7-2021

Effect of Harvest Time on the Incidence of Red Drupelet Reversion and Development of Tetraploid Linkage Maps in Blackberry

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Effect of Harvest Time on the Incidence of Red Drupelet Reversion and Development of Tetraploid Linkage Maps in Blackberry

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Horticulture

by

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Bachelor of Science in Crop Sciences, 2018

July 2021
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ABSTRACT

The cultivated eastern U.S. blackberry (*Rubus* L. subgenus *Rubus* Watson) has gone through tremendous strides in both trait improvement and market outreach at the University of Arkansas System Division of Agriculture (UA System). What began as primarily a pick-your-own local fruit found mostly in the wild, has become a commercialized year-round product in most major U.S. grocery retailers. This could not have been achieved without decades of diligent breeding efforts. Although the genetic improvement of fresh-market blackberries has advanced, there are still issues that need to be addressed. One issue is the prevalence of red drupelet reversion (RDR), a physiological disorder where the drupelets of a fully black berry begin to turn red after harvest. A two-year study was done at the UA System to discover if harvesting at different times of day and/or harvesting genotypes with different levels of firmness might influence the incidence of RDR in blackberries after one week of cold storage (5 °C). Less RDR occurred when fruit was harvested at earlier times in the day, especially at 7:00 AM, when there is cooler ambient temperature. RDR was also sharply reduced when fruit was harvested from firmer selections such as A-2453. Another pressing issue is the lack of molecular breeding strategies provided for blackberries. The cultivated blackberry is an autotetraploid where there are four sets of homologous chromosomes that follow a multisomic pattern of inheritance. As a result, blackberries have high heterozygosity and lack saturated molecular maps reliable for gene discovery. An F_1 population and the parents were genotyped with new strategies optimized for autopolyploids to yield two saturated genetic linkage maps of the parents with 3,942 markers in total across 65 linkage groups. The blackberry population was aligned to a recently released diploid ‘Hillquist’ (*R. argutus* Link.) reference genome and showed a high degree of collinearity,

highlighting its potential as a new tool for future comparative analyses of Rosaceous crops in molecular research.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Margaret Worthington, for giving me an incredible opportunity to work under her wing as her graduate assistant. Her knowledge, guidance, and most of all, her patience has greatly contributed to my success in finishing this colossal work. I can truly say my time at the University of Arkansas would not have been so successful without her continuous compassion and encouragement to do better.

I would also like to thank my committee members: Dr. John Clark, Dr. Renee Threlfall, and Dr. Ainong Shi for passing on their respective pieces of knowledge to me with open arms. I especially want to thank Dr. Clark for generously providing time to impart words of wisdom and encouragement to me throughout my highs and lows when learning the ropes of graduate school.

Special thanks to Lacy Nelson, Carmen Johns, and Autumn Brown for helping me out in the lab and providing great company and advice. I cannot imagine how much more mind-numbing lab work would have been otherwise. Thank you to Cindi Brownmiller and Dr. Luke Howard for allowing me to use your lab space. I also want to thank the crew at Fruit Research Station for your hard work and great memories.

Thank you to Dr. Bode Olukolu, Ryan Kuster, and your lab crew in spending a considerable amount of time in educating me on using your protocol for my project and giving me the opportunity to work alongside you all. Special thanks to the NCSU GSL staff for their hard work. Also, I would like to extend a thanks to Dr. Mackay and the entire Horticulture Department staff for the fond memories and great friendships I will have long after I leave.

Most of all, I want to thank my family and friends for their fundamental role in shaping me to reach, not just for greatness, but for satisfaction in life. True happiness comes from within.

DEDICATION

To Grandpa Lee,

From a life of hardship, tragedy, and determination, begins a legacy of prosperity, happiness, and success. This work is but a small example of your lasting impact in achieving the American Dream.

May you find Peace.

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OVERALL INTRODUCTION

The cultivated blackberry (*Rubus* L. subgenus *Rubus* Watson) is a member of the diverse Rosaceae family that includes other important food and ornamental crops such as apples (*Malus domestica* Borkh.), peaches (*Prunus. persica* (L.) Batsch), plums (*P. domestica* L.), sweet (*P. avium* L.) and sour cherries (*P. cerasus* L.), almonds (*P. dulcis* Mill.), strawberries (*Fragaria x ananassa* Duch.), roses (*Rosa* spp. L.), hawthorns (*Crataegus* spp. Tourn.), and red raspberries (*R. idaeus* L.). Blackberries lack a species epitaph as a result of interspecific hybridization between other of blackberry and raspberry species (Clark and Finn, 2011; Graham and Jennings, 2009). Recently, blackberries have emerged as an economically important horticultural crop with a burgeoning worldwide market. From its humble beginning as a plant scavenged by local gatherers in the wild to becoming the fourth most profitable small berry crop, the blackberry has made great strides in the last few decades (Clark, 2005; Clark and Finn, 2011; Finn and Clark, 2011).

The overall market value of blackberries in the United States in 2018 was over $634 million in sales with the majority of production concentrated in the Pacific Northwest. Oregon is currently the top producer for processed blackberries with 26,472 Mg of berries harvested and valued at $21 million. (California Strawberry Commission, 2018; NASS, 2017). The value of the U.S. fresh-market industry was estimated at $100 million with California as the main producer with an annual revenue of $79 million in 2016 (County of Monterey Agricultural Commissioner, 2016; County of Santa Barbara Agricultural Commissioner, 2016; County of Santa Cruz Agricultural Commissioner, 2016). Other states, such as Texas, Arkansas, and Georgia, also have thriving industries for the blackberry fresh market (Ballington, 2016; Clark, 2005).

Two major blackberry breeding programs exist in the United States that continue to release new cultivars to the market. The University of Arkansas System Division of Agriculture (UA) blackberry breeding program, which started in 1964 under the leadership of Dr. James N. Moore, has been instrumental in the substantial market growth for fresh-market blackberries (Clark, 2016). The program released many important blackberry cultivars focusing on introgressing and improving traits such as erect cane architecture, long-term storage capability, thornlessness, disease/pest resistance, sweeter flavors, and primocane-fruiting (Clark, 1999, 2005; Clark and Finn, 2008). The USDA-ARS program at Corvallis, OR, started in 1928 by George Darrow, developed cultivars catered towards the blackberry processing industry. Cultivars released by the USDA-ARS program are primarily derived from trailing and semi-erect blackberry plants that have highly aromatic flavor profiles (Finn and Clark, 2011; Finn and Strik, 2016).

Postharvest quality and the ability to withstand long-distance shipping are key traits in fresh-market blackberries that the UA blackberry breeding program continues to improve in order to keep up with growing market demand. A recurring issue associated with blackberries is red drupelet reversion (RDR). RDR is a physiological disorder that commonly appears during postharvest when black drupelets turn bright red or maroon after cold storage (Clark and Finn, 2011; Finn and Clark, 2012). This condition can negatively impact consumer perception of blackberries and hinder market growth. The causal mechanisms of RDR are hypothesized to be from physical and temperature-related damage to the cell wall and vacuolar membrane (Salgado and Clark, 2016). Various proposed methods on how to reduce the incidence of RDR are currently under investigation with previous studies suggesting changes in cultural practices, such as earlier harvest times and selecting for firm or ‘crispy’ fruit texture for future blackberry cultivars (Edgley et al., 2019; McCoy et al., 2016; Yin, 2017).

In addition, fresh-market blackberries used in the UA program are genetically classified as autotetraploids possessing four sets of chromosomes. Advancements in molecular breeding for blackberries has been a slow process due to its status as an autotetraploid (Foster et al., 2019). The creation of genetic linkage maps for blackberries often requires specialized software with more powerful statistical models to better handle its complex inheritance pattern and to properly identify allele dosage for multiple heterozygous classes (Bourke et al., 2018; Hackett et al., 1998). To date, only a few linkage maps for tetraploid blackberry have been created with no high-resolution integrated linkage map available yet (Castro et al., 2013; Weber, 2014). The creation of an ultra-dense linkage map with reliable genetic markers for key traits can potentially simplify the breeding process for blackberries and to more efficiently select for quantitative traits (Yin, 2017).

Objectives

1. To evaluate the incidence of RDR in seven genotypes harvested at 7:00 AM, 10:00 AM, 1:00 PM, and 4:00 PM to determine whether harvest time and/or fruit firmness impacts the rate of RDR in blackberries.

2. To construct a dense linkage map of tetraploid blackberry using high-resolution markers developed from a novel GBS pipeline, GBSapp, optimized for polyploid species and highly heterozygous populations.

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CHAPTER I

LITERATURE REVIEW

General Taxonomy

Blackberry (*Rubus* L. subgenus *Rubus* Watson) belongs to the diverse Rosaceae family. This family also contains many other economically important food crops such as apples (*Malus domestica* Borkh.), pears (*Pyrus* spp. L.), plums (*Prunus domestica* L.), peaches (*P. persica* (L.) Batsch), sweet cherries (*P. avium* L.), strawberries (*Fragaria x ananassa* Duch.), almonds (*P. dulcis* Mill.), as well as some species in ornamental horticulture such as roses (*Rosa* spp. L.) and hawthorns (*Crataegus* spp. Tourn.). Blackberries, red raspberries (*R. idaeus* L.), black raspberries (*R. occidentalis* L.), and the hybrids derived from crossing these species are all members of the *Rubus* genus (Clark and Finn, 2011; Graham and Jennings, 2009). Collectively, they are categorized as caneberries due to their botanical similarities (Hummer, 2010). Although blackberries are classified within the subgenus *Rubus*, they lack a species epitaph because most cultivars are derived from multiple species of blackberry or raspberry as a result of interspecific hybridization (Clark and Finn, 2011; Foster et al., 2019; Thompson, 1997). Most blackberry species are polyploids, and the majority of cultivars grown in the eastern United States are tetraploids (2n = 4x = 28) (Clark, 2005a; Clark et al., 2007).

Blackberry is a perennial crop with biennial canes. During the first year of development, canes that emerge from the crown of the plant are called primocanes. Primocanes typically remain vegetative before becoming dormant over the winter months to become second-year canes called floricanes. The floricanes bear fruit during the summer months (Strik et al., 2007). Concurrently, a new set of primocanes will emerge each spring to repeat the cycle of growth for

any individual blackberry plant (Clark, 2005a, 2008; Clark and Finn, 2011). All along the floricanes, buds will form that will develop into flowers. The flowers are perfect with many stamens and an apocarpous gynoecium that produce numerous drupelets on the same fruit to create an aggregate (Graham and Jennings, 2009). Each drupelet contains a single seed surrounded by a lignified endocarp or pyrene inside a fleshy mesocarp (Graham and Jennings, 2009; Moore and Skirvin, 1990; Tomlik-Wyremblewska et al., 2010). The drupelets are attached to a central receptacle or torus. Blackberries differ from raspberries in that the area of abscission is located at the base of the torus, and the torus remains attached to the fruit when picked (Clark and Finn, 2011).

The ripening process for blackberries begins when the fruit is green before going through a series of color changes from partial red to full red, then from partial black to full black. Berries that are fully black are shiny before developing a dull appearance (Burdon and Sexton, 1993; Perkins-Veazie et al., 2000). Fruit at these later stages are typically ready to harvest as sugars and anthocyanins accumulate to marketable levels, and the receptacle tissue has softened due to increased ethylene production (Edgley, 2019; Perkins-Veazie et al., 1996, 2000). Glucose and fructose make up most of the sugar content in fully black fruit, while cyanidin-3-glucoside is the predominant anthocyanin making up 79-90% of the total profile (Edgley et al., 2019a; Kim et al., 2019; Perkins-Veazie and Clark, 2011; Perkins-Veazie et al., 2000).

The cane architecture of blackberries is classified as erect, semi-erect, or trailing. Primocanes from erect cultivars emerge from buds located at the crown or on the roots and have a natural tendency to stand upright. Primocanes for semi-erect and trailing types only emerge from buds on the crown where they appear more lateral (Strik et al., 2007). The erect and semi-erect

cultivars are generally used for fresh-market production while trailing types are used primarily for processed markets (Strik and Finn, 2012).

Economic Importance

Cultivated blackberry has recently obtained the title of "fourth" berry since becoming the fourth most important fresh-market berry crop behind strawberries, blueberries (*Vaccinium* spp. Rydb.), and red raspberries (Clark, 2005b; Clark and Finn, 2011; Finn and Clark, 2011). The market for both processed and fresh-market blackberries has grown to new heights within the past few decades. The U.S. blackberry market is valued at over $634 million in sales with a 7.0% increase in market revenue compared to sales in 2017 (California Strawberry Commission, 2018). The Pacific Northwest contributes to much of the domestic production with 90-95% of its industry catering towards processing. Oregon is the top producer for processed blackberries in the United States with over 2,800 ha of blackberries planted and approximately 26,472 Mg of berries harvested bringing in sales totaling $21 million (Finn and Strik, 2016; NASS, 2017). California is the largest producer of fresh-market blackberries with over 752 ha grown and a revenue of $79 million in 2016 (County of Monterey Agricultural Commissioner, 2016; County of Santa Barbara Agricultural Commissioner, 2016; County of Santa Cruz Agricultural Commissioner, 2016). The southeastern United States also produces blackberries, although area and production are not well documented. North Carolina was once briefly the leading producer in blackberries over a century ago at 720 ha (Williams, 1961). Substantial fresh-market production for shipping has expanded in states such as Texas, Arkansas, and Georgia (Ballington, 2016; Clark, 2005b). When considering the overall trend, the fresh-market industry in the United States is valued at around $100 million (County of Monterey Agricultural Commissioner, 2016;

County of Santa Barbara Agricultural Commissioner, 2016; County of Santa Cruz Agricultural Commissioner, 2016).

Internationally, the largest producer for processed berries is Serbia with over 5,000 ha, accounting for 69% of the cultivated area in Europe (Clark and Finn, 2014; Finn and Clark, 2011; Strik et al., 2007). According to Strik et al. (2007), global blackberry production was estimated at 20,035 ha of commercial production with an additional 8,000 ha of wild-harvested plants to produce more than 140,000 Mg of produce. Mexico is currently the largest producer of blackberries with over 153,000 Mg produced on 12,000 ha in 2014, mostly from the state of Michoacán (Pérez-Pérez et al., 2018). Most of the blackberry crop in Mexico is for fresh-market production that is exported to the United States and Europe primarily from October to June (Clark and Finn, 2014). The cultivars grown in Mexico have traditionally been floricane-fruiting cultivars with low chilling requirements and dormancy is chemically broken for production during the U.S. off-season. Primocane-fruiting cultivars were in the early years of production in Mexico as of 2017 (J.R. Clark, personal communication). Blackberry yields generally range from 8,000-20,000 kg/ha, depending on production practice and cultivar (Clark and Finn, 2014).

Breeding Efforts

Different species of blackberry can be found across the northern hemisphere in cool temperate regions where they were historically used as a wild source of food for people and animals (Hummer, 2010). The blackberry gradually became a commercial crop starting with breeding efforts in the mid to late 1800s (Clark, 2016). Wild cultivars were first selected for novel characteristics starting with ‘Dorchester’ in 1841 as the first-named cultivar (Hedrick, 1925). The first cultivars derived from a breeding program were released beginning in 1880 (Hall, 1990). Organized public breeding programs appeared around the turn of the century.

The Texas Agricultural Experiment Station at Texas A&M University (College Station, TX) began the first public blackberry breeding program in 1909 directed by Helge Ness. In 1959, 'Brazos' was released from the program and became commercially important because of its low-chilling requirement, large berry size, and increased yield (Clark et al., 2007; Darrow, 1937; Moore, 1984, 1997). Soon thereafter, other public breeding programs began breeding for more desirable agronomic traits for both processed and fresh markets. Large fruit size, yield potential, thornlessness, plant hardiness, and disease resistance were common goals for most fresh-market blackberry breeding programs (Clark, 2005b). The USDA-ARS program (Corvallis, OR) was initiated in 1928 by George Darrow and he worked with the Oregon Agricultural Experiment Station to develop trailing cultivars for the processing industry. The release of 'Marion', with its highly aromatic fruit, in 1956 revolutionized the blackberry industry and became the market standard for the trailing blackberry industry (Finn and Clark, 2011; Finn and Strik, 2016; Waldo, 1957). The John Innes Horticultural Institute in England released 'Merton Thornless', which has provided a source for thornlessness in all future tetraploid cultivars, including the economically important 'Chester Thornless' (Clark and Finn, 2011; Scott and Ink, 1966). 'Chester Thornless' was released by the USDA-ARS (Beltsville, MD) as a high yielding semi-erect, thornless cultivar with good fruit firmness for shipping (Galletta et al., 1998).

Beginning in the 1990s, many other public breeding programs began to notice the marketability of blackberries and have introduced improved cultivars. In 1990, 'Tupy' was introduced by EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) Brazil as a high-quality cultivar that has a low-chilling requirement. 'Tupy' has replaced 'Brazos' to become the most common cultivar for fresh-market production in Mexico (Clark and Finn, 2002, 2011). Similarly, private breeding programs began to surface after realizing the increased market

potential in blackberry production. Driscoll's, Inc., based in Watsonville, CA, established its blackberry breeding program in 1991 to help cater for the increasing consumer demand for blackberries around the world with the intent to incorporate better flavor into their germplasm (Finn and Clark, 2012). Overall, there are at least 15 blackberry breeding programs around the world that served as the source of over 50 new cultivars for the past 20 years (Finn and Knight, 2002).

University of Arkansas Blackberry Breeding Program

The University of Arkansas System Division of Agriculture (UA) blackberry breeding program, which was started in 1964 by Dr. James N. Moore, has released many important erect blackberry cultivars derived from eastern North American blackberry species (Clark, 2016). The primary goal for the breeding program is to improve the overall quality of blackberries in the fresh-market and shipping industry. Specific traits that the program initially focused on included erect cane architecture, enhanced fruit quality, sweeter flavors, long-term storage capability, thornlessness, early ripening, broader environmental adaptations, and disease/pest resistance (Clark, 1999, 2005b; Clark and Finn, 2008). When the program started, the fresh market for eastern blackberries was solely local and used for home gardens. Production began to expand when 'Comanche' and 'Cherokee' were released in 1974 followed by 'Cheyenne' in 1976. All three cultivars stemmed from a cross made by Dr. Moore in 1964 between 'Darrow' and 'Brazos' (Moore, 1997). 'Cherokee' also showed improved postharvest handling and became a shipping industry standard prior to the 1990s, particularly from Chile (Finn and Clark, 2011). The 1984 release of 'Shawnee' was the program's first cultivar protected by a plant patent followed by the early maturing 'Choctaw' in 1988 (Moore, 1997).

Although UA cultivars helped expand and diversify the eastern blackberry market, almost all the production still came from pick-your-own or local sales through the early 1990s. Cultivars that were commonly used lacked significant postharvest handling capabilities (Clark, 1992, 2005b). 'Navaho', released in 1988, was the first thornless, erect cultivar that had excellent shelf life for shipping (Moore and Clark, 1989; Perkins-Veazie et al., 1997, 1999a, 1999b). The creation of 'Navaho' proved to be a major achievement in blackberry breeding, since thornlessness was previously thought of as an "intractable" trait in combination with erect canes (Clark, 2005a). Since the release of 'Navaho', more focus has been placed on developing thornless, erect cultivars that can withstand shipping long distances, such as the subsequent releases of 'Arapaho' and 'Apache' in 1994 and 1999, respectively (Clark, 2016; Clark and Moore, 1999; Moore and Clark, 1993). All such releases contained the thornless allele from 'Merton Thornless'. Other cultivars derived from 'Merton Thornless', such as 'Thornfree' and 'Smoothstem', were sources for thornlessness in UA cultivars (Clark and Finn, 1999, 2006; Scott and Ink, 1966). In the 21st century, more UA releases of thornless, erect cultivars with superior shipping quality include: 'Ouachita', 'Natchez', 'Osage', and 'Caddo' (Clark, 2013; Clark and Moore, 2005, 2008; Clark et al., 2019).

Another development in blackberry breeding that has potential to dramatically change the industry is primocane-fruiting (PF). Plants that possess the PF trait can develop flowers and fruit on primocanes in addition to normal fruiting on floricanes (Clark, 2008; Clark and Finn, 2011; Keep, 1988). The PF allele originally came from a wild diploid accession found near Ashland, VA called 'Hillquist' (Jennings et al., 1991; Thompson, 1995). This specimen was donated to the New York State Agricultural Experiment Station at Geneva, NY in 1949 for further study and

named after the individual who discovered the unique plant, L.G. Hillquist (Lopez-Medina et al., 2000). ‘Hillquist’ is the sole source of the PF allele in all PF blackberry cultivars.

According to Clark (2008), PF in blackberries were not sought after until the 1990s. The potential benefits of PF cultivars for the program were not realized until more recently. In Arkansas and other parts of the United States, floricanes typically produce fruit during the summer months, whereas primocanes produce fruit from July to October. As a result, the growing season can be extended for more flexibility in production (Strik and Finn, 2012). A double-crop system can be implemented with floricanes producing in the normal growing season and primocanes bearing fruit during the fall months (Clark and Salgado, 2016). A single-crop system can also be implemented with only primocanes producing fruit (Strik et al., 2007). With the single-crop system, blackberry production can expand to areas with insufficient chilling to break dormancy in floricanes (Clark and Finn, 2011; Finn and Clark, 2011). PF cultivars could also be grown in areas where floricanes experience winterkill and cold damage because primocanes do not need to overwinter (Clark, 2008). Pruning can be simplified by mowing the canes for reduced labor costs, while reducing any overwintering pests (Clark, 2005b). No- or low-chill environment production areas such as Mexico should experience major expansions of production due to the low maintenance costs and lack of chemical manipulation of PF cultivars (Clark, 2016; Clark and Finn, 2014). In addition, PF cultivars can especially benefit the U.S. market from September to November, when the commercial blackberry supply is low, and Mexican imports are not yet substantial (Carvalho et al., 2010).

The first two PF cultivars were released in 2004 as ‘Prime-Jan®’ and ‘Prime-Jim®’ for home garden production (Clark et al., 2005). The first PF blackberry with improved postharvest handling suitable for long-distance shipping and commercial production was ‘Prime-Ark® 45’,

released in 2009 (Clark and Perkins-Veazie, 2011). The release of APF-77, also known as 'Black Magic™', in 2013 was another PF cultivar for home garden production with exceptionally soft berries (Clark et al., 2014). Two thornless PF cultivars were subsequently released, 'Prime-Ark® Freedom' in 2014 and 'Prime-Ark® Traveler' in 2015. 'Prime-Ark® Traveler' also has improved postharvest storage capability (Clark, 2014, 2015; Clark and Salgado, 2016). In 2020, 'Prime-Ark® Horizon' was released as a PF cultivar with excellent shipping potential and longer fruiting period for an extended harvest season (J.R. Clark, personal communication). The release of these cultivars has allowed blackberry production to expand in a similar manner to PF red raspberries (Clark and Perkins-Veazie, 2011).

Red Drupelet Reversion

Red drupelet reversion (RDR) is a postharvest disorder that occurs when fully colored black drupelets turn red during or after cold storage (Clark and Finn, 2011; Finn and Clark, 2012). This condition can have a negative impact on consumer preferences, which can be detrimental to the blackberry fresh-market industry. The visual appearance of blackberries in clamshells, such as uniform color and glossiness, are important traits that consumers base their purchases (Threlfall et al., 2016a, 2016b). More recent surveys found the majority of consumers prefer purchasing clamshells containing large, oblong berries with little to no RDR present (Threlfall et al., 2020, 2021). Blackberries have generally been a perishable commodity with almost 40% of the produce lost due to postharvest mishandling (Pritts and Handley, 1989). Such losses can have major economic and environmental ramifications that makes finding a more sustainable solution to a highly sought-after goal (Molina-Bravo et al., 2019).

It has been speculated that the cause of RDR is physical, and temperature-related damage to the cell wall and vacuolar membrane causes the contents of the vacuole to release into the

cytoplasm (Salgado and Clark, 2016a). The vacuole is a cell organelle that takes up about 90% of the cytoplasmic space of a cell and accumulates macromolecules such as sugars, flavors, aromas, and anthocyanins, which heavily influence the growth stages of the cell (Fontes et al., 2011). As these contents are released into the cytoplasm, biochemical reactions are facilitated that degrade or transform the anthocyanins from a monomeric into a polymeric form to produce the characteristic color change (Hager et al., 2008; Pérez-Pérez et al., 2018; Salgado and Clark, 2016a). Fruit ripeness is associated with the polysaccharide components of the primary cell wall and the middle lamella. The middle lamella is a pectin layer that is responsible for maintaining cell-cell adhesion and structure (Brummell, 2006). According to Atkinson et al. (2012), the ripening in apples is concurrent with the disintegration of the middle lamella leading to reduced intercellular adhesion, increased cell separation, and softer fruit texture. Edgley et al. (2019a) observed the skin of reverted drupelets as more shriveled in appearance with large intracellular spaces between cells compared to unaffected drupelets.

Cultural practices during production that result in lower quality fruit are among the leading causes of postharvest problems like RDR. Excessive nitrogen fertilization can increase vegetative growth at the expense of fruit yield and quality, leading to an increased susceptibility to mechanical damage and physiological disorders (Lee and Kader, 2000; Mengel et al., 2001; Nelson and Martin, 1986). In two studies conducted by Edgley et al. (2018, 2019b), higher nitrogen application rates increased the incidence of RDR on ‘Ouachita’ blackberries. Lime-based applications containing calcium have been related to longer postharvest shelf life and firmer fruit by helping to maintain the integrity of the cell wall (Ali, 2012; Ferguson et al., 1999; Strik, 2017).

Another important factor that influences fruit quality where the plant is grown is the weather and climate. Fruit can soften and have higher susceptibility to mechanical stress before and after harvest following heavy rainfall (Clark and Finn, 2014; Finn and Clark, 2011; Kader, 2002; Perkins-Veazie and Clark, 2005; Salgado and Clark, 2016a). Temperature may play a role in determining susceptibility to RDR. McCoy et al. (2016) and Yin (2017) each conducted a one-year study focusing on harvesting blackberries at different times of day and found that RDR is reduced when harvesting before 10:00 AM and 12:00 PM, respectively. Edgley et al. (2019b, 2019c) also found reduced RDR on fruit when harvested before 10:00 AM or on cooler days. A possible cause for this trend is due to a decline in turgor pressure in response to heat stress that can decrease the mechanical stiffness of the cell (Hertog et al., 2004; Hussein et al., 2018). One study has shown that blackberries harvested with a skin temperature exceeding 25 °C are more apt to bruise from impact damage as a result (Edgley et al., 2019d). The use of shade cloth and more delicate harvesting practices are ways that can reduce impact damage done to blackberries when undergoing heat stress. Fruit that was harvested by hand into buckets had an average of 85% RDR on at least one drupelet per berry, while fruit that was harvested without handling had an average of 6% (Edgley et al., 2019c).

Temperature management during postharvest storage is critical to maintaining high-quality blackberries (Bolda et al., 2012). Signs of physical damage to fruit can appear within 24 h after harvest (Edgley et al., 2019d). Fruit deterioration can be slowed by keeping the storage temperature between -2 ºC to 0 ºC and preventing fruit from warming after cold storage. Most importantly, fruit should not have a long period of delay between harvest and cooling (Maxie et al., 1959; Robbins and Moore, 1992). Blackberries should be gradually acclimated to the final storage temperature to prevent chilling injury from occurring and to further minimize signs of

RDR (Edgley et al., 2019d; Felts et al., 2020; Salgado and Clark, 2016b). In addition, the methods of transport and handling can influence the final quality of the fruit. Perkins-Veazie et al. (1997) concluded that 'Navaho' blackberries were suitable for overseas shipment that heavily depended on storage temperature and handling conditions. Another study done by Pérez-Pérez et al. (2018), observed that blackberries exposed to a certain level of vibration frequencies at different lengths of times can induce mechanical stress to facilitate RDR. Sections of the fruit that showed no RDR contained cells with greater order and integrity than affected areas where RDR was present (Pérez-Pérez et al., 2018). Changing the punnet design for clamshells was a suggested solution to minimize injury during transport (Edgley et al., 2019c).

Genetic factors also play an important role in determining the incidence and severity of RDR. Most blackberry cultivars have soft and fragile skin with high respiration and transpiration rates that can cause decay in storage (Joo et al., 2011). The UA blackberry breeding program has been working to release cultivars that can withstand the stress of postharvest storage by selecting for increased fruit firmness. Fruit firmness is a difficult trait to improve and varies depending on the cultivar, stage of ripeness, and storage duration (Clark, 2005a; Lawrence and Melgar, 2018; Perkins-Veazie et al., 1996). In previous studies, a high degree of firmness was correlated with a lower incidence of RDR (Perkins-Veazie et al., 1996). Unusually firm selections at the UA were found in the early 1990s that were referred to as 'crispy' with significantly lower incidence of RDR (Clark, 2016; Felts et al., 2020; Salgado and Clark, 2016c; Segantini et al., 2017).

Salgado and Clark (2016a) conducted a study to determine the relative firmness of multiple genotypes (cultivars and advanced breeding selections) and examine the incidence of RDR after seven days in cold storage at 5 ºC. The crispy genotypes retained a higher level of firmness than the non-crispy genotypes and had a lower incidence of RDR. The crispy genotypes retained the

integrity of their middle lamella and had structurally stable mesocarp tissues (Salgado and Clark, 2016a). Atkinson et al. (2012) discovered that certain genotypes of apple have reduced polygalacturonase (PG) activity, which depolymerizes pectin contained in the primary cell wall. Less PG activity allows the integrity of the middle lamella to be maintained (Atkinson et al., 2012). The same result was found in raspberries where firmer cultivars went through a reduced degree of pectin hydrolysis compared to softer cultivars (Stewart et al., 2001). Firmer blackberry genotypes may also have reduced PG activity, though this has not been confirmed. Drupelets on crispy genotypes were observed to contain denser cells with thicker cell walls to support a more robust tonoplast resistant to temperature-related damage during storage (Salgado and Clark, 2016b).

Changes in fruit physiology were shown to make a difference, where generally smaller fruit size was positively correlated with firmness. Smaller fruit contains roughly the same number of cells as larger fruit, but develop in a dense structure (Ali, 2012). A denser fruit may contribute to firmer fruit with reduced RDR. According to multiple sensory panels, A-2453, a crispy genotype, was rated the most firm and had the highest liking for berry color compared to other non-crispy genotypes (Segantini et al., 2017; Threlfall et al., 2016a, 2016b). McCoy et al. (2016) and Yin (2017) both found a clear inverse relationship between firmness and RDR with A-2453 outperforming the other cultivars in the studies in terms of firmness and RDR.

Genetic Mapping in Autopolyploids

Genetic research in polyploid crops has been a slow and difficult effort. Much of the knowledge gained in mapping and constructing high quality linkage maps for diploid species has not met with the same success for polyploid crops (Leach et al., 2010; Molina-Bravo et al., 2019; Ripol et al., 1999). Polyploids differ from diploids in that they possess more than two sets of

chromosomes. The polyploids most commonly found possess even-numbered ploidy levels, with tetraploids constituting the largest group (Comai, 2005). They can be further categorized as autopolyploids and allopolyploids based on their pattern of inheritance. Autopolyploids are thought to possess duplicated chromosomes from one original diploid progenitor species that originated from unreduced gametes during meiosis. Autopolyploids display polysomic inheritance, where chromosomes can recombine with any homologous chromosomes during meiosis. Allopolyploids possess chromosomes hybridized from two or more related species and display disomic inheritance during meiosis, where chromosomes will preferentially pair with homologs that are more closely related (Glover et al., 2016; Harlan and de Wet, 1975). Organisms that are intermediate between allo- and autopolyploids and experience incomplete preferential pairing are referred to as segmental allopolyploids (Grandke et al., 2014; Stebbins, 1947). Eastern U.S. blackberries are autopolyploids or segmental allopolyploids based on previous research (Clark et al., 2007).

Creating linkage maps is important for the genetic advancement of horticulturally important polyploid crops. Breeders can use linkage maps to identify the positions of genetic loci controlling important traits to develop molecular markers for genomic breeding (Bourke et al., 2018a). Breeding for quantitative traits is especially difficult in polyploid crops because many are perennial with long breeding cycles or have reduced fertility that can slow the traditional breeding process (Grandke et al., 2014). Linkage maps can be used for future quantitative trait loci (QTL) analyses to study regions linked to quantitative traits for more efficient breeding strategies.

The tools developed for linkage mapping in diploids can be applied to allopolyploids, but other tools and techniques must be applied in autopolyploids for better resolution (Bourke et al.,

2018a; Pereira et al., 2018). Linkage mapping in autopolyploids is complicated by several factors. The primary issue is the existence of multiple heterozygous classes, referred to as allelic dosages, resulting from polysomic inheritance (Bourke et al., 2018a; Molina-Bravo et al., 2019). In tetraploids, there are five possible dosages: nulliplex (aaaa), simplex (Aaaa), duplex (AAaa), triplex (AAAa), and quadruplex (AAAA). The existence of multiple heterozygotes can create complicated recombination frequency estimations between marker dosages that require complex statistical software to calculate (Hackett et al., 1998). The genomes of autopolyploids are generally more complex and heterozygous than diploids and their heterozygosity can be maintained for much longer in cycles of self-pollination (Soltis and Soltis, 2000).

Linkage maps of diploid relatives can be a useful tool for comparison in polyploid species (Bourke et al., 2018a). Diploid red raspberry was the first species in the genus *Rubus* to have a linkage map created, using single sequence repeat (SSR) and expressed sequence tag-SSR (EST-SSR) markers (Graham et al., 2004). This map, and subsequent maps of raspberry (Pattison et al., 2007; Sargent et al., 2007; Spencer, 2012; Ward et al., 2013) and black raspberry (Bushakra et al., 2012, 2015) were used as tools for comparative mapping with other relatives in Rosaceae, including the first linkage maps for blackberry (Castro et al., 2013; Weber, 2014).

Single-dose restriction fragments (SDRF) were originally analyzed in pseudo-testcross mapping, where simplex x nulliplex (Aaaa x aaaa or aaaa x Aaaa) markers are used to create two parental haplotype maps (Wu et al., 1992). The use of SDRF markers and pseudo-testcross mapping is advantageous in that the markers segregate at a 1:1 ratio in the progeny, no dosage calling is required, and software designed for diploid species can be used to generate the linkage maps (Bourke et al., 2018a). However, the utility of pseudo-testcross mapping is limited because many bi-parental markers are needed to saturate all parental haplotype linkage groups for

successful integration into a consensus map (Grattapaglia and Sederoff, 1994; Kim et al., 2012). In addition, estimated recombination events can generate a biased view among the progeny in the process (Pereira et al., 2018). Reference genomes of diploid relatives provide a major advantage in further saturating maps for polyploid crops using comparative mapping strategies to infer marker placement and mapping QTL (Bourke et al., 2018a; Ripol et al., 1999; van Geest et al., 2017; Wu et al., 1992). Since then, linkage maps that include higher dose markers (e.g. duplex x nulliplex and simplex x simplex) alongside SDRF markers have been used to develop fully integrated maps (Hackett et al., 1998). TetraploidMap, a specialized software program for autotetraploid species, was developed to create integrated linkage maps based on dominant and codominant marker information between parents and offspring of a test population and facilitate QTL mapping (Hackett and Luo, 2003; Hackett et al., 2007). Linkage maps have successfully been made with TetraploidMap in alfalfa (*Medicago sativa* L.) (Julier et al., 2003), potato (*Solanum tuberosum* L. ssp. *tuberosum*) (Bradshaw et al., 2008), rose (*Rosa hybrida*) (Gar et al., 2011), and highbush blueberry (*Vaccinium corymbosum* L) (McCallum et al., 2016). Castro et al. (2013) also constructed a map using the software for tetraploid blackberry consisting of 119 SSR markers distributed across seven linkage groups for each of the parents of the population. Unfortunately, TetraploidMap can only handle up to a maximum of 800 markers overall with 50 markers per integrated linkage group, making it unsuitable for next-generation sequencing datasets (Hackett et al., 2017).

Codominant markers, such as biallelic single nucleotide polymorphism (SNP) markers generated from fixed SNP arrays, can be used to detect different dosage classes in autopolyploids (Bourke et al., 2018a). SNP arrays are used to detect polymorphisms between parental samples and their progeny or between multiple genotypes of a crop. The signals generated from SNP

arrays are then transformed into discreet dosage calls using software such as ClusterCall (Schmitz Carley et al., 2017) and fitTetra (Voorrips et al., 2011) in R. ClusterCall assigns dosage scores to clusters under a complete tetrasomic model using the expected segregation ratios of an F_1 population. This allows accurate genotype calling for autotetraploids that follow a pattern of complete tetrasomic inheritance (Schmitz Carley et al., 2017). FitTetra works well with autotetraploids that deviate from complete tetrasomy (Bourke et al., 2018a; Voorrips et al., 2011). Fixed SNP arrays were used to generate biallelic SNP markers with heterozygote dosage information and to create integrated genetic maps for crops such as rose (Bourke et al., 2017), potato (Hackett et al., 2013), and chrysanthemum (*Chrysanthemum* x *morifolium* Ramat.) (van Geest et al., 2017).

Genotyping-by-sequencing (GBS) has been used to generate large quantities of markers to develop dense linkage maps in many crop species. GBS reduces genome complexity using restriction enzymes and incorporates SNP discovery and genotyping in one step (Elshire et al., 2011; Kim et al., 2016). Linkage maps created by GBS include alfalfa (Li et al., 2014), barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) (Poland et al., 2012), rice (*Oryza sativa* L.) (Spindel et al., 2013), muscadine grape (*Vitis rotundifolia* Michx.) (Lewter et al., 2019), red raspberry (Hackett et al., 2018; Jibran et al., 2019; Ward et al., 2013), and black raspberry (Bushakra et al., 2015). A limitation to using GBS in autotetraploid linkage mapping is accurately determining allele dosage due to issues such as missing data and limited read depth. As the ploidy level increases, the ability to distinguish between heterozygote dosage classes becomes increasingly difficult. This problem can be minimized by providing high sequencing coverage for the region of interest (Foster et al., 2019; Grandke et al., 2014; Kim et al., 2016; Pereira et al., 2018). Well-established reference genomes for the target plant will also

significantly increase genotyping accuracy (Kim et al., 2016). A new protocol, called GBSpoly, can increase coverage and optimize GBS for highly heterozygous data in polyploid crops (Wadl et al., 2018). This protocol was used to create an ultra-dense linkage map of hexaploid sweetpotato (*Ipomoea batatas* (L.) Lam.) (Mollinari et al., 2020).

Several new software applications, including TetraploidSNPMap (Hackett et al., 2017), polymapR (Bourke et al., 2018b), and MapPoly (Mollinari and Garcia, 2019) have been developed specifically for linkage mapping using allele dosage scores in autopolyploids. TetraploidSNPMap works specifically with autotetraploids and follows a model which assumes that the four homologous chromosomes will randomly pair as bivalents (RCSA). TetraploidSNPMap can be used for linkage mapping and QTL mapping. Linkage maps are made using dosage-scored SNP data with dominant and codominant markers (Hackett et al., 2013). Several disadvantages exist with this software. Since recombination is assumed to have only random bivalent pairing in TetraploidSNPMap, the map order and estimated distance may be distorted by ignoring double reduction (Bradshaw et al., 2008). Another issue is this software is only compatible with mapping in tetraploids. Linkage maps were successfully made with TetraploidSNPMap in cultivated potato (Manrique-Carpintero et al., 2018; Massa et al., 2018), guinea grass (*Megathyrsus maximus* Jacq.) (Deo et al., 2020), and signalgrass (*Urochloa decumbens* (Stapf) R. D. Webster) (Ferreira et al., 2019).

PolymapR (Bourke et al., 2018b) is an R-based software package that creates linkage maps based on dosage-scored SNP data with a similar high-speed ordering algorithm to TetraploidSNPMap. In addition to mapping in autotetraploids, PolymapR can also be used to generate linkage maps of polysomic triploids, hexaploids, and segmental allotetraploid populations. The software is tolerant of preferential chromosomal pairing and takes into account

changes in gametic frequencies caused by double reduction from random chromatid assortment (Bourke et al., 2018a). A couple of disadvantages associated with the software are a lack of user-friendliness and the requirement of some experience in R coding. Some linkage maps created using PolymapR include potato (Bourke et al., 2016), rose (Bourke et al., 2017; Zurn et al., 2020), chrysanthemum (van Geest et al., 2017), lime (*C. medica* L. *x C. micrantha* Wester) (Ahmed et al., 2020), and kiwifruit (*Actinidia chinensis* var. *chinensis*) (Tahir et al., 2020).

MapPoly (Mollinari and Garcia, 2019) is another R-based software package that enables increasingly complex linkage maps to be made with even ploidy levels up to 12, depending on the statistical model used. This software can estimate multipoint linkages using the hidden Markov model (HMM) to accurately determine linkage phase information from multiple markers with incomplete or missing information, resulting in denser maps and smoother likelihood profiles for QTL (Hackett et al., 2018; Lander and Green, 1987; Mollinari and Garcia, 2019). A limitation for this software is that mapping populations with ploidy levels higher than eight are too computationally demanding for HMM-based estimations and simpler two-point marker analysis must be used for map construction. The data is also assumed to have complete polysomic inheritance with no double reduction present, which will limit accuracy in some polyploid crops (Mollinari and Garcia, 2019). Linkage maps for sweetpotato (Mollinari et al., 2020) and highbush blueberry (Cappai et al., 2020) were created using this software.

To date, there are no high-resolution integrated linkage maps of tetraploid blackberry. Existing maps include SSR-based parental linkage maps of ‘Prime-Jim®’ and ‘Arapaho’ (Castro et al., 2013) and pseudo-testcross maps of ‘Chester Thornless’ and ‘Prime-Jim®’ constructed with restriction site-associated DNA sequencing (RAD-Seq) (Weber, 2014). Multiple issues continue to complicate advancements in molecular breeding for blackberry such as polyploidy,

multisomic inheritance, and heterozygosity (Foster et al., 2019; Worthington et al., 2020). The development of new diploid blackberry reference genomes from ‘Burbank Thornless’ and ‘Hillquist’ (Worthington et al., 2020), GBS protocols optimized for autopolyploid species (Wadl et al., 2018), and specialized software for developing integrated genetic maps using dosage information in polyploid crops (Bourke et al., 2018b; Hackett et al., 2017; Mollinari and Garcia, 2019) all make the construction of high-resolution tetraploid linkage maps possible today.

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CHAPTER II

EFFECT OF HARVEST TIME AND FRUIT FIRMNESS ON RED DRUPELET REVERSION IN BLACKBERRY

Abstract

Red drupelet reversion (RDR) is a postharvest disorder of blackberries (*Rubus* L. subgenus *Rubus* Watson) where fully black drupelets revert to red after harvest. This disorder can negatively impact consumer perception of fresh-market blackberries. The cause of RDR is hypothesized to be related to intracellular damage sustained because of mechanical and environmental stress during and after harvest. Cultivars differ in susceptibility to this disorder and cultural factors, such as nitrogen rate, harvest and shipping practices, as well as weather and climate during harvest, influence RDR severity. In this two-year study (2019-2020), seven genotypes (cultivars and advanced selections) developed in the University of Arkansas System Division of Agriculture (UA) blackberry breeding program with a range of fruit textures were evaluated to determine whether firmness was correlated with RDR. In addition, fruit was harvested at four different times (7:00 AM, 10:00 AM, 1:00 PM, and 4:00 PM) to investigate whether harvest time influences RDR. All seven genotypes were harvested at the four times on two harvest dates per year and evaluated for RDR and firmness after one week of cold storage (5 °C). Fruit harvested early in the day had less RDR, with 7:00 AM harvests having the least RDR in both years. Significant genotypic differences in RDR and fruit firmness were found in each year. Firmness was negatively correlated with RDR in 2018 and 2019. These results indicate that growers may be able to reduce the prevalence of RDR by choosing cultivars with firm fruit texture and harvesting early in the day.

Introduction

The global fresh-market blackberry industry has grown dramatically during the past few decades. From 2000 to 2010, the amount of blackberries shipped to domestic markets increased from 4,500 kg to 54,545 kg (Clark and Finn, 2014). In 2018, the U.S. market for blackberries reached a value of over $634 million in sales with a 7.0% increase in market revenue compared to sales in 2017 (California Strawberry Commission, 2018). The expansion of the fresh-market blackberry industry can be attributed to multiple causes. Newer cultivars have been released with improved characteristics that allow for long-distance shipping, extended harvest season, higher quality fruit, and expanded production area. Better production practices and postharvest handling have also helped decrease crop loss. Blackberries have many similarities to raspberries, which can allow raspberry growers to easily transition into blackberry production. Blackberry plantings typically do not need to be replanted as often as raspberries and have lower disease pressure, which provides an economic incentive for growers. Additionally, consumer demand for blackberries has increased as a result of perceived health benefits associated with high levels of anthocyanins and antioxidants (Clark and Finn, 2008, 2011, 2014; Clark et al., 2007).

The continued expansion of the fresh-market blackberry industry is dependent on whether berries retain flavor and quality after harvest (Clark, 2016). Unfortunately, blackberries are one of the most perishable horticultural crops because of their high respiration rates and fragile skin. Up to 40% of blackberry production is lost due to postharvest mishandling (Joo et al., 2011; Pritts and Handley, 1989). Red drupelet reversion (RDR) is a postharvest disorder of blackberries that occurs when black drupelets on ripe berries turn red during and after cold storage (Clark and Finn, 2011; Finn and Clark, 2012). Reverted drupelets typically have a more shriveled appearance upon closer inspection with broken pistils surrounding the fruit compared

to fully black drupelets (Edgley et al., 2019a). Whole shipments of blackberries can be rejected if over 10% of produce is not fully black or blue colored according to United States Department of Agriculture (USDA) marketing standards (USDA-AMS, 2016). Consumers tend to primarily base in-store purchases of blackberries on visual appearance such as uniform black color, glossiness, and freshness (Threlfall et al., 2016a, 2021). According to an online survey done by Threlfall et al. (2020), the vast majority of consumers prefer larger, oblong shaped berries, with 73% of the respondents preferring solid black fruit with no reverted drupelets. When presented with three randomized clamshells filled with berries having varying levels of RDR, only 19% of consumers preferred the clamshell with the highest RDR in a consumer preference study conducted in person (Threlfall et al., 2021).

It has been speculated that RDR is caused by intracellular damage to the cell wall and vacuolar membranes that causes contents of the vacuole to spill out into the cytoplasm (Edgley et al., 2020). Tissue within reverted drupelets typically has larger intercellular spaces and ruptured cells indicative of widespread damage to the upper mesocarp (Salgado and Clark, 2016a). The vacuole can take up 90% of the cytoplasmic volume in a cell where it accumulates aromatics, anthocyanins, sugars, and tannins, which influence cellular development (Fontes et al., 2011). Edgley et al. (2019a) also used electrolytic leakage to measure damage to the plasma membrane of fruit tissue with significant increases from fully black drupelets (65%) to partially red (85%) and fully red drupelets (90%). The anthocyanins that are sequestered longer in the vacuole in damaged drupelets are susceptible to degradation, though different structural features of particular anthocyanins may affect their susceptibility to degradation (Edgley et al., 2019a; Edgley et al., 2020).

Previous studies have investigated physiochemical changes in reverted drupelets and have found that the anthocyanin content is significantly lower in reverted drupelets compared to fully black drupelets (Edgley et al., 2019a; Kim et al., 2019). Kim et al. (2019) harvested berries from 'Apache', 'Ouachita', and 'Triple Crown' and observed a 39-43% decrease in total anthocyanin content in red drupelets after a week in cold storage. A 42% average decrease in cyanidin-3-glucoside, the dominant anthocyanin present in blackberries, was also found in all three cultivars (Kim et al., 2019). Edgley et al. (2019a) found a 58% decrease in total anthocyanins and a 60% average decrease in cyanidin-3-glucoside between black and red drupelets in a similar analysis performed with 'Ouachita' blackberries. This reduction in anthocyanins is suspected as the reason black drupelets turn red during storage (Edgley et al., 2019a; Edgley et al., 2020).

Increasing fruit firmness is an important objective for fresh-market blackberry breeding programs. Fruit firmness is a quantitative trait that is typically evaluated after storage for postharvest retention (Clark and Finn, 2011). Perkins-Veazie et al. (1996) first observed an inverse relationship between fruit firmness and RDR using multiple genotypes (cultivars and advanced selections) after 7 d in cold storage (2 °C). Fruit firmness was partially dependent on cultivar with the firmest genotype having the highest quality retention (Perkins-Veazie et al., 1996). The UA System Division of Agriculture blackberry breeding program has intensely selected for firm-fruited genotypes to increase postharvest storage capacity, which has led to the discovery of especially firm genotypes with 'crispy' texture. Salgado and Clark (2016a) compared four 'crispy' genotypes and 11 standard-textured genotypes and showed that berry firmness was much higher and 28% fewer berries were affected by RDR in the 'crispy' genotypes compared to the standard-textured genotypes. Subsequent studies have compared the 'crispy' breeding selection, A-2453, with other cultivars and repeatedly shown that it has

significantly lower rates of RDR, even after one to two weeks in cold storage (Felts et al., 2020; McCoy et al., 2016; Yin, 2017). Segantini et al. (2017) compared A-2453 with 10 other genotypes and found it had the lowest RDR (0.7%), highest firmness (9.6 N), and most uniform/glossy appearance. Consumer panels have also placed A-2453 as having the highest liking for berry color, which shows much promise for 'crispy' selections (Threlfall et al., 2016a, 2016b).

The effects of cultural practices on RDR prevalence have been investigated. Excessive nitrogen fertilization increased bruise susceptibility of multiple species of fruit while decreasing fruit quality (Hussein et al., 2018; Lee and Kader, 2000; Mengel et al., 2001). 'Ouachita' blackberries had increased incidence and severity of RDR when fertilized with high levels of nitrogen (Edgley et al., 2018, 2019b). Based on these findings, applying a proper amount of nitrogen fertilizer is likely an important step during early fruit development in preventing RDR.

The methods of handling fruit during and after harvest can further influence RDR development (Perkins-Veazie et al., 1997). Up to 85% of 'Ouachita' berries that were hand harvested following standard industry practices developed RDR, compared with only 6% of fruit that was harvested without handling by cutting the pedicle above the fruit receptacle and placing the berries into cotton wool-lined cells (Edgley et al., 2019c). Edgley et al. (2019d) induced RDR in berries harvested without handling by exposing them to a point of impact injury. Over 95% of the fruit that was injured had some degree of color change, whereas the control samples not subject to injury had 5% RDR. Most of the color change occurred within 24 h of initial injury (Edgley et al., 2019d). Mechanical stress during shipping and transportation can be another factor contributing to RDR. Blackberries exposed to vibrations with a 10 Hz frequency and an

amplitude of 0.5 g for 10 or 30 min had significantly more RDR after 2 d of storage at 3 °C than fruit that was not subjected to vibration (Pérez-Pérez et al., 2018).

Climate conditions before, during, and after harvest can also affect fruit quality and RDR. Fruit is softer and more susceptible to mechanical damage during and after harvest following a heavy rainfall (Clark and Finn, 2014; Finn and Clark, 2011). Proper temperature management must be considered when storing berries after harvest (Bolda et al., 2012). Ideally, berries should instantly go through a cooling process after harvest to minimize heat exposure (Robbins and Moore, 1992). However, Edgley et al. (2019d) found that 'Ouachita' berries exposed to impact damage at warmer initial temperatures (>25 °C) before instantly cooling to 2 °C prior to a week in cold storage had increased rates of RDR, as opposed to berries that went through a more gradual cooling process. Salgado and Clark (2016b) also theorized the rapid change of temperature as a contributing factor leading to degradation of the tonoplast and cellular membrane fragmentation. These findings indicate that there may be an ideal rate of cooling after harvest for blackberries and that berries should be harvested with as much care as possible during cooler times of day to minimize RDR (Edgley et al., 2019d). Lawrence and Melgar (2018) concluded that cultivar selection and environmental conditions at harvest impact how fruit will respond postharvest.

Three separate single-year studies conducted in Clarksville, AR by McCoy et al. (2016), Yin (2017), and Felts et al. (2020) have investigated whether harvesting blackberries at different times of day impacted rates of RDR. McCoy et al. (2016) found that harvesting at earlier times of day, especially before 10:00 AM, resulted in significantly lower RDR rates, and Yin (2017) also found that harvesting before noon significantly reduced RDR. Felts et al. (2020) compared RDR in nine genotypes harvested at 7:00 AM and 12:00 PM, but found no significant impact of

harvest time on RDR. However, the fact that fruit harvested at 7:00 AM was stored in an ice chest for 5 h longer than fruit harvested at 12:00 PM before sorting and placing the fruit in storage at 10 °C may have impacted those results. Edgley et al. (2019c) conducted another single-year study in 2016 investigating the effect of temperature and harvest time on RDR in 'Ouachita' berries grown under a high tunnel in Tasmania. They observed lower rates of RDR when mean berry temperatures were below 23 °C, which was typically possible at 10:00 AM or before when the ambient temperature was cooler during the peak of the Tasmanian blackberry season. The results of these studies suggest that in warm climates, berries harvested in the morning before the ambient air temperature increases may have less severe RDR.

A multi-year study is needed to further investigate the impact of harvest time on the development of RDR. Thus, the objective of this study was to evaluate the incidence of RDR in seven genotypes harvested at 7:00 AM, 10:00 AM, 1:00 PM, and 4:00 PM to determine whether harvest time and fruit firmness impact the rate of RDR in blackberries.

Materials and Methods

Plant material and cultural practices. The study was conducted at the UA System Fruit Research Station, Clarksville [west-central Arkansas, lat. 35º31'5"N, long. 93º24'12"W; U.S. Department of Agriculture (USDA) plant hardiness zone 7b (USDA, 2012); soil type Linker fine sandy loam (Typic Hapludults)] in 2018 and 2019. Seven genotypes were harvested including: A-2453, 'Black Magic™', 'Natchez', 'Ouachita', 'Osage', 'Prime-Ark® 45', and 'Prime-Ark® Traveler'. Six of the seven genotypes in this study are commercial cultivars, whereas A-2453 is an advanced breeding selection that has been used in previous studies on 'crispy' texture. These genotypes were chosen to represent a range of fruit firmness from the soft home garden cultivar,

'Black Magic™', to the 'crispy'-textured A-2453. Each genotype was harvested from a single 3.3 m plot containing five plants spaced 0.6 m apart.

Standard production practices were applied to all plots harvested for the experiment. The plots were drip irrigated as needed and fertilized regularly. Nitrogen fertilizer was annually applied early in the spring during bud break in the form of ammonium nitrate (56 kg•ha^{-1} N). A fertigation system applied 20N-4.4P-17K every two weeks, beginning at berry development until harvest. Liquid lime sulfur fungicide (94 L•ha^{-1}) was applied during bud break for control of anthracnose [*Elsinoë veneta* (Burkh.) Jenkins]. Two additional fungicide applications, about five and three weeks before first harvest, were made to control anthracnose, botrytis fruit rot (*Botrytis cinerea* Pers.: Fr), and cane and leaf rust [*Kuehneola uredines* (Link) Arthur]. Multiple labelled insecticidal sprays containing zeta-cypermethrin, bifenthrin, and malathion were applied weekly for control of spotted wing drosophila (*Drosophila suzukii* Matsumura) starting at the beginning of berry development in late April until floricane harvest in late June. An additional labelled insecticide containing bifenthrin was applied annually in October to control for raspberry crown borer (*Pennisetia marginata* Harris). The plants were trained to a four-wire, horizontal T-trellis system where the two lower wires were 0.5 m above the soil level and 0.5 m apart while the upper two wires were about 1.0 m high and 0.8 m apart. Plants were pruned once floricane harvest was complete in August and tipped at 1.1 m height in mid-May as the canes grew 8 to 15 cm above the trellis. Black plastic mulch at the base of the plants was used for weed control.

Harvest. The fruit was harvested on 14 and 19 June in 2018 and 18 and 27 June in 2019 at four times (7:00 AM, 10:00 AM, 1:00 PM, and 4:00 PM). Two replicate 0.24 L vented clamshells (FormTex Plastics Corp., Houston, TX) were collected at each harvest time. Fruit was harvested when the genotypes included in the study were in the early to mid-season for harvest and all

harvested berries were at the shiny black stage of development and free of defects. The harvested fruit was placed inside clamshells with enough berries to fill the entire container without any of the berries touching the lid. A filled clamshell represented a single replicate for each genotype from the same plot.

The fruit temperature of the blackberries in the clamshell was recorded after harvest using a Raytek Raynger ST infrared crop temperature meter (Raytek Corp., Santa Cruz, CA). The temperature of the fruit for each clamshell was calculated from an average of five measurements taken a distance of 15 to 17 cm from the berries in the clamshell. Harvested clamshells of fruit were then placed in vented cardboard flats within a portable cooler filled with ice packs until each harvest was finished. The clamshells were placed in cold storage for 7 d at 5 ºC and 90% relative humidity.

Red drupelet reversion. After 7 d, the clamshells were removed from cold storage and allowed to return to room temperature (22 °C). The total number of berries in each clamshell was recorded before each berry was inspected for RDR. Moldy and diseased berries were discarded and not included as part of the total berry count. Drupelets were considered reverted if they were red or maroon in color. Many of the reverted drupelets were shriveled or showed signs of leakage. Drupelets that had a dried up, shriveled appearance, but were not discolored, were assumed to be damaged by a pathogen and not counted as reverted. Following the protocol from Clark and Perkins-Veazie (2011), berries with three or more reverted drupelets were scored as reverted, while berries with two reverted drupelets or fewer were not counted as reverted. The number of reverted berries was divided by the total number of berries in each clamshell to calculate the percent reverted berries for each clamshell (replicate).

Firmness. Texture was measured on 10 randomly selected berries from each replicate following red drupelet assessment. Individual berries were placed on a platform horizontally where they were compressed using a Stable Micro Systems TA.XT.plus Texture Analyzer (Texture Technologies Corporation, Hamilton, MA) with a 5 kg load cell. A 7.6 cm diameter cylindrical and plane probe was used to compress each fruit 5 mm. Fruit firmness was measured in Newtons (N).

Composition. Three berries were selected at random from each clamshell, placed in labelled storage bags, and frozen (-10 °C) after postharvest evaluation for composition analysis. The juice from each sample was analyzed to determine total soluble solids (SS) and titratable acidity (TA). The juice from each sample was extracted by thawing the berries and using cheesecloth to extract the juice. Soluble solids of the juice was measured using a Bausch & Lomb Abbe Mark II refractometer (Scientific Instrument, Keene, NH). Titratable acidity was measured by a Titrino plus 862 compact titrosampler (Metrohm AG, Herisau, Switzerland) and prepared using 6 g of juice from each sample diluted with 50 mL of deionized, degassed water. A solute of 0.1 N sodium hydroxide was used as the titrant to an endpoint of pH 8.2 to measure the citric acid content. Soluble solids and TA were both expressed as percentages.

Drupelet diameter. Prior to composition analysis for samples in 2019, three berries in each storage bag were used to measure drupelet diameter. For each berry, five drupelets were randomly selected to measure the diameter using digital calipers (Pittsburgh®, Camarillo, CA). Drupelet diameter was measured without removing the individual drupelets from the berry, and average value was calculated for all measurements per replicate.

Anthocyanins. High-performance liquid chromatography (HPLC) was performed for the 2019 samples using the remaining juice extracted from composition analysis. Samples from the four

different harvest times that belonged to the same genotype and harvest date were combined for a total of 14 samples. Three milliliters of sample from each genotype per day were dried using a Speed Vac concentrator (ThermoSavant, Holbrook, NY) and resuspended in 1 mL of 3% formic acid. The samples were then put through 0.45-µm polytetrafluoroethylene (PTFE) syringe filters (Varian, Inc., Palo Alto, CA) before HPLC analysis. The analysis was performed based on previous methods (Cho et al., 2004). A Waters HPLC System® (Waters Corporation, Milford, MA) was used that contained a 600 pump, a 717 Plus autosampler, and a 996-photodiode array detector. Separation was done using a 4.6 mm x 250 mm Symmetry® (Waters Corporation, Milford, MA) C18 column with a 3.9 mm x 20 mm Symmetry® C18 guard column. Anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-xyloside, cyanidin-3-malonylglucoside, and cyanidin-3-dioxalylglucoside) were all quantified as cyanidin-3-glucoside equivalents (C3GE). Total monomeric anthocyanin results were expressed as mg C3GE/100 mL berry juice.

Statistical analysis. Data was analyzed as a three-way factorial with a completely randomized design using the GLIMMIX Procedure in SAS v. 9.4 (SAS Institute, Inc., Cary, NC). Clamshells served as the experimental units. Genotype, harvest time, year, and their respective interaction terms served as fixed effects, while the harvest date was nested within year as a random effect. Pooled anthocyanin data from 2019 was analyzed using the MIXED Procedure in SAS v. 9.4 (SAS Institute, Inc., Cary, NC) with genotype as a fixed effect and harvest date as a random effect. Mean separation was performed with Tukey's Honestly Significant Difference (HSD) ($\alpha = 0.05$). Pearson's correlation coefficient was used to test the significance of the correlation between the severity of RDR and firmness of each genotype. Only significant r values were presented in the results.

Results

Climate conditions. The 2018 and 2019 growing seasons had different levels of precipitation and temperature during blackberry harvest (Fig. 2.1). Total monthly rainfall was recorded between the months of April and June during each season. In 2018, monthly rainfall was 131 mm in April, 84 mm in May, and 71 mm in June. Rainfall in 2019 was much higher than in 2018; April had 164 mm, May had an especially heavy rainfall with 349 mm, and June had 207 mm of rain. During the first season of data collection in 2018, 0.8 mm of rain was recorded within 5 d before the first harvest date while none was recorded for the second harvest date. During 2019, no rainfall was recorded within 5 d of the first harvest date, but 113.5 mm of rain fell within 5 d before the second harvest date. According to data collected from the Fruit Research Station weather station, ambient air temperature was similar in both years (Fig. 2.1). The surface temperature of the fruit at harvest varied depending on time of day in both years (Tables 2.1 and 2.2). In 2018 and 2019, the average berry temperature was lowest at 7:00 AM (22-25 °C), intermediate at 10:00 AM (29-32 °C), and highest at 1:00 PM (30-36 °C) and 4:00 PM (30-36 °C). Air temperature followed a similar pattern. In 2018, there was no difference in berry temperature or air temperature measured at 1:00 PM and 4:00 PM. However, air temperature was higher at 4:00 PM than 1:00 PM in 2019. Berry temperature and air temperature had strong positive correlations for both years (r = 0.93 and 0.87, respectively).

Red drupelet reversion. Significant year x genotype interactions were found for many variables measured in this study including RDR and firmness. Therefore, data for 2018 and 2019 are presented separately throughout the results. Overall, the severity of RDR was higher in 2019 than 2018. Rates of RDR differed significantly between harvest times for both years (Table 2.3). Later harvest times had higher rates of RDR with each harvest time increasing in sequential

order in 2018. The 1:00 PM harvest had the highest RDR rate in 2019 (30.28%). Although the 7:00 AM harvest in 2019 had the lowest rate of RDR (9.02%), it was not significantly different from the 4:00 PM harvest (15.37%).

The genotypic effect on RDR was also significant for both years. In 2018, A-2453, 'Osage', 'Ouachita', 'Prime-Ark® 45', and 'Prime-Ark® Traveler' had low rates of RDR between 1.42% to 5.20%. 'Natchez' (10.36%) and 'Black Magic™' (41.86%) had higher rates of RDR. All genotypes had a greater percentage of RDR during 2019; however, the difference in RDR between the first and second years were more pronounced in some genotypes than others. In 2019, A-2453 (3.30%) and 'Osage' (6.06%) had the lowest rates of RDR, while 'Prime-Ark® Traveler' (9.00%) and 'Ouachita' (9.29%) had intermediate RDR. 'Prime-Ark® 45' increased from 3.29% RDR in 2018 to 21.05% in 2019. 'Natchez' had 33.74% reverted berries in 2019, while 'Black Magic™' had the highest RDR of all genotypes at 79.83%. There was no significant harvest time x genotype interaction effect in either year for RDR. Air temperature and berry temperature were positively correlated with RDR in both years. Air temperature and RDR had a similar correlation in 2018 (r = 0.24) and 2019 (r = 0.27), while berry temperature was slightly less correlated with RDR in 2018 (r = 0.22) compared with 2019 (r = 0.35).

Firmness. There were no significant effects of harvest time or harvest time x genotype interaction on berry firmness in either year. Genotypes differed significantly in firmness for both years (Table 2.3). 'Black Magic™' was less firm than the other six genotypes in both 2018 and 2019, measuring 2.78 N and 2.27 N, respectively. A-2453 was firmer than all other genotypes in both years (13.92 N in 2018 and 10.71 N in 2019), and 'Prime-Ark® Traveler' was the second-firmest genotype in both years. 'Natchez', 'Osage', 'Ouachita', and 'Prime-Ark® 45' had intermediate firmness both years. Fruit firmness ratings for all genotypes in 2019 were lower

than 2018. Berry firmness and RDR were negatively correlated in 2018 (r = -0.53) and 2019 (r = -0.36) (Fig. 2.2).

Composition. There were no significant effects for harvest time when evaluating SS and TA in 2018 or 2019. A significant harvest time x genotype interaction was found for TA in 2019. Data for TA was pooled given the F statistic for genotype (F = 20) was an order of magnitude greater than the F statistic for the harvest time x genotype interaction effect (F = 2.03) (Data not shown). Our composition data indicates that fruit was within commercially acceptable ranges, and that fruit collected at different harvest times had similar levels of maturity.

Soluble solids varied significantly among genotypes in each year, though overall SS was higher in 2018 than 2019. 'Ouachita' had the highest SS in 2018 (15.12%) and 2019 (12.33%), respectively, with statistically similar levels in 'Prime-Ark® 45' (11.63%), 'Osage' (11.41%), and 'Black Magic™' (11.21%) in 2019. SS was negatively correlated with RDR in 2019 (r = -0.21), but no correlation was detected between SS and RDR in 2018. There were significant genotypic differences for TA in both years. 'Black Magic™' and 'Natchez' had the highest levels of TA (0.81% and 0.78%, respectively) in 2018 and 'Black Magic™' had significantly higher TA than any other genotype in 2019 (0.88%). The genotypes with the lowest TA in 2018 included 'Prime-Ark® Traveler', A-2453, and 'Ouachita', and in 2019 'Prime-Ark® Traveler', A-2453, 'Prime-Ark® 45', and 'Osage' were in the lowest acidity group. Berry reversion was positively correlated to TA in 2018 (r = 0.38) and 2019 (r = 0.46).

Drupelet diameter. The diameter of individual drupelets measured in 2019 varied across genotypes and harvest times, but no significant harvest time x genotype interaction effect was found. A-2453 (5.45 mm) and 'Ouachita' (5.34 mm) had the highest average drupelet diameter and 'Black Magic™' had the lowest (4.49 mm). Berries harvested later in the day had smaller

drupelet diameters, with an average length of 4.90 mm at 1:00 PM and 4:00 PM compared with drupelet diameters measuring 5.17 mm and 5.15 mm at 7:00 AM and 10:00 AM, respectively. Drupelet diameter was negatively correlated with RDR and positively correlated with firmness in 2019 (r = -0.58 and 0.40, respectively).

Anthocyanins. Total anthocyanins of the juice ranged from 22.95 to 74.85 mg/100 mL, but did not differ among genotypes (Table 2.4). Cyanidin-3-glucoside was the dominant anthocyanin in all genotypes and ranged from 17.60 to 66.65 mg/100 mL in 'Black Magic™' and 'Natchez', respectively. Cyanidin-3-rutinoside was the only individual anthocyanin that varied significantly among genotypes. However, no statistical differences among treatment means were detected using Tukey's Honestly Significant Difference (Table 2.4). No correlation was found between the level of cyanidin-3-rutinoside and RDR. 'Osage' had 5.85 mg/100 mL cyanidin-3-rutinoside, while 'Ouachita' had only 0.25 mg/100 mL and 'Prime-Ark® Traveler' had no measurable cyanidin-3-rutinoside. Cyanidin-3-malonylglucoside ranged from 0.85 to 1.80 mg/100 mL and cyanidin-3-dioxalylglucoside levels ranged from 0 to 2.85 mg/100 mL.

Discussion

Environmental effects on RDR. Significant main effects for genotype and harvest time on RDR were observed in both years of the study, with no significant interaction between these factors. SS and TA were within commercially acceptable ranges for all genotypes and harvest times. There were no differences in the firmness, SS, or TA of berries harvested at 7:00 AM, 10:00 AM, 1:00 PM, and 4:00 PM in either year, indicating that berries harvested at different times were equally ripe and that time of harvest did not impact any of these variables. Berries harvested at 7:00 AM had the lowest RDR at 2.67% in 2018 and 9.02% in 2019. The highest RDR rates occurred in fruit harvested at 1:00 PM and 4:00 PM. This finding agrees with the results of

McCoy et al. (2016), Yin (2017), and Edgley et al. (2019c), who found RDR increased for harvests at 10:00 AM or later.

Temperature changes are suggested to play a major role in influencing RDR severity at different harvest times (Edgley et al., 2019c; McCoy et al., 2016; Yin, 2017). In this study, average air temperature increased throughout the day with the greatest change occurring between 7:00 AM and 10:00 AM. Yin (2017) and McCoy et al. (2016) observed similar weather patterns in their research which was also conducted at the UA System Fruit Research Station. Yin (2017) found that air and berry temperature increased sharply between 7:00 AM and 12:00 PM before leveling out from 12:00 PM to 4:00 PM. McCoy et al. (2016) also found a 6.1 °C increase in air temperature between harvests at 7:00 AM and 10:00 AM, with no significant difference in temperatures during later harvest times. In this study, average air temperature started out 1.0 to 1.5 °C higher than average berry temperature at 7:00 AM before converging at 10:00 AM. From 1:00 PM onwards, average berry temperature was similar to air temperature. Previous research indicated that 'Arapaho' blackberries maintained equal stomatal conductance in temperatures ranging from 20 to 35 °C (Stafne et al., 2001), which may allow blackberry plants to maintain relatively stable canopy temperatures even in very warm conditions. Edgley et al. (2019c) also found that fruit temperature increased more than air temperature during the day in a study of blackberries grown in a high tunnel in Tasmania and attributed this effect to solar activity warming the fruit. The low correlation between temperature and RDR might be caused by different environmental or canopy conditions producing a confounding effect at each harvest.

Lawrence and Melgar (2018) suggested that other factors such as relative humidity, plant water status, and harvest date could also influence RDR severity. Precipitation particularly affected results in this study. Rainfall was greater during harvest in 2019 and likely impacted

firmness, RDR, and SS content. Firmness and SS content were lower while RDR rates were much higher in 2019. The 113.5 mm of rain that fell 5 d prior to the second harvest in 2019 may have affected the quality of berries collected that day. Heavy rainfall has been linked to decreased fruit firmness and growers are advised to postpone harvest for 4 d after significant rain events (Perkins-Veazie and Clark, 2005). McCoy et al. (2016) and Salgado and Clark (2016a) both reported that a wetter harvest season had negative impacts on overall fruit firmness. A future study looking into the firmness and RDR rate of berries grown in a high tunnel or rainout shelter with different overhead irrigation rates applied shortly before harvest may be useful to determine the effects of rainfall on RDR and develop harvest recommendations for growers.

Genotypic differences in RDR. Significant genotypic differences in RDR were observed in both years of this study, and berry firmness and RDR were negatively correlated in 2018 (r = -0.53) and 2019 (r = -0.36). 'Black Magic™' was significantly less firm than all other genotypes and had the highest RDR in both 2018 and 2019. McCoy et al. (2016) also found that 'Black Magic™' was the least firm and had the highest RDR of all cultivars and genotypes tested. 'Black Magic™' is a home garden cultivar that is not recommended for long-term shipping as it has repeatedly had poor postharvest performance (Clark et al., 2014). The 'crispy' selection A-2453 performed as expected, with significantly higher firmness than all other genotypes in the trial. A-2453 was among the group of genotypes with the lowest RDR in each year, as other researchers have shown (Felts et al., 2020; McCoy et al., 2016; Salgado and Clark, 2016a, 2016b, 2016c; Segantini et al., 2017; Yin, 2017).

Firmness gradually decreases during the ripening phase of physiological maturity for multiple fruit crops as the polysaccharide components of the primary cell wall and the middle lamella begin to degrade to reduce intercellular adhesion (Brummell, 2006). Soft blackberries have

higher susceptibility to bruising and cellular damage, leading to increased RDR. Fortunately, breeders have selected for blackberry genotypes that retain firmness during ripening (Clark, 2005). The relationship between fruit firmness and RDR has been documented in previous studies (Felts et al., 2020; McCoy et al., 2016; Perkins-Veazie et al., 1996; Salgado and Clark, 2016a, 2016b; Segantini et al., 2017; Yin, 2017). Ripe berries from the 'crispy' breeding selection, A-2453, had much greater cell-cell adhesion, thicker cell walls, and more uniform cellular structure than the standard-textured cultivar 'Natchez' (Salgado and Clark, 2016a, 2016b). A-2453 also had the least weight loss of all other genotypes during storage (Yin, 2017). In addition, Segantini et al. (2017) evaluated multiple blackberry genotypes for postharvest storage potential and found that weight loss was negatively correlated to firmness (r = -0.68). The increased integrity of cellular membranes and reduced weight loss in storage of firmer genotypes likely protect them from some of the cellular damage and bruising that causes RDR.

Other factors may also contribute to genotypic differences in RDR. In fact, only 28.4% and 12.7% of the genotypic variation in RDR was explained by firmness in 2018 and 2019, respectively. 'Osage' and 'Ouachita' had lower RDR in both years than anticipated based on berry firmness. While A-2453 was over twice as firm as 'Osage' and 'Ouachita' in both years, RDR levels for 'Osage' and 'Ouachita' in 2018 were not significantly different from A-2453 and 'Osage' was also not significantly different from A-2453 in 2019. McCoy et al. (2016) also found that 'Osage' had the second lowest rate of RDR after A-2453. On the other hand, 'Natchez' had the second highest level of RDR after 'Black Magic^{TM}' but was significantly more firm than 'Osage' and 'Ouachita' in both years. 'Osage' was previously reported as slightly firmer than 'Natchez' upon release (Clark, 2013). One explanation for this inconsistency is that other confounding variables influence RDR levels in addition to firmness. Environmental

conditions like precipitation likely affect firmness due to its quantitative nature (Clark, 2005; Salgado and Clark, 2016a).

Titratable acidity was correlated with RDR in both years and SS was negatively correlated with RDR in 2019. 'Black Magic™' and 'Natchez' had the highest TA and RDR in both years. It is possible that the higher acidity of these cultivars was a result of the intercellular damage that caused RDR (Fontes et al., 2011; Salgado and Clark, 2016a). Edgley et al. (2019a) found no differences in TA, but a lower pH, in fully reverted drupelets than fully black drupelets. A decline in pH below 3.0 will cause anthocyanins to shift to their red flavylium ion in isolated conditions and affect the color of purified solutions (Castañeda-Ovando et al., 2009). However, given the high concentration of anthocyanins in blackberries and their co-pigmentation with other polyphenols, it is unlikely that low pH results in the drastic color change seen in reverted drupelets (Edgley et al., 2019a). Blackberry genotypes vary widely in their acidity (Clark, 2005) and this correlation between RDR and TA is likely an artifact of the small number of genotypes selected for this study.

Genotypes with larger drupelets tended to have less RDR in 2019. A-2453 and 'Ouachita' had the largest drupelet diameter of the genotypes in this study, while 'Black Magic™' had the smallest diameter. The larger drupelet diameters of A-2453 and 'Ouachita' may be related to increased turgor pressure and cellular membrane integrity resulting from varying cuticle properties or respiration rates specific to each genotype (Hertog et al., 2004; Yin, 2017). Average drupelet diameter across genotypes decreased later in the day when air and berry temperatures were the highest. Transpiration rates are expected to increase as the plants are exposed to more sunlight and heat during the day. As transpiration rates increase, water leaves the cell, and the elastic modulus decreases (Hertog et al., 2004; Johnston et al., 2001; Yin, 2017). Higher

transpiration and water loss might contribute to the smaller drupelet diameter of berries harvested at 1:00 PM and 4:00 PM compared to the morning harvests and higher rates of RDR at these harvest times.

However, drupelet diameter was only measured for the 2019 harvest season, and the observed negative correlation between RDR and drupelet diameter could be an artifact of the small number of genotypes used. Although overall fruit size and weight were not measured, A-2453 was previously shown to be smaller and lighter than the other blackberry cultivars in this study while 'Natchez' was the largest and the heaviest (Felts et al., 2020; Threlfall et al., 2016a, 2016b). The negative relationship between drupelet diameter and fruit size might be the result of resource allocation, as smaller fruit may have a denser cellular structure (Ali, 2012). The positive correlation found between drupelet diameter and firmness supports this suggestion. Smaller fruit with fewer drupelets were also reported to experience less RDR than larger berries in a study conducted with 'Ouachita' (Edgley et al., 2018, 2019b). Similarly, smaller fruit of peach [*Prunus persica* (L.) Batsch] and apple (*Malus domestica* Borkh.) cultivars were reported to have less impact damage during harvest resulting in less bruising (Ericsson and Tahir, 1996; Maness et al., 1992). A multi-year study with a larger set of genotypes is needed to further examine the relationship between RDR, drupelet diameter, and fruit size.

The anthocyanin content and composition of different genotypes may also impact their susceptibility to RDR. Edgley et al. (2019a) and Kim et al. (2019) both found significant reductions in total anthocyanins in red drupelets compared to black drupelets. Anthocyanins vary in their stability depending on the sugars and other functional groups attached to the anthocyanidin (Welch et al., 2008) and cyanidin-3-glucoside is suspected to encounter the most chemical changes during color reversion as polymeric anthocyanin derivatives are created

(Pérez-Pérez et al., 2018). Edgley et al. (2019a) found that cyanidin-3-rutinoside and two of the acylated anthocyanins [cyanidin-3-dioxalylglucoside and cyanidin-3-(6"-malonylglucoside)] detected in ‘Ouachita’ blackberries were not significantly reduced in red drupelets compared to black drupelets, suggesting that these compounds may be somewhat protected from degradation during RDR.

In this study, juice samples were combined from the four harvest times and individual anthocyanins were measured in these pooled samples during the 2019 season to investigate whether differences in anthocyanin composition among the tested genotypes could explain any of the observed variation in RDR. Total anthocyanin levels did not vary between genotypes and cyanidin-3-glucoside was the most common anthocyanin found for all the samples, representing 77% to 90% of the anthocyanins measured. Cyanidin-3-rutinoside was the only anthocyanin found to vary between genotypes, though none of the genotypes were significantly different from each other according to Tukey’s Honestly Significant Difference. Edgley et al. (2019a) suggested that the disaccharide sugar compounds in cyanidin-3-rutinoside could inhibit nucleophilic cleavage and preserve the anthocyanin during reversion. Cyanidin-3-rutinoside was also shown to have better stability during thermal treatment at 95 °C and storage than other anthocyanins in black currant (*Ribes nigrum* L.) (Rubinskiene et al., 2005). While no significant correlation was found between cyanidin-3-rutinoside content and RDR in this study, the relatively high cyanidin-3-rutinoside content of ‘Osage’ (13.7% of total anthocyanins) might contribute to its lower than anticipated RDR rates given its relatively low firmness. Kim et al. (2019) also found genotypic differences in cyanidin-3-rutinoside among ‘Apache’, ‘Ouachita’, and ‘Triple Crown’. Thus, it may be possible to breed for increased cyanidin-3-rutinoside content, among other beneficial compounds, in blackberry (Cho et al., 2004). The anthocyanin data was collected for only one

season with only two replicates per sample. Future multi-year studies should evaluate the anthocyanin composition of a wider selection of blackberry genotypes in reverted and non-reverted drupelets to determine whether selection for cultivars with increased concentration of acylated and disaccharide anthocyanins could reduce the severity of RDR.

Conclusion

The results of this study add further support to the relationship between fruit firmness and RDR, which was documented in previous studies. The 'crispy' genotype, A-2453, had the lowest RDR of the genotypes evaluated, while the soft-fruited home garden cultivar, 'Black Magic™', had the highest RDR in both years. Other factors, including acidity, drupelet diameter, and composition of anthocyanins with greater stability than cyanidin-3-glucoside may also contribute to genotypic differences in susceptibility to RDR. However, future research with a greater number of genotypes is needed to determine the potential effect of these factors on RDR. As previously reported by McCoy et al. (2016) and Yin (2017), berries harvested earlier in the day had significantly less RDR after a week in cold storage. RDR rates were lowest for the 7:00 AM harvest, when average air and berry temperatures were lowest, signifying that cooler temperatures during harvest have a positive effect on fruit quality. Other environmental factors, including precipitation, likely also affected RDR and fruit firmness in this study. Our results indicate that growers may be able to reduce the severity of RDR by choosing cultivars with firm fruit texture and harvesting early in the morning.

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Tables and Figures

Table 2.1. Air temperature and fruit surface temperature of seven University of Arkansas System Division of Agriculture blackberry genotypes measured during each harvest date and time, Clarksville, AR (2018 and 2019).

Year	Harvest date	Harvest time	Air temp[z] (°C)	Fruit surface temp[y] (°C)
2018	14 June	7:00 AM	26.11	25.45
		10:00 AM	31.67	31.98
		1:00 PM	35.00	35.91
		4:00 PM	34.44	36.19
	19 June	7:00 AM	25.00	22.35
		10:00 AM	29.44	29.07
		1:00 PM	30.56	30.03
		4:00 PM	31.11	30.36
2019	18 June	7:00 AM	23.33	21.50
		10:00 AM	28.33	29.67
		1:00 PM	30.00	32.67
		4:00 PM	32.22	31.11
	27 June	7:00 AM	25.00	24.58
		10:00 AM	30.00	30.41
		1:00 PM	31.67	33.80
		4:00 PM	32.78	34.05

[z] Air temperature measured at each harvest time per harvest date.

Table 2.1 (Cont.)

[y] Average fruit surface temperature of seven genotypes collected at each harvest time per harvest date, each genotype had two replicate clamshells harvested with five berries per clamshell measured directly after harvest.

Table 2.2. Main effect means for harvest time for air temperature and fruit surface temperature of seven University of Arkansas System Division of Agriculture blackberry genotypes, Clarksville, AR (2018 and 2019).

	2018		2019	
Effect	Air temp[z] (°C)	Fruit surface temp[y] (°C)	Air temp (°C)	Fruit surface temp (°C)
Harvest time				
7:00 AM	25.56 a[x]	23.83 a	24.15 a	22.96 a
10:00 AM	30.54 b	30.48 b	29.15 b	30.02 b
1:00 PM	32.71 c	32.81 c	30.82 c	33.21 c
4:00 PM	32.73 c	33.14 c	32.50 d	32.54 c
P value	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*

[z] Average air temperature measured at each harvest time over all harvest dates.
[y] Average fruit surface temperature at each harvest time over all harvest dates.
[x] Means with different letter(s) are significantly different (α=0.05) using Tukey's honestly significant difference.

Table 2.3. Main and interaction effect means for harvest times and seven University of Arkansas System Division of Agriculture blackberry genotypes for red drupelet reversion, firmness, soluble solids, titratable acidity, and drupelet diameter after 7 days storage at 5 °C, Clarksville, AR (2018 and 2019).

	2018				2019				
Effects	Red drupelets (%)	Firmness (N)	Soluble solids (%)	Titratable acidity (%)	Red drupelets (%)	Firmness (N)	Soluble solids (%)	Titratable acidity (%)	Drupelet diameter (mm)
Harvest Time									
7:00 AM	2.67 b[z]	7.25 a	13.52 a	0.58 a	9.02 c	4.90 a	11.07 a	0.59 a	5.17 a
10:00 AM	3.94 b	6.97 a	12.89 a	0.63 a	16.47 b	5.12 a	11.01 a	0.51 a	5.15 a
1:00 PM	5.00 ab	7.12 a	13.40 a	0.59 a	30.28 a	5.06 a	11.23 a	0.57 a	4.90 b
4:00 PM	8.99 a	7.59 a	13.33 a	0.63 a	15.37 bc	5.39 a	11.06 a	0.56 a	4.90 b
P value	*0.0003*	*0.1855*	*0.1595*	*0.5357*	*<0.0001*	*0.2799*	*0.8809*	*0.2233*	*<0.0001*
Genotype									
A-2453	1.42 c	13.92 a	12.82 bc	0.57 cd	3.30 d	10.71 a	10.72 bcd	0.45 cd	5.45 a
Black Magic™	41.86 a	2.78 e	13.65 b	0.81 a	79.83 a	2.27 e	11.21 abcd	0.88 a	4.49 d
Natchez	10.36 b	8.35 bc	13.05 bc	0.78 ab	33.74 b	5.04 cd	10.30 cd	0.67 b	4.94 c

Table 2.3 (Cont.)

	2018				2019				
Effects	Red drupelets (%)	Firmness (N)	Soluble solids (%)	Titratable acidity (%)	Red drupelets (%)	Firmness (N)	Soluble solids (%)	Titratable acidity (%)	Drupelet diameter (mm)
Genotype									
Prime-Ark® 45	3.29 c	8.22 c	12.73 bc	0.61 bc	21.05 b	5.20 c	11.63 ab	0.44 d	5.12 bc
Prime-Ark® Traveler	5.20 bc	9.72 b	12.25 c	0.42 d	9.00 c	7.84 b	10.18 d	0.45 cd	5.08 bc
P value	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
Harvest time x genotype (*P* value)	*0.2161*	*0.7756*	*0.1319*	*0.5418*	*0.0873*	*0.7853*	*0.2874*	*0.0160*	*0.1213*

[z] Means with different letter(s) are significantly different (α=0.05) using Tukey's honestly significant difference.

Table 2.4. Main effect means[z] of anthocyanins of the juice[y] for seven University of Arkansas System Division of Agriculture blackberry genotypes, Clarksville, AR (2019).

Genotype	Cyanidin-3-glucoside (mg/100 mL)	Cyanidin-3-rutinoside (mg/100 mL)	Cyanidin-3-xyloside (mg/100 mL)	Cyanidin-3-malonylglucoside (mg/100 mL)	Cyanidin-3-dioxalylglucoside (mg/100 mL)	Total anthocyanins (mg/100 mL)
A-2453	24.3	1.05	0.95	0.85	0.80	28.20
Black Magic™	17.60	2.85	1.05	0.80	0.65	22.95
Natchez	66.65	3.30	0.45	1.50	2.85	74.85
Osage	33.85	5.85	0.30	1.10	0.90	42.10
Ouachita	44.65	0.25	1.80	1.80	1.05	49.55
Prime-Ark® 45	22.05	4.20	0.50	1.30	0.00	28.15
Prime-Ark® Traveler	24.50	0.00	0.70	0.75	1.15	27.20
P value	*0.1573*	*0.0434*	*0.5161*	*0.7020*	*0.0842*	*0.1667*

[z] No genotypic differences (α=0.05) were found for any attribute using Tukey's honestly significant difference.
[y] Anthocyanin results expressed as mg cyanidin-3-glucoside equivalents/100 mL of juice.

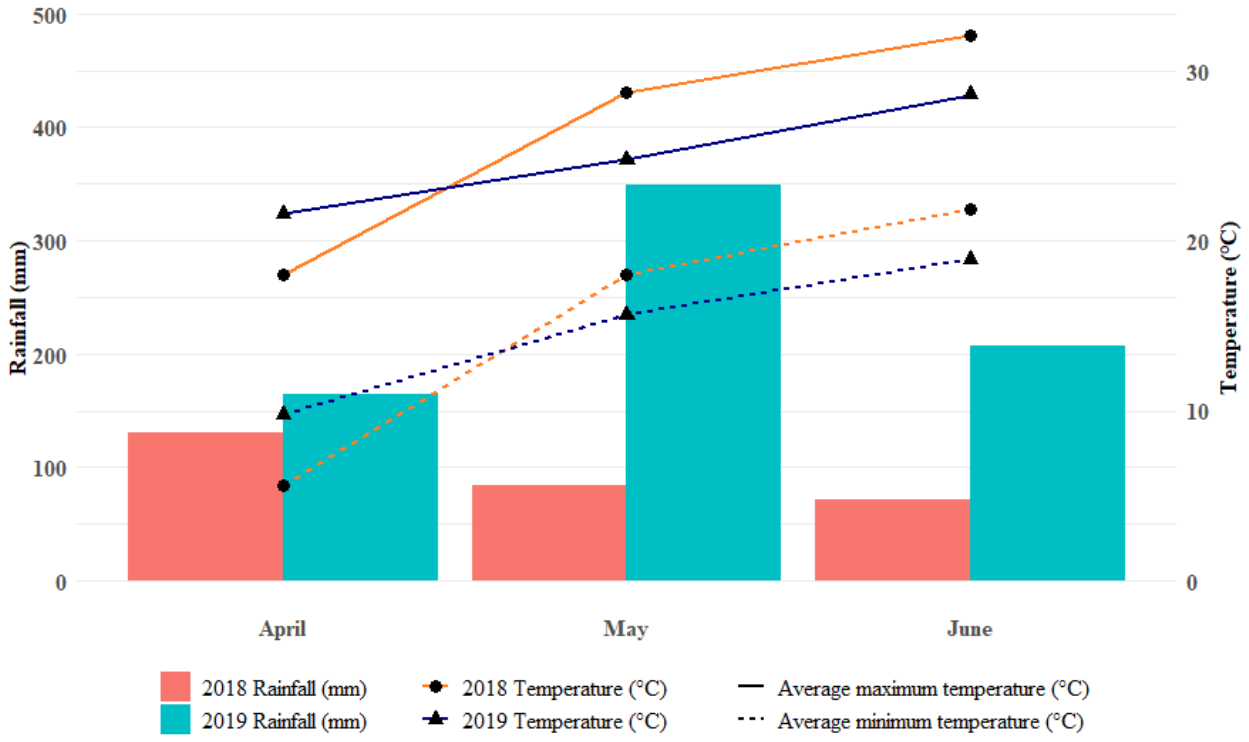

Fig. 2.1. Monthly rainfall and ambient air temperature at the University of Arkansas System Division of Agriculture Fruit Research Station, Clarksville, AR (2018 and 2019).

Fig. 2.2. Pearson's correlation coefficient for red drupelet reversion and firmness of blackberry genotypes harvested from the University of Arkansas System Division of Agriculture Fruit Research Station, Clarksville, AR (2018 and 2019). Hollow marker on a solid line indicates 2018 data. Solid marker on a dotted line indicates 2019 data.

CHAPTER III

DEVELOPMENT OF TETRAPLOID LINKAGE MAPS IN BLACKBERRY

Abstract

The fresh-market blackberry (*Rubus* L. subgenus *Rubus* Watson) is a horticulturally important crop that has experienced extensive market growth in recent times. As demand continues to increase, the need for more efficient molecular tools in blackberry research must be met. The high heterozygosity and multisomic inheritance of autotetraploid blackberries creates many challenges in generating reliable genetic maps for trait improvement. In this study, well-saturated genetic linkage maps were created for the maternal and paternal parents of an F_1 blackberry population using a novel genotyping-by-sequencing (GBS) pipeline to accurately score dosage calls for alleles. Processed reads were aligned to the black raspberry (*R. occidentalis* L.) genome and a new chromosome-scale reference genome of the diploid blackberry accession 'Hillquist' (*R. argutus* Link.). The resulting maps contained 3,942 markers in total across both parents with 65 linkage groups. Linkage groups ranged from 1.03 cM to 146.65 cM in length with an average density of 1 marker per 0.82 cM for the maternal haplotype map and 1 marker per 1.58 cM for the paternal haplotype map. A high degree of collinearity between 'Hillquist' and the tetraploid mapping population was confirmed, which shows the potential for this new blackberry reference genome in future genetic studies in *Rubus* crops.

Introduction

The fresh-market blackberry (*Rubus* L. subgenus Watson) has rapidly grown in market prevalence over the past few decades. In 2018, the U.S. blackberry market was valued at over $634 million in sales following a 7% market increase compared with sales the previous year (California Strawberry Commission, 2018). Despite the growing economic importance of this crop, few genomic resources exist for blackberries. The primary barrier to the development of molecular breeding tools for blackberries is their autopolyploid inheritance (Foster et al., 2019). Blackberries range from diploid (2n = 2x = 14) to 12x (2n = 2x = 84) (Meng and Finn, 2002), but fresh-market blackberries are primarily bred at the tetraploid (2n = 4x = 28) level (Clark et al., 2007).

Cultivated eastern U.S. blackberries are commonly classified as autopolyploids and display polysomic inheritance, where chromosomes can recombine with any homologous chromosomes during meiosis (Clark et al., 2007; Glover et al., 2016; Harlan and de Wet, 1975). Genetic research in blackberries and other polyploid crops has been a slow and difficult effort. Much of the knowledge gained in mapping and constructing high quality linkage maps for diploid species has not met with the same success for polyploid crops (Bourke et al., 2018a; Molina-Bravo et al., 2019; Ripol et al., 1999).

Creating linkage maps is important for the genetic advancement of horticulturally important polyploid crops. Breeders can use linkage maps to study patterns of inheritance and preferential pairing and to identify the positions of genetic loci controlling important traits to develop molecular markers for genomic breeding (Bourke et al., 2018a). Breeding for quantitative traits is especially difficult in polyploid crops because many are perennial with long breeding cycles or have reduced fertility that can slow the traditional breeding process (Grandke et al., 2014).

Linkage maps can be used for future quantitative trait loci (QTL) analyses to study regions linked to quantitative traits for more efficient breeding strategies.

The tools developed for linkage mapping in diploids can be applied to allopolyploids, but other tools and techniques must be applied in autopolyploids for better resolution (Bourke et al., 2018a; Pereira et al., 2018). Linkage mapping in autopolyploids is complicated by several factors. The primary issue is the existence of multiple heterozygous classes, referred to as allelic dosages, resulting from polysomic inheritance (Bourke et al., 2018a; Molina-Bravo et al., 2019). In tetraploids, there are five possible dosages: nulliplex (aaaa), simplex (Aaaa), duplex (AAaa), triplex (AAAa), and quadruplex (AAAA). The existence of multiple heterozygotes can create complicated recombination frequency estimations between marker dosages that require complex statistical software to calculate (Hackett et al., 1998). The genomes of autopolyploids are generally more complex and heterozygous than diploids and their heterozygosity can be maintained for much longer in cycles of self-pollination (Soltis and Soltis, 2000).

Linkage maps of diploid relatives can be a useful tool for comparison in polyploid species (Bourke et al., 2018a). Diploid red raspberry was the first species in the genus *Rubus* to have a linkage map created, using single sequence repeat (SSR) and expressed sequence tag-SSR (EST-SSR) markers (Graham et al., 2004). This map, and other early maps of red raspberry (Pattison et al., 2007; Sargent et al., 2007; Spencer, 2012; Ward et al., 2013) and black raspberry (Bushakra et al., 2012, 2015), were used as tools for comparative mapping with other relatives in Rosaceae, including the first linkage maps for blackberry (Castro et al., 2013; Weber, 2014).

Genotyping-by-sequencing (GBS) has been used to generate large quantities of markers to develop dense linkage maps in many crop species. GBS reduces genome complexity using restriction enzymes and incorporates single nucleotide polymorphism (SNP) discovery and

genotyping in one step (Elshire et al., 2011; Kim et al., 2016). Linkage maps created by GBS include alfalfa (Li et al., 2014), barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) (Poland et al., 2012), rice (*Oryza sativa* L.) (Spindel et al., 2013), muscadine grape (*Vitis rotundifolia* Michx.) (Lewter et al., 2019), red raspberry (Hackett et al., 2018; Jibran et al., 2019; Ward et al., 2013), and black raspberry (Bushakra et al., 2015). A limitation to using GBS in autotetraploid linkage mapping is accurately determining allele dosage due to issues such as missing data and limited read depth. As the ploidy level increases, the ability to distinguish between heterozygote dosage classes becomes increasingly difficult. This problem can be minimized by providing high sequencing coverage for the region of interest (Foster et al., 2019; Grandke et al., 2014; Kim et al., 2016; Pereira et al., 2018). Well-established reference genomes for the target plant will also significantly increase genotyping accuracy (Kim et al., 2016). A new protocol, called GBSpoly, can increase coverage and optimize GBS for highly heterozygous data in polyploid crops (Wadl et al., 2018). This protocol was recently used to create an ultra-dense linkage map of hexaploid sweetpotato (*Ipomoea batatas* (L.) Lam.) (Mollinari et al., 2020).

Early autopolyploid maps were constructed using pseudo-testcross mapping, where single-dose simplex x nulliplex (Aaaa x aaaa or aaaa x Aaaa) markers are used to create two parental haplotype maps (Wu et al., 1992). The use of single-dose restriction fragment (SDRF) markers and pseudo-testcross mapping is advantageous in that the markers segregate at a 1:1 ratio in the progeny, no dosage calling is required, and software designed for diploid species can be used to generate the linkage maps (Bourke et al., 2018a). The pseudo-testcross strategy is also theoretically simple to use for highly heterozygous organisms, and is an effective method for detecting unique marker reads for genetically divergent individuals (Grattapaglia and Sederoff, 1994). However, the utility of pseudo-testcross mapping is limited because many bi-parental

markers are needed to saturate all parental haplotype linkage groups and these maps are not optimal for QTL mapping in multisomic polyploids (Hackett et al., 2007). The number of bi-parental markers can significantly increase through the use of intraspecific hybrids (Kim et al., 2012).

Several new software applications, including TetraploidSNPMap (Hackett et al., 2017), polymapR (Bourke et al., 2018b), and MapPoly (Mollinari and Garcia, 2019) have been developed specifically for developing integrated maps in autopolyploids using higher dose markers (e.g. duplex x nulliplex and simplex x simplex) with allele dosage scores. TetraploidSNPMap works specifically with autotetraploids and follows a model which assumes that the four homologous chromosomes will randomly pair as bivalents (RCSA). Linkage maps were successfully made with TetraploidSNPMap in cultivated potato (Manrique-Carpintero et al., 2018; Massa et al., 2018), guinea grass (*Megathyrsus maximus* Jacq.) (Deo et al., 2020), and signalgrass (*Urochloa decumbens* (Stapf) R. D. Webster) (Ferreira et al., 2019).

PolymapR (Bourke et al., 2018b) is an R-based software package that creates linkage maps based on dosage-scored SNP data with a similar high-speed ordering algorithm to TetraploidSNPMap. In addition to mapping in autotetraploids, PolymapR can also be used to generate linkage maps of polysomic triploids, hexaploids, and segmental allotetraploid populations. Some linkage maps created using PolymapR include potato (Bourke et al., 2016), rose (Bourke et al., 2017; Zurn et al., 2020), chrysanthemum (van Geest et al., 2017), lime (*C. medica* L. *x C. micrantha* Wester) (Ahmed et al., 2020), and kiwifruit (*Actinidia chinensis* var. *chinensis*) (Tahir et al., 2020).

MapPoly (Mollinari and Garcia, 2019) is another R-based software package that enables increasingly complex linkage maps to be made with even ploidy levels up to 12, depending on

the statistical model used. This software can estimate multipoint linkages using the hidden Markov model (HMM) to accurately determine linkage phase information from multiple markers with incomplete or missing information, resulting in denser maps and smoother likelihood profiles for QTL (Hackett et al., 2018; Lander and Green, 1987; Mollinari and Garcia, 2019). Linkage maps for sweetpotato (Mollinari et al., 2020) and highbush blueberry (*Vaccinium corymbosum* L.) (Cappai et al., 2020) were created using this software.

To date, there are no high-resolution integrated linkage maps of tetraploid blackberry. Existing maps include SSR-based parental linkage maps of 'Prime-Jim®' and 'Arapaho' (Castro et al., 2013) and pseudo-testcross maps of 'Chester Thornless' and 'Prime-Jim®' constructed with restriction site-associated DNA sequencing (RAD-Seq) (Weber, 2014). Multiple issues continue to complicate advancements in molecular breeding for blackberry such as polyploidy, multisomic inheritance, and heterozygosity (Foster et al., 2019). The development of new diploid blackberry reference genomes of 'Burbank Thornless' (*R. ulmifolius* Schott) and 'Hillquist' (*R. argutus* Link.) (Worthington, 2020; Worthington et al., 2020), GBS protocols optimized for autopolyploid species (Wadl et al., 2018), and specialized software for developing integrated genetic maps using dosage information in polyploid crops (Bourke et al., 2018b; Hackett et al., 2017; Mollinari and Garcia, 2019) all make the construction of high-resolution tetraploid linkage maps possible today. The objective of this project was to construct a dense linkage map of tetraploid blackberry using a novel GBS pipeline to create high-resolution markers, and to further supplement genetic mapping resources for molecular research in blackberry.

Materials and Methods

Plant material and cultural practices. The A-2551TN x APF-259TN mapping population for this study was grown at the University of Arkansas System Division of Agriculture (UA) Fruit

Research Station, Clarksville [west-central Arkansas, lat. 35º31'5"N, long. 93º24'12"W; U.S. Department of Agriculture (USDA) plant hardiness zone 7b (USDA, 2012). Both parents were thornless breeding selections from the UA blackberry breeding program with distinctive shortened internodes (Fig. 3.1). The female parent, A-2551TN, was not primocane fruiting, while the male parent, APF-259TN, was primocane-fruiting. The two parents were crossed in 2015 to create 164 F_1 individuals. The cross was repeated in 2016 to create 86 F_1 individuals, for a total population of 250 progeny.

The progeny were planted in 3-L pots with a custom soil mix containing granular osmocote, hardwood mulch, and clay. Sulforix (18.9 L) were sprayed over the population in Feb. 2018 to control pathogens. The plants were watered using drip irrigation and were fertilized once in Apr. and May 2018 with 5.7 g of granular fertilizer (19N-19P-19K) on each plant. Three applications of liquid nitrogen fertilizer (24N-8P-16K) were applied every two weeks after tipping. An insecticidal spray containing zeta-cypermethrin (1.6 oz•ha^{-1}) was applied in Feb. 2019 to control for Japanese beetle (*Popillia japonica* Newman).

Genotyping-by-sequencing. DNA was extracted from young leaf samples harvested from parents and progeny following a modified CTAB protocol (Porebski et al., 1997). The extractions were quantified by a Qubit® fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to a concentration of 200 ng/µL in 30 µL wells. Genotyping-by-sequencing (GBS) library preparation and sequencing was performed at the Genomic Sciences Laboratory at North Carolina State University (Raleigh, NC). Libraries were prepared using the modified GBSpoly protocol optimized for polyploids and highly heterozygous genomes to produce uniform coverage across samples and loci as described by Wadl et al. (2018) and Mollinari et al. (2020). Briefly, the rare-cutter restriction enzymes, *Cvi*AII and *Tse*I, were used to digest the DNA

samples. The digested DNA samples were purified with AMPure® XP magnetic beads (Beckman Coulter Inc., Brea, CA) and the resulting fragments were ligated to barcoded adapters, with 10 bp buffer sequences upstream of the barcodes, which ranged from 6-9 bp. The buffer sequences were included to decrease the base call error rate in the barcode region and increase the percentage of reads that could be assigned to individual progeny after demultiplexing. A post-ligation digest with *Cvi*AII/*Tse*I was then performed to eliminate chimeric sequences. Following the second digest, the pooled libraries were purified with AMPure® XP magnetic beads and selected for 300-400 bp fragments using Pippin Prep (Sage Sciences Inc., Beverly, MA) to minimize PCR bias.

The 250 progeny and parents were first pooled in groups of 48 samples. Each pool was sequenced on a HiSeq 2500 (Illumina, San Diego, CA) lane with parents sequenced at 8x higher coverage than the progeny to ensure accurate parental dosage calls could be made for all polymorphic SNPs. Sequencing read depth was uneven across samples and inadequate for many genotypes in this first sequencing run. Thus, 188 progeny and parents (2x) were pooled in groups of 96 samples sequenced on two NovaSeq™ 6000 System (Illumina, San Diego, CA) lanes.

Genotype calling. Raw Fastq files were preprocessed using ngsComposer (Kuster et al., 2021). Preprocessing steps included trimming, demultiplexing, adapter removal, quality threshold filtering, artifact removal, and error correction. The GBSapp pipeline was then used for SNP-calling and filtering as described by Wadl et al. (2018). Processed reads were aligned to black raspberry (*R. occidentalis* L.) (VanBuren et al., 2018a) and a diploid blackberry accession ‘Hillquist’ (*R. argutus* Link.) (Worthington, 2020) reference genomes using BWA-MEM (Li, 2013). Alignment files were then processed with SAMtools before variant calling with GATK HaplotypeCaller (DePristo et al., 2011). Genotypes with less than 25 reads for each variant were

called as missing because a greater number of reads are required to make accurate genotypic calls in tetrasomic polyploids than diploid populations. Markers (SNPs and InDels) and genotypes with greater than 20% missing data were initially removed as well as markers that deviated from expected segregation ratios at $P < 0.001$.

Pseudo-testcross mapping. Separate genetic linkage maps of A-2551TN and APF-259TN were created in JoinMap 4.1 following the two-way pseudo-testcross strategy (Van Ooijen, 2011). Only markers that were heterozygous in the simplex condition (1/0/0/0) in A-2551TN and homozygous in the nulliplex condition (0/0/0/0) in APF-259TN were used to construct the maternal map. The paternal map was constructed with markers that were homozygous in the nulliplex condition (0/0/0/0) in A-2551TN and heterozygous in the simplex condition (1/0/0/0) in APF-259TN. Prior to map construction, single-dose markers segregating in each parent were inspected and individuals with 20% or more of missing data in either parental mapping dataset were excluded. Individuals with ratios of homozygote to heterozygote calls greater than 2:1 or less than 1:2 in either parental mapping dataset were identified as possible selfed progeny of A-2551TN or accidental outcrosses with contaminant pollen from other sources and removed from the mapping dataset. Identical markers and markers that deviated from expected segregation ratios according to the χ^2 test ($P < 0.10$) were excluded from mapping. JoinMap 4.1 can only handle datasets of 4,000 or fewer markers. Because the number of markers that passed initial missing data and segregation distortion thresholds for the female parents map far exceeded 4,000, 5% was set as the maximum allowable missing data for each marker. Markers with up to 20% missing data were included in the male parent map because the initial marker dataset was much smaller.

The threshold linkage logarithm of odds (LOD) for establishing initial groups was set to 9.0. Marker order and distances were then determined using the regression mapping algorithm with default settings and Haldane's mapping function. There was insufficient linkage in the data to create maps for several of the linkage groups that clustered together at LOD 9.0 in the female parent map. In these instances, higher LODs (ranging from 10-17) were selected for establishing groups with sufficient linkages for mapping. The JoinMap 4.1 regression mapping algorithm can only be used to order linkage groups of up to 250 markers. Therefore, in instances where more than 250 markers were assigned to a linkage group, markers with greater than 95% similarity were excluded from mapping. Charts of genetic linkage maps were drawn using MapChart 2.1 (Voorrips, 2002). Plots aligning the parental maps to the 'Hillquist' reference genome were generated in the R package *ggplot2* (Wickham, 2009).

Results

Genotype calling. A total of 495.2 million sequencing reads were obtained for the parents and progeny from HiSeq 2500 and NovaSeq™ 6000 System sequencing runs. After demultiplexing, processing, and quality filtering, we obtained 6.5 million reads for A-2551TN, 7.0 million reads for APF-259TN, and an average of 1.9 million reads for each of the progeny (Appendix 3.1). On average, 85.9% of reads were mapped to unique positions on the 'Hillquist' genome and 67.3% of reads mapped to unique positions on the black raspberry genome. 1,811,617 and 2,022,664 polymorphic markers were identified when these reads were aligned to the black raspberry and 'Hillquist' genomes, respectively, using the GBSapp pipeline. Only the markers identified using the 'Hillquist' reference genome were used for mapping. Two hundred and two of the original 250 progeny and 14,492 markers passed the initial filters for missing data and segregation distortion. Of these markers, 8,699 (58%) were classified as single-dose markers segregating in

A-2551TN, 2,003 (13%) were classified as single-dose markers segregating in APF-259TN, 2,198 (15%) were classified as double-simplex (single-dose markers segregating in both parents), and 2,092 (14%) were classified as multiplex (Fig. 3.2).

Genetic linkage maps. Only 119 of the original 250 progeny remained in the mapping dataset after filtering for greater than 20% missing single-dose markers and for ratios of homozygote to heterozygote calls greater than 2:1 or less than 1:2 in either parental mapping dataset. Because JoinMap 4.1 can only handle datasets with fewer than 4,000 markers and 8,699 single-dose markers segregating in A-2551TN passed initial quality filtering, we excluded all markers with greater than 5% missing data for the maternal map. Of the 3,796 markers used for linkage mapping in the maternal haplotype map, 470 were removed because they were identical, 201 were ungrouped, and 3,125 were placed in 30 linkage groups (Table 3.1; Fig. 3.3). Originally 395 markers were placed in linkage group 6a and 219 markers in linkage group 6b, but the regression mapping algorithm in JoinMap 4.1 could not process the ordering of over 250 markers per groups so markers with over 95% similarities were removed. Therefore, the final maternal haplotype map was composed of 2,935 markers, with between 5 and 249 markers per linkage group. The total map length was 2,411.81 cM with linkage groups ranging from 18.61 cM to 146.65 cM in length and an average of 1 marker every 0.82 cM.

A total of 1,588 markers were used for construction of the paternal haplotype map because 450 of the 2,003 markers classified as single-dose markers segregating in APF-259TN deviated from expected segregation ratios according to the χ^2 test ($P < 0.10$). Of these 1,588 markers, 194 were removed because they were identical, 274 were ungrouped, and 1,125 were placed in 35 linkage groups (Table 3.1; Fig. 3.3). 326 markers (29% of total mapped markers) were all assigned to linkage group 2a, but markers with over 95% similarity were removed so that the

final linkage group 2a consisted of 208 ordered markers. The final paternal haplotype map consisted of 1,007 markers placed in 35 linkage groups with between 5 and 208 markers each. The total map length was 1,587.17 cM and the individual linkage groups were between 1.03 and 96.71 cM in length. The paternal haplotype map density was one marker per 1.58 cM.

The physical positions of the mapped markers in the 'Hillquist' reference group were used to identify homologous linkage groups for each of the seven base chromosomes of blackberry (Fig. 3.4). In general, the genetic and physical maps were strongly collinear, with no major translocations or inversions (Fig. 3.4). Four homologous linkage groups were found as expected for chromosomes 1, 2, 3, 4, and 6 in the maternal haplotype map, but five homologous linkage groups corresponding to chromosomes 5 and 7 were identified. While many of the linkage groups in the A-2551TN maternal haplotype map contained markers that aligned to physical positions across the length of the chromosome, 10 linkage groups had markers aligned to physical positions spanning less than 10 Mbp in the 'Hillquist' genome. Based on the physical positions of these markers on short linkage groups, it is likely that linkage groups 7b and 7d and linkage groups 5c and 5e actually belong to the same haplotype of A-2551TN. In the more fragmented paternal haplotype map, we found four homologous linkage groups corresponding to chromosomes 1, 3, and 7, but only three homologous linkage groups for chromosome 2, five linkage groups for chromosome 4, seven for chromosome 5, and eight for chromosome 6 (Table 3.1; Fig. 3.3; Fig. 3.4). Only 12 of the 35 linkage groups on the paternal haplotype map cover physical distances of over 10 Mbp.

Discussion

In this study, we developed the densest genetic linkage maps of tetraploid blackberry available to date. Our final parental linkage maps consisted of 3,942 markers total, with 2,935

markers in 30 linkage groups across 2,411.81 cM in the maternal haplotype map and 1,007 markers placed in 35 linkage groups across 1,587.17 cM in the paternal haplotype map (Table 3.1). These maps are a drastic improvement over the previous SSR-based map 'Prime Jim®' x 'Arapaho' (Castro et al., 2013), which consisted of 119 markers. Our maps are similar in density to the RAD-Seq based pseudo-testcross maps of 'Chester Thornless' x 'Prime Jim®', which consisted of 3,877 markers total, with 2,118 markers in 29 linkage groups in the maternal haplotype map and 1,759 markers in 31 linkage groups in the paternal haplotype map (Weber, 2014).

Interestingly, while the number of markers in the 'Chester Thornless' x 'Prime Jim®' parental haplotype maps were roughly even (Weber, 2014), we had nearly three times the number of mapped markers placed in the maternal haplotype map of A-2551TN than the paternal haplotype map of APF-259TN and over four times the number segregating single-dose allele markers identified in A-2551TN than APF-259TN. The most likely explanation for the differences in marker density in the A-2551TN and APF-259TN parental maps is that APF-259TN is more inbred and has lower heterozygosity across the genome. Common parents, especially APF-1, 'Arapaho', and 'Prime Jim®', appear multiple times in the maternal and paternal pedigree of APF-259TN (Fig. 3.1). Furthermore, two grandparents, A-2278 and A-2307, in the maternal and paternal lineages of APF-259TN are full siblings. In contrast, the female parent of A-2551TN, A-2364, is more distantly related to its male parent, APF-174T. Thus, it is not surprising that the overall level of heterozygosity and the number of single-dose allele markers segregating in A-2551TN would be higher than in APF-259TN.

The relatively high levels of inbreeding in this population, particularly in the male parent, likely contributed to the low number and uneven distribution of multiplex markers segregating in

this mapping population (Fig. 3.2). The paucity of multiplex markers segregating in this mapping population and their uneven distribution across the genome made it impossible to create integrated phased linkage maps using new polyploid mapping tools that employ multiplex markers with discrete dosage calls including PolymapR (Bourke et al., 2018b), TetraploidSNPMap (Hackett et al., 2017), and MapPoly (Mollinari and Garcia, 2019). However, it may still be possible to create an integrated map for this population using a hybrid approach incorporating probabilistic allele dosage calls and manual curation of marker order with genomic information and the unconstrained multi-dimensional scaling (MDS) algorithm (Preedy and Hackett, 2016) in the software package MapPoly. The development of an integrated and phased linkage map would enable us to assess preferential pairing of homologs and map QTL for important horticultural traits segregating in the progeny (Bourke et al., 2018a).

The linkage maps developed in this study also demonstrate the utility and quality of the new chromosome scale assembly of 'Hillquist' (Worthington, 2020). Until the development of this diploid blackberry genome, the closest reference genome available for blackberry researchers was black raspberry (VanBuren et al., 2018b). While these species are both in the *Rubus* genus, black raspberry belongs to subgenus *Idaeobatus*, and blackberries belong to subgenus *Rubus*. Species belonging to subgenus *Rubus* diverged from other subgenera including *Idaeobatus*, *Chamaebatus*, *Cylactis*, *Dalibardastrum*, and *Malachobatus* approximately 15-20 MYA (Carter et al., 2019). An average of 85.9% of sequencing reads generated in this study mapped to unique positions in the 'Hillquist' genome, while only 67.3% of reads mapped to unique positions in the black raspberry assembly (Appendix 3.1). Furthermore, 2,022,664 polymorphic markers were identified using the 'Hillquist' genome, compared to 1,811,617 markers using the black raspberry genome. Marker order was also highly collinear between the physical map of

‘Hillquist’ and the parental haplotype maps of tetraploid blackberry generated in this study (Fig. 3.3). A high degree of collinearity between the diploid ‘Hillquist’ reference and the tetraploid mapping population was expected considering that the sequenced ‘Hillquist’ accession is the sole source of the PF allele in all PF blackberry cultivars (Lopez-Medina et al., 2000) and is highly represented in the pedigrees of both parents of our mapping populations (Fig. 3.1). Still, the agreement between the physical map of ‘Hillquist’ and the tetraploid pseudo-testcross maps developed in this study validates the order and orientation of the HiC-based chromosome scale assembly of ‘Hillquist’ and its utility for genomic breeding research in polyploid fresh-market blackberries.

Conclusion

While polyploidy, multisomic inheritance, and high heterozygosity complicate genetic research in blackberry (Foster et al., 2019), new tools and strategies make molecular breeding in this specialty crop a more realistic prospect in the coming years. The development of new diploid blackberry reference genomes (Worthington et al., 2020) and GBS protocols/analysis pipelines optimized for autotetraploid species (Wadl et al., 2018) enabled the development of the dense tetraploid linkage maps presented in this study. The A-2551TN and APF-259TN parental haplotype maps developed here demonstrate the quality and utility of the ‘Hillquist’ reference genome. The development of an integrated, phased genetic map of tetraploid blackberry suitable for QTL mapping and estimation of preferential pairing and inheritance mechanisms is the next challenge that remains to be confronted.

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Tables and Figures

Table 3.1. Distribution of single-dose allele markers across the University of Arkansas System Division of Agriculture blackberries, A-2551TN and APF-259TN, parental haplotype maps.

	A-2551TN			APF-259TN		
Linkage group[z]	Number of markers[y]	Length (cM)	Physical positions of mapped markers (Mbp)	Number of markers	Length (cM)	Physical positions of mapped markers (Mbp)
1a	169	127.31	0.19-31.38	32	35.31	0.11-2.02
1b	58	76.42	0.96-46.67	16	34.38	9.75-16.47
1c	32	36.15	16.30-20.38	12	11.95	1.50-2.01
1d	24	18.61	3.54-4.82	7	1.03	0.19-0.19
2a	195	113.66	0.24-31.98	208	96.71	2.42-32.42
2b	100	65.26	1.89-36.99	18	53.15	24.57-36.72
2c	26	74.48	6.70-28.55	13	60.67	0.21-22.92
2d	14	61.52	0.23-26.53	-	-	-
3a	123	125.99	4.19-41.83	120	43.55	1.181-31.29
3b	120	146.65	2.06-41.46	14	66.86	2.09-12.20
3c	9	18.71	10.18-11.74	11	24.70	13.60-17.95
3d	5	28.60	16.22-19.49	5	54.49	15.99-20.47
4a	147	65.81	23.54-34.61	23	26.44	1.07-9.39
4b	119	72.76	13.49-32.96	22	45.17	10.72-31.72
4c	79	91.95	0.90-31.00	13	49.31	22.86-29.55
4d	48	44.16	22.37-27.44	8	67.515	25.48-30.55
4e	-	-	-	7	37.00	21.10-26.25
5a	170	97.21	1.64-34.58	53	43.56	0.07-5.62
5b	151	130.18	0.10-38.53	41	99.82	12.32-35.22
5c	89	27.73	0.98-3.57	38	43.47	0.22-5.15
5d	54	91.67	6.12-35.11	19	34.39	17.56-31.95
5e	32	43.87	29.96-37.62	7	65.40	9.96-17.42
5f	-	-	-	6	47.29	0.12-7.94
5g	-	-	-	5	27.18	18.02-27.26
6a	249	88.74	17.27-45.25	34	70.54	2.14-28.82
6b	175	144.62	0.32-45.43	27	22.24	38.23-41.59
6c	165	84.35	3.08-42.41	17	42.69	8.13-15.43
6d	102	88.86	9.16-45.25	14	11.46	44.21-45.37
6e	-	-	-	12	31.41	26.17-36.16
6f	-	-	-	11	58.00	29.22-43.83
6g	-	-	-	9	54.72	0.44-5.23
6h	-	-	-	5	28.70	32.06-37.64
7a	219	132.84	0.13-36.48	105	64.18	6.02-28.09
7b	134	70.18	0.09-25.41	43	61.77	31.80-33.67

Table 3.1 (Cont.)

	A-2551TN			APF-259TN		
Linkage group[z]	Number of markers[y]	Length (cM)	Physical positions of mapped markers (Mbp)	Number of markers	Length (cM)	Physical positions of mapped markers (Mbp)
7d	35	48.33	29.49-32.06	9	21.32	31.93-33.10
7e	25	106.83	0.38-36.94	-	-	-
Total	2935	2411.81	-	1007	1587.17	-

[z] Linkage LOD 9.0 was used to establish baseline linkage groups. Higher LOD thresholds were imposed in three cases where there was insufficient linkage in the data to create maps for linkage groups that clustered together at LOD 9.0 in the A-2551TN maternal haplotype map, including 5b and 5d (split at LOD 10), 6c and 6d (split at LOD 17), and 7b, 7c, and 7d (split at LOD 11).

[y] Originally 395 markers were placed in linkage group 6a and 219 markers in linkage group 6b in the maternal haplotype map and 280 markers were placed in linkage group 2a of the paternal haplotype map, but markers with over 95% similarities were removed because the regression mapping algorithm in JoinMap 4.1 could not handle ordering over 250 markers per group.

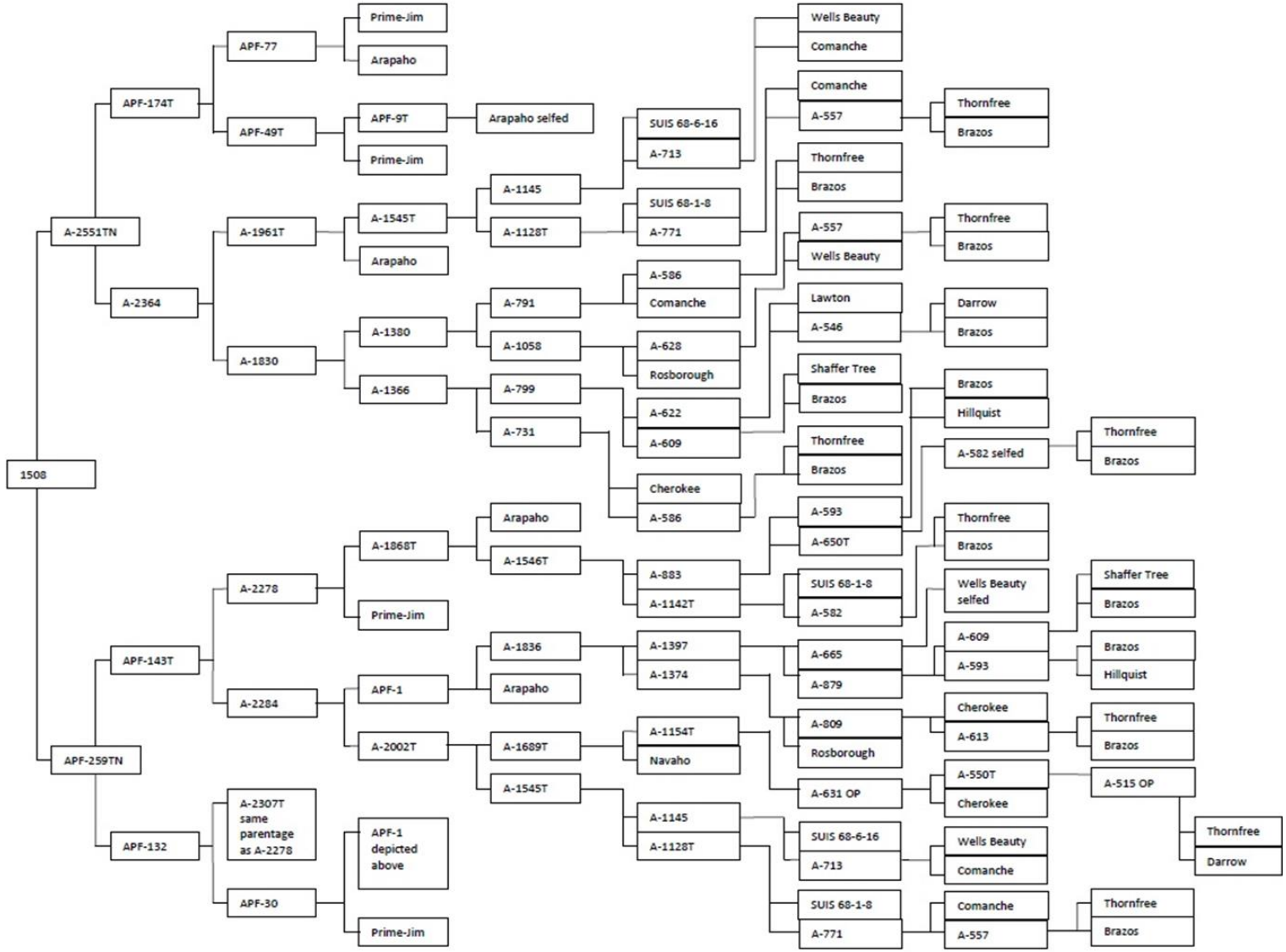

Fig. 3.1. Pedigree of the University of Arkansas System Division of Agriculture blackberries, A-2551TN x APF-259TN, mapping population.

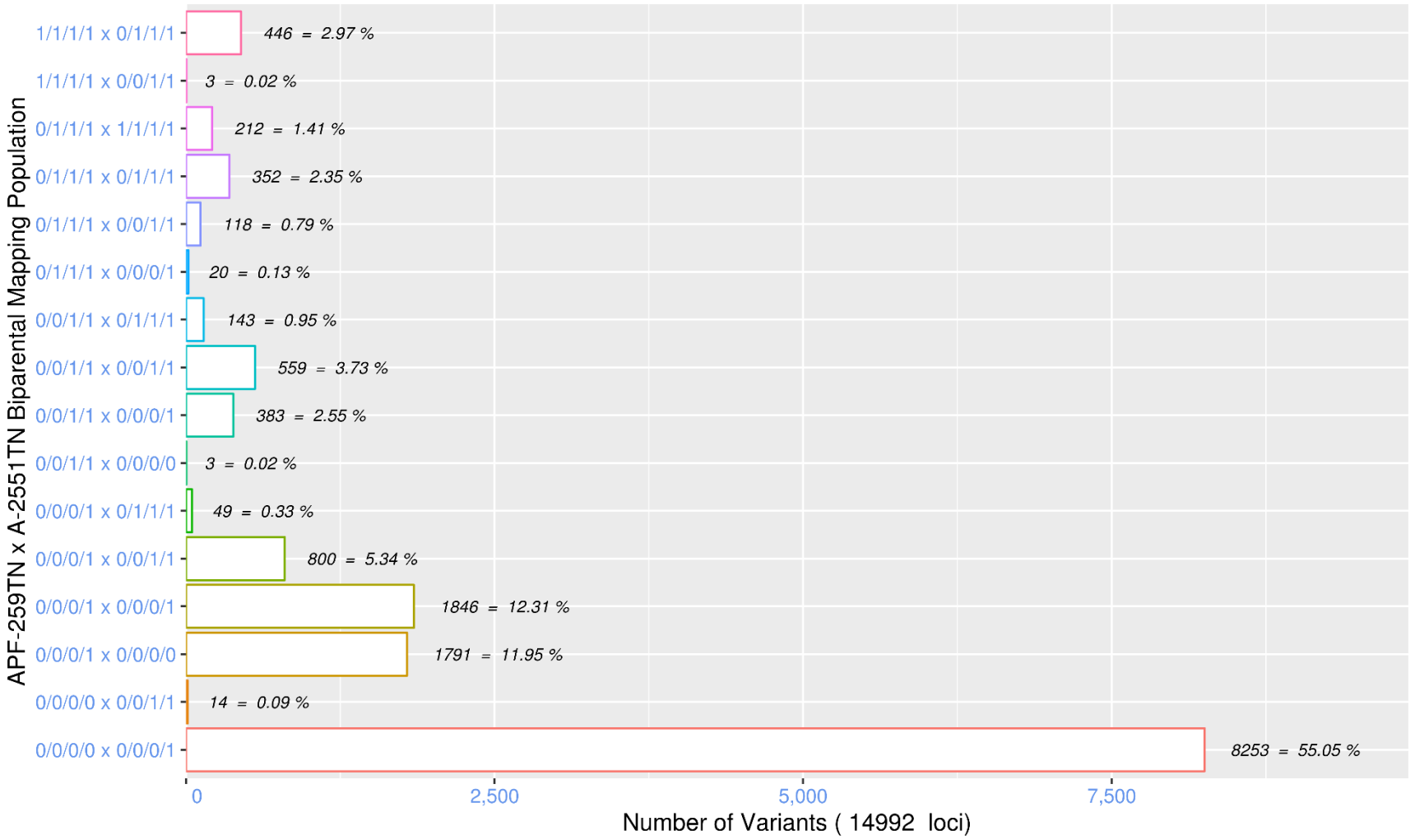

Fig. 3.2. Distribution of marker doses in University of Arkansas System Division of Agriculture blackberries, A-2551TN and APF-259TN genotypes.

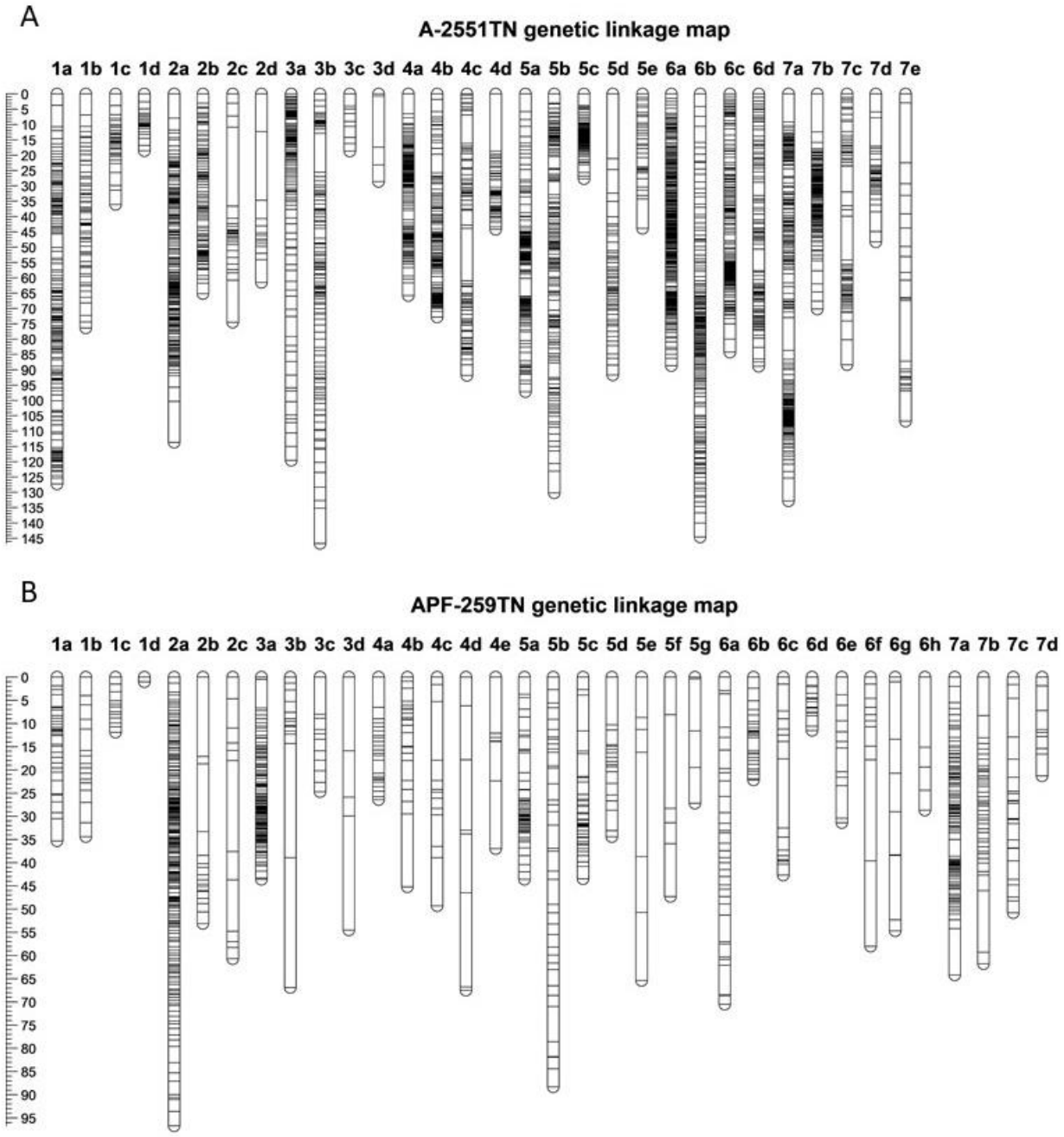

Fig. 3.3. The 30 linkages groups of the University of Arkansas System Division of Agriculture blackberries, A-2551TN maternal haplotype map (A) and the 35 linkage groups of the APF-259TN paternal haplotype map (B). Marker positions are expressed in cM.

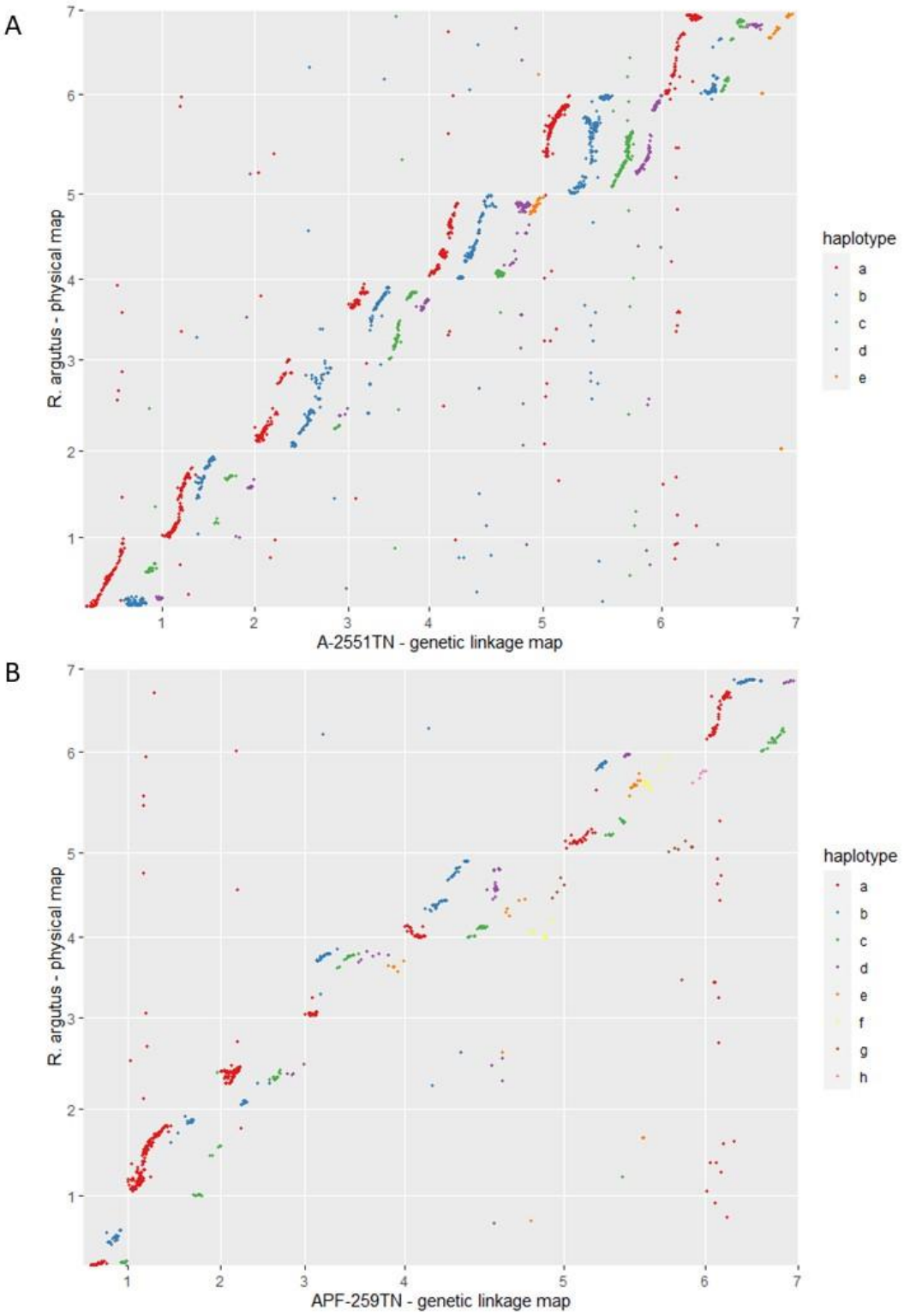

Fig. 3.4. Alignment of markers mapped to the University of Arkansas System Division of Agriculture blackberries, A-2551TN (A) and APF-259TN (B), genetic linkage maps with physical positions on the *R. argutus* diploid reference genome.

Overall Conclusion

The studies presented in this work contribute to the growing knowledge of the cultivated blackberry as a recently emerged world crop. Even as the blackberry market continues to grow in value and outreach each year, there continues to be longstanding issues concerning overall fruit quality such as red drupelet reversion (RDR). The incidence of RDR and the causal mechanisms of this disorder were investigated. The primary objective was whether earlier harvest times and/or selecting for firmer blackberry genotypes would decrease RDR prevalence after cold storage. Postharvest evaluations of blackberries from the University of Arkansas System Division of Agriculture blackberry breeding program revealed that blackberries harvested at earlier times, especially at 7:00 AM, resulted in less RDR from appearing. RDR particularly peaks at harvest times past 10:00 AM in this study and should be avoided to attain a more uniform harvest.

Firmer blackberry genotypes also showed a clear inverse correlation with the incidence of RDR. A-2453, a 'crispy' breeding selection, was the firmest genotype and consistently performed well with very low levels of RDR. This study, along with previous works comparing crispy textured selections with non-crispy cultivars, supports the view that crispy genotypes are a valuable source for resistance to RDR in blackberries. Based on these findings, earlier blackberry harvests and the use of firm-textured genotypes are recommended to minimize RDR. Additional studies that include more genotypes and investigate other factors, such as the environment and harvest technique, can further reinforce ways to prevent RDR in blackberries.

Improvements in molecular breeding for blackberries has also been a challenge. Since most fresh-market blackberries are tetraploids, the creation of dense genetic maps can be difficult. Two blackberry parental haplotype linkage maps were successfully made with markers created

by a new genotyping-by-sequencing protocol designed for autopolyploids. Processed reads were aligned to the diploid ‘Hillquist’ blackberry reference genome, with 85.9% of markers mapped to unique positions on the reference genome. This resulted in the densest linkage map for tetraploid blackberry to date containing 3,942 markers over a span of 65 linkage groups in total. High collinearity existed between the mapping population and the ‘Hillquist’ reference genome, which validates the usefulness of this genome for future mapping studies with blackberry and other related species. As polyploid mapping software and statistical tools continue to improve, the creation of a dense integrated, phased linkage map for tetraploid blackberry can be an achievable goal.

Appendix 3.1. Depth of genotyping-by-sequencing read coverage in the parents and F_1 progeny of University of Arkansas System Division of Agriculture blackberries, A-2551TN x APF-259TN, mapping population and percent of reads aligning to unique positions in the *Rubus argutus* and *Rubus occidentalis* reference genomes.

	R. argutus alignment			*R. occidentalis* alignment		
Genotype	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
A-2551TN	6478780	5453671	84.18%	6462605	4266663	66.02%
APF-259TN	6958380	6046229	86.89%	6942148	4767459	68.67%
1	1801505	1452765	80.64%	1798757	1041936	57.93%
3	2297995	1975470	85.96%	2293834	1578170	68.80%
4	1090755	834790	76.53%	1087914	631750	58.07%
5	3492524	2966980	84.95%	3484144	2320117	66.59%
6	1696329	1495461	88.16%	1692284	1193139	70.50%
7	1750578	1547493	88.40%	1746070	1230211	70.46%
8	1696006	1505879	88.79%	1691497	1213567	71.75%
9	1735215	1522589	87.75%	1730936	1226017	70.83%
10	1889128	1659359	87.84%	1884962	1345989	71.41%
11	2327118	1996924	85.81%	2321529	1542936	66.46%
12	2289843	1997518	87.23%	2283972	1563071	68.44%
13	2414845	2097325	86.85%	2408921	1637528	67.98%
14	2614043	2294346	87.77%	2606816	1785283	68.49%
15	3559818	3088087	86.75%	3551372	2398953	67.55%
16	1144771	990840	86.55%	1141364	749986	65.71%
17	1218395	1042251	85.54%	1214686	773673	63.69%
18	2049309	1767664	86.26%	2043976	1404026	68.69%
19	1686748	876619	51.97%	1683755	653956	38.84%

Appendix 3.1 (Cont.)

	R. argutus alignment			*R. occidentalis* alignment		
Genotype	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
20	2120728	1832286	86.40%	2114868	1466564	69.35%
21	2108185	1842547	87.40%	2103080	1459000	69.37%
22	2631291	2339327	88.90%	2624334	1814210	69.13%
24	2037021	1799033	88.32%	2031306	1411999	69.51%
25	2412700	2122540	87.97%	2406459	1631291	67.79%
26	647215	566087	87.47%	646404	461091	71.33%
27	2794200	2384800	85.35%	2788093	1882550	67.52%
28	2156451	1826438	84.70%	2152132	1420707	66.01%
30	2444076	1963354	80.33%	2440567	1448170	59.34%
31	2702104	2378009	88.01%	2695222	1829493	67.88%
33	2330408	2018642	86.62%	2323772	1569763	67.55%
34	2542476	2168845	85.30%	2536377	1694940	66.83%
36	1720637	1502815	87.34%	1715788	1158965	67.55%
37	1641905	1315450	80.12%	1638400	1030592	62.90%
39	1750646	1551963	88.65%	1746881	1255616	71.88%
41	2317526	2068045	89.24%	2312388	1671147	72.27%
44	2377999	2004242	84.28%	2373468	1511583	63.69%
45	1223738	1073427	87.72%	1220133	812462	66.59%
46	2600909	2255165	86.71%	2594871	1747827	67.36%
47	1705813	1507031	88.35%	1700787	1170584	68.83%
48	1775820	1566477	88.21%	1771711	1226241	69.21%
49	1667671	1464637	87.83%	1663089	1125781	67.69%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
50	1790076	1580533	88.29%	1784905	1269888	71.15%
51	1885412	1668932	88.52%	1881731	1342527	71.35%
53	2434536	2161162	88.77%	2427594	1688095	69.54%
54	1726687	1532591	88.76%	1721490	1236556	71.83%
56	2805953	2437997	86.89%	2798131	1904428	68.06%
57	1812253	1606206	88.63%	1807893	1277770	70.68%
58	2084694	1820898	87.35%	2079639	1431221	68.82%
59	2779291	2394797	86.17%	2772640	1862194	67.16%
61	2505385	2183181	87.14%	2499250	1693382	67.76%
62	2952712	2499663	84.66%	2946157	1958520	66.48%
63	2609383	2252963	86.34%	2603192	1745954	67.07%
64	2324293	1945010	83.68%	2319995	1563252	67.38%
65	1640348	1471668	89.72%	1636412	1178383	72.01%
66	2482437	2179059	87.78%	2475433	1705353	68.89%
67	1792949	1595307	88.98%	1788232	1282808	71.74%
68	1505726	1353020	89.86%	1501234	1082158	72.08%
69	1625999	1450657	89.22%	1621691	1182365	72.91%
70	2132534	1857081	87.08%	2126091	1446390	68.03%
71	2228757	1944619	87.25%	2221867	1503501	67.67%
72	1237638	1097561	88.68%	1234350	849923	68.86%
73	1108366	968909	87.42%	1105308	750772	67.92%
74	3259436	2828435	86.78%	3251064	2217629	68.21%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
76	3777	3217	85.17%	3775	2623	69.48%
78	2729300	2344048	85.88%	2723430	1847017	67.82%
79	2648184	2352855	88.85%	2642528	1856392	70.25%
81	2326466	1952178	83.91%	2320700	1530080	65.93%
84	521136	472548	90.68%	519409	362248	69.74%
85	1455773	1243985	85.45%	1451759	966720	66.59%
86	2541275	2230681	87.78%	2534442	1751936	69.13%
87	2485939	2159715	86.88%	2480168	1710073	68.95%
88	1695326	1465777	86.46%	1690998	1124094	66.48%
89	2981709	2529559	84.84%	2974434	1966819	66.12%
90	2859839	2404186	84.07%	2852104	1869811	65.56%
91	2442150	2083560	85.32%	2436546	1628888	66.85%
92	2093635	1851511	88.44%	2088294	1408208	67.43%
93	2210729	1935518	87.55%	2206527	1514039	68.62%
94	1729439	1473909	85.22%	1724731	1150778	66.72%
95	2305808	2014072	87.35%	2299326	1559472	67.82%
97	2341174	2074312	88.60%	2334666	1622638	69.50%
100	2782018	2307305	82.94%	2775011	1814041	65.37%
101	1405680	1188222	84.53%	1403804	859847	61.25%
102	2328809	2049607	88.01%	2323347	1604942	69.08%
103	2397219	2053428	85.66%	2392122	1592429	66.57%
104	1924321	1707276	88.72%	1919886	1342914	69.95%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
105	2120265	1687579	79.59%	2116474	1213840	57.35%
107	2107083	1786364	84.78%	2102194	1364764	64.92%
108	2259657	1985233	87.86%	2254883	1545532	68.54%
110	2469829	2165066	87.66%	2463730	1698556	68.94%
111	1797594	1119134	62.26%	1793624	848553	47.31%
112	1410808	1264031	89.60%	1407763	1009108	71.68%
114	1507357	982186	65.16%	1503771	745922	49.60%
115	1542370	1383390	89.69%	1537584	1109595	72.16%
117	2774287	2351593	84.76%	2767154	1831057	66.17%
118	1785384	1583449	88.69%	1780537	1269880	71.32%
119	1979021	1748447	88.35%	1973730	1389455	70.40%
120	1984459	1768552	89.12%	1978749	1392542	70.37%
121	2293327	1987207	86.65%	2286547	1536058	67.18%
122	2754623	2434154	88.37%	2746180	1880774	68.49%
123	1617952	1410927	87.20%	1613857	1108736	68.70%
124	1912516	1688800	88.30%	1907824	1335929	70.02%
125	1501399	1001993	66.74%	1498307	762697	50.90%
126	370809	331299	89.34%	370442	275243	74.30%
127	1326158	1183684	89.26%	1322038	961587	72.74%
129	1477469	1324199	89.63%	1472339	1063766	72.25%
130	1657065	1472495	88.86%	1652160	1183251	71.62%
131	2602240	2277729	87.53%	2595457	1790228	68.98%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
132	2423777	2098429	86.58%	2418300	1614744	66.77%
133	1160537	1017638	87.69%	1157089	750749	64.88%
134	1816994	1582119	87.07%	1813219	1256266	69.28%
135	134852	116823	86.63%	134686	97252	72.21%
136	2391688	2098702	87.75%	2386215	1640525	68.75%
137	969772	876502	90.38%	967309	700785	72.45%
138	2157778	1413952	65.53%	2153695	964172	44.77%
139	2670672	2289734	85.74%	2664339	1777333	66.71%
140	77240	69587	90.09%	77095	58833	76.31%
141	2276954	2006524	88.12%	2270870	1578149	69.50%
142	2072500	1731750	83.56%	2066608	1382840	66.91%
144	1069600	954164	89.21%	1067054	781142	73.21%
145	2611188	2190977	83.91%	2603991	1716602	65.92%
146	1473957	1322610	89.73%	1470208	1068371	72.67%
147	1591571	1394847	87.64%	1587696	1070837	67.45%
148	527729	468702	88.81%	526876	383353	72.76%
149	2268293	1950929	86.01%	2263057	1501575	66.35%
150	1643751	1470441	89.46%	1639637	1147617	69.99%
152	191171	171671	89.80%	190783	134930	70.72%
153	1742295	1529880	87.81%	1737190	1207717	69.52%
155	2036955	1763356	86.57%	2031115	1367365	67.32%
156	2278660	1974657	86.66%	2273151	1513489	66.58%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
157	1769810	1553226	87.76%	1763872	1231446	69.81%
159	431815	397560	92.07%	431013	333538	77.38%
160	1589257	1356038	85.33%	1584753	1040892	65.68%
161	1773524	1568960	88.47%	1768765	1265025	71.52%
162	1630231	1432705	87.88%	1626567	1135225	69.79%
163	1660328	1417970	85.40%	1656404	1123802	67.85%
165	2067314	1805779	87.35%	2061211	1410843	68.45%
166	1844392	1613225	87.47%	1839633	1291526	70.21%
167	2294678	2008260	87.52%	2289581	1504548	65.71%
168	2020872	1751810	86.69%	2015844	1391877	69.05%
169	1966070	1740528	88.53%	1960163	1404585	71.66%
170	2080805	1779127	85.50%	2074561	1426011	68.74%
172	1421527	1276637	89.81%	1418194	1018258	71.80%
173	2058785	1781803	86.55%	2052309	1428269	69.59%
174	1794700	1580242	88.05%	1790445	1232390	68.83%
175	1902363	1685715	88.61%	1896107	1343948	70.88%
176	2413516	2072098	85.85%	2407566	1612694	66.98%
177	2486671	2167341	87.16%	2479841	1657207	66.83%
178	1806049	1560343	86.40%	1801725	1246499	69.18%
179	1992530	1701348	85.39%	1987546	1309559	65.89%
180	2259243	1934932	85.65%	2253354	1518806	67.40%
181	1718145	1480846	86.19%	1713576	1166799	68.09%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
182	2066530	1804036	87.30%	2060864	1399106	67.89%
184	44162	39976	90.52%	43901	31330	71.37%
185	2007151	1742230	86.80%	2001699	1396936	69.79%
186	2648561	2292106	86.54%	2640993	1790038	67.78%
188	1810292	1585036	87.56%	1805230	1285849	71.23%
190	1512241	1338686	88.52%	1507870	1085008	71.96%
191	2166062	1822222	84.13%	2160240	1397598	64.70%
192	1904478	1650059	86.64%	1899691	1290992	67.96%
193	2324339	2042473	87.87%	2318064	1567295	67.61%
194	214408	188642	87.98%	213952	147451	68.92%
195	2103080	1845961	87.77%	2098356	1430241	68.16%
197	470861	426624	90.61%	469927	342089	72.80%
199	1178130	1028160	87.27%	1174791	756260	64.37%
200	1251075	1016132	81.22%	1247420	750156	60.14%
201	1692625	1480166	87.45%	1687788	1209633	71.67%
203	1990949	1770996	88.95%	1985354	1360290	68.52%
206	1910498	1663059	87.05%	1905243	1263081	66.30%
207	1027695	937234	91.20%	1024630	729225	71.17%
211	1572910	1338043	85.07%	1569084	1004178	64.00%
217	1751779	1500339	85.65%	1747173	1169131	66.92%
218	1929439	1231478	63.83%	1926025	835924	43.40%
219	2187576	1915255	87.55%	2181880	1454537	66.66%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
220	1276	1094	85.74%	1274	942	73.94%
222	2172682	1855129	85.38%	2166797	1481191	68.36%
225	1485547	1322679	89.04%	1482253	1074556	72.49%
226	2108076	1870742	88.74%	2102697	1465962	69.72%
227	1967829	1753253	89.10%	1963035	1421566	72.42%
228	1782602	1526496	85.63%	1778183	1235630	69.49%
229	1465969	820294	55.96%	1463772	619746	42.34%
230	2302532	2034016	88.34%	2296971	1621512	70.59%
231	1429652	988775	69.16%	1426797	749374	52.52%
233	2410994	2123295	88.07%	2405541	1699845	70.66%
234	630158	568330	90.19%	629451	470216	74.70%
235	2706940	2346638	86.69%	2700595	1854372	68.67%
236	1401982	983069	70.12%	1398650	745797	53.32%
237	2862717	2430670	84.91%	2856371	1902851	66.62%
238	1760800	1067567	60.63%	1756892	788414	44.88%
239	2803780	2442686	87.12%	2797030	1916392	68.52%
241	1727127	1526081	88.36%	1722877	1219034	70.76%
242	1474533	1284075	87.08%	1471001	980059	66.63%
243	1951124	1701168	87.19%	1945974	1353674	69.56%
244	2456048	2159339	87.92%	2449704	1699289	69.37%
245	1116072	946634	84.82%	1113126	714090	64.15%
246	1211276	1077399	88.95%	1208085	828685	68.59%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
247	2259107	1978751	87.59%	2253505	1548982	68.74%
248	2584533	2160499	83.59%	2577824	1670701	64.81%
250	1944366	1699515	87.41%	1939526	1334809	68.82%
251	2441392	2112896	86.54%	2436670	1667613	68.44%
253	951058	782075	82.23%	948948	598937	63.12%
254	1413531	1233689	87.28%	1410247	970394	68.81%
256	2278671	2023678	88.81%	2273321	1603334	70.53%
257	1413509	1209654	85.58%	1409221	908703	64.48%
258	2794198	2411275	86.30%	2786975	1895478	68.01%
259	2451748	2098891	85.61%	2444701	1653028	67.62%
260	1634187	963075	58.93%	1631096	727535	44.60%
261	1999764	1780292	89.03%	1995168	1374169	68.87%
262	2275078	2017287	88.67%	2269028	1572425	69.30%
263	2567	2283	88.94%	2572	1891	73.52%
265	2862575	2491651	87.04%	2855151	1937805	67.87%
266	1935028	1654653	85.51%	1930694	1258620	65.19%
269	1517885	1262835	83.20%	1513465	950597	62.81%
270	1740239	1538480	88.41%	1736589	1216220	70.03%
272	2062921	1709653	82.88%	2059670	1208991	58.70%
273	1369612	1160173	84.71%	1365520	846293	61.98%
274	1989208	1783274	89.65%	1984783	1375907	69.32%
276	1985408	1697609	85.50%	1981621	1220639	61.60%

Appendix 3.1 (Cont.)

Genotype	*R. argutus* alignment			*R. occidentalis* alignment		
	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
277	2158038	1897025	87.91%	2151240	1474112	68.52%
279	2665453	2351193	88.21%	2658661	1835203	69.03%
280	2021524	1498692	74.14%	2018169	1030199	51.05%
282	1150930	1002694	87.12%	1147535	748400	65.22%
283	1803506	1506327	83.52%	1799585	1178047	65.46%
285	1486674	1288556	86.67%	1483603	949590	64.01%
286	2055372	1817169	88.41%	2051004	1435892	70.01%
287	2254324	1945757	86.31%	2249339	1517146	67.45%
288	2388509	2095500	87.73%	2382780	1675027	70.30%
289	2823145	2459254	87.11%	2815234	1900019	67.49%
290	717255	650237	90.66%	715127	526099	73.57%
292	2420750	2093839	86.50%	2415218	1630365	67.50%
294	1911302	1596285	83.52%	1908507	1129653	59.19%
295	1790761	1529819	85.43%	1786457	1175563	65.80%
296	2574092	2265569	88.01%	2568478	1763905	68.68%
297	2192462	1932313	88.13%	2187318	1496142	68.40%
298	2291989	1990410	86.84%	2285542	1537679	67.28%
299	2608873	2289741	87.77%	2603907	1792737	68.85%
300	1899259	1478376	77.84%	1895589	1039093	54.82%
301	2410351	2039553	84.62%	2404154	1584910	65.92%
302	2654366	2242882	84.50%	2648527	1800242	67.97%
303	2308017	2016867	87.39%	2301590	1560181	67.79%

Appendix 3.1 (Cont.)

	R. argutus alignment			*R. occidentalis* alignment		
Genotype	Total Read Count	Mapped Reads	Percentage of Mapped Reads	Total Read Count	Mapped Reads	Percentage of Mapped Reads
304	2477190	2202200	88.90%	2471159	1726567	69.87%
305	2225092	1946660	87.49%	2220524	1544351	69.55%
306	2491978	2193329	88.02%	2484935	1728926	69.58%
307	2083868	1797595	86.26%	2077980	1443146	69.45%
309	1032756	905516	87.68%	1029946	691041	67.09%
312	660074	594457	90.06%	659088	481979	73.13%
313	2224422	1949000	87.62%	2218514	1510338	68.08%
314	2408216	2110219	87.63%	2401792	1630663	67.89%
315	1561010	1383503	88.63%	1558026	1107522	71.08%
318	3213837	2791600	86.86%	3205636	2169799	67.69%
319	1490569	1294438	86.84%	1486768	986804	66.37%
321	3177321	2816204	88.63%	3169751	2207669	69.65%