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Phenotypic Variation and Genetic Purity of the Original 'Prime-Jim®' x 'Arapaho' Population

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By

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Abstract

In 2013, Castro et al., produced the first linkage map for primocane-fruiting blackberries using the cross 'Prime-Jim®' x 'Arapaho.' This mapping population has been maintained since the original experiment with the hopes of conducting future studies. Further research was determined to be needed on the population to better characterize additional phenotypic traits, and since nine years had passed since the seedlings were established, there was a need to assess the continued genetic purity of the population using molecular methods. Phenotypic data was collected to analyze the variation of the population including soluble solids content, titratable acidity, average weight, shape, and firmness. While the population showed significant variation for each quantitative trait measured, the molecular results showed that none of the evaluated progeny matched the marker banding patterns expected based on previous results. Thus, the population was determined to be mislabeled or possibly some plants had grown together contributing to the impurity found. I recommended starting over with a new cross for future molecular mapping studies.

Introduction

Primocane-fruiting is the ability of blackberries (*Rubus* L. subgenus *Rubus* Watson) and red raspberries (*Rubus ideaus* L. *Idaeobatus*) to produce fruit on first-year canes (primocanes). Since the development of the primocane-fruiting trait in blackberry, breeding programs such as the University of Arkansas (UA) fruit breeding program have worked to improve the quality of primocane-fruit to help it reach its full potential. Studies such as Lopez-Medina et al. (2000) helped determine the inheritance of primocane-fruiting and it was found to be recessive.

Additional studies have since been conducted including a study that reported the first linkage map for primocane-fruiting blackberries from the cross ‘Prime-Jim®’ x ‘Arapaho’ (Castro et al., 2013).

On February 10th, 2015 Dr. John Clark contacted me about an honors thesis research project that would lay the foundation for future research within this same ‘Prime-Jim®’ x ‘Arapaho’ population that was used by Castro et al. (2013). The population was in Clarksville, AR at the University of Arkansas Fruit Research Station (FRS). The only data previously collected in the population was thornless and primocane fruiting traits. Thus, there were many questions left to answer about the phenotypic variation and molecular associations in the population.

Dr. Clark and Dr. Nahla Bassil (USDA- ARS, Corvallis, OR) believed that collection of further phenotypic and genotypic data work on this population would be beneficial. This information could then facilitate marker-assisted selection in blackberry breeding. However, because the population had aged since the original experiment, Dr. Clark felt the need to first identify the genetic purity of the population for future research. The purpose of my study was to phenotype the progeny of the cross ‘Prime-Jim®’ x ‘Arapaho’ by measuring traits that included soluble solids content, titratable acidity, average weight, shape, firmness. Additionally, to determine if the population could be used for further research, the population was tested for genetic purity using DNA extraction.

Literature Review

Blackberry History

Blackberries are a member of the Rosaceae family, which includes apples [(*Malus domestica* Borkh.), strawberries (*Fragaria x ananassa* Duch.) and red raspberries] (Kim, 2014; Mezzetti et al., 2016). Cultivation of blackberries in the United States (U.S.) began during the 1850s and breeding for commercial production began in the 19th century. Since then, blackberries have advanced rapidly within a short period (Kim, 2014). The initial classification of blackberries in the U.S. indicated that multiple species were involved in their evolution that contributed genetic variation for several traits (Finn and Clark, 2011). In addition, there was variation in ploidy level (Finn and Clark, 2011). It is from this genetic diversity that blackberries have developed and improved from the original wild blackberries, which had thorny canes, small fruit, large seeds and often bitter taste.

Recent genetic improvements in blackberries contributed to major production increases, causing production to almost double from 1995 to 2005 (Strik et al., 2008). Production in 2014 was valued at $50.1 million in the U.S. alone (AgMRC, 2015). Oregon is currently the primary producer within the U.S., with most production for processing (AgMRC, 2015). Likewise, there has been substantial growth in fresh-market production in recent years, especially in California and the Southern U.S. Growth in production occurred due to increases in population, overall blackberry quality improvement and the reported health benefits of blackberries (Debner, 2016). Research has shown that blackberries are an outstanding source of vitamin C (Debner, 2016). Blackberries can also contribute to a reduction in cholesterol, and are known to have anti-inflammatory and anti-cancer effects (Debner, 2016).

Plant and Fruit Characteristics

Botanically, the blackberry is classified as an aggregate fruit due to its structure being composed of multiple drupelets attached to an inner core also known as a receptacle or torus (Debner, 2016). The flowers typically have five petals along with numerous pistils and stamens with petal colors varying among cultivars (Kim, 2014). When berries are removed during harvest, the receptacle stays within the fruit, unlike red raspberries where the receptacle remains on the plant (Debner, 2016).

Blackberries have three typical cane growth habits: trailing, semi-erect and erect (Salgado, 2015). For each type of cane, various trellising methods are often used. For erect blackberries, the cane type of focus in the UA breeding program, most growers utilize a trellis to help direct the canes upward and provide support (Salgado, 2015). Blackberries have a perennial root system with biennial canes (Kim, 2014). The first-year canes are referred to as primocanes, while second-year canes are called floricanes. Most blackberries produce flowers and fruit only on the floricanes. However, there are cultivars of blackberries that are able to produce flowers and fruit on first-year canes, these plants are referred to as primocane fruiting. Primocane-fruiting blackberries have the potential to extend the harvesting period of the crop into the autumn, an additional fruiting period not possible in most regions utilizing only floricane-fruiting cultivars. In the UA breeding program, blackberries with primocane-fruiting have been a major focus for the last 20 years (Salgado, 2015). Thanks to the research efforts on primocane-fruiting blackberries, the quality of primocane-fruiting blackberries continues to improve.

Breeding in Eastern U.S. Blackberries

Improvements in breeding methods, as with any other horticultural crop, have helped unlock the genetic potential of blackberries. Whether breeders are focusing on the shipping quality, flavor components or the overall plant qualities, they are working towards new and improved cultivars for consumers. During the 1800s, wild plants were selected and grown that had improved traits including 'Dorchester,' and 'New Rochelle' (Finn and Clark, 2011). In the 1890s 'Loganberry' was released, a unique cross thought to be a combination of blackberry and red raspberry. (Clark and Finn, 2011; Finn and Clark, 2011). Developing a blackberry x raspberry cross opened the doors for blackberry breeders because of the large germplasm pool that the cross provided (Clark and Finn, 2011). In fact, the use of red raspberries within blackberry still occurs today, as seen in releases such as 'Newberry' (Clark and Finn, 2011).

The year 1908 marked the first time that blackberries were included in a public breeding program (Clark and Finn, 2011). The Texas Agricultural Experiment Station was the first of many blackberry breeding programs, followed by the New York State Agricultural Experiment Station and the USDA-ARS program in Georgia (Clark and Finn, 2011). Sadly, many of the programs that were established during this time have been discontinued, leaving limited public programs active (Clark and Finn, 2011). USDA-ARS in Oregon has been and continues to be active in blackberry breeding since 1928 (Clark and Finn, 2011). This program is known for contributing to the development of the trailing blackberry industry with cultivars such as the thorny 'Marion' and later 'Waldo', the first thornless, trailing cultivar (Clark and Finn, 2011). The UA breeding program is the other major public blackberry breeding program. While it originally focused on developing erect, floricane-fruiting blackberries, a significant effort now

focuses on improvement of primocane-fruiting blackberries (Clark and Finn, 2011).

Blackberry Molecular Research

Molecular techniques applied to commercial crops are often limited to those of high economic value. Unfortunately, the development of molecular techniques in blackberry has been substantially delayed when compared to other crops because of their limited economic value (Clark and Finn, 2011). In addition, the development of molecular markers is also confounded by the fact that blackberries are typically polyploid, which causes polymerase chain reaction (PCR) errors in both reproducibility and amplification (Stafne et al., 2005). Red raspberries on the other hand, are diploid, which makes the development of molecular techniques simpler (Clark and Finn, 2011). Minisatellite DNA probes were first used in 1989 to help differentiate red raspberry and blackberry cultivars from one another, which laid the foundation for additional studies towards use in minisatellite DNA fingerprinting (Clark and Finn, 2011). Other studies were conducted using Random Amplified Polymorphic DNA (RAPD) methodologies and raspberry simple sequence repeats (SSRs) to build genetic maps in blackberry (Clark and Finn, 2011; Stafne et al., 2005). While SSR markers were found to be transferable with other species within the Rosaceae family, the use of markers developed in species other than blackberries resulted in low levels of amplification (Castro et al., 2013; Clark and Finn, 2011).

In 2008, the first steps towards blackberry genetic mapping began with the development of an expressed sequence tag library and the first set of SSR markers designed in blackberries (Lewers et al., 2008). Later, the tetraploid blackberry linkage map was developed and used to locate the genetic regions controlling primocane-fruiting and thornless canes (Castro et al.,

2013). This research confirmed that primocane fruiting and thornlessness were not linked (Castro et al., 2013). Rather, the primocane-fruiting trait was in linkage group seven while thornlessness was located in group four (Castro et al., 2013). Of the 310 primer pairs tested in the population, only 30% were polymorphic in the parents (Castro et al., 2013). However, of the 107 polymorphic primer pairs, 261 polymorphic bands segregated in the 'Prime-Jim®' x 'Arapaho' population (Castro et al., 2013). Only 119 of the 261 PCR products had useful segregation ratios that could be used in cluster analysis (Castro et al., 2013). From these 119, 83 markers were polymorphic in both parents and mapped to the same chromosome in each parental map, which was substantial evidence for valid map creation (Castro et al., 2013). This research laid the foundation for future marker-assisted selection in blackberry breeding.

Without the use of marker-assisted selection, it can take several years for a plant to fully express its traits and for a breeder to analyze the phenotype of the plant. If marker-assisted selection is used, a breeder can collect DNA from seedlings and determine which plants have desirable traits. This reduces the number of plants retained for field planting, resulting in increased efficiency. For example, the marker indicating thornlessness, a highly desirable trait, could be used by breeders to select for thornless progeny at the seedling stage. Furthermore, a marker for primocane fruiting could identify progeny with this trait as segregation is often 35:1 or 5:1 of floricane-fruiting to primocane-fruiting progeny, depending on parents used (Lopez-Medina et al., 2000). It is essential to continue improving the tetraploid blackberry map because a majority of the map has not been completed and as additional genes are identified in the map the closer blackberry researchers come to understanding the entire blackberry genome and identifying useful markers for breeding.

Phenotyping Blackberries

Phenotypic traits are defined as the visible characteristics of an organism, in other words the form of an organism based on their set of genes in addition to environmental effects (Mahner and Kary, 1997). It was not until 1909 that the distinction between "phenotypes" and "genotypes" was developed by Wilhelm Johannsen during his research on field beans (Churchill, 1974; George, 2012). Since then, phenotyping has been used in various ways to understand organisms on a deeper level genetically. Phenotyping has been used commonly in numerous fruit crops, however the incorporation of quality phenotypic information with genotypic data in mapping populations has been limited for *Rubus*, compared to other crops within Rosaceae. Red raspberry mapping populations have been phenotyped for many traits of economic importance including fruit ripening, softening, thorniness of canes, root suckering, and also the 'crumbling' fruit characteristic (Graham et al., 2004; Graham et al., 2015; Simpson et al., 2017). Comparatively, blackberry has only had genetic studies on the characterization of thornless canes and primocane fruiting (Castro et al., 2013). The use of phenotypic data in development of molecular markers for important traits can one day lead breeders to select plants during immaturity based on their fruiting habit, but also for a range of other traits such as sweetness and acidity of berries, enhancing breeding efficiency even more.

Origins of 'Prime-Jim®' and 'Arapaho'

'Prime-Jim®'

'Prime-Jim®' was produced from a cross of 'Arapaho' x Ark. 830 in 1994 in the UA program. (Clark et al., 2005). This cultivar is unique because it is one of the first primocane-

fruiting blackberry cultivars ever developed (Clark et al., 2005). Primocane-fruiting was first reported in the wild plant designated ‘Hillquist’ (Lopez-Medina et al., 2000). ‘Prime-Jim®’ berries have been classified as glossy and conic to blocky in shape, similar to ‘Arapaho’ (Clark et al., 2005). ‘Prime-Jim®’ quality is not as desirable as ‘Arapaho’ due to its softer berries and often bitter flavor (Clark et al., 2005).

‘Arapaho’

In 1982, Ark. 631 x Ark. 883 were crossed, producing ‘Arapaho’ in the UA breeding program (Moore and Clark, 1993). Originally, ‘Arapaho’ was created with the intention of having a plant similar to ‘Navaho’ with erect and thornless canes (Moore and Clark, 1993). However, because ‘Arapaho’ ripens prior to ‘Navaho,’ the combination of cultivars created a longer harvest period (Moore and Clark, 1993). ‘Arapaho’ berries are conic, short, firm and glossy (Moore and Clark, 1993). ‘Arapaho’ has been used as a parent in numerous crosses in the UA program to and played a role in the development of several cultivars including ‘Natchez’, ‘Prime-Ark® 45’, ‘Prime-Ark® Freedom’ and ‘Prime-Ark® Traveler’ (Clark, 2011; Clark, 2014; Clark and Moore, 2008; Clark and Salgado, 2016).

Objectives

1. To phenotype 96 progeny and their parents from the cross ‘Prime-Jim®’ x ‘Arapaho’, for the variables soluble solids content, titratable acidity, average weight, shape, and firmness.
2. To extract DNA from the parents and progeny and react in PCR to determine the genetic purity of the population using simple sequence repeat (SSR) markers.

Materials and Methods

The population used in my study, ‘Prime-Jim®’ x ‘Arapaho’, was located at the University of Arkansas Fruit Research Station (FRS) (35° 32' 2.148" N, 93° 24' 20.725" W), Clarksville, AR. The progeny were planted 1 m apart in 2006 directly in the soil without any trellising or plastic mulch and were dormant pruned each winter. Summer tipping was not performed on the population. The original population consisted of 200 plants, but some plants died between 2006 and 2015 and there were concerns of some plant mixing due to root growth and sprouting canes in adjacent plant locations. Ninety-six of the most uniform plants in size and health were chosen for this study. However, as data was collected only 79 of the progeny could produce adequate sample sizes for this experiment. During the selection of progeny, plants were labeled with two different names, one for the original Castro et al. experiment and another for use in the field. To best confirm the identification system used, plants were selected based on their ability to primocane-fruit and whether they were thorny or thornless in correlation to Castro et al.’s (2013) previous data on the population’s characteristics.

Study One - Phenotypic Data Collection

Fruit was harvested from each plant for up to five times during the growing season, to achieve a minimum of 10 ripe berries from the plant. Each individual berry was then measured for weight, length and width. Firmness was measured for each berry with a TA.XTPlus Texture Analyzer (Texture Technologies Corp. Hamilton, MA) using a cylindrical and plane probe of 7.6 cm diameter, with each fruit compressed 5 mm. The berries collected throughout the harvesting period were then combined into a single mixture for each plant and frozen at -20 °C.

Berries were thawed, and two replications for each plant were then created by selecting five berries randomly from the mixture of collected berries. The berries were crushed and strained through a cheesecloth to collect a minimum of 4 ml of juice and juice was brought to room temperature. A drop of juice was used to measure soluble solids and 2 ml for pH. The 2 ml used to measure pH was then reused to measure titratable acidity with 4 ml of juice. Both pH and titratable acidity were measured with an 877 Titrino Plus autotitrator (Metrohm AG, Herisau, Switzerland) using sodium hydroxide (0.1 N). Soluble solids content was measured using a Bausch & Lomb Abbe Mark II refractometer (Scientific Instrument, Keene, NH).

After data collection was completed, average values for phenotypic traits were calculated for each of the progeny. The Shapiro-Wilk W Test was then performed to test the normality of distribution each variable for the population. Graphs were then created using the averages along with a boxplot to show the values based on quartiles.

Study Two – Validation of the Genetic Purity of the Population

Leaf Collection and DNA Extraction

Young leaf tissue was collected from each plant in May, 2015, placed in coin envelopes and lyophilized for 24 h in a Labconco® Lyph-Lock 6 Freeze Dry System (Kansas City, MO). Once lyophilized, >50 mg of tissue was extracted using the aseptic technique and placed in a 96-tube array. The extraction protocol was a modified procedure from Edge-Garza et al. (2014). Silica beads were incorporated into each well and shaken for 3 min with a 25/sec frequency, rotated in the shaker and repeated. The 96-tube array was then centrifuged to prevent contamination when removing the caps. Using the silica bead method, DNA was extracted with an extraction buffer created by mixing 100 mM Tris, 50 mM EDTA and 1.25% SDS followed by preheating the buffer to 65 °C. A volume of 500 µl was placed inside of each of the 96 wells and then incubated at 65 °C for 30 min. The array was then placed in a -20 °C freezer for 15 min followed by incorporating 6 M of ammonium acetate into each well and placing the plate inside of the -20 °C freezer for the same amount of time. The samples were then centrifuged at 1,700 rcf for 20 min and then moved to a new 96-well plate. After transferring the DNA into a new plate, 240 µl of isopropanol was placed into each well for 15 min followed by centrifuging the plate at 4,000 rpm until the DNA was concentrated at the bottom of each well. The plate was then slowly flipped over to ensure the DNA stayed attached to the bottom of the well and was then washed twice with 500 µl of ethanol. After the samples were dry, 125 µl of double distilled water was used to complete the silica bead DNA extraction method. DNA was then quantified using the NanoDrop spectrophotometer (Thermo Scientific, Sunnyvale, CA).

Polymerase Chain Reaction (PCR)

DNA was then diluted with 1 x TE buffer to 30 $ng.\mu l^{-1}$ (Stafne, 2005). The components of the master mix included 640 µl nuclease-free water, 300 µl buffer 5X, 120 µl dNTP 2.5 mM, 120 µl $MgCl_2$ 25 mM and 50 µl GoTaq 5 µl. From this master mix, 13.5 µl was then distributed amongst each of the 96 cells. Seven primers were selected by Dr. Nahla Bassil based on her prior evaluation for producing polymorphisms for this population, and these were dyed with FAM, HEX and NED based on their distance between base pairs (Table 1). A separate mixture was then created with 75 µl of the M13 Tag (TGTAAAACGACGGCCAGT) as recommended by Schuelke (2000), 75 µl reverse primer 10 µM and 18 µl forward primer 10 µM. A 96-well thermocycler (BIO RAD, model T100, Hercules, CA) was programmed following Bassil et al. (2016). The cycle began with denaturation for 3 min at 94 °C. After the denaturation cycle, 10 rounds of 40 sec at 94 °C; 45 sec at 62 °C with a decline by 1 °C each cycle; and 45 sec at 72 °C were performed. This was followed by 20 more cycles at 94 °C for 40 sec; 52 °C for 45 sec; and 72 °C for 45 sec. Finally, eight more cycles were done starting at 94 °C for 40 sec; 53 °C for 45 sec and ending at 72 °C for 45 sec. After the PCR cycle was completed, samples were stored at 4 °C. PCR products were then prepared for gel electrophoresis by incorporating 5 µl of PCR products with 5 µl of gel loading buffer (bromophenol blue). Samples were carefully inserted into a 2% agarose 6M gel using the Liberty 120 Gel System (Neuvitro Corporation, Vancouver, WA). For each of the seven primer pairs, a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems™, Foster City, CA) was used to transfer 2 µl of each PCR product and prepare the samples to be lyophilized. The Labconco® Lyph-Lock 6 Freeze Dry System (Kansas City, MO) was used to lyophilize the samples for 24 h followed by suspension of the samples in

HiDi® Formamide. Samples were then transported to the Clemson University Genomics Institute (Clemson, SC) where a Size Standard Gene Scan™ LIZ 600® (0.2 ul) dye (Applied Biosystems™, Foster City, CA) was used to measure the fragments of each sample. Peak Scanner™ 2 (Thermo Scientific, Sunnyvale, CA) was then used to visualize the fragments and to score the peaks of each sample.

Table 1. SSRs selected for this experiment and the base pair (bp) sequences for 'Prime-Jim®' and 'Arapaho'.

SSR name	Forward sequence (5'-3')	Reverse sequence (5'-3')	'Prime-Jim®' product size (bp)	'Arapaho' product size (bp)
RH_MEa0008cF01	TGTAAAACGACGGCCAGTAGATGGAATTCCTAGGGCGT	TTGGCTTCAATTCTCCCATC	162, 165, 168	162, 168
RH_MEa0003dF05	TGTAAAACGACGGCCAGTTCCCCGGTCTACATATTCCA	GTCTGGCAGTTGGAGCAGTT	212, 218	212, 218
RH_MEa0013bC12	TGTAAAACGACGGCCAGTGTTGTGACCAAGCAAGAGCA	AGCTGTTTTTGTTGGGGTTG	227, 230	227, 230
RH_MEa0016aD11	TGTAAAACGACGGCCAGTTACCCTCATGTCCTCCCAAG	TTCAGCTTCTTCTTCTGCTGG	248	248, 251
RH_MEa0007aG06	TGTAAAACGACGGCCAGTCTTCCCCCTATAAATCCCGA	CGTCTCTCTGCAATTCCTCC	143, 149, 152, 162	124, 149, 152, 162
RH_MEa0013dA06	TGTAAAACGACGGCCAGTTCCATCTCTATCCCGAAACG	GTGATGACGGTGATGGACAG	236, 244, 252	236, 244, 250
RH_MEa0011dG03a	TGTAAAACGACGGCCAGTTCCATCTCTATCCCGAAACG	CCAATTTCTGCAGGGTTGTT	356, 359	356, 359, 365, 368

Results

Study One

Results indicated that berry length and width both had normal distributions, based on the Shapiro-Wilk W Test. The p-values for both berry length and width were 0.89 (Table 2; Figs. 1-A and 1-B). The segregation of these variables was as expected due to the range of berry shape and size anticipated from 'Prime-Jim®' and 'Arapaho.' The overall mean berry length for the population was 21.1 mm (Table 2; Fig. 1-A). The population ranged from 11.0 - 35.0 mm and the standard deviation for length was 4.4 mm (Table 2). Berry width ranged from 13.2 - 24.5 mm, with a mean berry width of 18.8 mm and a standard deviation of 2.3 mm (Table 2). Berry weight had a non-normal distribution ($P < 0.05$) and was skewed towards smaller berry weights (Table 2; Fig. 1-C). The largest berry weight within the population was 7.6 g with a mean of 3.8 g and minimum of 1.2 g. The standard deviation for berry weight was 0.01 g (Table 2).

Titratable acidity and pH were both non-normally distributed, with the data skewed toward more acidic fruit (Figs. 1-D and 1-E). The Shapiro-Wilk W test for pH showed a p-value of 0.03 with a distribution ranging from 4.7 - 3.6 in pH levels, a mean of 4.0 and standard deviation of 0.03 (Table 2). Titratable acidity also had a small p value ($p < 0.01$; Table 2) with an overall mean of 0.8%, a maximum of 1.4%, minimum of 0.5% and standard deviation 0.2 % (Table 2). Soluble solids content was normally distributed ($P = 0.72$) with a mean content of 9.5%, a maximum of 13.4%, minimum of 5.1% and a standard deviation of 0.72% (Table 2; Fig. 1-F).

Lastly, firmness (N) had a non-normal distribution ($P < 0.01$; Table 2; Fig. 1-G). The mean firmness within the population was 5.6 N with a standard deviation of 1.4 N and minimum

of 2.9 N (Table 2). Of note is the extreme value of 11.3 N, this outlier is not within the three quartiles of the boxplot and is shown as the dot to the far right in the population (Table 2; Fig. 1-G). The berry was not immature; however, it is hypothesized that the texture analyzer measured the firmness of the seeds rather than the overall berry. If this outlier was excluded from the data, firmness would have a normal distribution.

Table 2. Distribution of progeny ranges including the maximum, minimum, mean, standard deviation and Shapiro-Wilk W P-value based on phenotypic data.

	Maximum	Minimum	Mean	Standard deviation	Shapiro-Wilk W P-value
Length (mm)	35.0	11.0	21.1	4.4	0.89
Width (mm)	24.5	13.2	18.8	2.3	0.89
Weight (g)	7.6	1.2	3.8	0.01	0.0101
Soluble solids (%)	13.4	5.1	9.5	0.72	0.7193
pH	4.7	3.6	4.0	0.03	0.0294
Titratable acidity (%)	1.4	0.5	0.8	0.2	0.0001
Firmness (N)	11.3	2.9	5.6	1.4	0.0086

Fig. 1 A-G. Berry phenotypic characteristics and genotype percentages for progeny in a population of 79 plants of 'Prime-Jim®' x 'Arapaho', in 2015, Fruit Research Station. Above each graph is a boxplot that is broken into three quartiles, the box in the center represents the second quartile while the diamond represents the mean of the population. The dots on the boxplot illustrate the outliers in the data.

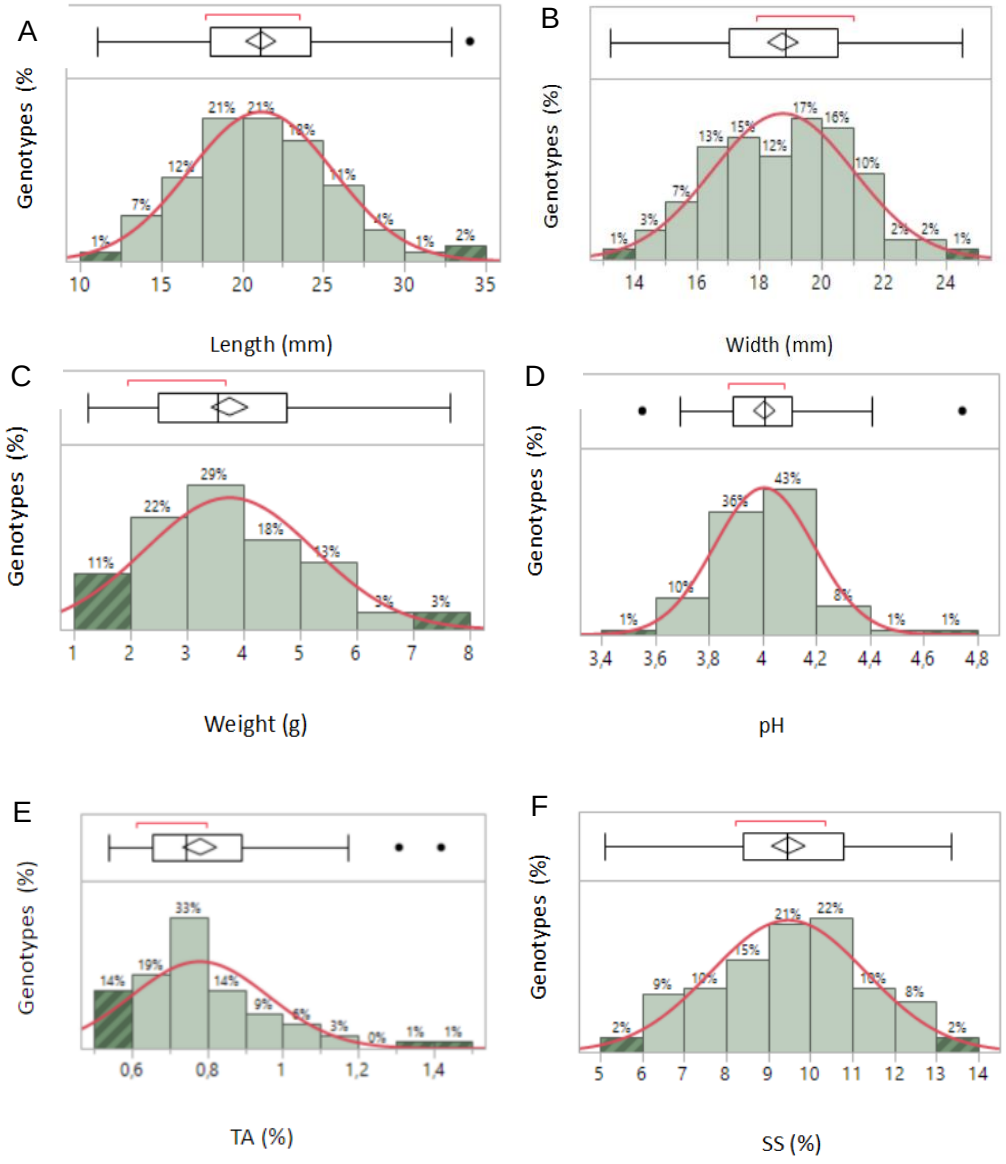
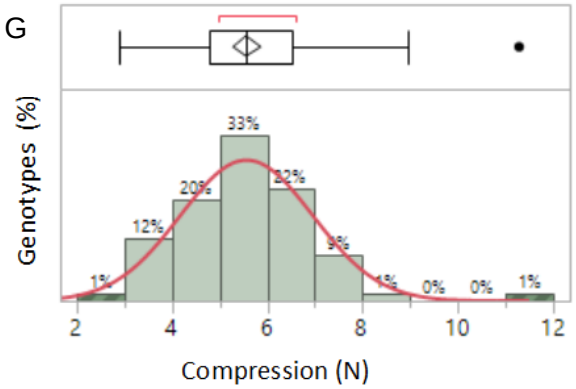


Fig 1. A-G (Continued)



Study Two

After scoring the PCR band sizes for each of the seven markers used in the population, the results were compared to allelic data from Castro et al. (2013). Thirty-seven of the 79 genotypes evaluated in the present study failed to amplify PCR results for any of the SSR primer pairs. Therefore, I was only able to compare the identity of 42 progeny from the 'Prime Jim®' x 'Arapaho' population based on SSR marker banding patterns. Additionally, marker RH_MEa0013dA06 (13dA06) was a failed reaction and did not provide any data. In total, 17 alleles generated from six SSR primer pairs were used to corroborate the identity of 42 progeny from the 'Prime Jim®' x 'Arapaho' population.

For marker RH_MEa0007aG06 (7aG06) there were five alleles of interest at 124, 143, 149, 152 and 162 bp. For the 124 bp allele, Castro et al. (2013) found 14 progeny amplified this band while I found only 10 progeny with this allele. Of those 10 progeny with the band, only four were originally reported to carry this allele by Castro et al. (2013), indicating that only 28.6% of the plants matched the previous study (Table 3). For the allele at 143 bp, 40.9% percent

of progeny matched the original study, yet the original study had 22 of 42 plants with this allele while my current study only had 13 (Table 3). Similarly, 40.5% of the progeny had matching scores for the band at 152 bp, but the original study had 37 plants with bands, while I found only 16 plants that produced bands (Table 3). Both bands at 149 and 162 bp had similar results with 32.0% of progeny matching 149 bp and 31.0% matching for 162 bp allele scores, and fewer progeny with bands than were originally reported (Castro et al., 2013 and Table 3).

Table 3. Summary table comparing the number of progeny that match the original data collected for Castro et al. data.

	Allele size (bp)	Percent of progeny with allele present		Percent of individuals with matching allele scores
		Castro et al.	Luther	
RH_MEa0007aG06	124	33.3	23.8	28.6
	143	52.4	31.0	40.9
	149	59.5	45.2	32.0
	152	88.1	38.1	40.5
	162	69.0	26.2	31.0
RH_MEa0011dG03a	356	100.0	33.3	33.3
	359	76.2	38.1	28.1
	365	73.8	14.3	16.1
	368	66.7	28.6	28.6
RH_MEa0008cF01	162	76.2	23.8	25.0
	165	52.4	33.3	22.7
	168	85.7	35.7	41.7
RH_MEa0003dF05	212	45.2	40.5	36.8
	218	78.6	47.6	48.5
RH_MEa0013bC12	227	73.8	61.9	61.3
	230	92.9	61.9	64.1
RH_MEa0016aD11	251	35.7	31.0	26.7
Average		68.2	36.1	35.6

Marker RH_MEa0011dG03a (11dG03a) had four alleles; 356, 359, 365 and 368 bp. While the results were similar to those of 7aG06, the percentage of matching alleles was lower for this primer. For example, 365 bp had originally appeared in 31 of the progeny selected for this experiment, yet only five of the plants matched the results of the previous study. The pattern of fewer progeny producing expected band sizes compared to Castro et al. (2013) continued throughout all of the 11dG03a alleles as well as SSR primers RH_MEa0008cF01 (8cF01), RH_MEa0003dF05 (3dF05) and RH_MEa0016aD11 (16aD11). None of the primers listed above had any alleles with more than 50% of progeny matching the previously reported banding

patterns. The highest percentage of matching progeny scores was found for 3dF05 with 48.5% matching at the 168 bp allele (Table 3).

While the majority of primers had far fewer individuals scored as positive for any given allele compared to the previous study, RH_MEa0013bC12 (13bC12) had results closer to expected patterns. Primer 13bC12 only had two alleles of interest at 227 and 230 bp. Castro et al. (2013) reported that 31 progeny carried the 227 bp allele, and we found 26 progeny in the population with this allele. However, only 19 of these plants matched previous results, meaning that 61.3% of progeny fit the expected results (Table 3). Likewise, 64.1% of the progeny had matching results for the allele at 230 bp (Table 3). While primer 13bC12 had a higher percentage of matching progeny, it is important to note that 0% of the population matched the previous data for all 17 alleles. Additional details can be found in the supplementary materials section.

Discussion

Study One

The findings from the phenotyping of berries indicated substantial variation for all variables for the progeny. Berry length and width means were as anticipated, with overall length of berries being higher than width. Although the parents were not measured in my study, these characteristics were reported for the parents when they were released (Clark et al., 2005; Moore and Clark, 1993). The average berry weight reported for ‘Arapaho’ was 5.1 g and Prime-Jim®’ 4.6 g at FRS, while the mean of the population of these parents was slightly less than either parent at 3.8 g. My findings agreed with previous reports of quantitative inheritance of fruit weight in blackberry, with partial dominance for smaller berries (Caldwell and Moore, 1982).

However, many of the berries were particularly small; 33% were 2.0 g or less in weight. This could indicate that some plants in the population were not healthy or able to produce normal berries as anticipated.

Values reported at release for soluble solids in ‘Arapaho’ was 9.1% while ‘Prime-Jim®’ was 7.9% (Clark et al., 2005; Moore and Clark, 1993). The mean soluble solids for this population was 9.5%, which exceeded both the parents’ reported soluble solids scores. However, the soluble solids data for the parents was collected in previous years, indicating that the distribution could be different due to environmental factors or crop load on the plants. The distribution of soluble solids within the population ranged from 5.1 to 13.4%. Titratable acidity and pH were not measured for the parent cultivars in their release information, but values for the progeny in my study were similar to what was expected for blackberries grown at this site (J.R. Clark, personal communication). Compression was not measured on the parents either, so no comparison is possible. The population did show substantial segregation for both very firm and soft berries. ‘Prime-Jim®’ is considered a much softer berry than ‘Arapaho’, as the latter has been shown to have good postharvest potential in commercial production while ‘Prime-Jim®’ is recommended only for the home garden (J.R. Clark, personal communication).

Study Two

None of the 42 progeny genotyped in the present study matched the expected results for all 17 SSR alleles as reported in Castro et al. (2013). While there are several hypotheses which could contribute to mixed identity of progeny such as the DNA extraction, summer tipping or sanitation, my conclusion is that the labeling system for this experiment is the primary reason for

the unexpected results. The labeling system for the progeny used by Castro et al. (2013) did not match the numbering system that was used to identify plants in the field. Measurements were taken at the beginning of the experiment to ensure that progeny matched the previous experiment's data for thorniness. However, the population was primarily thorny plants (5:1 thorny to thornless), meaning that the purity of the population could not be verified easily without molecular markers. Maintaining an effective labeling system is essential when working with populations for molecular research. It is recommended that plants should initially be spaced farther apart than in my study to contribute to longer-term separation of the plants. Also, plants should be labeled with identification on the plant crowns. Finally, annual examination of each seedling should be done to determine if the seedling plants are off type, based on presence or absence of thorns, or possibly other phenotypic characteristics. Furthermore, the identification method must be used amongst all researchers to ensure consistency and resolve any discrepancy that could occur.

While the molecular procedures did have some error, as shown in the failed PCR results, the observed allele peaks were strong and distinct. None of the progeny had all 17 alleles present, however if the bands were light or blurred, this would indicate errors in DNA extraction or PCR were the primary cause of the discrepancies between my results and the data previously reported by Castro et al. (2013). Additionally, the molecular results did not show any adjacent plants with identical DNA making the summer tipping hypothesis less likely. Thus, it seems more likely that the use of two different labeling systems in the field and in the Castro et al. (2013) project caused the misidentification of progeny and compromised my ability to add further markers or phenotypic information to this mapping population. If researchers are considering using this population for future projects, additional DNA tests should be conducted to determine that the

results collected during this experiment adequately represent the population. Otherwise, it is recommended that a new cross is created between ‘Prime-Jim®’ x ‘Arapaho’ and the original population be removed.

Conclusions

Phenotypic data for the population ‘Prime-Jim®’ x ‘Arapaho’ showed adequate variation within the population, indicating that it could be useful for improving the blackberry tetraploid map. Sadly, the current population should not be used in any additional research due to the lack of genetic purity that was found. The primary hypothesis for the discrepancy between results is likely due to the numerous labeling systems created for the population. Additional DNA tests could be conducted on the population to validate the results from this experiment. But, the cost and time of an additional DNA testing for a population that is likely contaminated is unnecessary. Rather, starting fresh with a new cross and more precise labeling system for the population is recommended to ensure purity during experimentation.

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Supplementary Material

Table 4. Data comparison of the results found by Castro et al. (2013) and the current study. Each of the alleles are organized based on primers used. The sample name is the name given by Castro et al., while the numbers column was based on the labels in the field.

		RH_MEa0007aG06					
		Castro et al.	Luther	Castro et al.	Luther	Castro et al.	Luther
sample name	number	124		143		149	
100Y	1	0	0	0	143	149	149
101Y	2	0	0	0	143	0	149
106Y	7	0	0	143	143	0	149
112Y	15	124	0	0	0	149	0
116Y	18	0	0	0	0	149	0
119Y	22	0	124	143	0	0	149
11Y	23	0	124	143	0	0	149
120Y	24	124	124	143	143	0	149
122Y	26	0	0	0	0	149	149
125YPF?	29	0	124	143	143	149	149
193YPF	32	0	124	0	0	149	0
133YPF	39	0	0	143	143	149	149
194Y	42	124	124	0	143	0	149
146Y	54	0	0	0	0	149	0
196Y	63	124	0	143	0	149	0
168Y	79	124	124	143	0	149	0
171YPF?	83	0	0	143	143	149	149
198Y	85	0	0	143	143	0	149
19Y	95	0	0	143	0	149	0
184Y	98	124	0	143	143	149	149
186YPF?	100	0	124	143	0	0	0
187Y	101	0	0	0	0	149	0
189YPF?	103	0	0	143	0	149	0
1Y	104	0	0	143	0	149	0
22Y	108	124	124	0	0	0	149
23Y	109	124	0	0	0	149	0
25Y	111	124	0	143	0	149	0
28Y	114	124	0	0	0	0	0
2Y	116	0	0	0	0	149	0
32Y	118	124	0	0	0	0	0
33Y	119	124	0	0	0	0	0
44T	132	0	0	0	143	0	149
45Y PF?	133	0	0	143	0	0	0
46Y	134	124	0	143	143	0	149
49T	137	0	124	143	143	0	149
55Y	143	124	0	0	0	149	0
60Y	148	0	0	0	0	149	0
62Y	150	0	0	143	0	149	0
63Y	151	0	0	143	0	0	0
64Y	152	0	0	0	0	149	0
67Y PF?	155	0	0	0	0	149	149
8Y PF	178	0	0	143	0	149	149
total present		14	10	22	13	25	19
total similar			4		9		8
percent matching			28.57		40.91		32

		RH_MEa0007aG06				RH_MEa0013dA06	
		Castro et al.	Luther	Castro et al.	Luther	Castro et al.	Luther
sample name	number	152		162		236	
100Y	1	152	152	0	0	236	0
101Y	2	152	152	162	0	236	0
106Y	7	152	0	162	0	236	0
112Y	15	152	0	162	0	236	0
116Y	18	152	0	162	0	0	0
119Y	22	152	152	0	0	0	0
11Y	23	152	152	162	0	236	0
120Y	24	152	152	162	0	0	0
122Y	26	0	0	162	162	0	0
125YPF?	29	152	152	0	0	236	0
193YPF	32	152	152	0	162	0	0
133YPF	39	0	152	162	162	0	0
194Y	42	152	152	162	0	236	0
146Y	54	152	0	162	0	236	0
196Y	63	152	0	0	0	236	0
168Y	79	152	152	162	162	236	0
171YPF?	83	152	152	0	0	236	0
198Y	85	152	0	162	162	0	0
19Y	95	152	0	0	0	236	0
184Y	98	152	152	0	162	0	0
186YPF?	100	152	0	162	0	236	0
187Y	101	152	0	162	0	236	0
189YPF?	103	152	0	0	0	236	0
1Y	104	152	0	0	0	0	0
22Y	108	152	152	162	162	236	0
23Y	109	152	0	0	0	236	0
25Y	111	152	0	0	0	236	0
28Y	114	152	0	162	0	0	0
2Y	116	0	0	162	0	236	0
32Y	118	152	0	162	0	236	0
33Y	119	152	0	162	0	236	0
44T	132	152	152	162	162	236	0
45Y PF?	133	0	0	162	0	236	0
46Y	134	152	0	162	162	236	0
49T	137	152	0	162	162	236	0
55Y	143	152	0	162	0	0	0
60Y	148	152	0	162	0	236	0
62Y	150	0	0	0	0	0	0
63Y	151	152	0	162	0	236	0
64Y	152	152	0	162	0	0	0
67Y PF?	155	152	152	162	162	236	0
8Y PF	178	152	152	162	0	0	0
total present		37	16	29	11	28	0
total similar			15		9		0
percent matching			40.54		31.03		

		RH_MEa0011dG03a							
		Castro et al.	Luther	Castro et al.	Luther	Castro et al.	Luther	Castro et al.	Luther
sample name	number	356		359		365		368	
100Y	1	356	356	0	359	365	365	368	368
101Y	2	356	356	0	359	365	365	368	368
106Y	7	356	356	0	359	0	0	0	0
112Y	15	356	0	359	0	365	0	368	0
116Y	18	356	356	359	359	365	0	368	0
119Y	22	356	356	359	359	365	0	0	368
11Y	23	356	356	0	359	365	0	368	368
120Y	24	356	356	359	359	365	365	368	368
122Y	26	356	356	0	359	365	0	368	0
125YPF?	29	356	356	0	359	0	0	0	0
193YPF	32	356	0	0	0	365	0	368	0
133YPF	39	356	356	359	359	0	0	0	0
194Y	42	356	0	359	0	365	0	368	0
146Y	54	356	0	359	0	365	0	368	0
196Y	63	356	0	359	0	365	0	368	0
168Y	79	356	0	359	0	0	0	0	0
171YPF?	83	356	356	359	0	0	365	0	0
198Y	85	356	356	359	359	365	0	0	368
19Y	95	356	0	359	359	0	0	0	0
184Y	98	356	0	359	0	365	0	368	0
186YPF?	100	356	0	359	0	365	0	368	0
187Y	101	356	0	359	0	365	0	368	0
189YPF?	103	356	0	359	0	365	0	368	0
1Y	104	356	0	0	0	365	0	0	0
22Y	108	356	0	0	359	365	0	368	368
23Y	109	356	0	359	0	365	365	368	0
25Y	111	356	0	359	0	365	0	368	0
28Y	114	356	0	359	0	365	0	368	0
2Y	116	356	0	359	0	0	0	0	0
32Y	118	356	0	359	0	365	0	368	0
33Y	119	356	0	359	0	365	0	368	0
44T	132	356	0	359	0	0	0	0	368
45Y PF?	133	356	0	359	0	365	0	368	368
46Y	134	356	356	359	359	365	365	368	368
49T	137	356	0	359	0	365	0	368	0
55Y	143	356	0	359	0	365	0	368	0
60Y	148	356	0	0	0	0	0	0	0
62Y	150	356	0	359	0	365	0	368	0
63Y	151	356	0	359	0	0	0	0	0
64Y	152	356	0	359	0	365	0	368	0
67Y PF?	155	356	356	359	359	365	0	368	368
8Y PF	178	356	0	359	359	0	0	0	368
total present		42	14	32	16	31	6	28	12
total similar			14		9		5		8
percent matching			33.33		28.13		16.13		28.57

		RH_MEa0008cF01						RH_MEa0003dF05	
		Castro et al.	Luther	Castro et al.	Luther	Castro et al.	Luther	Castro et al.	Luther
sample name	number	162		165		168		212	
100Y	1	162	162	0	0	168	0	0	0
101Y	2	0	162	165	0	168	0	212	0
106Y	7	162	0	165	0	168	0	0	0
112Y	15	162	0	0	165	168	168	0	212
116Y	18	unknown	0	unknown	0	unknown	0	212	212
119Y	22	162	162	165	0	168	168	212	212
11Y	23	162	162	165	0	168	168	0	212
120Y	24	162	0	0	165	168	0	212	212
122Y	26	unknown	162	unknown	0	unknown	0	212	0
125YPF?	29	162	0	0	165	168	168	212	212
193YPF	32	162	0	0	165	168	168	0	0
133YPF	39	162	0	0	165	168	168	0	0
194Y	42	162	0	165	165	168	168	212	0
146Y	54	162	0	165	0	168	0	212	212
196Y	63	162	0	165	0	168	168	0	212
168Y	79	162	0	165	0	168	0	0	212
171YPF?	83	162	162	0	165	168	168	212	212
198Y	85	0	0	165	0	168	0	212	0
19Y	95	162	0	165	165	168	168	0	212
184Y	98	0	0	165	0	168	0	212	0
186YPF?	100	162	0	165	0	168	0	0	212
187Y	101	162	0	165	0	168	0	0	0
189YPF?	103	0	0	0	0	168	0	212	0
1Y	104	162	0	0	0	168	0	212	0
22Y	108	162	162	0	0	168	168	0	0
23Y	109	unknown	0	unknown	0	unknown	0	0	0
25Y	111	162	0	0	0	168	0	212	0
28Y	114	unknown	0	unknown	0	unknown	0	0	0
2Y	116	162	0	165	0	168	0	unknown	0
32Y	118	0	0	165	0	168	0	0	0
33Y	119	162	0	0	165	168	0	0	212
44T	132	162	162	0	0	168	0	0	212
45Y PF?	133	162	0	165	0	0	0	212	0
46Y	134	162	162	0	165	168	168	0	212
49T	137	162	0	0	165	168	168	212	0
55Y	143	162	0	0	0	168	0	212	0
60Y	148	162	0	165	0	168	0	0	0
62Y	150	162	0	165	165	0	0	212	212
63Y	151	162	0	165	0	168	0	0	0
64Y	152	162	0	165	0	168	0	212	0
67Y PF?	155	162	162	165	165	168	168	0	212
8Y PF	178	0	0	165	165	168	168	0	0
total present		32	10	22	14	36	15	19	17
total similar			8		5		15		7
percent matching			25		22.73		41.67		36.84

		RH_MEa0003dF05		RH_MEa0013bC12				RH_MEa0016aD11	
		Castro et al.	Luther	Castro et al.	Luther	Castro et al.	Luther	Castro et al.	Luther
sample name	number	218		227		230		251	
100Y	1	218	218	0	0	230	0	0	251
101Y	2	218	0	227	0	230	230	0	251
106Y	7	218	0	227	227	230	0	0	0
112Y	15	218	0	0	0	230	0	0	0
116Y	18	0	0	227	227	230	0	unknown	251
119Y	22	218	218	227	227	230	230	251	251
11Y	23	218	218	227	227	230	230	0	251
120Y	24	218	218	227	227	230	230	0	0
122Y	26	218	0	227	0	230	230	251	0
125YPF?	29	0	218	227	227	230	230	0	0
193YPF	32	218	218	227	227	230	230	251	251
133YPF	39	218	218	227	227	230	230	0	0
194Y	42	218	0	227	227	230	230	251	0
146Y	54	0	218	227	227	230	230	251	0
196Y	63	218	0	227	0	230	230	unknown	0
168Y	79	218	218	227	227	230	230	0	251
171YPF?	83	0	0	227	227	230	230	251	251
198Y	85	218	218	0	227	230	230	251	0
19Y	95	218	218	227	227	230	0	0	0
184Y	98	0	0	0	227	230	230	251	251
186YPF?	100	218	218	227	227	230	0	0	0
187Y	101	218	0	227	227	230	0	251	0
189YPF?	103	218	0	227	0	0	0	0	0
1Y	104	0	0	0	227	230	0	251	0
22Y	108	218	218	227	0	230	230	0	251
23Y	109	218	0	227	0	230	230	0	0
25Y	111	218	0	227	0	230	230	251	0
28Y	114	218	0	unknown	0	unknown	0	unknown	0
2Y	116	unknown	0	227	0	230	0	0	0
32Y	118	218	0	227	227	230	230	unknown	0
33Y	119	218	218	0	227	230	230	251	0
44T	132	218	218	0	227	230	230	unknown	0
45Y PF?	133	218	0	227	0	230	0	0	0
46Y	134	218	218	227	227	230	230	0	0
49T	137	0	218	227	227	230	230	251	0
55Y	143	218	0	227	0	230	0	0	0
60Y	148	218	0	0	0	230	0	0	251
62Y	150	0	218	0	227	230	230	0	251
63Y	151	218	0	227	0	230	0	0	0
64Y	152	218	0	227	227	230	0	251	0
67Y PF?	155	218	218	unknown	227	unknown	230	251	0
8Y PF	178	218	218	227	0	230	230	0	251
total present		33	20	31	26	39	26	15	13
total similar			16		19		25		4
percent matching			48.49		61.29		64.10		26.67