Pentanthera Webs: Interspecific and Interploid Hybridization among Sympatric Azaleas in the Southern Appalachian Mountains

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Summary

Modern evolutionary research suggests that new species often arise rapidly from hybridization and chromosome doubling, augmenting the slow, divergent processes originally detailed by Darwin. Relationships between kindred species are thus best represented not by a simple branching candelabra or tree, as pictured in our old biology texts, but by a complex web of exchanges and ploidy variations. Such complexities seem especially evident in the highly compatible and multiploidal Southeastern deciduous azaleas, especially on those spectacular and much-visited mountaintops called "balds." We have conducted a survey of 92 samples taken from the azalea swarm on the bare top and adjacent woodlands of Gregory Bald within Great Smokies National Park. Flow cytometry was used to determine ploidy and published component traits of recognized species were used to elucidate mating interactions at that highly diverse site. A similar study was undertaken with 12 samples from along the interfaces between the *Rhododendron calendulaceum* (tetraploid) and *R. arborescens* (diploid) colonies on Wayah/Wine Spring Bald, to the south of the Park. Cytometry revealed no tetraploids within the open bald area of Gregory, belying previous suggestions that *R. calendulaceum* is directly involved in

that famous swarm. There were tetraploids keying out to *R. calendulaceum,* some with unusual variations (e.g., fragrance), within nearby woodlands. The traits of several of the samples from Wayah/Wine Spring gave evidence for genetic exchanges between the diploid and tetraploid species; one triploid, such as might serve as a two-way bridge between the azalea species, was in fact found. In addition, triploids had previously been discovered from *R. calendulaceum-R. periclymenoides* hybrids in northwestern South Carolina. Thus mechanisms for gene exchange between diploid and tetraploid species do indeed exist. Examination of the complex diploids of Gregory was still more suggestive. Flower colors, odors, and forms evince origins in *R. cumberlandense* and *R. arborescens* and possibly *R. viscosum*. Diploid plants consistent with *R. cumberlandense* at this site could not, based on flower sizes and forms, be distinguished from tetraploid *R. calendulaceum*. The lack of differentiating morphological traits between *R. cum-berlandense* and *R. calendulaceum* and similarity in average chromosome size suggests that tetraploid *R. calendulaceum* at this site is primarily derived from the diploid *R. cumberlandense* with limited genetic infusions from other species. Moreover, the indeterminacy of species upon

this bald suggests that the present azalea species in the Southeast are likely to be recent products of cycles of migration and interaction attending the as many as 20 glaciations and warm periods since the beginning of the Pleistocene. Our contemporary list of recognized azalea species that seem so distinct and certain may simply be a momentary snapshot of a complex and rapidly changing evolutionary web that is the *Pentanthera*.

Introduction

Beginning with Charles Darwin (Darwin 1859), the evolutionary process typically has been illustrated as a tree of life with radiating limbs that branch and diverge but never reconnect. This concept is rooted in the notion of reproductively isolated populations accumulating genetic variation over time. Subject to natural selection, these populations become increasingly distinctive as they gradually evolve along a one-way course without intersections. Current research provides ample evidence that this tree-of-life illustration is an oversimplification at best (Arnold 2006; Arnold and Larson, 2004). The process of evolution is more aptly depicted as a reticulate web of genetic exchange integrating periodic hybridization among nascent "species."

Interspecific hybridization and introgression are common among plants and are a significant evolutionary mechanism that can give rise to new evolutionary lineages and species (Arnold 1997, 2006; Barrier et al. 1999; Grant et al. 2005; Rieseberg and Carney 1998; Soltis et al. 2009). By some estimations, at least 25% of plant species hybridize naturally (Mallet 2005) and as many as 50-70% of all flowering plants are of hybrid origin (Ellstrand et al. 1996; Rieseberg 1997).

This process of introgressive hybridization can afford evolutionary benefits by enhancing genomic diversity. Through the development and combination of novel adaptations, introgressive hybridization can enhance fitness, colonization success, and adaptive radiation in new and changing environments (Arnold 2006; Grant et al. 2005; Lewontin and Birch 1966; Lindqvist et al. 2003; Rieseberg and Carney 1998; Soltis et al. 2009).

Rhododendron species of the sub-section *Pentanthera* are known for their widespread natural hybridization, including over 18 different combinations that sometimes involve multiple species (Millais 1924; Skinner 1955, 1961; Galle 1968; Leach 1958). Leach (1958) remarked that "hybridity is the most conspicuous single feature of the entire Azalea population of our Southern mountains." Skinner (1961) also emphasized what he considered to be the reticulate nature of the evolutionary lineages within this section.

The development of polyploids is also a fundamental evolutionary process in flowering plants. Most angiosperms are believed to have undergone whole genome duplication events, often followed by rediploidization, throughout their evolutionary history (Soltis et al. 2009). Polyploids have numerous traits that can contribute to successful speciation and radiation, including a greater number of potential alleles, greater potential heterozygosity, tolerance of deleterious mutations, novel gene expression(s), and enzymatic multiplicity (Adams and Wendel 2005; Comai 2005; Hegarty and Hiscock 2008; Osborn et al. 2003; Soltis et al. 2003; Soltis and Soltis 1993). The formation of polyploids can also contribute to the development of more abrupt speciation as a result of reproductive isolation. Once tetraploids arise in a population, they generally hybridize readily with other tetraploids. Crosses with diploids are typically less successful. Successful crosses between tetraploids and diploids typically yield triploids with low or nonexistent fertility (Ehlenfeldt

and Vorsa 1993; Ramsey and Schemske 1998). This reproductive barrier between cytotypes can foster sympatric divergence. Yet little is known about the potential for *in situ* interploid gene flow among coexisting taxa of different cytotypes.

Hybrid zones can provide an opportunity to study the degree of reproductive isolation and the presence of interspecific and interploid crossing within mixed populations. Pentanthera azaleas native to the Southern Appalachian Mountains often form hybrid zones or "swarms" with unusual phenotypic diversity indicative of natural hybridization (Galle 1987; Kron 1993; Rehder 1921; Skinner 1955, 1961; Towe 2004). A number of studies have confirmed interspecific hybridization and introgression among these azaleas, but these have been limited to diploid cytotypes (King 2000; Kron et al. 1993).

A well-known hybrid zone of azaleas is located on Gregory Bald in the Great Smoky Mountains National Park on the Tennessee-North Carolina border near Cades Cove. Based on morphology and the overlapping ranges of numerous species, this swarm has been thought to represent a complex hybrid zone among *Rhododendron arborescens* (Pursh), *Rhododendron cumberlandense* (E.L. Braun) Copeland, *Rhododendron calendulaceum* (Michx.) Torr., and *Rhododendron viscosum* (L.) Torrey (Hyatt 2001). Potential hybridization events in this population are of particular interest on account of the complex genetic interactions that may be occurring between species that vary in ploidy level. Of the four species found in that region, *R. arborescens, R. cumberlandense,* and *R. viscosum* are diploid ($2n = 2x = 26$), while *R. calendulaceum* is tetraploid ($2n = 4x = 52$) (Jones et al. 2007; Sax 1930). Approximately 47 kilometers to the SE of Gregory Bald, Wayah Bald and Wine Spring Bald are located in the Nantahala National Forest in North Carolina. Both Wayah Bald and Wine Spring Bald host sympatric populations of *R. calendulaceum, R. arborescens,* and their potential hybrids.

Although these grassy balds have certainly been disturbed and impacted by human beings (Lindsay 1976), they contain hybrid zones of deciduous azaleas and provide model sites to study ongoing genetic exchange.

The species found on these balds are characterized and may be differentiated in the following ways (Galle 1987; Kron 1993; Luteyn et al. 1996; Willingham, Jr. 1976) (also see Table 1):

•*Rhododondron arborescens* is known as the sweet azalea due to the sweet clove or heliotrope-like fragrance. Flowers are white, sometimes with a pink or reddish blush, 4.0-5.2 cm across, with or without a yellowish blotch. The transition from tube to corolla is gradual; sepals typically have a fringed margin. Pistil and stamens are characteristically reddish.

•*Rhododendron calendulaceum,* the flame azalea, is named for its brilliant flower color which can range from yellow to orange to red. Flowers are often larger than the flowers of other species, 4.0-6.5 cm across, and have a blotch on the upper lobe. There is an abrupt transition from tube to corolla, no substantial fragrance, and typically a fringed sepal margin. Flowers ($2n=4x=52$) often bloom before leaf emergence (though highly variable). *Rhododendron calendulaceum* is the only tetraploid species of the four taxa considered here (Jones et al. 2007; Li 1957; Sax 1930).

•*Rhododendron cumberlandense (R. bakeri),* the Cumberland azalea, closely resembles *R. calendulaceum* but is diploid. This species has been described as having smaller flowers, 3.8-4.5 cm across, a bristly sepal margin, and it typically blooms after leaves expand. The pedicel is typically eglandular.

•*Rhododendron viscosum (var. montanum),* the sticky azalea, has fragrant white flowers, 2.5-4.0 cm across, a glandular/viscid tube, a gradual transition from tube to corolla, and a strong spicy fragrance. *Rhododendron viscosum* can be confused with *R. arborescens* as they both have fragrant white flowers; however, unlike the *R. arbo-*

Table 1. Morphological characters of four deciduous azaleas (*Rhododendron* sp.) and putative hybrids sampled at Gregory, Wayah and Winespring Bald.

Taxa		Genome Size (pg)	Ploidy Level	Color	Blotch	Corolla Length + Corolla Tube (mm)	Corolla Width (mm)	Tube Flare	Petal Margin	Fragrance (Y/N)	Style & Filament Color	Sepal Margin	Pedicel Hairs
							Flower						
Rhododendron arborescens (Pursh) Torr.		1.65 ± 0.05	2x	White/Pink	Varies	36-48	40-52	G	F	Y	Red	F	G
R. calendulaceum (Michx.) Torrey		3.14 ± 0.09	4x	Yellow Orange Red	Present	34-45	40-65	A	W	N	Yellow Orange Red	F	G (E)
R. cumberlandense (E.L. Braun) Copeland		1.63 ± 0.02	2x	Yellow Orange Red	Present	29-39	38-45	A	W	N	Yellow Orange Red	B	E (G)
R. viscosum (L.) Torrey		1.67 ± 0.04	2x	White Pink	Absent	28-43	25-40	G	F	Y	White Green-White	F	G
Gregory Bald													
ID#	GPS Coordinates												
A001*	35°31.230'N, 83°51.880'W	1.65 ± 0.03	2x	Orange Red	N	32.03	37.43	A	F	N	Red	B	E
A002*	35°31.230'N, 83°51.881'W	1.61 ± 0.04	2x	Pink	Yellow	37.64	44.13	A	W	N	Pink	B	E
A003*	35°31.230'N, 83°51.880'W	1.63 ± 0.04	2x	Red Orange	N	39.20	38.70	A	W	N	Red	B	E
A004*	35°31.226'N, 83°51.880'W	1.60 ± 0.07	2x	Pink	N	44.13	37.33	G	W	Y	Pink	F	G
A005*	35°31.229'N, 83°51.884'W	1.62 ± 0.03	2x	Pink	Orange	37.30	40.50	A	W	N	Pink	F	G
A006*	35°31.226'N, 83°51.884'W	1.64 ± 0.05	2x	Dark Red	Some Orange	31.16	37.01	A	W	N	Red	B	G
A007*	35°31.240'N, 83°51.870'W	1.65 ± 0.02	2x	Pink and Yellow	Yellow fade	37.27	37.61	A	W	N	Pink/Red	B	G
A008*	35°31.235'N, 83°51.863'W	1.58 ± 0.07	2x	Pink	orange	31.28	35.21	A	W	N	Dark Pink	B	E
A009*	35°31.217'N, 83°51.884'W	1.62 ± 0.02	2x	Orange Peach	N	44.91	42.30	A	W	N	Dark Pink / White to Pink	B	E
A010*	35°31.216'N, 83°51.883'W	1.63 ± 0.04	2x	Yellow Pink	N	38.76	41.80	A	W	N	Yellow with White	B	G
A011*	35°31.213'N, 83°51.887'W	1.61 ± 0.03	2x	Pink	Orange	45.71	34.43	A	W	Y	Pink/Red	B	G
A012*	35°31.213'N, 83°51.884'W	1.60 ± 0.04	2x	Orange	N	U	U	A	W	N	Red	B	E
A013*	35°31.210'N, 83°51.886'W	1.63 ± 0.01	2x	Red	N	36.93	48.69	A	W	N	Red	B	E

rescens, R. viscosum does not typically have the tell-tale, red pistil and stamens and is a low, stoloniferous shrub.

In order to better differentiate these species (e.g., *R. calendulaceum* and *R. cumberlandense*) and to understand interploid hybridization, it is essential to know the ploidy levels of the plants in question. Cytological determination of chromosome numbers and ploidy levels for *Rhododendron* is notoriously difficult because of the small chromosome size. However, the recent development of flow cytometry provides for rapid measurement of genome size (DNA content) and associated ploidy levels and has been used extensively for *Rhododendron* (Jones et al. 2007). This method allows for rapid and accurate screening studies of cytotype distribution (Kron et al. 2007).

The objective of this study was to utilize morphological and genome size data to determine the presence of interspecific and interploid hybridization in selected hybrid zones of Pentanthera azaleas in the Southern Appalachian Mountains.

Materials and Methods

Flower and foliage samples were collected from 92 plants growing on and around Gregory Bald and 12 samples from Wayah Bald and Wine Spring Bald in mid-June 2011. Samples were collected throughout the sites in an attempt to represent a broad range of phenotypes that were present including plants that appeared to have hybrid phenotypes. Samples for cytometric analyses were placed in

bags with moist paper towels and were transported in coolers. Photographs and GPS coordinates were taken for each plant to allow for follow-up research. Plant locations and cytotype distributions were mapped (Google Earth, Google Inc., Mountain View, Calif.). Additional samples were collected from selected plants and pressed for herbarium vouchers that are now maintained at the NC State Herbarium, Raleigh, N.C. (K. Shearer, 1-38). Morphological characters, listed in Table 1, were determined. Presence of fragrance was confirmed by three individuals.

Genome sizes of all collected samples were determined using flow cytometry. Approximately 1 cm² of tissue was finely chopped in a petri dish with 400 µL of

Table 1 continued.

A014*	U	1.60 ± 0.02	2x	Orange	N	32.14	28.37	A	W	N	Red	F	E
A015*	35°31.219'N, 83°51.887'W	1.62 ± 0.02	2x	White	Yellow	48.06	51.87	A	W	Y	Red	F	E
A016*	35°31.204'N, 83°51.958'W	1.61 ± 0.02	2x	Red Orange	N	33.00	37.42	A	W	N	Red	B	E
A017*	35°31.197'N, 83°51.959'W	1.65 ± 0.01	2x	White Pink	Yellow	37.94	36.13	A	W	Y	Pink-red/Pink-white	B	G
A018*	35°31.191'N, 83°51.960'W	1.64 ± 0.03	2x	Orange Pink	N	41.46	47.03	A	W	N	Orange-pink	B	E
A019*	35°31.179'N, 83°51.954'W	1.62 ± 0.03	2x	Yellow	N	34.22	41.53	A	W	N	Yellow	B	E
A020*	35°31.179'N, 83°51.954'W	1.62 ± 0.001	2x	White	Yellow	38.48	38.41	A	W	N	White	F	G
A021*	35°31.169'N, 83°51.963'W	1.62 ± 0.02	2x	Orange	Yellow	28.05	28.74	A	W	N	Red	B	E
A022	35°31.178'N, 83°51.961'W	1.63 ± 0.02	2x	Pink	Orange	40.04	50.80	A	W	N	Red	B	E
A023	35°31.178'N, 83°51.967'W	1.63 ± 0.04	2x	Orange Red Yellow	N	39.54	42.29	A	W	N	Red	F	E
A024*	35°31.181'N, 83°51.974'W	1.62 ± 0.03	2x	Pink	Yellow	40.74	46.76	G	W	Y	Pink	F	G
A025*	35°31.184'N, 83°51.971'W	1.62 ± 0.02	2x	White	Yellow	43.54	37.29	A	W	Y	Red	B	E
A026*	35°31.194'N, 83°52.038'W	1.62 ± 0.03	2x	Orange	N	36.74	43.14	A	W	N	Red	B	E
A027*	35°31.196'N, 83°52.038'W	1.63 ± 0.04	2x	Pink Red	N	39.14	47.18	A	W	N	Red	F	E
A028	U	1.62 ± 0.02	2x	Orange	Yellow-orange	32.07	35.17	A	W	N	Both Pink-red	B	G
A029	35°31.240'N, 83°51.948'W	1.61 ± 0.04	2x	Light Peach	Yellow	38.53	35.08	A	W	N	Both Peach-pink	F	G
A030	35°31.240'N, 83°51.940'W	1.60 ± 0.04	2x	Peach	Orange	37.21	42.84	A	F	N	Red	B	E
A031	35°31.240'N, 83°51.948'W	1.61 ± 0.02	2x	Orange Red	N	35.53	38.81	A	W	N	Pink/Red	B	E
A032	35°31.228'N, 83°52.395'W	3.21 ± 0.03	4x	Orange	N	42.21	49.77	A	W	N	Orange	B	E
A033	35°31.229'N, 83°52.394'W	3.20 ± 0.02	4x	Orange	N	42.93	49.47	A	W	N	Yellow	F	E
B001*	35°31.254'N, 83°51.864'W	1.60 ± 0.02	2x	Pink	Yellow/Orange	40.44	52.13	A	W	N	Red	B	U
B002*	35°31.263'N, 83°51.877'W	1.61 ± 0.05	2x	Light Yellow	Dark yellow	37.14	40.77	A	F	N	White-red/White	B	U
B003*	35°31.266'N, 83°51.868'W	1.66 ± 0.03	2x	Pink	Orange/yellow	37.00	44.00	G	W	N	Pink	B	E
B004*	35°31.276'N,	1.60 ± 0.04	2x	Pink	Orange	33.83	32.38	A	W	N	Red	B	E

nuclei extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer, Partec, Münster, Germany) using a sharp razor blade. The suspensions were then filtered through 50 µm filters and nuclei were stained with 1600 µL 4', 6-diamidino-2-phenylindole (DAPI) staining buffer (CyStain UV Precise P Staining Buffer, Partec). Relative genome sizes were determined using a flow cytometer (Partec PA-II, Partec) using *Pisum sativum* 'Ctirad' (8.75 pg) (Doležel et al. 1998) as an internal standard. Ploidy levels were determined based on genome size as determined by Jones et al. (2007).

Results and Discussion
Gregory Bald (A & B Group)

Of the samples collected on and around Gregory Bald, 83 were diploid, while the remaining nine were tetraploid (Table 1). No triploids were identified at this site. Plants that were sampled in open areas at the top of the bald were exclusively diploids (Fig. 1; Google Earth Map, http://www.ces.ncsu.edu/fletcher/mcilab/publications/pentantheramap.kmx. You must first download Google Earth software to view this map: http://www.google.com/earth/index.html. Click on pins to show images of each plant.) Li (1957) completed chromosome counts on eight plants from the same location and also found them to all be diploid.

A number of diploid species were clearly represented on the bald. Many plants had phenotypes consistent with *R. cumberlandense* (e.g., A006, A021, B006, B009). There were also many plants that were consistent with *R. cumberlandense* but lacked a well-defined blotch (e.g., A001, A003, A009, A012, A013, A016, A018, A019, A023, A026, B016, B018, B020, B021, B024, B032, B033, B036, B037, B038, B042, B044, B046, B048, B052, B054), a trait some authors consider to be a primary characteristic of *R. cumberlandense* (Kron 1993). Other plants were largely consistent with *R. arborescens* (e.g., A015, A025, B031) and included a yellow blotch. We did not observe plants consistent with *R. viscosum*, but they have been reported as present in the past (Kron 1993).

Many of the diploids displayed morphological characters that were consistent with hybrids. For example, diploid plants with pink/peach flowers, with or without a blotch, sometimes with fragrance, and sometimes with a red pistil and stamens

Table 1 continued.

	83°51.840'W												
B005*	35°31.274'N, 83°51.831'W	1.60 ± 0.04	2x	Orange with Red Striations	N	33.60	40.00	A	F	N	Red	B	G
B006*	35°31.289'N, 83°51.830'W	1.62 ± 0.001	2x	Orange Red	Orange	35.68	39.18	A	W	N	Red	B	E
B007*	35°31.258'N, 83°51.793'W	1.61 ± 0.0001	2x	White	Yellow	41.27	39.28	A	W	Y	Red/White	F	G
B008*	35°31.239'N, 83°51.712'W	3.13 ± 0.09	4x	Light Yellow Pink blush	N	41.55	42.22	A	W	N	Yellow	F	G
B009*	35°31.234'N, 83°51.750'W	1.56 ± 0.03	2x	Red	Orange	33.92	44.57	A	W	N	Red	B	E
B010*	35°31.254'N, 83°51.806'W	1.63 ± 0.02	2x	Pink	Orange	37.95	42.90	A	F	N	Pink/Red	F	E
B011*	35°31.254'N, 83°51.804'W	1.63 ± 0.01	2x	White	Yellow	40.67	32.30	A	F	N	Red/White	F	G
B012*	35°31.246'N, 83°51.806'W	1.63 ± 0.01	2x	Pink Yellow	Orange	38.19	49.93	A	W	N	Yellow/White	B	E
B013*	35°31.227'N, 83°51.821'W	1.63 ± 0.01	2x	Pink with White Stripes	yellow	40.39	43.71	A	F	Y	Light Pink	B	E
B014*	35°31.216'N, 83°51.851'W	1.59 ± 0.04	2x	Red Orange Yellow	N	38.08	53.15	A	W	N	Orange	B	G
B015	35°31.207'N, 83°51.854'W	1.58 ± 0.04	2x	Peach	N	37.38	32.37	A	W	N	Red	B	G
B016	35°31.257'N, 83°51.943'W	1.58 ± 0.02	2x	Red Orange	N	38.75	33.03	A	W	N	Red	F	E
B017	35°31.263'N, 83°51.956'W	1.68 ± 0.02	2x	Pink	Yellow	33.45	35.19	A	F	N	Pink	B	G
B018	35°31.227'N, 83°51.990'W	1.55 ± 0.003	2x	Red	N	31.41	33.67	A	F	N	Red	F	E
B019	35°31.216'N, 83°52.030'W	3.22 ± 0.03	4x	Orange Yellow	N	39.29	41.42	A	W	Y	Orange/Red	B	E
B020	35°31.209'N, 83°52.035'W	1.64 ± 0.04	2x	Orange Red	N	34.52	42.84	A	W	N	Red	F	E
B021	35°31.192'N, 83°52.043'W	1.63 ± 0.03	2x	Orange	N	U	U	A	F	N	Pink-red	F	G
B022	35°31.192'N, 83°52.045'W	1.63 ± 0.02	2x	Orange Red	N	44.90	50.04	A	F	N	Red	F	G
B023	35°31.173'N, 83°52.043'W	1.65 ± 0.02	2x	Orange Red	N	36.67	43.27	A	F	N	Red	F	E
B024	35°31.186'N, 83°52.031'W	1.64 ± 0.06	2x	Peach Red	N	43.26	42.19	A	W	N	Red	B	E
B025	35°31.184'N, 83°52.029'W	1.63 ± 0.04	2x	Orange Red	N	36.75	44.31	A	W	N	Red	F	G
B026	35°31.176'N,	1.60 ± 0.04	2x	Orange	N	36.79	27.21	A	W	N	Peach/Orange	B	G

were common (e.g., A002, A004, A005, A007, A011, A022, A024, A029, A030, B004, B010, B013, B015, B017, B022, B027, B029, B030, B040, B041, B044, B045, B049, B051). Based on morphology, these are most likely hybrids between *R. arborescens* and *R. cumberlandense*. Plants with very similar appearance were discovered in Vogel State Park at Neel Gap, Georgia in 1934 and were named *R. furbishii* (Lemmon 1941). Leach (1958) later recreated this general phenotype by hybridizing *R. arborescens* and *R. cumberlandense* and concluded that *R. furbishii* was really an interspecific hybrid. Leach (1958) further speculated that blotched *R. arborescens* may be the result of introgression from *R. cumberlandense*. Other plants, including B002 (yellow flowers, no fragrance, but with red stamens and

pistil) and B011 (white flowers and no fragrance), are also most likely advanced hybrids between *R. arborescens* and *R. cumberlandense*. Evidence for hybrids involving other species was difficult to discern based on morphology. These observations support the premise that there is considerable interspecific hybridization among diploid species on Gregory Bald, most commonly between *R. arborescens* and *R. cumberlandense* (Galle 1987; Kron 1993).

Tetraploids were only found in understory conditions in areas surrounding and below the open bald, often in association with diploids. Many of these tetraploids were consistent with *R. calendulaceum* (e.g., A032, A033, B008, B053, B055, B058, B059), though none of these plants had distinctive blotches, a

common trait in *R. calendulaceum* (Kron 1993). Two tetraploids (B019 and B056) were noteworthy in that they had phenotypes consistent with *R. calendulaceum* but were also fragrant. These plants appear to represent introgression of fragrance from a diploid species (e.g., *R. arborescens*) into *R. calendulaceum*.

Without considering genome size and ploidy level, *R. cumberlandense* and *R. calendulaceum* were virtually indistinguishable at this site. Various traits that have been presented as key distinguishing characteristics, including flower size and sepal margins (Table 1), were variable within both cytotypes. Both taxa were blooming well after leaves had emerged at this site and displayed a range of flower colors from yellow to dark red. In a more extensive survey of *R. calendulaceum* and *R.*

Table 1 continued.

	83°52.014'W			Yellow									
B027	35°31.191'N, 83°52.006'W	1.61 ± 0.03	2x	Pink	Yellow	33.53	33.48	A	W	N	Pink	B	E
B028	35°31.191'N, 83°52.001'W	1.58 ± 0.06	2x	Red	N	31.19	37.65	A	W	N	Red	B	E
B029	35°31.190'N, 83°51.982'W	1.63 ± 0.02	2x	Pink	Yellow	42.03	44.16	A	W	N	Pink/Red	F	E
B030	35°31.187'N, 83°51.974'W	1.64 ± 0.03	2x	Pink	Orange	38.97	40.73	A	W	Y	Pink-red	B	E
B031	35°31.184'N, 83°51.974'W	1.64 ± 0.002	2x	White	Yellow	42.53	44.97	A	W	Y	White-red	B	E
B032	35°31.211'N, 83°51.952'W	1.64 ± 0.01	2x	Red	N	31.30	36.55	A	W	N	Red	F	E
B033	35°31.211'N, 83°51.926'W	1.64 ± 0.01	2x	Pink Orange	N	34.00	33.10	A	W	N	Red	B	E
B034	35°31.205'N, 83°51.930'W	1.62 ± 0.06	2x	Pink	Orange	33.40	35.20	A	W	Y	White-pink	B	G
B035	35°31.206'N, 83°51.924'W	1.61 ± 0.02	2x	White Yellow	N	32.00	28.60	G	F	N	White-pink/Red	F	G
B036	35°31.229'N, 83°51.898'W	1.67 ± 0.08	2x	Orange Red	N	35.56	43.26	A	W	Y	Pink-red	F	E
B037	35°31.248'N, 83°51.924'W	1.64 ± 0.02	2x	Orange	N	29.47	31.80	A	W	N	Pink-Red	F	E
B038	35°31.231'N, 83°51.901'W	1.65 ± 0.04	2x	Red	N	38.41	42.32	A	W	N	Red	F	E
B039	35°31.227'N, 83°51.887'W	1.60 ± 0.01	2x	Pink Orange	N	34.06	50.26	A	W	N	Red	B	E
B040	35°31.227'N, 83°51.880'W	1.63 ± 0.01	2x	Pink	N	42.76	47.91	A	W	Y	Red	F	G
B041	35°31.226'N, 83°51.883'W	1.62 ± 0.01	2x	Orange Yellow Peach	N	33.09	37.36	A	W	Y	Orange	F	G
B042	35°31.230'N, 83°51.877'W	1.61 ± 0.03	2x	Red Orange	N	35.95	51.77	A	F	N	Red	F	E
B043	35°31.228'N, 83°51.879'W	1.61 ± 0.003	2x	Orange	N	32.47	33.38	A	W	N	Peach	B	E
B044	35°31.237'N, 83°51.868'W	1.61 ± 0.04	2x	Pink	Orange	31.48	39.54	A	W	N	Pink to Red	F	E
B045	35°31.241'N, 83°51.861'W	1.60 ± 0.01	2x	Yellow Peach	N	33.73	34.26	A	F	N	Red	F	G
B046	35°31.248'N, 83°51.866'W	1.59 ± 0.004	2x	Pink	N	33.80	41.26	A	W	N	Pink	B	E
B047	35°31.247'N, 83°51.855'W	1.59 ± 0.02	2x	Pink	Yellow	38.70	36.03	A	F	Y	White to Pink/Red	B	E
B048	35°31.251'N, 83°51.858'W	1.64 ± 0.04	2x	Red Orange	N	30.22	31.33	A	F	N	Red	F	E
B049	35°31.257'N,	1.62 ± 0.02	2x	Pink	N	38.94	38.34	G	W	Y	Yellow to Red	F	G

cumberlandense in the Nantahala Mountains of N.C., Willingham, Jr. (1976) found it was difficult to separate the two species in the field—their flowering period overlapped—and the cited distinguishing characters (Bowers 1968; Galle 1968; Lemmon 1937) were of little value to the point of making field identification difficult or impossible. Perhaps introgression between these two species in Western North Carolina has made it particularly difficult to clearly separate them there.

There has been considerable debate and discussion on the origin of the tetraploid, *R. calendulaceum.* Li (1957) indicated that *R. calendulaceum* had two distinct sizes of chromosomes, suggesting it was an allotetraploid hybrid, possibly between *R. cumberlandense* and *R. prinophyllum* (Small) Millais (King 1977;

Kron 1993). However, more recent work by Jones et al. (2007) using flow cytometry showed that the base genome size (1Cx) for Pentanthera azaleas is highly conserved and that there is no discernible difference in average chromosome size between *R. cumberlandense, R. prinophyllum,* and *R. calendulaceum.* King (1977) found, in a limited sampling, that *R. cumberlandense* and *R. calendulaceum* varied slightly in flavonoid constituents; yet his phylogenetic analyses, based on flavonoid constituents, showed them to be closely allied. Phylogenetic analysis based on morphological, phenological, and chemical characters also show *R. cumberlandense* and *R. calendulaceum* to be very similar (Kron 1993). Considering these two species are virtually indistinguishable morphologically at the Gregory Bald site,

we suggest that *R. calendulaceum* there are primarily derived from *R. cumberlandense,* with limited introgression from other species, i.e., the two species represent a primarily autopolyploid series. However, it is not unlikely that elsewhere tetraploid *R. calendulaceum*-like plants have arisen, and continue to arise, through independent genome duplication events involving *R. cumberlandense* by itself or in various combinations with other diploid species. This scenario would certainly contribute to the great range of morphological variation noted in *R. calendulaceum* (Willingham 1973; Kron 1993).

Wayah Bald (W1-W4)

Of the samples collected at Wayah Bald, one plant was a tetraploid and consistent with *R. calendulaceum* (W4),

Table 1 continued.

ID	Coordinates	Genome size	Ploidy	Flower Color	Blotch			Tube Flare	Petal Margin		Color	Sepal Margin	Hairs
	83°51.858'W												
B050	35°31.260'N, 83°51.853'W	1.62 ± 0.04	2x	Apricot	N	26.09	26.15	G	W	N	Yellow to Pink	F	G
B051	35°31.263'N, 83°51.856'W	1.61 ± 0.01	2x	Pink	Orange	27.37	25.43	G	F	N	Pink	F	E
B052	35°31.207'N, 83°52.066'W	1.58 ± 0.01	2x	Red Orange	N	35.35	40.11	A	F	N	Red	B	E
B053	35°31.229'N, 83°52.287'W	3.18 ± 0.06	4x	Yellow Apricot	N	40.45	45.24	A	W	N	Peach	F	E
B054	35°31.215'N, 83°52.309'W	1.64 ± 0.03	2x	Red Orange	N	40.34	49.99	A	F	N	Red	B	E
B055	35°31.212'N, 83°52.306'W	3.22 ± 0.04	4x	Apricot Yellow	N	38.38	49.51	A	W	N	Peach	B	E
B056	35°31.247'N, 83°52.328'W	3.26 ± 0.03	4x	Yellow	N	39.53	50.06	A	W	Y	Yellow	F	E
B057	35°31.702'N, 83°52.003'W	1.60 ± 0.04	2x	Orange	N	42.86	55.10	A	W	Y	Red	B	E
B058	35°31.680'N, 83°52.146'W	3.20 ± 0.06	4x	Orange	N	34.10	40.00	A	F	N	Peach	B	G
B059	35°31.756'N, 83°52.296'W	3.17 ± 0.01	4x	Orange Pink	N	42.54	50.89	A	W	N	Yellow	B	G
Wayah Bald													
W1	35°10.638'N, 83°33.826'W	1.62 ± 0.03	2x	White	N	40.28	38.52	G	W	Y	Red/White to Red	B	G
W2	35°10.637'N, 83°33.827'W	1.58 ± 0.04	2x	White	Yellow	47.69	46.62	G	W	Y	White to Red/White	B	G
W3	35°10.640'N, 83°33.822'W	1.61 ± 0.04	2x	White	Yellow	47.43	44.55	G	W	N	White to Red/White	B	G
W4	35°10.677'N, 83°33.808'W	3.17 ± 0.06	4x	Orange Yellow	N	42.09	43.99	A	W	N	Light Pink/Dark Pink	F	G
Wine Spring Bald													
W5	35°10.501'N, 83°34.883'W	3.21 ± 0.09	4x	Red	N	37.31	44.87	A	W	N	Red	B	E
W6	35°10.399'N, 83°34.857'W	3.28 ± 0.10	4x	Orange	N	38.72	49.05	A	W	N	Peach	F	E
W7	35°10.395'N, 83°34.883'W	3.26 ± 0.03	4x	Orange	N	37.03	36.37	A	W	N	Orange	F	E
W8	35°10.391'N, 83°34.902'W	1.63 ± 0.03	2x	Yellow with Dark Yellow Striations	N	50.52	44.11	A	W	N	Red	F	G
W9	35°10.378'N, 83°34.796'W	3.19 ± 0.05	4x	Red Orange	N	39.03	44.99	A	W	N	Red-orange	B	E
W10	35°10.078'N, 83°34.832'W	2.41 ± 0.04	3x	Orange Pink	Orange	43.52	35.68	A	W	N	Red/Pink	F	E
W11	35°10.077'N, 83°34.835'W	3.17 ± 0.04	4x	Red	U	U	U	U	U	N	U	F	E
W12	35°10.064'N, 83°34.851'W	3.18 ± 0.07	4x	Orange	N	37.52	36.38	A	W	N	Red	B	G

Table Key
Unavailable (U)
Blotch: No (N)
Tube Flare: Abrupt (A), Gradual (G)
Petal Margin: Flat (F), Wavy (W)
Sepal Margin: Bristly (B), Fringe (F)
Flower/Pedicel Hairs: Eglandular (E), Glandular (G)
(*) indicates that voucher for specimen is available at NCSU Herbarium, accession numbers 130739 - 130776

Triploid
Tetraploid
Diploid

though lacking a blotch (Table 1, Fig. 2, Google Earth Map, http://www.ces.ncsu.edu/fletcher/mcilab/publications/pentantheramap.kmx). Three other plants from Wayah were diploid and fairly typical of *R. arborescens* (W1, W2, and W3). However, all three had wavy petal margins, W2 and W3 had yellow blotches, and W3 lacked any fragrance, possibly indicating some introgression from either *R. calendulaceum* or *R. cumberlandense* (if it has existed in this area).

Wine Spring Bald (W5-W12)

Of the plants surveyed at Wine-spring Bald, six were tetraploid, one was diploid and one was triploid. The tetra-ploid plants (W5, W6, W7, W9, W11, and W12) were fairly typical of *R. calendulaceum*, yet most lacked a blotch, and W5, W6, and W7 had eglandular pedicel hairs. The diploid plant W8 had a yellow flower with red pistil and stamens and an unusually long corolla (50.52 mm), suggesting hybridization between *R. arborescens* and either *R. calendulaceum* or *R. cumberlandense*. The triploid specimen, W10, had an orange-pink flower with an orange blotch and red pistil and stamens, consistent with an interploid hybrid between *R. calendulaceum* and *R. arborescens*. Although triploid plants do appear to be rare, Li (1957) also reported apparent triploids ($2n \sim 30\text{-}40$) on Wayah Bald. Other naturally occurring triploid Pentanthera azaleas have been reported by Jones et al. (2007). We have also documented three triploids (e.g., *R.* 'Chameleon') in hybrid zones of *R. calendulaceum* and *R. periclymenoides* found along Lake Keowee, S.C. (Towe 2004; unpublished data).

Introgression between diploid and tetraploid plants can occur in various ways. Diploid plants may produce an unreduced gamete ($2n = 2x = 26$) that combines with a reduced gamete from a tetraploid ($1n = 2x = 26$) to produce a tetraploid with balanced genetic contributions from each parent (Bretagnolle and Thompson 1995; Galletta and Ballington 1996; Ramsey and Schemske 1998). B019 and B056 from

Gregory Bald could have arisen in such a manner with an unreduced gamete from *R. arborescens* or an *R. arborescens* hybrid crossed with *R. calendulaceum* to produce a fragrant tetraploid in either F_1 or subsequent generations. Unreduced gametes are relatively common in *Rhododendron* spp. (Widrlechner et al. 1982; Widrlechner and Pellett 1983). In many cases, hybrids have been found to produce unreduced gametes at higher frequencies than parental species (Ramsey and Schemske 1998).

Alternatively, triploids can also serve as genetic and reproductive bridges. Triploid *Rhododendron* spp., though somewhat infertile, can produce unreduced gametes at relatively high (>5%) frequencies (Jones and Ranney 2009), often at higher frequencies than diploids (Dweikat and Lyrene 1988; Veilleux 1985). The union of an unreduced gamete from a triploid ($2n = 3x = 39$) with a normal reduced gamete from a diploid ($1n = 1x = 26$) offers an alternative pathway, sometimes called a triploid bridge, for diploids to introgress/transform into tetraploids (Ramsey and Schemske, 1998).

Introgression from triploids and tetraploids into diploid populations is less common, but can occur. In some cases, tetraploids can produce diploid offspring through the parthenogenetic development of reduced gametes, a form of apomixis sometimes referred to as polyhaploidy (de Wet 1971; Shoemaker 1986). The production of $1x$ or ~$1x$ gametes by triploids and tetraploids through complement fractionation, asynchronous meiotic rhythms, chromosome elimination, random chromosome assortment, fertile aneuploids, and other unusual meiotic pathways may also allow for introgression from higher to lower ploidy levels (Avers 1954; Burton and Husband 2001; Husband 2004; Lim et al. 2005; Norrmann and Quarin 1987; Peckert and Chrtek Jun. 2006; Ramsey and Schemske 1998; Risso-Pascotto et al., 2004; Smith-White 1948; Widrlechner et al. 1984).

Although instances of interspecific and interploid hybridization may initially be rare, once they do occur, they can provide a gateway for introgression. In studies of hybrid Iris zones, hundreds of advanced hybrid genotypes were detected (e.g., F_2, F_3, BC_2 BC_3) but no F_1s were found (Arnold 2006). This observation suggests that F_1 hybrids may represent the most restrictive bottleneck for genetic exchange, but once they are formed, they serve as bridges for further exchange. Rare events of interspecific/interploid hybridization can thus be extremely important and have substantial evolutionary impacts (Mallet 2005). Infrequent triploids, like the one found at Wine Spring Bald, may play a key role in introgressive hybridization between *R. calendulaceum* and *R. arborescens* there.

Habitats can change dramatically over both the short- and long-term. Natural events like succession, fire, and climate shifts lead to ever-changing distribution patterns and new species associations. As recently as 18,000 years ago, during the last glacial maximum, the Southern Appalachian region consisted of boreal and tundra environments (Delcourt and Delcourt 1975; Shafer 1988). Reconstructed paleovegetation maps for the Holocene epoch illustrate the continuous nature of climate-induced changes in vegetation biomes in this region, often without modern analogs (Overpeck et al. 1992). On an even broader scale, throughout the Pleistocene epoch, the flora of Southeastern North America were subjected to multiple glacial cycles (as many as 20 over a period of 2 million years) (Davis 1983; Hays et al. 1969). Temperate species, including Pentanthera azaleas, probably survived these glacial periods on higher terrain at refugial sites throughout southern Tennessee, southeastern and northern Louisiana, southwestern Mississippi, southwestern Georgia, Alabama, and northern Florida (Davis 1976; Delcourt 1980; Parks et al. 1994; Watts and Stuiver 1980). Over time, surviving lineages appeared to have undergone cycles of southern migrations followed by re-radiation that contributed to a reticulate pattern of evolution. These periods of melting-pot sympatry ostensibly led to rehybridization and reinforcement of reproductive compatibility that effectively compromised divergence (Levin et al. 1996; Rieseberg 1997). These interludes would also have generated novelty and diversity and spread successful alleles that allowed for enhanced fitness in new and varied habitats during expansion phases (Arnold 1997; 2006; Riseberg and Carney 1998). Although this ongoing genetic mixing appears to have contributed to a poorly differentiated web of species, this retained capacity for hybridization most likely afforded evolutionary and adaptive benefits for the common Pentanthera gene pool, a mechanism ideally suited to a rapidly changing climate.

Accumulated evidence suggests that hybridization among the Pentanthera azaleas is more than a taxonomic muddlement and botanical curiosity; rather, it reflects a pervasive and ongoing evolutionary process that has provided these plants with a successful adaptive outcome over recent millennia. The process has been less of a breakaway divergence of individual lineages and more of an ongoing, intertwined, evolutionary exchange.

Conclusion

Results of this study provide new evidence of both interspecific and interploid hybridization among species of Pentanthera azaleas. Despite sympatric populations of diploids and tetraploids with overlapping blooms, triploid hybrids were rare at these sites. However, rare triploids can provide bridges for genetic exchange between diploids and tetraploids. Other reproductive mechanisms, particularly unreduced gametes, may also allow for direct genetic exchange between ploidy levels. Furthermore, the substantial reproductive compatibility among species, ongoing hybridization, and the phytogeographic history suggest that the Pentanthera azalea species in the Southeastern United States continue to evolve with a high level of genetic exchange through a successful evolutionary web.

Figure 1. Topography map of Gregory Bald showing the location of sampled plants. Triangle symbols indicate tetraploids and squares indicate diploids.
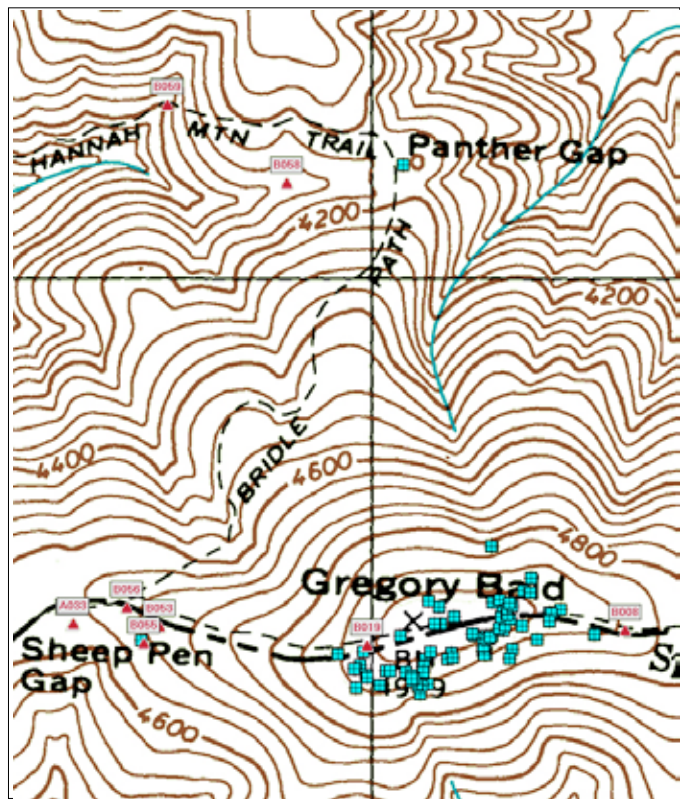

Figure 2. Topography map of Wayah and Wine Spring Balds showing the location of sampled plants. Triangle symbols indicate tetraploids and squares indicate diploids with the exception of W10 being a triploid.

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