# An Intersectional Point of View

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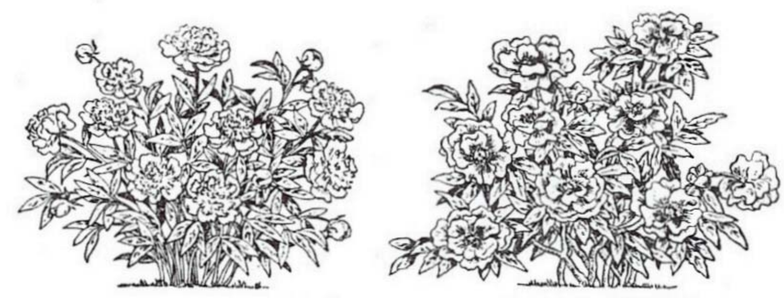

Herbaceous x Tree Peony

## *Chromosomes, Nuclear DNA and the Intersectional Peony*

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One of the things that has frustrated me for many years is the lack of basic knowledge concerning the ploidy level of the intersectional hybrids. I have stated numerous times my belief that these hybrids are all sterile triploids with 3 sets of chromosomes; two sets from the tree peony side and a single (haploid) set from the herbaceous side. This has always seemed like the most reasonable assumption based on the morphological characteristics of these plants. This assumption is further supported by the fact that these hybrids are so highly infertile. Although this hypothesis has been quite generally accepted, there is no solid, irrefutable scientific data to support this theory. Frankly, I am very surprised that no chromosome counts have ever been performed on this popular new group of hybrids. For years I have been hoping that someone would come along to fill this void, especially considering that peony chromosomes are so large and easy to count. By the middle of last summer, however, I had grown tired of waiting for someone else to provide these answers. So, in August 2005, I initiated and funded a modest scientific study to try to provide some more definite data to address the ploidy level of the intersectional hybrids. A summary of the approach and results of this activity are given below.

Last summer while doing some on-line research looking for possible sources for chromosome tests, I came across an exciting new technique for determining the ploidy level in plants, called Flow Cytometry. Following this lead, eventually led me to the Oregon State University Seed Laboratory and Dr. Sabry Elias. After a phone call and several email exchanges, Dr. Elias agreed to help me in a collaborative study to see whether this method could be useful in determining ploidy levels when applied to intersectional hybrid peonies.

# Nuclear DNA Content From Flow Cytometry

Flow cytometry is a fast and accurate method to determine the ploidy level in plant tissues by measuring the nuclear DNA content in living cells. This technique has been successfully used for ploidy determination of plants and seeds in Europe for over a decade and has become extremely popular in recent years. One of the key advantages of this method of ploidy analysis is that only a small amount of leaf tissue is required rather than the need for dividing cells, thus the tests can be done at any stage of plant growth. There are several basic steps to the experimental process. These are described briefly below:

1. Nuclear DNA of the plant sample under investigation is prepared (pulverized) and stained with a DNA-specific fluorescent dye.
2. Inside the flow cytometer, the microscopic particles are put into suspension and constrained to flow in single file within a fluid stream through the focus of intense light where the stained nuclear DNA will fluoresce.
3. The ploidy level is determined by measuring the fluorescent light intensity emitted from the stained molecules of a particular sample. The emitted light is proportional to the chromosomal DNA content of the sample.

The result of the analysis is usually displayed in the form of a histogram that plots the number of nuclei for each relative fluorescence intensity. The relative fluorescence intensity corresponds to the relative DNA content. A typical flow cytometer histogram from this study is shown in Figure 1. Because the particles are analyzed individually and at very high speed, large populations can be measured in a short time. With a typical measurement rate of about 1000 cells per second, more than 10,000 cells (nuclei) are analyzed in a fraction of a minute during a typical measurement. Since there is no need to employ tissues with dividing cells, the ease of sample preparation, and the ability to quickly measure the DNA content of thousands of cells, have made flow cytometry an extremely attractive alternative to microspectrophotometry and chromosome counting.

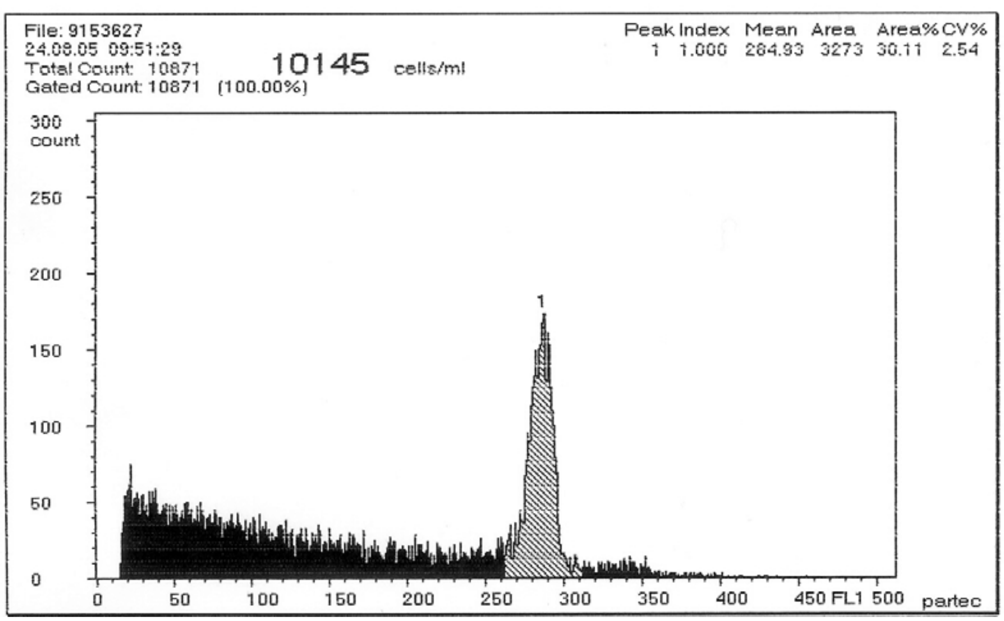


**Figure 1.** Histogram of the fluorescence intensity of leaf tissue from the lactiflora variety MARTHA W. as measured by a flow cytometer. The histogram plots the number of nuclei versus the relative nuclear DNA content for many thousands of cells.

## Sample Collection and Experimental Approach

Last summer, I collected leaves from 37 different peony cultivars for Dr. Elias to analyze using the Partec Flow Cytometer at the Oregon State University Seed Testing Laboratory. All samples were collected as mature leaves during August of 2005. These samples included various herbaceous, and tree peony cultivars as well as a number of intersectional hybrids. The herbaceous peonies were all lactiflora varieties that had proved to be successful seed parents in the intersectional cross. The tree peony cultivars included numerous Japanese tree peonies (i.e., p. suffruticosa) and lutea hybrids (i.e., p. suffruticosa x p. lutea). The hybrid tree peonies were $F_1$ and advanced generation (AG) hybrid cultivars. The advanced generation hybrids included both fertile and non-fertile hybrids. All six (6) fertile AG hybrids have been successfully used as pollen parents in the intersectional cross. The 37 peonies analyzed in these tests are listed in table 1 below.

**Table1.** Peony cultivars analyzed by DNA flow cytometry

| **Lactiflora Varieties** | **Suffruticosa Varieties** | **Fertile Adv. Gen. Lutea Hybrid Tree Peonies** | **$F_1$ Lutea Hybrid Tree Peonies** | **Intersectional Hybrids** |
|---|---|---|---|---|
| | | | | |
| Martha W. | Stolen Heaven | Golden Era | Goldfinch* | Yellow Emperor |
| Gertrude Allen | Renkaku | Reath's A-198 | Terresa | Singing in the Rain |
| Dewey's HP1-61 | Taiyo | Alice in Wonderland | Age of Gold | Smith Family Jewel |
| | Adzuma-Shibori | Unreg. Smith's Yellow | | Unnamed Sdlg R1P5* |
| | Fushakin | Boreas | | Unnamed Sdlg R3P1 |
| | Howzan | Zephyrus | | Unnamed Sdlg R3P14 |
| | Konron-Koku | | | Unnamed Sdlg R4P1 |
| | Unknown Suffrut-1 | | | Unnamed Sdlg R4P18 |
| | | | | Unnamed Sdlg R4P24 |
| | | | | Unnamed Sdlg R5P17 |
| | | | | Unnamed Sdlg R6P1 |
| | | | | Unnamed Sdlg R6P8 |
| | | | | |
| | | | | |
| | | **Non-fertile Adv. Gen. Lutea Hybrids** | **$F_1$ Potaninni Hybrid Tree Peonies** | **Reverse Cross Intersectional Hybrids** |
| | | | | |
| | | Leda | Helene Martin | Reverse Magic |
| | | Hephestos | | Impossible Dream |
| | | | | |
| 3 | 8 | 8 | 4 | 14 |

* These cultivars were eliminated from the summary results presented in Table 2 due to unusually large variances.

## Ploidy Level Determination

It is important to note that flow cytometry does not directly count or measure the number of chromosomes. Instead, this technique measures the amount of DNA in the chromosomes of the nucleus rather than the actual number of chromosomes in the nucleus. It does this by measuring the amount of nuclear (chromosomal) DNA in a very large number of cells individually as opposed to counting the chromosomes in a single or at most a few cells under a microscope. In most cases, the DNA amounts are expected to increase in direct proportion to the ploidy level. Therefore, tetraploids are expected to show double the amount of nuclear DNA compared to diploids. However, this expectation is not obeyed in all polyploids. The literature is full of examples where chromosome size in polyploids is smaller than expected. As you will see later, one of the exceptions to this rule occurs in the genus paeonia.

In cases where chromosome sizes are nearly the same for all samples, flow cytometry is a simple and unambiguous way to quickly and easily identify polyploids from diploid cultivars. Unfortunately, as mentioned above, this is not always the case. There are numerous examples in various genera, including paeonia, where chromosome size was found to decrease with increasing ploidy level and thus the relative DNA contents for polyploids and diploid samples cannot be directly compared to determine relative ploidy levels. As a result, it will be much more difficult to arrive at definitive conclusions regarding the ploidy level of the various peony cultivars included in this study without at least some additional information from actual chromosome counts.

## Flow Cytometry Results

The results of the DNA flow cytometer measurements for the 37 cultivars are summarized and presented in Table 2 below. The samples were measured in four separate batches over a period of about 2 weeks. Several samples (including MARTHA W.) were used as control samples and were therefore measured multiple times so that the various batches could be referenced to each other. The internal calibration standard and the various settings for the cytometer were the same for all runs. The relative DNA contents for all of the known diploid samples (i.e., the lactiflora and suffruticosa cultivars) were within a fairly narrow range of values as indicated in Table 2. I believe we can safely assume that the lactiflora and suffruticosa cultivars measured in this study are, in fact, diploids. This establishes a reference point against which the other cultivars can be compared in order to estimate the ploidy level of the other samples whose ploidy levels are unknown.

## Ploidy Levels of the Tree Peony Hybrids

As can be seen from the table, the $F_1$ lutea hybrid tree peonies all had DNA contents that were also within this same (diploid) range of values and these are, therefore, also presumed to be diploids. The advanced generation (i.e., $F_2$'s and BC's, etc.) lutea hybrids, on the other hand, split into two distinct groups based on their fertility. The first group contained two backcross AG hybrids (LEDA and HEPHESTOS), which are infertile. These fell well within the range of values expected for diploid cultivars and are, likewise, also assigned to the diploid level. The remaining AG tree peony hybrids are all fertile and as a group had DNA contents that were significantly higher than any of the other measured samples. On average, the DNA amounts for this group were about 25% higher than those of the diploid groups. In normal cases, these DNA values would correspond to a chromosome count of

12-13 (i.e., 1.25 x 10 = 12.5) as compared to the normal diploid count of 10. However, as mentioned previously, there is solid evidence to suggest that peonies don't follow the polyploid "rules". So, despite the fact that the measured DNA amounts for this group are not even close to twice those for the diploid groups, I am tentatively assigning these hybrids to the tetraploid level. This conclusion is based on several factors not the least of which is the high degree of fertility exhibited by this group, especially when used as pollen parents in the intersectional cross. I know, at this point, many of you are getting ready to abandon ship and look for the nearest lifeboat. This must sound like a pretty wild assumption based on the results presented in table 2. Nevertheless, this conclusion can be correct and yet still fit the present DNA data, as long as the chromosomes in these AG hybrids are substantially smaller than those in the diploid cultivars. Specifically, since it is assumed there are twice as many chromosomes (20) and the total compliment of chromosomes contains only 25% more DNA than the diploid set, it then follows directly that the polyploid chromosomes need to be smaller by a factor of ~1.6 in order to fit the DNA data in Table 2. OK, but is this a reasonable or even feasible assumption? I believe that it is and some of the data that supports this conclusion are discussed below.

**Table 2.** Summary of the DNA Flow Cytometry results for 35 peony cultivars

| Peony Type | # of Cultivars | Range of Relative DNA amounts | Average Relative DNA amount | Likely Ploidy Level |
|---|---|---|---|---|
| Intersectional Hybrid | 12 | 290-323 | 308.3 | 3n |
| Lutea Hybrid AG - Fertile | 6 | 318-352 | 340.3 | 4n |
| Lutea Hybrid AG - Infertile | 2 | 276-300 | 288.3 | 2n |
| Lutea Hybrid $F_1$ | 3 | 254-291 | 264.6 | 2n |
| Suffruticosa | 8 | 277-298 | 290.0 | 2n |
| Suffruticosa x Lactiflora | 1 | 272-282 | 276.9 | 2n |
| Lactiflora | 3 | 235-294 | 273.1 | 2n |

My conclusion that the fertile AG lutea hybrids are tetraploid is supported by numerous other observations and experimental results. For example, similar decreases in chromosome size with increasing ploidy level have been reported in a variety of other genera. In the genus Pratia, for instance, the size of the haploid set of chromosomes (genome) in the diploid species p. macrodon was found to be 2.5 times larger than the haploid set in the tetraploid species p. padunculata. Consequently, the amount of chromosomal DNA of the tetraploid species is actually 20% less than that of the diploid species. More importantly, this same pattern has also been observed in peonies. Recently, absolute haploid nuclear DNA amounts (i.e., C-values) were measured for 16 different species of paeonia, including 4 tetraploid species. By comparing the 4 tetraploids with the diploid species, we find that the genome size(s) in the diploid species are always larger than the genome size(s) in the tetraploid species by factors that vary between 1.02-1.53 with the average being about 1.3. These patterns suggest that there is some type of mechanism(s) in plants that tries to prevent genomic obesity by reducing excess or non-essential repetitive nuclear DNA in polyploids. It makes sense that there should be some limit to the amount of DNA that can be contained in the nucleus of cells. Plants cannot continue to increase their nuclear DNA as higher and higher-level polyploids are created without eventually encountering some serious genetic consequences. It seems this should be especially true for peonies, which have chromosomes that are among the largest in the plant world. When all of the scientific, morphological and fertility data are considered, I believe that this evidence supports the conclusion that the fertile AG lutea hybrids are tetraploids. However, it is clearly not the only possibility and thus this conclusion must be treated as preliminary, pending confirmation by actual chromosome counts.

## Ploidy Level Determination for the Intersectional Hybrids

Next, is the question of the ploidy level of the intersectional hybrids. Altogether, there were 14 intersectional hybrid samples that were analyzed as a part of this experiment. Twelve were normal (forward direction) hybrids and two were from reverse crosses. These cultivars are listed in Table 1. One of these samples (R1P5) was eliminated due to anomalously high variance. The results for the 13 remaining intersectional samples are summarized in table 2. Twelve of these 13 samples, including the reverse cross hybrid, REVERSE MAGIC, fell into a fairly narrow range of DNA values between 290-323. The one exception was the unique reverse cross (suffruticosa x lactiflora) hybrid, IMPOSSIBLE DREAM, which is, therefore, listed separately in table 2.

As a group, these 12 intersectional hybrids had DNA contents (307 ± 17) that were consistently higher than the two groups of known diploids (288 ± 10 and 265 ± 30) by about 10-15%. While this difference is significant, it is still substantially less than the 50% that would normally be expected, if these hybrids were triploid as previously assumed. However, this expectation is based on the assumption that the genome size is the same in polyploids as it is in the diploids. If this assumption were true, we would have to conclude, based on the DNA content, that these hybrids are aneuploids with an extra chromosome and a chromosome count of 11. Aneuploids with a single extra chromosome (2n +1) are known as trisomes. In nature, plants are far more tolerant of aneuploidy than animals. In *Homo sapiens*, trisomes for the X chromosome survive to birth and are responsible for serious problems such as Down syndrome. In plants, the consequences of an extra chromosome are usually much less serious. Here, the presence of an extra chromosome results in an odd number of chromosomes and thus causes infertility in addition to other morphological differences that depend on which particular chromosome is duplicated.

However, based on the discussion above regarding the AG lutea hybrids, there is another explanation that is consistent with the data and seems far more feasible. If we accept the conclusion stated earlier that the fertile AG lutea hybrids are, in fact, tetraploids, then we are forced to draw an entirely different conclusion in the case of the intersectional hybrids. One definitive conclusion, which can be drawn from the results of this study is that the DNA values for the intersectional hybrids as a group fall just about midway between the values for their parent groups. This is, of course, exactly what is expected for any cross that involves the union of normal haploid gametes. Specifically, in this case, the intersectional hybrids have DNA contents that is very close to one half the sum of the values for two parent groups (340.3 + 273.1)/2 = 613.4/2 = 306.7. This is compared to the average measured value of

308.3. In this way, the ploidy level of the intersectional group depends on the ploidy levels of both parent groups. Therefore, if we buy into the idea that the fertile AG lutea hybrid tree peonies are tetraploids (4n), then the intersectional cross becomes primarily a (2n x 4n) cross and should result in 3n progeny (1n + 2n = 3n). This leads to the conclusion that the intersectional hybrids are triploid with 15 chromosomes, despite having only a modest (~12%) increase in total nuclear DNA. This, in turn, requires that these 15 chromosomes be, on average, smaller (308.3/15 = 20.5) than the 10 chromosomes (273.1/10 = 27.3) present in the diploid seed parents, yet larger than those found in the tetraploid pollen parents (340.3/20 = 17.0). The end result is a clear pattern of decreasing chromosome size with increasing ploidy level.

## A Special Case – The Impossible Dream

Finally, I must address the question of the ploidy level of the one intersectional hybrid that stands apart from all the others. This is the unique reverse cross hybrid, IMPOSSIBLE DREAM. It originates from the cross (suffruticosa x lactiflora). Based solely on its morphological characteristics, I have previously suggested that this cultivar could be a diploid (APS Bulletin, No. 330). This suggestion now seems to be confirmed based on the results of this study. This plant was measured twice at different times and both measurements (271.5, 282.3) were well within the diploid range of DNA values. In addition, both parents, STOLEN HEAVEN (292.0) and MARTHA W., (276.7 ± 23) were also measured in this study. Ultimately, this determination may be the most important finding of the entire study.

## Comments and Conclusions

I began this study in the hope that this technique would provide a cheap, fast and effective alternative to counting chromosomes using the root tip squash method as a way of determining the ploidy level of intersectional peonies. In the end, this hope was largely unfulfilled as this method proved unsuccessful in providing a <u>definite</u> determination of the ploidy level of the intersectional hybrids. Although the results are consistent with other similar measurements on peonies and can easily be interpreted in a way that supports the previous presumption that the intersectional hybrids are triploids, they do not stand by themselves and therefore, cannot be used as a substitute for actual chromosome counts in peonies.

In the final analysis, this study has only increased my frustration with the lack of chromosome count data on the intersectional hybrids. In addition, it has highlighted the fact that such data is also lacking for the lutea hybrids as well. On the other hand, it has provided some intriguing new information even without yielding a definite answer to the basic ploidy level question. When everything has been said and done, however, this study has mostly served to emphasize the need and importance for actual chromosome counts on a number of increasingly popular peony cultivar groups. It has probably raised as many questions as it has answered. As a result, in recent months, I have been trying to find a university laboratory or commercial source for chromosome tests where I can have chromosome counts done on one or more intersectional hybrids. So far, this search has been unsuccessful. I would be extremely grateful to anyone who knows where such tests could be done or has a connection to someone with the facilities and expertise who might be willing to attempt to count the chromosomes of a few selected intersectional or fertile lutea hybrid peonies.