Investigating Pollen Compatibility of Commercial Sweet Cherry Cultivars by DNA Analysis

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Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma and, to the best of my knowledge, contains no copy or paraphrase or material published or written by any other person, except where due reference is made in the text of this thesis.

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Abstract

Sweet cherries (*Prunus avium* L.) are largely self-incompatible, which is determined by a gametophytic self-incompatibility system that is controlled by a multi-allelic *S*-locus. Commercial cherry orchards select cross-compatible cultivars based on synchronous flowering and cross-compatible *S*-alleles to maximise pollination success. My project evaluated molecular approaches to: 1) determine the unknown *S*-allele profile in three sweet cherry cultivars (Simone, Sweet Georgia and Reid Fruits in-house cultivar; SLK) by DNA sequencing and 2) identify the pollinisers of Kordia and Regina that are notorious for below average fruit set (<20%) using *S*-locus genes and microsatellite markers (SSRs). These molecular techniques have been tested in other plant models, however, there are limited examples in commercial sweet cherry orchards.

DNA sequencing unequivocally identified that the previously unknown *S*-alleles of Simone and Sweet Georgia are S_1 and S_4'. Interestingly, this is consistent with industry reports that both Simone and Sweet Georgia are self-compatible. SLK, had S_1 and S_3 alleles, supporting suggestions that it could be a mutation of the Regina cultivar. This new information means that producers can utilise these cultivars with confidence in their genetic compatibility.

Primers targeting specific alleles of the *SFB* gene (S_1, S_3, S_4, S_4', S_6, S_9, S_{12}, S_{13} and S_{36}), revealed the pollen donor candidates for Kordia and Regina seeds in the open-pollinated orchard, but failed to discriminate among candidates that shared *S*-alleles. Five out of thirteen microsatellites were identified to amplify small size differences between candidate pollen donors but require accurate sizing using a DNA sequencer to be practical as agarose gel electrophoresis did not provide reliable discrimination. This was beyond the resources available for this project.

The primers we designed can be developed into a multiplex PCR to make this technique even faster and cheaper. Orchardists can use these methods, which are also applicable to other *Prunus* crops, to optimise their orchard design and assist in the selection and introduction of new cultivars.

1. Literature Review

1.1 Introduction

1.1.1 Sweet cherry production

Globally, sweet cherries are an important crop that are enjoyed as a fresh product. In 2019, 68 countries produced 2.6 M tonnes of sweet cherry fruit in temperate, Mediterranean and sub-tropical regions (Webster and Looney, 1996, FAO, 2021). Often, the most successful cherry growing regions are close to a large body of water which acts to buffer against temperature extremes (Jackson et al., 2011).

The most recent FAO statistic for annual producer price places Australian sweet cherry prices fourth out of 46 countries (Table 1). Australian sweet cherries are regarded highly for premium quality fruits with an annual farm gate value of $7,409 USD/t in 2019; which, has grown from $2840 USD/t in 1991 (FAO, 2019). The demand for Australian sweet cherries is stimulating growth in the industry.

Table 1**:** Top 10 global sweet cherry producer prices in 2019 (FAO, 2019).

Country	Annual producer price (USD/t)
Japan	$15494
Israel	$9346.3
Norway	$8505.7
Australia	$7409.1
China, mainland	$6770.6
South Africa	$5833.9
Austria	$4883
France	$4552
Cyprus	$4527.9

The annual production in Australia has nearly doubled from ~7460 t/year between 1960-1970 to ~14460 t/year between 2010-2020, reaching a record high of 20,000 t for the year 2019 (FAO, 2019), from which more than 30% was exported to other countries particularly Hong Kong and China (Hort Innovation, 2021). Most of the sweet cherry varieties grown in Australia start flowering during the months of September to October, depending on the cultivar (James, 2011).

Intensive management of this high-value crop is required to mitigate the effects of uncharacteristic seasonal variation, pest, disease, and extreme rainfall events resulting in significant crop damage. By implementing crop covers, retractable roof technology and timely chemical applications, orchardists can produce more consistent yields to meet market demands (Kappel et al., 2012, James, 2011, Lang et al., 2016).

1.1.2 Botany

Sweet cherry (*Prunus avium* L.) plants are diploid ($2n = 2x = 16$) and typically form large pyramidal trees that can reach a height of 20m. The width of their leaves is generally half their length (7.5 – 12.5 cm) and petioles are long with reddish glands. They produce white flowers that form singly or in clusters of five on the previous year's growth and are commonly 2.5 cm in diameter. Cross-pollination is required in this species because individuals are generally unable to self-pollinate. Successful pollination produces drupe type fruits that are roundish, displaying a red or black skin colour, and are about 2 cm in diameter (Webster and Looney, 1996).

1.1.3 History

Domesticated sweet cherry varieties are thought to have originated in Europe or Asia Minor and dispersed throughout Europe by the Greek and Roman civilisations (Hedrick et al., 1915, Faust and Surányi, 1996). They are believed to be one of the oldest fruit crops, with evidence of cherry stones found at archaeological sites dating back to 5000 – 4000 BCE (Bargioni, 1996). The access or consumption of sweet cherries throughout history has served as an indication of luxury or affluence to a number of cultures (Webster and Looney, 1996). Due to demand, global cultivation of sweet cherries increased in the 1900s after European germplasm was introduced to North America (Dondini et al., 2018). Now, Sweet cherry breeding programs have successfully introduced many modern varieties closely related to the predominant landraces of the 1800's (Guajardo et al., 2021). Of particular interest to sweet cherry producers is pest and disease resistance, rain induced cracking resistance, hybrid rootstocks that promote precocity or dwarfing, and marketable fruit characteristics (Dondini et al., 2018).

1.1.4 Fruit characteristics

Domesticated varieties are selected for qualities that make them ideal for fresh consumption. Large fruit size, rich colour, firm texture, and flavour are sweet cherry traits that are important to consumers and are sought after in new cultivars (Dever et al., 1996, Dondini et al., 2018). Additionally, there is a growing body of research that highlights the nutraceutical properties of sweet cherries. Among the nutritional components of sweet cherry fruit (Table 2) are the occurrence of anthocyanins (responsible for the fruits red skin colour), flavonols and hydroxycinnamic acids, which exhibit antioxidant properties (Ferretti et al., 2010, Blando and Oomah, 2019). Moreover, diets containing these chemicals are purported to promote preventative health benefits against cancer, cardiovascular disease, diabetes, inflammatory diseases and Alzheimer's disease (McCune et al., 2010).

Table 2: Mean nutrient and bioactive compounds content of sweet cherries per 100g fresh weight. From Blando and Oomah (2019).

Nutrients and bioactive compounds	Per 100g of sweet cherry (fresh weight)
Energy (kcal)	63
Fiber (g)	2.1
Total sugars (g)	12.8
Sucrose (g)	0.15
Glucose (g)	6.6
Fructose (g)	5.3
Vitamin A (IU)	64
Vitamin C (mg)	7
Vitamin E (µg)	70
Potassium (mg)	222
ß-carotene (µg)	38
Anthocyanins (mg)	171.4
Flavonols (mg)	2.64
Flavanols (mg)	1507
Hydroxycinnamic acids (mg)	87.8
Phenols (mg GAE)	174.9

Sweet cherry production is important to consumers for their nutrition and cultural characteristics. It is likely that the demand for this fruit will continue to grow, and producers will benefit from improvements in crop yield. However, despite intensive management of this high value crop, fruit set for some varieties can be highly variable. One of the main reasons of poor fruit set is inadequate pollination resulting in low fruit yield and quality. This review will

firstly provide an overview of the pollination biology of sweet cherry followed by the main factors influencing pollination performance of sweet cherry. Secondly explore the molecular research in recent decades that explains the mechanisms responsible for the successful pollination in sweet cherries. The concept of sweet cherry parental analysis using molecular methods will also be explored. The last section will discuss how pollen donor research will impact optimal orchard management.

1.2 Pollination biology of sweet cherry

Sweet cherries are the first fruits of the summer season, and the reproductive process is stimulated by seasonal change from Winter to Spring: as cold conditions subside, plants enter a flowering phase (Webster and Looney, 1996, Campoy et al., 2019). Early in the flowering period, pollen grains dehisce during anthesis. The majority of pollen is dispersed within a 30 m radius among neighbouring flowers typically by bee pollinators; and, in some cases reaching distances up to 500 m in very low proportions (García et al., 2007, Shivanna, 2015). However, under commercial conditions, yields can be reduced by 52 – 59% for individuals greater than 9 m from a polliniser tree (Núñez-Elisea et al., 2008).

One to five days post-anthesis, the stigma is most receptive to pollen; on the stigma surface, papillae begin to produce an exudate that hydrates pollen grains (Zhang et al., 2018). Pollen grains germinate, extending a pollen tube to the base of the style after two to three days and reach a viable ovule a day later (Radunić et al., 2017). Fertilisation is a result of the pollen tube penetrating the ovule, producing a zygote and initiating fruit development (Figure 1; Cresti et. al., 1992).

Lack of pollination is a significant contributor to poor fruit yields in sweet cherry orchards. Weather conditions, pollinator activity, genetic compatibility and flowering synchrony are all limiting factors regarding the fruit set in sweet cherry (Hedhly et al., 2004, Hedhly et al., 2005, Radičević et al., 2015, Shivanna, 2015).

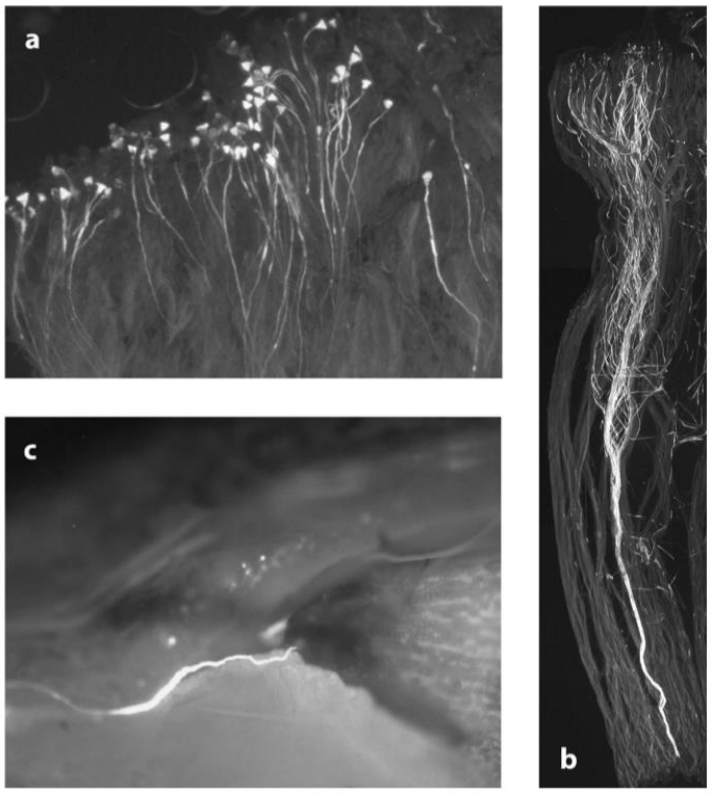


Figure 1: Pollen germination on the stigma (a), pollen tubes growth through the style (b) and through the ovary to the ovula (c). From Radunić et al. (2017).

1.2.1 Seasonal dormancy

Sweet cherry plants respond to seasonal changes with varying stages of dormancy, paradormancy, endodormancy and ecodormancy (Figure 2); this annual cycle promotes reproductive success and to allow them to survive through winter (Luedeling et al., 2013, Campoy et al., 2019, Rothkegel et al., 2020, Vimont et al., 2021). Paradormancy is a short period of dormancy that stops the growth of lateral buds after spring and is sustained by extended daylight hours in summer (Smita et al., 2021). Endodormancy describes the internal biological processes that lead to flower development during the winter months. Lastly, ecodormancy describes the period that the plant remains dormant as a response to unfavourable Spring temperatures, regardless of sufficient flower development (Rothkegel et al., 2020). The exact biological pathways relevant to sweet cherry dormancy isn't completely understood and is a current field of research (Fadon et al., 2018).

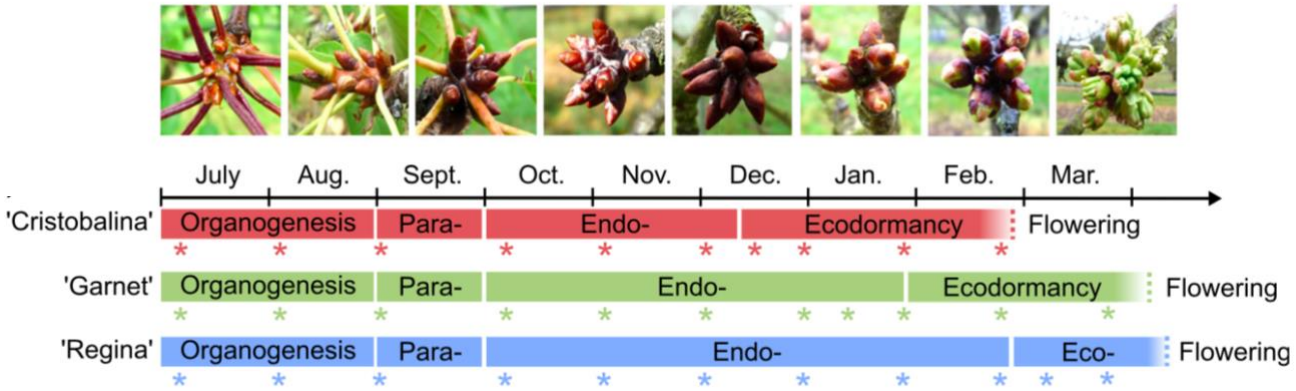


Figure 2: Dormancy stages of sweet cherry through the months from Vimont et al. (2019).

Regarding endodormancy, a recent study indicates that cold sensing mechanisms are triggered by DNA methylation which stimulates dormancy machinery during winter temperatures (Rothkegel et al., 2020). During the endodormancy phase, sweet cherry plants have evolved an internal means to recognise the accumulation of time at cold periods (2.4 – 9.1 ˚C), known as chill portions, which correlate with subsequent bud development (Luedeling et al., 2013, Campoy et al., 2019). If chill requirements are not met, flowering and fruit set are considerably reduced during spring warming; in fact, warmer winters lead to abnormal blooming times (Luedeling et al., 2013, Azizi-Gannouni and Ammari, 2020). Interestingly, Fadon et al. (2018) quantified an accumulation of starch in the flower primordia over winter that correlated with chilling requirements. This finding opens a window for future research to determine if Spring flower development is fuelled by these compounds and if starch accumulation is the main requisite for successful flowering.

During endodormancy, the chill requirement mechanism prevents premature budbreak in warm winter periods which can expose the sensitive tissues to ensuing cold extremes (Campoy et al., 2019, Rothkegel et al., 2020, Azizi-Gannouni and Ammari, 2020). In contrast, ecodormancy prevents the onset of the blooming period to avoid flower exposure to late winter conditions extending into Spring months (Fadon et al., 2018). This period is known as Spring forcing, which is comparable in the importance of chill requirements to sweet cherry producers because it determines when growers can supply produce to the market (Luedeling et al., 2013). For this reason, popular sweet cherry cultivars differ by growing region because each cultivar is suited to a particular climate (James, 2011). Due to changing climate conditions, irregular dormancy periods can be experienced by orchardists and has led to the development of flower inducing

compounds e.g., hydrogen cyanimide (sold as various products including Dormex®; Ionescu et al., 2016). The application of dormancy breakers can help orchardist to synchronise full bloom in otherwise asynchronous cultivar crosses and has been shown to advance flowering up to 13 days in cv. Burlat (Godini et al., 2008).

1.2.2 Effect of temperature on pollination

Extreme temperatures have detrimental impacts on sweet cherry productivity. An example of this is the slowing of pollen tube growth at high (>25˚C) and low temperatures (<10˚C) during the progamic phase (Hedhly et al., 2004, Hedhly et al., 2005, Radunić et al., 2017). Extreme cold spring temperatures can also damage flowers (Jackson, 2011). Furthermore, irregular winter and spring temperatures can interfere with flower development (Luedeling et al., 2013, Campoy et al., 2019).

1.2.3 Managed honeybee pollinators

Sweet cherry plants naturally rely on biotic pollinators (particularly insects) to ensure fruit set; by producing nectar, the plants provide a food incentive for insects to visit each flower, resulting in pollen distribution among a group of individuals (Shivanna, 2015). Honeybees (*Apis mellifera*) are intensively used in sweet cherry production because they display fidelity for target species and can be managed using hive boxes which can be transported to farms at the time of flowering. It is common practice to use 2 – 3 hives per hectare to ensure adequate fruit set (Somerville, 1999).

Poor pollination performance from bees can result from competition, weather conditions and colony strength. For example in Australia, Capeweed, Patterson's Curse and White Clover often flower at the same time as sweet cherry and, if abundant, can distract bees from pollinating the crop (Somerville, 1999). Also, temperatures below 13˚C, strong winds and rainfall events have been found to sharply decrease, if not, cease bee activity (Jackson, 2011). Furthermore, in Australia, colony strength can be impacted by several diseases and parasites including American foulbrood, black queen cell virus, Kashmir bee virus, chalkbrood disease, sacbrood virus and small hive beetle which can lead to unproductive hives and colony collapse (Oldroyd et al., 1989, Neumann and Elzen, 2004, Fung et al., 2018, Khan et al., 2020).

1.2.4 Flowering patterns

Variable blooming times can lead to decreased yield due to insufficient or incomplete fertilisation (Ganji Moghadam et al., 2009). This is because blooming times of compatible cultivars must overlap for sufficient pollen transfer to occur in order to produce a profitable yield (Békefi, 2004). For this reason, it is common to find cultivars with a range of blooming times in cherry orchards. To promote optimal fertilisation rates, cultivars are categorised into groups (early, early-mid, mid, mid-late or late) that describe their flowering times which assists orchardists to select cultivars with compatible blooming characteristics (Ganji Moghadam et al., 2009, Radičević et al., 2015). An example of this is shown in Figure 3, where the full bloom time of Lapins (mid-early blooming) fails to overlap with Regina (late blooming); in this case, pollen transfer between the two cultivars is unfeasible in open-pollinated orchards.

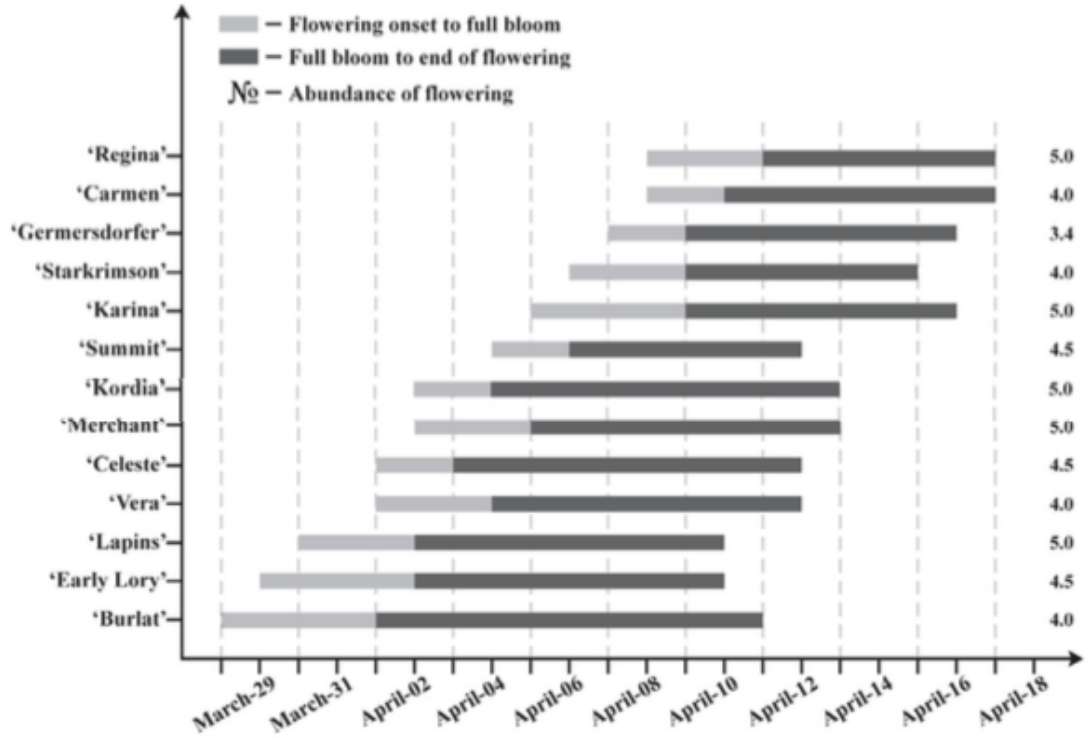


Figure 3: Blooming times of sweet cherry cultivars, from Radičević et al. (2015).

Five to eight days of full bloom overlap of a polliniser and a target cultivar is required for optimum pollination, including two to three days at the beginning of full bloom for the target cultivar (Radičević et al., 2015). The reason for this is that pollen tube growth through the style is greatly improved in the initial days of full bloom due to stigma receptivity (Radunić et al., 2017). However, lack of pollination can occur regardless of synchronous blooming characteristics. If the deposited pollen is genetically similar to the pistil, pollination will likely

fail due to the self-incompatibility (SI) system found in many flowering plants (Crane and Lawrence, 1929). Therefore, cherry orchards require a combination of cultivars with compatible flowering phenology and SI characteristics to ensure adequate pollination is achieved. The genetic basis for SI is very important to the sweet cherry industry because most economically important cultivars are self-sterile. Important developments in sweet cherry breeding programs has seen the introduction of 40 self-compatible varieties into circulation as a response to the SI limitation (Dondini et al., 2018).

1.3 Genetic self-incompatibility in Angiosperms

The SI phenomenon is produced by a multi-allelic gene region (*S*-locus) and is thought to have a single origin ancestry for all present eudicot species (Igic and Kohn, 2001, Vieira et al., 2008, Tao and Iezzoni, 2010, Ramanauskas and Igic, 2017). Gametophytic self-incompatibility (GSI) and sporophytic self-incompatibility (SSI) have been identified as the two mechanisms that regulate pollination failure between genetically similar plants of the same species. Both GSI and SSI favour the reproduction of genetically different individuals, diminishing the effects of inbreeding depression within the population. From an evolutionary perspective, the selection for genetically diverse offspring can confer versatility to a fluctuating environment and improve tolerance to environmental stresses (de Nettancourt, 1977). Table 3 shows examples of SI systems recorded in some important agricultural genera.

SSI plants recognise genetically similar pollen and inhibit the incompatible pollen germination or block the penetration of pollen tube growth through the stigma (Allen et al., 2011, Sehgal and Singh, 2018). *Brassica* species have been extensively studied as a model system for SSI and it is postulated that papilla cells on the stigma are the site where self-pollen recognition occurs, triggering a chain reaction which inhibits pollen hydration (Sehgal and Singh, 2018). Moreover, the inheritance of *S*-alleles differs in SSI species compared to GSI species. The occurrence of dominant, co-dominant and recessive *S*-alleles, leads to varying levels of SI (e.g., Class I – IV interactions in *Brassica*) making this system more complex compared to GSI (Kowyama et al., 2008, Sehgal and Singh, 2018).

Table 3: Three self-incompatibility (SI) systems in various angiosperm: GSI = gametophytic self-incompatibility, SSI = sporophytic self-incompatibility and DSI = diallelic self-incompatibility.

Genera	SI system	Female determinant	Male determinant	Model	Ref
Prunus	GSI	*S*-Rnase	*SFB*	Self-recognition triggers *S*-RNase cytotoxicity of pollen tube in the style	Matsumoto and Tao (2016)
Malus *Pyrus*	GSI	*S*-Rnase	*SFB* B_x^z	Nonself-recognition inhibits *S*-RNase cytotoxicity of pollen tube in the style	Matsumoto and Tao (2016)
Petunia *Nicotiana* *Solanum*	GSI	*S*-Rnase	SLF_x^z	Nonself-recognition inhibits *S*-RNase cytotoxicity of pollen tube in the style	Matsumoto and Tao (2016)
Papaver	GSI	PrsS	PrpS	Self-recognition to inhibit pollen tube penetration via pollen modification	Iwano and Takayama (2012)
Brassica	SSI	SRK SLG	SP11/ SCR	Self-recognition to inhibit pollen tube penetration via stigma modification	Sehgal and Singh (2018)
Ipomoea	SSI	SE1, SE2 and SEA	AB2	Self/nonself-recognition to inhibit pollen tube penetration via stigma modification	Kowyama et al. (2008), (Rahman et al., 2007)
Squadilus	SSI	uncertain	uncertain	Self-recognition to inhibit pollen tube penetration via stigma modification	Allen et al. (2011), Lou (2018)
Olea	DSI	uncertain	uncertain	Self-recognition that reduces pollen tube growth	Sánchez-Estrada and Cuevas (2019)

Extensive research has been conducted in numerous plant families exploring the mechanisms of GSI, including Rosaceae, Solanaceae and Papaveraceae (Iwano and Takayama, 2012, Matsumoto and Tao, 2016). Plants that exhibit GSI display retardation or immobilisation of genetically similar pollen tube growth in the style tissue (Igic et al., 2008). Studies exploring GSI within the plant family Rosaceae, provide insight into diversification of SI systems. Within *Prunus* species for example, incompatible pollen tubes are recognised in the style tissue, triggered by a single set of female and male genes i.e., the product of the female gene recognises the product of the male gene of the same *S*-allele, resulting in pollen rejection. *Malus* species differ to *Prunus* because multiple male *S*-genes ($SFBB_x$) produce pollen proteins that are responsible for supressing the inhibitory response of several style proteins; except for the protein produced by the same *S*-allele (Matsumoto and Tao, 2016).

Heteromorphic and diallelic SI are additional classifications of SI that are currently being discussed for families that don't fit the abovementioned models. Examples of these categories have been identified in buckwheat (*Fagopyrum esculentum*) and olive (*Olea europaea*). Buckwheat is purported to conform to the SSI model; however, the *S*-locus is closely linked to flower morphology traits which contribute to the failure of incompatible pollen i.e., variation of pollen morphology from 'pin' flowers (long styles and short stamens) and 'thrum' flowers (short styles and long stamens; Sánchez-Estrada and Cuevas, 2019). The diallelic SI model proposed for olive displays characteristic of both GSI and SSI; however, is believed to conform to the SSI model (Saumitou-Laprade et al., 2017, Sánchez-Estrada and Cuevas, 2019). Olive cultivars reported to be diallelic because they exhibit only two *S*-genotypes (S_1S_1 or S_1S_2) where S_2 is dominant over S_1 which is a key feature of SSI (Saumitou-Laprade et al., 2017). Interestingly, there is evidence that pollen tube growth is arrested in the style in olive cultivars, which is a characteristic of GSI (Sánchez-Estrada and Cuevas, 2019).

1.3.1 Gametophytic self-incompatibility in sweet cherry

S-alleles in sweet cherry code for stylar specific ribonuclease (*S*-RNase) proteins (Bošković and Tobutt, 1996 and Tao et. al., 1999) and pollen haplotype-specific F-box (*SFB*) proteins (Yamane et. al. 2003). Following pollination, if a haploid pollen grain (with a single *S*-allele) matches either of the two *S*-alleles of the stigma it lands on, the pollen tube growth is slowed or stopped after the pollen germination (Figure 4). This system is known as the one-allele match

model and is also observed in the tetraploid sour cherry; whereby, diploid pollen are rejected if either pollen allele matches any pistil allele (Hauck et al., 2006).

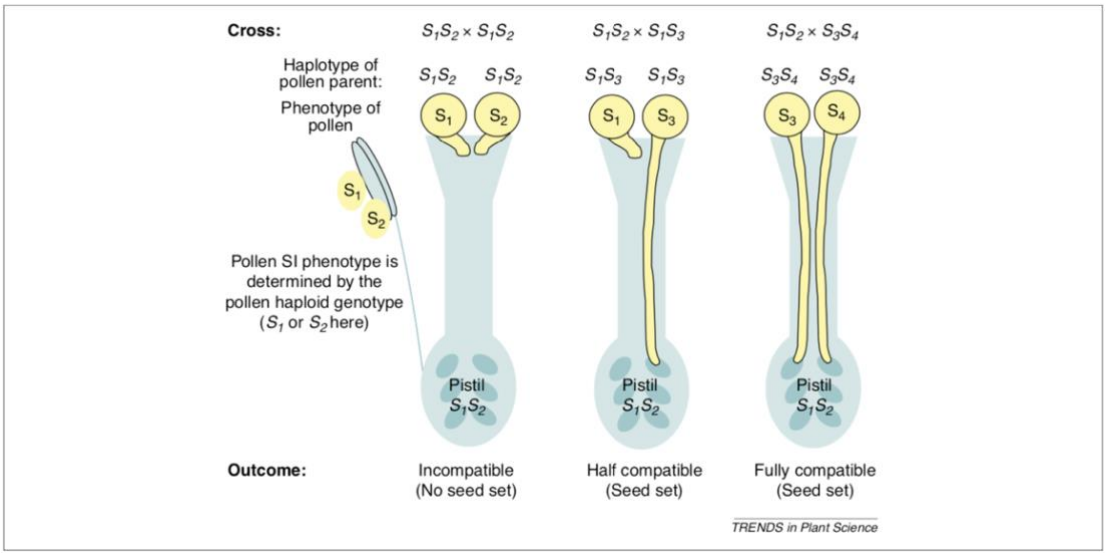


Figure 4: Progress of the pollen germ tube from the stigma to the ovary. If the *S*-allele of the haploid pollen matches either of the diploid pistil *S*-alleles, pollen tube growth will be inhibited, preventing fruit development (Franklin-Tong and Franklin, 2003).

In the case of incompatible pollen germination, *S*-RNase proteins act as a cytotoxin to genetically similar pollen tubes and it is believed that *SFB* proteins catalyse this effect. The model that describes this mechanism is known as the general inhibitor (GI) model (Figure 5; Matsumoto and Tao, 2016 and Sassa, 2016). The *SFB* protein confers substrate specificity to a self *S*-RNase as part of a RING-type E3 ubiquitin ligase that is responsible for the degradation of a theoretical GI that prevents the cytotoxic effect of *Prunus S*-RNases (Deshaies and Joazeiro, 2009, Matsumoto and Tao, 2016). Incompatible pollen tubes are arrested in the style displaying thick callosic walls and appear to be burst at their tip (Cresti et al., 1992).

Interestingly, pseudo-self-compatibility challenges the abovementioned model for GSI in sweet cherry. Sometimes self-pollen is not rejected by the style due to the malfunction of pistil or pollen *S* determinants (e.g., low levels of *S*-RNase transcription). Self-compatible (SC) cultivars do exist however and are a result of mutations in the SFB gene (e.g., S_3' involves a deletion in the *SFB* gene and S_4' involves a frame shift in the *SFB* gene) that prevent recognition of the *SFB* protein by the corresponding *S*-RNase (Company et al., 2015).

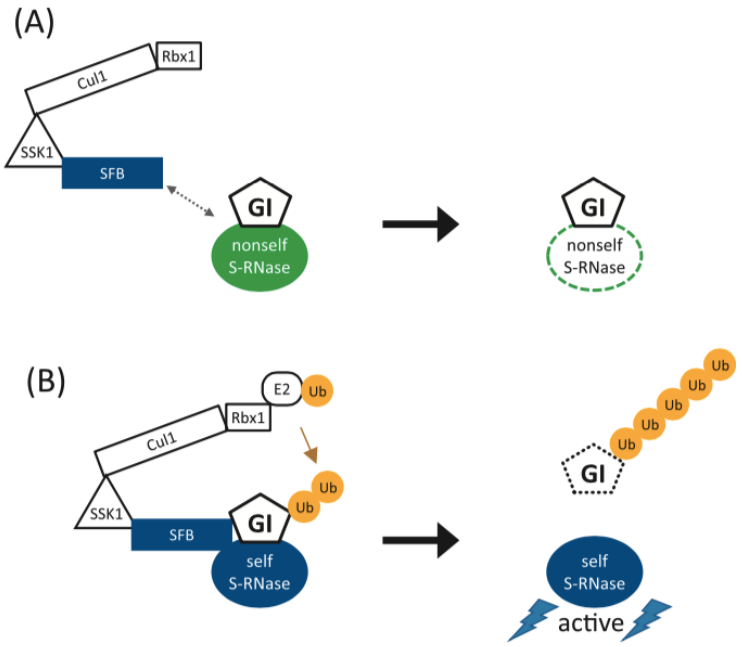


Figure 5: General inhibitor (GI) model from Matsumoto and Tao (2016): *S*-RNase activity is initiated in the case where the GI is degraded by the ubiquitin ligase complex. The *SFB* protein can only attach to a like *S*-RNase.

Understanding the GSI characteristics of sweet cherry cultivars is valuable to producers because the information can improve productivity through orchard design. Recently, Schuster (2017) compiled a list of sweet cherry cultivars and their *S*-alleles. The list of 1203 varieties include important cultivars, reporting 60 incompatibility groups (i.e., cultivars with matching *S*-alleles), detailing every known combination of *S*-alleles 1-22 (excluding 8, 11 and 15 which are duplicates of previously described alleles; Sonneveld et. al., 2003). This includes a group of universal donors that exhibit novel *S*-allele combinations.

1.3.2 Development of GSI analysis in sweet cherry

The methods used to analyse the GSI system have evolved significantly over the last century. Crane and Lawrence (1929) are credited as the first authors to record GSI in sweet cherry. Their study focussed on manual cross-pollination of various cultivars, taking note of pollen tube growth, and identifying incompatible crosses. Further work of Crane and Brown (1938), continued the controlled crossing trial for another six years to define *S*-alleles 1 – 6 and in-compatible crosses were categorised into groups with matching *S*-alleles.

As an attempt to further understand the incompatibility gene construct, Lewis and Crowe (1954) implemented X-ray irradiation on cultivars from various incompatibility groups to be used in crossing experiments. Progeny of the mutant cultivars were reported to be both self-fertile and self-sterile. The subsequent crosses (progeny x progeny, parents x progeny and progeny x parents) produced varying pollination success. It was concluded that mutant self-fertile offspring that displayed permanent loss of SI was due to mutations in the genes encoding the pollen or style SI determinants, although the proteins involved were not identified.

Bošković and Tobutt (1996) were the first to use DNA blot analysis to investigate *S*-alleles in sweet cherries using isoelectric focusing, polyacrylamide gel electrophoresis (IEF-PAGE). They identified active *S*-RNase proteins in stylar material which were absent in leaf material, making them an ideal candidate for *S*-locus products. Furthermore, variations of *S*-RNase proteins were observed in cultivars of varying incompatibility groups. Separation of the various *S*-RNases by their isoelectric point (pI), allowed the correlation of each *S*-RNase with known *S*-alleles. A caveat of this study was that IEF-PAGE potentially confuses S_4 and S_5 RNases due to their similar pI values.

Tao et al. (1999) similarly used DNA blot analysis to determine *S*-alleles 1 – 6. sodium dodecyl sulphate-PAGE and IEF-PAGE were used to separate *S*-RNases based on pI and protein size. This study further explored RNA sequencing of each *S*-RNase providing a new perspective on *S*-allele identification. RNA extracted from style material was reverse-transcribed and successfully cloned. The DNA sequences, generated from the amplified cDNA, were used to develop oligonucleotide primers that are necessary for polymerase chain reaction (PCR) based methods for *S*-RNase identification. The same process was used to identify *SFB* proteins as the pollen *S* determinant (Yamane et al., 2003).

PCR amplification and DNA sequencing techniques have since enabled high-resolution analysis of the *S*-locus using vegetative material such as leaves, rather than flower material (Sonneveld et al., 2001, Sonneveld et al., 2003). These methods are now used to identify the *S*-allele profile in new and old cultivars that have not been recorded (RoSBREED, 2021). The information is invaluable to the sweet cherry industry because orchardists can avoid planting incompatible cultivars together; historically, this is an issue orchardists have discovered when poor fruit yield is observed at crop maturity, four or five years after planting (Jackson et al., 2011).

1.4 Optimisation of cultivar cross-pollination in the orchard

1.4.1 Sweet cherry orchard design

The right cultivars need to be selected before an orchard is planted. Genetically compatible cultivars with synchronous flowering periods that produce economically viable fruit, are sought after. Because it can be difficult to find two main cultivars that meet all the mentioned requirements, specific polliniser cultivars can be incorporated into the orchard design that produce an acceptable economic crop (James, 2011). The next consideration is how many polliniser plants to include and how many spaces away from the main cultivar should each polliniser plant be?

A common cherry orchard design incorporates polliniser cultivars planted intermittently with the main cultivar at a 1:9 ratio i.e., a polliniser cultivar is planted every third space in every third row (Figure 6). This means each target plant is no more than one space from a polliniser cultivar and is used in low-density orchards to ensure optimal pollen availability for bee dispersal amongst target cultivars (Webster and Looney, 1996, James, 2011).

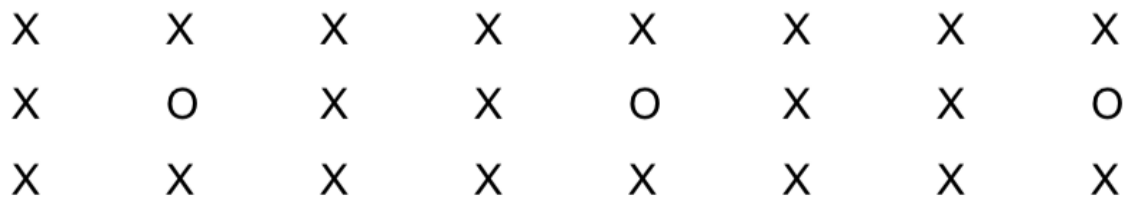


Figure 6: Low density orchard design example for the intermittent use of a polliniser cultivar (O) with a target cultivar (X) at a 1:9 ratio. From James (2011).

High-density planting systems are now possible with the implementation of specialised rootstocks and advances in canopy architecture. An example of this is the use of Gisela root stocks that can decrease a cultivar's time to maturity and limit tree size, combined with a trellis training system (e.g., upright fruiting offshoot canopy). In a review, Lang (2019) discusses how the use of rootstocks and canopy architecture can increase production efficiency including increased light interception and decreased labour costs. This review, however, doesn't discuss the role of polliniser abundance or spacing.

In the case of high-density orchards, it is less common to see the orchard design in Figure 6. In contrast, the planting of intermittent polliniser cultivars can be increased to a ratio of 1:5 (polliniser to main cultivar) i.e., one polliniser plant, every sixth plant, in every row. Otherwise, it is common to see alternating rows of two compatible main cultivars planted in varying ratios (e.g., 1:1, 1:2 or 2:2; Figure 7; James, 2011).

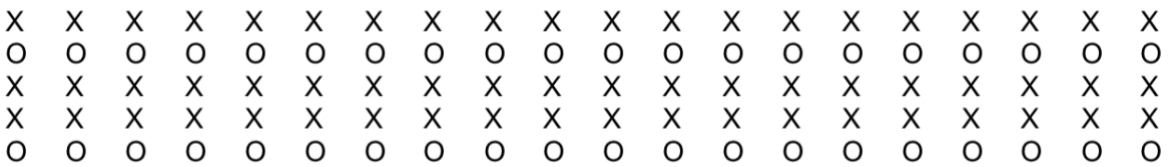


Figure 7: High-density orchard design implementing the uneven planting of two main cultivars.

It is important to note that the orchard layouts that are discussed here are from James (2011), which provides no supporting references to validate their efficacy. A similar rule of thumb is presented in Webster and Looney (1996), whereby figure 6 is recommended for polliniser spacing, continuing to suggest that more polliniser plants would produce better results (e.g., planting the main cultivar and the polliniser cultivar intermittently in a 1:1 ratio down each row). Similar to James (2011), the recommendation of Webster and Looney (1996) is supported by no references. Perhaps the best practice for the use of polliniser cultivars is tacit knowledge, generated by the trial and error of producers themselves (Evans et al., 2017).

There are limited empirical studies which explore the effects of polliniser abundance and distance on the main cultivar's pollination success. For example, Núñez-Elisea et al. (2008) provides a succinct study that suggests a negative correlation between the number of tree spaces to a polliniser and main cultivar yield per tree. A caveat of this study is that, out of the four polliniser cultivars implemented; it isn't possible to determine if there were differences in pollination success between them. To the best of my knowledge, this study is the first explicit knowledge available that can inform the best practice for polliniser use in cherry. I will now discuss recent research in orchard design that use genetic paternity testing on the seeds of fruit trees. The new methods can elaborate on the effectiveness of various polliniser cultivars, distance, and abundance; and demonstrate the potential to save growers time and money when introducing new cultivars to their orchards.

1.4.2 Methods for paternity analysis in fruit crops

Genetic analysis of offspring can determine the pollen source that is responsible for successful fruit set in a mother plant. Isozymes, microsatellites, and *S*-locus alleles have all been used to determine pollen donors in SI fruit crops.

Isozymes refer to catalytic enzymes that function equally regardless of being different on a molecular level: amino acid differences lead to variations in overall net charge among isozymes (Kumar et al., 2009, El-Esawi, 2017). Once the enzymes are extracted from somatic tissues, they can be separated on a gel substrate using electrophoresis to visualise their structural differences (Kumar et al., 2009). Because progeny inherit the enzyme variations found in their parents, isozymes have been used to successfully determine paternity in offspring (El-Esawi, 2017). Isozyme analysis is specific to plant tissues and life-stage. Moreover, the biochemical assays are limited to a minute coverage of the genome, leading to a paucity of markers when compared to more recent genetic analyses (Sulkowska, 2012).

Microsatellites commonly describe repetitive DNA sequences of one to six nucleotide bases: for example, the dinucleotide repeat, ACACACACACAC or $(AC)_6$ (Bhargava and Fuentes, 2010). The intriguing characteristic of microsatellites that makes them effective in assessing genetic variability is the high rate of length polymorphisms at the same locus among individuals within a species (Zane et al., 2002). Two mechanisms are known to cause microsatellite polymorphisms. Firstly, an unequal crossing over of repeat motifs during meiosis and secondly, slippage during DNA replication, can increase or decrease the motif repetition length (Park et al., 2009). The inheritance of the polymorphic repeat regions make microsatellite analysis a great tool for paternity analysis (Bhargava and Fuentes, 2010). The PCR-based methods require a small amount of template DNA and specific primers that are designed to amplify highly variable regions. Before microsatellite analysis can be done, time consuming testing is required to exclude primers that produce null alleles or poor polymorphism in target samples (Park et al., 2009).

In the case of SI species, paternity analysis can be explored through the inheritance of *S*-alleles (Sebolt and Iezzoni, 2009). Like microsatellite analysis, *S*-allele analysis is a PCR-based method that is relatively fast and inexpensive. Particularly in cherry, the known *S*-alleles have been recorded with DNA sequences of *S*-locus genes, allowing easy selection of primers to categorise target samples (Sonneveld et al., 2003). A limitation of *S*-allele analysis is that it is

not possible to discriminate between potential pollen donors with the same *S*-alleles. In this case, microsatellite analysis can be incorporated as a post-hoc test (Gasi et al., 2020). The combination of the two methods minimises the time and cost limitations of microsatellite primers because the primers only need to discriminate between two or three varieties as opposed to several. I will now discuss how these methods have been used in various fruit crops to explore paternity of seeds and their implications on future orchard design.

1.4.3 Polliniser performance in other crops

Paternity has been analysed in various crops Table 4. The results from these studies provide an insight into effective polliniser cultivars, effective polliniser distribution and explore produce quality as a function of polliniser cultivar.

Table 4: The molecular methods used in various crops to determine polliniser performance.

Crop	**Molecular marker type**	**Reference**
Avocado	Isozyme	(Sulaiman et al., 2004)
Apple	*S*-locus	(Schneider et al., 2005)
Olive	SSR	(Arbeiter et al., 2014, Mariotti et al., 2021)
Chestnut	SSR	(Nishio et al., 2019)
Macadamia	SSR	(Richards et al., 2020)
Plum	SSR	(Meland et al., 2020)
Pear	*S*-locus and SSR	(Gasi et al., 2020)
Apricot	*S*-locus	(Boubakri et al., 2021)

Sulaiman et al. (2004) is an example of isozyme analysis being used to determine the most effective polliniser for the avocado cultivar Gwen. Avocado display a less strict phenological self-incompatibility system as opposed to the GSI in *Prunus* species. Type A cultivars are pollinated more effectively by type B cultivars because type A flowers undergo anthesis when type B flower stigmas become receptive and vice versa. Out of six potential pollinisers, the study concluded that one cultivar was the pollen source for at least half of Gwen offspring. The six isozyme markers failed to adequately discern between potential pollen donors because only three alleles were generated for each marker. This is the reason that half of the seeds could only be narrowed to 2 or 3 potential pollen donors. The study recommends that genetic methods be used to further investigate the pollen source of avocado offspring.

The recent microsatellite study of nine plum orchards in Norway by Meland et al. (2020), is an excellent example of how genetic paternity analysis can benefit orchard design. The selection of seven microsatellite markers produced a total of 84 alleles across the eleven cultivars being investigated; the high number is related to the hexaploid nature of the cultivars. The results identified the pollen parent for each plum kernel sample from important Norwegian cultivars. One cultivar was found to be the most efficient polliniser despite it not being the most abundant, which was attributed to high pollen production and fast pollen tube growth rates. Meland et al. (2020), provides a good comparison of complex cultivar combinations, thus demonstrating the utility of microsatellite analysis. In this study, the polliniser spacing was uniform across all orchards. By manipulating this variable, further research could inform the efficacy of the different plum varieties under different orchard design.

Gasi et al. (2020) provides another example of microsatellite analysis in diploid pear cultivars. Three orchards with a different combination of two or three polliniser cultivars (five polliniser cultivars in total) were compared to recommend the most effective polliniser cultivars in Norway for the pear cultivar, Celina. It was concluded that two out of the five cultivars were responsible for siring most Celina seeds. Interestingly, *S*-allele PCR analysis was used in this study only to identify the parent cultivars *S*-alleles. Five out of the six cultivars all shared a single *S*-allele (S101), the other *S*-alleles in each cultivar were independent of each other. In this case, *S*-genotyping of the seeds would have been sufficient to determine paternity. However, it is mentioned that the SI response is known to breakdown in Celina due to environmental conditions and that self-pollination is possible. Instead, 11 microsatellite markers were used to amplify 45 different alleles across the six cultivars.

1.4.4 Polliniser performance in cherry

In the present study, pollinisers of Kordia and Regina are investigated. These cultivars produce premium fruit qualities, including attractive size and colour, firm texture and desirable flavour profiles (James, 2011, Dondini et al., 2018). Yields from Kordia and Regina are often observed to be disappointing, which may be due to low temperature sensitivity and short ovule viability, which, in turn, leads to pollination issues (Granger, 2004, Dondini et al., 2018). To determine if pollination issues are present under current orchard design in Tasmania this study draws from previous research in sweet cherry that use isozyme analysis (Granger, 2004), *S*-allele genotyping (Sebolt and Iezzoni, 2009, Hedhly et al., 2016, Guajardo et al., 2017) microsatellite

analysis (Schueler et al., 2003, Cottrell et al., 2009, Jolivet et al., 2012) and single nucleotide polymorphism (SNP) analysis (Fernandez et al., 2012, Rosyara et al., 2014, Guajardo et al., 2015, Shirasawa et al., 2017). Although SNPs are tending to replace microsatellites for phylogenetic research, it is unclear if SNP genotype libraries are available for Australian sweet cherry varieties and is beyond the scope of the current study. I will now discuss studies that exemplify the use of these methods for the investigation of polliniser performance in cherry orchards.

Granger (2004) provides an example for the isozyme analysis to determine if variable fruit production observed in certain cultivars was related to differences in polliniser genotypes. By comparing the offspring isozyme genotypes with that of potential pollen donors, it was found that only a portion of selected pollinators were effective pollen sources for difficult to set cultivars.

In their *S*-locus analysis of sweet and sour cherries, Sebolt and Iezzoni (2009) provide a practical template for pollen donor determination. A prior condition of this method is knowing the *S*-haplotypes of potential pollinators. An example of their method as a laboratory exercise for students called, "What set your crop?", can be seen in Figure 8. In the study, cv. Emperor Francis (S_3S_4) was the mother plant, and cv. Rainier (S_1S_4) and cv. Gold (S_3S_6) were the pollen donors. The *S*-haplotypes S_4 and S_3 from Rainier and Gold, respectively, are incompatible with Emperor Francis. This means the presence of an S_1 or S_6 allele in Emperor Francis seeds is due to Rainier or Gold pollen. Utilising *S*-allele analysis to determine pollen parents is a valuable tool for the cherry industry; however, the method is limited when potential pollen donors share *S*-alleles.

Lastly, Guajardo et al. (2017), conducted a similar experiment within a sweet cherry nursery to determine the predominant pollen source of cv. Rainier seedlings. Five cultivars were identified as potential pollen donors due to overlapping flowering time and location within the orchard. The *S*-alleles of seedlings were used to infer paternity similar to Sebolt and Iezzoni (2009) and two cultivars were found to be responsible for 49% of all fruit-set. The paper concludes that *S*-allele genotyping combined with SSR analysis can confidently determine the parents of sweet cherry fruits.

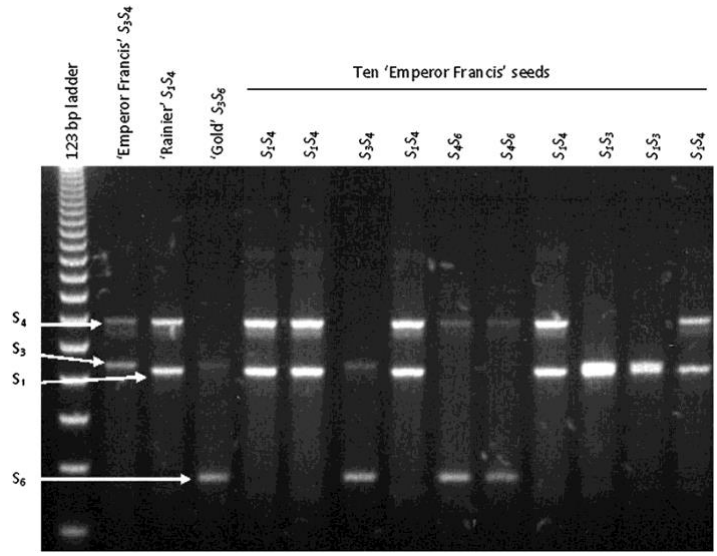


Figure 8: An agarose gel image of the S-alleles in the "What set your crop" exercise, with Emperor Francis as the mother plant and Rainier and Gold as the pollen donors (Sebolt and Iezzoni, 2009).

1.5 Conclusion

The PCR based paternal analysis in sweet cherry cultivars can identify ineffective polliniser cultivars and enhance orchard design for optimum fruit set in sweet cherry cultivars. To meet market demands, it is important that sweet cherry orchards can produce consistent yields which require the selection of compatible cultivars. This necessitates the collaboration between researchers and sweet cherry producers to explore how orchard design may be altered to improve the quality and quantity of production.

2. Introduction

Sweet cherry is a significant economic crop within Australia. Between June 2019 and June 2020, Australian cherry growers produced 14,720 t, valued at $184 m (Hort Innovation, 2021). Currently, Tasmanian produces 25% of Australia's total cherry output (Hort Innovation, 2021) and has a competitive edge with the island's fruit fly pest-free status for Queensland Fruit Fly (Cherry Growers Australia Inc., 2015). This means Tasmanian cherries qualify for export to countries with tough import regulations such as Japan (Cherry Growers Australia Inc., 2015). The export of Tasmanian cherries reaches more than 20 countries in Asia, Middle East and Europe (Cherry Growers Australia Inc., 2015). Due to the relatively mild temperate climate, Tasmanian orchards are able to grow varieties that produce large, flavoursome fruit (James, 2011). Cherry growers select cultivars that display high yield, good fruit quality (size, colour, firmness and sweetness), such as Lapins, Kordia, Regina, Simone, Sylvia and Sweet Georgia (James, 2011).

Most cherry varieties are self-incompatible due to genetic factors (*S*-alleles) and require a cross-compatible cultivar to set fruit (Crane and Lawrence, 1929, Bošković and Tobutt, 1996). Recent research has identified the importance of classifying the *S*-alleles of cherry varieties new and old (Schuster, 2017, Patzak et al., 2019). By cataloguing cherry *S*-alleles, the industry can optimise pollination efficiency and simplify crop management. Also, cherry breeding programmes can eliminate synonymous cultivars and improve the quality of new varieties.

As of 2017, the *S*-alleles of 1203 cherry cultivars have been described (Schuster, 2017). In Tasmania, a sweet cherry producer, Reid Fruits, has identified a new cultivar variation in one of their orchards (SLK) which requires *S*-allele profiling and there remain two important cultivars that have not been genetically catalogued for the *S*-locus (Simone and Sweet Georgia). This project aims to use DNA sequencing to identify the unknown *S*-alleles of the cultivars Simone, Sweet Georgia and SLK. The project also aims to investigate the pollen donors for the cultivars Kordia and Regina in an open-pollinated orchard. These cultivars deliver premium grade fruit that is ideal for export trade; however, they can produce low yield quality because of poor fruit set (Granger, 2004, Bound et al., 2014, Sagredo et al., 2017). We use PCR methods to identify the cultivars responsible for the successful pollination of Kordia and Regina and to identify if the pollen compatibility of pollinisers is contributing to their observed poor fruit set.

The findings of this project will improve the ability of the cherry industry to evaluate cultivar crosses and make informed decisions on orchard design.

3. Materials and Methods

3.1 Sample collection and locations

All cherry leaf and fruit samples were obtained from one of two locations: Hansen Orchards (Huonville, Tas – 43˚00’03’’S 147˚06’06’’E) and Reid Fruits (Jericho, Tas – 42˚22’56’’S 147˚16’46’’E).

3.1.1 Experiment 1 – collection of leaf samples for Simone, Sweet Georgia and SLK

To determine the unknown *S*-alleles of Simone, Sweet Georgia and SLK, fully expanded leaves were collected from each cultivar on the 5/10/2020, placed in a Ziplock bag and directly transferred to a cooler. Samples were returned to the laboratory and stored at -20 °C. Leaf material was also collected as reference material for all potential pollen donors.

3.1.2 Experiment 2 – collection of Kordia and Regina fruits with reference leaf material

To determine the pollinisers of Kordia and Regina plants, Kordia fruit was collected on the 19/01/2021 and Regina fruit was collected on the 5/02/2021. Thirty Kordia and Regina fruits were selected from four locations (n = 240) at Reid Fruits, Jericho to include in the *S*-genotyping analysis. Sample locations A, B, C, D and F (Figure 9) for each cultivar were selected to represent areas of varying proximity to other cultivars grown on the property. Only plants in the centre of rows were chosen to avoid potential edge effects due to proximity to beehives. For comparison, 30 Regina fruits were collected from a single location at Hansen Orchards (Location F; Figure 9) where a single polliniser cultivar (Sylvia) was present. Once harvested, fruit samples were kept in paper bags, transferred to a cold cooler then stored in a laboratory fridge at 4 °C. The seed was removed from each fruit using a cherry pitter, placed on paper towel, and dried at room temperature (~20 °C) for four days. Seeds were returned to paper bags and stored at room temperature awaiting further processing. For a genetic reference to compare Kordia and Regina seed DNA to cross-compatible cultivars within the orchard (Table 5) leaf samples were collected as above.

Table 5: sweet cherry cultivars present at Reid Fruits orchard

Cultivar	***S*-allele**
Regina	S_1S_3
Sylvia	S_1S_4
Lapins	S_1S_4,
Sweetheart	S_3S_4,
Kordia	S_3S_6
Fertard	S_3S_6
Penny	S_6S_9
Johanna	S_1S_{12}
Kentish[a]	$S_6S_{13}S_B$

[a] as reported in Tobutt et al. (2004)

3.2 DNA extraction

Leaf and seed DNA was extracted and purified following the method described in Yuskianti et al (2014).

3.2.1 Leaf material

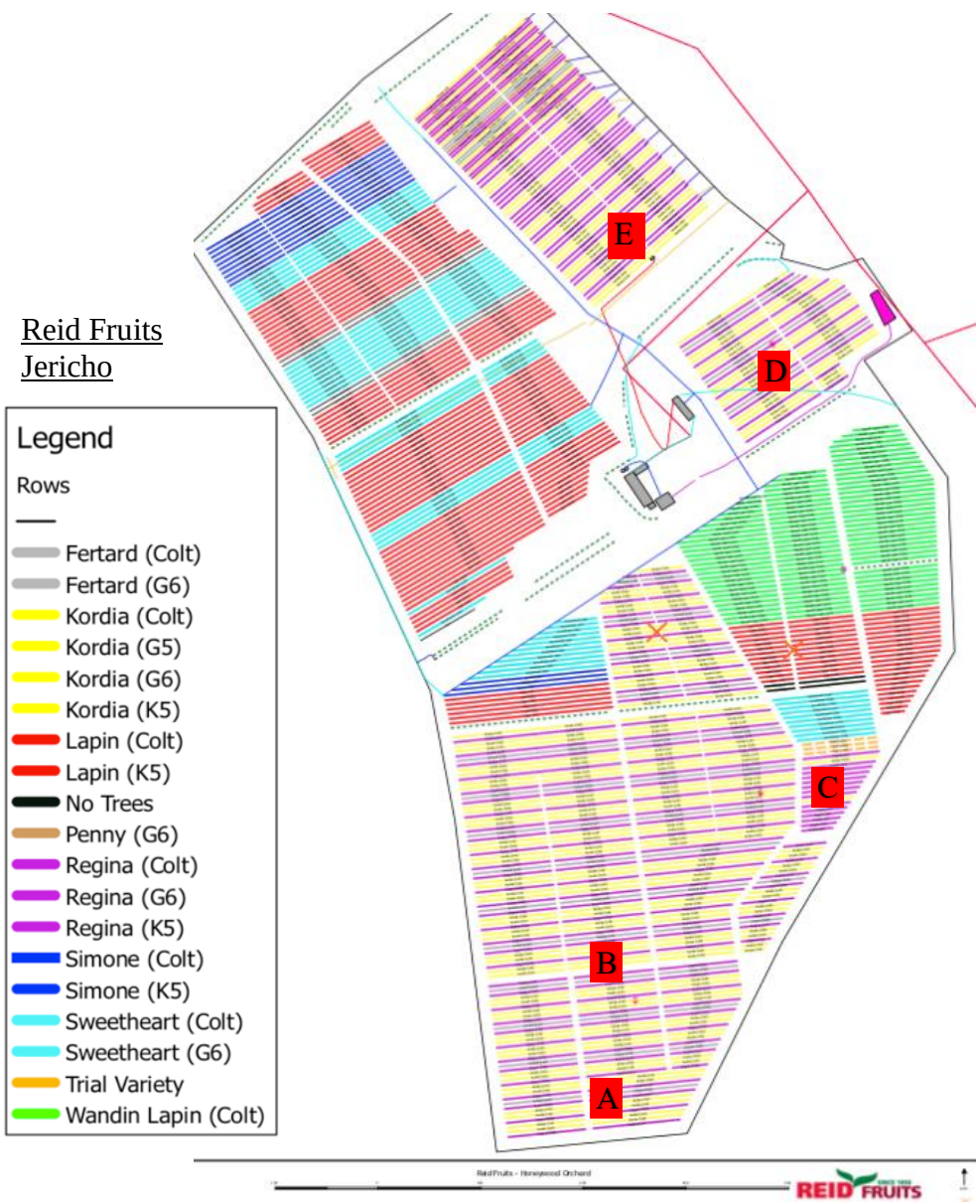
Approximately 1 cm^2 (>50 mg) of leaf material was cut from each sample leaf using a clean scalpel blade. The remaining leaf samples were returned to the freezer at -20 °C. Following extraction and purification, leaf genomic DNA was eluted in 50 μl TE buffer and diluted 1:10 in TE buffer prior to PCR.

3.2.2 Seed material

Seeds (containing the embryo) required separation from the shell using a mortar and pestle. Seeds were soaked for 2-3 hours in a small volume of water, grouped by location, to soften the testa (seed coat) for removal, as this contains only maternal DNA. The seed isolated from the shell and testa was initially cut in half using a clean scalpel blade and DNA was extracted as above. In DNA samples extracted from half a seed the supernatant containing the DNA could not be successfully separated from seed debris (particularly lipids) due to their buoyant nature. A smaller quantity (~20 mg) of seed material was therefore used in the DNA extraction protocol. DNA was eluted in a final volume of 50 μL TE buffer. Initial trial PCR reactions of seed genomic DNA with dilutions of 1:2, 1:4 and 1:10 DNA in TE buffer found that the 1:4 dilution was optimal for amplification of seed DNA.

3.3 PCR amplification

Polymerase chain reaction was utilised to isolate and amplify the *S*-locus genes for seed and leaf samples. Five μL of genomic DNA (diluted in TE buffer as described above) was used in a 25 μL PCR reaction; a negative control reaction without template DNA was included in each PCR batch. In all reactions the reagents and their concentrations were as follows: 2 mM of $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, 1 U of *Taq* DNA polymerase (Meridian Bioscience, Cincinnati, USA e), 0.2 mg/ml BSA and 1 × PCR buffer (Meridian Bioscience, Cincinnati, USA). PCR reactions were prepared in a UV treated laminar flow cabinet with all reagents and DNA kept on ice. PCR tubes were transferred to a Bio-Rad T100™ thermal cycler once the hot lid had preheated to 105 °C. A specific thermal cycler program was used for each primer pair.



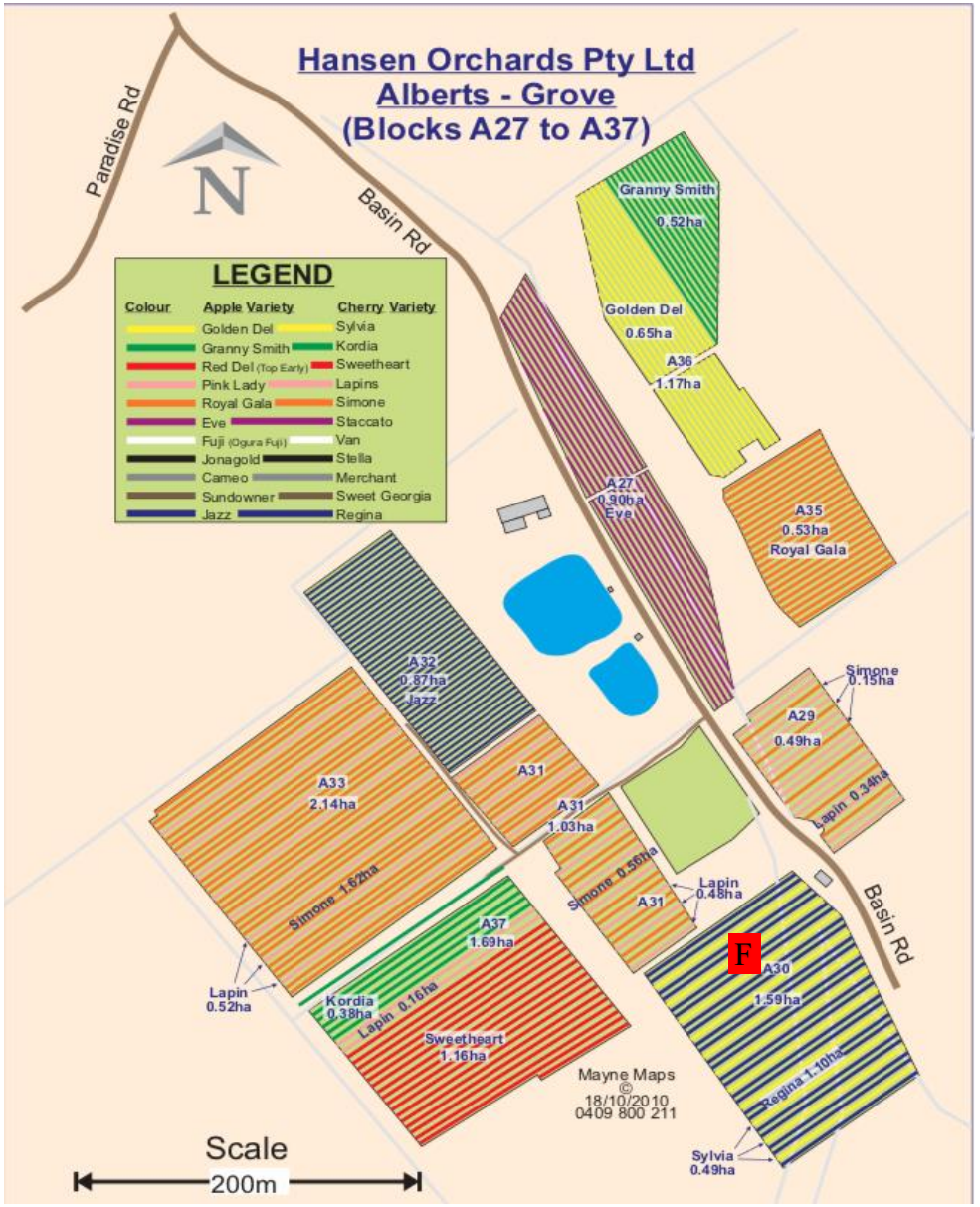


Figure 9: Sampling locations for cv. Kordia and cv. Regina Fruit at Reid Fruits, Jericho, and Hansen Orchards. Grove. All locations are open air plots except location E, which is located under a retractable roof. Location F only included cv. Kordia fruit samples.

3.4 Experiment 1 – *S*-locus amplification and sequencing of cultivars with unknown *S*-alleles to identify their genotype

3.4.1 *S*-RNase consensus primers

Using two consensus primers sets (Table 6) designed by Sonneveld et al. (2003), leaf material was used as template DNA to amplify the first and second introns of the *S*-locus. It was important to test both primer sets for their ability to produce different sized amplicons that represented a single *S*-allele for subsequent sequencing. A gradient PCR was run using leaf genomic DNA to optimise the annealing temperature. i.e., 95°C for 3 mins, 35 cycles of (95 °C for 30 s, 52-60 °C for 2 mins and 72 °C for 1 min) and 72 °C for 5 mins. The PCR products were separated on a 1.3% agarose gel (15 cm x 15 cm) at 100 V for 30 mins alongside a molecular weight ladder (Bioline Easyladder I, Meridian Bioscience, Cincinnati, USA).

Table 6: Oligonucleotide sequences for the *S*-RNase consensus primers used for PCR amplification.

Target gene	**Primer[a]**	**Primer sequence 5' → 3'**	**Ta (˚C)**	**PCR product size**
***S*-RNase 1st intron**	PaConsI-F	(C/A)CT TGT TCT TG(C/G) TTT (T/C)GC TTT CTT C	54	Signal peptide to C2
	PaConsI-R	CAT G(A/G)A TGG TGA A(A/G)T (T/A)TT GTA ATG G	54	
***S*-RNase 2nd intron**	PaConsII-F	G GCC AAG TAA TTA TTC AAA CC	54	C2 to C5
	PaConsII-R2	GCC ATT GTT GCA CAA ATT GA	55	

[a]F = forward, R = reverse

3.4.2 Sequencing with Macrogen

The consensus primers PaConsI-F and PaConsI-R were chosen to amplify the first intron of the *S*-locus for Simone, Sweet Georgia and SLK with the protocol outlined above. The PCR product was separated on a 3% Hi-Res agarose gel (20 cm x 15 cm) at 60V for 3 hours. The gel was transferred to a UV Transilluminator where a clean scalpel was used to cut a single band out of the gel and transferred to a clean 1.5 mL tube. The process was repeated until a gel slice for each band was collected in a labelled 1.5 mL tube, representing two alleles for each of Simone, Sweet Georgia, and Reid's In-house Cultivar. The samples were sent to Macrogen (Seoul, South Korea) for purification and Sanger sequencing. After purification of the DNA from the gel slice, the DNA from all but one of the samples was too low in concentration to proceed with sequencing. Therefore, DNA for each allele was re-amplified as described below.

3.4.3 Sequencing at the University of Tasmania

A PCR reaction was carried out as described earlier with PaConsI-F and PaConsI-R. Whilst the PCR product was separated on a 3% Hi-Res agarose gel (20 cm x 15 cm) at 60V for 3 hours, another PCR master mix was prepared as described earlier for each fragment to be sequenced (n = 6). The gel with separated bands was transferred to the UV Transilluminator. A sterile pipette tip was used to pierce a gel band and was 'rinsed' in the corresponding PCR tube, filled with PaConsI PCR master mix. This step was repeated for each band. The new PCR tubes were transferred to the thermal cycler and run with the same thermocylcer program. The PCR product was purified using an UltraClean PCR Clean-Up kit (MoBio, Carlsbad, CA, USA) then sent to staff at the Central Sciences Molecular Lab, University of Tasmania, for sequencing. A forward and reverse sequencing reaction was prepared for each sample, containing: 1 μL of BigDye™ Terminator v1.1, 3.5 µL of BigDye™ v3.1 5 x sequencing buffer, 3.2 *p*mol of primer (PaConsI-F for forward reactions and PaConsI-R for reverse reactions), 10 μL deionised water and 2 μL of template DNA (7 – 14 *n*g/μL in concentration). The reaction samples were transferred to a thermal cycler and run at 96 °C 1 min and 25 cycles of (96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 mins). The sequencing reactions were ethanol precipitated, washed with 70% ethanol, dried and re-suspended with 10ul of HiDi loading solution before being separated on the Applied Biosystems 3500 Genetic Analyzer.

3.4.4 Sequencing the *SFB* gene

Initial findings using specific *SFB*-allele primers indicated that Simone and Sweet Georgia possessed an S_4 allele. To determine whether Simone and Sweet Georgia carried a self-compatible mutation of

the *SFB* gene (*SFB*$_4$'), a new *SFB* specific primer set was developed. Using reference sequences (AY649873.1 and AY649872.1) published by Sonneveld et al. (2003) and Sonneveld et al. (2005), the new primers were designed to encompass the gene region where a 4bp deletion exists in the *SFB*$_4$' gene (Table 7). Simone and Sweet Georgia genomic DNA was used in a PCR reaction, as described for the specific *SFB* primers including both negative control and blanks samples. The PCR products were purified and sequenced as previously described.

3.4.5 Sequence alignment and identification

The sequencing process generated DNA trace files that were visualised and edited in 4Peaks© (by A. Griekspoor and Tom Groothuis, nucleobytes.com). The forward and reverse chromatograms were inspected for signal quality. Noisy sequences at the beginning or end of the chromatograms were cut from the final sequence. Forward and reverse sequences for each sample were aligned in Clustal X 2.0 (Larkin et al., 2007) and the consensus sequence was saved in .fasta format. The international nucleotide sequence databases (INSDs) were searched for matching sequences using BLAST (Altschul et al., 1990) on NCBIS (National Centre for Biotechnology Information). Search results were aligned with new sequences to confirm each sequence identity.

3.5 Experiment 2 – identification of pollen donor cultivars for Kordia and Regina fruit.

3.5.1 Specific *SFB* allele primer design

Primers were designed to detect each of the specific *SFB* alleles in each of the reference leaf samples. Reference sequences for each allele were obtained from GenBank (Appendix A) and aligned in Geneious Prime© (www.geneious.com) using the Clustal method to identify polymorphisms that differentiated each allele. Primers were designed to have GC content of 38-60%, length of 18-24 nucleotides and with an annealing temperature as close as possible to 60 °C using online tools (http://biotools.nubic.northwestern.edu/OligoCalc.html, https://sg.idtdna.com/pages/tools). Table 7 describes the specific *SFB* primer sets used in seed PCR reactions.

The Kentish cultivar belongs to an ambiguous group of sour cherries, as such, it was important to identify the *S*-alleles of the cultivar present at the Reid Fruits orchard. By comparing publications that report sour cherry *S*-alleles, the *SFB* specific primers were developed for S_6, S_{13}, S_{26} and S_{36a+b} as likely candidates for the cv. Kentish found at Reid Fruits (Bošković et al., 2006, Tsukamoto et al., 2010, Tsukamoto et al., 2008).

Each specific primer set was designed for an optimal annealing temperature of 60˚C and the thermocycler program used was: 95 °C 3 mins, 40 cycles of (95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) and 72 °C for 7 mins. Primer sets were tested using genomic DNA from nine sweet cherry cultivars (Table 5) to determine their specificity to the target *SFB* allele. Each trial reaction was separated on a 1.3% agarose gel (15 cm x 15 cm) at 100 V for 30 mins, with a size marker ladder (Bioline Easyladder I), positive control and blank sample included.

3.5.2 Specific *SFB* PCR for Kordia and Regina seeds

A PCR reaction for each seed sample was run using primer sets for SFB_1, SFB_2, SFB_3, SFB_4, SFB_6 SFB_9, SFB_{12}, SFB_{13} and SFB_{36a+b} on a thermal cycler program and electrophoresed as described above. Seed genotypes were recorded by the presence of a positive band after electrophoresis of PCR products. Paternal alleles inherited by the seeds were determined by selecting seed *S*-alleles that were not present in the maternal genotype. Cultivars that had a genotype containing the corresponding paternal seed *S*-allele, were then considered to be a potential pollen donor for that seed sample. Considering the effective pollination period of Kordia and Regina reported by Radičević et al. (2015), cultivars were excluded as a potential pollen donor if their full bloom date was ±10 days of either Kordia (i.e., Sweetheart) or Regina (i.e., Sweetheart and Lapins).

3.5.3 Statistical analysis

Kordia and Regina seed genotypes found at each location were tested using Fisher's Exact Test to determine if there were any difference in proportions between the sample locations. The One Sample Proportion Test was used to compare the proportion of paternal *S*-alleles inherited by Kordia and Regina seeds to a proportion of 0.5 at each location.

Table 7: Specific *S*-allele primers designed to amplify the *SFB* region in sweet cherry for alleles: S_1, S_3, S_4, S_4', S_6, S_9, S_{12}, S_{13}, S_{26}, S_{36a+b}, S_{36a}, S_{36b}.

Target gene	Accession number	Primer[a]	Primer sequence 5' → 3'	GC%	Salt adjusted Ta (˚C)	PCR product size
***SFB*$_1$**	AY805048.1	FB1-F2	AGG GAA CAC AGA ACA TTA TGG G	45	60	147
		FB1-R2	GTT AAT GTT GGT GCT GAT TGG TG	43	60	
***SFB*$_3$**	AY805057.1	FB3-F2	GAG CAG TTC TCC AAT TTA AGC C	45	60	210
		FB3-R	CCC AAA TTG GAG AGA AAC ATG G	45	60	
***SFB*$_4$**	AY649872.1	FB4-F2	TAC AAG TTA AAT CAT CCC TTA GGC	39	60	212
		FB4-R2	CTG GGG TGG AAC CCA AAC T	58	60	
***SFB*$_4$'**	AY649873.1	SFB4-Fp	TTG CGA GGA GAA GGG TAT GC	55	60	106
		SFB4-Rp	TAC CGA GTG TAC CAT AAT AAT GAG	38	60	
***SFB*$_6$**	AY805051.1	S6-C2F2	GGC TTA GTT TGC ATT TCG GAG	48	60	190
		S6-C3R2	CAT CCT TAC AGC CTT ATA GTC G	45	60	
***SFB*$_9$**	DQ422809.1	FB9-F	AGG CGG AAA TTG TTG TGC	50	60	178
		FB9-R	GCC AAC TTA ATT CCT GTT TCT TG	39	60	
***SFB*$_{12}$**	AY805054.1	FB12-F	CGA TTT CTG TGT ACA TCC AAG TT	39	60	290
		FB12-R	CGA AAC GCA AAC TAA ACC GT	45	60	
***SFB*$_{13}$**	AY805055.1	FB13-F	GGA GTG ATT TGA TTG GAA GCT TG	43	60	251
		FB13-R	TTA ATC CAT TGC TTG AGC CAT AC	39	59	
***SFB*$_{26}$**	EU035977.1	FB26-F	CCC TCC TTC GGT TTC TTT CT	50	61	325
		FB26-R	CTA TCG AAA TTC AGT ATC TCA TCG	38	60	
***SFB*$_{36a+36b}$**	consensus	FB36-F	GGT TTC TGT GTA CAA GCA AGT CT	43	61	358
		FB36-R	GGG GTT CTA AAT TTC CTG ATC G	45	60	
***SFB*$_{36a}$**	EU042131.1	FB36A-F	AC AAG CAA GTC TTG GAG TGA TC	45	60	347
		FB36-R	GGG GTT CTA AAT TTC CTG ATC G	45	60	
***SFB*$_{36b}$**	EU042132.1	FB36B-F	GGC AGC ATC AAA TGA GTA CA	45	60	206
		FB36B-R	CTC ACA ACC ATA GAA TCT AAA AGG	38	60	

[a]F = forward, R = reverse

3.5.4 Further paternal discrimination using microsatellite markers

For some seeds, it was not possible to determine the pollen donor due to the presence of two or more pollinisers having the same *S*-allele. For these samples, SSR analysis was undertaken to investigate which loci could discern between the potential pollen donors. Thirteen SSR markers from published papers were selected for their capacity to generate variation in product sizes between cultivars and their significant discrimination power in previous studies (Table 8). Prior to using SSR markers on Kordia and Regina seeds, a trial was conducted including all control leaf DNA. A PCR reaction for each primer was prepared using the same reagents and concentrations used for previous PCR reactions. The initial thermal cycler program used was 95 °C for 2 mins, 35 x (95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s) and 72 °C for 5 mins, and the PCR product was separated on a 3% Hi-res agarose gel (15 cm x 20 cm) at 50 V for 8 hours.

Table 8: SSR markers that were included in the present study because of their potential to discriminate between individuals.

SSR name	Primer sequence (5′→ 3′)	Repeat motif	No. of alleles	Size range (bp)	Discrimination power	Reference
BPPCT-05	GCT AGC AGG GCA CTT GAT C ACG[F] CGT GTA CGG TGG AT[R]	$(AG)_{10}$	6	179 – 185	0.79	Dirlewanger et al. (2002)
BPPCT-026	ATA CCT TTG CCA CTT GCG[F] TGA GTT GGA AGA AAA CGT AAC A[R]	$(AG)_8GG(AG)_6$	6	140 – 190	0.85	
BPPCT-028	TCA AGT TAG CTG AGG ATC GC[F] GAG CTT GCC TAT GAG AAG ACC[R]	$(TC)_{15}$	3	151 – 173	0.63	
BPPCT-034	CTA CCT GAA ATA AGC AGA GCC AT[F] CAA TGG AGA ATG GGG TGC[R]	$(GA)_{19}$	6	224 – 258	0.84	
BPPCT-037	CAT GGA AGA GGA TCA AGT GC CTT[F] GAA GGT AGT GCC AAA GC[R]	$(GA)_{25}$	5	142 – 156	0.80	
BPPCT-038	TAT ATT GTT GGC TTC TTG CAT G TGA[F] AAG TGA AAC AAT GGA AGC[R]	$(GA)_{25}$	5	101 – 133	0.85	
BPPCT-039	ATT ACG TAC CCT AAA GCT TCT GC[F] GAT GTC ATG AAG ATT GGA GAG G[R]	$(GA)_{20}$	5	134 – 150	0.87	
BPPCT-040	ATG AGG ACG TGT CTG AAT GG AGC[F] CAA ACC CCT CTT ATA CG[R]	$(GA)_{14}$	6	122 – 146	0.84	
UCD-CH11	TGC TAT TAG CTT AAT GCC TCC C[F] ATG CTG ATG TCA TAA GGT GTG C[R]	$(CT)_{15}$	4	134 – 151	0.68[X]	Struss et al. (2003), Korecký et al. (2017)
UCD-CH12	AGA CAA AGG GAT TGT GGG C[F] TTT CTG CCA CAA ACC TAA TGG[R]	$(CA)_{14}$	5	173 – 200	0.83[X]	
UCD-CH26	CTG TCG AAA TGC CTA TGC[F] ATG AAT GCT GTG TAC ATG AGG C[R]	$(CT)_{10}$	4	110 – 124	Not reported	
PMS-30[Z]	CTG TCG AAA GTT TGC CTA TGC[F] ATG AAT GCT GTG TAC ATG AGG C[R]	Not reported	11	119 – 175	0.97	Cantini et al. (2001)
PMS-67[Z]	AGT CTC TCA CAG TCA GTT TCT[F] TTA ACT TAA CCC CTC TCC CTC C[R]	Not reported	13	144 – 191	0.98	

[F] = Forward primer, [R] = Reverse primer

[X] = data from 48 wild cherry varieties, [Z] = data from 59 tetraploid cherry varieties

4. Results

4.1 Experiment 1 – *S*-locus amplification and sequencing of cultivars with unknown *S*-alleles to identify their genotype

4.1.1 *S*-RNase consensus primers

A gradient PCR tested annealing temperatures between 52 and 60 °C for the consensus primers PaConsIF/PaConsIR and PaConsIIF/PaConsIIR. Amplification was confirmed from leaf material of two varieties at all tested temperatures (Figure 10) and the most stringent temperature, 60 °C, was used in subsequent PCR reactions.

Amplification of the cultivars with unknown *S*-alleles, Sweet Georgia, Simone and SLK, with the consensus primers was expected to produce two fragments representing the two alleles from each cultivar. The two fragments from the PaConsIF/PaConsIR PCR varied sufficiently in size to be separated when electrophoresed on a 1.3% agarose gel at 7 V/cm for 30 minutes, whereas PCR products amplified with PaConsII-F and PaConsII-R co-migrated on the gel (Figure 10).

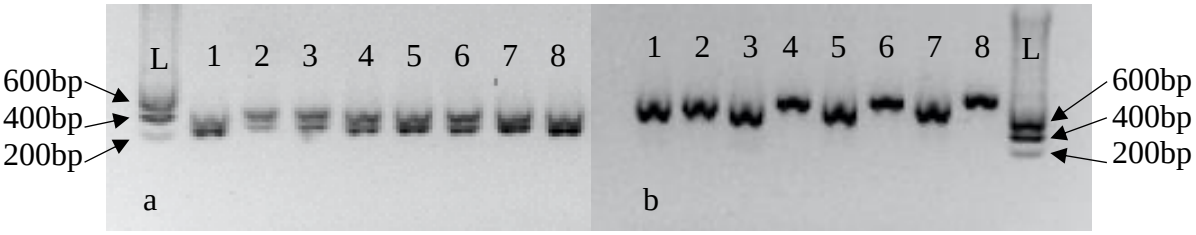


Figure 10**:** PCR amplification using primers PaConsIF/PaConsIR (a) and PaConsIIF/PaConsIIR (b) with SLK leaf genomic DNA. Lanes 1 – 8 represent an annealing temperature gradient of 52 – 60°C (left to right) and L represents HyperLadder 1kb (Bioline).

Simone, SLK and Sweet Georgia DNA was again amplified using PaConsIF/PaConsIR and 10 µL of PCR product was loaded onto a 3% agarose gel then electrophoresed at 3 V/cm for 3 hours. Bands were excised using a new scalpel blade and sent to Macrogen for elution and sequencing, but insufficient template DNA was recovered.

Amplification was repeated and after separation in 3% agarose (no photo available), each allele was re-amplified separately. Master mix was aliquoted into tubes before the gel was placed on a transilluminator and template DNA was obtained by inserting a pipette tip into the fragment

of interest then placing the tip into the previously aliquoted PCR master mix and stirring. Re-amplification of single target alleles was confirmed by electrophoresis of a 5 µL aliquot on 1.3% agarose at 7 V/cm for 1 hour (Figure 11).

Figure 11: Samples that were amplified using PaConsIF/PaConsIR and template DNA extracted from gel bands of a previous PaConsIF/PaConsIR reaction using Simone, SLK and Sweet Georgia genomic DNA. Bands represent *S*-alleles for Simone (lanes 1 – 4), SLK (lanes 5 – 8) and Sweet Georgia (lanes 9 – 10), and L represents HyperLadder 100bp (Bioline).

The rest of the re-amplified PCR products (20 µL still in the tube) were purified and sequenced in both directions, using the same primers, at the Central Sciences Molecular Genetics laboratory at the University of Tasmania. Chromatograms were edited and trimmed, and matching sequences were found by a BLAST search of INSDs.

4.1.2 *S*-RNase sequence alignments

The largest PCR product found in Simone and Sweet Georgia (~450 bp in Figure 11) had the highest match with GenBank accession AJ635288.1, the *S*-RNase gene for ribonuclease S_4 from *P. avium* cv. Napoleon (S_3S_4). Both Simone S_4 and Sweet Georgia S_4 sequences (402 bp and 450 bp, respectively) were identical to that of AJ635288.1 (Figure 12), which includes exons 1 and 2 of the S_4-RNase gene.

The PCR product of ~400 bp (Figure 11) found in Simone, SLK and Sweet Georgia had the highest match with GenBank accession AJ635282.1, the *S*-RNase gene for ribonuclease S_1 from *P. avium* cv. Early Rivers (S_1S_2). The S_1 sequences for Simone (379 bp), SLK (378 bp) and Sweet Georgia (379 bp) were all identical to AJ635282.1 (Figure 13) which includes exons 1 and 2 of the S_1-RNase gene

Lastly, the PCR product only found in SLK (~250 bp in Figure 11) had the highest match with GenBank accession AY571663.1, the *S*-RNase gene for ribonuclease S_3 from *P. avium* cv. Cristobalina (S_3S_6). The SLK S_3 sequence (231 bp) was identical to AY571663.1 (Figure 14).

4.1.3 *SFB*$_4$ sequence alignments

The PaConsIF/PaConsIR sequence obtained for Simone and Sweet Georgia did not allow discrimination between S_4 and S_4', so primers were designed to span a region of the *SFB* gene that includes the 4 bp deletion that impairs the function of the S_4 *SFB* gene product. Simone and Sweet Georgia DNA was amplified using FB4-Fp and FB4-Rp and were sequenced as above, producing sequences of 106 bp. Both sequences were identical to GenBank accession AY649873.1, the S-Fbox gene for S_4' from *P. avium* cv. Sonata (S_3S_4'). Compared to the GenBank accession AY649872.1, the *SFB* gene for S_4 from *P. avium* cv. Inge (S_4S_9), both sequences only shared a sequence identity of 96.36% (Figure 15).

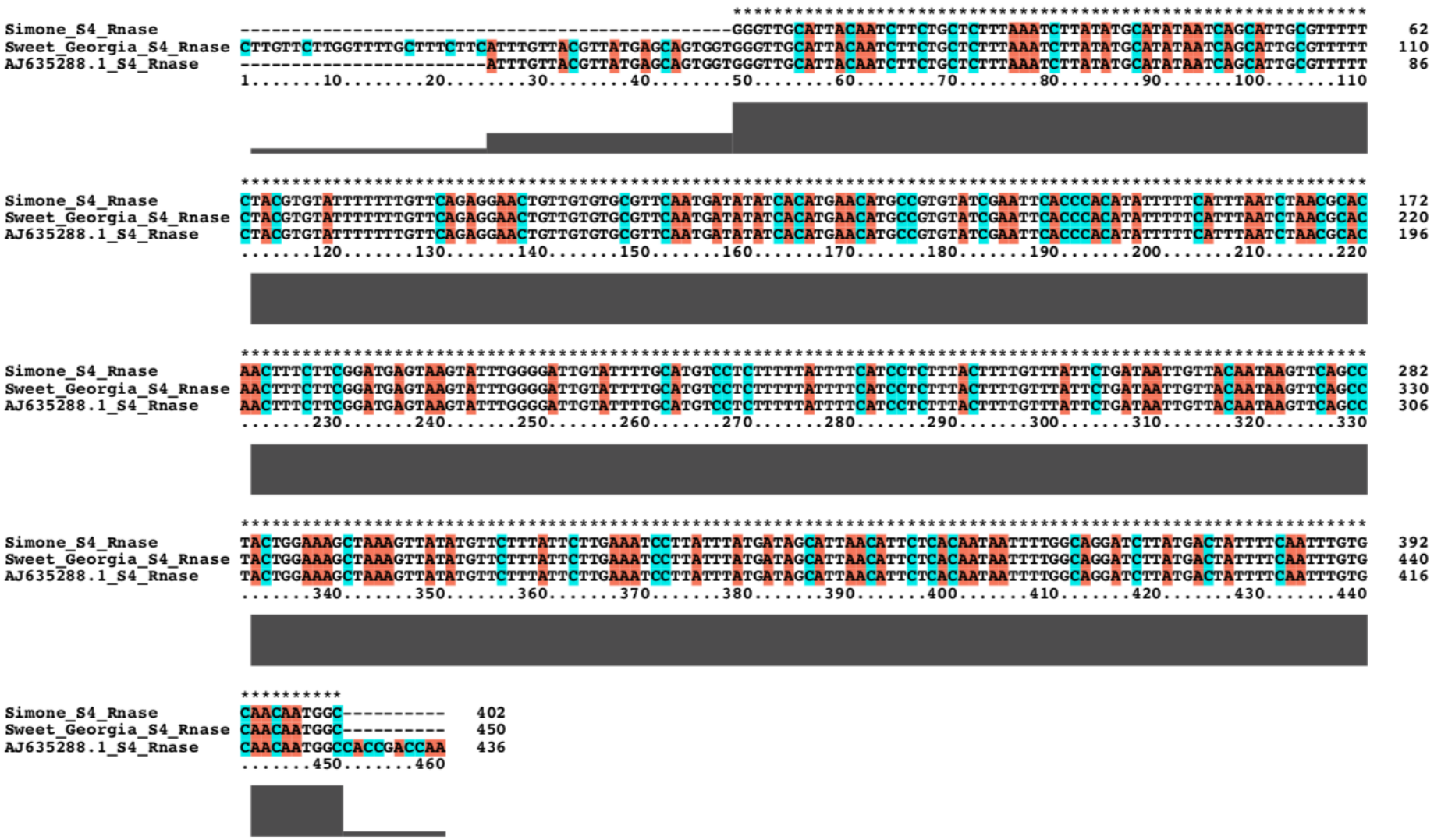


Figure 12: Clustal X 2.0 alignments comparing Simone and Sweet Georgia S_4 ribonuclease with *S*$_4$-RNase of *P. avium* cv. Napoleon (*S*$_3$*S*$_4$; GenBank: AJ635288.1).

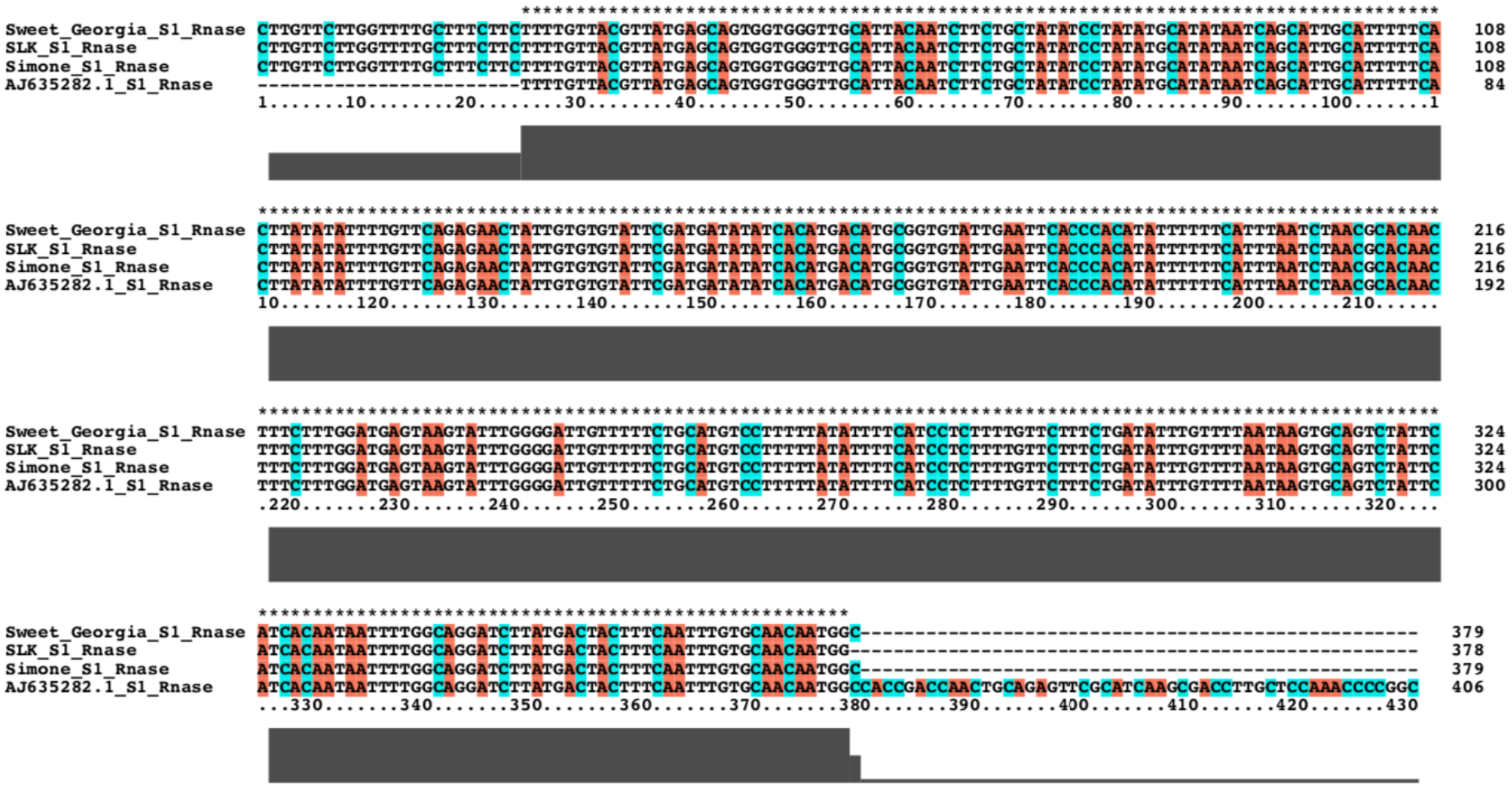


Figure 13: Clustal X 2.0 alignments comparing Simone, SLK and Sweet Georgia S_1 ribonuclease with *S*$_1$-RNase of *P. avium* cv. Early Rivers (*S*$_1$*S*$_2$; GenBank: AJ635282.1).

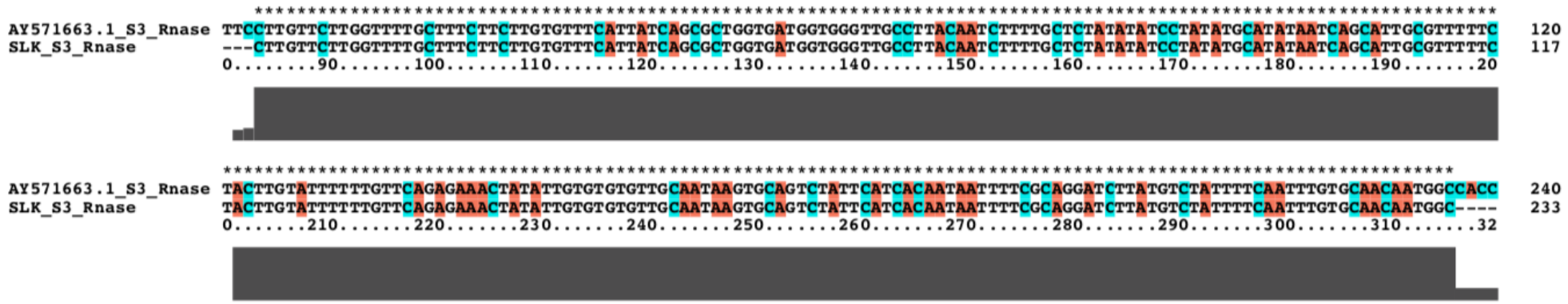


Figure 14: Clustal X 2.0 alignments comparing Simone, SLK and Sweet Georgia S_3 ribonuclease with S_3-RNase of P. avium cv. Cristobalina (S3S6; GenBank: AY571663.1).

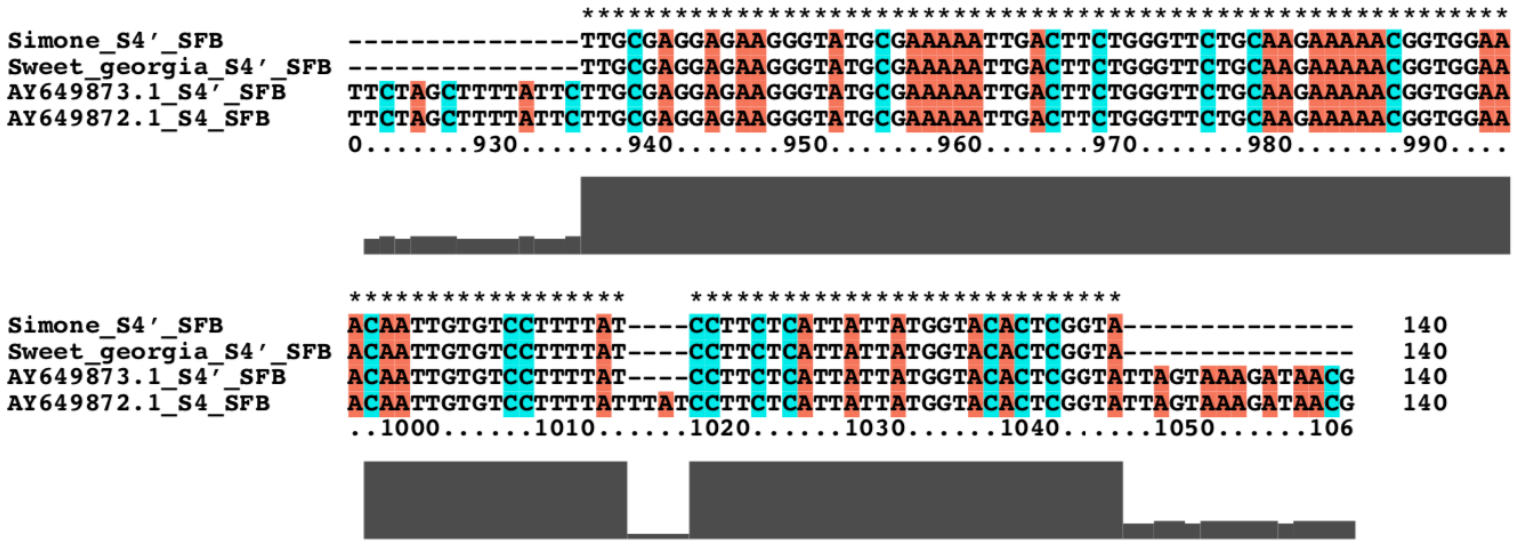


Figure 15: DNA sequence alignments comparing the Simone and Sweet Georgia *SFB* gene fragment with the *SFB*$_4$' F-box gene of *P. avium* cv. Sonata (*S*$_3$*S*$_4$'; GenBank: AY649873.1) and the *SFB*$_4$ F-box gene *P. avium* cv. Inge (*S*$_4$*S*$_9$; GenBank: AY649872.1).

4.2 Experiment 2 – identification of pollen donor cultivars for Kordia and Regina fruit.

4.2.1 Confirmation of primer specificity to specific *SFB* alleles

To test the specificity of *SFB* primers, each primer was tested using a positive control (DNA of varieties with the *S*-allele), multiple negative controls (DNA of varieties lacking the target *S*-allele) and an internal control (primer without template DNA). Each specific *SFB* primer only amplified DNA from cultivars with the target alleles (Figure 16 and Figure 17). Internal and negative control samples commonly produced low molecular weight primer dimers (product size <100 bp) but not the target amplicon.

4.2.2 Confirmation of Kentish *S*-alleles

The *SFB* primers for *S*-alleles that were deduced to be candidates for the Kentish cultivar found at Reid Fruits were used in a PCR reaction with all cultivar leaf samples. The Kentish cultivar in this study tested negative for S_1, S_3, S_4, S_9, S_{12} and S_{26}, and positive amplification for S_6, S_{13} and S_{36} was observed on agarose gel (Figure 16 and Figure 17). Importantly, only Kentish produced a positive amplicon for the S_{13} and S_{36} allele.

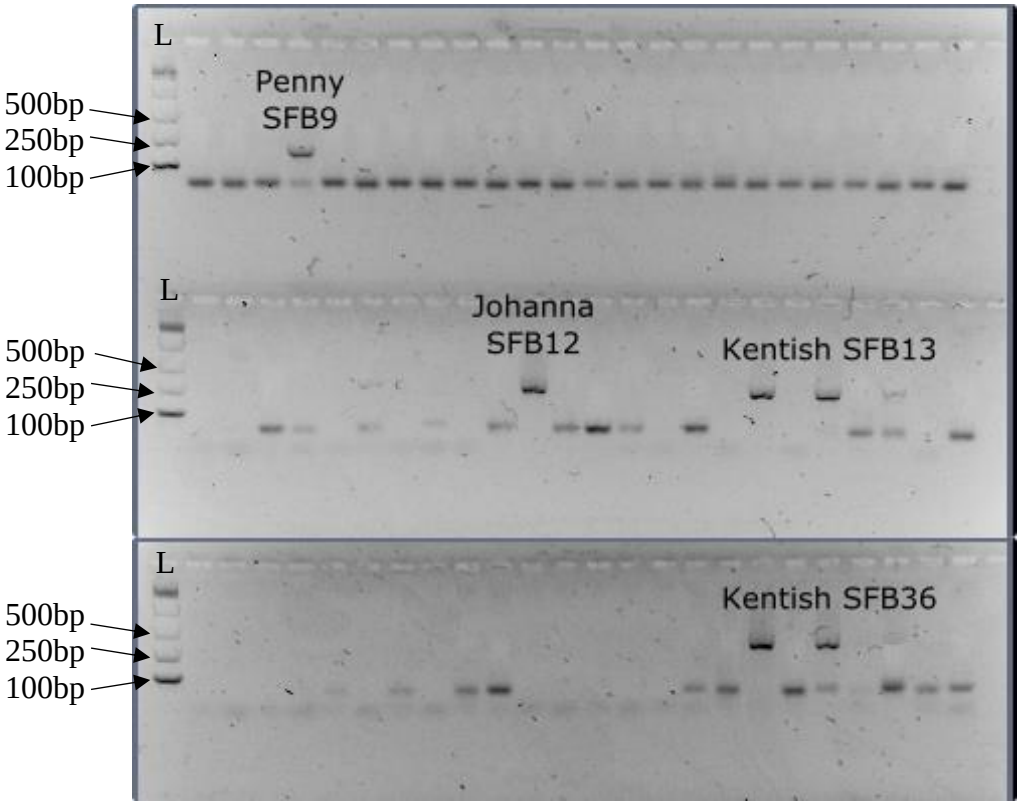


Figure 16: Agarose gel image of DNA amplified using specific primers for *SFB*$_9$, *SFB*$_{12}$, *SFB*$_{13}$, *SFB*$_{26}$ and *SFB*$_{36}$. The template DNA used in PCR reactions were Kordia, Penny, Sylvia, Sweetheart, Fertard, Johanna, Regina, Lapins and Kentish (dilutions – ½, ¼ and $^1/_{10}$; in order form left to right). Top row – Blank, interspersed with *SFB*$_9$, middle row – *SFB*$_{12}$, interspersed with *SFB*$_{13}$ and bottom row – *SFB*$_{26}$, interspersed with *SFB*$_{36}$. L represents EasyLadder 1 (Bioline).

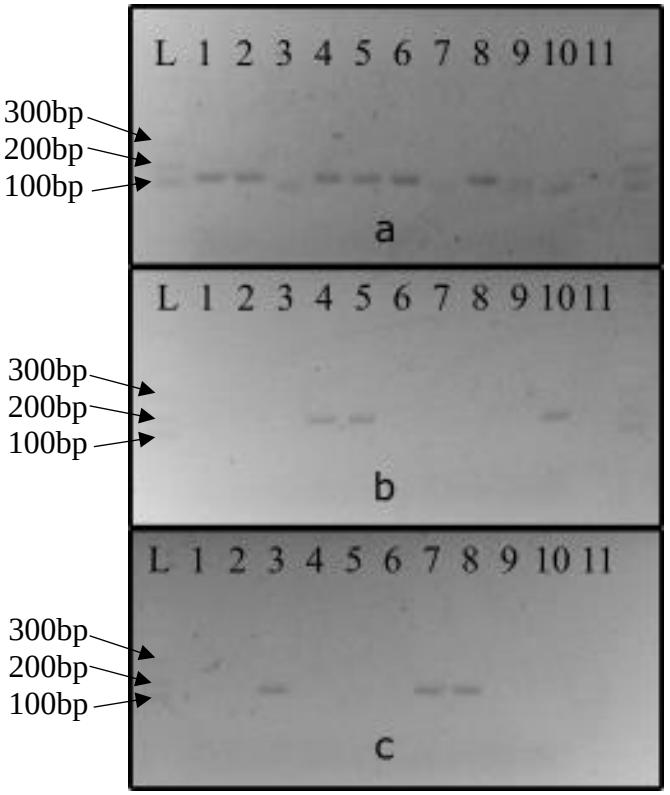


Figure 17: Agarose gel image of DNA amplified using specific primers for *SFB*$_1$ (a), *SFB*$_4$ (b) and *SFB*$_6$ (c). Lane numbers represent: 1, Lapins; 2, Regina; 3, Kordia; 4, Sweet Georgia; 5, Simone; 6, SLK; 7, Kentish; 8, Kordia seed; 9, Fertard seed; and 10, Regina seed. The PCR reaction using FB3F/FB3R failed and was subsequently repeated successfully (no image available). L represents HyperLadder 100bp (Bioline).

4.2.4 Specific *SFB* PCR for Regina seeds at Hansen Orchards

The Hansen Orchards block of Regina plants (location F, Figure 9) allowed us to test the specific *SFB* primers on open pollinated seed samples that had a single pollen source i.e.., a Sylvia polliniser row that was planted every third row. Although *S*-locus cross-compatible cultivars were present in surrounding blocks (Lapins, Simone and Sweetheart) they were excluded as potential pollen sources because of their distance to sample trees (≥ 50 m) and asynchronous flowering times (> 11 days) compared to Regina. Out of the 29 Regina seeds sampled at Hansen Orchards, 100% inherited a single paternal allele, S_4. Considering the orchard design, the S_4 alleles inherited by Regina seeds correlate with Sylvia (S_1S_4) pollen.

4.2.5 Specific *SFB* PCR for Regina seeds at Reid Fruits

We confirmed the paternal *S*-alleles present in Regina seeds at Reid Fruits (location A, C, D and E): S_4 from Sylvia (S_1S_4) pollen, and S_6 from Kordia (S_3S_6) and Fertard (S_3S_6) pollen, where the proportion of S_4 to S_6 varied between locations (Table 9; Fisher's Exact Test – p = <0.01). Although Sylvia flowers were synchronous with Regina flowers, Sylvia pollen accounted for 17 %, 19 % and 40 % of Regina offspring at location E, A and D (Table 9). The skewed inheritance of S_6 alleles in these locations, particularly E and A, is likely due to the high abundance of Kordia and Fertard plants that reached full bloom five and two days prior to Regina. At location E, two Kordia rows are planted to two Regina rows and at location A and D, rows are planted in a ratio of 4:3:1 Kordia, Regina and Fertard plants, respectively. Compared to Sylvia, which is planted every 10th space in Regina rows and every 20th space in Kordia rows. Interestingly, we observed a skewed inheritance of S_4 alleles in Regina seeds at location C where eight Regina rows are planted to one Kordia row (Table 9), providing further evidence that the high inheritance of S_6 alleles in Regina seeds is due to a high abundance of Kordia pollen. Indeed, Fertard must also be considered as a pollen donor, although, if polliniser abundance is the main determinant of seed paternity, then Fertard must play a minor role in the pollination of Regina flowers at these locations. Moreover, Kentish is planted every 20th space in Regina rows, flowers synchronously with Regina and produces S_6 pollen, so can be considered a potential pollen donor. However, we found no evidence of S_{13} or S_{36} alleles in Regina seeds that would be expected if Kentish was an active polliniser of Regina flowers. To address the precise pollen source for Regina seeds that inherited an S_6 allele, further analysis using SSRs is required.

Table 9: Proportion of paternal alleles inherited by Regina seeds at Reid Fruits.

	S_4		S_6		Proportion test (H_0 = 50%)
Location	**Proportion (*n*)**	**95% CI**	**Proportion (*n*)**	**95% CI**	**p-value**
A	19% (5)	7-39%	81% (22)	61-93%	<0.01
C	63% (17)	43-80%	37% (10)	20-58%	0.25
D	40% (12)	23-59%	60% (18)	41-77%	0.36
E	17% (5)	7-37%	83% (24)	64-93%	<0.01

4.2.6 Specific *SFB* PCR for Kordia seeds at Reid Fruits

We confirmed the paternal *S*-alleles present in Kordia seeds at Reid Fruits (location A, B, D and E): S_1 from Regina (S_1S_3) and Sylvia (S_1S_4) pollen, and S_4 from Sylvia (S_1S_4) pollen, where the proportion of S_1 to S_4 was not significantly different between locations (Table 10; Fisher's Exact Test – p = 0.38). We observed a significantly skewed inheritance of S_1 alleles at all locations (Table 10). The lack of variation between location is to be expected because the orchard design remains consistent across all locations (as described above). Considering both Sylvia and Regina reached full bloom five days after Kordia, it is likely that the high frequency of S_1 alleles in Kordia seeds is due to the high abundance of Regina plants compared to Sylvia plants. However, like the Regina seeds, SSR analysis is required to determine the proportion of S_1 alleles that can be attributed to Regina and Sylvia pollen.

Table 10: Proportion of paternal alleles inherited by Kordia seeds at Reid Fruits.

	S_1		S_4		Proportion test (H_0 = 50%)
Location	**Proportion (*n*)**	**95% CI**	**Proportion (*n*)**	**95% CI**	**p-value**
A	93% (27)	76-99%	7% (2)	1-24%	<0.01
B	87% (26)	68-96%	13% (4)	4-32%	<0.01
D	83% (25)	65-94%	17% (5)	6-36%	<0.01
E	77% (23)	57-89%	23% (7)	11-43%	<0.01

4.2.7 Microsatellite markers

It was not possible to identify exactly which cultivars were the pollen donors at Reid Fruits based on *S*-alleles alone, so SSR markers were tested to see if they could distinguish among each group – Sylvia, Lapins and Regina for S_1 or S_4 in Kordia seeds, and Kordia, Fertard, Penny and Kentish for S_6 in Regina seeds. Seven out of thirteen SSR primers successfully produced amplicons for these groups; Table 11 shows their approximate fragment sizes, and Figure 20

shows the fragments on agarose gel after electrophoresis. Appendix B shows approximate size fragments produced by seven SSRs for the nine sweet cherry cultivars used in this study.

BPPCT-034, BPPCT-038, BPPCT-039, PMS-30, UCD-CH12 and UCD-CH26 failed to produce amplicons for all potential pollen donors. To determine if improved results could be achieved by PCR optimisation, each marker was retried using a new DNA dilution (1:20), a lower annealing temperature (53 °C) and a higher annealing temperature (57 °C). BPPCT-039 amplified successfully when the annealing temperature was increased to 57 °C (Figure 18). Other than BPPCT-039, these trials did not improve SSR amplification.

The BPPCT-039 trial in Figure 18 produced fragments of different sizes for Kordia and Fertard which is necessary to differentiate between Regina offspring with the paternal S_6 allele. Subsequently, the primers were used to amplify DNA from Regina seeds (Figure 19). The resulting electrophoresis did not clearly discriminate fragment sizes for Kordia and Fertard to infer pollen origin.

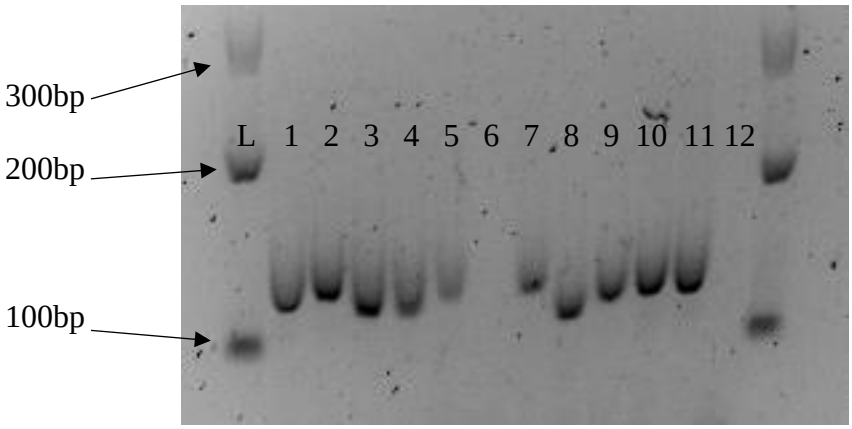


Figure 18: SSR marker trial using leaf genomic DNA with BPPCT-039. Lane numbers represent: 1) Regina; 2) Lapins; 3) Kordia; 4) Johanna; 5) Fertard; 6) blank; 7) Sylvia; 8) Sweetheart; 9) Penny; 10-11) Kentish (dilutions 1/4 and 1/10, respectively); and 12) blank (water). L represents HyperLadder 100bp (Bioline). Amplicons were electrophoresed on 1.3% agarose at 7 V/cm for 3 hours.

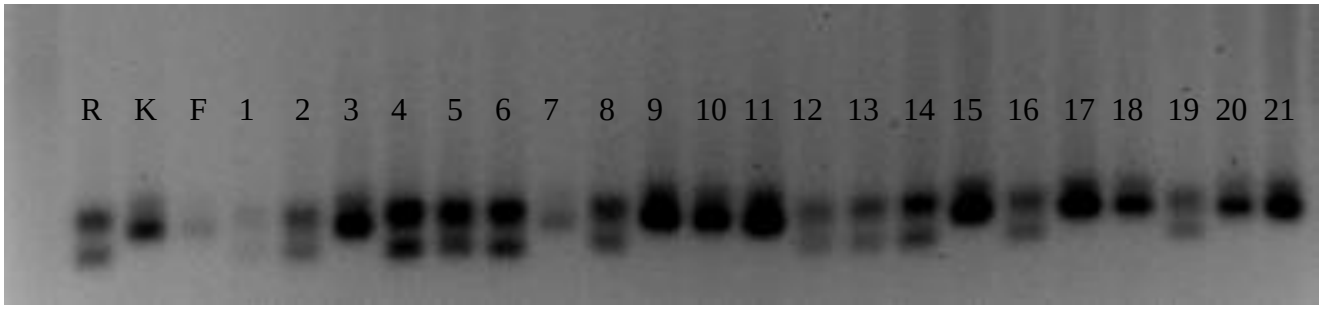


Figure 19: Amplification of microsatellite locus BPPCT-039 from leaf DNA of Regina (R), Kordia (K) and Fertard (F) compared to DNA from Regina seed samples (lanes 1 – 21).

Of the seven SSR markers, five markers could be used to determine pollen origin for Kordia and Regina seeds (Table 11). Three primers were estimated to amplify a distinct size fragment for Regina (BPPCT-005 and BPPCT-026) and Sylvia (BPPCT-026 and BPPCT-037) when comparing potential pollen donors for Kordia seeds. To compare the potential pollen donors for Regina seeds, four SSR markers were estimated to amplify distinct size fragments for Fertard (BPPCT-005 and PMS-67), Kordia (PMS-67) and Kentish (BPPCT-026, BPPCT-028, BPPCT-037 and PMS-67).

Although the SSR markers displayed promising amplicon size variation among potential pollen donors, the range of size differences are too small to allow accurate visual assessment using agarose gel electrophoresis.

Table 11: SSR amplicon sizes of potential pollen donors for Kordia and Regina, estimated from agarose gel fragments following electrophoresis. Asterisks (*) indicate size fragments that are distinct within the potential pollen donor group of cultivars for a particular SSR marker.

	Estimated fragment sizes (bp)				
	Potential pollen donors for Kordia seeds		Potential pollen donors for Regina seeds		
SSR marker	Regina	Sylvia	Kordia	Fertard	Kentish
BPPCT-005	147*	159	159	153*	159 133 113
BPPCT-026	179* 157*	162*	179 157	157	172* 113*
BPPCT-028	175 143	143	143	143	164*
BPPCT-037	152	147*	152	152	158*
BPPCT-040	147 129*	147 119	147 129	147 129	129
UCD-CH11	168 149	168 149	168* 149	149	157 123
PMS-67	146	146	146*	149*	161*

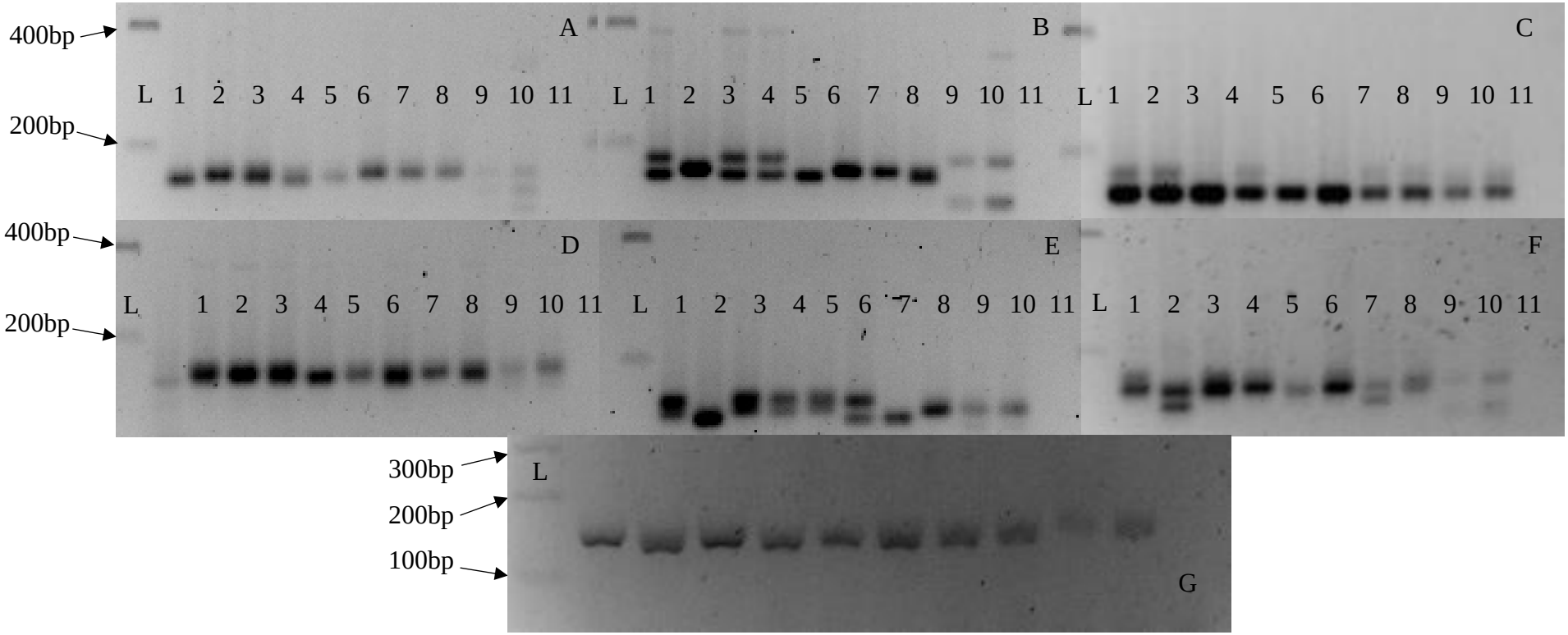


Figure 20: SSR marker trial using leaf genomic DNA with SSR markers BPPCT-005 (A), BPPCT-026 (B), BPPCT-028 (C), BPPCT-037 (D), BPPCT-040 (E), UCD-CH11 (F) and PMS-67 (G). Lane numbers represent: 1, Regina[1]; 2, Lapins; 3, Kordia[2]; 4, Johanna; 5, Fertard[2]; 6, Sylvia[1]; 7, Sweetheart; 8, Penny; 9-10, Kentish[2] (dilutions 1/4 and 1/10, respectively); and 11, blank (water). L represents HyperLadder 1kb (Bioline; A-F) and HyperLadder 100bp (Bioline; G).

[1] Potential pollen donor for Kordia seeds
[2] Potential pollen donor for Regina seeds

5. Discussion

5.1 Experiment 1 – *S*-locus amplification and sequencing of cultivars with unknown *S*-alleles to identify their genotype

In this work we unequivocally confirm that the unknown *S*-alleles for Simone, Sweet Georgia and SLK are S_1S_4', S_1S_4' and S_1S_3, respectively. The cherry industry has long recognised both Sweet Georgia and Simone as self-pollinating early flowering cultivars (James, 2011). Sweet Georgia is recorded as being a whole plant mutation or a 'sport' of a 'Lapins' tree grown in Tasmania (IPAustralia, 2009, James 2011) whereby a sport is defined as a clonal selection that is found to be different to the mother plant (Dondini et a., 2018). Therefore, the fact that this study found Sweet Georgia to have the same genotype as Lapins (S_1S_4') is not surprising. The origin of Simone is uncertain but the literature suggests that Simone is also a mutation of Lapins (Granger, 2004). However, to determine the precise pedigree of these cultivars, further molecular approaches such as the SSR analysis would be required (Rosyara et al., 2014, Ivanovych and Volkov, 2018).

In this study, an additional primer pair, FB4-Fp and FB4-Rp was required to correctly identify the pollen part *SFB* mutation in Simone and Sweet Georgia that confers self-compatibility. These primers amplify the gene region encompassing the 4bp deletion that inhibits the self-incompatibility mechanism in S_4' pollen, making them ideal for sequencing. The sequencing approach was adopted in preference to other methods of characterising this gene region due to the small scale of this study. For example, Muñoz-Espinoza et al. (2017) reports a single step, high throughput analysis using a qPCR-HRM approach that is capable of distinguishing between S_4 and S_4' alleles based on the different melting temperatures of amplicons with and without the 4 bp indel. This methodology is economical when processing large quantities of samples (e.g., commercial laboratory services[3]); however, the PCR method that I used in this study is more cost effective to amplify and sequence a few samples. Ikeda et al. (2004) and Ushijima et al. (2004) used a nested PCR followed by digestion with restriction enzymes, a methods that is also more labour-intensive than sequencing. Regarding FB4-Fp and FB4-Rp, it was unfeasible for the primers to bridge the 4bp indel of SFB_4' so that amplification might be specific to the S_4' haplotype because the indel region is of low complexity with a low GC content.

[3] https://www.rosbreed.org/breeding/dna-tests/cherry/cross-compatibility is an example of a company that provides commercial DNA analysis services for *Rosaceous* species in the US.

5.1.1 Selection of *S*-RNase consensus primers

The first intron *S*-RNase primers, PaConsI-F and PaConsI-R, were selected over the second intron *S*-RNase primers, PaConsII-F and PaConsII-R, for the PCR amplification of Simone, Sweet Georgia and SLK as the former option provided higher variation in amplicon size between the two alleles. We now know that SLK's *S*-genotype is S_1S_3 and the reason that PaConsII-F and PaConsII-R produced fragments that comigrated together is because the expected size fragments for S_1 and S_3 using these primers is 874bp and 898bp, respectively (Sonneveld et al., 2003). Although PaConsII-F and PaConsII-R may have been used to separate the S_1 (874bp) and S_4 (1064bp) alleles of Simone and Sweet Georgia, the second intron PCR products are much larger than the first intron PCR products using PaConsI-F and PaConsI-R (S_4 – 523 bp, S_1 – 456 bp and S_3 – 303 bp), making them time consuming to separate using electrophoresis (Sonneveld et al., 2003). Interestingly, the trial PaConsI-F and PaConsI-R PCR reaction using a gradient of annealing temperatures found that 60 ˚C was an optimum annealing temperature. This is contrary to the 54˚C annealing temperature used by Sonneveld et al. (2003) and is likely due to the higher concentration of $MgCl_2$ used in this study.

An alternate method that has been used to sequence *S*-alleles in cherry is that used by Vaughan et al. (2008). To identify new *S*-alleles the primers PaConsI-F and PaConsII-R were used to amplify the first and second intron of the *S*-locus. By extracting the gel bands that represent each *S*-allele and inserting the PCR product into a plasmid for cloning, enough target DNA was amplified to further separate each intron of each allele for sequencing. Prior to this method further steps were required to isolate the individual allele in the form of RNA from flower material before cloning and sequencing (Sonneveld et al., 2001). The methods used in this study circumvent the need for DNA cloning; however, had the agarose gel electrophoresis failed to separate the two alleles, cloning would have been required.

5.2 Experiment 2 – identification of pollen donor cultivars for Kordia and Regina fruit.

5.2.1 Evaluation of S-allele specific *SFB* primers

We designed seven *SFB* primer sets to specifically amplify S_1, S_3, S_9, S_{12}, S_{13}, S_{26} and S_{36} (Table 7) because of there exists a limited number of published primers for *SFB* haplotypes (Yamane et al., 2003, Ikeda et al., 2005, Tsukamoto et al., 2006, Tsukamoto et al., 2010). Additionally, we included the S_3 and S_6 specific *SFB* primers reported by Yamane et al. (2003) to determine the seed genotypes of Kordia and Regina seeds. Table 7 reports S6-C2F, S6-C3R and FB3F with

additional nucleotides (G, C and TG, respectively) compared to the original primer sequence; by increasing their GC content we aligned the annealing temperature of all primer sets making them suitable for multiplex PCR. For the same reason, new primers were designed for *SFB*$_{13}$ and *SFB*$_{36}$ even though primers for these haplotypes have been published previously (Tsukamoto et al., 2006, Tsukamoto et al., 2010). An alternate approach to genotyping the *S*-locus of sweet cherry plants is to use the well-established *S*-RNase (Sonneveld et al., 2003) and *SFB* (Vaughan et al., 2006) consensus primer sets to infer *S*-allele identity based on amplicon sizes using capillary electrophoresis (Sharma et al., 2016, Marchese et al., 2017, Ivanovych et al., 2018). Due to the small scale of this study, these high throughput methods were less cost-effective compared to the use of specific *S*-allele primer sets. We provide evidence that the new primer sets produce the correct amplification of positive samples, and no false positives were observed in all reactions. Additional screening for false positives is recommended before using these primers to discriminate against additional *S*-alleles.

5.2.2 Putative identification of the *S*-alleles of Kentish sour cherry

The *S*-allele profile of the Kentish polliniser cultivar that was planted in one part of the Reid Fruits orchard (A Hall 2021, pers. comm.), was tentatively identified as *S*$_6$*S*$_{13}$*S*$_{36}$. In the literature, Kentish is described as one of three subspecies of sour cherry (*Prunus cerasus* L.) and is synonymous with Amarelles (Dondini et al., 2018). There is little consensus regarding the cultivar identity of Kentish. A report of three Kentish varieties; Kentish Morello, Kentish Red A and Kentish Red C, can be found in Tobutt et al. (2004). Although the exact identity of the Kentish cultivar in our study is unclear, we report the *S*-alleles to be *S*$_6$*S*$_{13}$*S*$_{36}$. Compared to the varieties used in Tobutt et al. (2004), the Kentish cultivar used in the current study corresponds with Kentish Morello or Kentish Red C, both reported as *S*$_6$*S*$_{13}$*S*$_B$. The *S*$_B$ allele was later sequenced by Bošković et al. (2006) and assigned to *S*$_{36}$ by Tsukamoto et al. (2010). The presence of an *S*$_{36}$ allele for the Kentish cultivar in this study is reported using a consensus *SFB*$_{36}$ primer set and would require sequencing to confirm the precise *S*-locus sequence identity. Furthermore, although we designed *SFB* primers for *S*$_{36a}$ and *S*$_{36b}$ (Table 7), we did not test them as no *S*$_{36}$ alleles were detected in Kordia or Regina offspring.

5.2.3 Identification of Kordia and Regina pollinisers

The paternal *S*-allele was successfully identified for Kordia and Regina seeds using the *SFB* primer sets. At Hansen Orchards a single polliniser was present (Sylvia) for Regina flowers, and this was

detected by a single paternal allele being present in all Regina seeds. A greater number of cultivars were present at Reid Fruits and were excluded as potential pollen donors based on their distance from sampling sites and full bloom dates. It is common for most pollen to be dispersed within a 30 m radius among neighbouring flowers and Regina yields have been reported to be reduced by 52 – 59% for individuals greater than 9 m from a polliniser tree (García et al., 2007, Núñez-Elisea et al., 2008). Moreover, the flowering overlap of two cultivars that is required for pollination is approximately five to eight days (Radičević et al., 2015). For these reasons, we deduce that pollen donors at Reid fruits for Kordia seeds to be Sylvia and Regina, and Regina seeds to be Kentish, Kordia, Fertard and Sylvia. For example, although Lapins (S_1S_4') is genetically compatible with Kordia, these plants reached full bloom seven days prior to Kordia (poor flowering overlap) and were ~300m from the closest sample location (figure 9). A limitation of this study is that seed samples could have been sired by more than one polliniser cultivar (e.g. Kordia or Fertard for Regina seeds), and as suggested by Sebolt and Iezzoni (2009), paternity in this case can be determined using SSR markers. Similar to Guajardo et al. (2017) we narrowed the potential pollen donors for target cultivars using *S*-genotyping that makes the SSR analysis less time consuming and requires SSR loci.

5.2.4 Microsatellite marker evaluation

Analysis of potential pollen donors with the same *S*-alleles using SSR markers revealed that out of the thirteen markers tested, BPPCT-005, BPPCT-026, BPPCT-028, BPPCT-037 and PMS-67 produced independent alleles within potential pollen donor groups (Table 11). Unfortunately, the resolution of SSR products separated on agarose gel was not sufficient in most cases to precisely discern between the allele size differences (<10bp). Consequently, it is unclear if any of the reported homozygous alleles were in fact heterozygous alleles that were close in product size. Furthermore, BPPCT-034, BPPCT-038, BPPCT-039, PMS-30, UCD-CH12 and UCD-CH26 failed to successfully amplify template DNA from all cultivars, regardless of attempts to optimise the PCR conditions. The current study uses all the SSR markers (BPPCT-026, BPPCT-038 PMS-30 PMS-67, UCD-CH11, UCD-CH12 and UCD-CH26) that Guajardo et. al. (2017) reports for the paternity analysis of cv. Rainier seeds in an open pollinated orchard. This study doesn't report amplicon sizes, or the thermocycler program used; unfortunately, it is not possible to compare my results with this study or whether the SSR markers that failed in this study were a facet of the cultivars being researched. It is unclear whether further optimisation will produce amplification in all cultivars or if the lack of amplification is simply due to the occurrence of null alleles at these

loci. The results that we report must be considered as estimates to be confirmed using capillary electrophoresis that can more accurately size the alleles present in all cultivars as used in the linkage mapping study by Guarino et al. (2009).

Interestingly, a trial using leaf DNA and the marker BPPCT-039, produced amplicons that were visually distinct between Kordia and Fertard. However, when used as controls on a gel with Regina seed samples the amplicons for Kordia and Fertard could not be discriminated. This is most likely due to slight changes in electrophoresis conditions; the first gel being run at 7 V/cm for 3 hours, and the second was run at 4 V/cm for 6 hours. Higher resolution methods, e.g., capillary electrophoresis, is required to accurately determine the product sizes of BPPCT-039 for the cultivars used in this study.

6. Conclusion and industry recommendations

In this study, DNA sequencing confirmed that both Simone and Sweet Georgia have the same *S*-allele profile as another important self-pollinating commercial cultivar, Lapins (S_1S_4'). Clarification of the *S*-genotype, particularly of Simone, will help orchardists make informed decisions about pollination compatibility of new cherry cultivars. That said, in terms of pollination performance, while all three cultivars offer the same *S*-alleles, it is important to recognise that the use of these cultivars as pollinisers is not based on *S*-allele profile alone. Other factors to consider include crop load, evenness of fruit maturity and fruit quality, which differ amongst Simone, Sweet Georgia and Lapins. Ultimately, the sweet cherry industry will continue to utilise all three cultivars to minimise risk of seasonal variation in pollination performance (N Owens 2021, pers. comm.).

I also confirmed that SLK had the same *S*-allele profile as cv. Regina (S_1S_3), supporting suggestions that it could be a 'sport' of a Regina plant. Regina is considered a late flowering cultivar and therefore has a limited number of potential pollinisers (Radičević et al., 2015). As such, the identification of a new cultivar that has similar desirable characteristics as Regina but with earlier flowering (A Hall 2021, pers. comm.) will offer industry a valuable alternative option in orchard design.

Although we report the proportion of paternal *S*-alleles that were inherited by Kordia and Regina seeds, a limitation of this study is that we were unable to definitively confirm which polliniser is responsible for a majority of Kordia or Regina fruit set. Therefore, unlike previous studies (Granger, 2004, Sebolt and Iezzoni, 2009, Guajardo et al., 2017, Gasi et al., 2020, Meland et al.,

2020), we were unable to make any conclusions regarding polliniser performance of the cultivars for seeds collected from Jericho. However, the fact that both Kordia and Regina seeds contained the paternal *S*-alleles of cultivars that were planted with intention of being active pollen donors (A Hall 2021, pers. comm.), suggests that poor fruit set reported by Reid Fruits must be the result of factors other than pollen compatibility.

In terms of molecular approaches to examine parental analysis in sweet cherry, my results confirm that both *S*-allele specific *SFB* primers combined with the use of microsatellites can be used to investigate pollination performance of other important cherry cultivars. In particular, the allele-specific detection can be further developed for multiplex PCR that can generate fast and economic information about pollination success in open pollinated orchards like that offered by the US company RoSBREED (RoSBREED, 2021).

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8 Appendices

Appendix A - Alignment of *SFB* primers and sequences[4]

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SFB_1           TGGTGAGACTACCTACAAAGTCCCTCGTTCGATTTCTGTGTACATGCAAGTCATGGAGTG
SFB_3           TAGTAAGACTACCTGCAAAATCCCTCATTCGGTTTCTATGTACATGCAAGTCGTGGAGTG
SFB_4           TAGCAAGACTACCAGCAAAATCCCTCATTCGGTTTCTTTGTACATGCAAGTCATGGAGTG
SFB_4p          TAGCAAGACTACCAGCAAAATCCCTCATTCGGTTTCTTTGTACATGCAAGTCATGGAGTG
SFB_6           TAGTAAGACTACCTGCAAAATCCCTTGTTCGGTTTCTGTGTACATGCAAGTCATGGATTG
SFB_9           TAGCAAGACTACCTACAAAATCCCTTGTTCGGTTTCTTTGTACATGCAAGTCATGGAGTG
SFB_12          TGGTAAGACTACCTGCAAAATCACTCGTTCGATTTCTGTGTACATCCAAGTTATGGAGTG
SFB_13          TAGTAAGACTACCTGCAAAATCCCTCGTTCGATTTCTGTGTACATGCAAGTCATGGAGTG
SFB_26          TAGTAAGACTACCTGCAAAATCCCTCCTTCGGTTTCTTTCTACATGCAAGTCATGGAGTG
SFB_36a         TAGTAAGATTACCTGCAAAATCCCTCGTTCGGTTTCTGTGTACAAGCAAGTCTTGGAGTG
SFB_36b         TAGTAAGATTACCTGCAAAATCCCTCGTTCGGTTTCTGTGTACAAGCAAGTCTTGGAGTG
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SFB_1           ATTTTATTGGCAGCTCGAGTTTTGTTAGCACACACCTTGATAGGAATGTCACAAAACATG
SFB_3           ATTTGATTGGCAGCTCAAGTTTTGTTCGCACACACCTTCATAGGAATGTCACAAAACATG
SFB_4           ATTTGATTGGCAGCTCGAGTTTTGTCAGCACACACCTTCATAGGAATGTCACAAAGCATG
SFB_4p          ATTTGATTGGCAGCTCGAGTTTTGTCAGCACACACCTTCATAGGAATGTCACAAAGCATG
SFB_6           ATTTGATTGGCAGCTCCAGTTTTATCAGCACACACCTTCATAGGAATGCCACAAAACATA
SFB_9           ATTTGATTGGCAGCTCGAGCTTTGTTAGCACACACCTTCATTGGAATGTCACAAAACATG
SFB_12          ATTTGATTGGCAGCTCGAGCTTTGCTAGCATGCACCTTCATAGGAATGTCGCAAAACATG
SFB_13          ATTTGATTGGAAGCTTGAGTTTTGTGAGCACACACCTTCATAGGAATGTCACAAAGCATG
SFB_26          ATTTGATTGGCAGCTCGAGTTTTGTCAGCACACATCTTCATAGGAATGTCACAAAACATA
SFB_36a         ATCTGATTGGCAGCTCGAGTTTTGTTTGCACACACCTTCATAGGAATGTCGCAATACATG
SFB_36b         ATTTGATTGGCAGCTCGAGTTTTGTTTGCACACACCTTCATAGGAATGTCACAATACATG
                ** * ***** ****  ** ***    ***  ** *** ** ****** * ***  ***

SFB_1           CCCATGTCTATCTACTCTGCCTCCACCACCCAAATTTTGAATGTCACGTCGACCCTGATG
SFB_3           CTCATGTCTATCTACTTTGCCTTCACCACCCACAATTTGAACGTCAGAACGACAATGATG
SFB_4           ATCATGTCTATCTACTTTGCCTCCACCACCCAAATTTTGAACGTTTGGTCGACCCTGATA
SFB_4p          ATCATGTCTATCTACTTTGCCTCCACCACCCAAATTTTGAACGTTTGGTCGACCCTGATA
SFB_6           CCCATGTCTATCTACTATGCCTCCACCACCCAAATTTTGAACGAAACGACGACCCTGATG
SFB_9           CCCGTGGCTATCTACTTTGTCTCCACCACCCAGATGTTGAACGTCAAGCCGACCCTGATG
SFB_12          CCCATGTCTATCTCCTCTGCCTCCACCACCCAAATGTTAGACGTCAGGTCCACCCTGATG
SFB_13          ACCATGTCTATCTACTTTGCCTCCACTACTCAAATTTTGAACTTCAGGCTGATCCCGATG
SFB_26          CCCATGTCTATCTACTCTGCCTACACCACCCAAATTTTGAACGAAACGAGGATCCTGATG
SFB_36a         CCCATGTCTATCTACTTTGCCTCCACCCCTCAAATTTTGAATGGGCGGTCGATCCTGATG
SFB_36b         CCCATGTCTATCTACTTTGCCTCCACCCCTCAAATTTTGAATGGGCGGTCGATCCTGATG
                  * ** ****** ** ** ** ***  * ** *  **  *          *    ***

SFB_1           ACCCATATGTTAAAAAAGAATTTCAATGGTCTCTTTTTCCCAATCAAACATGTGAGGTGT
SFB_3           ACCCATATGATATAGAAGAACTTCAGTGGTCACTTTTTTCCAATGAAAAGTTTGAGCAGT
SFB_4           ACCCATATTTTAAAAAGGAATTTCAATGGTCTCTTTTTTCCAATGAGACATTTAAGCAGT
SFB_4p          ACCCATATTTTAAAAAGGAATTTCAATGGTCTCTTTTTTCCAATGAGACATTTAAGCAGT
SFB_6           ACCCATATGTTGAACAAGAATTTCAATGGTCTCTTTTTTCCAATGAAACATTTGAGGAGT
SFB_9           ACCCATATGTTAAACAAGAACTTCAATGGTCTCTTTTTTCCAACGAAACATTTGAGGAGT
SFB_12          ACCCATATGTTCAACAAGAATTTCAATGGTCTCTTTTTTCCAATGAAACATCTGAGAAGT
SFB_13          ACCCACATGTTAAACAAGAATTTCAATGGTCTCTTTTTTCCAATCAAACATTTGAGGAGT
SFB_26          ACCCATATGTTGAACAAGAATTTCAATGGTCTCTTTTTTCGAATGAAACATTTGAGGAGT
SFB_36a         ACCCATATGTTAAACAAGAACTTCAATGGTCTCTTTTCTCCAATGAAACATTTGAGAAGT
SFB_36b         ACCCATATGTTAAACAAGAACTTCAATGGTCTCTTTTCTCCAATGAAACATTTGAGAAGT
                ***** **  *  * * *** **** ***** *****  * **  * *  * * **  **
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[4] Highlighted nucleotides indicate primer oligonucleotides

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SFB_1          TCTACAAGTTAAGCCATCCCTTAGGGAACACAGAACATTATGGGATATATGGTTCAAGCA
SFB_3          TCTCCAATTTAAGCCATCCTTTAGAAAACACAGAGCATTTTAGAATATATGGTTCAAGCA
SFB_4          GCTACAAGTTAAATCATCCCTTAGGCAGCACAGAACATTATGTGATATATGGTTCAAGCA
SFB_4p         GCTACAAGTTAAATCATCCCTTAGGCAGCACAGAACATTATGTGATATATGGTTCAAGCA
SFB_6          GCTCCAAGTTAAGCCATCCCTCAGGGAGCACAAAACATTATGTGATATATGGCTCAAGCA
SFB_9          GCTCCAAGTTAAGCCATCCCTTAGGTAGCACAGAACATTATATGATATATGGCTCAAGCA
SFB_12         GTTTCAAGTTATGCCATCCCCTAGGGAGCACAGAATATTTTGTGATATATGGTTCAAGCA
SFB_13         GCTCCAAGTTAAGCCATCCCTTAGGGATCACAGAACATTATGTGATGTATGGCTCAAGCA
SFB_26         GCTCCAAGTTAAGCCATCCCTTAGGGAGCACAGAACATTATGTGATATACGGTTCAAGCA
SFB_36a        GCTTTGAGTTAAGACATCCCATAGGGAGCACAGAACATTACGGAATATATGGTTCAAGCA
SFB_36b        GCTTTGAGTTAAGACATCCCATAGGGAGCACAGAACATTACGGAATATATGGTTCAAGCA
                 *   * ***   *****   **  * **** *  ***     ** ** ** *******

SFB_1          ATGGTTTAGTTTGCATTTCGGATGAGATACTGAATTTCGATAGTCCTATACACATATGGA
SFB_3          ATGGTTTAGTTTGCATGTCGGATGAGATATTGAATTTCGATAGTCCTATACAAATATGGA
SFB_4          ACGGTTTAGTTTGCATTTCTGATGAGATATTGAATTTTGATAGTCCTATACACATATGGA
SFB_4p         ACGGTTTAGTTTGCATTTCTGATGAGATATTGAATTTTGATAGTCCTATACACATATGGA
SFB_6          ATGGCTTAGTTTGCATTTCGGAGGAGATATTGAATTTCGATAGTCCAATACACATATGGA
SFB_9          ACGGTTTAGTTTGCATTTCGGATGAGATACTGAATTTCGATAGTCCTATACACATATGGA
SFB_12         ACGGTTTAGTTTGCGTTTCGGATGAGATATTGAATTTCGATAGTCCTATACACATATGGA
SFB_13         ATGGATTAATTTGCATTTCGGATGAGATACTGAATTTTGATAGTCCTATACACATATGGA
SFB_26         ATGGTTTAGTTTGCATTTCCGATGAGATACTGAATTTCGATAGTCCTATACACATATGGA
SFB_36a        ATGGCTTAGTTTGCATTTCGGATGAGATATTGAATTTCGATAGTCCTATACACATATGGA
SFB_36b        ATGGCTTAGTTTGCATTTCGGATGAGATATTGAATTTCGATAGTCCTATACACATATGGA
               * ** *** ***** * ** ** ****** ******* ******** ***** *******

SFB_1          ACCCATCGGTTAGGAAACTTAGAACCACACCAATCAGCACCAACATTAACATGAAATTTA
SFB_3          ACCCATCGGTTAGGAAATTCAGGACTCTTCCAATGAGCACCAACATTAACATGAAATTTT
SFB_4          ACCCATCGGTTAGGAAATTTAGGACCCCTCCAATGAGCACTAGCATTAACATTAAATTTA
SFB_4p         ACCCATCGGTTAGGAAATTTAGGACCCCTCCAATGAGCACTAGCATTAACATTAAATTTA
SFB_6          ACCCCTCGGTTAAGAAATTTAGGACTCCTCCAATGAGCACCAACATTAACATTAAATTTA
SFB_9          ACCCATCGGTTAGGAAACTTAGAACCACTTCAATGAGCACCAACATTAACATTAAATTTA
SFB_12         ACCCATCGGTTAGGAAATTTAGGACCATTCCAATGAGCACCAATACTAACATCAAATTTG
SFB_13         ACCCATCGGTTAGGAAACTTAGAACCACCCCAATCAGCACCAACATTAACATTAAATTTA
SFB_26         ACCCATCGGTTAGGAAACTTAGATCCACTCCAATCAGCACCAACATTAACATTAAATTTA
SFB_36a        ATCCATCGATCAGGAAATTTAGAACCCCTCCAATGAGCACCGACATTAACATTAAACATA
SFB_36b        ATCCATCGATCAGGAAATTTAGGACCCCTCCAATGAGCACCGACATTAACATTAAACATA
               * ** *** * * **** * **  *     **** *****    * ****** ***  *

SFB_1          GCCTTGTTTCTCTTCAATTTGGGTTCCACCCCGTGGTTAATGACTATAAGGCTGTACGGA
SFB_3          CCCATGTTTCTCTCCAATTTGGGTTCCACCCCGGGGTTAATGACTACAAGGCTGTAAGGA
SFB_4          ATTATATTGCTCTCCAGTTTGGGTTCCACCCCAGGGTTAATGACTACAAGGCTGTAAGGA
SFB_4p         ATTATATTGCTCTCCAGTTTGGGTTCCACCCCAGGGTTAATGACTACAAGGCTGTAAGGA
SFB_6          GCCATGTTGCTCTCCAATTCGGGTTCCACCCTGGGGTTAACGACTATAAGGCTGTAAGGA
SFB_9          GCCATGTTGCTCTCCAATTCGGGTTCCACCCTGGGGTTAATGACTACAAGGCTGTAAGGA
SFB_12         CCTATCTTGCTCTCCACTTCGGGTTCCACCCTGGGATTAATGACTACAAGGCGGTAAGGA
SFB_13         GCCACGTTGCTCTCCAATTCGGATTCCACCCCGGAGTTAATGACTGCAAGGCTGTAAGGA
SFB_26         GCCATATTGCTCTACAATTCGGGTTCCACCCCGGGGTTGATGACTACAAGGCTGTAAGGA
SFB_36a        GTTATGTTGCTCTCCAATTCGGGTTCCACCCCCGGGTTAATGACTACAAGATTGTAAGAA
SFB_36b        GTTATGTTGCTCTCCAATTCGGGTTCCACCCCCGGGTTAATGACTACAAGATTGTAAGAA
                     ** **** ** ** ** ********     ** * ****  ***   *** * *

SFB_1          TGATGCGTACCAACAAAGGTGCCTTGGCGGTTGAGGTTTATAGTCTTAGAACAGACTCTT
SFB_3          TGATGCATACCAACAAAGGTGCCTTGGCAGTTGAGGTTTATAGTCTTAAAACAGATTGTT
SFB_4          TGATGCGTACGAACAAAGATGCCTTGGCGGTTGAGGTTTATAGTCTTAGAACAGACTCTT
SFB_4p         TGATGCGTACGAACAAAGATGCCTTGGCGGTTGAGGTTTATAGTCTTAGAACAGACTCTT
SFB_6          TGATGCGTACCAACAAAAATGCCTTGGCAGTTGAGGTTTATAGTCTCAAAACAGACTCTT
SFB_9          TGATGCGTACCAACAAAAATGCCTTGGTGGTTGAGGTTTATAGTCTCAGAACAGACTCTT
SFB_12         TGATGCGTACCAACAAAAATGCCTTGGCGGTTGAGGTTTATAGTCTTAGAACAGACTCTT
SFB_13         TGATGCGTACCAACAAAAATACCTTAGCGGTTGAGGTTTATAGTCTTAAAACAGACTCTT
SFB_26         TGATGCGTACCAACAAAAATGCCTTCGCTGTTGAGGTTTATAGTCTTAAAACAGACTGTT
SFB_36a        TGATGCGTACCAACAAAGATGCCTTCGCCGTTGAGTTTTTTAGTCTTGGAACGGACTCTT
SFB_36b        TGATGCGTACCAACAAAGATGCCTTCGCCGTTGAGGTTTTTAGTCTTGGAACGGACTCTT
               ****** *** ******  * **** *  ****** *** ******   *** ** * **
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SFB_1           GGAAGATGATTCAAGCAATTCCTCCTTGGTTAAAATGCACTTGGCAGCATCATAAGGGTA
SFB_3           GGAAGATGATTGAAGTAATTCCTCCTTGGTTAAAATGCACTTGGAAGCATCATAAGGGTA
SFB_4           GGAAGATGATTGAAGCAATTCCTCCTTGGTTAAAATGCACTTGGCAGCATCATAAGGGTA
SFB_4p          GGAAGATGATTGAAGCAATTCCTCCTTGGTTAAAATGCACTTGGCAGCATCATAAGGGTA
SFB_6           GGAAGATGATTGAAGCAATTCCCCCTTGGTTAAAATGCACTTGGCAGCATCATAAGGGTA
SFB_9           GGAAGATGATTAAAGCAATTCCTCCTTGGTTAAAATGCACTTGGCAGCCTTTTAAGGGTA
SFB_12          GGAAGATGATTGAAGCTATTCCTCCATGGTTAAAATGCACTTGGCAGCATCATAAGGGTA
SFB_13          GGAAGATGATTGAAGCAATTCCTCCTTGGTTAAAATGCACTTGGCAGCATCTTAAGGGTA
SFB_26          GGAAGATGATTGAAGCAATTCCTCCGTGGTTAAAATGCACTTGGCAGCATCATAAGGGTA
SFB_36a         GGAAGATGATTGAAGCAATTCCGCCTTGGTTAAAATGCACTTGGCAGCATCAAATGAGTA
SFB_36b         GGAAGATGATTGAAGCAATTCCGCCTTGGTTAAAATGCACTTGGCAGCATCAAATGAGTA
                *********** ***  ***** ** ****************** *** *   * * ***


SFB_1            CATTTTCTAATGGAGTAGCATACCACATCATTGAGAAAGGTCATATAATCAGCATTATGT
SFB_3            CATTTTTTAATGGAGTAGCATACCACATCATTGAGAAAGGTCCTATATGCAGCATTATGT
SFB_4            CGTTTTTTAATGGAATATCATACCACATCATTGAGAAATGTCCTATATTCAGCATTATGT
SFB_4p           CGTTTTTTAATGGAATATCATACCACATCATTGAGAAATGTCCTATATTCAGCATTATGT
SFB_6            CATTTTTTAATGGAGTAGCATACCATATCATTGAGAAAGGTCCTATATGCAGCATTATGT
SFB_9            CATTTTTTAATGGAGTAGCATACCACATCATTCTGAAAGGTCCTATATTCAGCATTATGT
SFB_12           CATTCTTTGATGGGGTATCATATCACATCATTGAGAAAGGTCCTATATTCAGTATTGTGT
SFB_13           CAATTTTTAATGGAGTAGCATACCACATCATTCAGAAAGGTCCTATATTCAGCATTATGT
SFB_26           CATTTTTTAATGGAATAGCATACCACATCATTGAAAAAGGTCCTATATTCAGCATTGTGT
SFB_36a          TATTTTCTAACGGAGTAGCGTACCACCTCCTTAGGAAAGGTCCTATATTCAGCATTATGT
SFB_36b          CATTTTCTAACGGAGTAGCATACCACCTCCTTAGGAAAGGTCCTATATTCAGCATTATGT
                    * * * * **  ** * ** **  ** **   *** *** ****  *** *** ***

SFB_1            CATTTAATTCAGGCAGCGAAGAATTTGAAGAGTTCATAGCACCAGATGCCGTTTGTAGTT
SFB_3            CATTCGATTCAGGCAGCGAAAAATTCGAAGAATTCATAACACCAGATGCCATTTGCAGTC
SFB_4            CATTCGATTCAGGCAGTGAAGAATTCGAAGAGTTCATAGCACCAGATGTCATTTGCAGTT
SFB_4p           CATTCGATTCAGGCAGTGAAGAATTCGAAGAGTTCATAGCACCAGATGTCATTTGCAGTT
SFB_6            CATTCGATTCAGGCAGTGATGAATTCGAAGAATTCATAGCACCGGATGCCATTTGCAGTC
SFB_9            CATTCGATTCAGACAGTGAAGAATTCGAAGAATTCATAGCACCAGATGCCATTTGCCATT
SFB_12           CCTTTGATTCAAGCAGCGAAGTATTCGAAGAATTCATAGCACCAGATGCCATTTGCCGTC
SFB_13           CTTTTGATTCAGGCAGTGAAGAATTCGAAGAATTCATAGCACCAGATGCCATTTGCAGTT
SFB_26           CATTCGATTCAGGCAGTGAAGAATTCGAAGAATTCATAGCACCGGATGCCATTTGCACTT
SFB_36a          CATTCGATTCAGGCAGTGAAGAATTCGAAGAATTCATAGCACCAGATGCCATTTGCAGTT
SFB_36b          CATTCGATTCAGGCAGTGAAGAATTCGAAGAATTCATAGCACCAGATGCCATTTGCAGTT
                 * **  *****  *** **   *** ***** ****** **** **** * ****   *

SFB_1            CATGGAGGTCATGCATCGAGGTTTACAAGGAACAAATTTGTTTGCTTCTTGACTTTTATC
SFB_3            CACGGGAGTTATGCATTGACGTCTACAAGGAACTAATTTGCTTGATTTTTGGATTTTATG
SFB_4            CATGGGGGTTATTTATTGACCTTTACAAGGAACAAATTTGCTTGCTTTCTAGCTTTTATT
SFB_4p           CATGGGGGTTATTTATTGACCTTTACAAGGAACAAATTTGCTTGCTTTCTAGCTTTTATT
SFB_6            CATCTGAGTTATGTATTGACATTTACAAGGAACGAGTTTGCTTGCTTTTTAGCTTTTATT
SFB_9            CATGGGAGTTATGTATCGATGTTTACAAGGAACAAATTTGCTTGCTCTTTAGCTGTTATT
SFB_12           CATTTGCTTTATGTATTGATGTTTTCAAGGAGCAAATTTGCTTGCTTTTTAGATATTATT
SFB_13           CATGGGGGTTATGTATTGACGTTTACAAGGAACAAATTTGCTTGCTTCTTAAGTTTTATT
SFB_26           CAGTGGGGTTATGGATCGACGTTTACAAGGACCAGATTTGCTTGCTTTTTAAATGTTACG
SFB_36a          CATGGGGGTTATGTATTGACGTTTACAAGGAACACATATGCTTGCTTTTTAGATTCTATG
SFB_36b          CATGGGGGTTATGTATTGACGTTTACAAGGAACACATATGCTTGCCTTTTAGATTCTATG
                 **      * **  ** **  * * ****** *   * ** ***     *   *  **

SFB_1            CTTGTGAGGAGGAGGGCATGGAAAAATTTGACTTGTGGGTTCTGCAAGAAAAACGGTGGA
SFB_3            GTTGTGATGAGGAGGGCATGGACAAAGTTGACTTGTGGGTTCTGCAGGAAAAACGGTGGA
SFB_4            CTTGCGAGGAGAAGGGTATGCGAAAAATTGACTTCTGGGTTCTGCAAGAAAAACGGTGGA
SFB_4p           CTTGCGAGGAGAAGGGTATGCGAAAAATTGACTTCTGGGTTCTGCAAGAAAAACGGTGGA
SFB_6            CTTGTGACGAAGAGGGCATGGTACCAAATGACTTATGGGTTCTGCAAGAAAAACGATGGA
SFB_9            CTTGTGAAGAGGAGGACATGGAAAAAGTTGACTTATGGGTTCTGCAAGAAAAAAGGCGGA
SFB_12           CTTGTGCGGAGGAGGACATGGCAAAAAATGACTTATGGGTTCTGGAAGAAAAACGGTGGA
SFB_13           CTTGTGAGGTGGAAGGCATGAAGAAAATCGACTTATGGGCTCTGCAAGAAAAACGGTGGA
SFB_26           GTTGTGAGGAGGAGGGCATGGATAAAGTTGATTTATGGGTTCTGCAAGACAAACGGTGGA
SFB_36a          GTTGTGAGGAGGAGGGCATGGAACAAGTTGACTTATGGGTCCTAAAAGAAAAACGGTGGA
SFB_36b          GTTGTGAGGAGGAGGGCATGGAACAAGTTGACTTATGGGTCCTAAAAGAAAAACGGTGGA
                  *** *  *   * *  ***     *   ** ** ****  **  * ** *** *  ***
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SFB_1           AACAATTGTGCCCTTTTATTTAT---TCTTCAGATTATTGTTATCGTACAATCGGGATTA
SFB_3           AACAATTGTGTCCTTTTATTTTT---CCTTTGAATCATTGTCATCGTACAATCGGGATTA
SFB_4           AACAATTGTGTCCTTTTATTTAT---CCTTC---TCATTATTATGGTACACTCGGTATTA
SFB_4p          AACAATTGTGTCCTTTTAT-------CCTTC---TCATTATTATGGTACACTCGGTATTA
SFB_6           AACAATTGTGTCCTTTTATTTAT---CCTGC---TGGTAGTTATGGTACAATCGGGATAA
SFB_9           AATTGTTGTGCCTTTTATTTAT---CCTTTGGGTTATGATTATCGTCCAATCGGGATTA
SFB_12          AACAAATGTGTCCTTTTATTTAT---CCTCT---TGATAGTTATGGTACAATCGGGATTA
SFB_13          AACAATTGTGTCCTTTTACTTTT---TCTTTGGATTACAATTATCGTACAATCGGGATTA
SFB_26          AACAATTGTGTCCGTTTATTTCT---TCTTT---TGATTGTTGCGGTCCAGTCGGAATCA
SFB_36a         AACAATTGTGTCCTTTTATTTAT---CCCCGG---AGCTGTTATCGGACAATGGGGATTA
SFB_36b         AACAATTGTGTCCTTTTATTTAT---CCCCGG---AGCTGTTATCGGACAATGGGGATTA
                **    **** ** ****         *           *    *  ** * ** ** *
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Appendix B – Estimated fragment sizes for SSR loci in sweet cherry cultivars

	Approximate fragment sizes (bp)								
SSR marker	Regina	Lapins	Kordia	Johanna	Fertard	Sylvia	Sweetheart	Penny	Kentish
BPPCT-005	147	159	159	147	153	159	159	159	159/133/113
BPPCT-026	179/156	164	179/156	179/156	157	162	162	157	172/113
BPPCT-028	175/143	175/143	143	175/143	143	143	175/143	175	164
BPPCT-037	152	152	152	147	152	147	152	152	158
BPPCT-040	147/129	119	147/129	147/129	147/129	147/119	119	129	129
UCD-CH11	168/149	149/123	168/149	168/149	149	168/149	149/130	157	157/123
PMS-67	146	141	146	146	149	146	149	153	161