*	0.4 b	*R. canescens*	1.0 b
Trt.	Solution-only	0.9 b	Solution-only	8.8 b	*R. canescens*	13.2 b	Solution-only	0.1 b	Non-treated	0.3 b
	Non-treated	0.8 b			Solution-only	6.4 b			Solution-only	0.1 b
Pr<F		0.0053		0.0082		0.0011		0.008		0.0038

[z] Surviving adult female lacebugs at 120 hours. Each replication included 4 adult female lace bugs.
[y] Frass on treated side of leaves at 120 hours.
[x] Frass on non-treated side of leaves at 120 hours.
[w] Eggs on treated side of leaves at 120 hours.
[v] Eggs on non-treated side of leaves at 120 hours.
[u] The recipient of treatments.

[t] The donor of leaf wax, solution-only only treatment, or non-treated.
[s] All reported values are means over five replications.
[r] Mean separation based on least significant difference (LSD) at $\alpha = 0.05$. Means followed by the same letters are statistically similar.

Table 3-2. Degrees of freedom, mean difference, standard deviation, t-value, and probability of *t* values for pairwise comparison of frass deposition and oviposition counts on treated versus untreated sides of azalea foliage. Data for the resistant genotypes *R. periclymenoides* and *R. canescens* were combined, as were the data for the susceptible genotypes 'Buttercup' and 'My Mary'.

			Frass deposition					Oviposition				
Donor	Recipient	D.F.	Mean	SD	*t* -value	Pr > | *t* |		Mean	SD	*t* -value	Pr > | *t* |	
Susceptible	Resistant	9	1.45	6.02	0.76	0.4658	*ns*	0.90	0.88	3.25	0.0100	**
Resistant	Susceptible	9	-20.90	21.96	-3.01	0.0143	**	-8.40	4.39	-6.05	0.0002	***
Susceptible	Susceptible	9	-15.35	32.79	-1.48	0.1729	*ns*	-0.65	3.22	-0.64	0.5385	*ns*
Resistant	Resistant	9	-3.30	5.24	-1.99	0.0776	*ns*	-0.90	0.84	-3.38	0.0082	**
Solution (control)	Susceptible	9	-1.25	11.51	-0.34	0.7392	*ns*	0.70	4.45	0.50	0.6312	*ns*
Solution (control)	Resistant	9	0.60	3.46	0.55	0.5970	*ns*	-0.25	0.86	-0.92	0.3809	*ns*

* Pr < 0.05
** Pr < 0.01
*** Pr < 0.001

Figure 3-1. Deciduous azaleas showing azalea lace bug feeding damage and frass deposition. 'My Mary' (susceptible) adaxial leaf surface treated with *R. canescens* (resistant) leaf wax solution on the left side of the midrib (left), 'My Mary' abaxial leaf surface treated with *R. canescens* leaf wax solution on the right side of the midrib (center), and *R. canescens* abaxial leaf surface treated with 'My Mary' wax extract on the left side of the midrib (right). Distance bar (top-left) represents 1.0 cm.

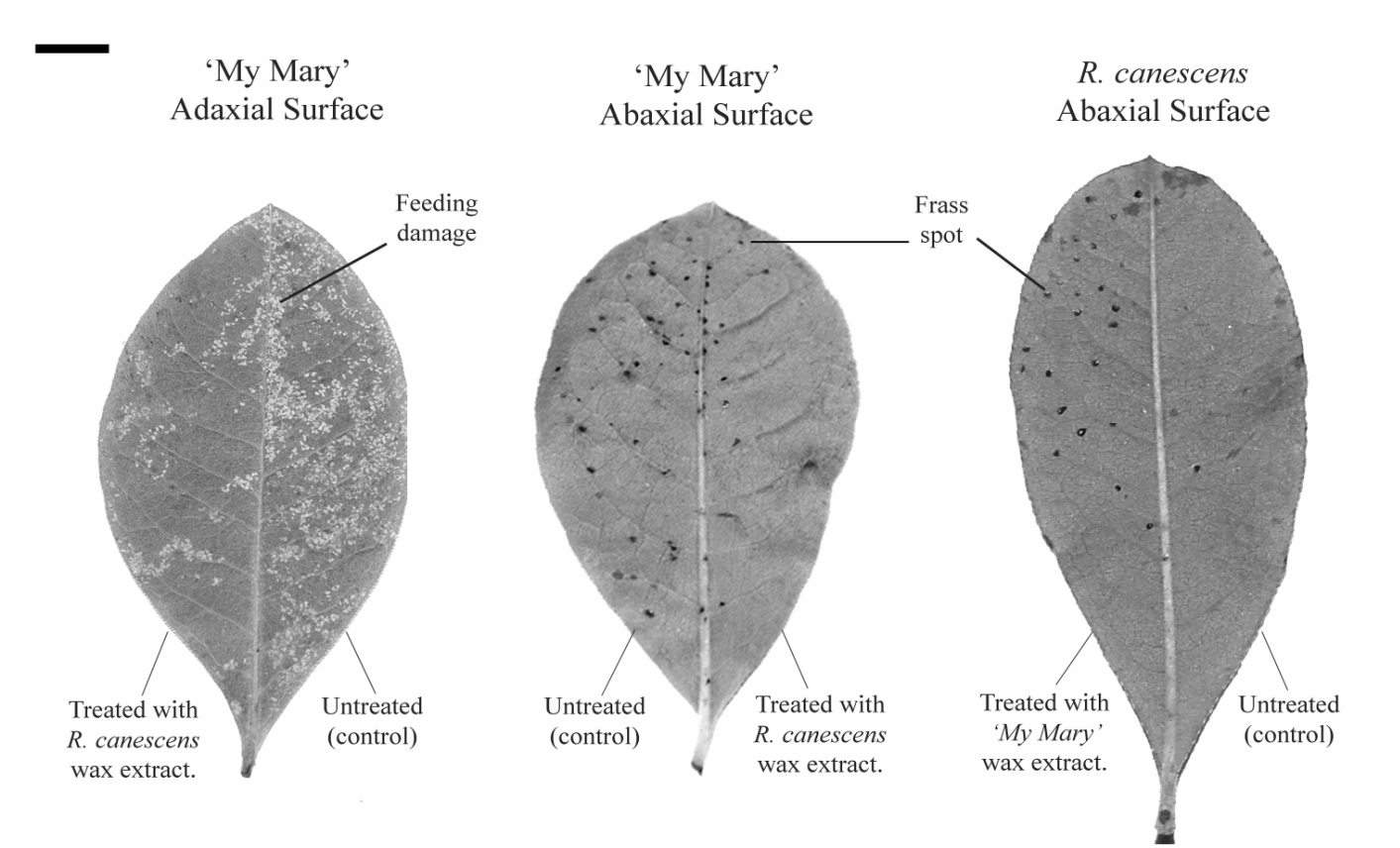
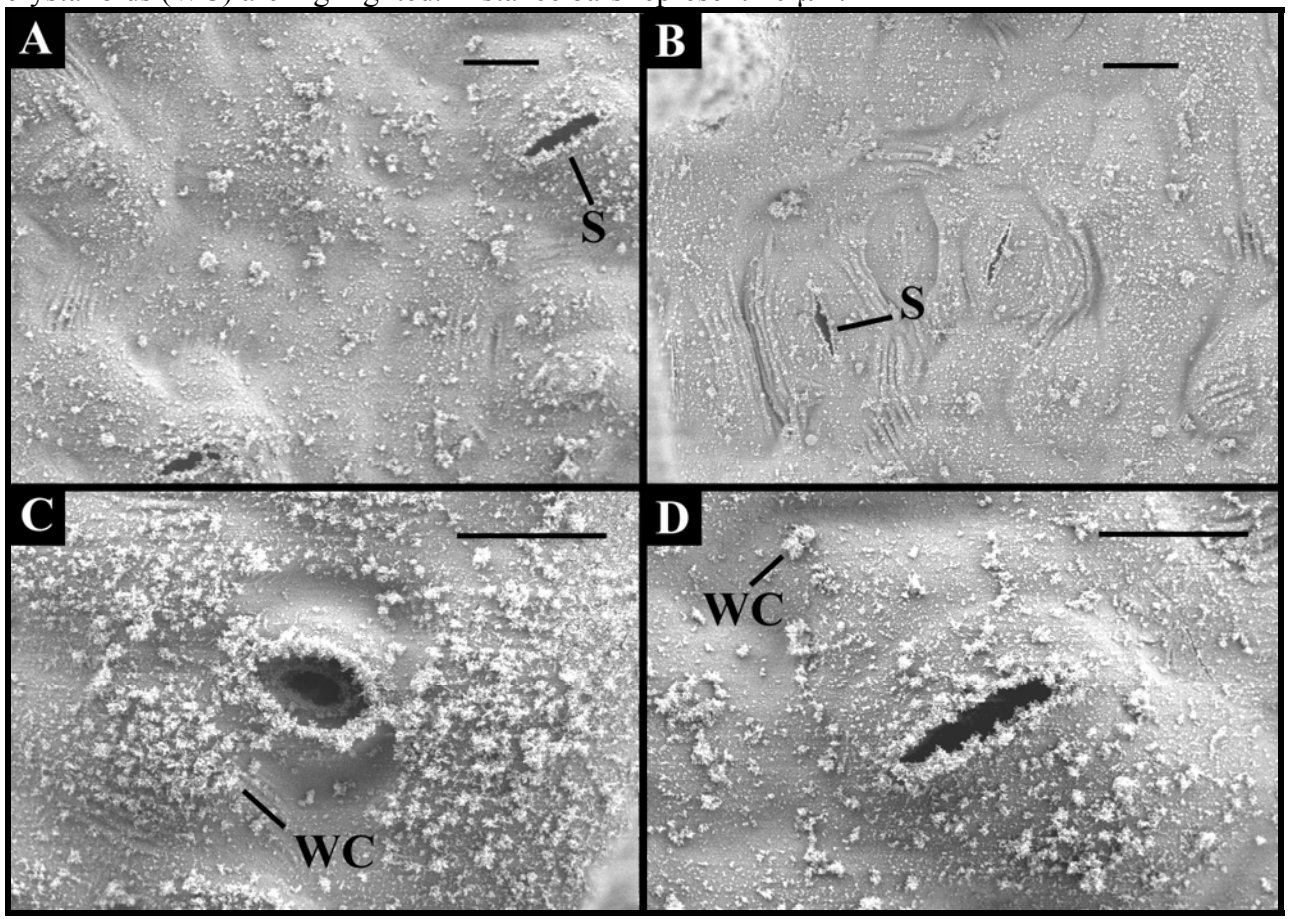


Figure 3-2. Scanning electron micrographs of azalea lace bug susceptible cultivar 'Buttercup' foliage treated with wax solution of *R. periclymenoides* (panel A and C) and control micrographs of non-treated 'Buttercup' foliage (panel B and D). Stomata (S) and wax crystalloids (WC) are highlighted. Distance bars represent 10 μm.



CHAPTER 4

CONCLUSIONS

Conventional thought regarding the level of diversity among and within members of *Rhododendron* section *Pentanthera*, based on ample phenotypic (Rehder, 1921; Sax, 1930; Wherry, 1943; Li, 1957; Skinner, 1961; Galle, 1967) and limited molecular analysis (Kron et al., 1993; Scheiber et al., 2000), has revolved around the idea that native deciduous azalea species are closely related. Despite the aforementioned work, our understanding of interspecific variation of North American deciduous azalea species was quite limited. Furthermore, no genetic studies had been conducted to ascertain the level of intraspecific or interpopulation genetic variation. The present study addressed in detail the issue of genetic diversity among and within seven individual species and twenty-five populations of North American deciduous azalea species through the use of amplified fragment length polymorphism (AFLP) analysis. Utilizing three primer pairs, 417 polymorphic bands were obtained that allowed for high resolution of genetic differentiation among and within species and populations. Based on analysis of molecular variance (AMOVA) and estimates of Nei's coefficients of gene diversity (H_S, H_T, and G_{ST}), the majority of variation in deciduous azalea occurs within populations. Both among species and among population variation was low, likely the effect of common ancestry as well as frequent introgression among members (and populations) of section *Pentanthera*. The majority of populations were grouped into species based on Nei's unbiased genetic distances viewed as a UPGMA phenogram. However, there was evidence of introgression within members (and populations) of section *Pentanthera*. Overall, three crucial ideas can be taken from this study in relation to the breeding of North American native azaleas. First, breeders should take precautions to ensure breeding material, if deemed to be a pure species, has not been compromised via introgression. Identification of populations consisting of one species without hybrid individuals, based on phenotypic keys, was difficult in the field. In several instances, multiple observations of

phenotypic characters indicated a population consisted of a single species, yet genetic data suggests introgression had occurred. Second, there is a low level of genetic diversity observed among populations within species. While genetic diversity does exist among populations, the relatively low level of diversity indicates that the importance of collecting individuals from multiple geographically isolated populations to obtain allelic diversity in parent stock is not critical. Third, results illuminate potential pitfalls in the conservation of native azaleas in the eastern United States. The low levels of genetic diversity, coupled with its entomophilous crossing nature, suggest that gene flow both among populations and among species (introgression) is important in maintaining the heterosis of populations and/or species. Fragmentation or isolation of populations due to anthropogenic activity could have a significant and swift negative effect on the health, vigor, and adaptability of individual populations and/or species. Studies should be conducted to identify those species that may be at risk either due to small geographic range (*R. flammeum*, *R. prunifolium*) or habitat preferences that collide with anthropogenic activities such as housing development (*R. atlanticum*, *R. flammeum*).

The mechanism of resistance and susceptibility of *Rhododendron* section *Pentanthera* species and cultivars to azalea lacebug (*Stephanitis pyriodes* Scott) was assumed by previous researchers to be linked to components of epicuticular leaf wax. However, studies disagreed upon specific wax compounds linked to resistance and/or susceptibility (Baldson et al., 1995; Wang et al., 1999). Azaleas are largely resistant to predation by insects, with the exception of ALB. Within deciduous azalea (*Rhododendron* section Pentanthera) varying levels of resistance to ALB is observed with a continuous distribution from susceptible to highly resistant. In this study, epicuticular leaf wax from two ALB resistant [*R. canescens* Michaux and *R. periclymenoides* (Michaux) Shinners] and two ALB susceptible (‘Buttercup’ and ‘My Mary’)

deciduous azalea genotypes was extracted and re-applied to fresh azalea foliage. Leaf wax extracted from ALB resistant genotypes and applied to ALB susceptible genotypes conferred a high level of resistance to ALB feeding and oviposition. Conversely, leaf wax extracted from ALB susceptible genotypes and applied to ALB resistant genotypes conferred susceptibility to ALB resistant genotypes. The results indicate that leaf wax serves as a primary mechanism of resistance of deciduous azalea to ALB.

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