

Understanding Polyploidy:

Insights Into the Evolution and Breeding of Azaleas

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Polyploidy is relatively common in plants and *Rhododendron* are no exception. This curious genetic phenomenon has provided an important pathway for evolution and speciation and continues to be a significant field of study. On a practical level, there are many opportunities for utilizing polyploidy as a valuable tool in azalea breeding programs.

What Is Polyploidy and How Does it Arise?

A polyploid is simply an organism that contains more than two complete sets of chromosomes. For animals, this is rare (though a polyploid rat, the first polyploid mammal ever identified, was recently discovered in Argentina). In plants, however, polyploidy occurs naturally and is widespread.

The term “ploidy” or “ploidy level” refers to the number of complete sets of chromosomes and is notated by an “ x ”. An individual with two sets of chromosomes is referred to as a diploid ($2x$), three sets would be a triploid ($3x$), and so on with tetraploid ($4x$), pentaploid ($5x$), hexaploid ($6x$), etc. It is sometimes also important to identify if one is referring to the reduced (gametophytic) chromosome number following meiosis as would be found in egg and sperm (denoted as “ n ”) or in non-reduced (sporophytic) tissue as would be found in a growing plant (denoted as “ $2n$ ”). Thus, for example, a tetraploid azalea would be presented as $2n=4x=52$.

Polyploidy can arise naturally in a number of different ways. In some cases a somatic (non-reproductive) event can occur, due to a disruption in mitosis, resulting in chromosome doubling in a meristematic cell(s) that will give rise to a polyploid shoot. These sports are sometimes evident on a plant by their enlarged “gigas” condition. Polyploids can also result from the union of unreduced gametes—eggs and sperm that have not undergone normal meiosis and still have a $2n$ complement.

The origin of polyploids can often determine if polyploids will be fertile and how they can best be used in a breeding program. If a tetraploid arises from spontaneous doubling in a shoot or from the union of unreduced gametes from two closely related (e.g., same species) diploid individuals, it will have four similar (homologous) versions of each chromosome. Despite different pathways, both of these polyploids behave similarly reproductively and are often referred to as autotetraploids (or polysomic tetraploids). Autopolyploids may or may not be fertile. In diploids, meiosis involves the pairing of homologous chromosomes, which eventually segregate to form two separate gametes, each with one set of chromosomes. Infertility can arise in

autopolyploids due to the fact that there are more than two homologous chromosomes. The presence of multiple homologous chromosomes often results in spurious pairing between multiple chromosomes, unpaired chromosomes, and gametes with unbalanced chromosome numbers (aneuploids).

Offspring that result from sexual reproduction between unreduced gametes or somatic doubling in a hybrid of different species are referred to as allopolyploids (or sometimes amphidiploids or disomic polyploids). These plants also have four versions of each chromosome, but the two from one parent are sufficiently different from the two from the other parent that they generally don’t pair during meiosis. Due to this composition, allopolyploids are typically fertile. During meiosis each chromosome can pair with its homologous partner, meiosis continues, resulting in fertile germ cells.

In many cases polyploids fall somewhere in between an autopolyploid and allopolyploid, whereby there is partial chromosome homology resulting in a combination of disomic and polysomic pairing and are referred to as segmental allopolyploids.

Role of Polyploids in Plant Evolution

In contrast to the gradual evolutionary process whereby new species evolve from isolated populations, new species of plants can also arise abruptly. The most common mechanism for abrupt speciation is through the formation of natural polyploids. Once a tetraploid arises in a population, it can generally hybridize with other tetraploids. However, these tetraploids are reproductively isolated from their parental species. Tetraploids that cross with diploids of the parental species will result in triploids that are typically sterile. This phenomenon provides a “reproductive barrier” between the polyploids and the parental species—a driving force for speciation.

Various estimates suggest that as many as 47-70 percent of flowering plants are of polyploid origin. There are a number of factors that may provide polyploids with adaptive and evolutionary advantages. Perhaps most importantly, polyploids can be significantly more heterozygous than their diploid counterparts. Polyploids can have four different genes (alleles) present at any given locus (location on a chromosome). The degree of heterozygosity may be a key factor in the growth, performance, and adaptability of a polyploid. Allopolyploids can have a much greater degree of heterozygosity (dissimilar genes) which can contribute to heterosis or hybrid vigor. Furthermore, this heterozygosity is somewhat fixed (chromosomes that originated from

a given species preferentially pair with similar homologous chromosomes during meiosis, ensuring that the genomes of both parental species will continue to be present). On the other hand, the addition of multiple copies of homozygous chromosomes (as would be the case with autopolyploids) does little to enhance genetic superiority and can actually reduce vigor and fertility by creating a more “inbred” situation.

One question that frequently arises is whether or not polyploids inherently have greater stress tolerance. For example, it has often been observed that disproportionate numbers of polyploids are found in cold, dry regions. Some argue that this is a spurious correlation or possibly the result of intermixing of species and formation of allopolyploids during glacial periods. Studies of *Rhododendron* did not find improved cold hardiness in induced tetraploids (Väinölä and Repo, 1999; Krebs, 2005). However, some polyploids may have certain characteristics that do provide certain adaptive benefits. Studies have demonstrated that allopolyploids exhibit “enzyme multiplicity” (Soltis and Soltis, 1993; Soltis et al., 2003). Since allopolyploids represent a fusion of two distinctly different genomes, these polyploids can potentially produce all of the enzymes produced by each parent as well as new hybrid enzymes. This enzyme multiplicity may provide polyploids with greater biochemical flexibility; possibly extending the range of environments in which the plant can grow (Roose and Gottlieb, 1976). Other changes in gene expression, altered regulatory interactions, and rapid genetic and epigenetic changes could further contribute to increased variation and new phenotypes (Osborn et al., 2003).

Polyploid Rhododendron

Jones et al. (2007) determined ploidy levels of a diverse collection of species, hybrids, and cultivars in the *Hymenanthes* (elepidote rhododendrons), *Rhododendron* (lepidote rhododendrons), *Pentanthera* (deciduous azaleas), and *Tsutsusi* (evergreen azaleas) subgenera. Polyploidy was found to be common in the genus *Rhododendron* and considerably more prevalent in the subgenus *Pentanthera* than previously known. As expected from past reports, all of the sampled species within the *Hymenanthes* were diploid. However, many interspecific hybrids were polyploids. Hybridity has been shown to increase formation of unreduced gametes even when the parental species might not exhibit the same characteristic. Concordant with previous findings, polyploidy was common among species and their hybrid derivatives from subgenus *Rhododendron*. *Rhododendron augustinii* and its hybrids were found to be tetraploids, while *R. maddenii* clones were found to be hexaploid or octoploid. ‘Bubblemum’ and ‘Northern Starburst’ were both tetraploids developed from in-vitro colchicine treatments. Polyploidy was not common among the evergreen azaleas with the exception of two chemically induced tetraploids. The majority of deciduous azaleas were found to be diploids as has been reported previously, and *R. calendulaceum* was confirmed as a tetraploid. However, these results indicated that natural polyploidy is more prevalent among deciduous azalea spe-

cies than once thought. Particularly noteworthy were the findings that *R. occidentale* includes both diploid and tetraploid individuals and that *R. atlanticum* and *R. austrinum* are predominantly tetraploid species. Many deciduous azalea cultivars were found to be polyploids including the tetraploids ‘Admiral Semmes’, ‘Gibraltar’, ‘Gold Dust’, ‘Lemon Lights’, ‘Marydel’, ‘My Mary’, ‘Klondyke’, ‘Snowbird’, and the octoploid ‘Fragrant Star’. Zhou et al. (2008) also found the newly described species, *R. colemanii*, to be tetraploid.

Polyploidy and Plant Improvement

Considering the profound importance of polyploidy in plant evolution, it is understandable that there was considerable interest in developing induced polyploids when mitotic inhibitors were first discovered in the 1930s. However, despite the fact that polyploids have been developed for many major crops, these plants are almost always found to be inferior to their diploid progenitors. Somatic doubling does not introduce any new genetic material, but rather produces additional copies of existing chromosomes. This extra DNA must be replicated with each cell division. Enlarged cell size is often associated with polyploids, which can result in anatomical imbalances. Other deleterious effects can include erratic bearing, brittle wood, and watery fruit. High-level polyploids (e.g. octaploids) can be stunted and malformed, possibly resulting from the extreme genetic redundancy and somatic instability that leads to chimeral tissue. Despite the drawbacks of induced autopolyploids, these plants may be valuable if they are in turn used in a breeding program to enhance the degree of heterozygosity and are further selected for desirable traits.

Opportunities for Breeding Polyploid Azaleas

Overcoming barriers to hybridization. In some cases, desirable crosses are difficult to obtain between parents with different ploidy levels. Although it is possible to hybridize across ploidy levels in azaleas, we have found that success is generally low and the resulting hybrids will most likely be sterile or highly infertile. Crosses among azaleas of the same ploidy level are typically more successful and result in fertile breeding lines. In some cases, increasing the ploidy level of one plant to match the ploidy level of the other parent may enhance success and future breeding options.

Restoring fertility in wide hybrids. It is not unusual for hybrids between distant taxa (e.g., different subgenera) to be sterile. This often occurs due to failure of the chromosomes to pair correctly during meiosis—referred to as chromosomal sterility. By doubling the chromosomes of a wide hybrid, each chromosome has an exact duplicate and chromosomal homology and fertility can be restored. This technique has been used successfully to restore fertility in *Rhododendron* ‘Fragrant Affinity’ (Contreras, 2007a), an unusual hybrid between *R. ponticum* and a fragrant deciduous azalea (Contreras, 2007b).

Enhancing pest resistance. Increasing the chromosome number and related gene dose can sometimes enhance the expression and concentration of certain secondary metabolites and defense chemicals. However, this is not always the case, and little is generally known about the relationship between gene dose, gene silencing, and expression of secondary metabolites. A more promising approach would be to create allopolyploids between plants with diverse endogenous secondary metabolites. This strategy could be particularly effective for combining pest (e.g., azalea lace bug) or disease (e.g., *Phytophthora* spp.) resistance characteristics and potentially contributing to a much broader, more horizontal form of pest resistance in azaleas.

Enlargement and enhanced vigor. Although enlarged cell size found in some polyploids can have undesirable effects, it can sometimes also be beneficial. Flower petals can be thicker and flowers can be longer lasting in polyploid plants including *Rhododendron* (Kehr, 1996).

Methods for Inducing Polyploidy

In the late 1930s it was discovered that colchicine inhibited the formation of spindle fibers and temporarily arrests mitosis at the anaphase stage. At this point, the chromosomes have replicated, but cell division has not yet taken place resulting in polyploid cells. Other mitotic inhibitors, including oryzalin, have also been identified and used as doubling agents.

Methods for applying these agents varies. One of the easiest and most effective methods is to work with a large number of seedlings with small, actively growing meristems. Seedlings can be soaked or the apical meristems can be submerged with different concentrations, durations, or frequencies of a given doubling agent. Shoots on older plants can be treated, but it is often less successful and results in a greater percentage of cytochimeras. Treatment of smaller axillary or sub-axillary meristems is sometimes more effective. Chemical solutions can be applied to buds using cotton, agar, or lanolin or by dipping branch tips into a solution for a few hours or days. Surfactants, wetting agents, and other carriers (dimethyl sulfoxide) are sometimes used to enhance efficacy.

Jones et al. (2008) evaluated the effectiveness of using repeated treatments of an oryzalin suspension in a warm agar solution, applied directly to apical shoots of *Rhododendron* seedlings, to induce polyploidy. Apical meristems of hybrid seedlings were subjected to 1, 2, 3, or 4 applications of oryzalin separated by 4-day intervals or left untreated (control). The results of this study demonstrated that the method of applying a suspension of oryzalin in warm, semi-solid agar to the shoots of *Rhododendron* seedlings was an effective method for inducing polyploidy. Although single applications resulted in some polyploid plants, multiple applications increased efficacy for some of the taxa studied. Treatments resulted in a range of ploidy levels, from $2x$ to $8x$, including cytochimeras.

Verifying Polyploidy Levels

Plants with increased ploidy levels are sometimes apparent by their distinct morphology. Increasing ploidy often results in increased cell size that in turn results in thicker, broader leaves and larger flowers and fruit. However, we have found it virtually impossible to identify polyploidy azaleas based on visual appearance. Other effective, but more time consuming, measures that indicate polyploidy include larger pollen size, greater number of chloroplasts per guard cell, and larger guard cells and stomates. Flow cytometry is a very useful tool for measuring DNA content, which can be correlated with ploidy level, and is very effective for *Rhododendron* (Jones et al., 2007). Traditional cytology is sometimes necessary to determine chromosomes' number and ploidy level, but is notoriously difficult for *Rhododendron* (Jones et al., 2007).

When testing and breeding polyploids, it is important to recognize that induced polyploids can sometimes be cytochimeras where the ploidy level varies in different types of tissue. Meristems are typically divided into three histogenic layers L-1, L-2, and L-3. Mutations and doubling agents may result in increased ploidy levels in one, two, or all three layers. For information on reproductive behavior, it is important to measure the ploidy level of L-2, or cortical layer, which is reflected in pollen size and chromosome counts from reproductive tissue (e.g. anthers). Root tips reflect the L-3 layer while the guard cells and the epidermis reflect the L-1 layer.

Summary

In the vast majority of cases, induction of autopolyploids will not, in of itself, result in substantially improved landscape plants. However, many opportunities exist, including overcoming barriers to hybridization, restoring fertility in wide hybrids, enhancing ornamental characteristics, increasing heterosis and vigor, and improving pest resistance. Recent developments in identifying existing polyploids and developing new ones provide an excellent foundation for breeding improved azaleas.

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Dr. Tom Ranney has been a faculty member at North Carolina State University since 1989 and is currently a Professor of Horticultural Science. He leads a research program at the Mountain Horticultural Crops Research and Extension Center in Fletcher, N.C. that focuses on the evaluation, selection, and development of new landscape plants.

Jeff Jones recently finished his Masters degree in Horticultural Science with Dr. Tom Ranney at North Carolina State University. His project took an in-depth look at polyploidy within the genus *Rhododendron*. He currently assists Dr. Ranney's ornamental plant breeding program as a Research Specialist.

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ASA Financial Position at December 31, 2007

INCOME STATEMENT

Income	
Azalean Income	3,915.00
Dues Income	28,989.00
Gift Income	1,512.00
Interest Income	4,150.22
Seed Exchange Income	243.00
Other Income	400.00
Total Income	39,209.22

Expenses	
Awards and Memorials	200.00
Azalean Expense	22,119.76
Dues Expense	3,007.29
Membership Roster	2,316.48
Website Expense	171.43
Other Postage	315.20
Other Exoebse	851.08
Total Expenses	28,981.24

Income - Expenses	10,227.98
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BALANCE SHEET

Assets	
Checking	10,600.63
Investments	76,236.43
Harding Garden	5,543.63
Total Assets	92,380.69

Liabilities and Reserves	
Liabilities	0.00
Operating Funds	21,687.56
General Reserve	51,081.08
Research Fund	14,068.43
Harding Garden	5,543.63
Total Liab. and Reserves	92,380.69

Respectfully Submitted,
Dan Krabill, Treasurer
April 28, 2008