Interploid Hybridizations in Ornamental Cherries Using *Prunus maackii*[1]

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Abstract

The United States National Arboretum has an ongoing flowering cherry (*Prunus*) breeding program aimed at broadening the genetic base of cultivated ornamental cherries by developing new cultivars with disease and pest resistance, tolerance to environmental stresses, and superior ornamental characteristics. Interploid crosses, specifically 2X × 4X, in ornamental *Prunus* would be beneficial in breeding because they could allow introgression of traits not available in the diploid germplasm (pest resistance, cold hardiness), and could result in the creation of seedless triploids that would not set nuisance fruit and possibly have extended bloom durations. This report documents successful hybridization of *P. maackii* (Manchurian or Amur cherry), a tetraploid species, with *P. campanulata*, *P.* 'Umineko', and *P. maximowiczii*, all diploid species. Chromosomes of one of these resulting triploid hybrids were successfully doubled using oryzalin in tissue culture to create a hexaploid plant.

Index words: flowering cherry, ornamental plant breeding, ploidy manipulation, oryzalin.

Species used in this study: Higan cherry (*Prunus subhirtella* Miq.); Taiwan cherry (*P. campanulata* Maxim.); *P.* 'Umineko'; Amur cherry (*P. maackii* Rupr.); Korean cherry (*P. maximowiczii* Rupr.).

Significance to the Nursery Industry

Flowering cherries (*Prunus* species) are popular plants in landscapes, and also have significant economic impact to the wholesale and retail nursery industries. Over one million plants are sold wholesale each year at a value of more than $22 million. Despite the large number of *Prunus* species with diverse origins and ornamental traits, the most widely cultivated flowering cherry trees planted in the United States represent only a few species.

The United States National Arboretum has an ongoing breeding program aimed at broadening this narrow genetic base of flowering cherry by developing new cultivars with disease and pest resistance, tolerance to environmental stresses, and superior ornamental characteristics. As part of this objective, we created interspecific hybrid trees using a cold-tolerant, pest resistant species, Amur cherry. Because this plant is tetraploid (containing four sets of chromosomes), and other ornamental species are diploid (containing two sets of chromosomes), the hybrids are triploid (containing three sets of chromosomes). These hybrids may have ornamental value per se, or may be useful as breeding stock to incorporate traits of interest that aren't found in the diploid ornamental species. We will continue to evaluate these crosses for ornamental potential, as well as to explore other methods to increase the genetic diversity of ornamental cherry cultivars in the landscape.

Introduction

Ornamental flowering cherry trees (*Prunus* species) are popular plants for street, commercial, and residential landscapes. Grown primarily for their spring bloom, flowering cherries have been in the United States since the mid 1850s (7), and gained in popularity after the historic Tidal Basin cherries were planted in Washington, DC, in 1912. Over one million plants are sold wholesale each year at a value of more than $22 million (18). Despite the large number of *Prunus* species with diverse origins and ornamental traits, the most widely cultivated flowering cherry trees planted in the U.S. represent only a few species, primarily *P. serrulata*, *P. subhirtella*, and *P.* ×*yedoensis*. The United States National Arboretum has an ongoing breeding program aimed at broadening this base by developing new cultivars of ornamental cherry with disease and pest resistance, tolerance to environmental stresses, and superior ornamental characteristics.

The basic chromosome number for most *Prunus* species is n = 8 (5). While some of the edible *Prunus* are tetraploid or hexaploid, most of the common ornamental species are diploid, which makes interspecific hybridizations relatively easy to carry out. In fact, spontaneous hybridization between native and cultivated species, as well as naturalization of species and selections beyond their native ranges, has led to some of the confusion as to the taxonomic and nomenclatural treatment of the ornamental *Prunus* (12, 15). Interploid crosses, specifically 2X × 4X, in ornamental *Prunus* would be beneficial in breeding because it could allow introgression of traits not available in the diploid germplasm (pest resistance, cold hardiness), and could result in the creation of seedless triploids that would not set nuisance fruit and possibly have extended bloom durations. *P. maackii* (Manchurian or Amur cherry) is a tetraploid species native to Korea that has been reported to be cold hardy to USDA Hardiness Zone 2 (10, 17). In addition, it has ornamental exfoliating bark, is relatively fast-growing in the nursery, and has shown consistent resistance to attack by Japanese beetles in the Washington, DC, area (Pooler and Kidwell-Slak, personal observation). In order to incorporate these traits into the existing ornamental *Prunus* germplasm, we hybridized *P. maackii* with several diploid ornamental *Prunus* species. This study reports on the success and status of these hybridizations.

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Table 1. ***Prunus*** **accessions used in this study, hybrids, pollination and seed information, and estimated nuclear DNA content.**

Parents	Species or cultivar	# seeds germinated (approx. # flowers pollinated)	Relative 2C nuclear DNA content (picograms)
NA 3695 H	*P. subhirtella* 'Shidare Higan'		0.52
418 ER (NA 69013)	*P. campanulata*		0.53
5 ER (PI 77313)	*P.* 'Umineko'		0.55
321M ER (PI 5108887)	*P. campanulata*		0.57
62 ER (NA 70773)	*P. maximowiczii*		0.55
19 ER (PI 135617)	*P. maackii*		0.96
416 ER (NA 68773)	*P. maackii*		0.97
417 ER (NA 68811)	*P. maackii*		0.97
Hybrids (*year*)			
46-80 (19 ER × 5 ER) *1980*	*P. maackii* × *P.* 'Umineko'	34 harvested (~100)	0.73
1-06 (19 ER × NA3695) *2006*	*P. maackii* × *P.* subhirtella	13 (130)	0.76
2-06 (19 ER × 418 ER) *2006*	*P. maackii* × *P. campanulata*	6 (95)	0.78
2-03 (416/417 ER[z] × 321M ER *2003*	*P. maackii* × *P. campanulata*	2 (~20)	0.78
6-97 (19 ER × 62 ER) *1997*	*P. maackii* × *P. maximowiczii*	3+ (101)	0.77
2-03 doubled (5142-ind) *2010*	*P. maackii* × *P. campanulata*	Oryzalin doubled	1.50

[z]Accession records for female parent indicated that it was either 416 or 417; hence ploidy measurements of both accessions were included.

Materials and Methods

Plant materials and hybridizations. *Prunus* accessions used in this study are listed in Table 1. Controlled crosses were performed in the field during the year indicated. Crosses were made by covering clusters of unopened flower buds with a pollination bag, and then hand pollinating flowers as they opened using a camel-hair paint brush. *P. maackii* reaches peak bloom usually 2–3 weeks after the diploid pollen parent used in the crosses, so pollen was collected from the pollen donor and stored in the refrigerator in gelatin capsules prior to use. In most cases the seed parent (*P. maackii*) was not emasculated prior to pollination. As seeds developed, they were covered with mesh bags to prevent loss, and harvested when ripe. Seeds were cleaned, sown in flats containing a soilless potting mix (milled sphagnum and course sand, 1:1 by vol), and then moist stratified for three months in the dark at 4C (40F). After stratification, flats were placed in a 21C (70F) greenhouse for seed germination. Seedlings were transplanted to containers and ultimately to the field.

In vitro polyploidy induction. Shoot tip cultures of *Prunus* accession 5142 were established in vitro from field-grown plants following previously published protocols (3). Shoot apices, each approximately 3–5 mm long, were harvested from actively growing cultures and leaves were removed. Shoot tips were placed into glass culture vessels containing 18 mls of liquid medium containing MS salts and vitamins (14), 3% sucrose, and 15 µM oryzalin. Cultures were placed on a rotary shaker at 25 rpm for two days, then rinsed with sterile distilled water and placed in the dark for two days on MS medium containing 3% sucrose and solidified with 4 g·liter^{-1} agarose and 2 g·liter^{-1} Gelrite (Sigma Chemical, St. Louis, MO). Treated shoot tips were then transferred to semi-solid MS medium supplemented with 0.5 mg·liter^{-1} 6-benzyladenine (BA) and 0.1 mg·liter^{-1} indole-3-butyric acid (IBA) and brought into the light under a 16-hour photoperiod. Surviving plantlets were subcultured every four weeks, and after three months, young leaves were collected for analysis on the flow cytometer. Plants that showed doubled nuclear DNA content compared to the control untreated plants were hardened off, transplanted to potting mix, and grown in containers in the greenhouse and polyhouses at the U.S. National Arboretum.

Ploidy analysis. DNA content was determined via flow cytometry using the mean of three leaf samples for each parent and hybrid tested. Nuclei isolation and staining followed a modified protocol provided by Partec (Partec GmbH, Münster, Germany). Approximately 0.3 cm^2 of newly expanded leaf tissue was chopped with a sharp razor blade in a polystyrene petri dish containing 400 µL of extraction buffer (CyStain UV Precise P; Partec). Nuclei were stained in two stages. After a 2–4 min incubation, 400 µL of staining buffer containing 4',6-diamidino-2-phenylindole (DAPI) (CyStain UV Precise P) was added to the petri dish. The suspension was filtered through a 30 µm nylon mesh and another 400 µL of staining buffer was added to the solution followed by 400 µL of deionized water. The suspension was analyzed using a flow cytometer with fluorescence excitation provided by mercury arc lamp (PAII Ploidy Analyzer, Partec). The mean fluorescence of each sample was compared with diploid *Prunus campanulata* (NA58778, PI 510887) and an internal standard of known nuclear DNA content (2C) [soybean, *Glycine max* 'Williams 82', 2C = 2.26 pg; (4)]. A minimum of 5000 nuclei were analyzed to calculate the ratio of a sample peak to the internal standard for determining relative nuclear DNA content [2C pg = (sample peak / soybean peak) × 2.26 pg]. Sample ploidy levels were calculated by dividing sample nuclear DNA content by that of diploid *P. campanulata* (ploidy = sample 2C pg / 2C *P. campanulata*).

Results and Discussion

Ploidy of parents and hybrids, as estimated by flow cytometry (Fig. 1), was consistent with published numbers for ornamental cherry and with our phenotypic assessment of hybrids. Variation among the three samples tested for each accession, as well as among accessions of the same species, was low (relative standard errors were < 2.2%). The stain used for flow cytometry in this study (DAPI) binds preferentially with AT base pairs, and therefore cannot be used reliably to determine absolute DNA content of the nucleus (6). However, our objective was to measure the total DNA content of the

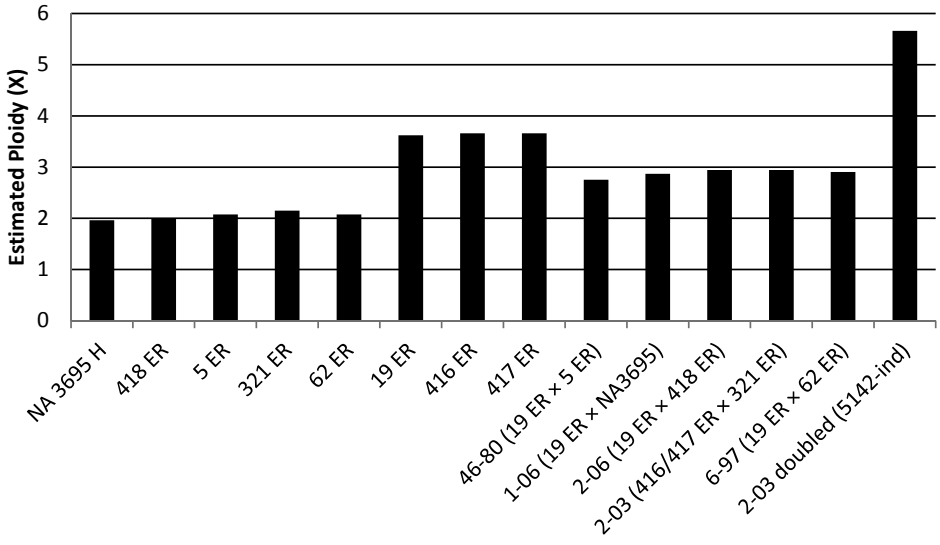

Fig. 1. Estimated ploidy of parents and hybrids. Ploidy levels were calculated by dividing sample nuclear DNA content (Table 1) by that of diploid *P. campanulata*.

samples relative to each other (Fig. 2), and to use this data to make estimations of ploidy. The estimated genome size of our samples, based on comparison with soybean, ranged from 0.52 pg for diploid species (*P. subhirtella*) to 1.50 pg for the derived hexaploid (Table 1). While these numbers cannot be construed as absolute genome size measurements, they are within the size range of the diploid peach (*P. persica*) genome, which is estimated to be 0.54–0.60 pg (1, 2).

The pollen parents used in these crosses were chosen based primarily on phenotypic traits that could complement the traits of the *P. maackii* phenotype. *P. campanulata* (Taiwan cherry) is a small tree or tall shrub with very deep pink, almost red campanulate flowers. Native to Southern Japan, it is only reliably hardy to USDA Hardiness Zone 8 (10). Our objective in using this species was to combine the dark flower color and early bloom time of *P. campanulata* with the disease resistance and cold hardiness of *P. maackii*. We used a double-flowering selection of *P. campanulata* in this study (NA69013, Fig. 3). *P. subhirtella* (Higan cherry) is a large tree with several well-known cultivars including the autumn-flowering cultivars. It is native to Japan, and hardy to USDA hardiness Zone 5–6 (10). The tree that we used as a pollen donor, a large weeping cultivar (NA3695H, Fig. 3), was chosen for its potential contribution of novel plant habit to the otherwise fairly upright *P. maackii*. P. 'Umineko' is a relatively upright cultivar with pure white, relatively thick-petaled flowers that resulted from a cross between *P. incisa* and *P. speciosa* (9; Fig. 3). We have used this accession in

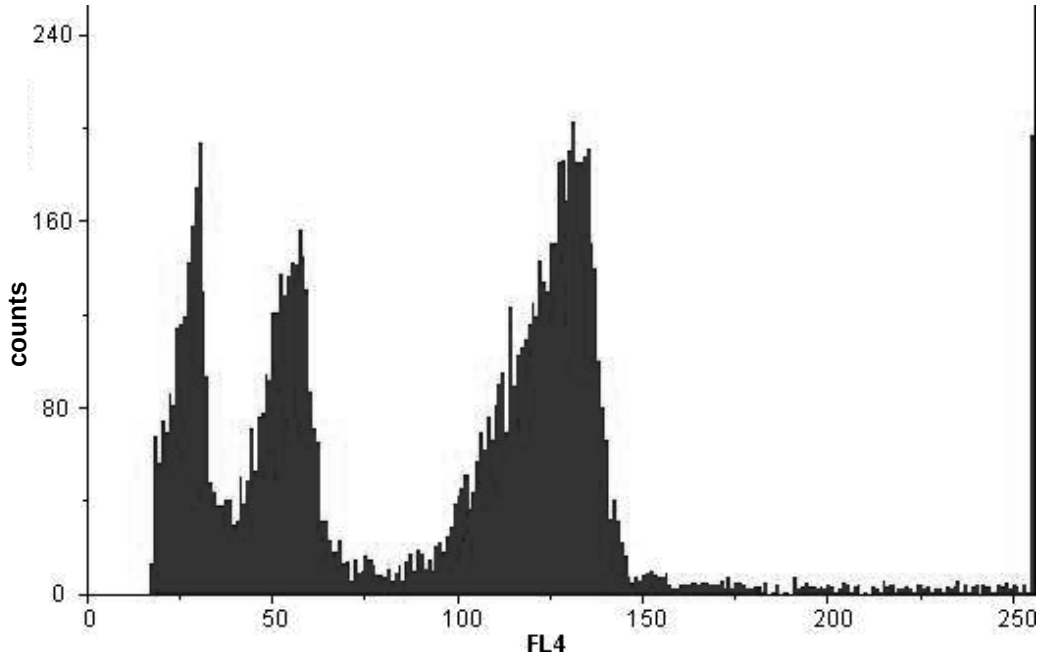

Fig. 2. Histogram of fluorescence intensity (x-axis) of nuclei from *P. subhirtella* (NA 3695H, peak 1 - diploid); *P. maackii* (19ER, peak 2 - tetraploid); and *Glycine max* (internal standard, peak 3).

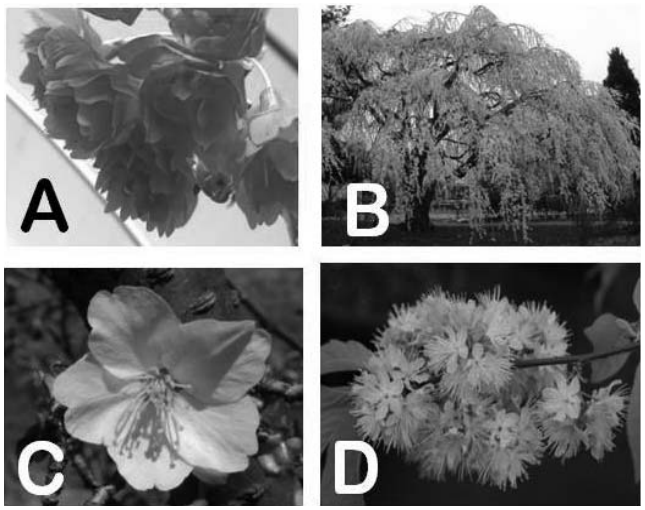

Fig. 3. Floral phenotypes of some of the ornamental *Prunus* used in this study. A) 418 ER - *P. campanulata*, B) 'Shidare Higan', C) 5 ER - P. 'Umineko', and D) *P. maackii* × *P. campanulata*.

many crosses because of the outstanding flower phenotype. *P. maximowiczii* is a small tree with cream white flowers that often occur at the same time as foliage appears in the spring. It is native to Japan, Korea, and Manchuria, and hardy to USDA Zone 5 (10). Relatively rare in cultivation, this species was used as a pollen parent primarily to broaden the genetic base of hybrids in our program, as this species is a member of subgenus *Cerasus*, section *Phyllomahaleb.*

Interspecific hybridizations in ornamental *Prunus* are generally successful, both through controlled crosses (13; unpublished data) and naturally occurring (11). We have created thousands of interspecific hybrid *Prunus* seedlings in the 30-year history of the flowering cherry breeding program at the U.S. National Arboretum. While most of these crosses have involved accessions within the Subgenus *Cerasus*, section *Pseudocerasus*, we have also attempted crosses between sections (using *P. maximowizcii*, subgenus *Cerasus*, section *Phyllomahaleb*) and across subgenera (using *P. maackii*, subgenus *Padus*). Wide crosses within *Prunus* could be advantageous not only in creating novel combinations of traits, but also, if the cross is wide enough, in creating sterile (seedless) progeny. Similarly, interploid 2X × 4X crosses would also result in seedless triploid progeny. We successfully created triploid interspecific hybrids using *P. maackii* and several diploid species (Table 1). These hybrids have been confirmed by phenotypic traits (inheritance of pink flower color from pollen parent, Fig. 2) or molecular markers (for hybrid 6-97; 13). While these triploid *P maackii* hybrids would be expected to be sterile, we have observed that they do set occasional fruit with viable seed. This could be caused by unreduced gametes, which are reported to be more frequent in hybrids than in nonhybrids (16). However, attempts to use these hybrids in crosses, either as pollen or seed parents, over several years, resulted in no seed set.

While seedless hybrid flowering cherry trees are desirable in the landscape, they pose an obvious problem in a breeding program, in that they cannot be used for further crosses. We are attempting to overcome this breeding 'dead end' by doubling the chromosome number of select *Prunus* accessions. One method to overcome sterility is to double the chromosomes of an infertile hybrid (8). We successfully doubled the chromosomes of one of our triploid *P. maackii* × *P. campanulata* hybrids to create a hexaploid. This plant has not flowered yet, so we cannot test its fertility, but we expect that it would at least be self-fertile, behaving as an allopolyploid with disomic inheritance, thereby allowing the creation of segregating F_2 populations that could yield desirable phenotypes. We have also doubled the chromosomes of diploid ornamental cherry accessions (data not shown) to allow breeding with *P. maackii* at the 4X level.

The interspecific and interploid hybrids reported here are only a small number of those that we have growing at the U.S. National Arboretum. We will continue to evaluate these crosses for ornamental potential, as well as to explore other methods to increase the genetic diversity of ornamental cherry cultivars in the landscape.

Literature Cited

1. Arumuganathan, K. and E.D. Earle. 1991. Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter 9:208–218.

2. Bennett, M.D. and I.J. Leitch. 1995. Nuclear DNA amounts in angiosperms. Ann. Botany 76:113–176.

3. Cheong, E. and M. Pooler. 2004. Factors affecting somatic embryogenesis in *Prunus incisa* cv. February Pink. Plant Cell Rep. 22:810–815.

4. Chesnay, C., A. Kumar, and S.R. Pearce. 2007. Genetic diversity of SIRE-1 retroelements in annual and perennial Glycine species revealed using SSAP. Cellular and Molecular Biology Letters 12:103–110.

5. Darlington, C.D. and A.P. Wylie. 1955. Chromosome atlas of flowering plants. George Allen & Unwin Ltd., London.

6. Dolezel, J., S. Sgorbati, and S. Lucretti. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Phys. Plant. 85:625–631.

7. Faust, M. and D. Suranyi. 1997. Origin and dissemination of cherry. Hort. Rev. 19:263–317.

8. Fehr, W. 1987. Principles of Cultivar Development. Macmillan Publishing Co., New York.

9. Ingram, C. 1970. A Garden of Memories. H.F. and G. Witherby Ltd., London.

10. Krussmann, G. 1984. Manual of Cultivated Broad-leaved Trees and Shrubs. Vol. II, Pru-Z. Timber Press, Portland, OR.

11. Kuitert, W. 1999. Japanese Flowering Cherries. Timber Press, Portland, OR.

12. Jefferson, R.M. and K.K. Wain. 1984. The nomenclature of cultivated Japanese flowering cherries (*Prunus*): The sato-zakura group. U.S. Dept. of Agriculture, National Arboretum Contribution No. 5.

13. Ma, H., R. Olsen, M. Kramer, and M. Pooler. 2009. Evaluation of flowering cherry species, hybrids, and cultivars using simple sequence repeat markers. J. Amer. Soc. Hort. Sci. 134:435–444.

14. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

15. Notcutt, R.C. and R.F. Notcutt. 1935. Flowering cherries. J. Royal Hort. Soc. 60:354–362.

16. Ramsey, J. and D.W. Schemske. 1998. Pathways, mechanisms, and rates of polyploidy formation in flowering plants. Annu. Rev. Ecol. Syst. 29:467–501.

17. U.S. Department of Agriculture (USDA). 1990 Plant hardiness zone map. USDA Misc. Publ. 1475.

18. U.S. Department of Agriculture (USDA), National Agricultural Statistics Service (NASS). 2001. 1998 census of horticultural specialties. Accessed September 13, 2100. http://www.agcensus.usda.gov/Publications/1997/Horticulture_Specialties/table13.pdf.