Genetic, Metabolite and Phenotypic Determination of Friction Discolouration in Pear

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ABSTRACT

Friction discolouration (FD) of pears is a postharvest disorder responsible for significant consumer discontent in markets because of the unattractive appearance of the fruit surface. New Zealand pear breeders are aiming to develop novel pear varieties with consumer desired fruit characters (skin colour, flavour and storability), with reduced susceptibility to FD. Therefore understanding the genetic control of FD is essential to enable development of new pear cultivars using genomics-informed breeding. FD is influenced by agronomic and genetic factors. Previous research on this disorder has been limited to a small number of commercial cultivars and no study has been done to understand its genetic basis. Biochemical constituents (polyphenol oxidase activity, phenolic compounds and ascorbic acid concentration) and skin anatomy have been proposed to play important roles on FD susceptibility in a limited number of cultivars. The Plant and Food Research (PFR) breeding population with hundreds of closely related seedlings is an ideal resource to test whether these previously identified associations hold true across multiple genotypes.

In this study, 241 genotypes from two segregating populations (POP369 and POP356) derived from interspecific crosses between Asian (*Pyrus pyrifolia* Nakai and *P. bretschneideri* Rehd.) and European (*P. communis*) pears were used to identify biochemical and genetic factors associated with susceptibility to FD. In 2013, a small replicated trial involving eight genotypes was conducted. Large variability for FD and other variables was recorded. Four different trends were observed for genotypes for which multiple harvests were obtained in a single season. Most of the genotypes were consistently low or consistently high throughout the season, but a proportion (26.1 %) showed an increase in FD susceptibility during the season and a further 15.7 % showed a decreasing trend in susceptibility. Twenty genotypes had multiple harvests in each of 2011 and 2012, and 13 of these showed consistent trends from year to year. These results indicate a significant genetic component to FD but with additional influence from the stage of fruit maturity at harvest and external environmental conditions.

Single nucleotide polymorphism (SNP)-based linkage maps suitable for QTL analysis were developed for the parents of both populations. The maps for population

POP369 comprised 174 and 265 SNP markers for the male and female parent, respectively, while POP356 maps comprised 353 and 398 SNP markers for the male and female parent, respectively. Phenotypic data for 22 variables measured over two successive years (2011 and 2012) were used for quantitative trait locus (QTL) analysis. QTLs linked to phenotyped variables were identified, including QTLs for FD on linkage groups 2, 3, 7, 10 and 14. A number of stable QTLs across the years were detected for some aspects of fruit quality as well as potential risk factors for FD incidence.

Overall, no single underlying phenotypic variable (enzyme or substrate) appeared to act as a rate limiting factor to susceptibility of FD in both populations and in 2013 trial. However certain phenolics consistently appeared to have weak negative association with FD. This suggests a separate role from their typical concept of being a substrate. Identification of stable QTLs controlling firmness, PPO activity, and phenolic compound concentration have also provided future opportunities for identification of candidate genes by utilizing the reference genome sequences of ‘Bartlett’ and ‘Dangshansuli’ pears and syntenic apple ‘Golden Delicious’. This study also demonstrated that FD is controlled by multiple small effect QTLs and genomic selection could be employed to select elite genotypes with reduced susceptibility to FD, early in the breeding cycle.

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Dedicated To My Parents

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LIST OF ABBREVIATIONS

AsA	Ascorbic acid
cDNA	Complementary DNA
cM	Centi morgan
Conc.	Concentration
contig	Contiguous sequence
DA	Discriminant analysis
Da	Dalton
DNA	Deoxyribonulceic acid
FD	Friction discolouration
FMF	Find molecular features
GBS	Genotyping by sequencing
GEBV	Genomic estimated breeding value
GS	Genomic selection
GxE	Genetic x environment
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LOD	Logarithm of odds
MAS	Marker assisted selection
NGS	Next generation sequencing
POP	Population
PPO	Polyphenol oxidase
QTL	Quantitative trait locus
RAD-seq	Restriction site associated sequencing
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
Spp	Specie
SSR	Simple sequence repeat
TSS	Total soluble solids

1 INTRODUCTION

1.1 Pear origin and history

Pear belongs to the tribe Maleae and genus *Pyrus* in the Rosaceae family and is believed to originate from the Northern hemisphere of the "Old World". The genus *Pyrus* contains about 22 species, half of them belong to Europe, North Africa, and Asia Minor; and the other half to Asia (Itai, 2007). The genus *Pyrus* originated in the western and south western mountains of China 65-55 million years back. Two subcentres (Central Asia and Eastern China) are supposed to be involved in the diversity of genus (Vavilov, 1952).

The domestication of a subset of species gave rise to a group of soft-fleshed European pear, and a group of crisp-fleshed and round-shaped Asian pears. Asian pears (e.g. *Pyrus pyrifolia, P. bretschneideri and P. ussuriensis*) are known to be domesticated in China from prehistoric times and have been grown from at least 3000 years (Itai, 2007). European pears (*Pyrus communis*) are believed to have originated in Europe as early as 1000BC. The first documented record for pear cultivation in Europe was from the Greek poet Homer, who mentioned a large orchard of pears and referred this fruit as "gifts of the gods" in his famous poem Odyssey written about 1000 BC (Hancock and Lobos, 2008). In the old Chinese medicine book 'Bencao Gangmu' (Shi-zhen Li, 1518-1593) pear was proposed as having various medicinal effects such as antitussive and anti-inflammatory, as well as some diuretic properties (Li, 1982; Cui et al., 2005).

1.1.1 Pear industry in New Zealand

The New Zealand pear industry is mostly located in the Nelson region (South Island) and Hawke's Bay (North Island) (Brewer and Hilton, 2005). Pear trees in dormant conditions can easily endure the New Zealand's cool temperature and rain which is not as severe as in other key growing areas in the world (Palmer, 2011).

Pear trees were introduced in New Zealand in 1819 by Samuel Marsden. The first experimental orchard of European pear was established in 1903 at Weraroa;

Manawatu Region, North Island (Wood, 1997). A number of *P. communis* varieties such as ‘Beurre Bosc’, ‘Doyenne du Comice’, ‘Packham’s Triumph’ and ‘Taylor’s Gold’ were planted in orchards around the country. Initially Asian pears, mainly *P. pyrifolia* ‘Nijisseiki’ were grown at a small scale in home gardens. In 1983, *P. pyrifolia* was given the status of commercial crop with 'Nashi' being the major cultivar with almost 30,000 trees planted and a cultivated area reaching 760 ha in 1989. Later the 'Nashi' growers removed most of their trees because they were not as profitable as other crops in terms of growth and income returns. In the early 2000s, the area under 'Nashi' pear cultivation was only 119ha, mainly located in the Auckland, Waikato and Bay of Plenty regions (White, 2001).

Recently, pear growers have shifted to more profitable crops such as apple and kiwifruit. According to Pipfruit NZ Inc. (2008) in 2002 a total of 965 ha were planted using European pear cultivars, which decreased to 722 ha and 412 ha in 2006 and 2008, respectively. Pear imports have increased in proportion to the decreased production, with pear imported from Australia comprising 71% of the total import, and the remainder coming from United States of America and China. New Zealand usually exports 60% of its pear production with cultivars ‘Beurre Bosc’, ‘Comice’, and ‘Taylors Gold’ dominating the exports (Brewer and Hilton, 2005). Pear exports have fluctuated in the last few years with a total of 5,777 tonnes in 2007 of pears (mainly *P. communis*), and 4,309 tonnes in 2011. Production has also declined from 2007 to 2011 (Table 1.1).

Table 1.1: Pear production, import and export in New Zealand

Year	2007	2008	2009	2010	2011
Area Harvested (Ha)	791	750	723	700	619
Production (tonnes)	35,000	32,500	31,800	30,000	27,000
Export Quantity (tonnes)	5,777	4,770	5,512	5,872	4,309
Export Value (1000 US$)	6,598	5,407	5,923	6,571	6,380
Import Quantity (tonnes)	2,919	3,739	3,158	3,873	1,510
Import Value (1000 US$)	4,079	5,790	3,961	5,201	2,236

1.1.2 Pear breeding programme at PFR

The New Zealand Institute of Plant & Food Research Ltd. (PFR) established a pear breeding programme in 1983. The initial focus of the programme was breeding Chinese (*P* × *bretschneideri* Rehd.), Japanese (*P. pyrifolia* Nakai) and European (*P. communis* Linn.) types of pear (White and Brewer, 2002; Brewer et al., 2008; Volz et al., 2008). However, the focus later changed towards the use of inter-specific hybrids (Volz et al., 2008). By combining the best characteristics from Asian and European species, PFR pear breeders have developed new visually distinct pear varieties with novel fruit characteristics such as crisp and juicy flesh, a great diversity of aromas, a long shelf life and that are suitable for eating with or without preconditioning (Brewer et al., 2008; Volz et al., 2008). In general, Asian types are crisper, while the flavour and aromas come from the European types. Along with fruit quality characters phenotypic variability for other traits such as resistance to disease and postharvest disorders became apparent.

1.2 Friction discolouration

In the 1940s, USA Pacific coast fruit shippers noticed that the handling of pears at cool temperature resulted in skin blemishes referred to “finger prints”, “belt burns” or “scald” (Smith, 1946). It was verified that refrigeration temperature was not responsible for increased susceptibility to skin blemishes, but rather that pear fruit lost their resistance to tolerate mechanical damage during the storage period (Smith, 1946; Gomila et al., 2011). Damage is often more extensive after long term storage.

It was discovered that dark surface marks are developed as a result of vibration during the transport, especially to loosely packed fruit which can move inside the package (Sommer, 1957). Mechanical damage can occur at any step during picking, sorting, processing or transportation (Mitcham et al., 2001; Feng et al., 2004). This phenomenon was therefore named “friction discolouration” as the damage is the result of friction force. The damage is usually only confined to the epidermal layer, and moisture loss is higher in injured fruit, leading to the shortening of shelf life. The mechanical damage in the fruit skin leads to the development of brown colour which involves enzymatic browning by the oxidation of phenolic compounds (Wang and

Mellenthin 1973). Fruit with an uneven surface are more prone to friction damage as compared to smooth skin fruit (Thompson, 2007).

Figure 1.1: Pear fruit showing incidence of friction discolouration on the skin.

This postharvest disorder has been described with various names ever since it was first documented (Table 1.2). I will use the term of "friction discolouration" (FD) to refer this disorder as browning induced in this study is through friction damage. On the basis of all these facts FD can be defined as:

"*Mechanical damage to the fruit skin due to pressing, rolling or rubbing against any hard surface or adjacent fruit that leads to enzymatic browning in damaged portion of the skin*".

Table 1.2: Different names for FD used in past

Name given to problem	Documented first time
Finger prints, Belt or brush burn or Scald	Early Pacific Coast pear shippers in USA as mentioned by Smith, 1946
Surface abrasion, Surface discolouration	(Smith, 1946)
Scuffing	(Wang and Mellenthin, 1973)
Friction discolouration	(Wang and Mellenthin, 1973)
Skin browning	(Mitcham et al., 2001)
Peel browning	(Feng et al., 2004)

1.2.1 Detrimental effects of FD

The nutritive value and flavour of fruit affected by FD remain unchanged, however the unattractive appearance of affected pear fruit poses problem to the industry. Along with the poor cosmetic value, mechanical injury enhances respiration and moisture loss from the injured area, which then increases ethylene production and results in rapid ripening and shorter shelf life than for non-damaged fruits (Sommer, 1957; Agar and Mitcham, 2000; Agar et al., 2000).

FD has been described as “one of the most serious postharvest problems in the pear industry” (Amarante et al., 2001a). The New Zealand pear industry commissioned a study from PFR to study the problem in a variety known to be highly susceptible ‘Comice’, (Palmer et al., 2007). Industry practices have been modified in efforts to alleviate this disorder: wearing of gloves for picking, use of smaller bins at harvest time and avoidance of abrasion sites on packing lines (Palmer at al. 2007). PFR pear breeders noticed that the problem showed signs of heritability in their breeding populations (Brewer et al., 2011). Further systematic study was required to understand the underlying genetic mechanism governing FD susceptibility in breeding populations.

1.2.2 Internal phenomenon of friction discolouration

1.2.2.1 Enzymatic browning

Cell disruption in plant tissue leads to the mixing of substrate and enzyme to generate coloured end products (brown, black or pink), this phenomenon is known as enzymatic browning. This discolourationwhich is the result of the enzyme catalysed reaction is generally unacceptable for the food industry in as it affects the cosmetic as well as nutritional quality. However in some cases enzymatic browning is desirable such as when it is involved in developing flavour and colour in prunes, dates, tea and tobacco (Nicolas et al., 2003).

The polyphenol oxidase (PPO) enzyme is usually located inside plastids while its substrate (polyphenols) are predominantly inside the vacuole (Barrett et al., 1991). When epidermal cells get injured, de-compartmentalization of both enzyme and substrate occurs and enzymatic browning proceeds in the presence of oxygen (Amiot et al., 1993; Amiot et al., 1995; Nicolas et al., 2003; Franck et al., 2007; Salta et al., 2010). The process of enzymatic browning take place in two steps: The first step involves the catalysis of conversion of monophenols or o-diphenols to oxidation products (o-quinones) in the presence of enzyme and oxygen, followed by a non-enzymatic step known as condensation or polymerization reactions in which unstable o-quinones quickly convert to complex melanin polymers as shown in Figure 1.2 (Lerner, 1953; Sánchez-Ferrer et al., 1995). The process is reversible at first step and o-quinones can be reversed back to respective phenolic compounds through involvement of coupled oxidation or antioxidative reactions (Goupy et al., 1995).

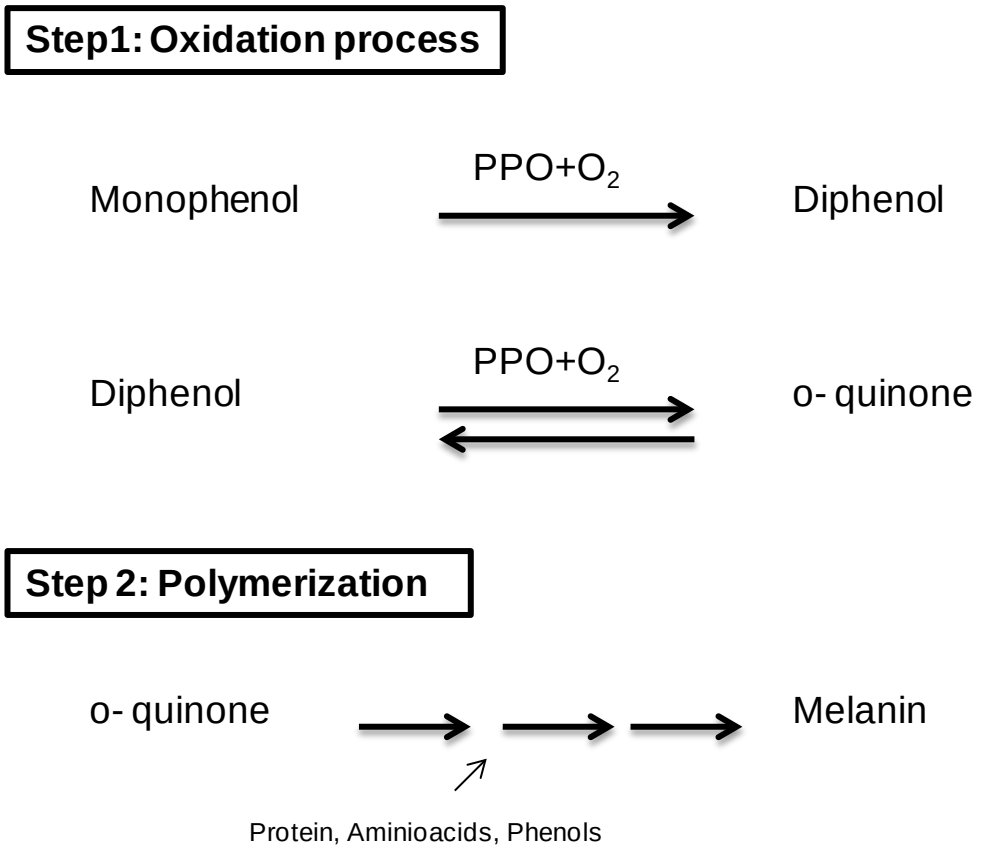


Figure 1.2: Enzymatic reactions catalysed by PPO (Lerner, 1953).

1.2.2.2 Factors involved in FD susceptibility

1.2.2.2.1 Polyphenol oxidase

Polyphenol oxidase (PPO; 1 ,2 benzenediol: oxygen oxidoreductase; EC 1.10.3. l) was first discovered in mushrooms in 1856 by Schoenbein (Schoenbein, 1856). This enzyme is also known by other names such as tyrosinase, phenolase, catechol oxidase, cresolase, or catecholase (Whitaker, 1994; Fraignier et al., 1995; Haruta et al., 1999). It is a copper containing plastid enzyme present in two forms: a bound form in which the enzyme is located on the thylakoid membrane of chloroplasts, anchored by its hydrophobic tail, and a soluble form present in soluble fractions of the cell (Nicolas and Potus, 1994). The enzyme activity is highly variable within the plant between organs and even within organs depending on the tissue type and development. In general, the PPO activity is higher in young green fruit where it is

present mostly in its bound form. The bound form generally decreases as fruit maturation proceeds and the proportion of soluble forms increases to reach a maximum in ripe fruit (Nicolas et al., 1994). The purification and extraction of PPO is difficult because of its aggregate form and its rapid reaction with phenols to form brown pigments. Gauillard and Richard-Forget (1997) found two isozymes and two latent forms of PPO in 'Williams' pear. Presence of intermediate products (quinones) and phenols in crude extracts of the enzyme make the situation even more intricate and this can be controlled by adding phenol-binding agents such as polyvinyl polypyrrolidone (Carbonaro and Mattera, 2001; Yoruk and Marshall, 2003).

1.2.2.2.2 Phenolic compounds

Phenols are a very important group of chemical compounds due to their role in colour and aroma development (Macheix et al., 1990). Phenols are involved in two important oxidative reactions i.e. enzymatic browning and as an antioxidant. The availability of these compounds along with enzyme (PPO) within the pear peel is fundamental for peel browning (Amiot et al., 1995).

Plant susceptibility to browning varies widely even within species and and is believed to be concomitant with both quantity and quality of their phenolic content. Browning intensity not only depends upon the amount but also the of type of phenolics used as susbtrate, for example end products of enzymatic oxidation while using catehin as susbstrate are darker as compared to chlorogenic acid (Goupy et al., 1995).

PPO is highly substrate specific, so from a wide range of phenolics only a few serve as direct substrate for the enzyme. Generally caffeic acid derivatives (including chlorogenic acid) and monomeric flavanols (catechin and epicatechin) are hypothesized to be the best substrates for PPO. Phenolic groups such as anthocyanins, flavonols, and condensed forms of flavanols (tannins), have low affinity for PPO. Flavones, flavanones, flavononols, chalcones, and dihydrochalcones also belong to the same category of less reactive substrates (Macheix et al., 1990; Kindl, 1994; Yoruk and Marshall, 2003).

Wang and Mellenthin (1973) reported that chlorogenic acid serves as the best substrate for PPO for the incidence of FD in pear. Goupy et al. (1995) elucidated that not only chlorogenic acid, but catechin and epicatechin also show high affinity to PPO during enzymatic browning. The content of phenolics such as hydrocinnamic derivatives and flavonoids are higher in the peel as compared to the cortex of pear (Amiot et al., 1995). Several studies have indicated the direct relationship of FD to substrate content (Wang and Mellenthin, 1973; Kvåle, 1979), however, Mitcham et al. (2001) indicated that PPO activity and phenol substrate concentration do not act as rate limiting factors for FD, but rather increase the browning intensity. Later, Burger et al. (2005) confirmed that FD susceptibility is not always associated with total phenol content or PPO activity and it is highly variable among cultivars.

The phenol content of fruits and vegetables is dependent on fruit maturity, pre- and postharvest operations and intraspecific genetic characteristics (Herrmann, 1976; Amiot et al., 1992). Phenolic contents vary among the cultivars and their amount can be connected to the browning intensity of the tissues. For example in peach, 'Sunbeam' contains 20 times less total phenolics than 'Elberta', and is less susceptible to peel browning than 'Elberta' (Nicolas et al., 2003). Amiot et al. (1995) studied the effect of maturity, storage conditions and cultivars on the interaction of phenol contents and browning in pears. Browning was correlated with increased amount of phenolic compounds, however their amount was variable depending on the storage and cultivar.

These variable results suggest that phenolics are important in the process of enzymatic browning in the context of FD susceptibility, however, they do not strictly act as rate limiting factors. Their opposing roles as pro-oxidant and antioxidant along with involvement of underlying genetic factors (genetic background) makes the situation even more involved. The situation is expected to be enhanced in the case when a wide variety of genotypes are considered.

1.2.2.2.3 Skin anatomy

Membrane disintegration as a result of mechanical damage plays a very crucial role in the process of enzymatic browning, as it leads to the mixing of enzyme and

substrate (Toivonen, 2004). Skin characteristics are important for induction of enzymatic browning. Total phenol contents and PPO activity had no direct correlation with FD in two European pear cultivars, rather it was dependent on the skin properties of cultivars along with their degree of permeation of gases (Amarante et al., 2011a). Coated pears showed reduced FD as compared to non-coated ones by delaying the ripening process and reduced entry of oxygen. Thick coating layers also helped to protect the fruit epidermis by providing an extra cushion against friction force applied to induce FD. In fresh-cut potato neither PPO activity nor phenolic contents served as limiting factor for enzymatic browning (Cantos et al., 2002).

Russeted pear varieties have cork cells in the skin due to phellogen deposit in the sub epidermal layer. This layer is less permeable and relatively more flexible than skin without phellogen deposits, hence it protects the fruit skin against water loss and adverse physical damage which eventually make the fruit less susceptible to FD (Khanal et al., 2013). Fruit with thinner epidermal layer and cuticle are at higher risk compared to fruits with thick cuticle, thick cuticles also serve as a barrier to water loss and physical damage (Khanal et al., 2013). Thus the incidence of FD depends on whether the cell wall displacement has exceeded the elastic limit, which eventually results in cell wall damage and loss in tissue integrity with leakage of cytosolic contents from respective membrane bounded organelles. Amarante et al. (2001a) illustrated that thick cuticle could prevent the fruit skin from extra water loss by transpiration. Cultivars with lower oxygen permeability were less prone to physical damage (Amarante et al., 2001a). However, FD is a complex phenomenon controlled by many factors, and we cannot assume that this hypothesis will always apply.

The number and position of lignified stone cells, which are made up of phenol polymers have also been suggested to play an important role in determining fruit shelf life. Stone cells provides strength and help to maintain the cell structure and also increase the cell impermeability (Tao et al., 2009). Amarate et al. (2001b) conducted a study on non-lignified ‘Bartlett’, ‘Comice’ and ‘Packham’s Triumph’ and lignified ‘Beurre Bosc’ pears. They reported that ‘Bosc’ with lignified stone cells in the epidermis had higher water and lower oxygen and carbon dioxide permeance through the epidermis than the non-lignified varieties. This suggests that the ripening process is slower in ‘Beurre Bosc’ compared to cultivars with non lignified

epidermis (Amarante et al., 2001b). Pears with non lignified stone cells in the epidermis e.g. 'Comice' had more interconnected cracks in the cuticle than 'Bosc'. These cracks might offer an extra water loss path through skin not found in intact cuticle (Amarante et al., 2001b). Excessive water loss from the skin gives rise to a shrivelled and uneven surface which enhances the chance of cellular damage by friction force.

During fruit softening, a series of cellular changes takes place which alters cell wall structure. The major event in fruit softening is cell wall disassembly which is largely responsible for a reduction in cell rigidity and a reduction in the strength of cell-to-cell adhesion. This disassembly can cause a reduction in turgor pressure which also may reduce tissue firmness (Brummell, 2006). Loss of turgor pressure and cell wall degradation may make fruit more prone to physical damage. However this phenomenon needs to be tested in genotypes with distinct textural characteristics to evaluate their potential role in the incidence of FD.

1.2.3 Factors observed to influence FD susceptibility

1.2.3.1 Cultivar susceptibility

FD is influenced by various environmental and genetic factors. Cultivar variability with respect to FD susceptibility might be due to number of underlying genetic factors which control the quantitative and qualitative characteristics of substrate, enzyme and skin properties. For example pears with different textural properties tend to have different degrees of susceptibility (Meheriuk et al., 1994).

Kvåle (1979) studied the effect of fruit maturity and phenolic content on FD susceptibility for two European pear cultivars. 'Moltke' was more susceptible than 'Herrepaere' and high FD susceptibility was associated with chlorogenic acid content. In a later study, a range of susceptibility to FD was observed among four cultivars (Kvåle, 1988). 'Amanlis' and 'Moltke' were more susceptible to FD than 'Philip' and 'Herzogin Elsa'. Amiot et al. (1995) studied 9 cultivars of which 'Abate Fetel', 'Comice', 'P2198' and 'Guyot' showed higher enzymatic browning, while 'Conference', 'Williams', '6.30.100' and 'Harrow Sweet' were among the less

susceptible ones. ‘Comice’ was more sensitive to FD compared to ‘Packham’s Triumph’ and their susceptibility was not associated to phenolic content or PPO activity (Burger et al., 2005). The effect of harvest maturity effect was proposed for ‘Packham’s Triumph’, i.e. late harvested fruit were more susceptible as compared to early harvested fruit. However, ‘Comice’ behaviour was complex and opposite to what was observed for ‘Packham’s Triumph’. ‘Conference’ was more susceptible to transport vibration damage than ‘Decana’ and ‘Abate Fetel’ regardless of vibration acceleration, position of the package on the floor and column of packages(Berardinelli et al., 2005).

Fruit phenol contents change from cultivar to cultivar depending upon the maturity and other postharvest factors and it could be one of the reasons for variation in susceptibility to browning. Other factors that could influence susceptibility to FD in different cultivars include PPO activity (Mathew and Parpia, 1971; Gauillard and Richard-Forget, 1997; Larrigaudiere et al., 1998; Brandelli and Lopes, 2005). Cultivars with strong epidermal cells may be more resistant to physical damage as compared to cultivars with thin skins. Skin properties also affect permeability to gases as explained in section 1.2.2.2.3. Cultivars with higher permeance will be more sensitive to FD (Crisosto et al., 1993; Amarante et al., 2001a; Tao et al., 2009). Fruit with uneven fruit surface are more sensitive to friction damage and cultivars having fruit with these characteristics will be more prone to FD (Meheriuk et al., 1994).

FD is known to be influenced by environmental factors. Amiot et al (1995) regarded ‘Abate Fetel’ pear being more susceptible to browning than ‘Conference’ whilst (Berardinelli et al., 2005) reported a reverse trend where ‘Conference’ is more susceptible than ‘Abate Fetel’. However these studies were conducted in different years (1995 and 2005 respectively) and at different locations (France and Italy) and experimental conditions and FD assessment methods also varied. Berardinelli et al. (2005) induced the physical damage by putting the fruit trays in a chamber and provided simulated shaking similar to the original transport vibration, while Amiot et al. (1995) conducted the peel browning test by chemical means.

1.2.3.2 Fruit maturity

Fruit maturity plays a vital role in the postharvest life of both fruit and vegetables (Kader, 1996). European pears are generally not allowed to ripen on the tree because they develop mealy texture and poor flavour. Generally European pears are harvested at proper maturity, but at an unripe stage and are conditioned at low temperature (Kingston, 1992). This cold temperature conditioning stimulates internal production of ethylene which will enhance ripening at room temperature after conditioning. Harvest maturity, conditioning temperature and number of days varies from variety to variety. On the other hand Asian pears do not need chilling pre-conditioning, rather they should be harvested at an almost ripe stage (Kingston, 1992).

Harvesting at improper maturity can lead to the development of several storage disorders. FD incidence is also highly dependent on harvest maturity (Mellenthin and Wang, 1974; Kvåle, 1979; Burger et al., 2005; Gomila et al., 2011). Studies related to the effect of fruit maturity in relation to susceptibility to FD have led to two opposing hypotheses: 1) FD increases as season advances meaning firmer fruits are less susceptible to FD, 2) FD decreases as season advances meaning that immature or firm fruits are more susceptible. The effect of maturity on the susceptibility to FD in different European pear cultivars has been reported, such as ‘d’Anjou’ (Mellenthin and Wang, 1974) ‘Bartlett’ (Mitcham et al., 2001), ‘Comice’ (Amarante et al., 2001a), ‘Packham’s Triumph’ and ‘Comice’ (Burger et al., 2005), ‘Abate Fetel’ (Gomila et al., 2011) and an array of other European cultivars by Kvåle (1988) and Amiot et al. (1995). Researchers have often explained relationship of FD to maturity in terms of variation in amount of phenolics, enzyme activity and skin properties.

Fruit harvested before or after the climacteric peak tend to be more susceptible to FD as compared to fruit harvested at proper maturity (Kvåle, 1979; Kvåle, 1988). Firmness decreases as the ripening process advances and late harvested fruits (soft fruits) were more susceptible to FD. In a study of ‘Bartlett’ pear, early harvested (firm) fruits were less prone to FD compared with late harvested (soft) fruit (Mitcham et al., 2001). Higher FD incidence was recorded in late harvests in both 1-MCP (1-methylcyclopropene) treated and control fruits (Gomila et al., 2011). Firmness effect on FD can be described in terms of ripening and cell turgor pressure

changes. As ripening proceeds, cell walls undergo a natural degradation process resulting in loss of cell wall integrity/strength and loosening of adjacent cells. Turgor pressure also decreases due to certain internal (osmotic solute accumulation) and postharvest factors which results in substantial water loss (Toivonen and Brummell, 2008). All these events contribute to loss in firmness and make fruit prone to physical damage.

Pear maturity indices are complex and generally not reliable across European and Asian pear cultivars (Tvergyak, 1985). Although the indices currently employed to determine maturity for harvest (firmness, total soluble solid content and ground colour) are the same as used for apple, the value of these indices that indicates harvest maturity appears to diverge among genotypes, orchards and seasons (Tvergyak, 1985).

Appropriate harvest maturity indices are required to be determined for each variety in a particular growing area and season. To overcome this complexity, the "Streif index" has been proposed as a more efficient tool for determining optimal pear maturity:

$$Streif\ index = \frac{Firmness}{Total\ soluble\ solids\ (TSS)} \times Starch\ index$$

The index is measured on a scale from 1 to 10 (DeLong et al., 1999). However, "Streif index" value for harvest maturity needs to be established for each cultivar in each growing location and season and is not helpful when considering a wide range of pear cultivars.

It can be hypothesized that pear harvest maturity plays a very important role for the susceptibility to FD and fruits harvested before (immature) or after (over mature) the climacteric rise in respiration may be more prone to FD regardless of variety, season and growing conditions. The role of harvest maturity in relation to FD can be explained through variation in underlying factors i.e. accumulation of phenols and PPO and change in skin properties as maturity advances, and would therefore be expected to vary in detail between different varieties.

1.2.3.3 Fruit size

There is an evidence for the involvement of fruit size in the susceptibility to FD (Chen and Varga, 1996). Mellenthin and Wang (1974) studied the susceptibility of pear fruit according to different size and maturity. They divided the fruit into three size groups: small with diameter less than 6.3 cm, medium with diameter between 6.4 and 7.9 cm and large with diameter greater than 8.0 cm. Small fruit were more susceptible to FD, at all maturity stages, as compared with medium and large fruit, respectively. Mitcham et al. (2001) reported similar results of high FD susceptibility in relation to small size pear fruit. Gorny et al. (2000) reported that freshly cut pear slices derived from small sized fruit showed more intense surface browning than slices cut from large fruit with the same maturity level.

Two hypothesis can be formulated for the influence of fruit size on browning. Smaller fruit could be immature ones (although harvested on the same date along with medium to large size fruit) and are more prone to physical damage due to a thin cuticle, as discussed in section 1.2.2.2.3. Alternatively, small fruit are likely to have tissues with small cells and with proportionally extra cell wall content, lower quantity of cell sap and lower volumes of intercellular air spaces. Therefore, the same degree of friction damage would injure more cells in smaller fruit as compared to large fruit and would trigger greater enzymatic oxidation of phenols.

1.2.3.4 Storage duration

An increase in FD susceptibility has been observed with lengthened storage duration (Mellenthin and Wang, 1974; Kvåle, 1988; Amiot et al., 1995; Mitcham et al., 2001; Burger et al., 2005; Gomila et al. 2011). Smith (1946) reported that susceptibility to FD during cold storage was more influenced by ripening stage rather than the handling temperature. ‘d’Anjou’ pear could be stored longer than ‘Bartlett’ without subsequent increase in susceptibility to FD. An increase of total phenol and chlorogenic acid content was observed for the first seven weeks of storage along with FD susceptibility (Wang and Mellenthin, 1973), and then became constant in later storage. Nevertheless, incidence of FD kept on increasing during storage. Since not all phenolic compounds serve as specific substrates for PPO in enzymatic browning,

a possible explanation might be that the other phenolic compounds continued to increase during later storage and caused higher FD. Amiot et al. (1995) noticed a prominent increase of flavanols rather than other phenols during storage, which could lead to a higher FD susceptibility, even if total phenolic concentration remains stable.

Another likely reason for the enhanced susceptibility during long storage could be ripening related anatomical changes. Amarante et al. (2001a) suggested that increased FD susceptibility with prolonged storage was a consequence of tissue softening, water loss and reduced turgor pressure, making tissues more susceptible to physical damage. Another reason for high FD during storage can be related to loss in firmness. As described in section 1.2.4.2, softer fruits are more prone to physical damage (Kvåle, 1988; Mitcham et al., 2001), which leads back to the phenomenon of loss in cell turgidity and elasticity.

1.2.3.5 Temperature

Susceptibility to FD is also influenced by the temperature of the fruit or the handling environment. Pear exporters in USA related the problem of FD to direct handling of the fruit at cold temperature, however studies on 'd' Anjou' and 'Bartlett' proved that it was not handling temperature alone that causes FD. Storage duration and ripening stages were also involved in this phenomenon. A contrasting argument was presented by Sommer et al. (1960) that warm fruits are more prone to FD than cold ones. In 1979, Kvåle reported that pear fruits which were handled immediately from storage at 0 °C were likely to brown more than fruits which were conditioned at 10-15 °C for 2 days before assessment of FD. Gomila et al. (2011) commented that lowest sensitivity temperature for 'Abate Fetel' to FD was between 7- 11°C while storage temperatures at 0 °C or above 15 °C made fruit more sensitive to FD.

Physical properties of warm and cold fruit may alter their susceptibility to physical damage. For example, cell elasticity and turgidity are temperature dependent and affect tensile strength (Banks et al., 1997). These results suggest that low temperature can slow down the enzyme activity and when fruit core temperature increases, enzyme activity will be restored and discolouration will occur immediately.

Some researchers have also related browning susceptibility to the preharvest growing environment. Zerbini et al. (2002) described how 'Conference' pears grown in the Mediterranean area (higher temperature) were significantly less susceptible to internal browning disorders than those grown in the northwest of Europe. A contrasting result was reported by Kvåle (1988) in relation to FD where high incidence of FD was recorded in relation to abnormally high temperature of the summer growing season.

Protection from a high temperature growing season may be similar to the reported reduction in chilling injury susceptibility in some fruit such as tomato; membrane integrity during storage may be linked to elevated temperatures before harvest (Ferguson et al., 1999), and if this phenomenon is found in pears it may leave the fruit less prone to membrane rupture when abraded. The opposite effect may be found if high pre-harvest temperatures result in excessive water loss and enhanced shrivelling during storage. Rough skin texture may create enhanced susceptibility to FD damage by increasing friction forces between epidermal tissue and contact surface (Amarante et al., 2001a).

1.2.4 Means of prevention of FD

1.2.4.1 Avoiding mechanical injury

One way to minimise the risk of FD is to avoid physical damage during pre- harvest, harvest, grading and packing and during storage and transport. Often fruit gets injured by striking against other fruit, twigs and field bins during harvest operations. Pear trees should be well pruned before and after flowering so that chances of fruit striking can be minimised. Fruit thinning can be another preventive measure to avoid rubbing and striking against neighbouring fruit. By adopting suitable mechanized harvesting procedure a high proportion of pears can be saved from FD damage. Extra care is required for highly sensitive cultivars such as 'Comice'. Trained pickers, clean and padded containers should be used, to avoid harvest injury. During hand picking, care should be taken while moving fruit from bags to field bins.

Pack house operations such as transfer from bins to packing line, washing, grading and segregation all need intensive care to minimise the chances of physical damage. Inexpensive modifications to pack house operations such as immersion dumping of the fruit with addition of some salt and reducing dropping height to less than 15 cm can significantly reduce impact and friction damage (Mitcham et al., 1994). Sodium sulphate or sodium lignin sulphate salts can be used in water dumps to make water density higher than that of the pears so that they float (Agar and Mitcham, 2000). Slowing down the speed of the packing line is also recommended (Meheriuk et al., 1994; Chen and Varga, 1996).

The next source of extensive damage is transport vibration (Smith, 1946; Sommer 1957; Berardinelli et *al*., 2005; Zhou et al., 2007). Proper packaging systems during transport are needed, especially when the roads are rough. Mitchel et al. (1968) introduced the 'tight fill' system by wrapping or packing of individual fruit rather than packing in bulk. However that packing could not ensure success for complete removal of the issue. Since box weights are variable, less filled boxes will suffer more damage than properly filled ones. Dimensions for different boxes are not exactly the same to absorb same tight fill density of these boxes. In some cases, corrugated boxes are over packed and instead of the package bearing the weight of overlying boxes, the bulging boxes allow the weight to be directly transmitted to the fruit. The issues related to packaging need to be addressed by designing adequate packaging systems for pear cultivars with distinctive shapes. By combining the strategies i.e. fruit wrapping inside the box in polyethylene bags which will help to reduce the moisture loss and suitable packaging boxes which can endure palletized effects could be effective means of minimising damage due to transport vibration.

1.2.4.2 Inhibition of enzymatic browning

Once efforts have been made to minimise the physical damage, next step is to minimise or prevent enzymatic browning. A number of methods have been evaluated to inhibit or slow down the enzymatic reaction (either physical or chemical). Some inhibitors can affect all components of reaction processes (enzyme, substrate and reaction product) while others may be reaction specific.

Oxygen concentration plays a crucial role in enzymatic browning. Low oxygen availability may reduce enzymatic browning, however may also create the risk of anaerobism (Nicolas et al., 2003). Controlled atmosphere (CA) conditions i.e. 1% O_2 and 1% CO_2 are considered to be effective in minimising browning, reducing phenol biosynthesis (Nicolas et al., 2003).

Surface coating by edible waxes and films have been proved to block skin lenticels and stomata. Blockage of epidermal pores can reduce the water loss and oxygen availability to the tissues (Amarante and Banks, 2010).

The application of antioxidants has also been studied as a means of minimizing the FD (Mellenthin et al., 1982, Amarante et al., 2001a, Mitcham et al., 2001, Feng et al., 2004). The mode of action in the case of the antioxidants is to counteract oxidative browning, even though cellular damage has occurred (Tomás-Barberán and Espín, 2001). In general, most coatings are applied to prevent or at least minimize, the physical damage that fruit will inevitably undergo during handling processes.

1.2.4.3 Effect of antioxidants

Antioxidants have been shown to serve as natural inhibitors against enzymatic browning for many years (Mathew and Parpia, 1971; Jang and Moon, 2011). Some antioxidants have been widely accepted for their inhibitory control of FD and reported by several authors, such as 2-mercaptobenzothiazole (Wang and Mellenthin, 1974), 4-hexylresorcinol (Dong et al., 2000), ascorbic acid and sulphur dioxide (Meheriuk et al., 1994).

Ascorbic acid (AsA) is a naturally occurring vitamin and it acts as reactive oxygen scavenger that minimises oxidative stress related damage. AsA plays an important role in fruit ripening as a cofactor of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, as well as in photosynthesis where it removes hydrogen peroxide from photosystem 1 in the chloroplast (Veltman et al., 2000). AsA been used extensively as a permitted inhibitor of enzymatic browning in the food industry (Golan-Goldhirsh and Whitaker, 1984; Martinez and Whitaker, 1995).

Veltman et al. (1999) conducted a study to observe the relationship of AsA concentration to internal browning of 'Conference' pears under various CO_2 and O_2 conditions. They reported that with the increase of CO_2, substantial decrease in fruit AsA amount was observed and the incidence of core browning was extensive after AsA amount dropped to certain threshold limits.

Arias et al. (2007) found an increased lag phase of the enzymatic browning of fresh cut pears after addition of ascorbic acid to the reaction mixture. AsA does not directly inactivate the PPO, rather it acts as antioxidant agent on oxidative substrate products (brown polymers) of the enzymatic reaction, acting on the reaction products and reduces them back to original products.

It is clear that AsA plays an important role in the prevention of enzymatic browning. Studies involved in the quantification of natural AsA have also reported that the antioxidative effect is temporary and enzymatic browning was restored with depletion of AsA (Arias et al., 2007). A potential inhibitory role of AsA in the case of FD incidence is not clear as no single study has quantified the natural AsA. In current study, AsA concentration has been quantified to evaluate the role of AsA in FD susceptibility.

1.3 Friction discolouration: physiology to genetics

FD is a complex postharvest disorder that impairs the cosmetic quality of the fruit In the literature mechanical damage to fruit skin tissues leading to phenol oxidation has been reported as major underlying phenomenon of FD. This phenomenon has been well studied in the past as described in detail in section 1.2. Some pear varieties are believed to be more susceptible than others (Section 1.2.4.1) which emphasises an underlying genetic control. Several postharvest techniques have been used to alleviate FD (Section 1.2.5). A number of biochemical (phenolics and AsA concentration and PPO activity) and physical (skin properties) factors are known to influence the FD susceptibility along with genetic characteristics (Section 1.2.3). This variation has been explained by changes in levels of underlying factors (i.e. phenolics, PPO and AsA) and these factors themselves are likely to be controlled genetically.

1.4 Quantitative genetics

Many agricultural and horticultural traits such as quality, yield and disease and disorder resistance are controlled by several genes with small effects. The study of these complex quantitative traits with continuously distributed variation which do not follow simple Mendelian inheritance rules is known as ‘quantitative genetics’. The phenotypic variation of quantitative traits is a complex combination of genetic and environmental factors (and their interaction), which makes the study of the effect of the underlying genes very challenging. Application of quantitative genetics is crucial to gain an understanding of phenotypic variation in animal and plant populations, especially for improvement using breeding (Falconer, 1960).

Until the 1980s quantitative variation resulting from genetic and non genetic effect was studied by using the statistical methods developed by Fisher (1918)) and Wright (1922) such as population means and variances, covariances of relatives and heritabilities. Such parameters were helpful to describe relatedness in the population but did not explain individual gene effects. Recent advances in biometrics and molecular genetics have made it possible to dissect quantitative traits into the specific regions of the genome that control them: the quantitative trait loci (QTLs). The concept of QTL has a long history back to Sax (1923) with first statistical approach proposed by Thoday (1961), however QTL mapping has only been applied widely for crop and animal species in the last 15 years thanks to the availability of molecular markers for the development of genetic maps, as well as new statistical tools.

1.4.1 QTL mapping in plants

Three main steps are involved in QTL mapping of plants: 1) Collection of phenotypic data from a fairly large number of seedlings from segregating populations for a particular trait; 2) genotyping of the same segregating population using markers covering the genome, followed by genetic map construction; 3) and statistical analyses using phenotypic and genotypic data to identify the loci that are associated with the trait(s) of interest.

1.4.1.1 Phenotyping

In genetic terms the phenotype is the expression of the genotype in a given environment. It is usually a measurable variable or factor such as plant height, susceptibility to disease or concentration of a plant metabolite in a given tissue. Phenotype itself is highly influenced by environment (E), genetics (G) and the interaction between genetics and environment (GxE). Collection of accurate and sufficient amounts of phenotypic data from segregating populations for a continuous trait is a prerequisite for QTL analysis. This is the statistical association of phenotype and genotype and is highly influenced by authenticity and quality of phenotype data. Complex traits such as yield and fruit quality are highly influenced by environment so special care should be taken into account during phenotype evaluation, such as using replicates and controls, and treating plants and samples in a very consistent way (Kearsey and Farquhar, 1998). This is a particularly important consideration for study of gene characters related to postharvest behaviour of fruit. Breeding populations can be used for QTL analysis as they are often obtained from biparental families of sufficient size (typically more than 100 seedlings). However phenotype data collected from breeding populations can be obtained from complex trials (multiple years and locations) with unbalanced experimental designs, which affects the QTL detection by producing false positives (i.e. the QTL is due to a response to the environment) (Wang et al., 2012) or false negative (i.e. no QTL detected because the environmental variation masks the genetic effect). One further complication with using breeding populations for QTL mapping is that plant breeders do not always replicate genotypes for phenotypic evaluation.

1.4.1.2 Genotyping and genetic linkage map construction

1.4.1.2.1 Genetic markers

Genetic markers are inherited variations in the genome. At the start of 20th century, scientists discovered that the units of inheritance described by Mendel are organised in a specific manner on cytogenetic structures of the nucleus called chromosomes. Genetic markers are useful to locate the position of genes controlling phenotypic traits. All markers have their own advantage and disadvantages; however selection of

markers depends upon many factors such as marker availability and transferability across the populations and species, polymorphism rate, and resources available (Gupta, 2007a).

Genetic markers can be grouped into three major classes on the basis of their nature or origin:

(1) Morphological markers are based on visible traits or characters. They are usually visually distinct phenotypic characters such as fruit skin and flesh colour. However such markers are not very useful because most of them are only expressed a specific development stage and tissue, such as flower colour. Therefore, they are of very limited interest for breeding applications such as marker-assisted selection for adult traits such as fruit friction discolouration. Furthermore, most of them are dominant markers as heterozygous and homozygous states are not distinguishable.

(2) Biochemical markers are based on secondary metabolites and isozymes. As compared to morphological markers, biochemical markers can be screened in a wider range of the plant developmental stages and tissues. Isozyme markers are multiple copies of enzymes which differ in amino acid sequence, but catalyse the same reaction. By the early 1980s, biochemical markers had been employed as a tool for QTL mapping (Weller, 1988), however their use is limited as they are restricted by their low number. Biochemical markers have regained a great interest in the last few years due to increase in throughput for chemical analysis. However, they are used as bio-indicators or bio-markers but not now as genetic markers for genetic analysis.

(3) Molecular markers are DNA-based and abundant in the genome of most organisms (Winter and Kahl, 1995; Jones et al., 1997). The major advantage of molecular markers is their abundance. They are consistent from generation to generation and as compared to morphological and biochemical markers are not highly influenced by environmental and developmental stage factors (Winter and Kahl, 1995). Molecular markers arise as a result of DNA polymorphisms such as point mutations (the most frequent), rearrangements or replication errors in DNA repeats (Paterson, 1996). Many techniques exist to identify and screen molecular

markers.

Restriction Fragment Length Polymorphisms (RFLP) are the earliest type of molecular markers introduced in early 1980s for human genetics (Botstein et al., 1980) and then later in plants (Helentjaris et al., 1986; Weber and Helentjaris, 1989). The RFLP technique basically relies on the digestion of DNA fragments by restriction enzyme at specific sites based on variation in length/ size of the DNA fragment (Figure 1.3). Polymorphisms such as point mutations, insertion/deletions, translocations, inversions and duplications can all result in gain, loss or relocation of restriction sites and give DNA fragment product of variable length. RFLP markers follow simple Mendelian segregation and are useful for construction of genetic maps; however this is labour intensive, especially if a large number of markers are required for saturating genetic maps.

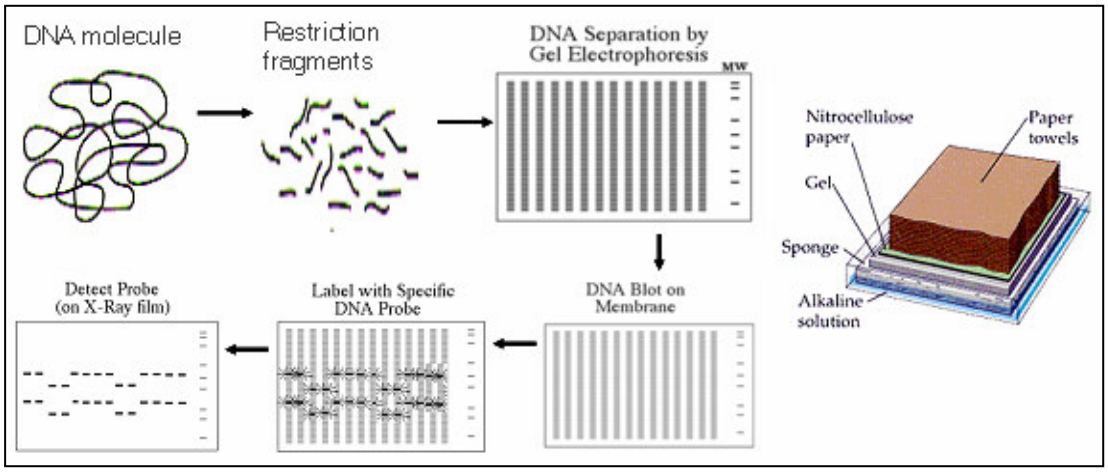


Figure 1.3. Pictorial representation of the RFLP technique 1. Digestion with restriction enzyme (RE), 2. Separation by agarose gel electrophoresis 3.Transfer of DNA to membrane, 4. Nucleic acid hybridization with labelled probe, 5.Visualization by autoradiography; modified from (Semagn et al., 2006a).

The PCR technique opened a new realm for molecular biology in the 1990s because smaller DNA templates could be used and specific DNA fragments can be replicated many times and more easily detected. The Amplified Fragment Length Polymorphism (AFLP) technique is similar to RFLP but with the additional advantage of PCR technology. Restriction enzymes are used to cut DNA, however

unlike RFLP, adaptors are ligated to the restricted fragments and then amplified using two rounds of PCR reactions (Jonah et al., 2011). The Random Amplification of Polymorphic DNA (RAPD) technique is another method that uses short arbitrary primers (normally 10 base pair) to amplify template DNA and which are separated by agarose gel electrophoresis. This method exploits the DNA sequence difference by presence or absence of the primer binding sites. RAPD and AFLP markers are relatively simple, fast and can produce multiple markers per reaction. These markers have extensively used in association mapping studies including pear(Iketani et al., 2001; Yamamoto et al., 2007). However due to the random nature of RAPD primers and AFLP restriction sites, both types of markers have some limitations in reproducibility and transferability across populations. Furthermore, these markers are dominant and less informative than co-dominant markers for genetic map construction and QTL mapping.

1.4.1.2.2 Microsatellites

Microsatellite or simple sequence repeats (SSR) are short (one to five) tandemly repeated nucleotide motifs. Microsatellites are found in the entire eukaryotic kingdom. The genetic variations arise as a result of variable numbers of tandemly repeated units, originating from a mutation mechanism during DNA replication known as “slip strand mispairing” (Figure 1.4). Microsatellites are valuable genetic markers because of their abundance, high polymorphism, co-dominant inheritance and the simplicity of assessing the variation using electrophoresis (Schlötterer and Tautz, 1992).

1.4.1.2.3 Single nucleotide polymorphism (SNPs)

SNPs are biallelic genetic markers representing sequence variation at the level of a single nucleotide. They are extensively used in modern genetics. SNPs are the smallest unit of inheritance and therefore the ultimate marker of choice, providing a high level of polymorphism in genomes including crop species. Maize has reported to have 1 SNP per 60–120 bp (Ching et al., 2002) as compared to humans with an estimated 1 SNP per 1,000 bp (Sachidanandam et al., 2001). Along with being abundant and having a high polymorphism rate, SNPs are also stable from generation

to generation. A number of new methods have been developed to genotype SNPs, such as allele specific oligunucleotide hybridization, primer extension, oligonucleotide invasion and cleavage.

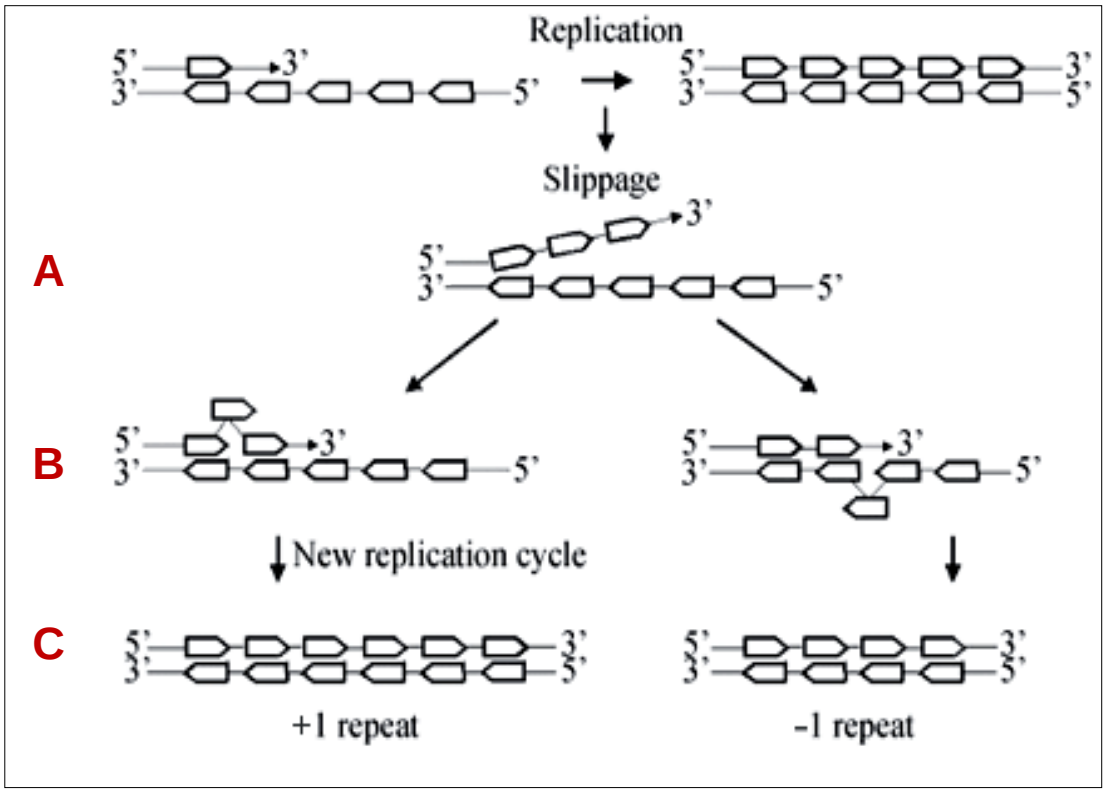


Figure 1.4: Pictorial representation of slippage process at microsatellite loci. DNA repeat units are symbolized by small boxes and small arrow illustrates direction of replication. a) slippage, b) misalignment of newly developed repeats c) new templates; adapted from (Madesis et al., 2013).

Such methods have now been scaled up to high throughput genotyping technologies where hundreds to thousands of SNPs can be genotyped simultaneously in one reaction, such as SNP arrays/chips, allele-specific PCR and genotyping by sequencing (Gupta et al., 2008). Such new tools provide plant geneticists with large sets of markers for construction of high density genetic maps which can further assist in speedy selection of new cultivars and association studies of various economically important complex traits.

Normally a two step process is required to develop a SNP marker assay for genome screening and then application: 1) SNP discovery on a few but diverse set of individuals, 2) validation on a larger set of individuals and number of populations, and 3) selection by genotyping of a large population. A comparison of different types of markers used for QTL analysis is provided in Table 1.3.

Table 1.3: Comparison of commonly used molecular markers for QTL analysis (Collard et al., 2005).

Marker	Nature	Advantages	Disadvantages
Restriction Fragment Length (RFLP)	C	Robust Reliable Transferable across species No sequence information required	Time consuming Laborious Expensive Large amount of DNA required
Random amplified DNA Polymorphism	D	Quick and Simple Inexpensive Multiple loci from a single primer possible	Can't be reproduced Non transferable
Amplified Fragment Length (AFLP)	D	Multiple loci High level of Polymorphism Transferable across the species No prior sequence information required	Large amount of DNA required Complicated methodology Non reproducible
Simple sequence repeat (SSR)	C	Technically simple Robust and reliable Transferable between the populations Highly polymorphic Good genome coverage	Time consuming and laborious job to develop primers Usually require sequence information Non transferable across species
Single Nucleotide Polymorphism (SNP)	C	Universal Abundant in nature Compatible with automotive techniques Mostly in non coding region not affecting phenotype	Bi allelic Less polymorphic amenable to high- and ultra-high-throughput automation

C=co-dominant; D= Dominant

1.4.1.3 Genotyping

Screening of whole populations, including the parents is performed after selection of polymorphic DNA markers and is known as 'marker genotyping'. The scoring methods for dominant and co-dominant markers will be different as co-dominant markers cannot discriminate between dominant homozygous and heterozygous individuals (Semagn et al., 2006b). Generally markers follow Mendelian segregation rules however segregation distortion of markers deviating fromMendelian ratios can

also be encountered (Xu et al., 1997; Sayed et al., 2002) and this influences QTL analysis.

1.4.1.3.1 Plant genome sequencing technologies

Availability of reference genome sequence provides the basis for understanding the evolutionary process and gene function. Over the last three decades plant sciences have made enormous progress in the areas of linkage mapping, population diversity, evolutionary studies, association studies and marker assisted breeding. In 1977 Sanger introduced the first sequencing method, which depended upon a combination of deoxy and dideoxy labelled chain terminator nucleotides. Using this sequencing technique, the first complete genome sequence was released for bacteriophage *phi X174* in the same year (Sanger et al., 1977). The first plant genome sequence (*Arabidopsis thaliana*) was reported in 2000 (The Arabidopsis genome intiative, 2000) and represents one of the most significant recent breakthroughs in plant biology. Following the Arabidopsis genome sequencing, more plant genomes were sequenced using Sanger sequencing: rice (Goff et al., 2002; Yu et al., 2002) and poplar (Tuskan et al., 2006). However a Sanger sequencing-based project would be too challenging for sequencing plants with larger genome size, such as wheat (~16 GB genome). Next Generation Sequencing (NGS) has replaced Sanger sequencing in the last few years. The first platform introduced in 2005 was the Roche 454 Life Sciences one, which relies on the principle of high throughput pyro-sequencing (Margulies et al., 2005). Together with other methods, such as Solexa (Illumina) and SOLiD, NGS based technologies have now almost replaced all the other technologies (Sanger sequencing) due to their high throughput assay and lower cost. The development of NGS technology has enabled sequencing of entire genomes for more complex plant species, including crops such as grape (Jaillon et al., 2007; Velasco et al., 2010), soybean (Schmutz et al., 2010), sorghum (Paterson et al., 2009), maize (Schnable et al., 2009), peach (Verde et al., 2012), apple (Velasco et al., 2010), potato (The potato genome consortium, 2011), tomato (The tomato genome consortium, 2012) and most recently, Chinese pear (Wu et al., 2013). NGS can also be used for high throughput SNP detection and genotyping.

1.4.1.3.2 SNP discovery, genotyping and array development

Medium to high throughput NGS has made de novo and reference based SNP discovery feasible for many plant species. SNP discovery using NGS has been reported in many plants, however presence of paralogs, incomplete and an inaccurate reference genome could create problems in SNP calling (Kumar et al., 2012a). Many genome complexity reduction strategies with restriction enzyme (REs) are used for simplified and cheap SNP discovery through NGS; RNA- Seq, complexity reduction of polymorphic sequences (CRoPS), restriction site associated sequencing (RAD-Seq) and genotyping by sequencing (GBS). GBS holds the best promise out of these methods as it allows simultaneous discovery and genotyping by simple and cost efficient methods. SNP genotyping has been greatly improved by NGS Array-based technologies such as Infinium® and Goldengate®. These arrays can detect tens of thousands to hundreds of thousands of SNPs per sample (Illumina, 2013). The Illumina Infinium® assay consist of silicon chip contains beads with attached oligonucleotides that are designed to target a DNA sequence immediately flanking the SNP. The protocol involves DNA amplification to create amplicons, which are fragmented by restriction enzyme digestion. DNA fragments are hybridized to DNA probes covalently attached to bead chips and allelic specificity is determined by enzymatic base extension and finally the product on bead chips is fluorescently stained. Fluorescent intensity of bead arrays is detected by a bead array Reader and provides information about allelic variation and are analysed by Illumina's software (GenomeStudio, Illumina Inc.) for automated genotype calling (Figure 1.5).

SNP arrays have been developed for many complex plant species including fruit species in the Rosaceae family such as apple, peach, cherry and strawberry. A SNP array for apple was developed by the International RosBREED SNP consortium (IRSC) (www.rosbreed.org) (Chagné et al. (2012a)) This 8K SNP array v1 enabled the screening of 5554 genome-wide polymorphic SNPs in apple, with its 7867 SNPs from apple. Furthermore, the International Peach SNP Consortium (IPSC) developed a 9K SNP array for peach including 8144 SNPs developed from re-sequencing of 53 genotypes along with other data. Almost 84.3% of those SNPs were polymorphic (Verde et al., 2012). As described by Peace et al. (2012), IRSC also led the development of a 6K SNP array for cherry.

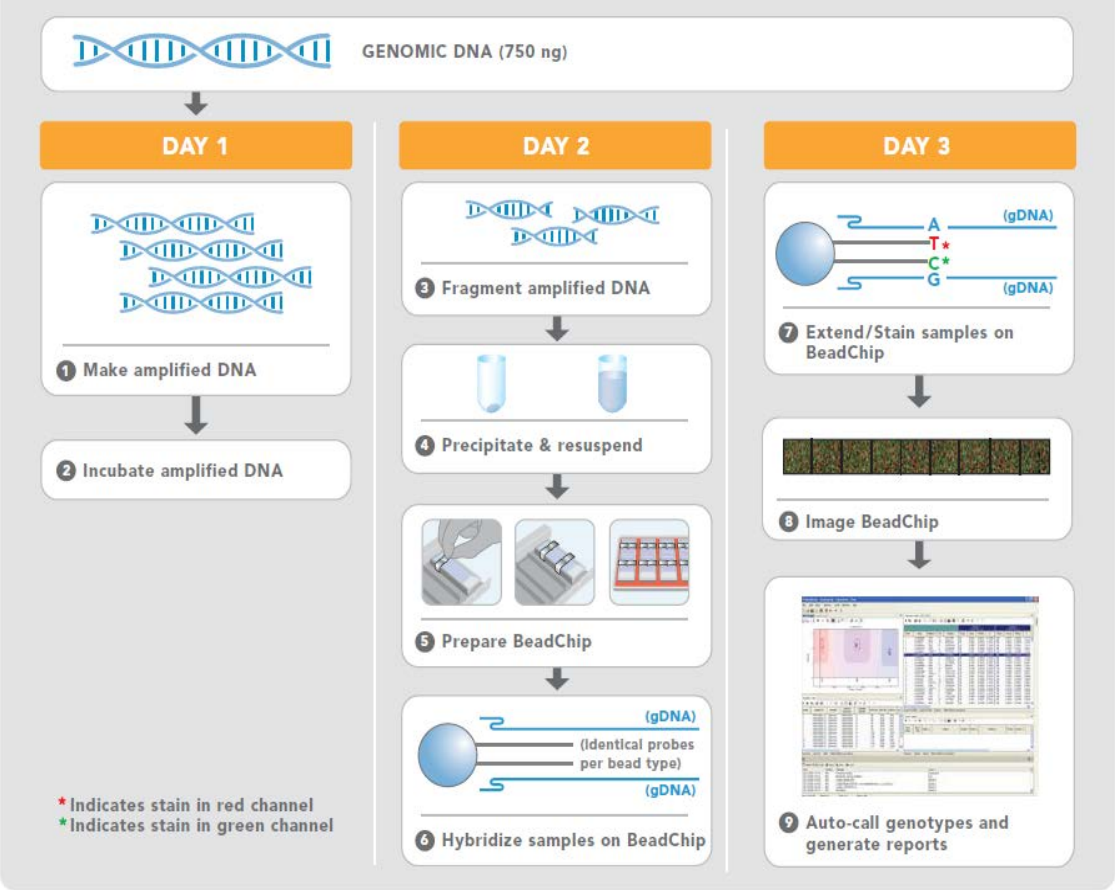


Figure 1.5: Illumina Infinium®II assay protocol (Illumina, 2006).

1.4.2 Linkage analyses and map construction

1.4.2.1 Mapping population

Development of a segregating population is the basic requirement for linkage map development and for subsequent QTL mapping. Genetic diversity of both parents is crucial as it decides the polymorphism rate in the population, i.e. how many genetic markers will be polymorphic and useful for map construction. Outcrossing species usually give higher polymorphism rates in their populations than inbreeding species where both parents are homozygous. Back cross (or testcross), double haploid, near isogenic and F2 populations from self pollinated and half-sib, full-sib families and two-way pseudo-testcross populations derived from cross pollinated species have

been used for genetic mapping in plant species (Doerge, 2002). The choice of mapping population applicable to some plants with long breeding cycles such as fruit trees is limited, and most genetic studies in fruit trees are based on one-generation full-sib families and the pseudo test cross strategy for map construction and QTL analysis (Zhao et al., 2013; Gardner et al., 2014; Jensen et al., 2014).

Population size (N) and heritability (h^2) of the trait influence the QTL effect, as the proportion of additive variance effect of QTL is inversely proportional to h^2N. Hence the prediction power for QTL (especially with small effects) for moderate to low heritability for smaller population (e.g. less than 100 segregating individuals) is low (Bradbury et al., 2011; Liu et al., 2012). Moreover smaller populations might also affect the resolution and accuracy of the linkage maps (Mohan et al., 1997). Denser maps are required for smaller populations.

1.4.2.2 Linkage analysis

This step involves the coding of each individual marker for a whole population and running of the linkage analysis by an appropriate software packages are presently available and have been used in recent years for genetic linkage mapping, including JoinMap (Stam, 1993), LINKAGE (Suiter et al., 1983), MAPMAKER/EXP (Lander et al., 1987), GMENDEL (Echt et al., 1992) and Map Manager QTX (Manly et al., 2001). This step involves the coding of each individual marker for a whole population and running of the linkage analysis by an appropriate software programme. A number of software packages are presently available and have been used in recent years for genetic linkage mapping, including JoinMap (Stam, 1993), LINKAGE (Suiter et al., 1983), MAPMAKER/EXP (Lander et al., 1987), GMENDEL (Echt et al., 1992)

Linkages between the markers are calculated by odds ratios (i.e. probability of linkage of two loci versus no linkage of two loci). This ratio is expressed in terms of logarithm of ratio and also known as logarithm of odds (LOD) value or LOD scores (Risch, 1992; Stam, 1993). The significant LOD scores used to create linkage groups are called ‘linklod’ (Stam, 1993; Ortiz et al., 2001) and marker groups having LOD score higher than critical ‘linklod’ are considered to be linked and vice versa. LOD

values of >3 have been used by many researchers as a minimum threshold level for linklod to see if the loci are linked or not. A LOD value of 3 specifies that chances of linkage between two markers is 1000 times higher in comparison to no linkage (i.e. 1000:1). However, higher and lower LOD threshold values can be used according to the situation, a lower LOD score will give few linkage groups with more markers but higher LOD score gives smaller fragments of chromosomes with fewer markers. However, a lower LOD threshold will give more false positives than a higher threshold, for example by merging groups that do not belong to the same chromosome. Conversely a high LOD threshold will generate more fragmented linkage groups, where more than one linkage group may be obtained for each chromosome. Ideally the number of linkage groups produced by linkage mapping should be the same as the number of haploid chromosomes for that species. When a genetic map reaches this stage it is said to be "saturated". Failure to saturate a map may be due to insufficient number of markers. If a linkage group has markers from different chromosomes that often indicates suspect linkages in the map.

1.4.2.3 Map distance and mapping function

Map distances are a measure of recombination frequency between markers. Distances are directly proportional to recombination frequency when the map distances are small (<10 cM; centimorgan), however it is not the case when map distances are higher than 10 cM. For recombination frequency of 10% or lower Haldane or Kosambi mapping functions give the same map distance, but at higher frequencies map distance will be higher for Haldane than the Kosambi function. The Kosambi mapping function is usually used to translate recombination frequency into map units (cM), as it takes into account the physical interference between chiasma and minimises double recombinants at short distances. One genetic map unit is equivalent to one percent of recombinant or 1 cM.

It is important to keep in mind that genetic map distance in cM and physical distance (in bp or kb) have no direct linear relationship. Relationship between genetic and physical distance are variable due to variable rates of recombination within a single chromosome (Ahn and Tanksley, 1993; Young, 1994; Künzel et al., 2000) as there are specific hot or cold spots on chromosomes according to recombination

frequencies (Faris et al., 2000; Ma et al., 2001; Yao et al., 2002). For example in rice 1 cM map distance on average equals to 258.5 kb (International rice genome sequencing project, 2005).

High quality genetic linkage maps serve many purposes such as a) QTL analysis and identification of genes responsible for economically important traits (Mohan et al., 1997; Doerge, 2002; Yim et al., 2002) b) introgression of favourable loci or genes (Cullingham et al., 2013); c) for comparative genome mapping (Ahn and Tanksley, 1993; Celton et al., 2009; Illa et al., 2011); d) for anchoring DNA sequence scaffolds (Chagné et al., 2014).

1.4.3 Statistical analysis to identify QTL

QTL analysis provides information on how many genes control a trait, what are their effects and how they interact. The basic principle of QTL analysis relies on the association between genotypic information and phenotype (expression of trait). There can be several QTLs influencing the same trait and they can be on the same or different chromosomes. Statistical tests are applied to test if there are any significant phenotypic differences between groups of genotypes for markers spanning the genome. For example if a heterozygous AB genotype for a marker has a higher mean phenotypic value than for the AA genotype, then that marker is linked to a QTL and the “B” allele may have an additive positive effect on the phenotype (Figure 1.6). Once we know where the QTL is located on the genetic map, it is possible to make use of the whole genome sequence to investigate whether this region of the genome codes for functional genes. This can be helpful to identify candidate genes that are involved in the expression of traits.

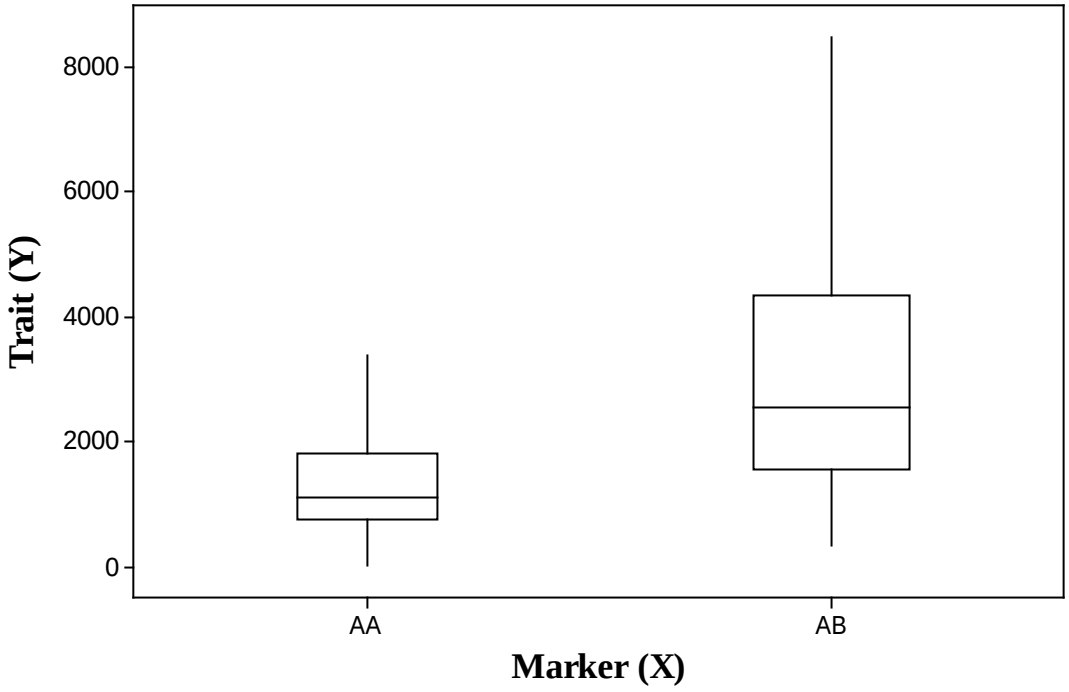


Figure 1.6: Graphical representation of quantitative trait locus (QTL). This QTL shows 2 groups of mapping population according to marker genotype "AA" and "AB" with significant difference between mean values of trait under study.

1.4.3.1 Methods to detect QTLs

1.4.3.1.1 Single-marker analysis (SMA)

The most simple method to determine whether an association exists between marker and phenotypic trait is known as single marker analysis or SMA (Sax, 1923; Ahn and Tanksley, 1993; Sofi and Rather, 2007). SMA can be conducted by a variety of statistical methods including t-tests, analysis of variance (ANOVA), linear regression and likelihood ratio and maximum likelihood estimation (Collard et al., 2005). However QTL detection by this method is not very accurate. The advantage of this method is that it does not require a complete linkage map and advanced software to run the analysis. However, failure to detect QTLs linked to distant markers is its

major disadvantage. High density genetic markers covering whole genome could be a used to minimise the bias posed by this approach. Results from single marker analysis are presented in tabular form as shown in Table 1.4 (all values presented here are hypothetical). A QTL is detected for the marker 'A' from linkage group (LG) 7 and the variation explained by this QTL is 80%. Another marker 'B' is also linked to QTL for the observed trait on LG7 which explained the 40% phenotypic variation. Whereas markers C and D from LG 5 and 2 respectively are also associated with QTL.

Table 1.4: Results from single marker analysis.

Marker	Linkage group	P value	*R2*
A	7	<0.0001	80
B	7	0.001	40
C	5	0.025	27
D	2	0.5621	2

1.4.3.1.2 Simple interval mapping

To overcome the disadvantages of the single marker approach, (Lander and Botstein, 1989) introduced the interval mapping approach based on association between QTL and flanking markers along the chromosome. This approach is statistically more powerful with use of maximum likelihood and or regression or combination of both. Both models have a similar approach; first detect the QTL by testing the hypothesis of presence (H1) or absence (H0) of a QTL at a certain position (cM) within in the interval between adjacent markers on the whole chromosome repeatedly and then plot the curve against the trait and map distance (Figure 1.7). The LOD position with highest score is regarded as the position of QTL on the map (Van Ooijen, 2004). The LOD score threshold used to detect and estimate the position of QTL is normally fixed arbitrarily in a range from 2-3. However it is recommended to calculate LOD threshold value for each experiment separately and this largely depends upon recombination frequency of population under study (Van Ooijen, 1999).

In addition, the problem of the recombination event between the markers and the QTL have been compensated by using linked markers (Lander and Botstein, 1989; Liu, 1998). Commonly used software packages to conduct SIM are MapMaker/QTL (Lincoln et al., 1993) and QGene (Nelson, 1997).

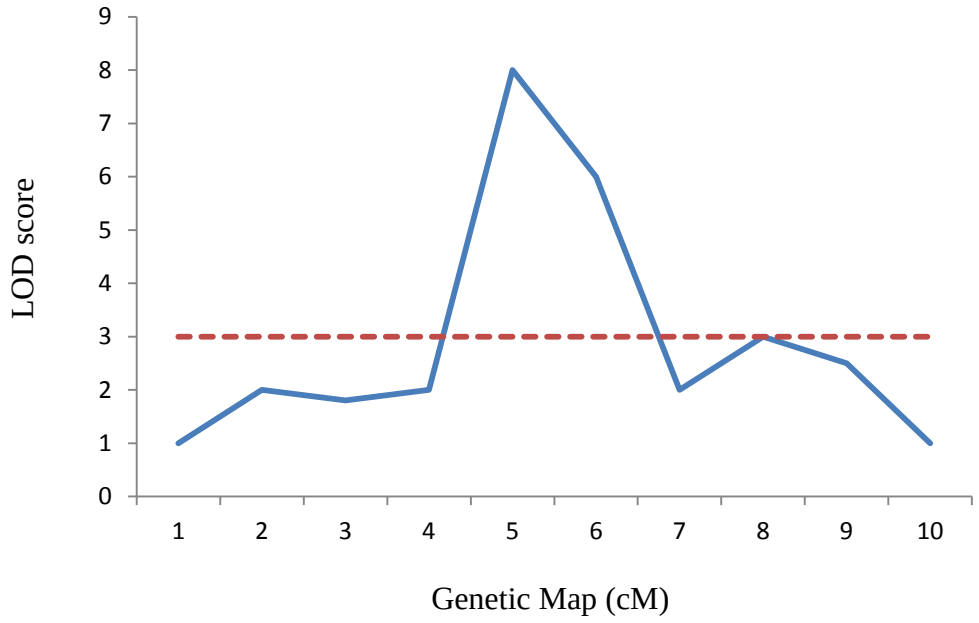


Figure 1.7: Graphical representation of an output of simple interval mapping for a typical chromosome (x axis) showing a LOD profile for trait (y axis). The dotted line in red indicates the threshold of significance and output peak indicates the location of QTL at marker 5. Best flanking markers are showing highest peak.

1.4.3.1.3 Composite interval mapping (CIM)

Simple Interval Mapping is used to detect single QTL so there is possibility of declaring a single QTL when in fact multiple QTLs are located in proximity. Multiple QTLs can be resolved using the composite interval mapping approach (or multiple interval mapping) as introduced by Zeng (1994). In this approach interval mapping is combined with multiple regression using significant markers for other QTLs on the same genome as cofactors. This is known as Cockerhams model (Gupta, 2007b; Sofi and Rather, 2007). This method can identify more than one QTL on the same chromosome at different locations and also provides the opportunity to find interactions between these QTLs. CIM gives benefits over

interval mapping due to more precise mapping with high power detection and reduced residual errors of effects and location of QTL (Jansen, 1993; Zeng, 1993; Jansen and Stam, 1994; Zhao-Bang, 1994). A number of software tools are used to perform CIM including MapQTL (Van Ooijen, 2004) Cartographer (Basten et al., 2004), MapManager QTX (Manly et al., 2001) and PLABQTL (Utz and Melchinger, 1996).

1.4.4 Factors affecting the QTL detection

Accuracy of QTL mapping depends not only on the appropriate statistical method, but also on biological constraints (Doerge, 2002). The genetic properties of QTL controlling the quantitative trait i.e. number and contribution of each QTL to genetic variance of trait also play an important role in QTL detection. QTLs controlled by several genes with small effect QTL are difficult to detect as compared with single and large effect QTL. Another genetic property is their relative genome positions on chromosomes and their interaction. A high resolution linkage map with large number and evenly spaced polymorphic markers is required to decrease the confidence interval (CI) for detection of several QTL on same chromosome (Liu et al., 2012). High resolution is also useful for preventing genetic drift during the process of introgression of wild genes using marker assisted selection. Plants with large genome size require more polymorphic markers for proper coverage of the genome. Errors in marker genotyping, such as missing information and markers with distortion can result in different arrangement of markers on the chromosome and eventually contribute to detection of "ghost" QTLs instead of real ones (Asíns, 2002).

Accurate phenotyping is also very important for accurate detection of QTL. Researchers should take special consideration while phenotyping, as any erroneous data will give more false positive results than actual QTLs (Danesh et al., 1994; Haley and Andersson, 1997).

Environment has a profound effect over the nature and expression of quantitative trait in a population, affecting the QTL detection. Specially designed experiments with replication over time (different years) and location are necessary to test the robustness of QTL detected (Price and Courtois, 1999; Hittalmani et al., 2002;

Jampatong et al., 2002; Lindhout, 2002; George et al., 2003).

Population type (double haploid, back cross) and size (number of individuals) also affects the power of QTL detection. There is no clear discussion to decide what is the number for high resolution QTL detection, however it is confirmed from many studies that higher the number of individuals (500) more statistical power, reliable small effect QTL detection and CI for the accurate location of QTL (Ahn and Tanksley, 1993; Haley and Andersson, 1997; Beavis, 1998). However limitation of less number of individuals can be overruled by using high density genetic linkage maps.

1.4.5 Genomics study in *Pyrus*

Pear (*Pyrus* spp.) belongs to the tribe Maleae in family Rosaceae together with apple. Almost all major cultivated pears are functionally diploid ($2n = 2x = 34$) with 17 as the basic chromosome number ($n = 17$). Genus *Pyrus* comprises almost 22 species which are widespread in pear growing areas of the world. However the three major species *P. communis.* L (European pear.), *P. pyrifolia* Nakai (Japanese pear) and *P. bretschneideri* Rehd (Chinese pear.) are the most popular and are grown commercially.

1.4.5.1 Pyrus genotyping

The advent of new high throughput technologies has enabled the availability of more and more sequenced plant genome and large numbers of SNPs discovery. QTL mapping has become easier and more popular for fruit plants than in previous years. Considerable amount of QTL mapping work in fruit crops have been performed in the last two decades, especially in the last few years (Chen et al., 1999; Etienne et al., 2002; Dondini et al., 2004; Arbelbide and Bernardo, 2006; Dunemann et al., 2009; Costa et al., 2010; Khan et al., 2012; Costa et al., 2013).

Recently, whole genome sequencing of Chinese pear (*Pyrus bretschneideri*) with a coverage 194× was reported by (Wu et al., 2013). The genome was sequenced using a combination of NGS and BAC-by-BAC sequencing technology and covered almost

97.1 % of the estimated genome size. The whole genome sequence of the European pear 'Bartlett' obtained using NGS technology has also been published recently (Chagné et al., 2014). Mapping of ‘Old Home’ and ‘Louise Bonne de Jersey’ low coverage sequence data to ‘Bartlett’ scaffolds enabled discovery of a total of of 3,893,643 putative SNPs. Average SNP frequency after filtration was one SNP per 674 bp. The ‘Bartlett’ genome assembly can be used to compare synteny of the pear genome with other related species, including other members from Rosaceae family. Comparison of ‘Bartlett’ protein cluster with 13 other species including *P. bretschneideri* Rehd and apple revealed that a set of 1,433 protein clusters present in both pear species (European and Chinese pear) and apple that were absent from other species and this set of proteins may include genes responsible for the pome fruit character (Chagné et al., 2014).

1.4.5.2 Construction of linkage maps in Pyrus

Construction of a high density map is a prerequisite in plant genetic improvement programmes. Pear genetics and genomics are not as advanced as in other major crops, including apple. Iketani et al. (2001) was the pioneer, developing the first pear linkage map, using Japanese pear (*P. pyrifolia* Nakai) varieties ‘Kinchaku’ and ‘Kosui’ and RAPD markers to develop maps. They developed two separate maps for each parent by using 82 individuals (Iketani et al., 2001). These maps were estimated to cover almost half of the pear genome when compared to apple maps. The second set of linkage maps was developed by Yamamoto et al. (2002, 2004) using 63 seedlings from interspecific crosses between ‘Bartlett’ (*P. communis*) and ‘Hosui’ (*P. pyrifolia*) pear. They used the AFLP and SSR markers from apple, pear and peach, isozymes and phenotypic markers to construct two maps. Their maps were first reported reference maps for pear eg ‘Bartlett’ had 256 loci spanning 1020cM. Average distance between the loci reported is 4 cM (Yamamoto et al., 2002; Yamamoto et al., 2004).

Linkage maps from two European pear cultivars ‘Passe Crassane’ and ‘Harrow Sweet’ were developed by Dondini et al. (2004). These maps were constructed by using 99 F1 individuals with SSR, MFLPs (microsatellite-anchored fragment length polymorphism), AFLPs, RGAs (resistance gene analog) and AFLP-RGA (amplified

fragment length polymorphism- resistance gene analog) markers from apple and pear origin. These maps were used to map QTL controlling resistance to fire blight, one of the important diseases of European pears. The map for 'Passe Crassane' comprised 155 loci in 18 groups over the distance of 912cM and for 'Harrow Sweet' comprised 156 loci spanning over distance of 930cM and 19 groups. Pierantoni et al. (2004) used two European populations to construct maps using 100 apple SSRs. Syntenic relationships between apple and pear was also confirmed by at least carrying two or more common SSR markers. Pierantoni et al. (2007) used one of the same population to construct a high density map from 157 AFLP and 41 SSR markers and to identify QTLs for scab resistance.

Genetic maps for Chinese pear 'Yali' and 'Jingbaili' were constructed by using AFLP and SSR markers from 145 F1 individuals by Sun et al. (2010) in order to identifyQTLs for leaf traits. Their maps consisted of 402 markers (AFLP and SSR) which covered 1395.9 cM area and average distance between pair of loci was 3.8 cM (Sun et al., 2009). Zhang et al. (2011) used a population of 97 F1 seedlings from a cross between the interspecific hybrid pear 'Bayuehong'(European × Chinese species) and the Chinese pear 'Dangshansuli', for construction of parental linkage maps for the purpose of QTL analysis of fruit traits. Both these maps consisted of 17 linkage groups with 214 and 122 markers spanning over 1,352.7 cM and 1,044.3 cM for Bayuehong'and 'Dangshansuli' respectively.

Recently Wu. et al (2014) published a dense integrated pear map consisting of 3143 SNPs and 98 SSR markers developed by restriction-association DNA sequencing (RAD). This map consisted of 17 LG spanning 2243.4 cM.

1.4.5.3 Pyrus *QTL analysis*

Extensive research involving QTL mapping has been reported in apple (Korban and Tartarini, 2009), however only a few studies have been reported for *Pyrus* species. Generally these researches were conducted to study the disease resistance, one about leaf characteristics and last focuses on fruit characters.

QTL mapping was carried out for fire blight resistance in European pears by Dondini et al. (2004). They identified four putative QTLs for control of fire blight (*Erwinia amylovora*) in a resistant parent 'Harrow Sweet', and no QTLs were detected in the susceptible parent 'Passe Crassane'. Another important disease for European pear is scab (*Venturia inaequalis*). Pierantoni et al. (2007) used the linkage maps from 'Abbé Fétel' × 'Max Red Bartlett' to identify QTLs linked to scab resistance. They detected two putative major QTLs for scab resistance on LG3 and 7 these QTLs explained a total of 88% of phenotypic variance.

QTL mapping in Chinese pear was reported by Sun et al. (2009) for vegetative traits including leaf length, width, leaf length/leaf width ratio and petiole length. So far two studies have been reported for fruit trait QTLs in pear. A population developed from interspecific hybrid pear 'Bayuehong' (*P. communis* × *P. bretschneideri*) and the Chinese pear 'Dangshansuli' has been used in two different studies for genetic map construction and QTL mapping for fruit traits (Zhang et al., 2011; Wu et al., 2014). Zhang et al. (2011) developed two parental linkage maps using AFLP and SRAP markers and detected nine QTLs for five fruit traits (fruit weight, transverse diameter of fruit, vertical diameter of fruit, soluble solids content and fruit shape index). Recently a total of 32 potential QTLs for 11 fruit traits including pedicel length, fruit weight, transverse diameter, vertical diameter, flesh colour, number of seeds, juice content, calyx status, skin colour, skin smoothness and TSS were identified using the same population (Wu et al., 2014).

1.4.6 QTL analysis for fruit disorders

Breeding for fruit quality has become a major priority for many breeding programs worldwide. A series of experiments has been conducted to understand the genetic and molecular mechanism of fundamental physiological processes involved in fruit quality. Only a few studies have been reported concerning QTL studies of disorders in fruit trees. such as chilling injury in peach (Martínez-García et al., 2013a), water-soaking, necrosis of the placental tissue, chilling injury, decay, over-ripening, flesh browning, hollow flesh disorder, and flavour loss during storage in melon (Fernández-Trujillo et al., 2007) and flesh browning in apple (Di Guardo et al., 2013).

1.5 Thesis aim and objectives

The aim of this project is to shed new light on the genetic control of friction discolouration (FD) in pear. The new knowledge gained during this project will make selection for elite pear seedlings for cultivar breeding more efficient. On the basis of understanding developed from the literature review, the following research objectives were designed:

1. To define the physiological and biochemical basis of FD in two pear segregating populations (POP369 and POP356) obtained from interspecific crosses and identify the underlying risk factors in these genotypes.
2. To construct high density genetic linkage maps for both populations covering all pear linkage groups.
3. To identify quantitative trait loci (QTLs) for FD and other fruit traits by using phenotypic and genotypic data from two populations over two years.

Chapter 2 describes the physiological and biochemical basis of FD and other fruit quality traits for pears from interspecific crosses. Fruit samples (241) from two populations (POP369 and POP356) were harvested at maturity to assess FD and traits such as fruit firmness, total soluble solids (TSS), polyphenol oxidase (PPO) activity, and phenolic and ascorbic acid (AsA) concentrations. Various statistical techniques were used for data interpretation. The phenotypic data collected were used for further QTL analysis.

Chapter 3 explores the tools for the construction of genetic linkage maps for populations POP369 and POP356 using apple and pear single nucleotide polymorphic (SNP) markers. Newly discovered SNPs in the pear genome by next generation sequencing (NGS) were included in the IRSC apple Infinium® II 8K SNP array. This array was evaluated for application in pear and used to construct the genetic maps.

Chapter 4 describes QTL mapping for FD and other fruit-related quality traits in both segregating populations. Genomic regions linked to some of the 22 phenotypic

variables including FD were identified in both populations. Robustness and potential implication of these QTLs is discussed.

Chapter 5 is a general discussion wrapping up whole research project, drawing together the qualifications and caveats of this study. The future prospects of the study are discussed, considering FD as a model for postharvest biology genetic analysis, along with the potential areas for extending this research in the future.

In addition to the thesis chapters, four published papers (1 Conference Proceedings and 3 peer reviewed journal) are enclosed in the appendix. The paper in the Conference Proceedings (Acta Horticulturae; Saeed et al., 2012) concerns phenotypic determinants of friction discolouration in pear. This paper is part of chapter 2 and I led the preparation of this paper. The first journal paper published in PLoS ONE (Montanari et al. 2013) describes the development and evaluation of SNP markers in pear. This paper, I equally prepared with two fellow PhD candidates (Sara Montanari and Mareike Knäbel), is an extension of Chapter 3 of this thesis, incorporating three other segregating populations. This paper includes genetic linkage maps from two segregating populations used to study FD. The second journal paper (Chagné et al., 2014; PLoS ONE) is about the sequencing of the European pear genome. My involvement in this paper was the use of my genetic linkage maps to anchor the pear genome scaffolds. The third journal paper (Saeed et al. 2014; BMC Plant Biology) presents part of the results from Chapters 2 and 4 of this Thesis, and I led the preparation of this paper.

2 CHARACTERIZATION OF PHENOTYPIC DETERMINANTS OF FRICTION DISCOLOURATION IN PEAR[*]

2.1 Introduction

Plant & Food Research (PFR) New Zealand established an interspecific pear breeding programme in 1983 to develop novel pear cultivars. Along with directional and non-directional selection for fruit quality parameters, breeders also screened populations for disease and disorder susceptibility (Brewer et al., 2008). Friction discolouration (FD) is an important disorder against which the pear breeding programme is applying strong selection pressure. In initial studies it was found that the narrow sense heritability estimate of FD was high (0.72), which implied that fair genetic gains could be achieved in development of FD-free pears (Brewer et al., 2011).

The mechanism of skin browning involves physical injury to the fruit skin from rubbing or pressing of fruit against a hard surface during harvest, packing, transportation or marketing (Thompson, 2007), followed by enzymatic oxidation of phenolic compounds causing diffuse brown skin marks (Kvåle, 1979; Feng et al., 2004). FD affects fruit quality and degrades the cosmetic appeal of fruit, reducing consumer acceptance (Raese, 1989) and storage life, resulting in economic losses which are significant. Fruits with an uneven surface get damaged more easily and sometimes a pattern of damage develops which can easily be identified, such as the pattern of fabric that may appear on skin when fruits are pressed against rough picking or packing bags. Some people erroneously take bruising and FD as the same phenomenon, however FD is generally restricted to the epidermal cells (Berardinelli et al., 2005), unlike bruising where fruit flesh is also damaged.

[*] Material from this chapter is included in papers: a) Saeed, M., McGhie, T., Brewer, L., Gardiner, S., Chagné, D., and Heyes, J. (2012). Characterization of phenotypic determinants of friction discolouration in pears. Acta Horticulturae, 1012, 1111-1118. b) Saeed, M., Brewer, L., Johnston, J., McGhie, T. K., Gardiner, S. E., Heyes, J. A., and Chagné, D. (2014). Genetic, metabolite and developmental determinism of fruit friction discolouration in pear. BMC plant biology, 14(1), 241. doi: 10.1186/s12870-014-0241-3. Fruit were harvested in Motueka according to my specified requests and shipped to me in Palmerston North; I did all the experimental work thereafter, and drafted the manuscripts.

Some cultivars are more susceptible to FD compared to others, which retain their resistance character even under conditions favourable to browning (Amiot et al., 1995; Bertolini and Trufelli, 2002). Both European and Asian cultivars are sensitive to FD, however Asian pears are regarded as more susceptible and very rarely up to one third of fruit can be affected (e.g. 'Huanghua' Chinese pear, (Zhou and Li, 2007). 'Abbe Fetel' and 'Doyenne du Comice' are regarded as susceptible European pears and require special handling (Burger et al., 2005; Gomila et al., 2011), however their susceptibility to FD varies due to influence of pre- and postharvest factors such as growing environment, season, harvest maturity and handling temperature. Nonetheless there is no agreement about how harvest maturity and handling temperature affects FD susceptibility in different cultivars. For example Mellenthin and Wang (1973) and Kvåle (1979) described that late harvested fruits are less prone to FD than early fruits during and after storage, while Mitcham et al. (2001) and Burger et al. (2005) commented that early harvested (firm) fruits are less prone to FD as compared to late harvested ones. It can be anticipated from these opposite trends observed by different authors that fruits harvested at proper maturity are less prone to damage as compared to pre or over mature fruits regardless of other factors. There is a general agreement that FD susceptibility increases with time in storage (Mellenthin and Wang, 1974; Gomila et al., 2011). The increase in pear susceptibility to FD with prolonged cold storage has been attributed to loss of turgidity of fruit (Kvåle, 1988; Amiot et al., 1995), enhanced ripeness (Volk et al., 1977; Li et al., 2012), or accumulation of the phenolic compounds present in pear skin (Wang and Mellenthin, 1973). FD was assesed using different methods including mechanical vibration (Feng et al., 2004; Burger et al., 2005), rolling (Kvåle, 1979; Gomila et al., 2011) and hand held rubbing (Wang and Mellenthin, 1973; Feng et al., 2004; Brewer et al., 2011). Method used in current study was based on the simple hand held method adopted by PFR breeders (Palmer et al., 2007; Brewer et al., 2011). This method is especially suitable to assess large number of genotypes from segregating populations as no extra equipment is required to induce browning and a single user can quickly develop a standardised approach.

Availability of specific substrate, polyphenols, to PPO is fundamental to the process of enzymatic browning. Study of individual phenolic compounds can provide information about which specific phenolic compounds serve as the best substrates for

PPO for induction of FD in two segregating populations used in this study. Phenolic composition of different plants varies greatly with cultivar, stage of maturity, and postharvest storage conditions (Herrmann, 1976; Spanos and Wrolstad, 1992). Along with these biochemical components (enzyme and substrate), skin anatomy may also play a role in the incidence of FD, as enzyme and substrate are located in different cellular compartments. As a result of physical damage, interaction of PPO and phenols leads to oxidative browning in the cell. Epidermal and hypodermal anatomy, and stone cell frequency and location have been proposed to contribute to susceptibility in some cultivars (Amarante et al., 2001a; Palmer et al., 2008). Ascorbic acid (AsA) is known for its strong antioxidant properties and has been used as an external coating to minimise the problem of FD (Mellenthin and Wang, 1974; Kvåle, 1979). As nobody has tested the role of endogenous AsA as a natural inhibitor of FD in pear to date, its concentration was monitored in this study.

Previous studies used a small number of unrelated genotypes and come up with different explanations of the factors that influence FD susceptibility. Hence, this study was performed using a wide range of related genetic material from an interspecific pear breeding programme. This work aimed to characterise the effect of physical and physiological factors on the susceptibility to FD in pear genotypes from two segregating populations (POP356 and POP369) from interspecific crosses of Asian and European pears. To complement this, in 2013 a small replicated trial was conducted to test the consistency of identified potential predictors of FD, using replicated trees and harvests.

2.2 Materials and methods

2.2.1 Plant material and fruit sampling

Two full sib families (POP356 and POP369) resulting from interspecific crosses between Asian (*P. pyrifolia* Nakai and *P. bretschneideri* Rehd.) and European pears (*P. communis* L.) (Figure 2.1) were grown at the Motueka Research Centre, PFR, Motueka, New Zealand. POP369 is a population of 1028 full-sib genotypes from a cross between POP369-female and POP369-male. POP356 is a population totalling 1285 full-sib genotypes from a cross between POP356-female and POP356-male

parent. Both families were planted on their own roots into the orchard in 2007 at row spacing of 3 m (meter) and in row spacing of 0.75 m. Plants received a standard fertiliser programme and any branches at least one metre above the wire trellis at a height of 1.8 m were bent down to the wire in January each year. Trees that had not begun to fruit were girdled in December with a Vaca cane girdler which removed a 4mm horizontal strip of vascular tissue below the 1.8 m wire.

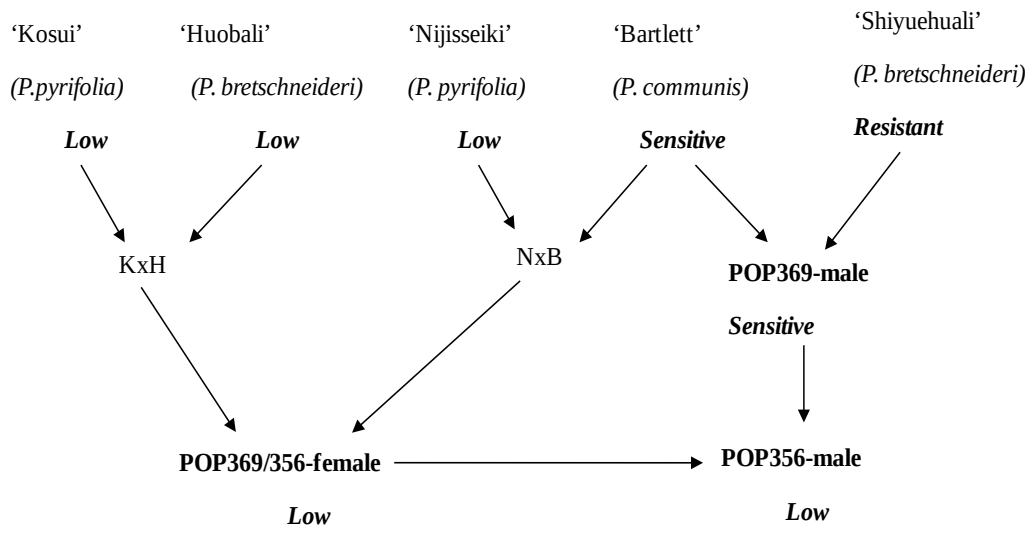


Figure 2.1: Genotypic information and FD potential concerning parents of POP369 and POP356.

Fruit from 241 individual genotypes were harvested in 2011 with some genotypes sampled multiple times – 206 fruit samples from 143 genotypes of family POP356 and 125 fruit samples from 98 genotypes of family POP369. In 2012, 177 fruit samples from 91 genotypes of the POP369 population were harvested, with multiple harvests (2 or 3 replicates for each genotype) where possible. Due to interspecific nature of fruit, it was impossible to follow any objective parameter for maturity assessment at the time of harvest. Fruit harvest for each genotype began when fruit had a green-yellow background colour and samples of 4-9 fruit were harvested every 7-12 days until fruit ran out. Fruit was stored for 90 to 100 days at 3 °C for initiation of ripening, and then transported to PFR Palmerston North by refrigerated truck for further analysis.

A comparatively smaller trial was conducted in 2013 with clonal replicates and repeated harvests in order to test their role for FD susceptibility. In 2013, 45 fruit samples from 8 seedlings including one parent and one grandparent of POP369 were collected. Each seedling was replicated (2 or 3 clonal replicates for each tree) and for most replicate trees three (3) harvests were made, with few exceptions where only one or two harvests were made due to low fruit load borne by some trees .

To assess FD, four representative, preferably unblemished fruit were selected per genotype, removed from the cool store and kept overnight at room temperature. FD was induced the next day by rubbing the fruit twice against a fibre tray cup surface (Brewer et al., 2011; Saeed et al., 2012). This method was developed by PFR breeders and I was trained by one of them to ensure the consistency of the approach. After another 24h at room temperature, browning area and intensity was recorded according to a 0-9 scale, where 0 is absence of FD and 9 is the highest FD score (Figure 2.2). The FD score was scored by the same single assessor (the author of this thesis) to reduce experimental error. FD score was averaged across all four fruit for each seedling and harvest date.

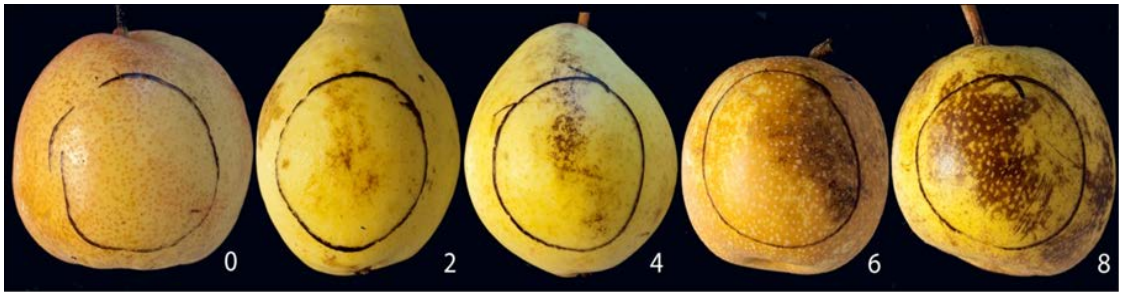


Figure 2.2: Visual scoring scale for friction discolouration (FD) assessment.

2.2.2 Total soluble solids and firmness

Total soluble solids content (TSS) and fruit firmness were measured using two of the same fruit on which FD was determined. Equal amount of juice from both ends of fruit was used to assess TSS (°Brix) using a digital refractometer (Atago, Japan). Compression firmness was measured in Newtons (N) at two points separated by 180°

around each fruit equator, using a texture analyser TA-XT Plus (Stable Micro System, Godalming, UK) fitted with a 7.9 mm probe.

2.2.3 Peel sample preparation and extraction for polyphenol and AsA quantification

Peel of 1 mm thickness, consisting of fruit skin with underlying flesh was removed from the equatorial area of 4-5 fruit per genotype (generally the undamaged part of the same fruit that were used for FD assessment). Peel tissues were snap frozen and then ground together with a pellet of dry ice, using a coffee grinder. Ground peel tissue was stored frozen at -80 °C until further analysis. Extraction solution (80:20:1 EtOH: H_2O: formic acid; 5 ml) was added to finely ground peel (1 g peel) and left for 24 h at 4 °C. After 24 h, culture tubes containing the extract were centrifuged (1000 *g*, 15 min) at 20 ± 2 °C and the extracts were sampled directly into high pressure liquid chromatography (HPLC) vials. Aliquots of the extract were diluted in cold solvent (50:50:1 MeOH: H_2O: formic acid) prior to polyphenol analysis.

2.2.4 Polyphenol quantification in pear peel

Polyphenol content of these extracts was analysed using a liquid chromatography mass spectrometry (LC-MS) system that comprised a Dionex Ultimate® 3000 Rapid Separation LC system and a micrOTOF-QII mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray source operated in negative mode. A high resolution mass spectrometer was used and the quantification was achieved using accurate mass ion chromatograms e.g. m/z 289.0712 (±0.02 Da). The analytical column was Zorbax™ SB-C18 HD, 2.1 x 150 mm, 1.8 µm (Agilent, Melbourne, Australia). Solvents used in 2011 were A = 90 % methanol, and B = 0.5 % formic acid in water (v/v), with a gradient of 5 % A, 95 % B, for 0-0.5 min; gradient to 40 % A, 60 % B, from 0.5-8 min; gradient to 75 % A, 25 % B, from 8-11 min; gradient to 100 % A, 0.0 % B from 11-12 min; the composition was then held at 100 % A, 0.0 % B, from 12-14 min; before returning to 5.0 %A, 95 % B, between14-14.2 min. The gradient of 5 % A, 95 % B was maintained until injecting the next sample. Total run time for each sample was 17 minutes.

In 2012, the method was slightly changed based on advice from Dr. McGhie (personal communication). Solvents used in 2012 and 2013 were A = 100 % acetonitrile, and B = 0.1 % formic acid with a gradient of 5 % A, 95 % B, for 0-0.5 min; gradient to 30 % A, 70 % B, from 0.5-10 min; gradient to 100 % A, 0.0 % B, from 10-14.50 min. The composition was then held at 100 % A, from 14.5-16.50 min; and returned to 5.0 % A, 95 % B, between 16.5-17 min and maintained until the next sample was injected. Total run time for each sample was 20 minutes.

Polyphenol components were quantified using QuantAnalysis (Bruker Daltonics, Bremen, Germany) by extracting accurate (±0.02 Da) mass ion chromatograms. As external standards were not available for all the detected compounds, peak area (response/ min) was uses for calculations involving phenolic concentrations for all the compounds.

2.2.5 Ascorbic acid quantification in pear peel

Ascorbic acid (AsA) content in pear fruit peel was quantified using HPLC (high performance liquid chromatography). Solvent based peel extracts (prepared for polyphenol quantification) were diluted 1:4 with tris-(2-carboxyethyl) phosphine and incubated in the dark for 90 minutes. The HPLC system used was an Alliance 2690 HPLC (Waters, Milford, MA, USA). AsA was resolved using a Synergi 4 µm Hydro 4.6 x 250 mm (Phenomenex, Torrance, CA) reversed phase column protected with a guard column of the same packing. Column temperature was set at 40°C. The solvents used were A= 0.5 % v/v phosphoric acid (98 %) and C= 70:30 methanol/Milli-Q water (2 %) and proportions remained the same throughout the run. Sample injection volume was 10 µL and flow rate was 0.8 mL per minute. Total run time for each sample was set at 9 min. An external calibration curve was constructed for AsA based on the basis of three standards with concentrations 10 µg/mL, 20 µg/mL and 50 µg/mL respectively. Quantification of AsA was based on peak areas determined at 240 nm in 2011 and 250 nm in 2012 and 2013. Choice of wave length was made on the basis of visual quality of the AsA peak. Chromatographic data was collected and manipulated using the Chromeleon® Chromatography Management System version 6.8. The AsA concentration derived

from the HPLC analysis was transformed from µg/mL (Cv) to µg/g (Cw) of fresh weight by dividing Cv by the fresh weight of each sample used.

2.2.6 PPO activity quantification in pear peel

Polyphenol oxidase (PPO) activity was measured spectrophotometrically (Mitcham et al., 2001; Burger et al., 2005) with a few modifications. Extraction solution (0.05 M sodium phosphate buffer, 1 M KCl, pH= 7, 9 ml) and 1 g polyvinylpolypyrrolidone (PVPP) was added to 1g finely ground frozen peel. This mixture was homogenised and centrifuged (14,000 x*g*) for 15 minutes at 4 °C. Supernatant was carefully pipetted to eight wells in a single column of a 96 well plate. Each sample well contained 25 µL extract, 220 µL reaction buffer (0.2 M sodium phosphate, 0.1 M sodium citrate, pH = 6.5) and 55 µL standard catechol solution (0.5 M catechol in a 10 fold dilution of the reaction buffer). Substrate was added to all samples simultaneously by using a multichannel pipette. Assay procedure was carried out at 20 °C with initial shaking for 2 seconds. Two or three replicated wells were used for each sample. The increase in absorbance at 420 nm was recorded by spectrophotometer (Molecular Devices Spectra Max Plus, Sunnyvale, CA, USA). Enzyme activity was calculated by using initial 20 sec gradient of curve in 2011 and initial 30 sec in 2012 and 2013. PPO activity is presented as the change in absorbance at 420 nm per gram fresh pear peel per minute ($A_{420}\, g^{-1} minute^{-1}$).

2.2.7 Anatomical study

A total of 40 randomly selected fruit samples from low and high FD susceptibility categories from POP369 and POP356 were used for the anatomical study. Fluorescent microscope (Olympus BH2 fluorescent microscope, Tokoyo, Japan) was used to examine transverse and tangential images. Hand sections of almost 10mm length were cut using a sharp blade and placed on the glass slide with one drop of distilled H_2O. To prepare stained transverse sections, a saturated solution of phloroglucinol in 20 % HCl was used to stain the tissue. Images were collected using a Leica digital camera (Leica DFC 500, Germany) and Leica application suite software. Images were examined for stone cell abundance, distance between stone

cells and epidermis and epidermal thickness with a minimum of 3 measurements per section for 3-4 sections per fruit sample.

2.2.8 Statistical analysis

Minitab® version 16.1.1 was used to calculate Pearson correlation coefficients for FD and other fruit variables and to perform analysis of variance with 95 % confidence interval for POP369, POP356 and data from replicated genotypes. Only data significant at $P < 0.05$ are discussed in the context. Genstat® version 14.2 was used to perform step wise discriminant analysis (DA) for POP369 and POP356.

2.3 Results and discussion

2.3.1 Friction discoloration variation in the pear segregating populations

Both populations (POP369 and POP356) displayed the full range of FD scores (Figure 2.2). Although there was a range of FD for different genotypes, there were clear patterns of FD between genotypes. Having more than one harvest from some genotypes provided the opportunity for comparison of FD susceptibility between fruit at different stages of maturity. FD susceptibility showed substantial variation between genotypes. Of 23 genotypes with multiple harvests in 2011, there were 16 genotypes that did not show any variation of FD score among the harvest dates, with 14 genotypes scoring consistently low, and two exhibiting a consistently high score (Figure 2.3).

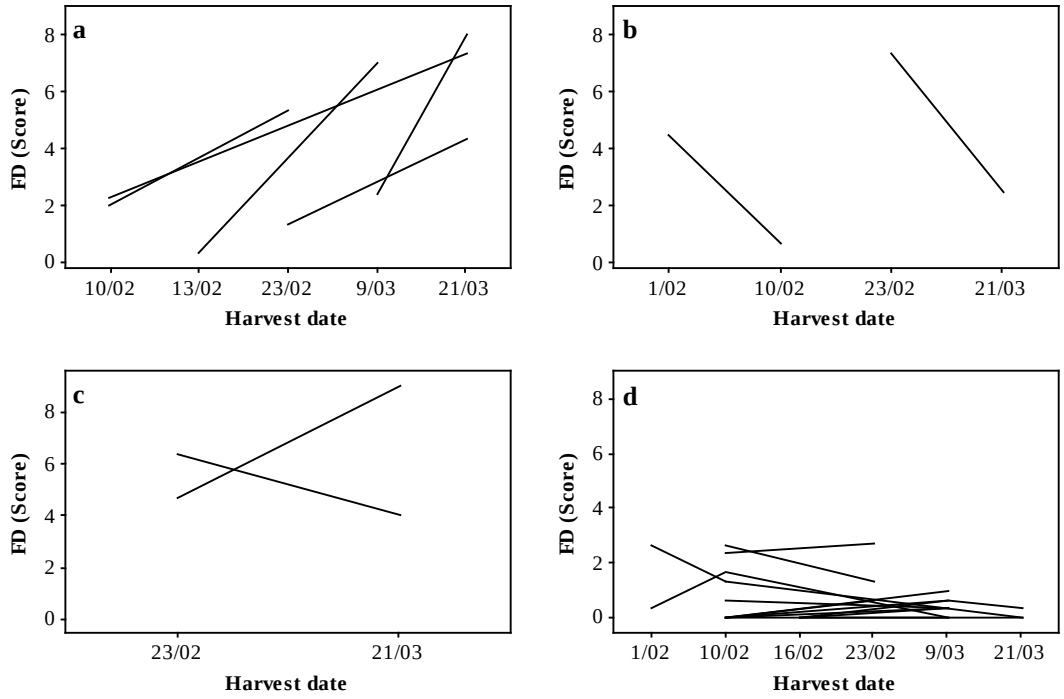


Figure 2.3: Mean FD scores arranged by harvest dates for multiple harvests of genotypes for POP369 (2011). Genotypes with multiple harvests for individual tree were divided into four distinct groups a) represents the seedlings with increasing FD trend during the season b) represents seedlings with decreasing trends c) represents consistent high FD susceptibility d) represents seedlings with consistent low FD susceptibility.

In addition, five genotypes of the POP369 population exhibited low FD at early harvests and high FD at later harvests; and two genotypes exhibited a decrease in FD susceptibility later in the season. In 2012, 63 genotypes from POP369 had multiple harvests. Of these, 19 showed a stable high susceptibility to FD, and 14 showed a stable low susceptibility. A further 23 genotypes showed an increasing susceptibility during the season and seven decreased in susceptibility, as the season advanced (Figure 2.4).

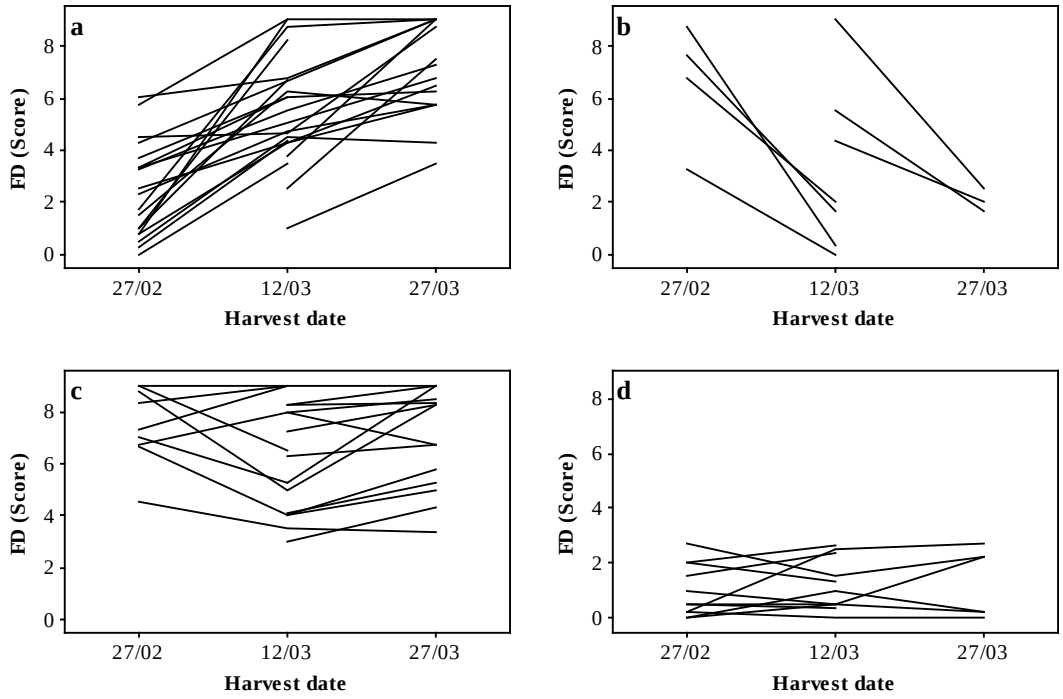


Figure 2.4: Mean FD scores arranged by harvest dates for multiple harvests of genotypes for POP369 (2012). Genotypes with multiple harvests for individual tree were divided into four distinct groups a) represents the seedlings with increasing FD trend during the season b) represents seedlings with decreasing trends c) represents consistent high FD susceptibility d) represents seedlings with consistent low FD susceptibility.

POP356 in 2011 had 48 genotypes with multiple harvests. 29 genotypes did not show any variation of FD score between the harvest dates, with 24 genotypes scoring consistently low, and five exhibiting a consistently high score (Figure 2.5), but there were also seven genotypes that showed increasing susceptibility during the season and 12 genotypes that showed decreasing susceptibility. POP356 had higher rate of genotypes exhibiting a decreasing trend during the season than POP369 in either year.

Over all in both populations across the years, over 50 % genotypes were consistent in their trend of susceptibility, either consistently low or consistently high.

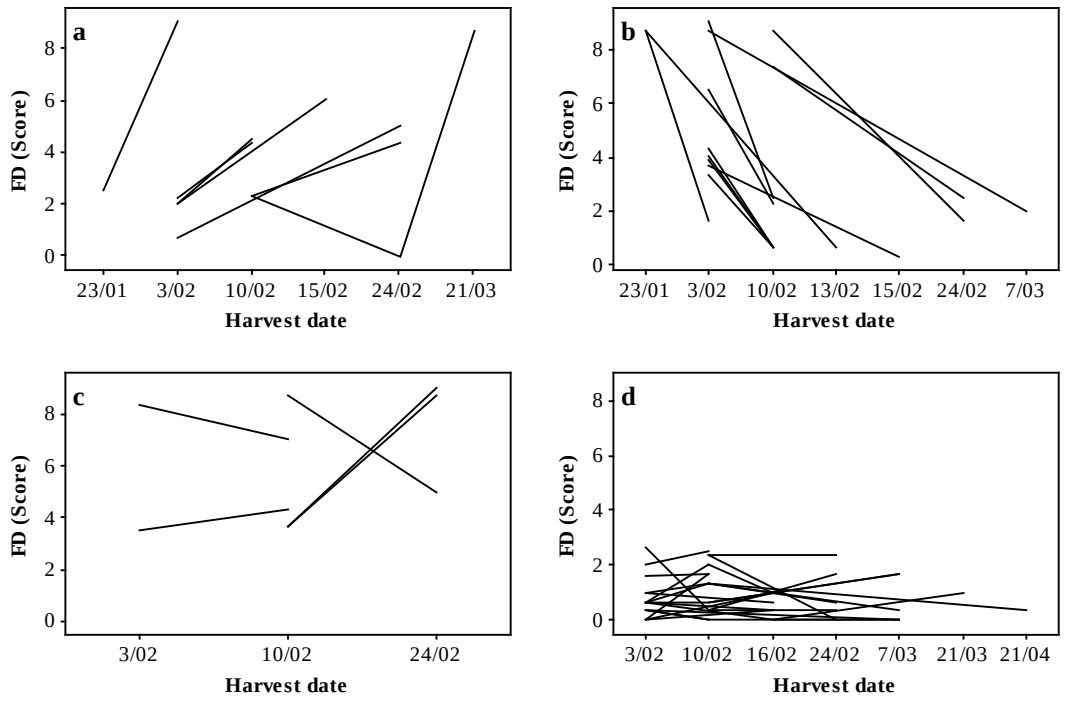


Figure 2.5: Mean FD scores arranged by harvest dates for multiple harvests of genotypes for POP356 (2011). Genotypes with multiple harvests for individual tree were divided into four distinct groups a) represents the seedlings with increasing FD trend during the season b) represents seedlings with decreasing trends c) represents consistent high FD susceptibility d) represents seedlings with consistent low FD susceptibility.

FD in pear is recognized to be a complex postharvest disorder that is highly influenced by both genetic factors and environmental factors (growing area, season etc), as well as factors related to development (harvest maturity). Results from 2011 and 2012 in both populations supported this view, as there was clear variation in susceptibility to FD among the seedlings of the two segregating populations (Figure 2.3-2.5), indicating that while there is involvement of genetic component of FD control, there is also a substantial non-genetic effect.

Different trends for FD among fruit harvested within a season have been discussed extensively by different researchers. Wang and Mellenthin (1974) reported that early harvested ‘d’Anjou’ pear fruits were more susceptible to FD than later harvested fruits. Kvåle (1979) proposed that fruits of ‘Moltke’ and ‘Herrepaere’ harvested before the climacteric peak were more susceptible to FD than late harvested or

mature fruit. Burger et al. (2005) reported that the relationship between FD and fruit maturity varies among genotypes. However, they studied two European pear genotypes only. 'Packham's Triumph' showed greater susceptibility to FD at late harvest compared with earlier harvest, while 'Comice' exhibited the opposite trend. There is however, another observation reported by Mitcham et al. (2001) and Gomila et al. (2010) that late harvested fruits are more prone to FD. These variable trends of different varieties reported by different researchers propose that role of environmental and developmental factors along with genetic factors cannot be neglected. Analysis of FD among hundreds of individual genotypes from two interspecific populations (POP369 and POP356) highlighted even more clearly the complexity of the relationship between FD and underlying genetic and environmental factors.

By ignoring the concept of four different seasonal trends in susceptibility as described earlier, overall correlations of r = 0.36 (P<0.0001) and r = 0.27 (P<0.0001) were observed between FD and harvest date in 2011 and 2012 respectively, in the POP369 population. No such significant correlation was found in the POP356 population in 2011 but careful inspection of the actual harvest dates in Figure 2.3-2.5 shows that POP356 is an earlier-maturing population than POP369. The significant correlation of FD with harvest date in population POP369 indicates that overall susceptibility of POP369 to FD increased as the harvest date (maturity) advanced. Low correlation values could be due to the presence of different trends in FD susceptibility as season advanced.

Maturity indices are certainly not reliable across species as European and Asian pear cultivars have quite different indices (Tvergyak, 1985); and it is therefore not at all clear what indices would be useful for interspecific progeny between Asian and European pears. Although the indices currently employed to determine maturity for harvest (firmness, total soluble solid contents and ground colour) are the same as used for apple, these indices may well differ from genotype to genotype, between orchards and seasons. In this study, fruit firmness and TSS were measured after storage in an attempt to evaluate fruit maturity of selected genotypes. In 2011, TSS for both populations ranged from 10 to 13.5°Brix and in 2012 starting from a slightly lower °Brix value of 9, to 14 for POP369. However no significant correlation was

observed between FD and TSS. Fruit firmness ranged from 15 to 45 N for both populations in 2011 and in 2012 the POP369 population fruit had a slightly narrower range of 15- 36 N. While these traits segregated in both progenies, no relationship with susceptibility to FD was identified, except a statistically significant but low correlation value (r= -0.21) observed for POP369 in 2012. A general trend of high FD for softer fruit was observed especially in POP369 (Figure 2.3-2.5), but clearly this relationship did not hold for genotypes that showed consistently low or high FD susceptibility regardless of time of harvest.

The role of genetic (genotype) and non-genetic factors influencing the expression of FD during repeated harvests was explored by comparing common genotypes with multiple harvests from the 2011 and 2012 data sets of POP369. POP369 had 20 common genotypes with at least two harvests each season. Out of these 20, 13 genotypes exhibited the same trend regardless of year. Most of these (11 genotypes) were in the consistently low or high category; the other two genotypes showed an increasing trend in susceptibility during each season (Figure 2.6).

These consistent trends covering most genotypes indicate the strong underlying genetic factor influencing FD susceptibility. Of the seven inconsistent genotypes across the years only one (P369T13) showed consistently low susceptibility in one year (2011) and consistently high in other year (2012). This contrasting trend could be attributed to maturity varying between years.

Results from this study support the argument that there is a strong genetic component to FD susceptibility, however some genotypes are able to be affected by seasonal or maturity related factors which can affect their susceptibility within season even. Seedlings from breeding populations POP369 with consistently low susceptibility to FD in 2011 and 2012 could offer a good genetic pool for breeding FD free seedlings.

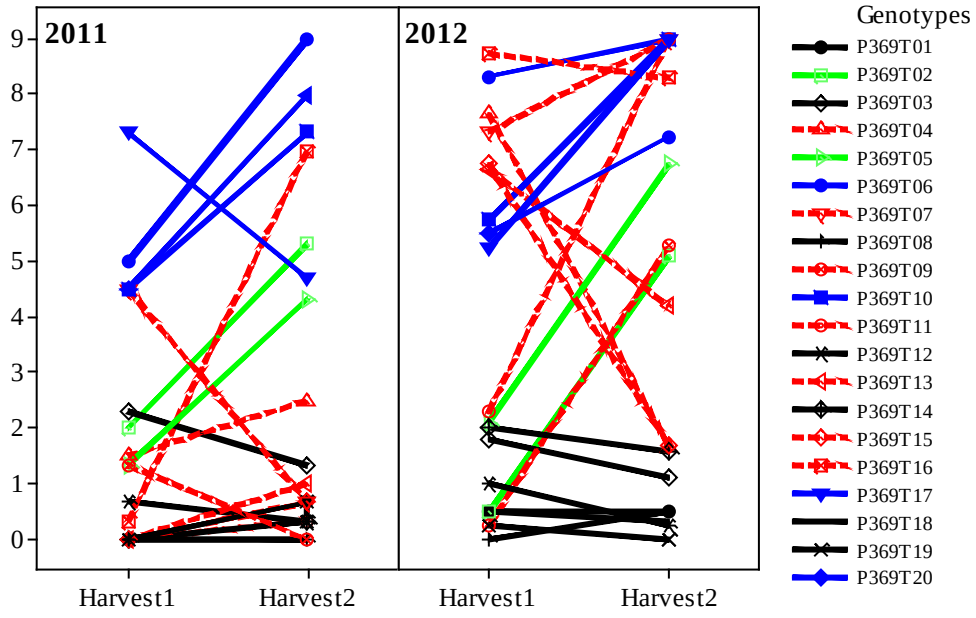


Figure 2.6: Seedlings from POP369 with multiple harvests from 2011 and 2012 plotted against FD. Genotypes with consistent behaviour across the years are represented by solid lines while inconsistent ones are represented by 'red dashed lines'. Different colours of lines represent different categories of seedling behaviours: 'black solid line' represents the category of consistently low, 'blue solid line' represents category of consistently high, and 'green solid line' represents the seedlings with low FD at earlier harvest and high FD at late harvest. The 'broken red line' represents the genotypes with inconsistent trend across the years.

In 2013, a narrow set of genotypes (8) with replicated trees and multiple harvests was assessed for FD and other fruit variables (firmness, TSS, AsA, PPO and phenolics). Fruit from these genotypes were collected in three harvests with an interval of 9 days between harvests. No difference in FD susceptibility was observed between tree replicates (Table 2.1), which confirms that clonal seedlings have identical FD susceptibility. Data from 2013 again verified that FD is highly variable amongst different genetic background (Table 2.2) as was observed in 2011 and 2012 (Figure 2.3;Figure 2.4;Figure 2.5).

Table 2.1: Analysis of variance to check the effect of genotype replicates for FD in 2013..A total of eight genotypes with two or three clonal replicates each were tested.

Source	DF	SeqSS	AdjSS	SeqMS	F	P
Genotype replicate	3	69	69	23	1.83	0.157
Error	39	489.6	489.6			
Total	42	558.7				

DF: degree of freedom, SeqSS: sequential sums of squares, AdjSS: adjusted sums of squares; SeqMS: sequential mean of squares

Table 2.2: Analysis of variance to check the effect of genotype for average FD in 2013. FD score was averaged for each of eight genotypes with two or three clonal replicates each.

Source	DF	SeqSS	AdjSS	SeqMS	F	P
Genotype	7	439.0	439.0	62.7	18.3	0.000
Error	35	119.7	119.7	3.4		
Total	42	558.7				

DF: degree of freedom, SeqSS: sequential sums of squares, AdjSS: adjusted sums of squares; SeqMS: sequential mean of squares

These results coincide with those reported in the literature (Kvåle, 1979; Meheriuk et al., 1994; Amarante et al., 2001a; Burger et al., 2005; Zhou and Li, 2007). Furthermore, harvest date did not have any effect on average FD score regardless of genotype (Table 2.3). All genotypes (each with 2 or 3 clonal replicate) showed consistent FD scores across the harvests i.e. either remained consistently high or consistently low (Table 2.2); with one exception, genotype named as tree 4, which changed from low FD on 1st harvest to high in 2nd harvest (Figure 2.7). Tree 3 that showed relatively high (3.7) FD score on the second harvest had chilling injury symptoms (peel and flesh browning) which may have contributed to the comparatively high susceptibility at this harvest.

Table 2.3: Analysis of variance to check the effect of harvest date on average FD in 2013. FD score was averaged for each of eight genotype with two or three clonal replicates each.

Source	DF	SeqSS	AdjSS	SeqMS	F	P
Harvest date	3	86.8	86.8	28.9	2.4	0.083
Error	39	471.9	471.9	12.1		
Total	42	558.7				

DF: degree of freedom, SeqSS: sequential sums of squares, AdjSS: adjusted sums of squares; SeqMS: sequential mean of squares

Tree 2, one of the parents of POP369, showed a high FD susceptibility in each year (6.7 in 2011; 8.8 in 2012 and 8.3 in 2013). This parent is presumably the source of high FD risk alleles in the population. Unfortunately the other parent (which is common to both populations) was no longer available for study and could not be included for comparison.

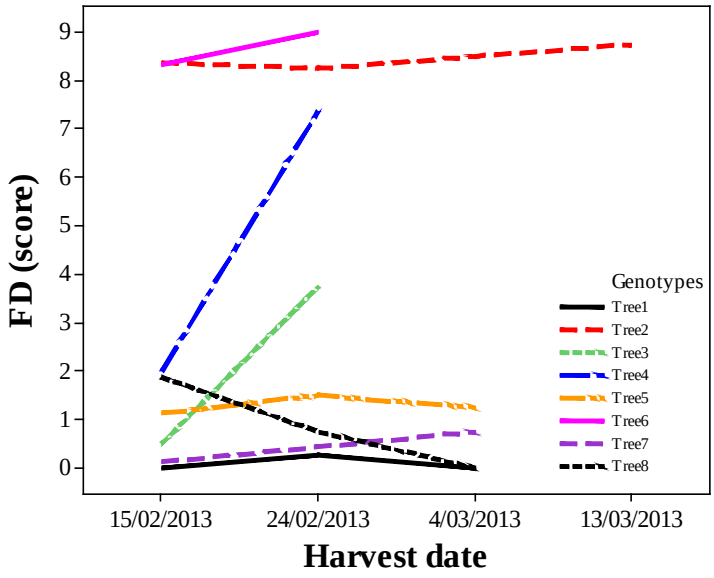


Figure 2.7: Mean friction discolouration of genotypes on different harvest dates in 2013.

2.3.2 Anatomical study

Microscopic transverse images from epidermis of susceptible and resistant seedlings from POP369 and POP356 were collected in 2011 in order to evaluate the role of skin properties for FD incidence. Previously there has been general agreement that a thick cuticle may serve as a barrier to transpiration and could also serve as barrier to

protect the fruit skin from physical damage (Amarante et al., 2001a; Khanal et al., 2013). Amarante et al. (2001) proposed that stone cells play an important role to prevent excessive water loss and gaseous permeance and varieties with high numbers of stone cells are less prone to FD susceptibility. Later Palmer et al. (2008) attributed the high FD sensitivity of 'Comice' as compared to 'Packham's Triumph' to the proximity of stone cells to the cell surface.

The role of the above mentioned skin properties was tested in 40 (20 fruit with high FD and other 20 with low FD) pear samples randomly selected from both populations. No consistent significant consistent differences were observed between skin properties of genotypes with a high or low susceptibility to FD. It is clear from Figure 2.8 that there was wide variation in skin anatomy among different genotypes from susceptible and resistant seedlings. Examples were found of abundant or scarce stone cells, close to or far from the epidermis and with thin or thick cuticles in fruit that were both highly susceptible to FD and fruit that were not. Similarly tangential sections from both extremes of FD sensitivity showed no consistent difference in stone cell distribution and cluster size in the skin (Figure 2.9). These results suggest that the previously reported associations between anatomical features and FD susceptibility were coincidental rather than causal.

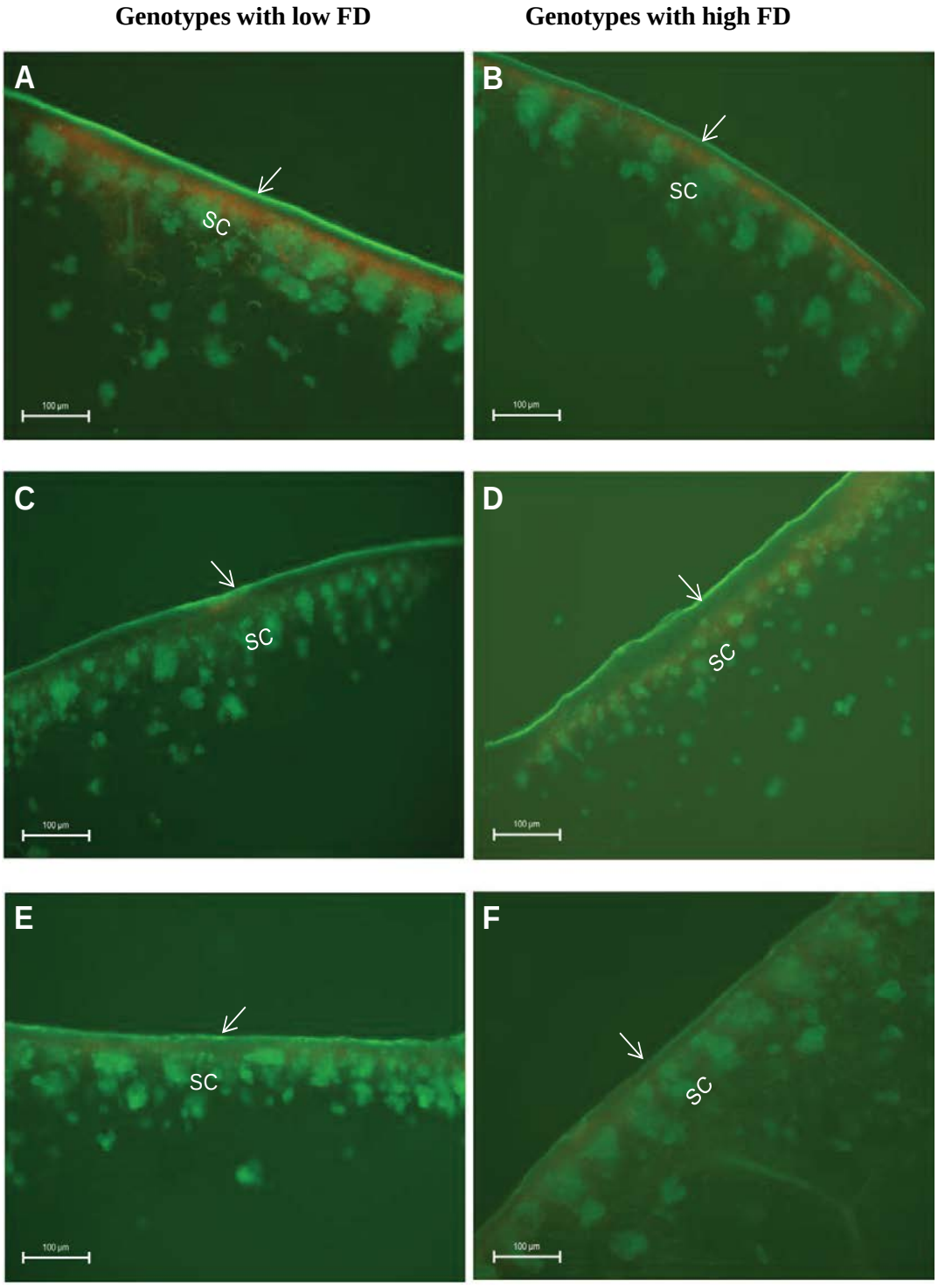


Figure 2.8: Fresh transverse sections of pear skin from genotypes with low and high FD. Size and location of stone cells (SC) from epidermis (arrows) are visible in each figure. A and B represent stone cells position in genotypes with blushed pear skins, C and D represent the genotypes with small sized stone cells and distant from epidermis while E and F represent pear genotypes with large stone cells closer to the epidermis.

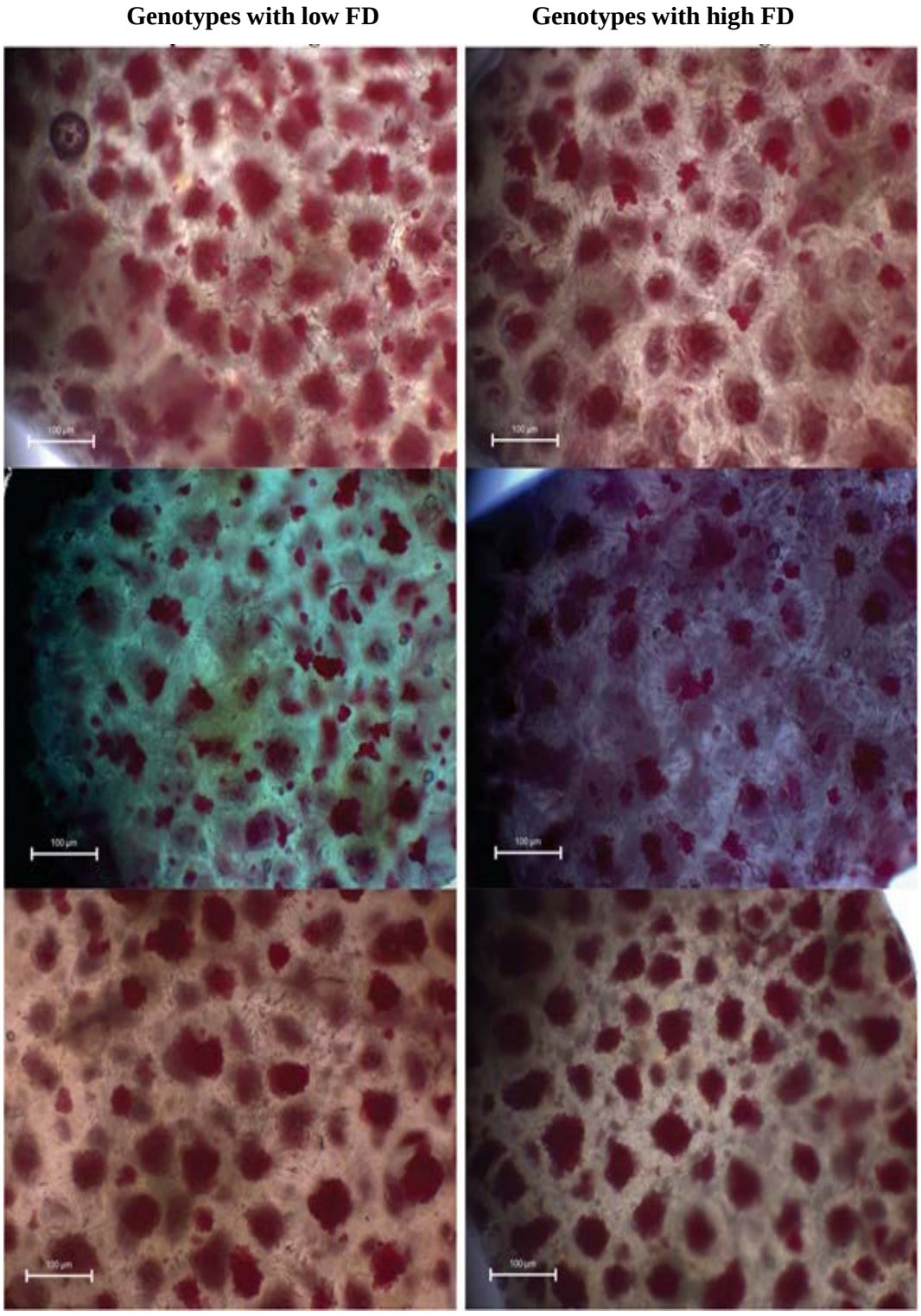


Figure 2.9: Comparison of stone cell size and clustering in the skin of genotypes with low and high FD. Tangential sections were stained with phloroglucinol in 20% HCl, stained stone cells are visible in each section.

2.3.3 Phenotypic variables affecting FD susceptibility

Pearson correlation coefficients were calculated to evaluate the relationships between FD and physical and biochemical variables averaged across harvest dates (Table 2.4). Multivariate analysis (Discriminant analysis; DA) was also performed to evaluate the relationship of multiple factors affecting FD susceptibility in POP369 and POP356 over the years (2011 and 2012). Again Pearson correlation was used to analyse the effect of different variables in relation to FD susceptibility of eight genotypes in 2013.

2.3.3.1 Selection of attributes from segregating populations

In 2011, a total of 72 candidate metabolite compounds were detected by using LC-MS in peel samples of POP369 and POP356. From these, 17 phenolic compounds were selected by performing multivariate analysis (principal component analysis: PCA) with the help of Profile Analysis software (Bruker Daltonics, Bremen, Germany). Only two categories of seedlings with low (<1.5) and high FD scores (>5.0), neglecting the middle range of FD, were used to select these compounds.

In 2012, three versions of the data were created, by processing the results in three ways. The first dataset of 8869 candidate metabolites was created using FMF (Find Molecular Features), the second was created by dividing the retention times into 0.5 minute bins, and the mass-to-charge ratio values into 5-unit bins to give a total of 4140 “buckets”, and the third was created by dividing the retention times into 0.5 minute bins, and the mass-to-charge ratio values into 2-unit bins to give a total of 10350 “buckets”. Each dataset was analysed separately but in the same way. First, the dataset was filtered for signals present in >40 samples. Threshhold intensity was set at 2000 units. Partial least squares regression (PLS) was applied to this filtered data to predict the FD score. The predicted FD scores were correlated with and plotted against the observed FD scores. The coefficients for each of these filtered signal data were plotted against mass-to-charge ratio to observe visual patterns in the results. However none of this data analysis led to a set of phenolics with strong influence on FD score.

Again 8869 candidate metabolites from FMF were filtered for signal present in >40 samples with values below a threshold of 10,000 intensity units, which resulted in a set of 37 candidate metabolites. These candidate metabolites were again checked for their peaks in chromatographs. Using this approach, a subset of 17 compounds was selected that included similar compounds (flavanols, flavonols, procyanidins and chlorogenic acid) to those detected in 2011. Chlorogenic acid was the most abundant polyphenol found in pear fruit peel in both populations in both years. Concentrations (expressed as peak areas) of all 17 polyphenolic compounds varied among individual progeny in both populations; however none of these compounds showed high significant correlations to FD (Table 2.4).

Although genotypes exhibited a wide range of PPO activity in 2011 and 2012, PPO activity was weakly correlated with FD for POP369 in 2012 only. However, a weak yet significant negative correlation between some polyphenol compounds and PPO activity was observed e.g. from the 2012 data of population POP369, PPO and epicatechin had a significant (P<0.01) negative correlation (r=-0.28). No significant correlation was observed between FD and AsA (Table 2.4).

Table 2.4: Correlation coefficient (r) for all trait data in relation to, FD, harvest date TSS and firmness for POP369 (2011, 2012) and POP356 (2011). NB: comp_417.12 (1) and comp_417.12 (2) are unknown compounds identified from LC-MS quantification analysis, represented by their molecular weight. Units for traits studied are following: FD (scale), TSS (°Brix), Firmness (N: newtons), PPO (ΔA_{420} $g^{-1}minute^{-1}$), AsA and polyphenol compounds (concentration).

	POP369 (2011)				POP369 (2012)				POP356 (2011)			
Trait	**FD**	**Harvest date**	**TSS**	**Firmness**	**FD**	**Harvest date**	**TSS**	**Firmness**	**FD**	**Harvest date**	**TSS**	**Firmness**
FD	0.36**				0.27**				ns			
TSS	ns	ns			0.16*	-0.34**			ns	ns		
Firmness	ns	ns	0.29**		-0.21**	-0.14*	0.16*		ns	ns	ns	
PPO	ns	-0.36**	ns	ns	0.20**	0.15*	0.28**	ns	ns	-0.25**	ns	ns
AsA	ns	0.31**	ns	ns	ns	ns	ns	-0.15*	ns	0.54**	ns	ns
Chlorogenic acid	-0.27**	-0.19*	0.18*	ns	ns	-0.31**	0.2**	ns	-0.12*	-0.18**	ns	0.27**
Cryptochlorogenic acid	-0.27**	ns	ns	ns	ns	-0.25**	ns	ns	ns	-0.2 **	ns	0.21**
Neochlorogenic acid	ns	ns	ns	ns	ns	-0.20**	ns	ns	ns	ns	ns	0.22**
Catechin	ns	ns	0.21*	0.44**	-0.21**	-0.19**	0.15*	0.30**	-0.20**	ns	ns	0.27**
Epicatechin	ns	ns	ns	0.42**	-0.15*	ns	0.30**	0.40**	-0.18**	ns	ns	0.15*
Procyanidin B2	-0.23**	ns	0.19*	0.27**	ns	-0.18*	0.19**	0.22**	-0.24**	ns	ns	0.21**
Isorhamnetin 3-galactoside	ns	-0.22*	ns	ns	-0.15*	-0.21**	ns	ns	ns	ns	ns	0.19**
Isorhamnetin rutinoside	-0.25**	-0.37**	ns	ns	ns	-0.30 **	ns	ns	ns	ns	ns	0.23**
p-coumaryl quinic acid	-0.2*	-0.21*	0.27**	0.23**	ns	-0.23**	0.20**	ns	ns	ns	ns	ns
Quercetin galactoside	ns	-0.17*	ns	ns	-0.15*	-0.18**	ns	0.23**	ns	-0.13*	ns	ns
Quercetin glucoside	ns	-0.25**	ns	ns	-0.20**	-0.32**	0.19**	0.25**	ns	-0.20**	ns	ns
Quercetin arabinoside	-0.21*	-0.22**	ns	ns	ns	-0.24**	ns	ns	ns	ns	ns	0.16**
Quercetin rhamnoside	-0.19*	-0.25**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Quercetin rutinoside	ns	-0.28**	ns	ns	-0.19**	-0.27**	ns	0.16*	ns	-0.20**	ns	ns
Quercetin	0.22**	0.34**	ns	ns	-0.17*	ns	ns	ns	ns	0.16**	ns	0.37**
Comp_417.12 (1)	-0.21*	-0.20*	ns	ns	ns	-0.24**	ns	ns	ns	ns	ns	0.17**
Comp_417.12(2)	-0.22**	ns	ns	ns	ns	-0.24**	ns	ns	ns	ns	ns	0.15*

Note: *= P<0.05 **=P<0.01 and ns =non-significant

Discriminant function analysis is used to determine which continuous variables discriminate between two or more naturally occurring groups. The full scale employed of FD assessment form 0-9 provided sufficient flexibility to allow grouping of the genotypes according to their susceptibility. In 2011, POP356 genotypes were divided into 3 groups containing at least 30 individuals on the basis of FD score: (1) low i.e. below 2.0 FD score, (2) medium i.e. in the range of 2.1 to 4.5 score and (3) high i.e. above 4.5 FD score. POP369 genotypes in 2012 fitted the same groups, but for the data from POP369 in 2011, grouping criteria had to be slightly changed, using 2.1-5.1 for the middle range, to ensure at least 30 individual samples per group (Table 2.5).

Table 2.5: Grouping criteria and number of samples in each group for discriminant analysis.

2011				2012	
POP356		POP369		POP369	
Grouping criteria	**No. of individuals**	**Grouping criteria**	**No. of individuals**	**Grouping criteria**	**No. of individuals**
< 2.1	113	< 2.1	54	< 2.1	45
2.1 - 4.5	58	2.1 - 5.1	30	2.1 - 4.5	47
≥4.5	35	≥ 5.1	38	≥ 4.5	84
Total	206	Total	122	Total	176

Dependent variables (TSS, firmness, PPO, AsA and phenolic compounds) were tested for their ability to predict which genotypes will fall in low, medium or high FD group. Stepwise linear discriminant function analysis was used to find variables that could help discriminate between these three groups. Standardized coefficients for selected variables from the analysis are provided for the first two discriminant functions. These coefficients represent the contribution of each variable to discrimination of groups. This analysis does not function with missing values, so prior to analysis, any zero values were replaced by half the smallest non-zero value for that variable, and then data were log10-transformed.

Discriminant analysis clearly discriminated between the low category and the other two categories (medium and high). Maximum variation (85 %) was explained by score 1 only (Appendix 1). Score 2 did distinguish between medium and high groups but is clearly of no use for comparison with low susceptibility (Figure 2.10).

Despite the clear separation of low FD fruit from those with medium (2.1-5.1) or high (≥5.1) FD, as shown by the non-overlapping 95% confidence intervals, only 55.1% of fruit were correctly classified into the low category (Figure 2.10). Clearly the overall discrimination is not particularly strong, emphasising that no single factor is emerging as the principal predictor of FD across this wide range of seedling genotypes.

A total of eight out of 21 variables contributed most strongly to the discrimination of low, medium and high FD groups in stepwise DA. A list of variables along with standardized coefficients is provided in Table 2.6. From this set of 8 variables, 6 are polyphenol compounds with largest variation being explained by isorhamnetin rutinoside.

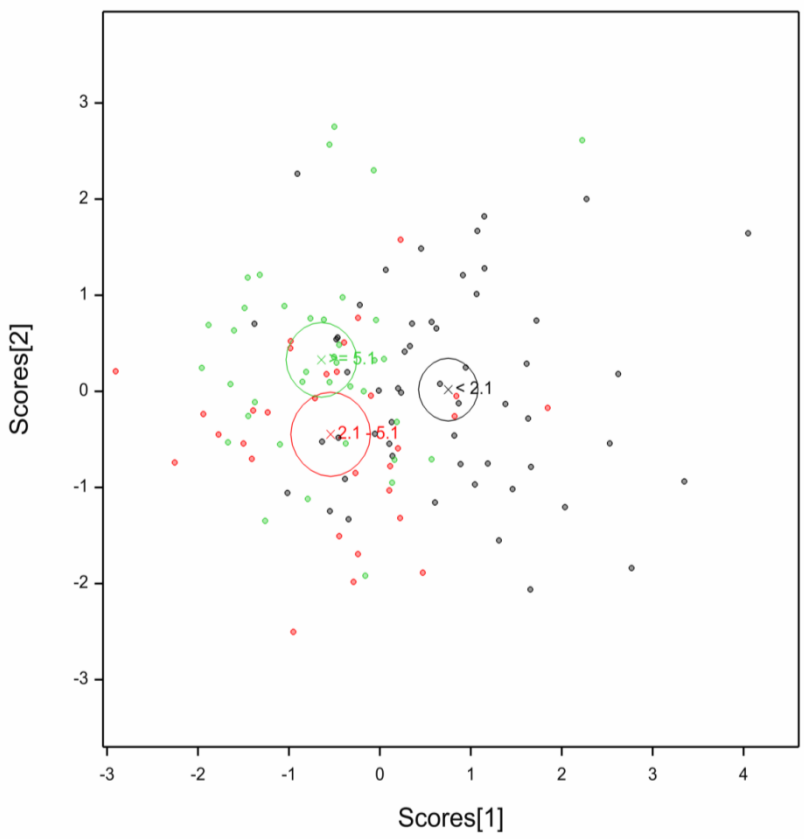


Figure 2.10: Graph of first two discriminant functions for the POP369 (2011) dataset. The circles show 95% confidence limits for the group means (marked with X). Key: black <2.1, red = 2.1- 5.1, green ≥5.1.

Table 2.6: Standardised coefficients in 2 discriminant function scores for 8 optimum variables for POP369 (2011) dataset.

Variable	Scores[1]	Scores[2]
Isorhamnetin rutinoside	3.44	1.107
Chlorogenic acid	1.6	2.972
Isorhamnetin galactoside/glucoside	-1.42	-2.187
AsA	-1.25	1.218
Quercetin	-1.23	0.567
Quercetin rutinoside	-1.09	0.779
Procyanidin B2	0.68	-2.391
417.12 (1)	0.41	0.305

To further investigate the role of variables best explaining the discrimination of FD groups in 2011, means of the first four best predictors (3 phenols plus AsA) from the genotypes with assigned FD groups were calculated and plotted against group categories (Figure 2.11). Isorhamnetin rutinoside, chlorogenic acid and isorhamnetin galactoside/glucoside were negatively associated with FD i.e. low FD groups had higher concentrations of phenolic compounds and AsA concentration showed a linear trend with FD i.e. low FD groups had a tendency to have lower AsA content while high FD groups had higher AsA contents (Figure 2.11).

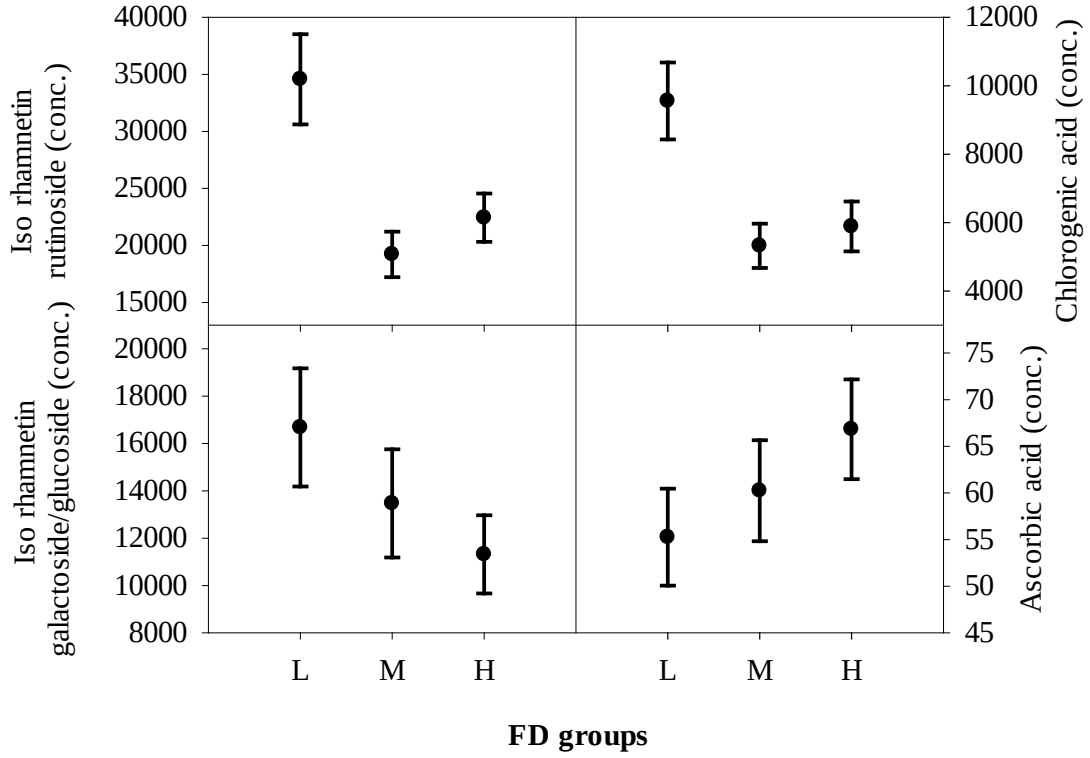


Figure 2.11: The four best predictors from discriminant analysis of POP369 (2011) plotted against three FD groups. Genotypes were divided into 3 groups on the basis of FD (1) L=low (<2.0); (2) M= medium (2.1-5.1) and (3) H= high (≥5.1) FD score. Error bars represent standard errors of the means.

In 2012, DA was again conducted to analyse POP369 data. A clear separation of low FD fruit from those with medium (2.1- 4.5) or high (≥4.5) FD was observed. However, only 48.8 % of fruit were correctly classified into the low category (Appendix 1). Most of the variation (91%) was explained by score 1 (Figure 2.12).

A total of six variables that contributed most strongly to the discrimination of low, medium and high FD are listed inTable 2.7. Four out of these six variables were phenolic compounds, however the highest variation was explained by TSS.

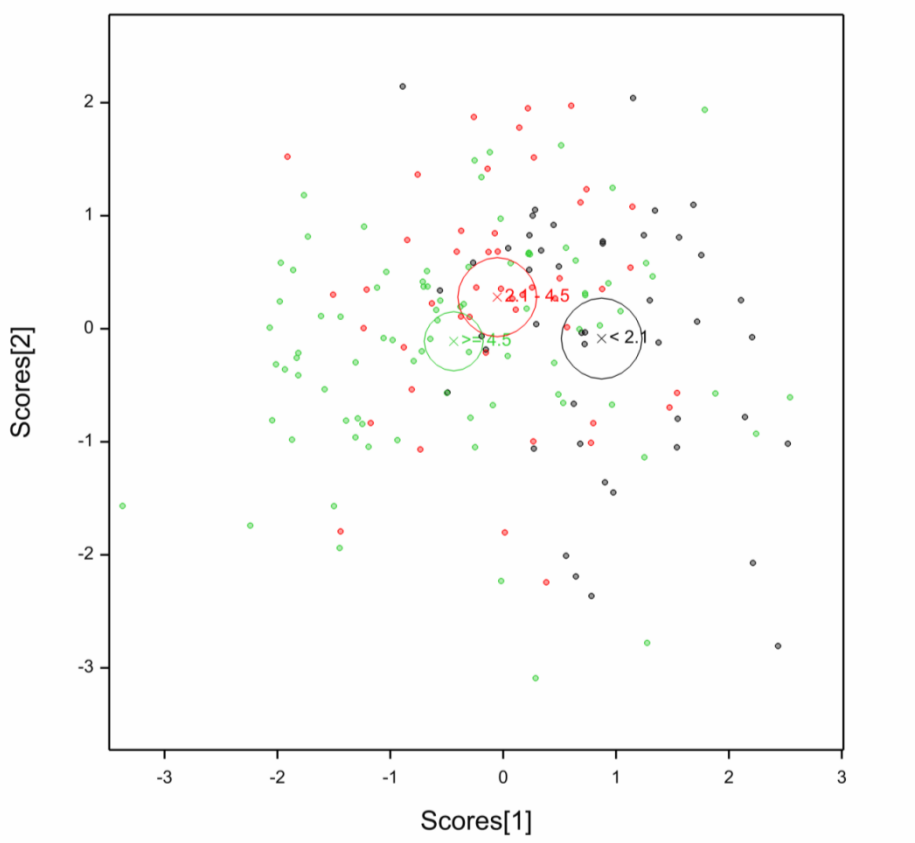


Figure 2.12: Graph of first two discriminant functions for POP369 (2012) dataset. The circles show 95 % confidence limits for the group means (marked with X). Key: black <2.1, red = 2.1- 4.5, green ≥4.5.

Table 2.7: Stepwise discriminant function scores for six optimum variables for POP369 (2012) dataset.

Variable	Scores[1]	Scores[2]
TSS	7.821	5.623
Procyanidin B2	-2.375	-0.389
PPO	-1.51	1.762
Catechin	1.408	-1.859
Quercetin	1.238	0.817
Quercetin rutinoside	0.898	1.224

To further investigate the role of variables that best explained the discrimination of FD groups in 2012 for POP369, means of the first four best predictors from the assigned FD groups to the genotypes were calculated and plotted against group categories (Figure 2.13). TSS and catechin were negatively associated with FD groups. PPO was positively associated with FD. Relationship of procyanidin B2 was complex, however the overall trend was again a negative association of compound contents to the FD.

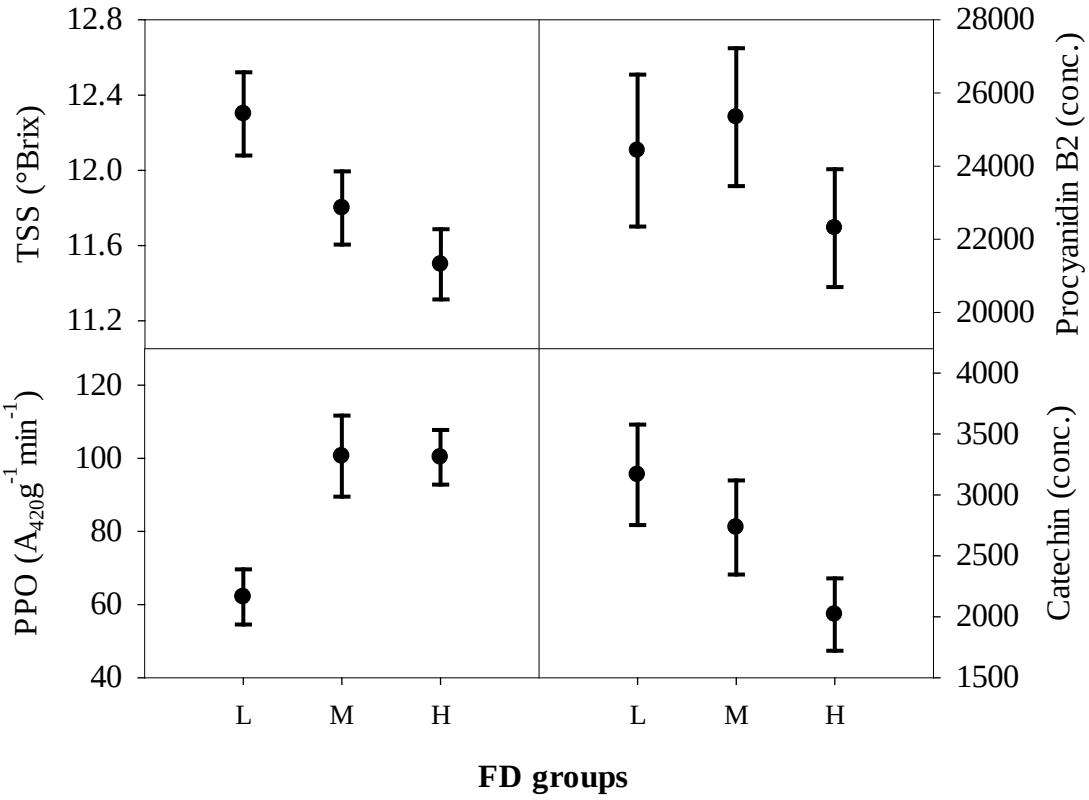


Figure 2.13: Four best predictors from discriminant analysis of POP369 (2012) plotted against three FD groups. Genotypes were divided into 3 groups on the basis of FD (1) L=low (<2.0); (2) M= medium (2.1 to 4.5) and (3) H= high (≥4.5) FD score. Error bars represent standard errors of the means.

The discriminant function analysis of the POP356 (2011) dataset yielded a single variable, epicatechin out of 21 variables. This variable gave a 49.4 % error rate in assigning observations to the three FD score groups (Figure 2.14). Variation was explained by score 1 solely (100 %). Concentration of epicatechin was inversely correlated to FD score of the genotypes (Figure 2.15).

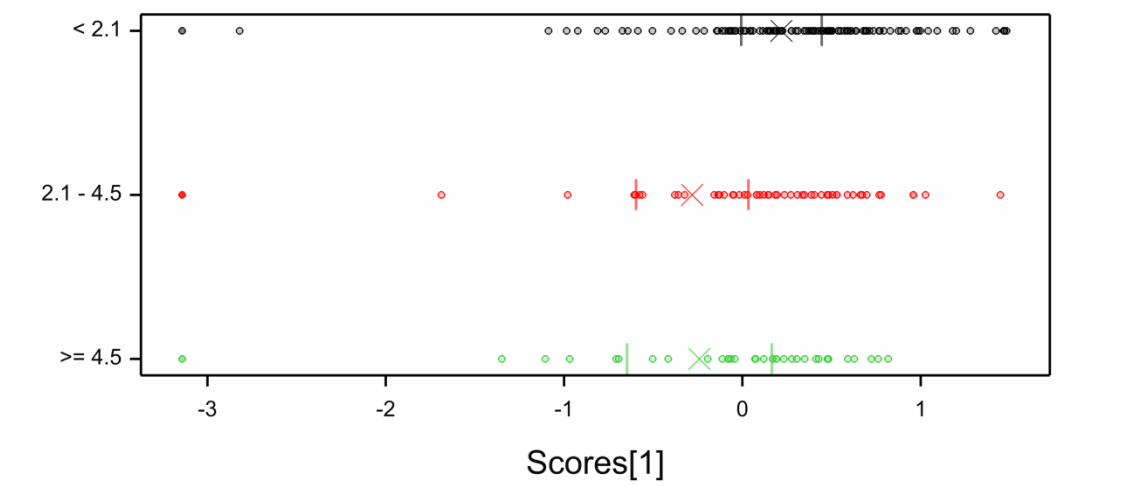


Figure 2.14: Graph of FD score group vs first discriminant function score for the POP356 (2011) dataset. The vertical lines show 95% confidence limits for the group means (marked with X). Key: black ≤ 2.1, red = 2.1- 4.5, green ≥ 4.5.

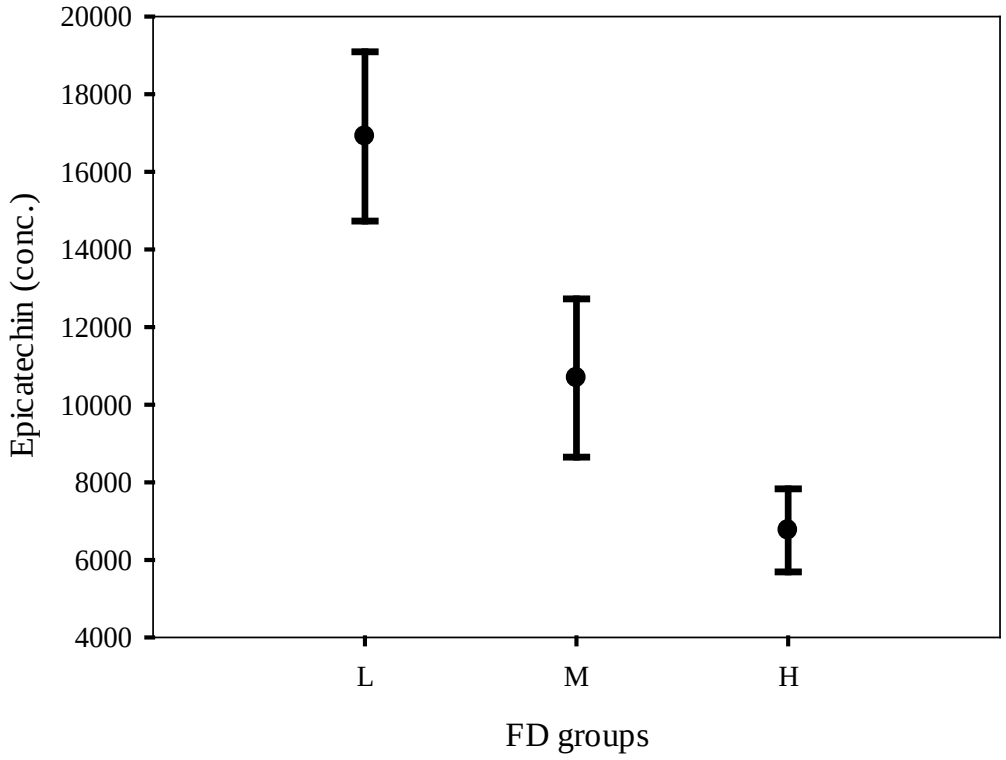


Figure 2.15: Single best predictor from disciminant analysis of POP356 (2011) plotted against three FD groups. Genotypes were divided into 3 groups on the basis of FD (1) L=low (<2.0); (2) M= medium (2.1-4.5) and (3) H= high (≥4.5) FD score. Error bars represent standard errors of the means.

Clearly the overall discrimination is not particularly strong, emphasising that no single factor is emerging as the principal predictor of FD across this wide range of seedling genotypes. However, despite the difference of various harvest dates and grouping criteria each year and across the populations, content of several phenolic compounds consistently appeared as an important factor associated with FD. This positive relationship between phenolic content and FD is in contrast to the hypothesis that the concentration of total phenolics or chlorogenic acid is inversely proportional to incidence of FD (Wang and Mellenthin, 1973; Kvåle, 1979). Later Burger et al. (2005) reported that high FD is correlated with high PPO rather than high concentration of total phenolics. They also noticed that PPO has an inverse relationship to the total phenolics i.e. low amounts of total phenolics were observed when PPO activity rate was high. A similar trend, although not highly significant, was observed in 2012 for population POP369, where PPO and epicatechin have significant (P<0.01) negative correlation (r=-0.28) and were in the top four variables that best explained the discrimination of FD groups (Table 2.7). Also the role of phenolics as antioxidative compounds cannot be ignored, this argument is discussed in detail in chapter 5 (Section 5.1.3). Moreover, this previous positive relationship between phenolic content and FD was established in only a few studies conducted using one or two pear genotypes each.

2.3.3.2 Relationship of FD with phenotypic variables in replicated genotypes

A targeted approach was used to select phenolic compounds to be included in the 2013 analysis of replicated genotypes. The molecular mass of 17 known compounds from 2011 and 2012 was added to Quant analysis software (Bruker Daltonics, Bremen, Germany) and peaks for all of these compounds were identified and concentration was calculated for all the samples, except quercetin arabinose, which was not detected in the majority of samples and quercetin which was more often absent than present. The mean FD of replicated trees was significantly correlated with TSS and a content of a few phenolic compounds, including chlorogenic acid (r=0.46; P<0.05;Table 2.8).

From the 2012 DA results, TSS best explained the discrimination among the low, medium and high FD groups. When plotted against means of these groups, TSS

showed negative relation to the FD, i.e. higher the TSS values lower the FD value (Figure 2.13). TSS after storage might be lower if fruit are picked past optimal maturity, this could be possible explanation of low TSS in the fruit that were more susceptible to FD.

Table 2.8: Correlation coefficient (r) for all trait data in relation to harvest date, FD, TSS and firmness. NB: comp_417.12 (1) and comp_417.12 (2) are unknown compounds identified from LC-MS quantification analysis, represented by their molecular weight. Units for trait studied are following: FD (scale), TSS (°Brix), Firmness (N: newton), PPO (ΔA_{420} $g^{-1}minute^{-1}$), AsA and polyphenol compounds (concentration).

	FD score	Harvest date	TSS	Firmness
FD score		ns		
TSS	-0.55**	ns		
Firmness	ns	ns	ns	
PPO	ns	ns	ns	ns
AsA	ns	ns	ns	0.61**
Chlorogenic acid	-0.46*	ns	ns	0.46*
Cryptochlorogenic acid	-0.47*	-0.45*	ns	ns
Neochlorogenic acid	ns	ns	ns	ns
Catechin	ns	ns	ns	ns
Epicatechin	ns	ns	ns	0.54**
Procyanidin B2	-0.45*	ns	ns	0.57**
Isorhamnetin 3-galactoside	ns	ns	ns	ns
Isorhamnetin 3-glucoside	ns	ns	ns	ns
Isorhamnetin rutinoside	ns	ns	ns	0.65**
p-coumaryl quinic acid	-0.46*	ns	ns	0.56**
Quercetin galactoside	ns	-0.45*	ns	ns
Quercetin glucoside	ns	-0.48*	ns	ns
Quercetin rhamnoside	ns	ns	ns	ns
Quercetin rutinoside	-0.44*	ns	ns	ns
Comp_417.12 (1)	ns	ns	ns	ns
Comp_417.118(2)	ns	ns	ns	ns

Note: *= P<0.05 **=P<0.01 and ns =non-significant

Interestingly, clonal replicates of eight genotypes did not exhibit any difference between replicated for chlorogenic acid concentration; note the clustering in Figure 2.16, except Tree 5. Phenolic compounds consistently correlate negatively with FD

incidence over the years and across the populations, and the same trend was again observed in the 2013 trial (Figure 2.16;Table 2.8).

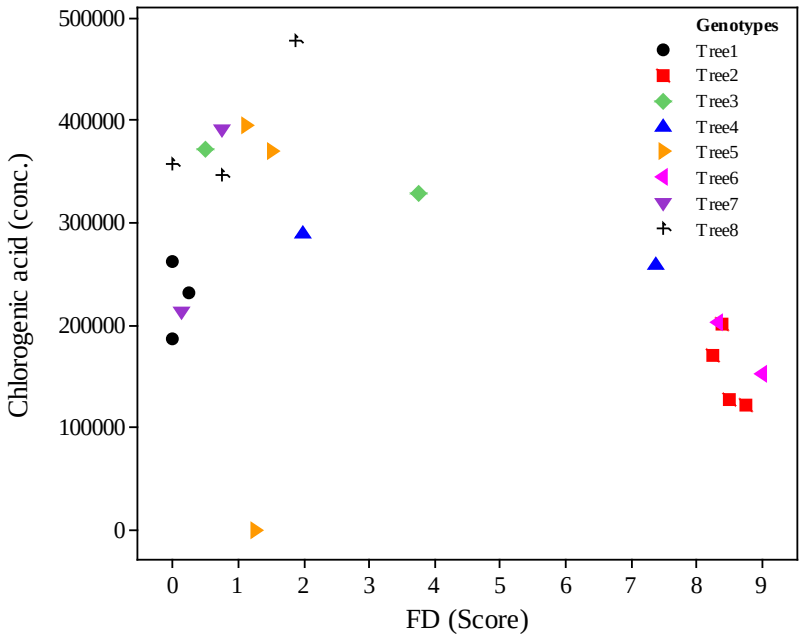


Figure 2.16: Relationship between FD score and chlorogenic acid content from data of year 2013.

In 2013, fruit that softened to a greater extent exhibited low concentrations of metabolites (Table 2.8). Firmness was not directly correlated with FD incidence, however its association with other metabolites could explain indirect relationships among firmness, metabolites and FD. Some phenolic compounds such as chlorogenic acid, procyanidin B2 and p-coumaryl quinic acid were positively correlated with firmness which may mean that firmer fruit have higher phenolic contents and as fruit become softer, phenolic compounds are degraded. Conversely the content of phenolics was inversely proportional to FD incidence.

It can be speculated from this indirect relationship between content of phenolics, firmness and FD susceptibility that relatively firm fruit are less prone to FD as compared to softer fruit, however this speculation may or may not be true as no before storage phenolics measurement is available for comparison.

2.4 Conclusion

Friction discolouration (FD) is a complex postharvest disorder influenced by both genetic and environmental factors. As the genotypes in current study are crosses between two totally different species (and each seedling is a different genetic entity), their behaviour in accumulation of phenols, enzyme activity, antioxidants as well as their skin anatomy is totally different. Therefore this should have been the ideal dataset to identify underlying trends of metabolites or enzymes that have a major contribution to FD susceptibility. The lack of strong associations identified emphasises that the FD phenomenon is multifactorial in nature. The presence of some weaker associations in terms of genotypes with consistently high or low risk of FD provides evidence for genetic predisposition of FD along with other risk factors. It seems that comparatively high contents of phenolics are associated with low FD and vice versa. Contradictory results have been repeatedly reported for relationships of FD and phenolic contents. This study suggests phenolics may act as antioxidants and in the case of physical injury, may reduce FD symptoms (Section 5.2.2). Activity of PPO was not significantly associated with FD, despite the fact that it is fundamental in the process of browning. Harvest maturity can have conflicting effects on FD of seedlings, which poses difficulty while working with multiple genotypes for which harvest maturity is not yet determined. This explanatory study of influence of different phenotypic variables on FD susceptibility will be used to identify quantitative trait loci (QTL) linked to FD and other variables utilizing statistical correlation of phenotypic data to genotypic information (Chapters 3 and 4).

3 CONSTRUCTION OF SNP BASED GENETIC MAPS FOR QTL MAPPING IN INTERSPECIFIC PEAR POPULATIONS [*]

3.1 Introduction

Genetic maps refer to the framework of markers which are useful to determine the genetic architecture of a plant's quantitative traits, the structure of the genome and identifying genomic regions linked to phenotypic traits (QTLs). High quality genetic maps enriched with co-dominant and reproducible markers with maximum coverage of the genome (i.e. high density of markers and covering as many linkage groups as haploid chromosomes) are a necessary prerequisite for the identification of QTLs.

A new improved sequencing method was introduced in 2005: next generation sequencing (NGS), which enabled researchers to produce large amounts of DNA sequence data. Due to its high throughput technology, NGS has steadily increased from one gigabase (Gb) of data in 2007 to terabase (Tb) in 2011 generated in a single sequence run (Illumina, 2013).

One major application of NGS is SNP discovery through whole genome (re) sequencing. Single nucleotide polymorphisms (SNPs) are today's marker of choice due to their high abundance in almost every living organism including higher plants. SNPs identified by NGS re-sequencing can be used to develop SNP arrays that are increasingly being used for large-scale and simultaneous SNP genotyping and for genome-wide analysis of polymorphism across large populations of non model plants. Such arrays include the the Infinium® II assay (Illumina Inc.) and Affymetrix SNP arrays have been developed for a range of fruit crops, including Rosaceae, such as apple, peach, cherry and strawberry. A SNP array was developed by the

[*]Material from this chapter is included in papers: a) Montanari, S., Saeed, M., Knäbel, M., Kim, Y., Troggio, M., Malnoy, M., Velasco, R., Fontana, P., Won, K., Eric Durel, C., Perchepied, L., Schaffer, R., Wiedow, C., Bus, V., Brewer, L., Gardiner, S. E., Crowhurst, R. N., and Chagné, D. (2013). Identification of Pyrus single nucleotide polymorphisms (SNPs) and evaluation for genetic mapping in European pear and interspecific Pyrus hybrids. PLoS ONE, 8(10), e77022.
b) Chagné, D., Crowhurst, R. N., Pindo, M., Thrimawithana, A., Deng, C., Ireland, H., Fiers, M., Dzierzon, H., Cestaro, A., Fontana, P., Bianco, L., Lu, A., Storey, R., Knäbel, M., Saeed, M., Montanari, S., Kim, Y. K., Nicolini, D., Larger, S., Stefani, E., Allan, A. C., Bowen, J., Harvey, I., Johnston, J., Malnoy, M., Troggio, M., Perchepied, L., Sawyer, G., Wiedow, C., Won, K., Viola, R., Hellens, R. P., Brewer, L., Bus, V. G. M., Schaffer, R. J., Gardiner, S. E., and Velasco, R. (2014). The draft genome sequence of European pear (Pyrus communis L. 'Bartlett'). PLoS ONE, 9(4), e92644.
[#]Work presented in the whole chapter except section 3.2.1, 3.2.1.1 and 3.3.1 was performed by me.

International RosBREED SNP consortium (IRSC) (www.rosbreed.org) for apple (Chagné et al. 2012). This 8K SNP Infinium® II v1 array contains 7867 genome-wide apple SNPs, of which 5554 are polymorphic in apple. The International Peach SNP Consortium (IPSC) developed a 9K SNP array for peach including 8144 SNPs with 84.3% of those SNPs showing polymorphism (Verde et al., 2012). As described by Peace et al. (2012), IRSC also led the development of a 6K SNP array for cherry, with 1825 verified polymorphic SNPs in sweet cherry and 2058 in sour cherry. To date pear genotyping has been limited to markers other than SNPs and of lower throughput such as microsatellites; these enabled the construction of a number of maps (Dondini et al., 2004; Yamamoto et al., 2004; Pierantoni et al., 2007; Yamamoto et al., 2007; Sun et al., 2010; Zhao et al., 2013), however none of them have used highly abundant and cost effective markers.

A set of 1096 pear SNPs identified by NGS technologies was included in the IRSC apple Infinium® II 8K SNP array. For my project, two interspecific pear hybrid populations were screened for polymorphic SNPs and four parental genetic linkage maps were developed. The goal of this chapter includes pear SNP discovery through NGS technology and the development of the Illumina Infinium® II 9K SNP array by adding the newly developed pear SNPs in to the apple Illumina Infinium® II 8K SNP array (Chagné et al., 2012a). These new pear SNPs, along with apple SNPs were evaluated for high-throughput genotyping and genetic map construction using two segregating interspecific pear hybrid populations. This chapter also describes the development of the four genetic linkage maps which were used to identify QTLs associated with FD and other fruit traits.

3.2 Materials and methods

3.2.1 Next generation sequencing (NGS)

A SNP detection panel of three European pear (*Pyrus communis* L.) cultivars was chosen for whole genome, low coverage sequencing. The accessions were ‘Bartlett’ (a.k.a. ‘William Bon Chrétien’, WBC), ‘Old Home’ (OH) and ‘Louise Bon Jersey’ (LBJ) and were chosen as WBC is a founder of most breeding programs worldwide, and OH and LBJ are the parents of a segregating population developed at Plant &

Food Research (PFR). Each accession was sequenced using one lane of Illumina GA II with 75 cycles per read and small insert paired-end sequencing as described in Chagné et al. (2012; Figure 3.1).

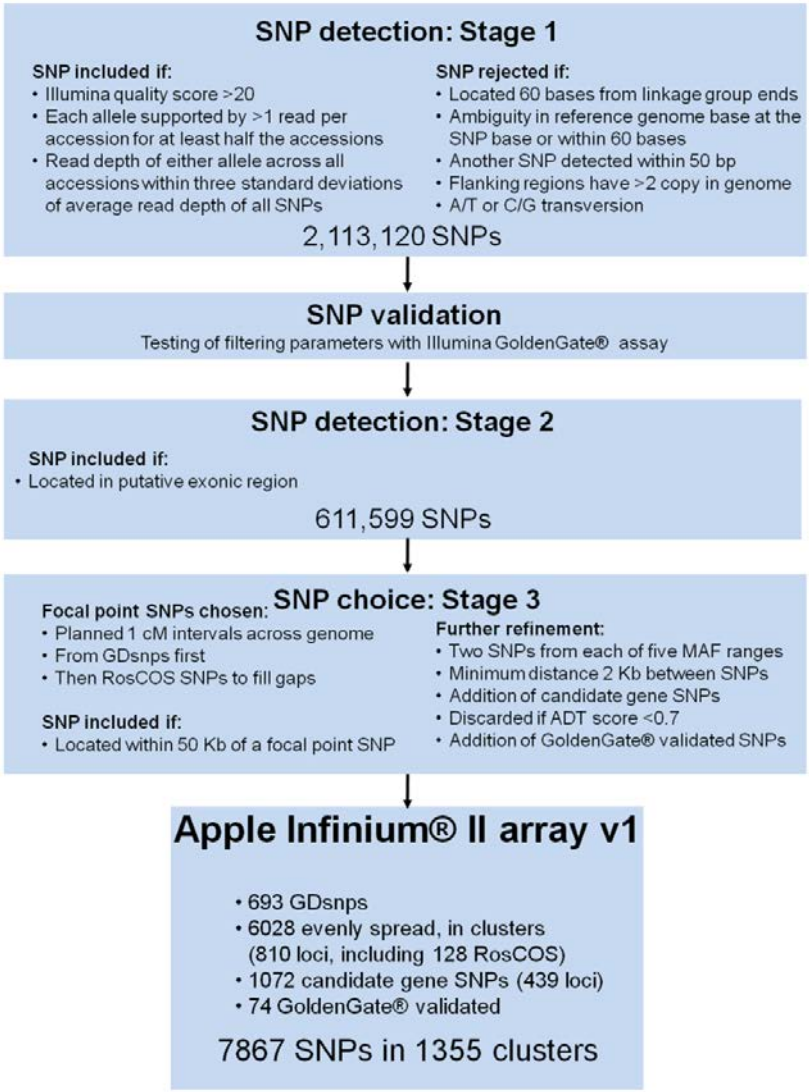


Figure 3.1: Workflow for single nucleotide polymorphism (SNP) detection, validation, and final choice employed for development of the IRSC apple 8K SNP array v1 (Chagné et al., 2012a). Key: MAF (Minor allelic frequency), GDsnp (Golden Delicious validated SNPs; RosCOS: (Rosaceae conserved orthologous set), ADT (Assay design tool).

3.2.1.1 Bioinformatics detection and selection of SNPs for array

A *de novo* assembly was performed for the sequencing data of 'Bartlett' using AbySS 1.2.1 (k = 43). Contigs of 600 bp or larger were used as a reference genome set. The sequencing data of OH and LBJ were mapped to the reference genome set of 'Bartlett' using *Soap2.20* (-p 8 -M 4 -v 5 -c 52 -s 12 -n 5 -r 2 -m 50 -x 600). *Soap* output files were split into a single file per contig and each contig file sorted by location of the mapped reads. *SoapSNP* was used for SNP detection and filtering with the same parameters as described in Chagné et al. 2012. The detected SNPs were then subjected to a filtering where calls were discarded when quality score was less than 20, fewer than 2 reads per genotype were present, overall coverage depth was greater than the average coverage plus three standard deviations, the site was at least 25 bases (50 bases in case of apple) away from another SNP call, and the SNPs were not located within regions associated with a set of candidate genes. This set was made of cDNA sequences of *Malus* full length genes and locations within pear defined by mapping these cDNAs to the reference genome set of WBC using *gmap* with command line options -K 3000 –L 50000. The Illumina Infinium assay design tool (ADT) was used on the detected SNPs within the candidate gene set, with a threshold of 0.7.

3.2.2 Plant material for SNP evaluation

Two populations, POP369 and POP356, interspecific crosses between Asian (*P. pyrifolia* and *P. bretschneideri*) and European pear (*P. communis*), were used to evaluate the Illumina Infinium® II 9K apple and pear SNP array. For the POP369 population, DNA from 94 full sibs and the pollen parent was purified from young leaves using a CTAB extraction method (Doyle and Doyle, 1987), followed by column purification using the NucleoSpin® kit (Macherey-Nagel GmbH & Co. KG). For the POP356 population, DNA from 123 full sibs and the pollen parent was extracted using the QIAGEN DNeasy Plant Kit (QIAGEN GmbH, Hilden, Germany). For both segregating populations, no DNA from the female parent (common between populations) could be prepared as this genotype no longer existed in the field. DNA quantifications were carried out using the NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific Inc.).

Genomic DNA (200 nanogram) from progeny and male parents was amplified and hybridized to the Apple and Pear 9K SNP array (Chagné et al., 2012a; Montanari et al., 2013) following the Infinium HD Assay Ultra protocol (Illumina Inc., San Diego, USA) and scanned with the Illumina iScan at AgResearch Invermay (NZ).

3.2.3 SNP genotyping and data analysis for normal and distorted alleles

The Illumina Infinium II® assay contains thousands of beads with attached oligonucleotides that are designed to bind DNA sequences immediately flanking the targeted SNP. A single base extension reaction is performed after hybridization, in parallel for all SNPs present on the BeadArray. The reaction uses dideoxynucleotides (ddNTP) and fluorescent two-colour dye chemistry to distinguish the two alleles for a given SNP. The DNA fragments bind to the complementary oligonucleotide, stopping one base before the SNP. ddNTPs (terminating nucleotides) are attached to the 3' terminus of the oligonucleotide through single base extension by DNA polymerase. A homozygous individual will generate a fluorescent signal for only one of the two colours (e.g. AA = all red or BB = all green), while a heterozygous individual will generate approximately equal fluorescent signals of both colours (e.g. AB = red and green and show yellow). The fluorescence intensity is detected by a fluorescent scanner and provides information about the allelic variation (Figure 3.2). This information can be simultaneously analyzed using specialized software (GenomeStudio, Illumina Inc.).

The extracted DNA was amplified and hybridized to the Apple SNP BeadChip following the Infinium HD Assay Ultra protocol (Illumina Inc., San Diego, USA). The BeadChip was then scanned with the Illumina iScan, which records high-resolution images of the light emitted from the fluorophores of the single-base extension products. These signals are converted to genotype calls using Illumina's GenomeStudio v 1.0 software Genotyping Module, setting a GenCall Threshold of 0.15. GenCall Score is the quality parameter for the reliability of called genotype, which depends on various factors including DNA quality. Lower GenCall means poor clustering and farther location of genotypes from centre of cluster (GenomeStudio TM Genotyping Module v1.0 User Guide). Genotype calling algorithm of software automatically determines the cluster positions of the

AA/AB/BB genotypes for each SNP and displays them in normalized graphs (Figure 3.3).

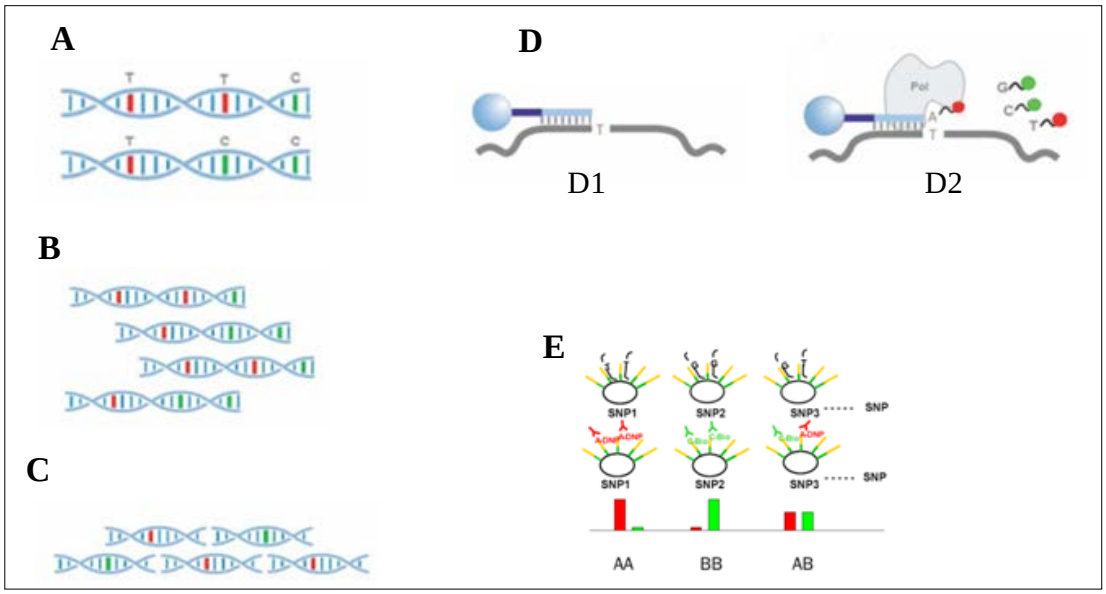


Figure 3.2: Principle of the Infinium II assay for whole genome genotyping. A: Genomic DNA (200-400 ng); B: Linear whole genome amplification; C: DNA fragmentation; D: Two step allele detection D1) Selectivity: Hybridization of unlabelled DNA fragment to 50mer probe, D2) Specificity: Enzymatic single base extension with labelled nucleotide; E: Array scanning and genotype scoring.

3.2.4 SNP data analysis

A systematic method was used to evaluate the SNP array data using quality metrics extracted from GenomeStudio (Illumina): GenTrain score ≥ 0.50, minor allelic frequency (MAF) ≥ 0.15 and call rate > 80 %. The GenTrain score represents a number between 0 and 1 and indicates how well the samples clustered for this locus. A population of samples (e.g. POP369 and POP356 in current study) generally exhibits three genotypes for each SNP and Genome studio can automatically determine the clustering position of these genotypes (Figure 3.3). These clustering positions determine the heterozygosity or homozygosity of particular SNP.

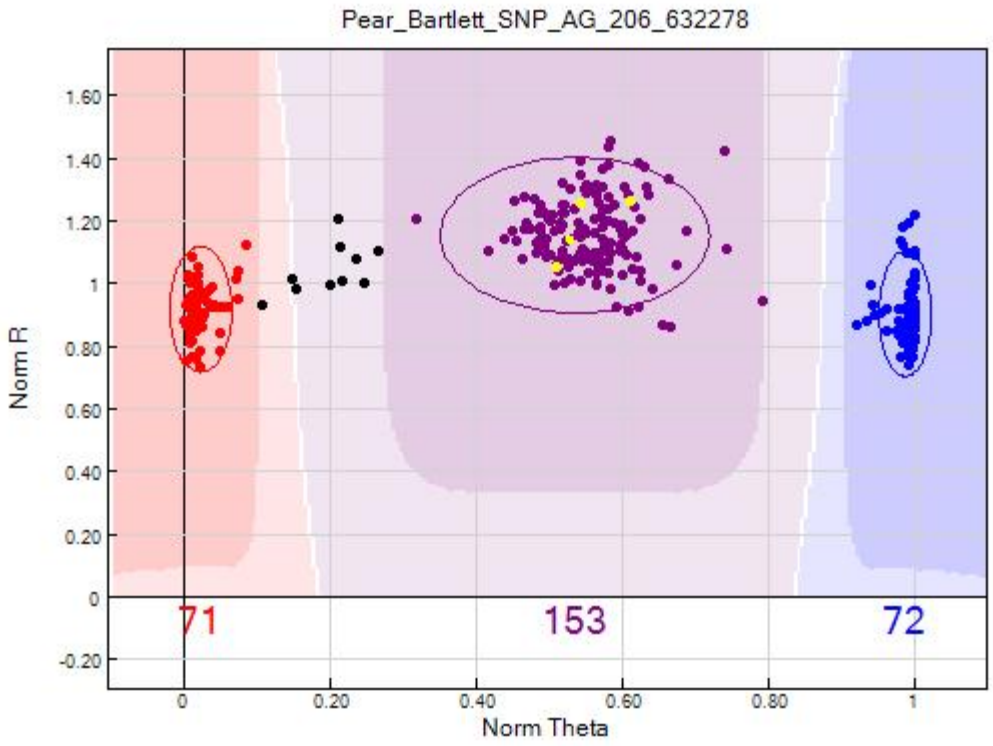


Figure 3.3: Example of a SNP graph in polar coordinates. The red cluster is identified as AA genotype, the purple cluster as AB genotype, and the blue cluster as BB genotype. Samples are coloured according to their genotype call. Samples in the lighter shaded regions fall below the user-specified Call Score Threshold set at the beginning, and are coloured black to indicate that they are classified as "No Calls". Yellow dots indicate the parent's replicates.

All 8786 SNPs were inspected visually to check the quality and clustering position of genotypes. The genotype of the female parent was inferred manually on the basis of the genotype of other parent and genotype of progeny i.e. if the progeny exhibits two genotype classes AB:AA with ratio of 1:1, this means 50 % of progeny has homozygous allele AA and 50 % heterozygous AB. If the genotype of the known parent is AB then the other parent must have homozygous allele information and hence the genotype of missing parent will be AA. All the SNPs were first sorted according to GenTrain Score, then SNPs with GenTrain less than 0.50 were edited if necessary. Manual re-location of clusters was also done where the automated algorithm failed to calculate the correct cluster position.

Misclassified SNPs, where the automated algorithm resulted in a wrong clustering position, were manually annotated and previously monomorphic or no call markers were genotyped as 1:1, 1:3 or 1:2:1 ratio (Figure 3.4). Furthermore, the SNPs for which 25 % and 50 % of the individuals were not called in clusters were manually edited by annotating the clustering positions to clearly separated and unambiguous groups of genotypes, since this situation could be due to null allele segregation (Figure 3.5). SNPs that seemed to exhibit a null allele segregation were marked with different identity number and missing genotypes were changed to their respective genotype based on their clustering position.

The population size necessary for a good clustering depends on the minor allele frequency of the SNPs: the lower the minor allele frequency, the more samples are required to achieve representation of all clusters. Typically a population size of 100 or more individuals is recommended, which is true in this study.

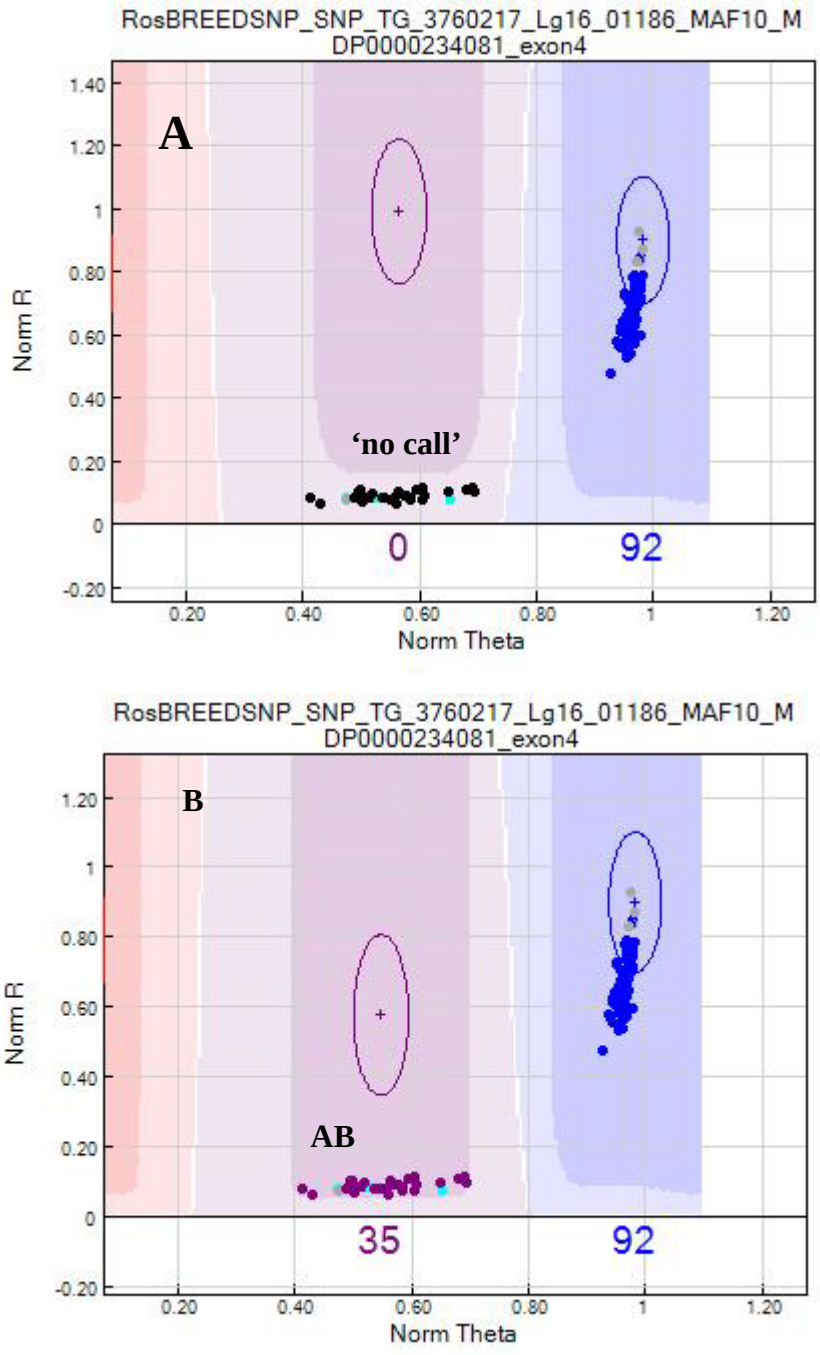


Figure 3.4: Example of manual annotation in GenomeStudio. A) represents marker in genome studio regarded as monomorphic with 25% 'no call' and B) represents same marker after manual annotation of AB cluster from 'no call', genotype of this marker is AB:AA with segregation ratio of 1:3.

3.2.5 Linkage mapping analysis

The genetic maps of both parents of both populations were built using JoinMap v3.0 software (Van Ooijen and Voorrips, 2001), based on the SNP data for each individual population. Linkage grouping was determined with a LOD score of 5 and higher for grouping and was used for map calculation. Recombination frequency was converted to map distance in centiMorgan (cM) using the Kosambi function. Maps were constructed initially by using only "normal" markers (i.e. no presumed null allele) segregating in the 1:1 and 1:2:1 ratio. Later, null alleles were added to the map. Markers segregating 1:3 were coded as dominant ab x ab and included in both parental maps. Events of double recombination were carefully inspected for each LG by transferring the marker data (AB, AA and BB) to the Excel sheet and the original calling was rechecked in GenomeStudio to avoid error. Markers with segregation distortion which created problems for map construction were checked for their genotype calls in the genome studio and corrected if possible; if the problem persisted on remapping then they were removed from the marker dataset. Markers deviating from Mendelian ratio were re-checked in GenomeStudio and eventually recoded. The map construction was deemed achieved when there was sufficient number of markers to saturate the pear genome with each chromosome containing at least four markers with map distance between two adjacent markers less than 30 cM. The parental maps of the four populations were drawn and aligned using MapChart v2.2 (Voorrips, 2002).

3.3 Results and discussion

3.3.1 SNP detection and selection for 1 K pear array

In total, 34,082,435, 35,687,533 and 25,167,853 paired-end reads were generated for 'Bartlett', OH and LBJ, respectively. The de novo assembly genome set of 'Bartlett' consisted of 78,748 contigs of 600 bp or greater in length, containing a total of 79,067,993 bases, with a maximum contig length of 15,094 bases, N50 of 1004 bases, N90 of 658 bases, and an average contig length of 1004 bases. A total of 73,214 SNPs were predicted by SoapSNP when reads of OH and LBJ were aligned to the genome of 'Bartlett' using the Soap aligner, corresponding to one SNP per

1079 bases. In total, 1,456 SNPs passed the filtering criteria and were then subjected to the Illumina assay design tool. This yielded 1107 SNPs, of which 1064 were included in the final SNP array. In addition to these, 69 SNPs experimentally validated by M. Troggio (unpublished data) and which passed the Illumina ADT design, were selected for inclusion in the SNP array. In total, 1133 pear SNPs were incorporated in the final array, making a grand total of 9000 attempted apple and pear SNPs combined.

3.3.2 Identification of polymorphic and null alleles

A total of 1177 polymorphic markers (excluding null alleles) including 621 apple SNPs and 556 pear SNPs were detected in both populations from a total of 8768 SNPs genotyped. Out of these, 815 polymorphic markers exhibited 1:1 segregation ratio and 362 exhibited 1:2:1 segregation ratio (Table 3.1). For population POP369, the total number of unique polymorphic markers including null alleles was 1144 with 849 apple and 296 pear markers. For population POP356, unique polymorphic markers including null alleles were 1147 with 763 apple and 384 pear markers. Of all the pear and apple SNPs on the array, 40 % and 16 % of pear and apple markers were polymorphic and useful for genetic map construction, respectively. Genetic marker transferability between apple and pear has been demonstrated in the past, however not using SNP markers. Pierantoni et al. (2004) tested marker transferability across the genera by using two pear genetic maps. Out of 112 polymorphic apple SSR markers used to create maps, 41 markers mapped successfully in 'Passe Crassane' × 'Harrow Sweet' and 31 in the 'Abbé Fétel' × 'Max Red Bartlett' map. Yamamato et al (2007) created genetic linkage maps of European pear based on pear and apple SSR and AFLP markers. Out of 111 apple SSR markers, 66 mapped successfully on the pear maps and were colinear with their position on the apple reference map. Celton et al. (2009) aligned two apple and two pear maps that were constructed using apple and pear SSR markers. These maps were aligned using 102 common markers. Out of these 102 common markers, 90 markers (53 pear and 37 apple) showed complete co-linearity between the apple and pear genomes.

Hybridization of pear DNA onto apple probes on the SNP array is not unexpected because apple and pear are closely related and must share high sequence similarity in

gene coding regions. However, the observed conservation of SNP sites is surprising. A total of 833 apple SNP markers were polymorphic in pear as null allele markers, and this can be interpreted as occurring due to polymorphism on the probe site flanking the target SNP rather than on the target SNP site itself.

The proportion of pear polymorphic markers mapping in interspecific populations was lower than for European pear populations. European pear parents have high polymorphic rates in the range of 29 % to 35.1 % while Asian x European parents displayed a lesser proportion of polymorphic markers i.e. in the range of 2.9 % to 21.4 % (Montanari et al., 2013). This could be due to the fact that the new pear SNPs had been developed from European pear. Hence polymorphic pear SNPs are either conserved between Asian and European pear or the SNP alleles were inherited from the European grandparent to the progeny. It is impossible to distinguish between two scenarios because SNPs are biallelic and the grandparents were not genotyped.

Analysis of SNP polymorphism pointed to the presence of potential null alleles. Null alleles refer to markers which partially fail to amplify during genotyping (Figure 3.2). They can be the result of deletions surrounding the target SNP site, secondary polymorphism at primary polymorphic site of sequence or triallelic form of SNPs (Crooks et al., 2013). By default the standard SNP calling algorithms of GenomeStudio clustered heterozygous A0 and B0 genotypes together with homozygous AA and BB genotypes, and called homozygous null genotypes (00) as missing genotypic calls (Figure 3.5). Null alleles deviated from the expected Mendelian ratio that might be expected from their parental genotype. Therefore, manual editing of all these potential null allele markers was performed and SNPs which displayed a clear clustering and for which genotypes could be unambiguously determined as containing potential null alleles were selected for further linkage analysis (Figure 3.5). Four types of null allele segregation were observed in populations POP369 and POP356: 00xA0, A0xA0, A0xB0, ABxA0 (Figure 3.5; Table 3.2).

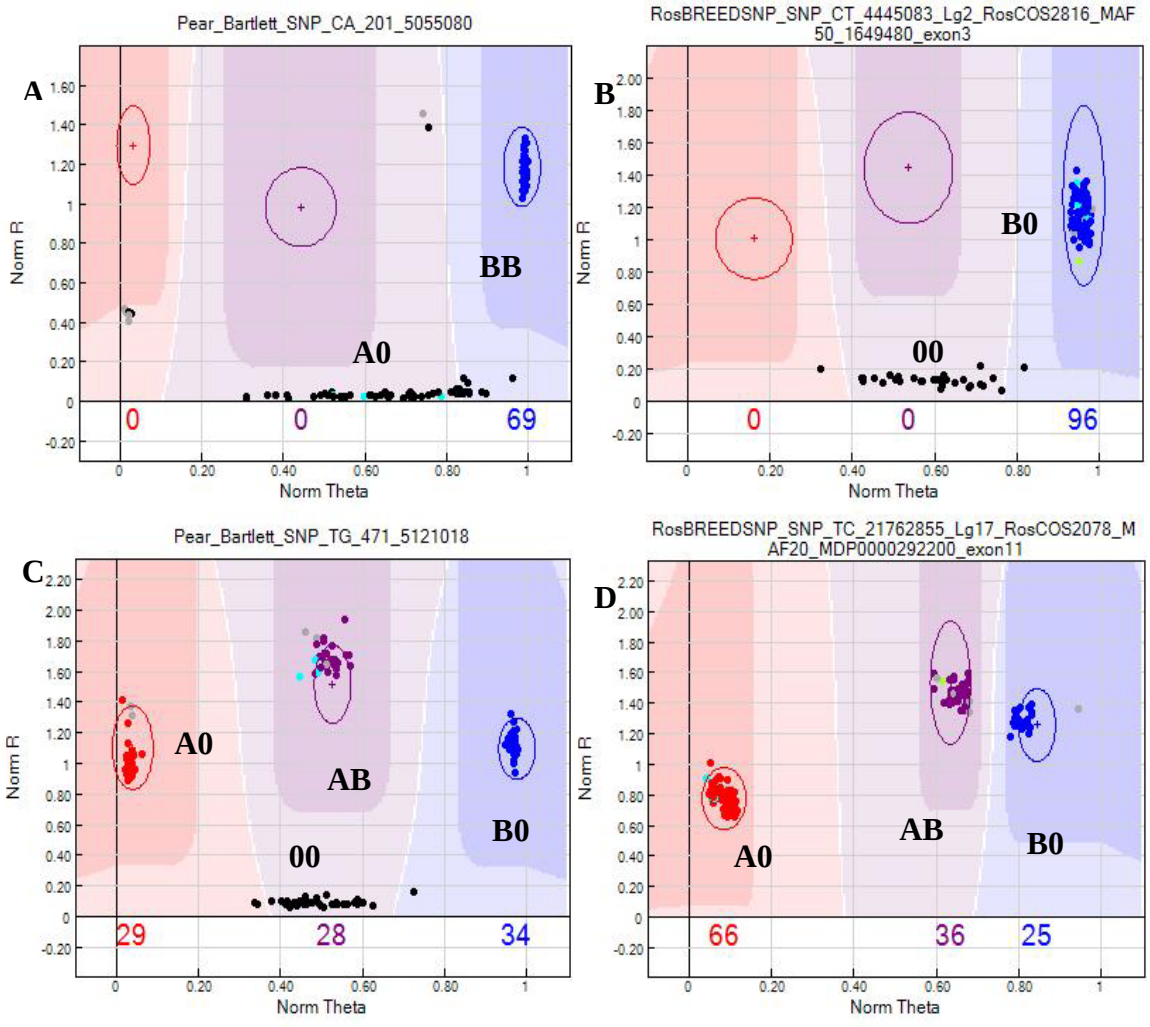


Figure 3.5: Examples of graphical display of null alleles from GenomeStudio software, with 123 individuals in total. A) A pear SNP (ss527788247) represented as A0xB0 with segregation ratio of 1:1. B) An apple SNP (ss475877058) represented as B0xB0 with segregation ratio of 1:3. C) A pear SNP (ss527789773) represented as A0xB0 in GenomeStudio with segregation ratio of 1:1:1:1. D) An apple SNP (ss475882196) represented as A0xAB with segregation ratio of 2:1:1.

A total of 937 unique null alleles were detected in both populations studied. The number of polymorphic null allele markers from attempted bead types was higher in population POP369 (702 SNPs), than in POP356 (450 SNPs) with A0xA0 and 00xA0 being most abundant segregation type in POP369 and POP356 respectively (Table 3.2). Percentage of apple and pear null alleles seemed to be similar for POP369: 9.5% and 7.5% for female and male parent and POP356: 4.9% for both.

Polymorphic null alleles were used to increase the density of the maps for the interspecific crosses for both populations. All the null allele markers were checked manually for genotype errors, missing values and double recombinants both before mapping and after mapping. The number of mapped null alleles was lower than the polymorphic alleles (Table 3.2). The proportion of SNPs with null allele was greater for interspecific hybrid parents as compared to European pear parents (Montanari et al., 2013).

The most likely reason for the high number of polymorphic null alleles in interspecific populations could be attributed to the fact that the discovery panel and genotyped samples are genetically different. In the current study the SNP array included apple and European pear SNPs (which means that both the target SNP and the sequences flanking the SNPs were from these species), while the genotyped populations are interspecific crosses among *P. communis*, *P. pyrifolia* and *P. bretschneideri*. In this scenario interspecific populations are likely to have high number of partially hybridized markers. It can be hypothesized that a particular SNP allele carried from the *P. communis* grandparent hybridized successfully to *P. communis* sequence on the array whilst the second allele from the *P. pyrifolia* grandparent failed to bind and was detected as no call (null allele). This phenomenon is explained in Figure 3.6. As heterozygous null alleles are significant sources of polymorphism, they were included in the map construction. A total of 243 and 332 null alleles were successfully mapped to POP369 and POP356 respectively. These polymorphic SNPs with null alleles helped to saturate maps of both populations and in particular maps from POP369. While the null alleles were challenging to score using the GenomeStudio software, they provided numerous markers useful for map saturation. The complex pedigree of the POP369 and POP356 with three species contributed to the generation of these useful null allele SNPs.

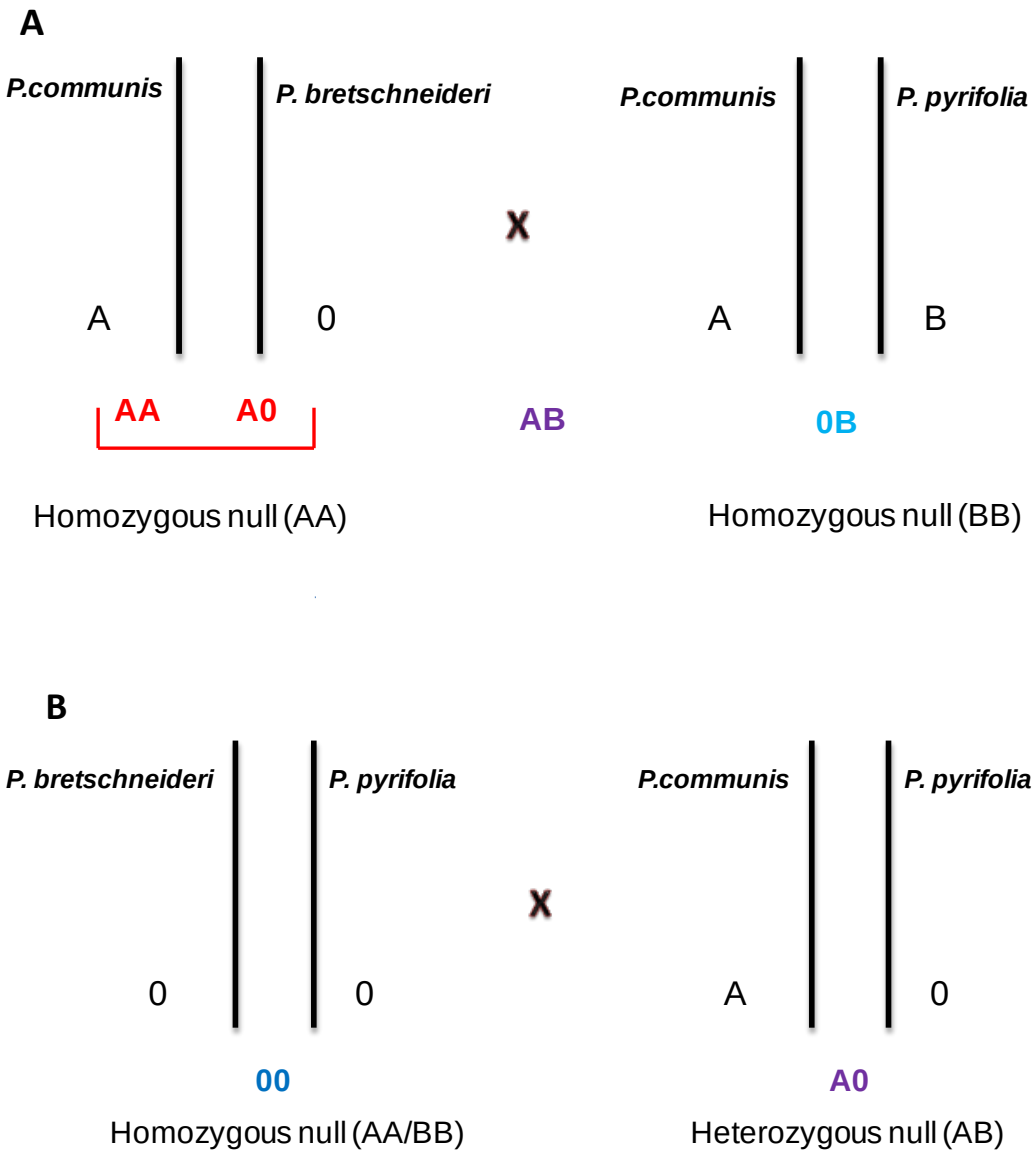


Figure 3.6: Null allele classes observed in the pear interspecific segregating populations with the possible combination of alleles from grandparents. Null alleles are represented by '0' (i.e. no hybridization to the array probe) while actual genotypes are provided for the target SNP as an A or a B allele. The observed genotype in the progeny are provided in brackets '( )'. A) SNP marker with a null allele following a segregation ratio of 2:1:1 in the progeny; B) SNP with null alleles following a 1:1 segregation ratio.

3.3.3 Segregation distortion

Careful examination of markers revealed that there were a number of markers that exhibited segregation distortion (i.e. significant deviation of marker from the expected Mendelian ratio) at $P<0.05$ in all four maps from both populations. The number of distorted markers was variable in both populations: POP356 had 40 and 54 distorted markers for the POP356-female and POP356-male parent, respectively while distorted markers for population POP369 were 8 and 9 for POP369-female and POP369-male parent respectively. Major regions with distorted markers were on LG1, 8, 11 and LG15 of POP356, while POP369 did not have such regions (Supplementary figure 3.1-3.4).

Segregation distortion is very common in plants and varies from species to species and population type (Huang et al., 2010). It has been widely reported that incidence of segregation distortion is very common in interspecific populations. Lin (2005) reported that in tetraploid cotton, 135 (18 %) and 10 (6.41 %) markers showed distorted segregation in interspecific and intraspecific populations, respectively. A significant number of SNPs with segregation distortion were identified in genetic maps constructed using POP356 (94 SNPs) while POP369 exhibited only 17 distorted SNPs (Supplementary figure 3.1-3.4). This variability could be due to a higher number of polymorphic markers in POP356 as compared to POP369, which could explain the higher number of distorted markers in POP356. POP356 exhibited few random distorted markers spread all over the chromosome which indicates almost no chance of inherent distorted region.

Segregation distortion can affect linkage mapping as well as QTL analysis and this effect depends upon distance of the QTL from the distorted loci and on the size of population (Vogl and Xu, 2000). Therefore it is important to know the type and location of distorted regions on the chromosome. In extreme cases of segregation distortion, the genetic map of a whole chromosome can be affected by the distorted locus and agronomic traits linked to that distorted locus in repulsion will be underrepresented or even absent from the phenotype during introgression of these traits (Alheit et al., 2011).

In the current maps, highly distorted markers which were causing major drift in genetic distance in linkage maps were removed at the 1st step of the filtering process and all maps were reconstructed without them. However other relatively less distorted markers were mapped in all four maps (Supplementary figure 3.1-3.4). Later, distorted loci which were posing a problem to QTL mapping were also removed from genetic linkage maps and maps were reconstructed once more prior to QTL analysis.

3.3.4 Genetic map construction

Parental genetic maps were constructed for the two segregating populations using the 1670 unique polymorphic SNPs (Supplementary figure 3.1-3.4) where 671 (40 %) markers were common to both populations. Out of these 1670 unique polymorphic SNPs 70 % (1167) mapped successfully in either parent. Highly distorted null alleles that created a problem for map construction were removed from the maps and reconstruction of maps were performed until maps were developed suitable for use in QTL analysis (Chapter 4).

For population POP369, both parental maps spanned 17 linkage groups of 1018 and 1101 cM and consisted of 371 and 255 SNP markers for the POP369-female and POP369-male parent, respectively (Supplementary figure 3.1-3.4; Table 3.1and Table 3.2). For population POP356 both parental maps spanned 17 linkage groups of 1485 and 1580 cM and consisted of 628 and 682 SNP markers for the POP356-female and POP356-male parent, respectively (Supplementary figure 3.1-3.4; Table 3.1 and Table 3.2). In total 1167 unique SNPs were mapped, including null allele markers. Average distance between two adjacent markers for population POP369 was 2.7 cM and 4.3 cM for POP369-female and POP369-male parent, respectively. For POP356 this was 2.3 cM for both female and male parents.

Table 3.1: Polymorphic and mapped markers in populations POP369 and POP356.

		Polymorphic markers			Mapped markers		
Population	**Marker segregation**	**Pear SNPs**	**Apple SNPs**	**Total**	**Pear SNPs**	**Apple SNPs**	**Total**
POP369	ABxAA/BB	179	83	262	131	66	197
(n=91)	ABxAB	28	67	95	15	43	58
	BB/AAxAB	12	73	85	11	52	63
	Total	**226**	**240**	**466**	**157**	**161**	**318**
POP356	ABxAA/BB	96	113	209	82	89	171
(n=123)	ABxAB	137	130	267	132	111	243
	BB/AAxAB	97	138	235	89	121	210
	Total	**330**	**381**	**711**	**303**	**321**	**624**

Table 3.2: Polymorphic and mapped null allele markers in population POP369 and POP356.

		Null- allele markers			Mapped null- allele markers		
Population	**Marker segregation**	**Pear SNPs**	**Apple SNPs**	**Total**	**Pear SNPs**	**Apple SNPs**	**Total**
POP369	00xA0/00xB0/BBx B0	30	193	223	24	123	147
(n=91)	A0xA0/B0xB0	40	421	461	10	76	86
	A0x B0	5	7	12	3	2	5
	A0x AB/B0xAB/ABxB0	2	4	6	2	3	5
	Total	**77**	**625**	**702**	**39**	**204**	**243**
POP356	00xA0/00xB0/BBx B0	32	213	245	18	134	152
(n=123)	A0xA0/B0xB0	13	169	182	12	156	168
	A0xAB	4	1	5	2	1	3
	A0xB0	6	12	18	3	6	9
	Total	**55**	**395**	**450**	**35**	**297**	**332**

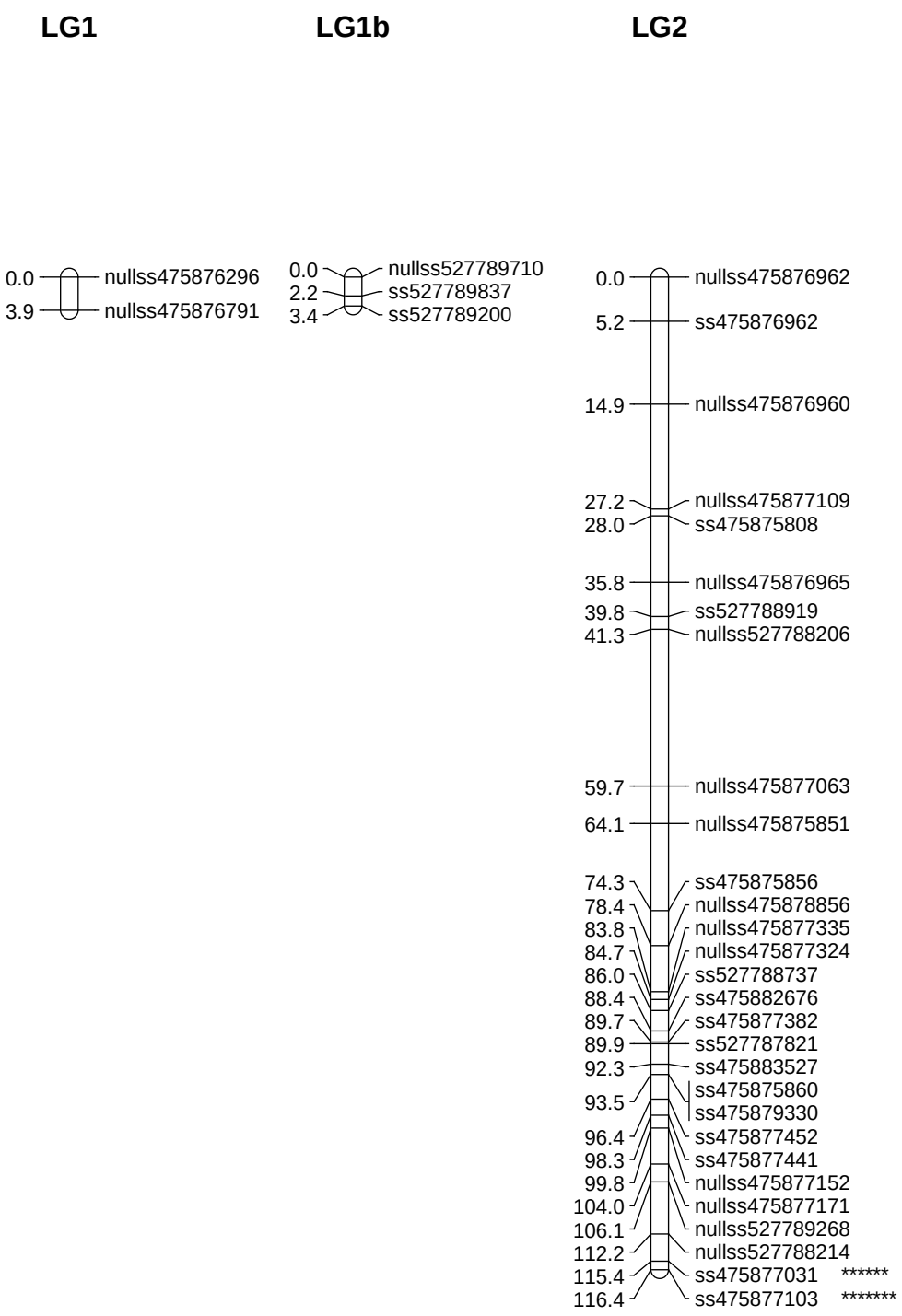
In population POP369, the number of polymorphic unique markers, excluding the null allele markers, was 466 with a GenTrain score ≥ 0.5, minor allelic frequency ≥ 0.15. Out of these 466 markers, 226 and 240 were pear and apple SNPs, respectively (Table 3.1). POP369-male parent had fewer pear (39) and more apple markers (140) while female parent of POP369 has the opposite case with more pear

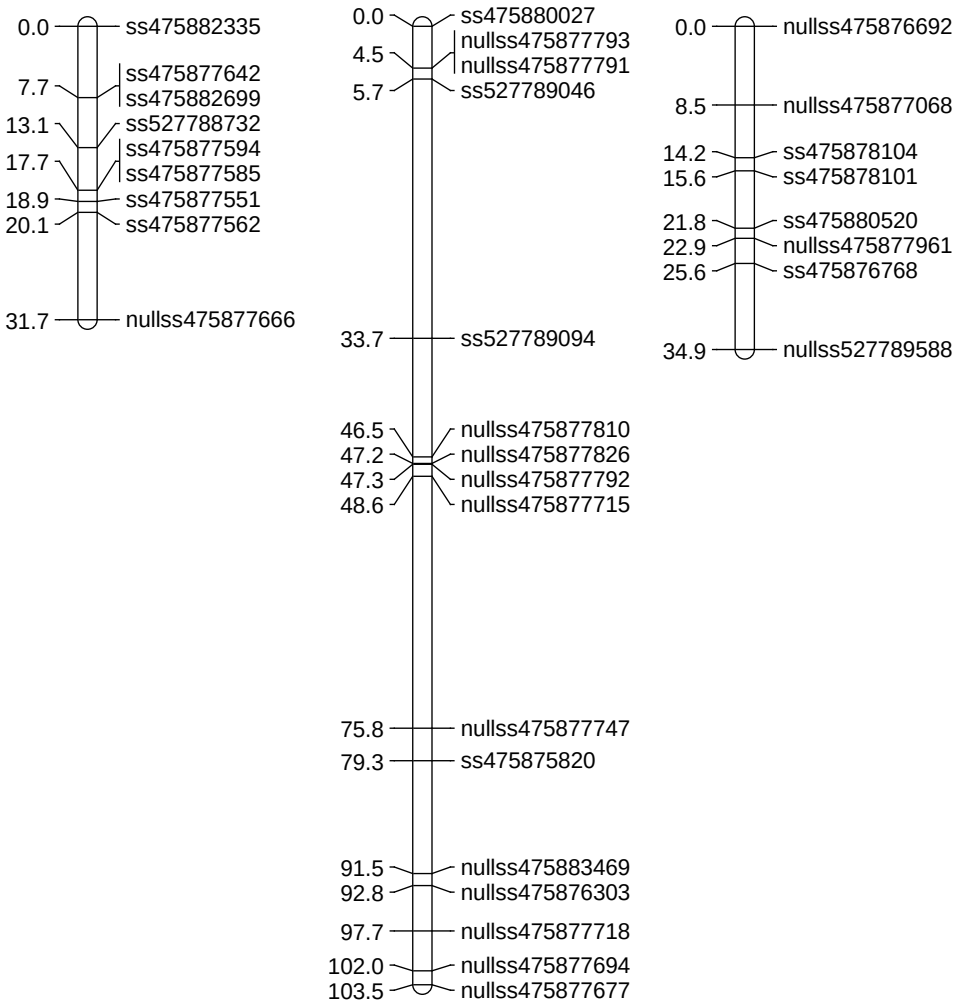
and fewer apple markers. Each linkage group was manually examined for double recombinants. Markers with frequent recombination present at short genetic distance were edited manually before re-mapping. Markers showing abnormal behaviour after rescoring were removed from the map permanently. A few markers that disturbed positions of loci drastically without any known reason were also removed from maps.

3.4 Conclusion

High resolution genetic maps saturated with reproducible and co-dominant markers are important for efficient and accurate QTL mapping and are transferable across labs and populations. Saturated pear linkage maps have been constructed in the past, mostly using AFLP and SSR markers (Yamamoto et al., 2002; Dondini et al., 2004; Yamamoto et al., 2004; Pierantoni et al., 2007; Sun et al., 2010; Zhao et al., 2013). Only one published map used SNP markers (Terakami et al., 2013). However, none of these maps was developed using SNP markers directly from *Pyrus* genome sequences. The first SNP-based genetic maps for two interspecific populations were constructed in this study. These maps contain pear as well as apple markers and could hence assist in the study of apple and pear synteny, including the comparison of the QTL location traits that are similar in pear and apple. These maps have been used to identify QTLs for friction discolouration (FD) and metabolites that might be associated with the development of FD, in order to develop genetic tools for use in the breeding of pears with low FD (Chapter 4).

Supplementary figure 3.1: Linkage map of male parent of POP369 population. SNPs are presented using the NCBI dbSNP accession number. Apple SNPs are represented with an accession number starting with '4' while pear SNPs accessions start with '5' Asterisks at the end of the marker name denote significantly distorted loci (* *P* < 0.05, ** *P* < 0.01, * *P* < 0.001, *****P* < 0.0001, ***** *P* < 0.00001).**

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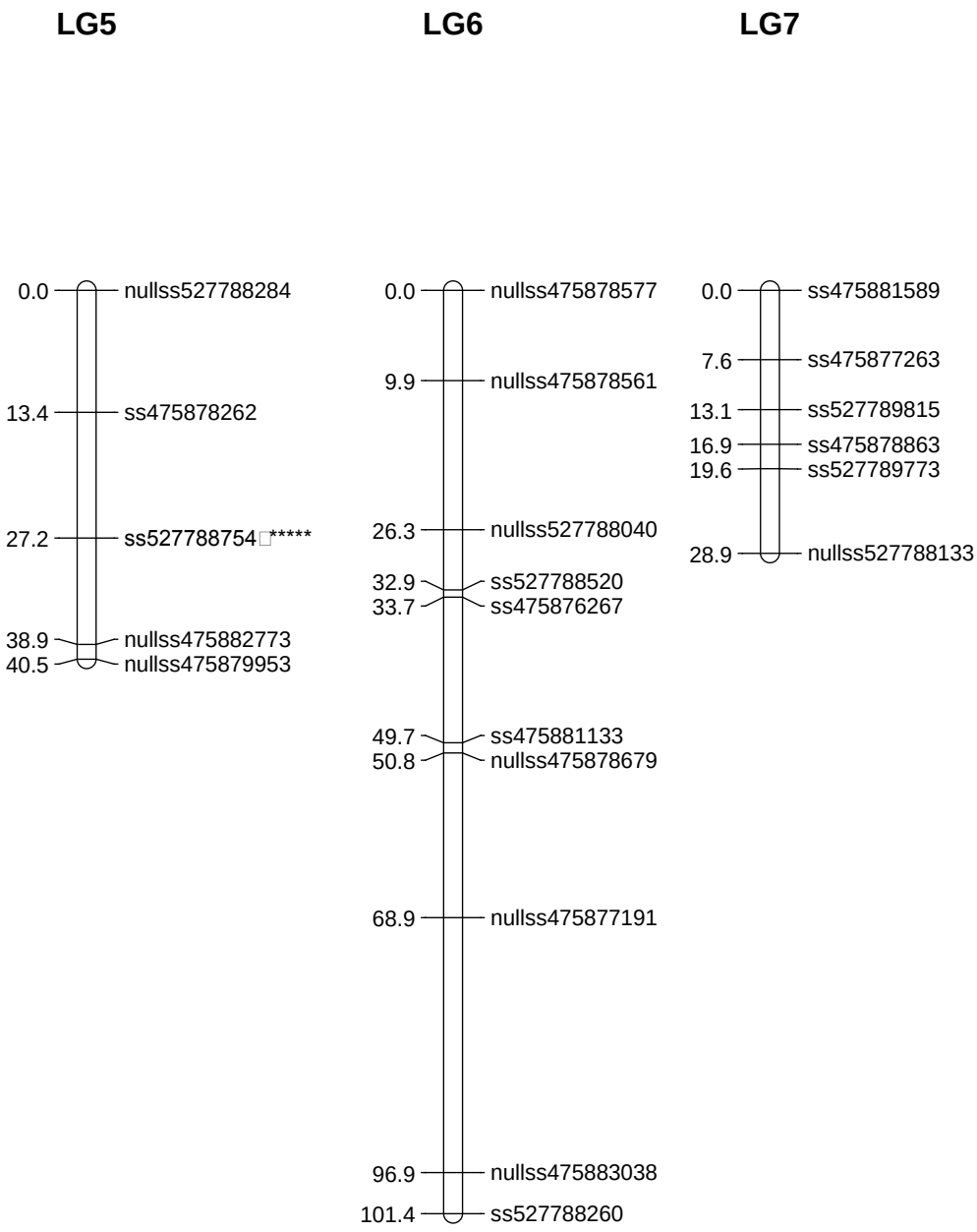
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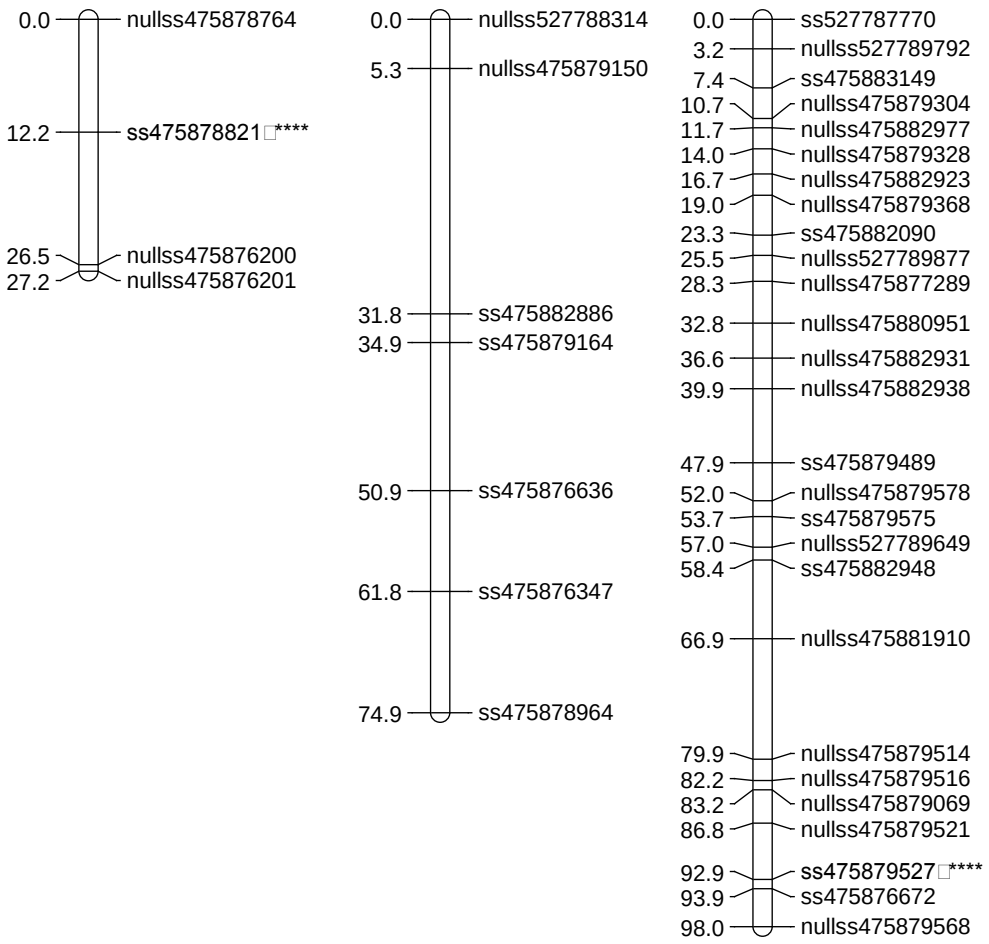
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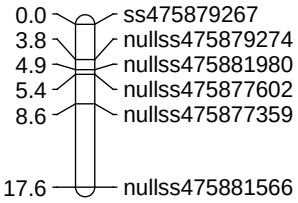

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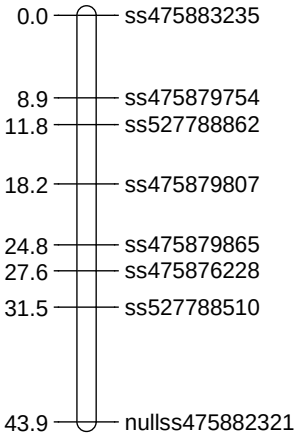

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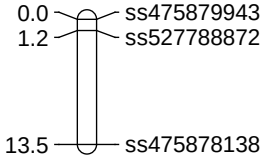

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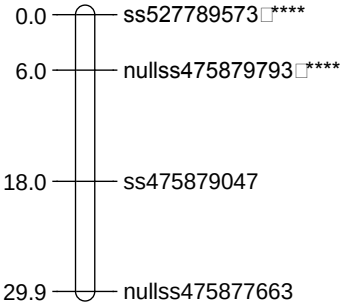

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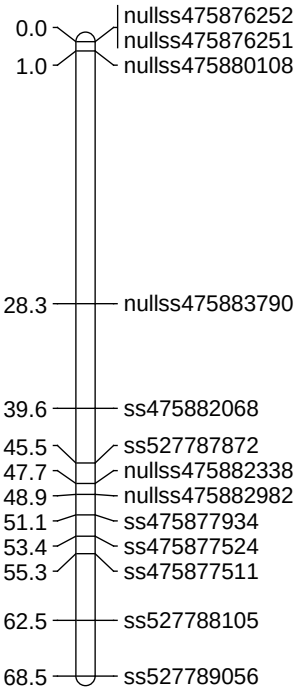

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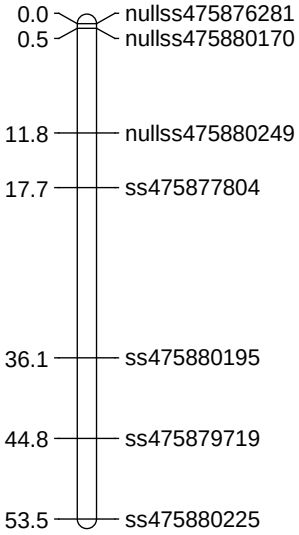

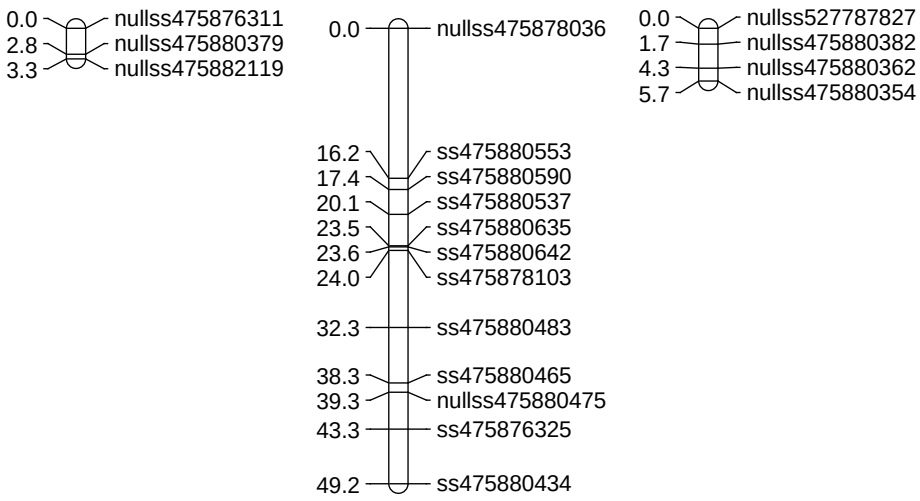
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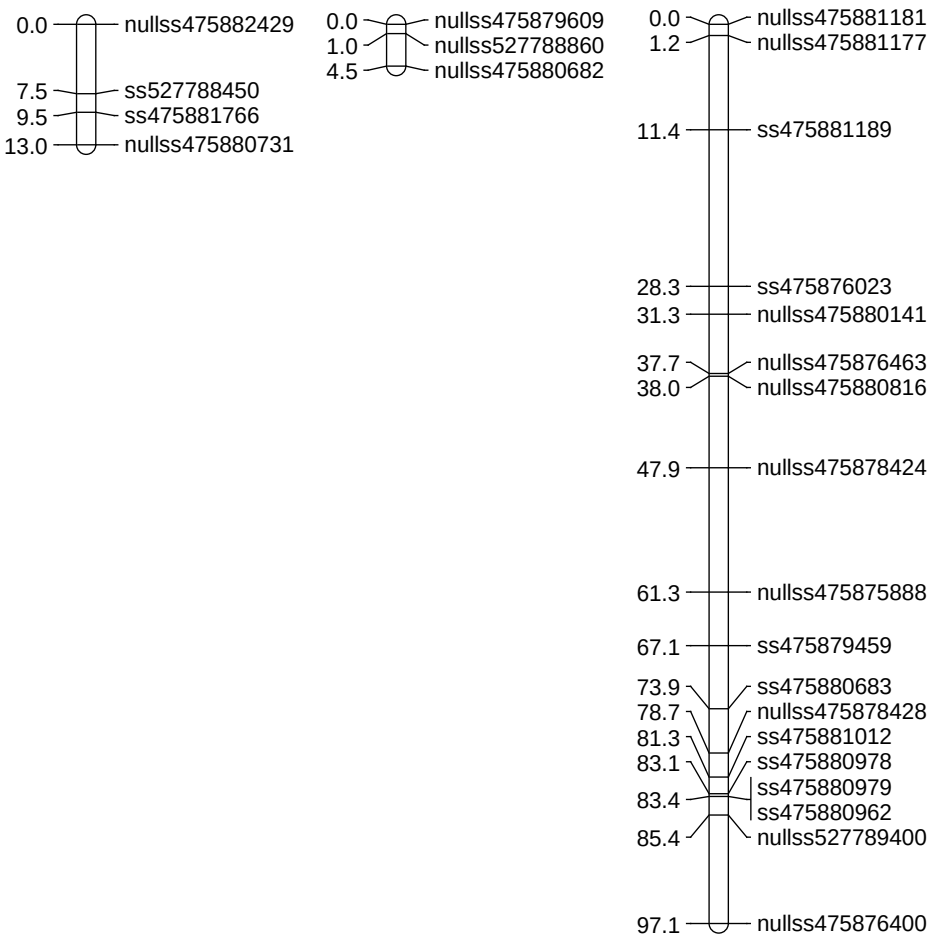
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47.9
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61.3
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67.1
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73.9
ss475880683
78.7
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81.3
ss475881012
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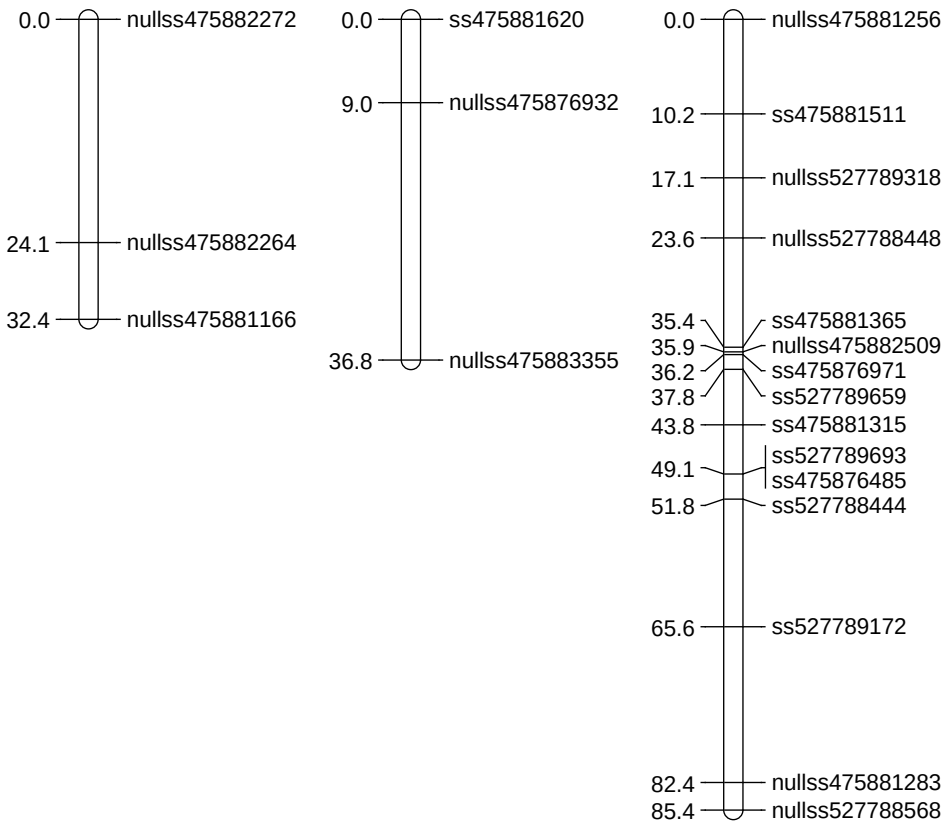
LG14b
LG15
Lg15b
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9.0
nullss475876932
36.8
nullss475883355
0.0
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10.2
ss475881511
17.1
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23.6
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35.4
ss475881365
35.9
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36.2
ss475876971
37.8
ss527789659
43.8
ss475881315
49.1
ss527789693
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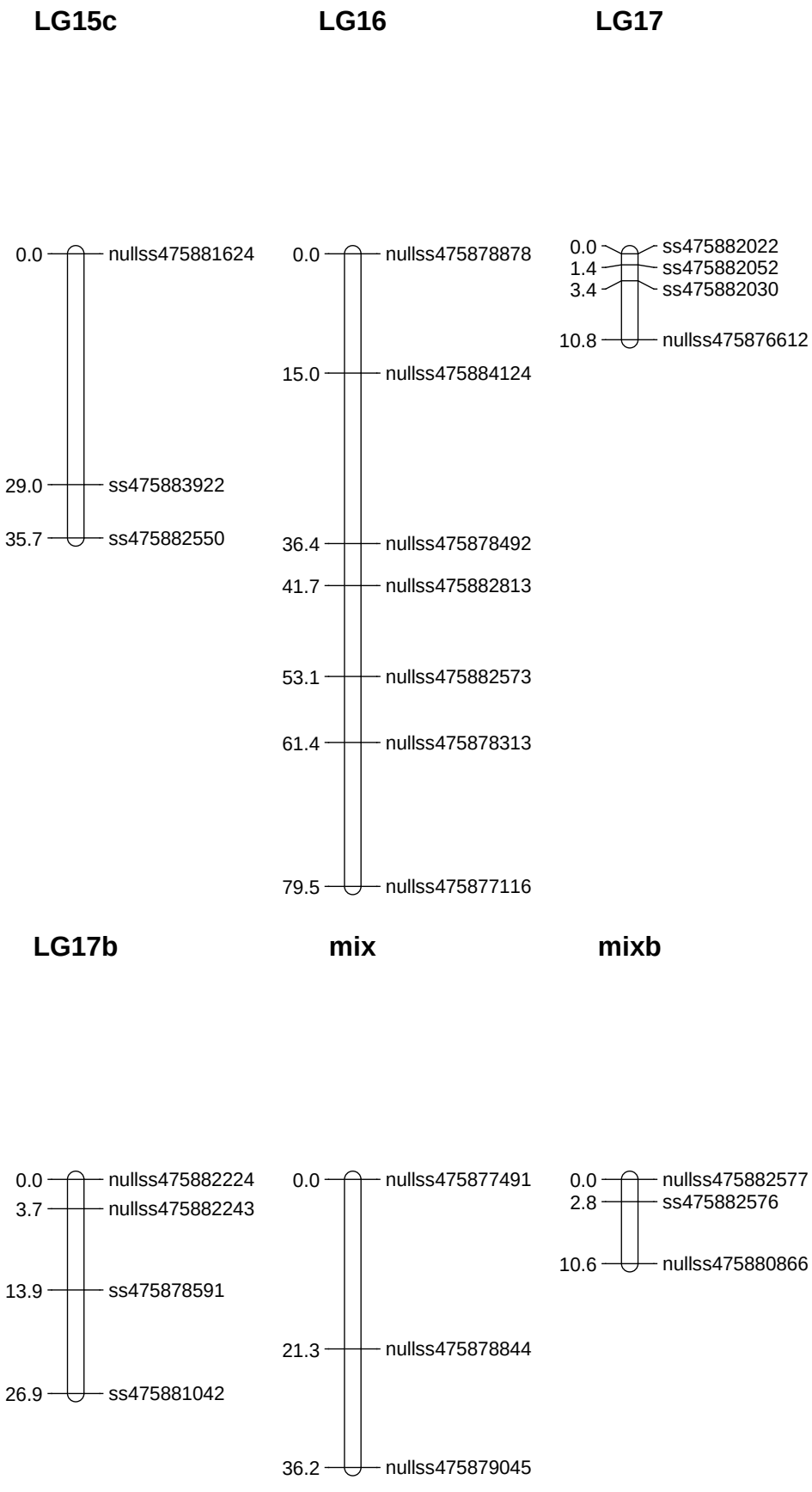
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LG16
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nullss475878492
41.7
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nullss475882573
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79.5
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LG17
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ss475882022
1.4
ss475882052
3.4
ss475882030
10.8
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LG17b
0.0
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3.7
nullss475882243
13.9
ss475878591
26.9
ss475881042
mix
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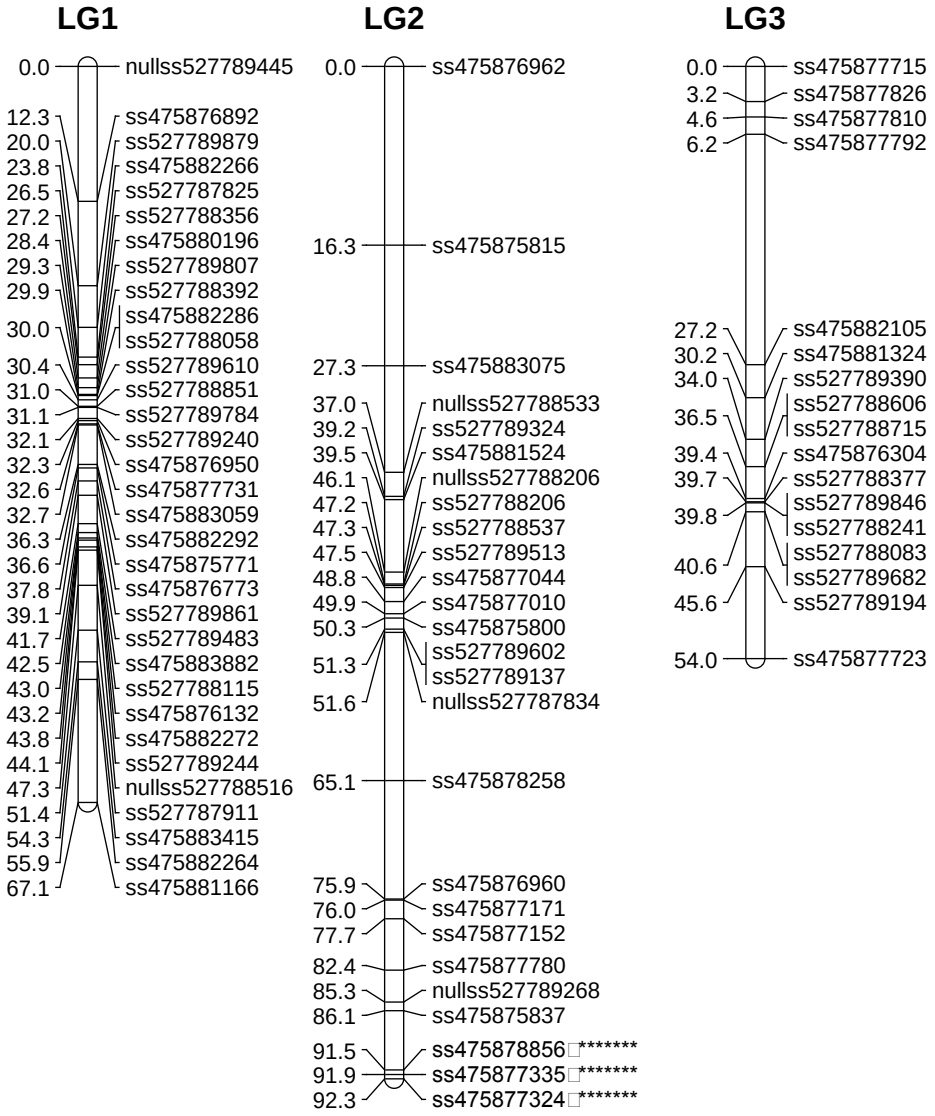
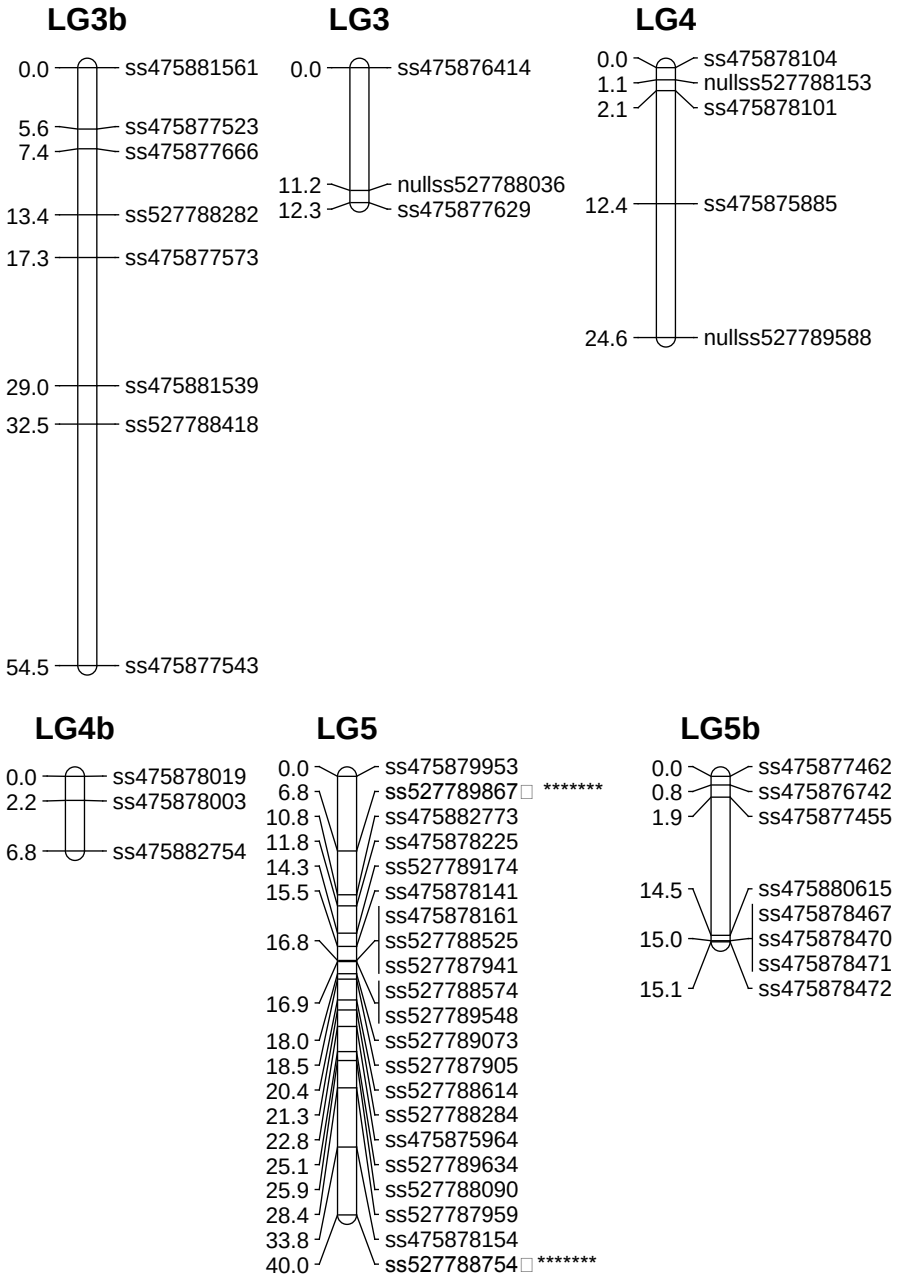
nullss475877491
21.3
nullss475878844
36.2
nullss475879045
mixb
0.0
nullss475882577
2.8
ss475882576
10.6
nullss475880866

Supplementary figure 3.2: Linkage map of female parent of POP369 population. SNPs are presented using the NCBI dbSNP accession number. Apple SNPs are represented with an accession number starting with '4' while pear SNPs accessions start with '5' Asterisks at the end of the marker name denote significantly distorted loci (* *P* < 0.05, ** *P* < 0.01, * *P* < 0.001, *****P* < 0.0001, ***** *P* < 0.00001).**

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29.0 ss475881539
32.5 ss527788418
54.5 ss475877543
LG3
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11.2 nullss527788036
12.3 ss475877629
LG4
0.0 ss475878104
1.1 nullss527788153
2.1 ss475878101
12.4 ss475875885
24.6 nullss527789588
LG4b
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2.2 ss475878003
6.8 ss475882754
LG5
0.0 ss475879953
6.8 ss527789867 *******
10.8 ss475882773
11.8 ss475878225
14.3 ss527789174
15.5 ss475878141
ss475878161
16.8 ss527788525
ss527787941
ss527788574
16.9 ss527789548
18.0 ss527789073
18.5 ss527787905
20.4 ss527788614
21.3 ss527788284
22.8 ss475875964
25.1 ss527789634
25.9 ss527788090
28.4 ss527787959
33.8 ss475878154
40.0 ss527788754 *******
LG5b
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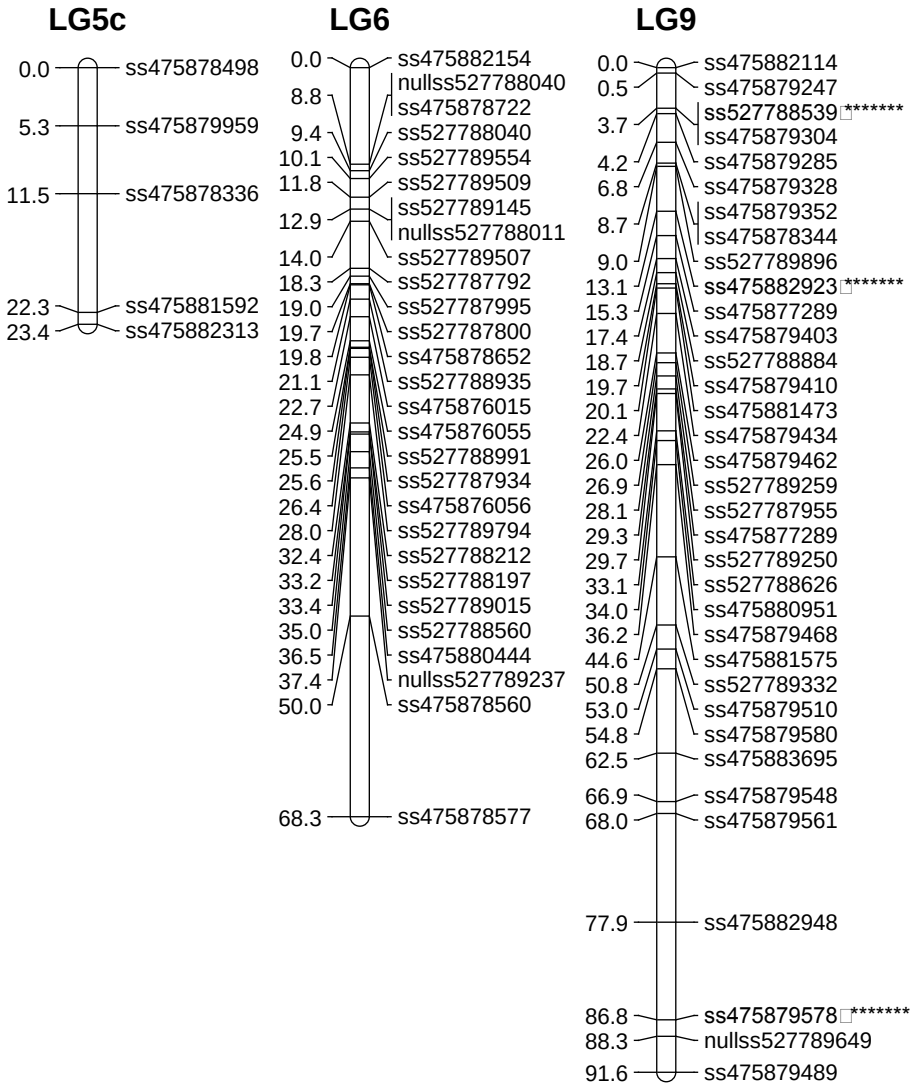
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14.0 ss527789507
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19.0 ss527787995
19.7 ss527787800
19.8 ss475878652
21.1 ss527788935
22.7 ss475876015
24.9 ss475876055
25.5 ss527788991
25.6 ss527787934
26.4 ss475876056
28.0 ss527789794
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33.4 ss527789015
35.0 ss527788560
36.5 ss475880444
37.4 nullss527789237
50.0 ss475878560
68.3 ss475878577
LG9
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0.5 ss475879247
3.7 ss527788539*******
ss475879304
4.2 ss475879285
6.8 ss475879328
8.7 ss475879352
ss475878344
9.0 ss527789896
13.1 ss475882923*******
15.3 ss475877289
17.4 ss475879403
18.7 ss527788884
19.7 ss475879410
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28.1 ss527787955
29.3 ss475877289
29.7 ss527789250
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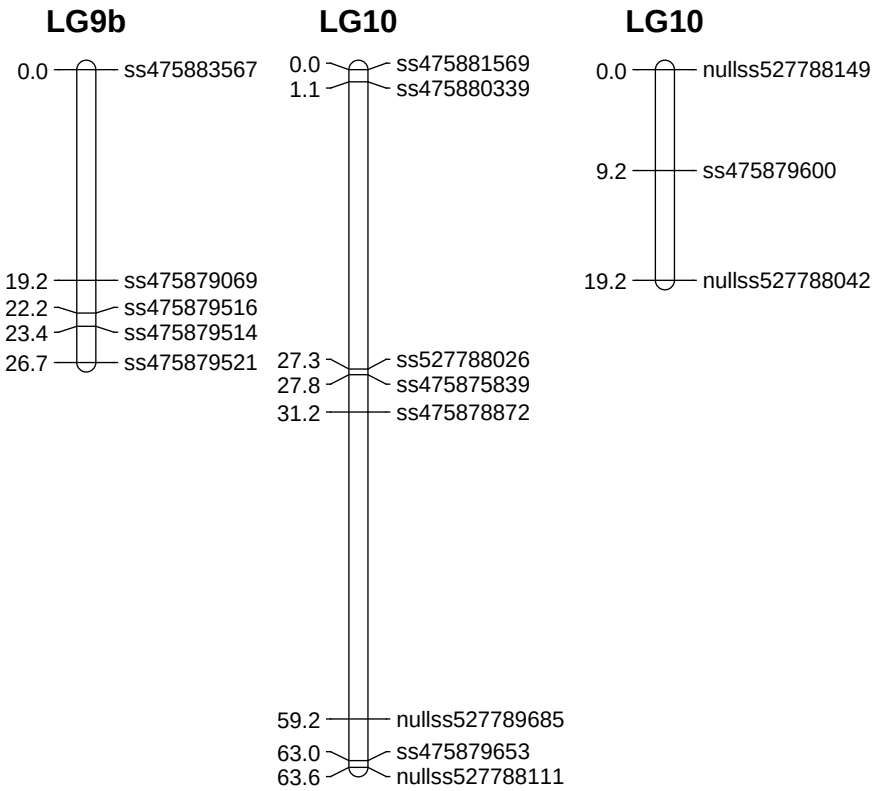
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ss475880339
27.3
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27.8
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ss475878872
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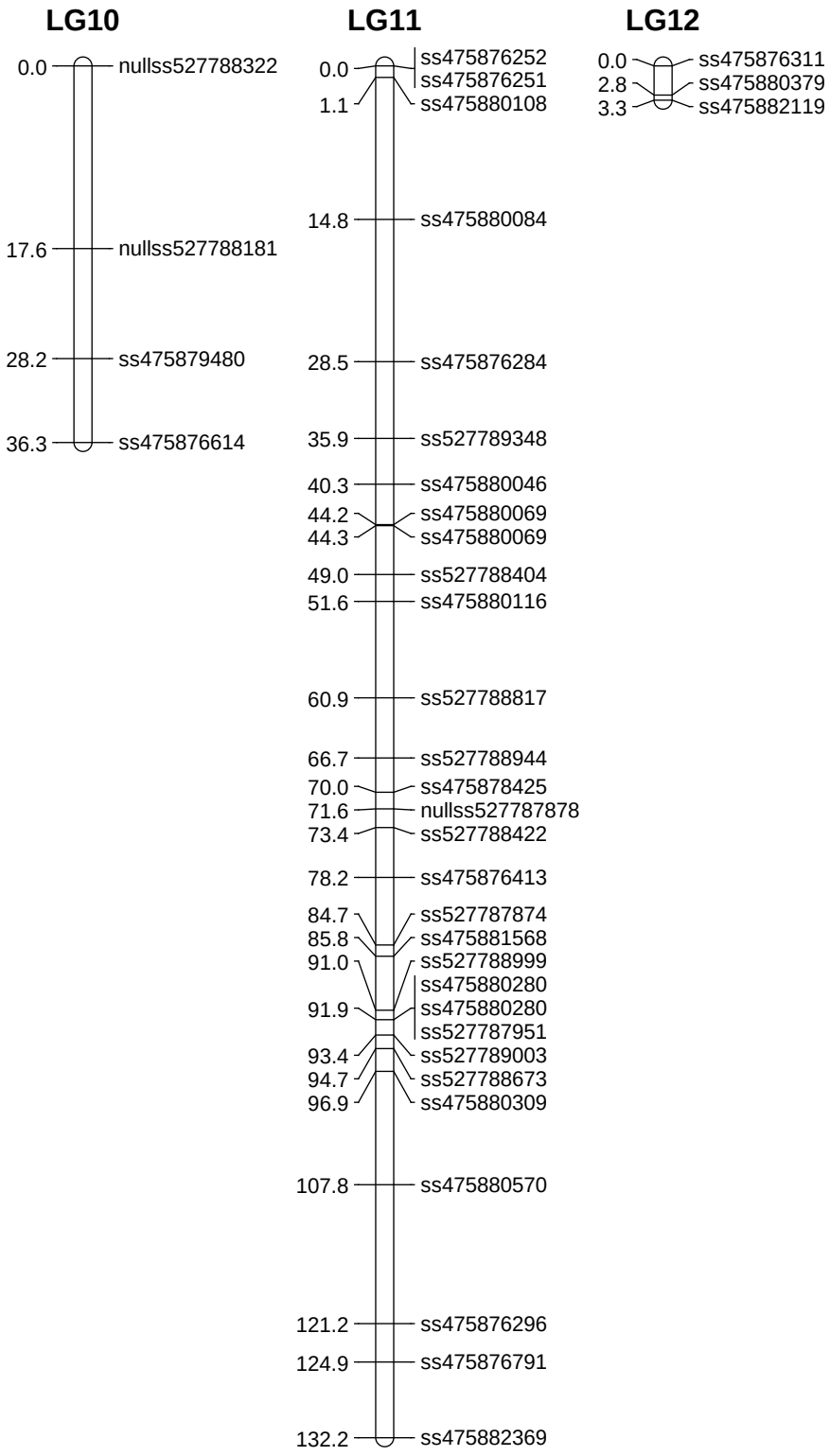
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28.2 ss475879480
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LG11
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ss475876251
1.1 ss475880108
14.8 ss475880084
28.5 ss475876284
35.9 ss527789348
40.3 ss475880046
44.2 ss475880069
44.3 ss475880069
49.0 ss527788404
51.6 ss475880116
60.9 ss527788817
66.7 ss527788944
70.0 ss475878425
71.6 nullss527787878
73.4 ss527788422
78.2 ss475876413
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85.8 ss475881568
91.0 ss527788999
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93.4 ss527789003
94.7 ss527788673
96.9 ss475880309
107.8 ss475880570
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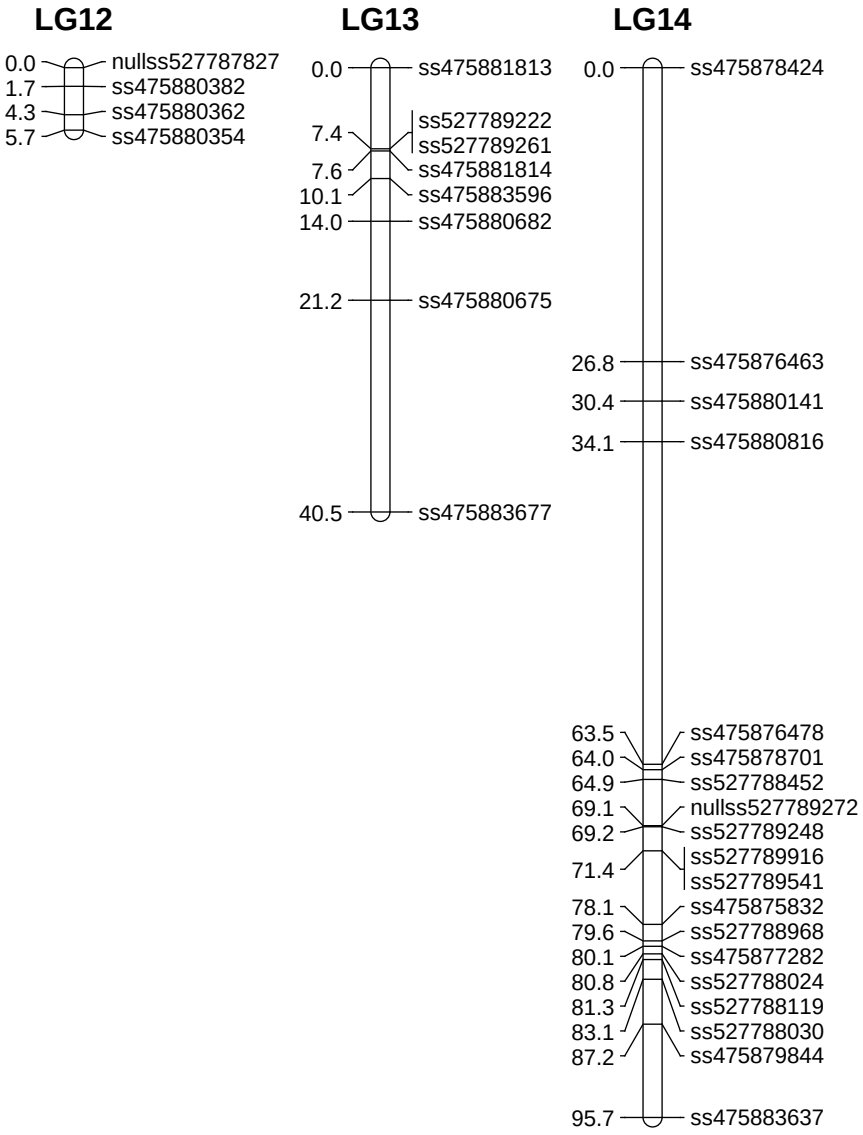
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7.6
10.1
14.0
21.2
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ss527789222
ss527789261
ss475881814
ss475883596
ss475880682
ss475880675
ss475883677
LG14
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26.8
30.4
34.1
63.5
64.0
64.9
69.1
69.2
71.4
78.1
79.6
80.1
80.8
81.3
83.1
87.2
95.7
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ss475876463
ss475880141
ss475880816
ss475876478
ss475878701
ss527788452
nullss527789272
ss527789248
ss527789916
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ss527788968
ss475877282
ss527788024
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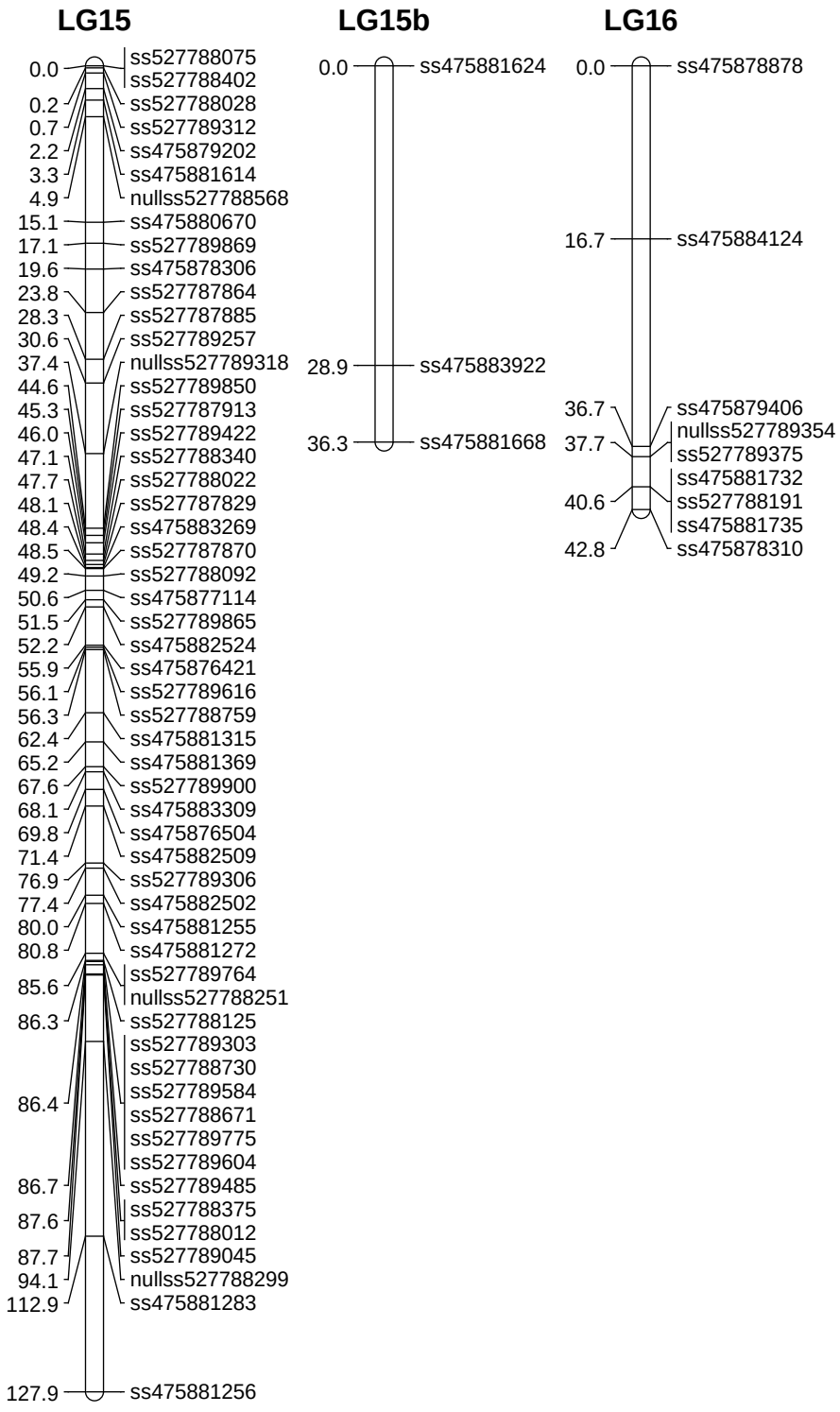
ss475883637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15.1 ss475880670
17.1 ss527789869
19.6 ss475878306
23.8 ss527787864
28.3 ss527787885
30.6 ss527789257
37.4 nullss527789318
44.6 ss527789850
45.3 ss527787913
46.0 ss527789422
47.1 ss527788340
47.7 ss527788022
48.1 ss527787829
48.4 ss475883269
48.5 ss527787870
49.2 ss527788092
50.6 ss475877114
51.5 ss527789865
52.2 ss475882524
55.9 ss475876421
56.1 ss527789616
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62.4 ss475881315
65.2 ss475881369
67.6 ss527789900
68.1 ss475883309
69.8 ss475876504
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77.4 ss475882502
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80.8 ss475881272
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ss527788730
86.4 ss527789584
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ss527789775
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86.7 ss527789485
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87.7 ss527789045
94.1 nullss527788299
112.9 ss475881283
127.9 ss475881256
LG15b
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28.9 ss475883922
36.3 ss475881668
LG16
0.0 ss475878878
16.7 ss475884124
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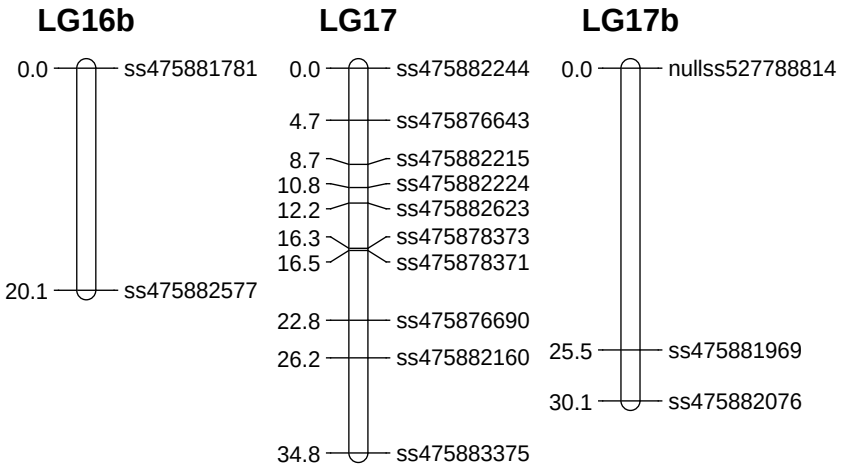
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LG17
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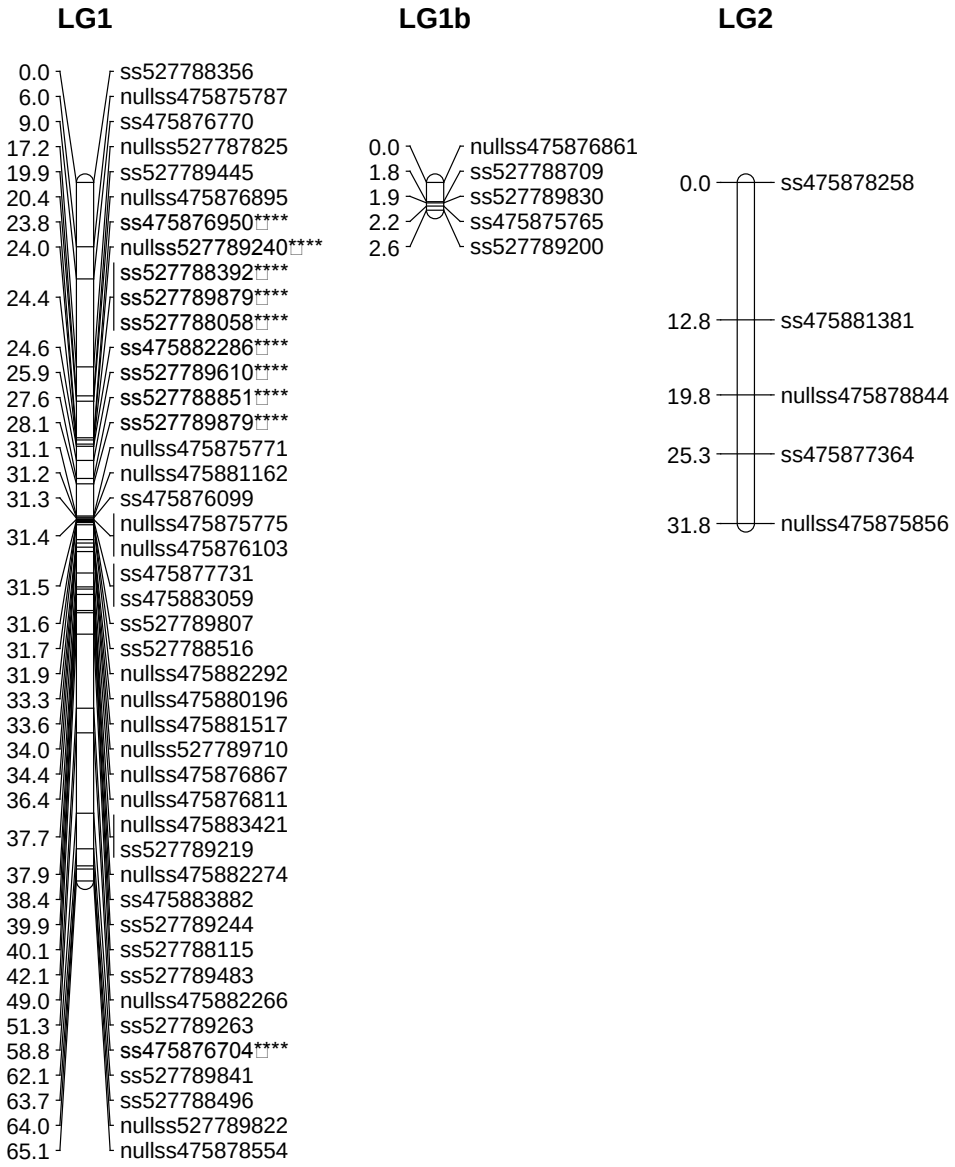
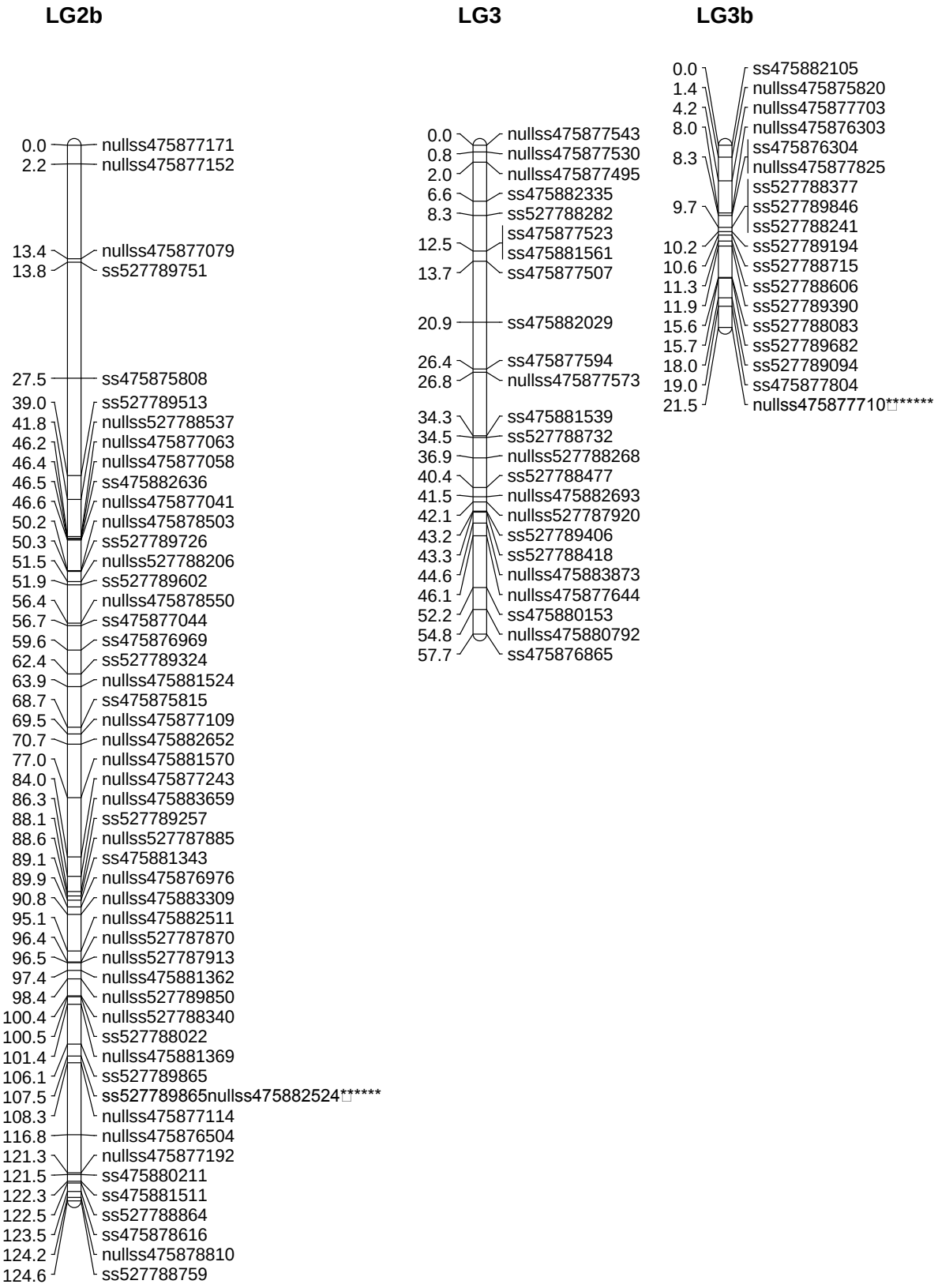
ss475876643
8.7
ss475882215
10.8
ss475882224
12.2
ss475882623
16.3
ss475878373
16.5
ss475878371
22.8
ss475876690
26.2
ss475882160
34.8
ss475883375
LG17b
0.0
nullss527788814
25.5
ss475881969
30.1
ss475882076

Supplementary figure 3.3: Linkage map of male parent of POP356 population. SNPs are presented using the NCBI dbSNP accession number. Apple SNPs are represented with an accession number starting with '4' while pear SNPs accessions start with '5' Asterisks at the end of the marker name denote significantly distorted loci (* *P* < 0.05, ** *P* < 0.01, * *P* < 0.001, *****P* < 0.0001, ***** *P* < 0.00001).**

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13.8 ss527789751
27.5 ss475875808
39.0 ss527789513
41.8 nullss527788537
46.2 nullss475877063
46.4 nullss475877058
46.5 ss475882636
46.6 nullss475877041
50.2 nullss475878503
50.3 ss527789726
51.5 nullss527788206
51.9 ss527789602
56.4 nullss475878550
56.7 ss475877044
59.6 ss475876969
62.4 ss527789324
63.9 nullss475881524
68.7 ss475875815
69.5 nullss475877109
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84.0 nullss475877243
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88.1 ss527789257
88.6 nullss527787885
89.1 ss475881343
89.9 nullss475876976
90.8 nullss475883309
95.1 nullss475882511
96.4 nullss527787870
96.5 nullss527787913
97.4 nullss475881362
98.4 nullss527789850
100.4 nullss527788340
100.5 ss527788022
101.4 nullss475881369
106.1 ss527789865
107.5 ss527789865nullss475882524******
108.3 nullss475877114
116.8 nullss475876504
121.3 nullss475877192
121.5 ss475880211
122.3 ss475881511
122.5 ss527788864
123.5 ss475878616
124.2 nullss475878810
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LG3
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2.0 nullss475877495
6.6 ss475882335
8.3 ss527788282
12.5 ss475877523
ss475881561
13.7 ss475877507
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26.4 ss475877594
26.8 nullss475877573
34.3 ss475881539
34.5 ss527788732
36.9 nullss527788268
40.4 ss527788477
41.5 nullss475882693
42.1 nullss527787920
43.2 ss527789406
43.3 ss527788418
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10.6 ss527788715
11.3 ss527788606
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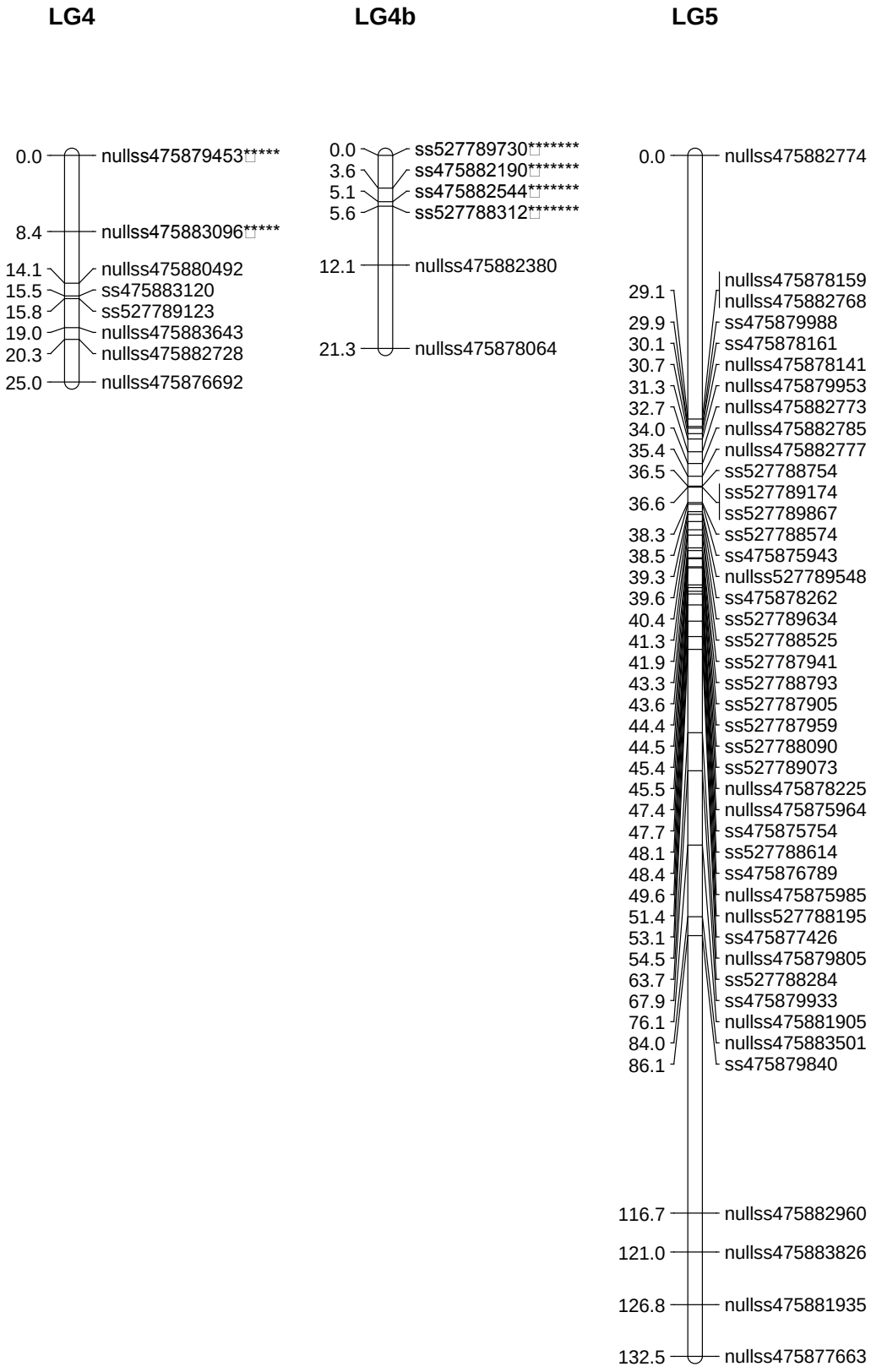
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25.0 nullss475876692
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3.6 ss475882190*******
5.1 ss475882544*******
5.6 ss527788312*******
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21.3 nullss475878064
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44.4 ss527787959
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53.1 ss475877426
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63.7 ss527788284
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76.1 nullss475881905
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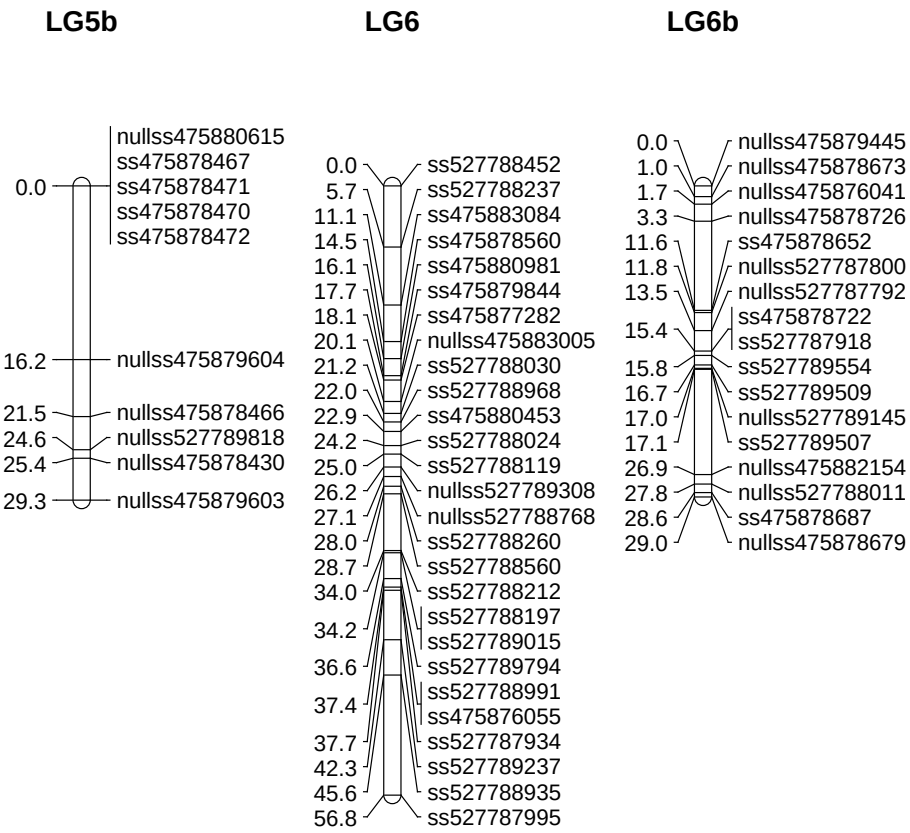
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LG6b
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24.6
25.4
29.3
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17.7
18.1
20.1
21.2
22.0
22.9
24.2
25.0
26.2
27.1
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28.7
34.0
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15.4
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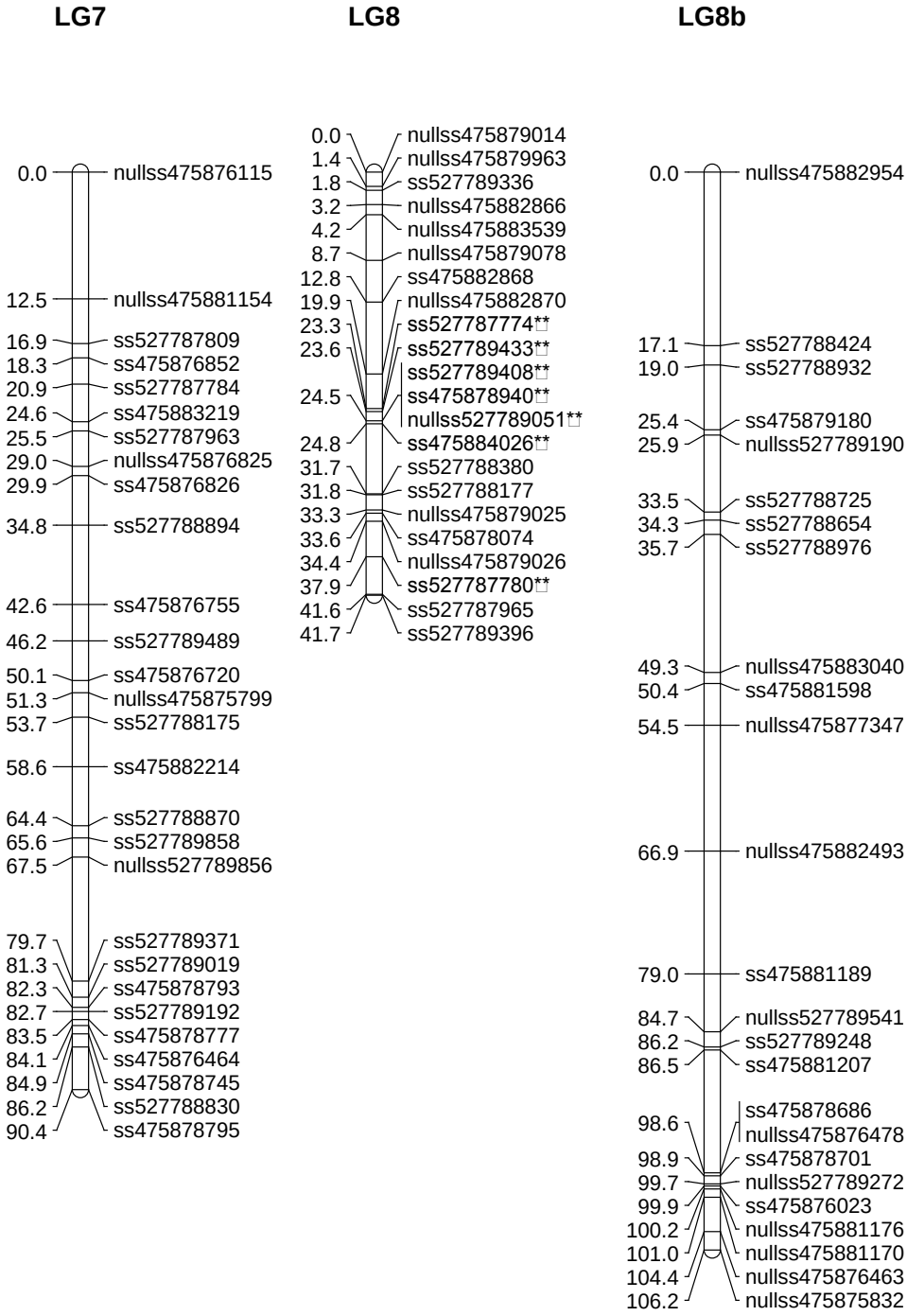
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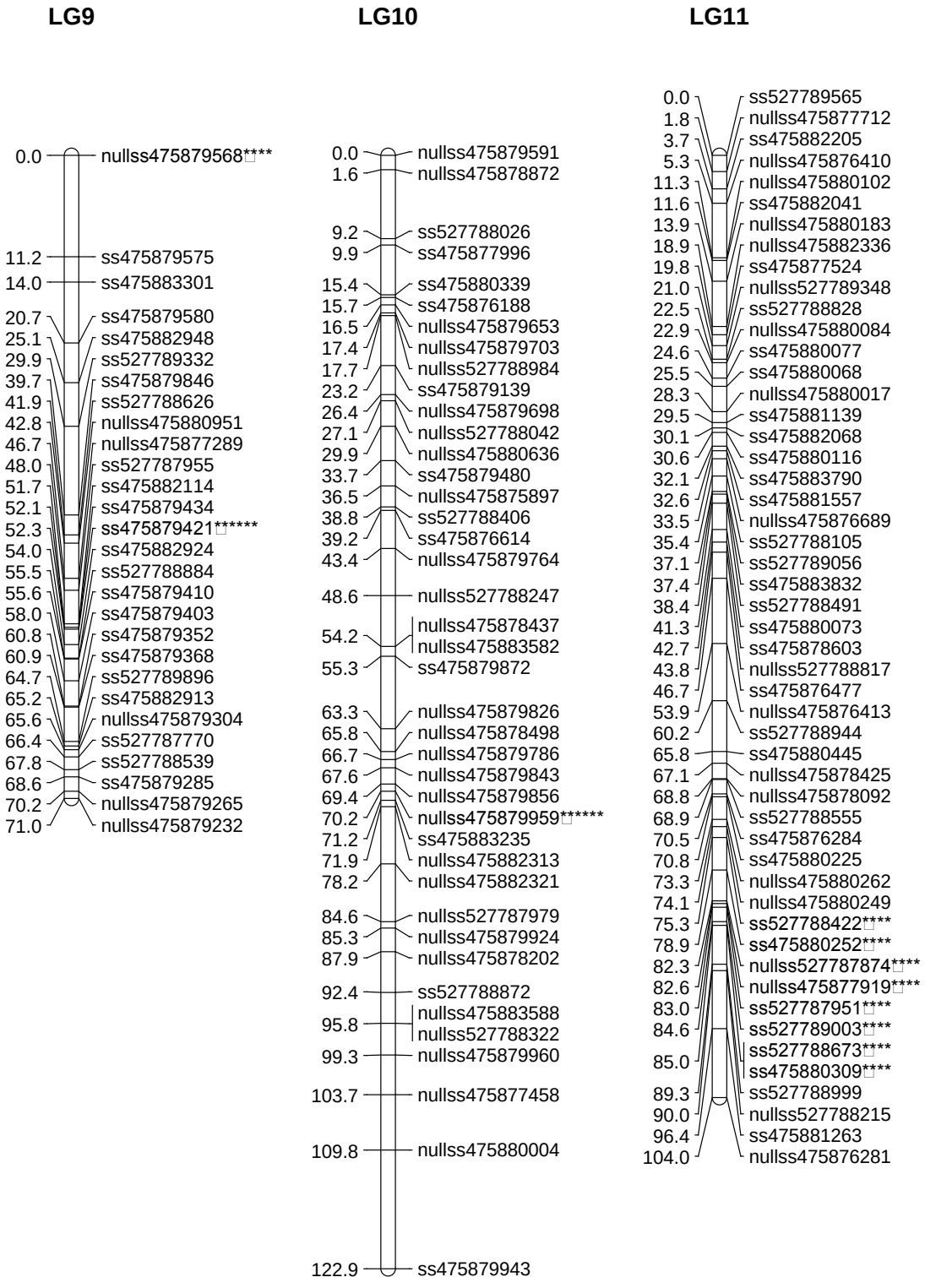
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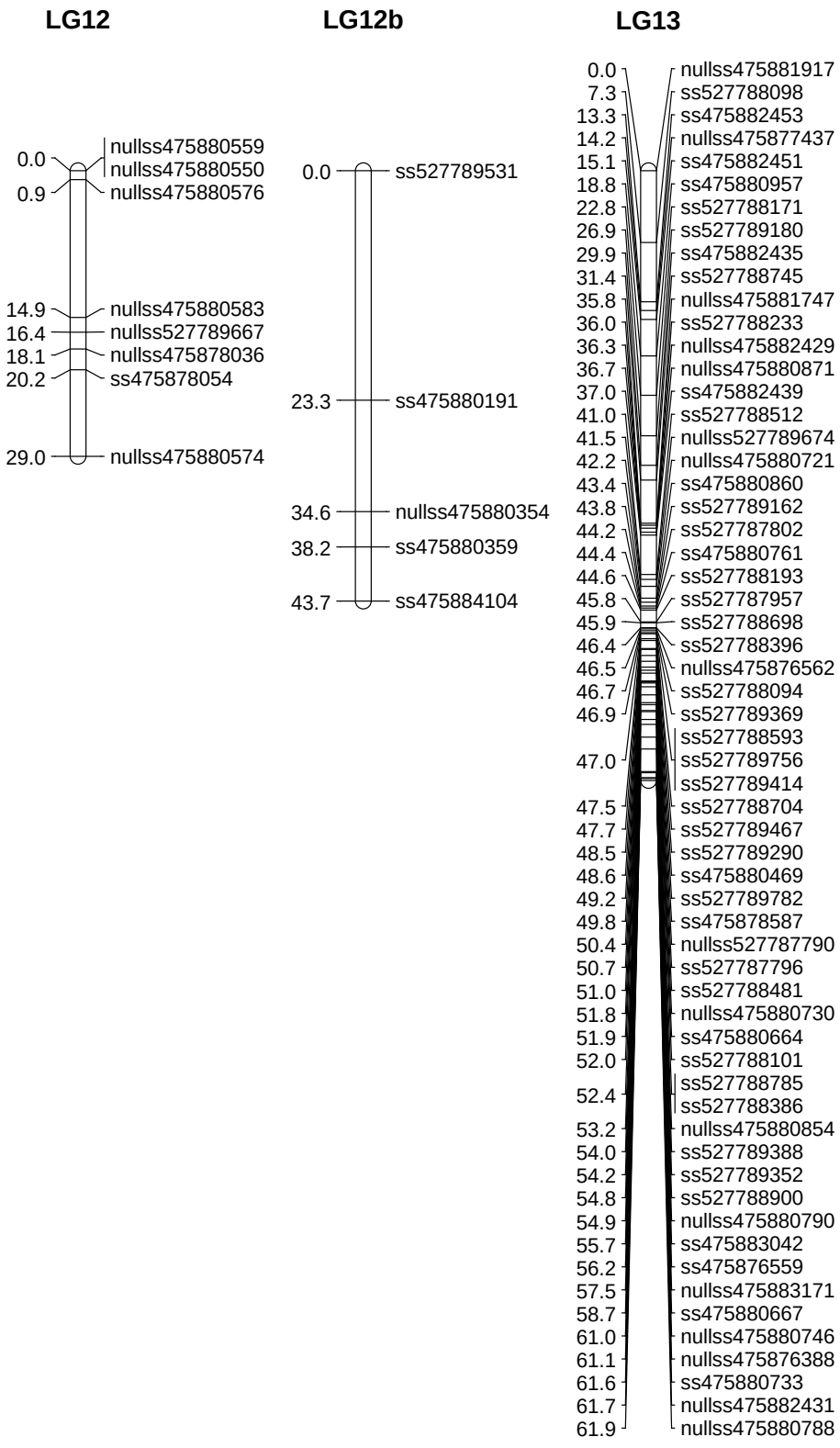
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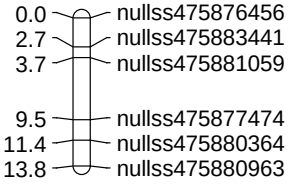

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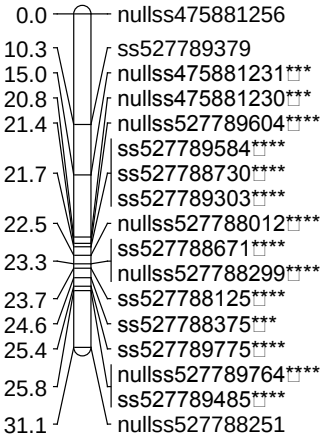
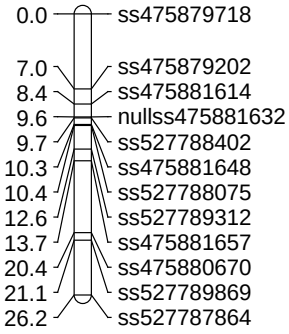
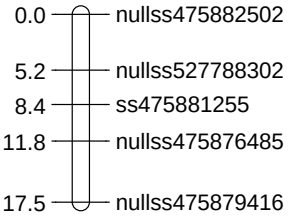


LG15b



LG15c

LG15d



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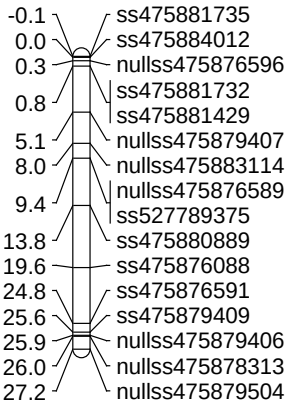

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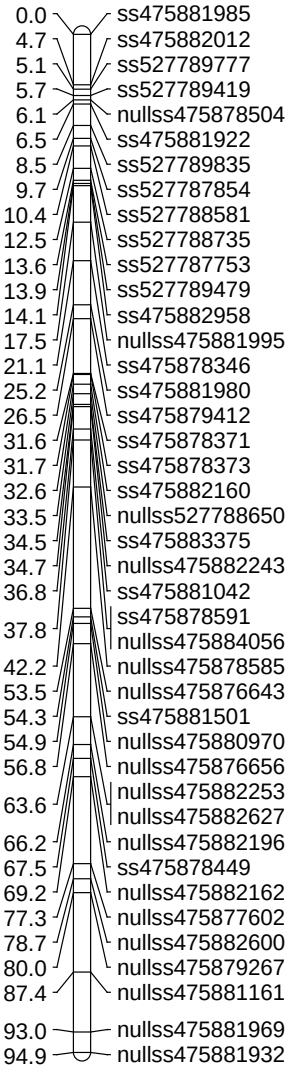
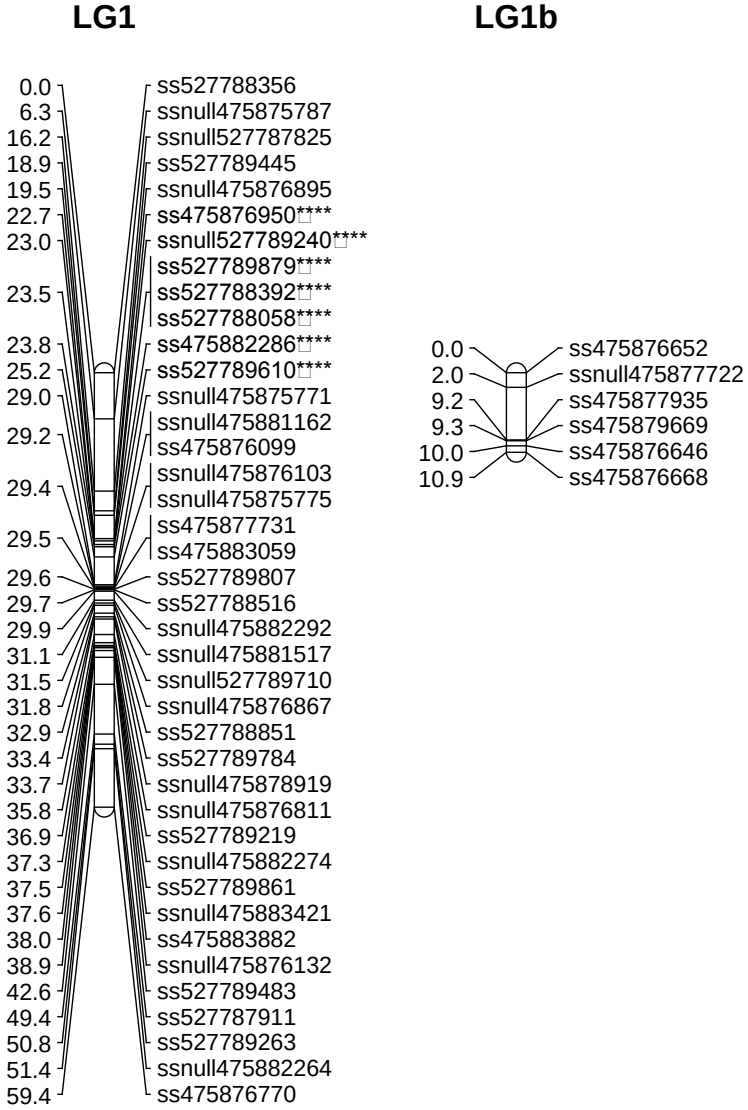
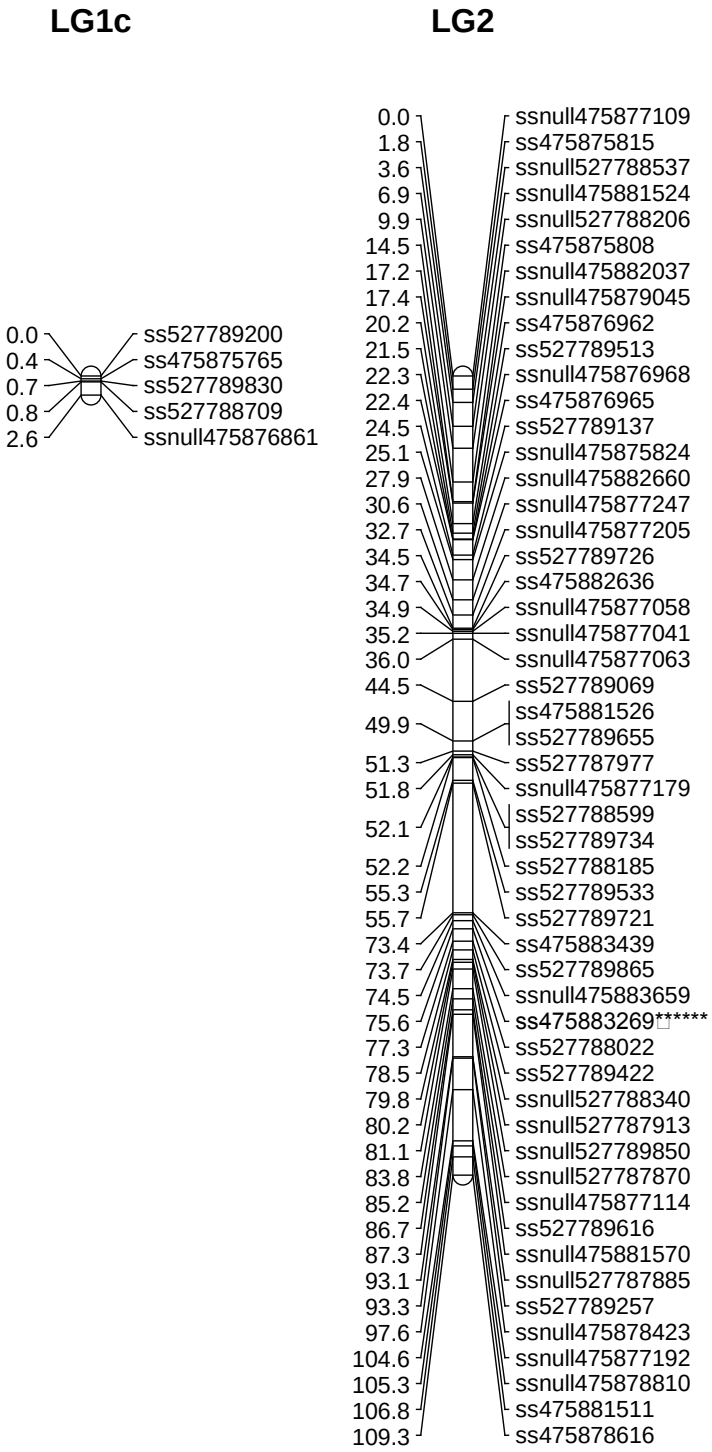


Supplementary figure 3.4: Linkage map of female parent of POP356 population. SNPs are presented using the NCBI dbSNP accession number. Apple SNPs are represented with an accession number starting with '4' while pear SNPs accessions start with '5' Asterisks at the end of the marker name denote significantly distorted loci (* *P* < 0.05, ** *P* < 0.01, * *P* < 0.001, *****P* < 0.0001, ***** *P* < 0.00001).**

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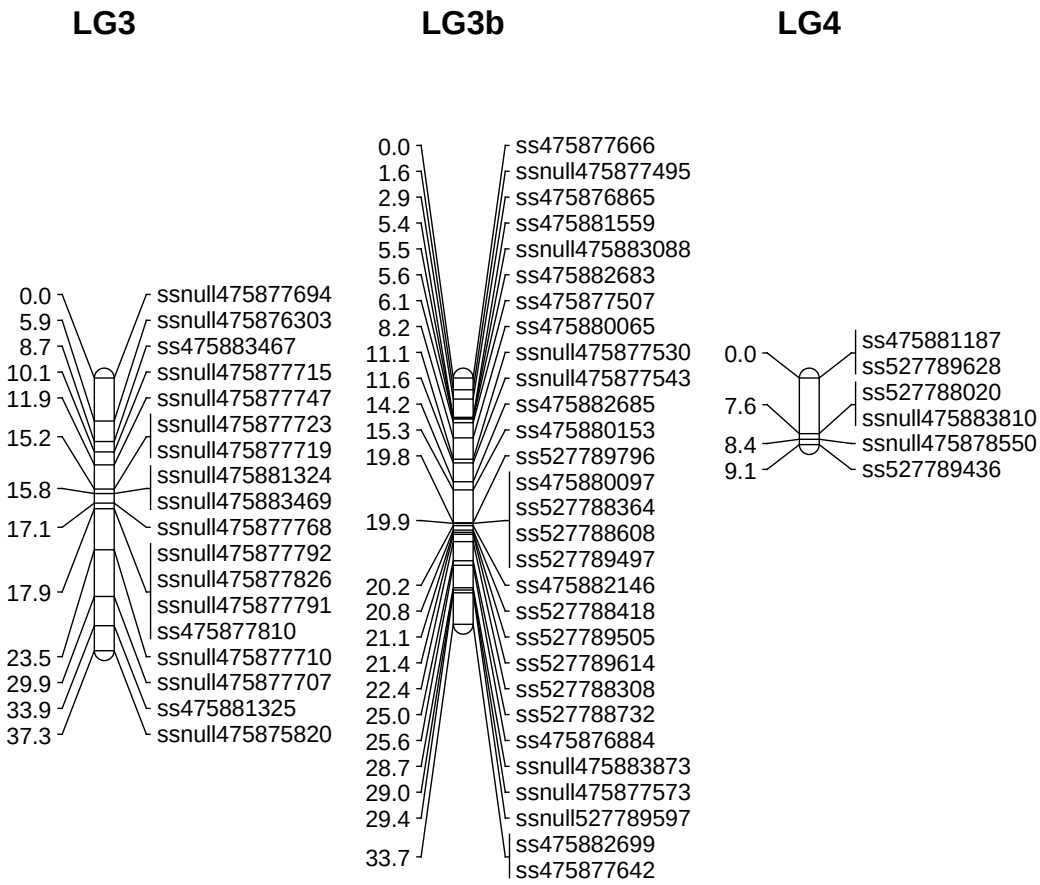
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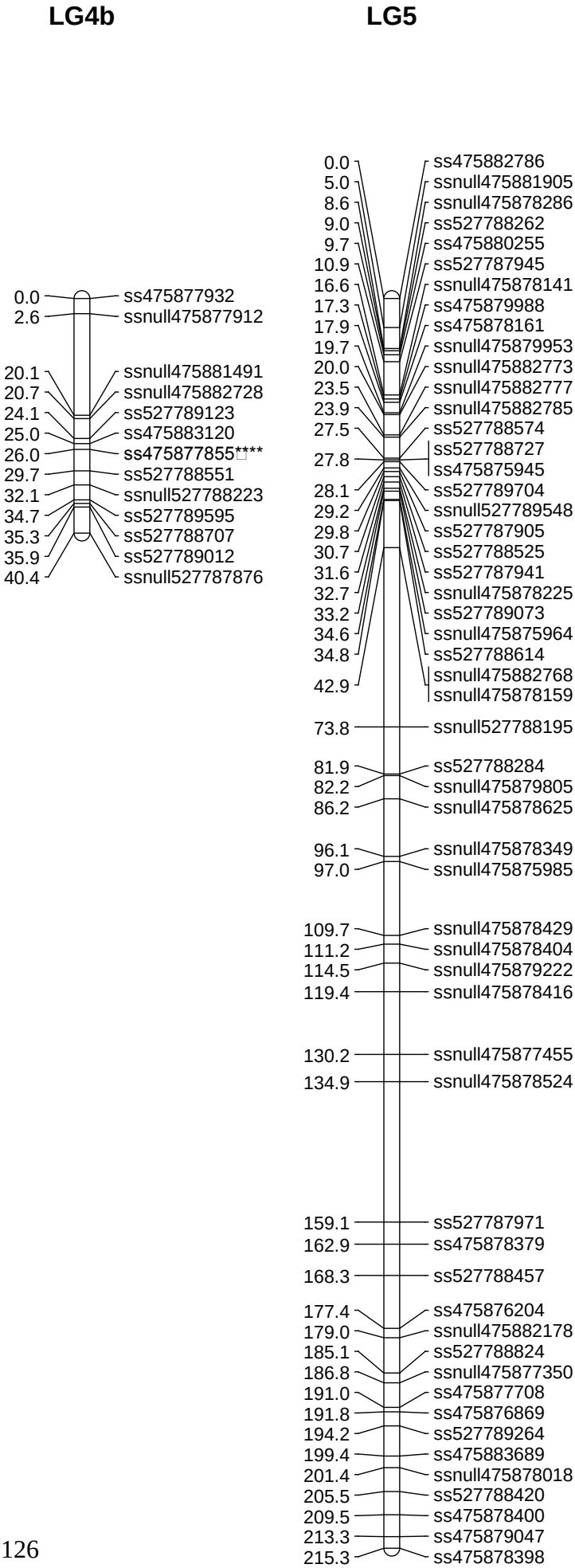
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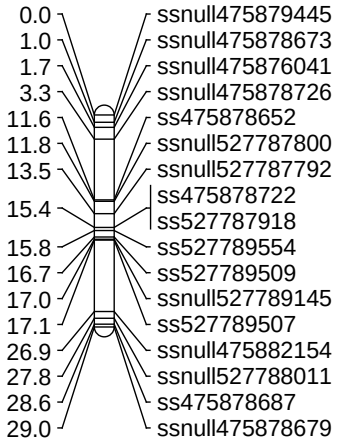
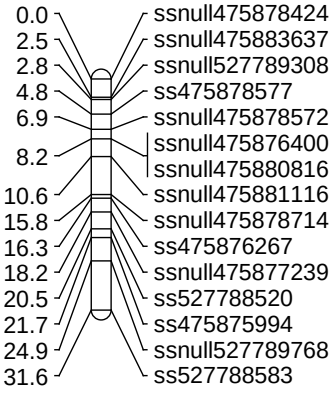
LG6



LG6b



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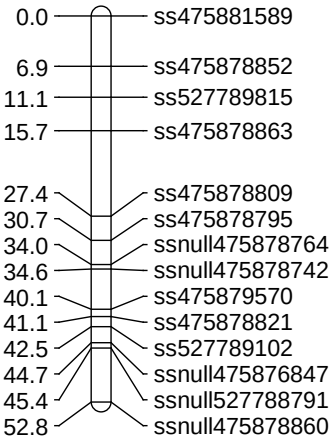

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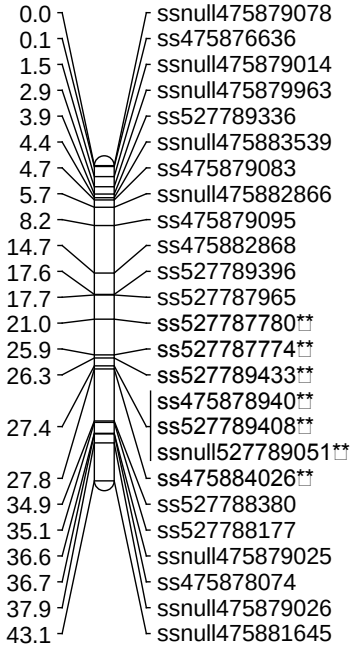

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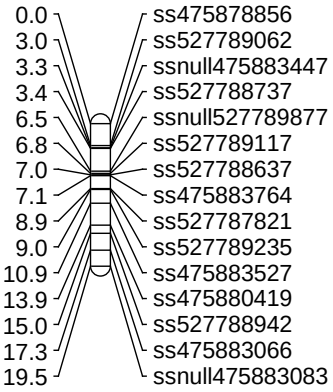

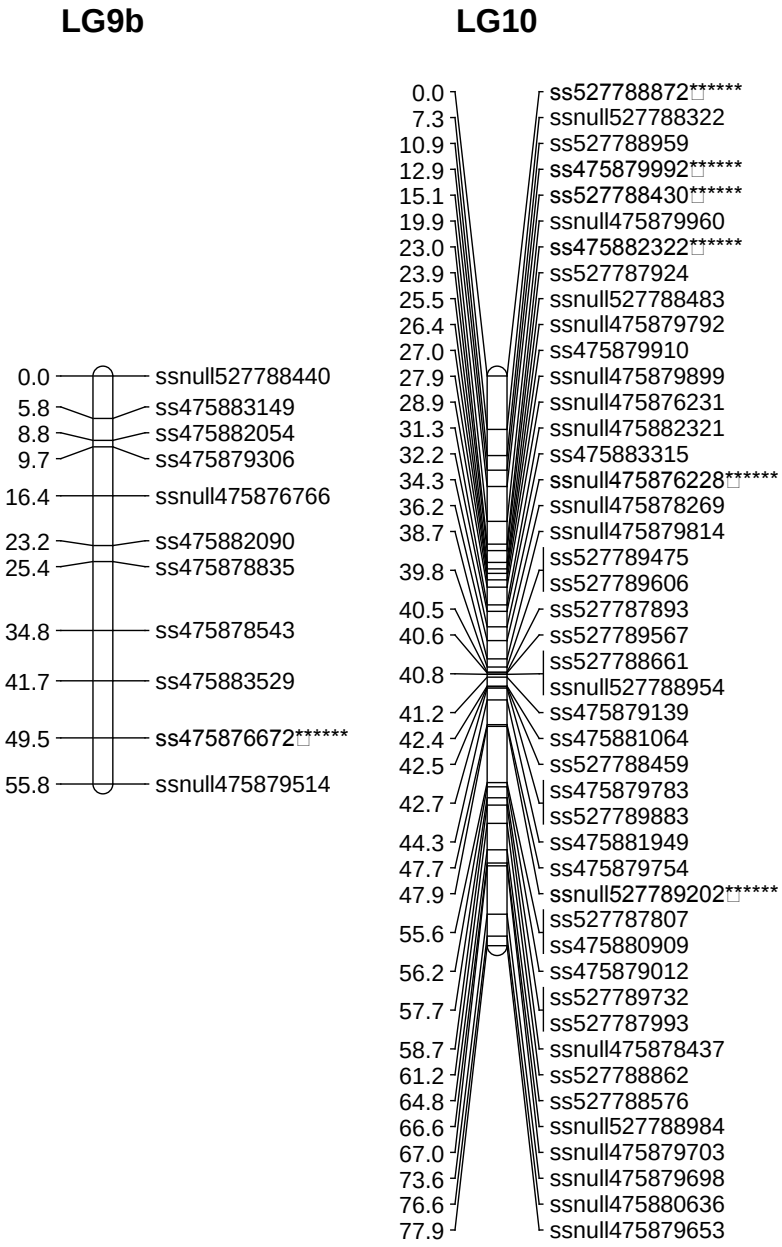
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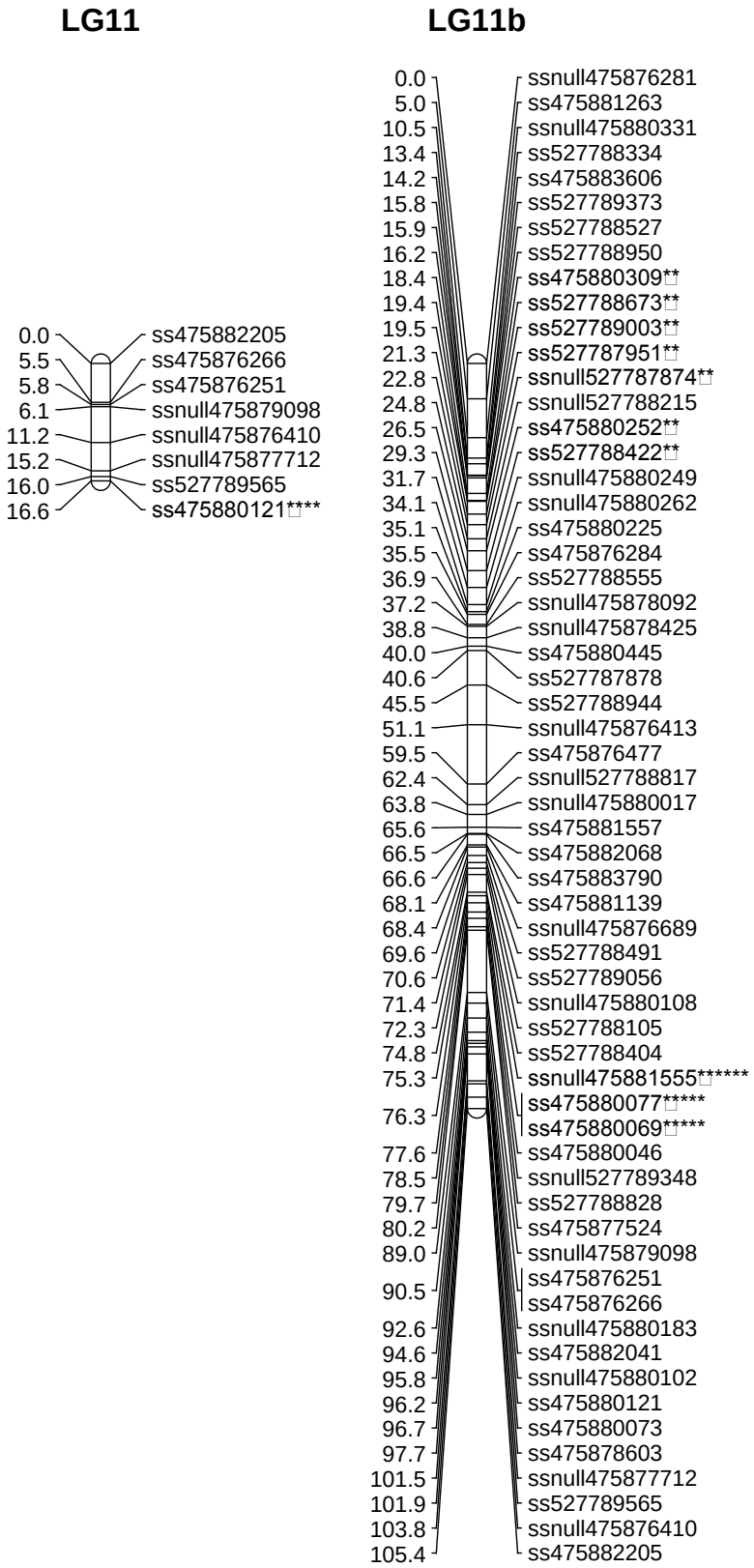
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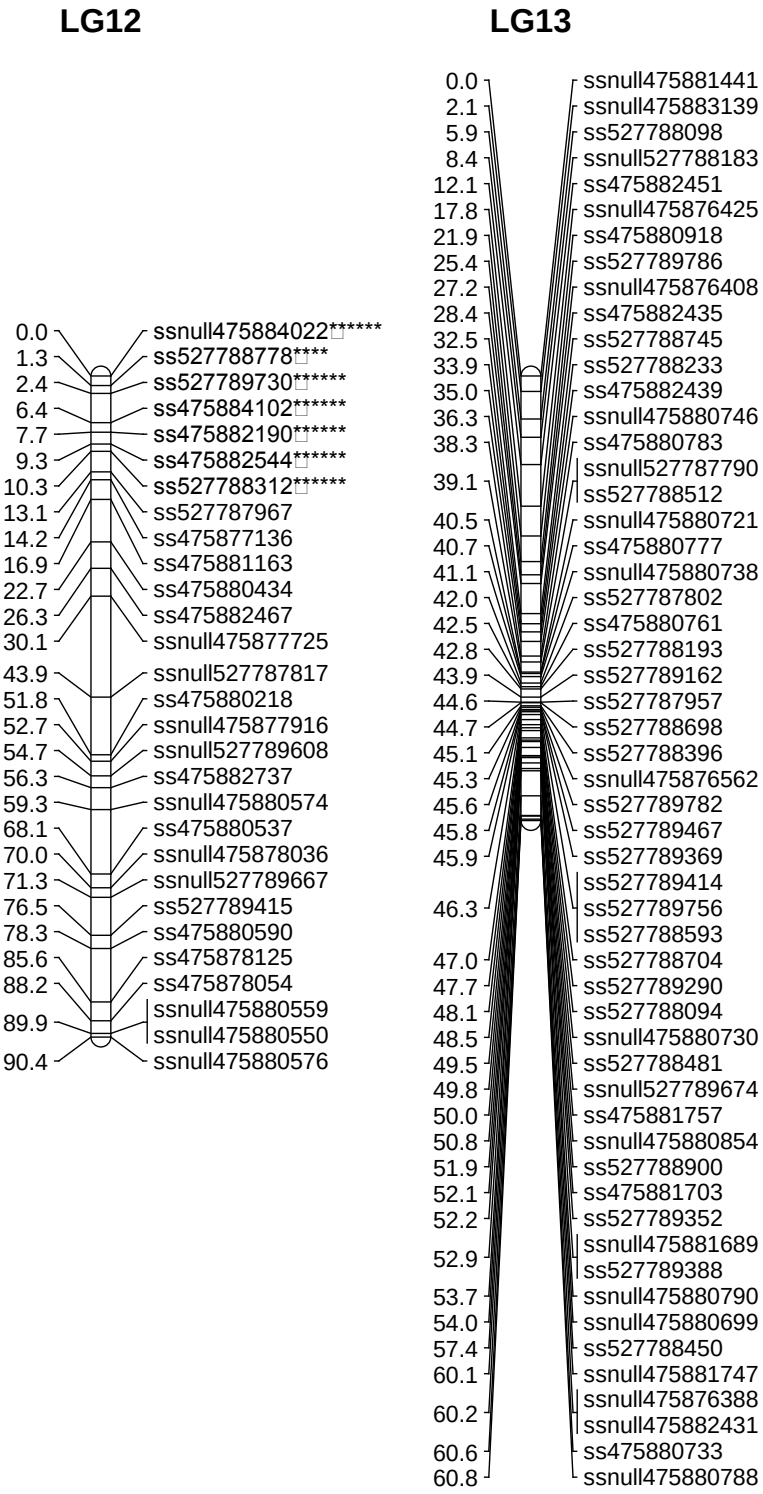
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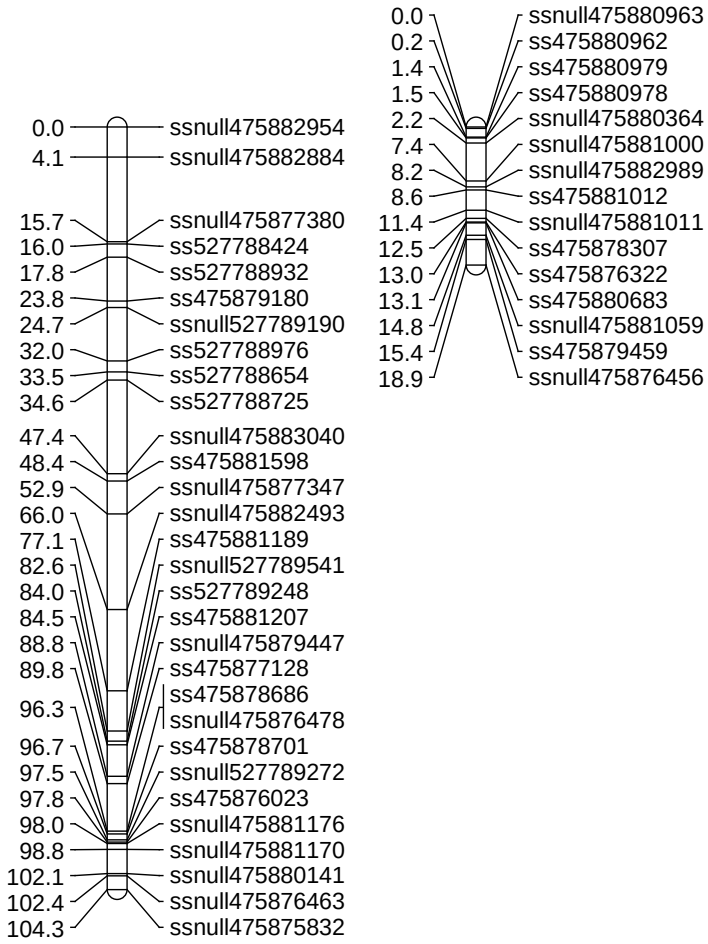
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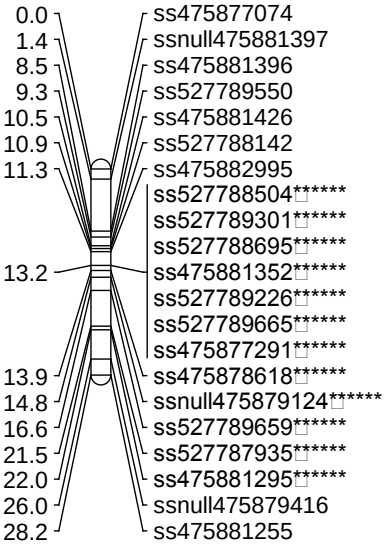
LG15



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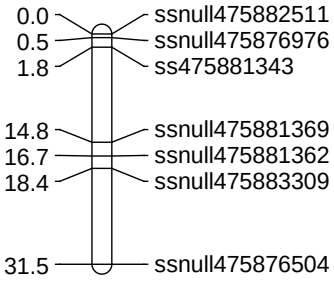

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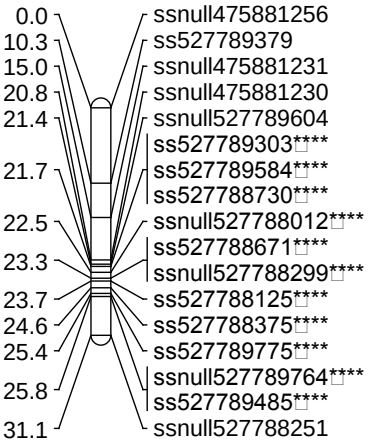

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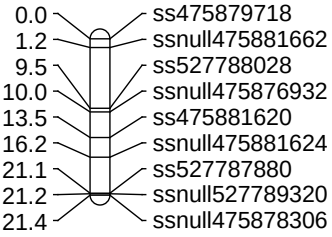
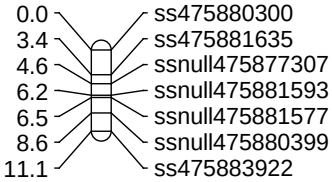
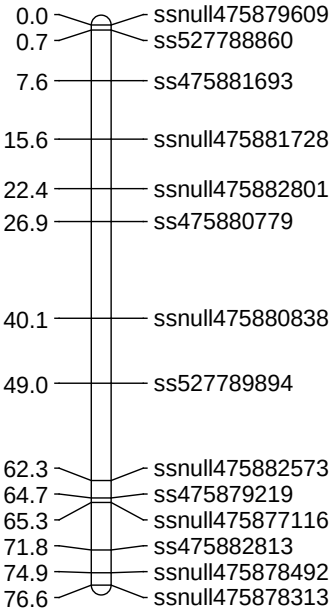


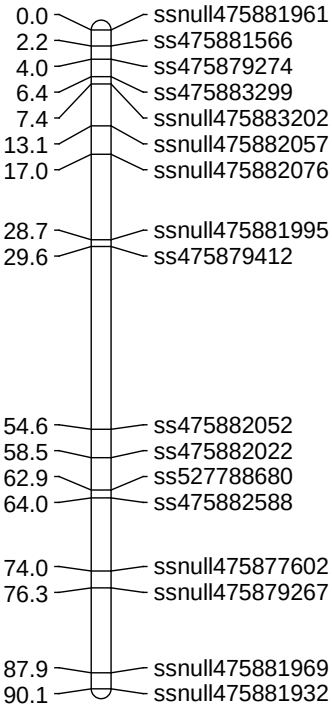
LG15e



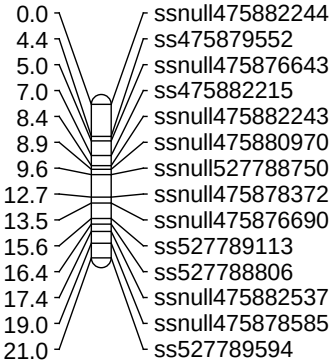
LG16



LG17



LG17b



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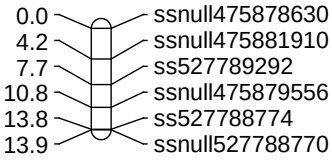

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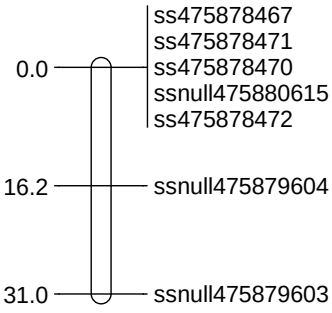

4 QUANTITATIVE TRAIT LOCI ANALYSIS FOR FRICTION DISCOLOURATION AND FRUIT TRAITS IN PEAR*

4.1 Introduction

Quantitative trait loci (QTLs) are the regions of genome (loci) containing the genes controlling or associated with quantitative traits. Availability of phenotypic data and high density genetic linkage maps are prerequisites for QTL mapping. In the past, QTLs for fruit traits, such as fruit shape, sugar content, acid content, vitamin C content, maturity, and fruit skin composition have been mapped in a range of fruit crops including tomato (Chen et al., 1999; Frary et al., 2000; Feng et al., 2004; Chapman et al., 2012), peach (Etienne et al., 2002; Dirlewanger et al., 2006; Martínez-García et al., 2013b), apple (King et al., 2000; Kenis et al., 2008; Dunemann et al., 2009; Chagné et al., 2012b; Costa et al., 2013; Longhi et al., 2013), strawberry (Zorrilla-Fontanesi et al., 2012), sweet cherry (Sooriyapathirana et al., 2010; Zhang et al., 2010), apricot (Salazar et al., 2013; Socquet-Juglard et al., 2013), and papaya (Blas et al., 2012), among others. Recently QTL analysis of pear fruit traits such as pedicel length, fruit weight, transverse diameter, vertical diameter, flesh colour, number of seeds, juice content, calyx status, skin colour, skin smoothness and TSS has been published using Chinese pear (*Pyrus bretschneideri*) cultivars 'Bayuehong' and 'Dangshansuli' (Wu et al., 2014). There are two reports in apple (Di Guardo et al., 2013; Kumar et al., 2013) and one in melon (Fernández-Trujillo et al., 2007) evaluating the QTLs associated with fruit physiological disorders, however, none of them has used a systematic approach to evaluate the genomic regions (QTLs) linked to the disorder as well as internal fruit characteristics (potential risk factors). Also this is the first study focusing on a genetic solution to any postharvest disorder in pear.

* Material from this chapter is included in paper: Saeed, M., Brewer, L., Johnston, J., McGhie, T. K., Gardiner, S. E., Heyes, J. A., and Chagné, D. (2014). Genetic, metabolite and developmental determinism of fruit friction discolouration in pear. BMC plant biology, 14(1), 241. doi: 10.1186/s12870-014-0241-3. I was responsible for all of the experimental work involved and drafting the manuscript

Although there are previous studies on postharvest aspects of FD (Wang and Mellenthin, 1973; Kvåle, 1979; Kvåle, 1988; Burger et al., 2005; Gomila et al., 2011), there has been no attempt to explore systematically the genetic basis and control of this disorder. This study has focussed first on developing an in depth understanding of the variation of phenotypes that might be associated with FD development (FD intensity, firmness, total soluble solids, PPO activity, and concentration of AsA and seventeen polyphenols) among the different genotypes in two mapping populations (Chapter 2). That phenotypic data and SNP based genetic linkage maps (Chapter 3) were used then to identify the QTLs linked to FD and other fruit variables in both related populations in which individuals segregated for susceptibility to FD. QTL stability across the years, parents and populations was also checked. Finally the possibilities to use these QTLs in a pear breeding programme have been discussed in this chapter.

4.2 Materials and methods

Standard methods for assessment of FD, TSS, firmness, quantification of PPO, polyphenols and AsA are detailed in chapter 2 (Section 2.2). DNA extraction and SNP screening methods are described in detail in chapter 3 (Section 3.2).

4.2.1 Genetic map construction and QTL mapping

The genetic maps of the four parents of the two populations were constructed using double pseudo test cross methodology (Grattapaglia and Sederoff, 1994) and JoinMap v3.0 software (Van Ooijen and Voorrips, 2001) based on the SNP data for the individuals in each population. Linkage groups were determined with a LOD score of 5 or higher for grouping and the Kosambi mapping function was used for genetic distance calculation.

The four parental maps were drawn and aligned using MapChart v2.2 (Voorrips, 2002). Linkage group numbering was determined using apple SNPs (Chagné et al., 2012a) anchored to the reference genome of ‘Golden Delicious’. Furthermore POP369 shares a common parent with a population published earlier (Montanari et al., 2013) that has 54 simple sequence repeats (SSR) mapped to enable LG

numbering that is consistent with previously published pear and apple maps (Saeed et al., 2014).

QTL analysis was performed using MapQTL 5.0 (Van Ooijen, 2004). For individual seedlings with more than one fruit harvest, both average and maximum score of the data were tested as phenotypic data, where FD score was expressed for each individual as maximum FD and average FD. The data distribution for each compound was verified before QTL analysis. QTLs were identified using the Kruskal-Wallis Test (KW) because most of the traits were not normally distributed. SNPs are presented using the NCBI dbSNP accession number (ss #) and SNPs with null alleles are represented with the prefix 'null'.

4.3 Results and discussion

4.3.1 Phenotypic data in the pear segregating populations

Phenotypic data in 2011 was collected from 143 genotypes from POP356 and 98 genotypes from POP369. In 2012, phenotypic data was collected from 91 genotypes of POP369 only (chapter 2). In both years phenotypic data were assessed for a total of 22 fruit traits including FD susceptibility score. Details about FD, phenol and AsA concentration and PPO activity are provided in chapter 2 (Section 2.2). Both populations displayed a range of FD scores, from no FD observed for some genotypes, to high FD scores observed in others (Figure 2.2).

Analysis of variance in the POP369 population indicated a significant effect of the year, explaining 4 % of the phenotypic variation in FD ($P<0.001$), whilst the effect of the genotype and harvest date accounted for a higher proportion of the phenotypic variation, at 54 % ($P<0.0001$) and 23 % ($P<0.0001$), respectively. Although interaction between genetics and harvest date was not significant for FD, the effect of the genetics x year interaction accounted for 22 % of the phenotypic variation in FD ($P<0.05$).

4.3.2 Genetic map construction

Parental genetic maps were constructed for POP369 and POP356 populations using a subset of 1144 and 1357 polymorphic SNPs, respectively. The genetic maps for QTL analysis were modified from (Montanari et al., 2013) and chapter 3, by removing dominant (distorted) markers with the segregation ratio 3:1 in order to improve their utility for QTL mapping (Appendix 2). Numbers and segregation types of mapped and revised for QTL map markers are provided in Table 4.1. The revised parental maps of the POP369 population comprised 174 and 265 markers for POP369-male and POP369-female parent, respectively. The male parental map covered a total of 858.2 cM (one SNP every 4.9 cM) and spanned 23 groups of 17 recognized LGs, because two LGs were constructed by JoinMap v3.0 software for chromosomes 2, 9, 11, 12, 13, 14 and 17. The POP369-female parental map constructed in the POP369 population spanned 1027.9 cM (one SNP every 3.3 cM) and 20 groups of 17 LGs, of which LG 10 and 13 were split into two and LG5 into three groups. The female parental map constructed in POP356 consisted of 398 markers covering 885.9 cM and had 28 groups of 17 LGs, of which LGs 3 and 8 consisted of two and LG15 consisted of three groups.

Table 4.1: Number and segregation type of markers in QTL maps of the POP369 and POP356 populations. POP369 map includes null allele markers in the map.

	POP369				POP356			
Marker Type	**Marker Segregation**	**Pear SNPs**	**Apple SNPs**	**Total**	**Marker Segregation**	**Pear SNPs**	**Apple SNPs**	**Total**
Polymorphic	ABxAA/BB	144	69	213	ABxAA/BB	90	95	185
	ABxAB	16	37	53	ABxAB	92	51	143
	BB/AAxAB	8	37	45	BB/AAxAB	97	127	224
	Total	**168**	**143**	**311**	**Total**	**279**	**273**	**552**
Null allele	00xA0/00xB0 /BBxB0	18	96	114				
	A0xA0/ B0xB0	3	31	34				
	A0xB0	1	2	3				
	A0/B0xAB /ABxB0	3	2	5				
	Total	**25**	**131**	**156**				

Common female parent between both populations shared 202 common markers. The POP356-male parental map comprised 353 SNPs covering 1114.6 cM and spanned 23 LGs, of which LGs 3, 5, 14 and 17 were split into two and LG2 was split into three groups.

4.3.3 Scope of QTLs identified for genetic control of fruit traits

QTLs were detected for 22 fruit traits, including FD score, TSS, fruit firmness, PPO activity and concentration of AsA and 17 polyphenolic compounds. A total of 105 QTLs with significance of $P<0.005$ were detected for the 22 traits over two years for the POP369 population (Supplementary table 4.1), and 77 QTLs for the POP356 population (Supplementary table 4.2). The largest cluster, which comprised 22 QTLs associated with fruit firmness, PPO activity, and concentration of AsA and five polyphenolic compounds (catechin, epicatechin, procyanidin B2, isorhamnetin rutinoside and quercetin), was identified on LG3 in POP369. The largest cluster for population POP356 is located on LG5, with 11 QTLs associated with the concentration of polyphenolic compounds (isorhamnetin galactoside/glucoside, quercetin arabinose/rhamnoside and compounds 417.12(1) and 417.12(2).

4.3.3.1 QTL for friction discolouration of fruit

As FD was non-normally distributed in both populations (Appendix 3), the Kruskal-Wallis test was used for QTL analysis. Despite the complexity of the FD disorder and strong influence of environmental and developmental factors, a total of 27 QTLs over 10 chromosomal regions (LG2, 3, 4, 7, 9, 10, 13, 14, 15, 16) were detected for FD, using the average and maximum (max.) score of multiple harvests in 2011 and 2012 for population POP369 (Table 4.2), with the proportion of genotypes explained by each QTL ranging from 3.5 % to 12.8 %. In general, the QTLs were in common for average and maximum FD scores (Table 4.2) however none of them could be regarded as a major QTL. As phenotypic data were collected for the POP369 population in two consecutive seasons (2011 and 2012) hence QTLs could be verified for their stability across years in this population.

The QTL detected on LG14 derived from the POP369-female parent was stable between years when either the maximum or average FD score data classes were used, with the homozygous AA genotype for marker ss527788030 linked to low FD score (Figure 4.1A). The QTL on LG7 of the POP369-male parent was not stable between years, as it only exhibited a strong effect in 2012; however a weaker effect QTL in 2011 was identified in another location of the same LG for the same parent (Figure 4.1B). The homozygous AA genotype for marker nullss475876200 from LG7 was linked to low FD score in 2012 (Figure 4.1B). The marker information from both stable QTLs on LG7 and LG14 from POP369 was combined into four possible genotypic combinations (Table 4.3) and compared with phenotype data from those multi-harvest date seedlings categorized into the four FD groups shown in Figure 2.3- 2.5 (i.e. consistently high and low FD score, increasing and decreasing FD score with advancing harvest). In 2012, seedlings lacking both LG7 and LG14 QTLs (AB genotype for both SNP markers) exhibited a consistently high FD score (10), increasing (22) or decreasing (4) with advance harvest date, with none that showed a consistently low FD. However, the seedlings with genotypes associated with low FD for both QTLs (AA genotype for both markers) had consistently low (6), decreasing (6) and increasing (9) FD scores during the season and there were no seedlings with consistently high FD. The trend was not as clear in 2011; however four seedlings having consistently high FD score also lacked the low FD QTL genotypes for both LG7 and LG14. Although the suggested FD QTL on LG7 is below the threshold of detection (Figure 4.1B) in 2011 a likely reason for lower significance could be the large environmental and developmental effect on FD incidence.

Table 4.2 Quantitative Trait Loci (QTL) detected for FD in the POP369 population. QTLs were identified using average and maximum (max) FD score from multiple harvests of the same seedling. SNPs are presented using the NCBI dbSNP accession number (ss#). Apple SNPs are represented with an accession number starting with '4' while pear SNPs accessions start with '5'. Similar markers are shaded with light grey colour.

Year	Data type	Parent	LG	Position	SNP marker	(K*)	Significance	Variance
2011	average	female	2	59.44	nullss475883075	8.9	P<0.005	11.37%
2011	average	female	15	6	ss527788075	6.3	P<0.05	10.26%
2011	average	female	14	4.93	ss527788030	6.1	P<0.05	6.22%
2011	average	female	3	48.09	ss527788418	5.8	P<0.05	8.30%
2011	max	female	2	59.44	nullss475883075	10.1	P<0.005	12.09%
2011	max	female	16	42.79	nullss475878310	6.7	P<0.01	7.30%
2011	max	female	14	4.93	ss527788030	5.1	P<0.05	5.50%
2011	max	female	3	48.09	ss527788418	5	P<0.05	6.86%
2012	average	female	3	26.02	ss527788282	6.2	P<0.05	8.74%
2012	average	female	14	8.8	ss527788968	3.7	P<0.1	8.16%
2012	max	female	3	26.02	ss527788282	9	P<0.005	12.85%
2012	max	female	14	4.93	ss527788030	3.8	P<0.1	5.72%
2012	max	female	10	36	nullss475879653	3.5	P<0.1	3.48%
2011	average	male	2	12.09	nullss475877109	8.2	P<0.005	10.05%
2011	average	male	14	3.4	ss527789200	6.9	P<0.01	8.92%
2011	average	male	13	2.75	ss475882576	5.1	P<0.05	6.07%
2011	max	male	2	12.09	nullss475877109	9.1	P<0.005	10.70%
2011	max	male	14	3.4	ss527789200	7.1	P<0.01	9.15%
2011	max	male	16	16.29	nullss475878313	5.7	P<0.05	6.29%
2011	max	male	13	2.75	ss475882576	5.3	P<0.05	6.74%
2011	max	male	7	16.79	ss475878863	5.2	P<0.1	9.07%
2012	average	male	7	42.04	nullss475876200	7.9	P<0.005	8.34%
2012	average	male	4	25.6	ss475876768	7.3	P<0.01	8.68%
2012	max	male	7	42.04	nullss475876200	7	P<0.01	8.67%
2012	max	male	2	20.08	ss475877562	6.9	P<0.05	8.34%
2012	max	male	4	25.59	ss475876768	6.7	P<0.01	7.16%
2012	max	male	9	9	ss527787770	4.3	P<0.05	5.02%

K* represents Kruskal-Wallis test value.

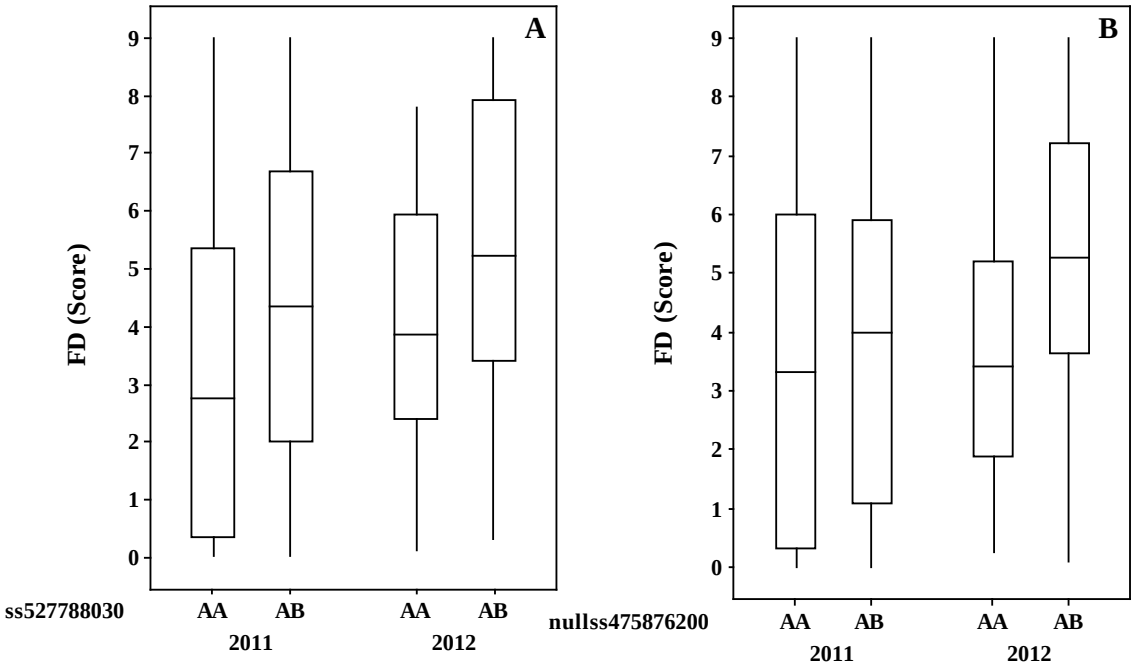

Figure 4.1: Graphical representation of QTL controlling FD (average) across the years. A) represents QTL for POP369-female parent on LG 14 and B) represents QTL on LG 7 from POP369-male parent.

Table 4.3: Genotypic effect of the FD QTLs detected in the POP369 population in 2011 and 2012. Seedlings are grouped according to their seasonal trend for FD susceptibility as illustrated in Figure 2.3-2.5. The markers with the most significant Kruskal-Wallis value were used (Table 4.2): ss527788030 and nullss475876200 for LG14 and LG7, respectively. Alleles favourable for a low FD score are marked with a "+".

	LG14	**AA(+)**	**AA(+)**	**AB**	**AB**
	LG7	**AA(+)**	**AB**	**AA(+)**	**AB**
2012	**Consistent High**	0	23	6	10
	Consistent Low	6	16	10	0
	Increasing	9	16	9	22
	Decreasing	6	0	2	4
2011	**Consistent High**	0	0	0	4
	Consistent Low	2	11	8	8
	Increasing	2	4	0	2
	Decreasing	0	2	0	2

Another FD QTL for female parent of POP369 is located on LG3, however the allelic trend is inconsistent between years (Figure 4.2), which implies that this QTL is an artefact.

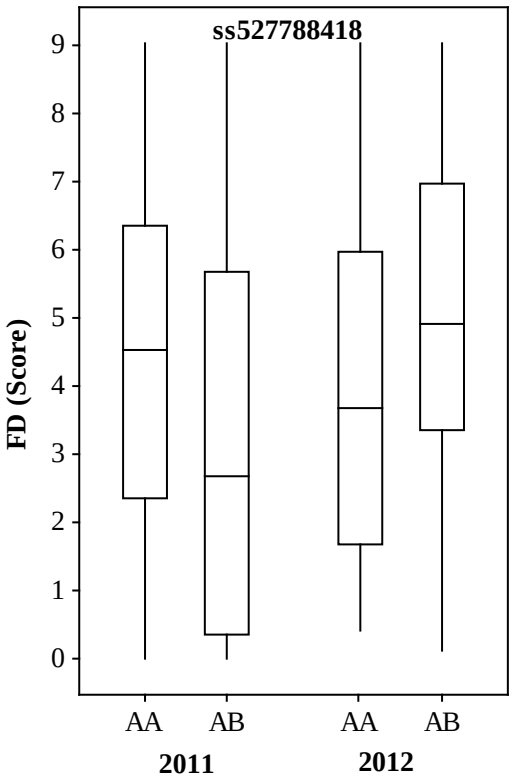

Figure 4.2: Graphical representation of QTL associated with FD on LG3 of female parent of POP369.

In total 12 QTLs over five chromosomal regions (LG2, 5, 11, 15, 16) were detected in the POP356 population using the average and maximum FD score, with genotype variation explained ranging from 4.4 % to 10.5 % (Table 4.4). Population POP356 had only one year (2011) of phenotypic data so it is not possible to verify QTL stability across years. The QTLs on LG 11 and 15 were common to both parental maps in the POP356 population. Common QTLs between populations are located on LG2 in the POP356-male parent and for both male and female parents of POP369, however for POP369-female parent this QTL was observed in 2011 only (Table 4.2; Table 4.4).

Table 4.4: Quantitative Trait Loci (QTL) detected for FD in the POP356 population. QTLs were identified using average and maximum (max) FD score from multiple harvests of the same seedling. SNPs are presented using the NCBI dbSNP accession number. Apple SNPs are represented with an accession number starting with '4' while pear SNPs accessions start with '5'. Similar markers are shaded with light grey colour.

Year	Data type	Parent	LG	Position	SNP	K*	Significance	Variance
2011	average	female	11	23.61	527788944	12.6	P<0.005	9.70%
2011	average	female	15	3.44	527789584	8.3	P<0.05	8.89%
2011	average	female	5	0	475879840	6.8	P<0.01	4.37%
2011	max	female	11	2.96	475880309	13.3	P<0.005	10.46%
2011	max	female	15	3.44	527789584	8.6	P<0.05	8.33%
2011	average	male	11	20.60	527788944	12.6	P<0.005	9.70%
2011	average	male	2	3.17	527788737	8.5	P<0.005	6.00%
2011	average	male	15	85.81	527789303	8.3	P<0.05	8.89%
2011	average	male	16	104.88	527789436	7.4	P<0.01	6.94%
2011	max	male	11	20.60	527788944	13.4	P<0.005	10.17%
2011	max	male	2	3.17	527788737	8.8	P<0.005	7.52%
2011	max	male	15	85.81	527789303	8.6	P<0.05	8.33%

K* represents Kruskal-Wallis test value.

QTL analysis in the current study indicated that FD is a polygenic trait controlled by many small effect QTLs, of which only a subset are stable across years. The QTLs on LG7 and LG14 provide closely linked markers which are candidates that might be theoretically used for MAS. However, these QTLs individually explain only 8 % of the phenotypic variation, which would provide only limited genetic gain if they were used for selection in a breeding population. However when the QTLs are considered in combination, none of the seedlings with the marker genotype associated with low FD exhibited a consistently high score for FD in 2012 and 2011. No seedlings with the other homozygous AB type allelic pair appeared to have a consistently low FD score in 2012, but in 2011, 8 seedlings of this group exhibited a consistently low

score (Table 4.3). These results point towards the possibility of using these QTLs in combination for MAS in bi-parental populations.

The polygenic control of FD by small effect QTLs suggests that genomic selection may be a more suitable approach to cull susceptible seedlings early in the breeding cycle. Genomic selection makes use of genome wide markers to predict total genetic value instead of phenotype and has been evaluated recently in apple (Kumar et al., 2012b). In genomic selection, a prediction equation is established from genotype and phenotype data collected from the 'training population' and this prediction equation is used later to estimate genomic estimated breeding values (GEBVs) of individual progeny in the 'selection population' (Meuwissen et al., 2001).

4.3.3.2 QTLs for fruit firmness, total soluble solids, PPO activity and ascorbic acid concentration

A QTL linked to fruit firmness identified at the top of LG3 for both parents of both populations is stable between 2011 and 2012 for the POP369-male parent of the POP369. Although TSS exhibited no stable QTL between years, TSS QTLs on LG2 and 16 were detected for both female and male parents of POP369 in 2012. A QTL associated with PPO activity identified on LG3 of parent POP369-male was stable across the years and was detected only in 2012 in the POPO369-female parent. The POP356 population had a QTL for PPO activity on LG2 for both parents, however no QTL was detected on LG3 as for POP369 (Supplementary table 4.1). Other QTLs associated with PPO activity that were unstable between years were located on LG5, 9 and 14 for POP369 and LG6 and 17 for POP356. QTLs influencing fruit AsA concentration were identified on LG3 of all four parental maps in 2011 only (Supplementary table 4.1- 4.2).

4.3.3.3 QTLs for phenolic compounds

A total of 86 and 64 QTLs were detected that were associated with the concentration of seventeen phenolic compounds in pear fruit for POP369 and POP356, respectively. QTLs detected for polyphenols were identified on all LGs, except LG4,

6 and 10 for population POP369, and LG4, 13 and 16 for population POP356 (Supplementary table 4.1- 4.2). The largest clusters of QTLs associated with polyphenol concentration are located on LG3 of POP369 and LG5 of POP356.

4.3.4 QTL stability between years and parents

Supplementary table 4.1 shows that major stable QTLs exhibited across the years for the POP369-male parent were for control of fruit firmness and PPO activity on LG3, as well as concentration of chlorogenic acid on LG9, cryptochlorogenic acid on LG1, catechin on LG3 and LG9, epicatechin on LG3, quercetin arabinose on LG5 and unknown compounds 417.12(1) and 417.12(2) on LG5. QTLs that were stable across 2011 and 2012 that were identified in the POP369-female parent of population POP369 were associated with concentration of chlorogenic acid on LG1 and LG13, cryptochlorogenic acid on LG1 and LG13, catechin on LG17, epicatechin on LG3 and LG14 and procyanidin B2 on LG14.

Clusters of QTLs that were identified on LG3 and associated with fruit firmness and epicatechin concentration were stable between 2011 and 2012 and between parents of each of the two populations, as well as across these populations. In addition, for population POP369, several other QTLs were conserved between parents, however were identified in one year only. Examples for 2012 include: QTLs on LG2 and LG16 for control of TSS, chlorogenic acid concentration on LG9, catechin on LG3 and procyanidin B2 on LG3. QTLs associated with iso-rhamnetin galactoside/glucoside concentration were observed on LG2 in 2011 only, as well as quercetin on LG3 and LG15. Population POP356 also exhibited QTLs conserved between the parents: control of fruit firmness on LG3, PPO activity on LG2, concentration of AsA on LG3, concentration of cryptochlorogenic acid on LG9, catechin and epicatechin on LG3, procyanidin on LG15, iso-rhamnetin galactoside/glucoside on LG5 and LG6, iso-rhamnetin rutinoside, quercetin galactoside and quercetin arabinose on LG5, quercetin rhamnoside on LG3, quercetin rutinoside on LG2 and LG7, quercetin on LG12 and unknown compounds 417.12(1) and (2) on LG5.

QTLs like other genes are also sensitive to environmental effect and it is likely to get environmental + genetic effect while doing QTL analysis which is most probable reason for the detection of non-reproducible QTLs across the years or environments. The data for fruit variable from genotypes with more than one harvest were averaged to conduct the QTL analysis but for some genotypes only a single harvest was obtained. A separate analysis was done using only the maximum FD score for each genotype, but the associations were not improved. This variation in data selection could be one of the reasons for the lower genetic variability of detected QTLs. Experimental error during phenotyping could also affect the QTL expression across the years or environments. In the current study, phenotypic data collected in both years (2011 and 2012) for POP369 is not strictly comparable however stable QTLs emphasis the fact that these QTLs are reproducible across the environments and could serve as valuable genetic resources for the pear breeders.

From epidemiological studies, there is evidence that consumption of dietary antioxidants through eating polyphenol-rich fruits and vegetables can enhance cellular defence and help to guard against diseases such as cancer, coronary heart disease and osteoporosis. Chlorogenic acid has strong antioxidant properties and is the most abundant type of polyphenol in pear. Breeders could use this QTL associated with chlorogenic acid to select genotypes rich in this compound. Furthermore, candidate genes controlling fruit firmness in pear might be identified by utilizing the stable QTL on LG3, to identify candidate genes in the aligned genome sequences of both Chinese and European pear, as well as apple.

4.3.5 QTL co-location between traits

Co-location of QTLs associated with different traits may mean that the QTLs for both traits are tightly linked, or even that the same gene controls both. In the second situation this helps provide a clue to as to the nature of the molecular control underlying both traits. In total, 10 genomic regions exhibited QTLs for different fruit traits that co-located (Supplementary table 4.1- 4.2). A QTL located on LG14 of POP369-female was for FD, PPO activity and chlorogenic acid concentration in 2011 (Figure 4.3). Although no highly significant statistical correlation among the phenotypes was observed as a whole, however a weak opposite trend between

chlorogenic acid and FD was observed through all years as described in detail in chapter 2. Considering only the QTL cluster on LG14 of POP369-female parent, it was observed that individuals in POP369 carrying the low FD allele exhibited both low PPO activity and a high concentration of chlorogenic acid (Figure 4.3). An opposing trend between chlorogenic acid and FD may indicate that although the substrate amount is not crucial in terms of browning in interspecific pears, the PPO activity may play an important role. In this scenario, it can be hypothesised that a candidate gene influencing PPO activity located in this genomic region of female parent of POP369 and POP356, but not male parents of both populations, might contribute to FD susceptibility via a stimulation of enzymatic browning in pear.

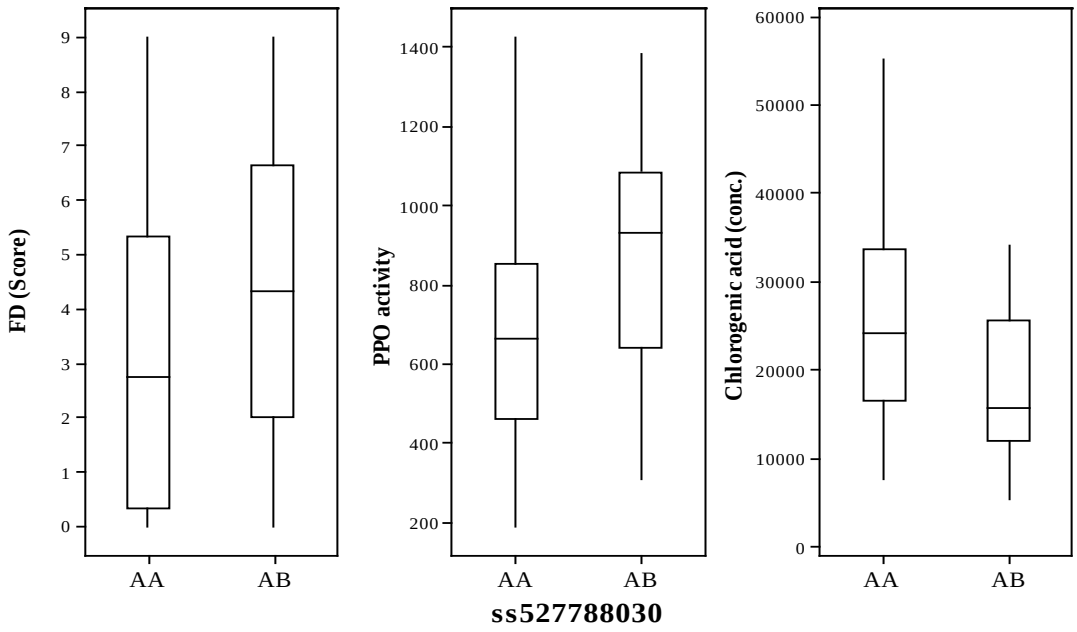

Figure 4.3: Common QTLs controlling FD and other variables on LG 14.

For POP369-male parent a stable QTL for epicatechin and procyanidin B2 is also located at the same location of LG14. For POP369-male, QTLs associated with firmness, PPO activity, and concentration of catechin and epicatechin in both years and procyanidin B2 in 2011 only, co-located on LG3 for both 2011 and 2012. For the POP356-female parent, QTLs co-locating at LG3 are associated with fruit firmness and concentration of AsA, catechin, epicatechin and quercetin rhamnoside. Similar

group of QTLs was also detected for POP356-male parent on LG3 (Supplementary table 4.2). QTLs controlling concentration of the flavanol isomers (catechin and epicatechin) were identified on LG3 in the same genomic location across the populations and between the two years of the study, except for POP369-female, where a potential QTL identified for catechin in 2011 was lower than the set threshold ($P<0.01$). This parent also exhibited stable QTLs on LG14 for epicatechin and procyanidin B2 between the two years. Parent POP369-male exhibited QTLs for catechin and epicatechin on LG3 across both years, and for procyanidin B2 only in 2012 on LG3, while the POP369-female parent exhibited QTLs on LG3 for epicatechin in both years, and in 2012 only for epicatechin and procyanidin B2. Potential QTLs associated with concentration of catechin and procyanidin on LG3 were detected in 2011, however, the significance was lower than the set threshold ($P>0.005$; data not shown). In POP356, both parents exhibited QTLs on LG3 associated with concentration of catechin and epicatechin, but not for procyanidin B2.

Polyphenols such as catechin and epicatechin are substrates for PPO during the enzymatic browning that characterizes FD. Clearly, there are opportunities for further analysis, including mining the European (Chagné et al., 2014) and Chinese (Wu et al., 2013) pear genome sequences in the QTL region to identify candidate genes. Although no candidate gene for the control of such compounds has been identified in the syntenic region on LG3 in the apple genome (Chagné et al., 2012b) to date, the apple genome is another clear source of information.

Polyphenol content of fruits and vegetables is dependent on fruit maturity, pre- and post-harvest operations, as well as genetic characteristics (Herrmann, 1976; Amiot et al., 1992) and firmness is one of the most reliable indicators of maturity in commercial European pear cultivars (Kingston, 1992). Our identification of stable QTL on LG3 across years and populations and in common for control of fruit firmness, PPO activity and polyphenol concentration, confirms reports of the physiological relationship between firmness and maturity in accumulation of polyphenols (Mellenthin and Wang, 1974; Kvåle, 1988; Mitcham et al., 2001).

4.3.6 QTLs orthologous between apple and pear

Pear belongs to the Pyreae subfamily of the Rosaceae, that also includes apple, and their genomes are syntenic which means both shares the preserved co-localization of genes on their chromosomes (Celton et al., 2009). Syntenic species (apple and pear) conserve QTLs for similar traits and this synteny information opens new possibilities for identification of candidate genes controlling similar traits across species. QTLs associated with concentration of chlorogenic acid and its isoforms i.e. cryptochlorogenic acid and neochlorogenic acid located on LG17 of the female parent from POP356 are orthologous to a QTL identified for control of chlorogenic acid concentration in apple (Chagné et al., 2012b). Interestingly, in the POP369 population both parents have QTLs for the same variables on LG9, which is homeologous to LG17 in apple (Velasco et al., 2010) and pear (Fan et al., 2013). This homology in the *Malus* and *Pyrus* genomes indicates that these QTLs may be derived from paralogous gene copies from the Pyreae whole genome duplication (Velasco et al., 2010). In apple, a QTL for chlorogenic acid is also located at the bottom of LG17, where the *HCT*/*HQT* (hydroxy cinnamate transferase/hydroxy quinate transferase) gene is located (Chagné et al., 2012b). The *Pyrus HCT*/*HQT* gene is therefore a strong candidate gene for the LG17 QTL from POP356-female parent.

A stable QTL governing pear fruit firmness is located on LG3 in the same region where a QTL for apple firmness has been detected earlier (Liebhard et al., 2003), however no apple candidate gene has yet been proposed for this QTL.

4.4 Conclusion

Unlike other more studied fruit species such as tomato and apple, genetic information about the control of expression of pear fruit characters has been scanty to date. Of few reported QTL mapping studies in pear, only one recent concerns physical fruit traits mostly (Wu et al., 2014). These new generation SNP maps do provide advantages as they are derived from the *Pyrus* genome sequence and hence identified QTLs can be used to detect the candidate genes, in the genome sequence involved in the mechanism of control of these traits. In current study, the first SNP-based genetic

maps in pear (Montanari et al., 2013) were used to identify QTLs for 22 variables, including friction discolouration (FD), using two interspecific segregating populations (POP369 and POP356). QTL clusters were found for all 22 variables with a number of QTLs being stable across years, parents and populations. QTLs associated with fruit firmness and concentration of AsA and phenolic metabolites are the first reported for pear. Most notably, the QTLs detected that influence susceptibility to FD are the first fruit disorder QTLs to be reported in pear and one of the few in tree species. This study clearly demonstrates that the postharvest disorder friction discoloration is controlled by multiple small effect QTLs, unlike fruit quality attributes such as firmness and skin biochemical composition that are controlled by small and medium effect QTLs. In the future, candidate genes for QTLs controlling firmness, PPO activity, and polyphenolic compound concentration will be identified utilizing the reference genome sequences of pears ‘Bartlett’, ‘Dangshansuli’ and syntenic apple ‘Golden Delicious’. The polygenic nature of FD genetic control also indicates that it will be difficult to apply marker-assisted selection, however genomic selection could be employed to select elite genotypes with lower or no susceptibility to FD early in the breeding cycle.

Supplementary table 4.1: List of QTLs for fruit traits except FD for POP369. The Kruskal-Wallis (K) test was adopted to identify the QTLs as almost all traits were non normal in distribution, QTLs for each trait are given for each parent and for both years. SNPs are presented using the NCBI dbSNP accession number (ss #). Apple SNPs are represented with accession number starting with '4' while pear SNPs accessions start with '5'.

Trait	Parent	Year	LG	Position	SNP Marker	K value	Significance	Variance (%)
Total soluble solids	POP369-female	2012	LG16	42.79	nullss475878310	15.2	P<0.0001	15.52
Total soluble solids	POP369-female	2012	LG2	29.34	ss527788537	11.2	P<0.001	15.04
Total soluble solids	POP369- male	2012	LG2	39.64	nullss475877063	13.3	P<0.0005	15.08
Total soluble solids	POP369- male	2012	LG16	36.59	nullss475878492	9.2	P<0.005	9.03
Total soluble solids	POP369- male	2011	LG11	8.50	ss527787872	8.7	P<0.005	15.53
Firmness	POP369- male	2011	LG3	1.10	ss527789094	11.3	P<0.005	15.87
Firmness	POP369- male	2012	LG3	4.88	nullss475877747	15.4	P<0.0001	15.34
Firmness	POP369-female	2012	LG3	85.63	ss527788715	16.1	P<0.0001	15.92
Firmness	POP369- male	2012	LG2	94.45	ss527788737	9.0	P<0.005	14.19
Polyphenol oxidase	POP369-female	2011	LG14	2.52	nullss475879844	13.2	P<0.0005	14.93
Polyphenol oxidase	POP369-female	2011	LG9	10.00	nullss475879514	13.0	P<0.0005	14.4
Polyphenol oxidase	POP369-female	2012	LG3	92.56	ss475876304	16.3	P<0.0001	20.37
Polyphenol oxidase	POP369-female	2012	LG5	9.08	ss527789867	10.3	P<0.005	10.54
Polyphenol oxidase	POP369-male	2011	LG9	39.99	nullss475879514	13.0	P<0.0005	14.4
Polyphenol oxidase	POP369-male	2011	LG3	1.10	ss527789094	11.6	P<0.005	13.7
Polyphenol oxidase	POP369-male	2011	LG14	15.00	nullss475876400	11.5	P<0.001	13.21
Polyphenol oxidase	POP369- male	2012	LG3	24.63	nullss475876303	14.8	P<0.0005	16.21
Ascorbic acid	POP369-female	2011	LG3	85.63	ss527788715	10.6	P<0.005	6.77
Ascorbic acid	POP369- male	2011	LG3	4.88	nullss475877747	10.0	P<0.005	6.51
Chlorogenic acid	POP369-female	2011	LG14	7.04	ss527788119	15.5	P<0.0001	10.01
Chlorogenic acid	POP369-female	2011	LG13	21.08	nullss475880675	11.0	P<0.001	5.61

Trait	Parent	Year	LG	Position	SNP Marker	K value	Significance	Variance (%)
Chlorogenic acid	POP369-female	2011	LG15	5.00	ss527788028	8.9	P<0.005	8.01
Chlorogenic acid	POP369-female	2011	LG1	0.00	nullss475876892	8.1	P<0.005	4.07
Chlorogenic acid	POP369-female	2012	LG9	20.25	nullss475881473	18.5	P<0.0001	21.86
Chlorogenic acid	POP369-female	2012	LG1	18.06	ss475882286	8.5	P<0.005	21.5
Chlorogenic acid	POP369-female	2012	LG9	20.25	nullss475881473	7.9	P<0.005	6.5
Chlorogenic acid	POP369- male	2011	LG14	15.00	nullss475876400	12.6	P<0.0005	8.67
Chlorogenic acid	POP369- male	2011	LG9	24.78	nullss475879328	9.0	P<0.005	11.43
Chlorogenic acid	POP369- male	2012	LG9	24.78	nullss475879328	9.8	P<0.005	14.12
Cryptochlorogenic acid	POP369-female	2011	LG1	18.45	ss527789610	11.6	P<0.001	5.66
Cryptochlorogenic acid	POP369-female	2011	LG13	7.19	ss527789222	11.2	P<0.001	5.4
Cryptochlorogenic acid	POP369-female	2011	LG14	7.04	ss527788119	9.7	P<0.005	9.1
Cryptochlorogenic acid	POP369-female	2012	LG1	18.06	ss475882286	12.1	P<0.001	15.83
Cryptochlorogenic acid	POP369-female	2012	LG9	20.25	nullss475881473	9.1	P<0.005	13.16
Neochlorogenic acid	POP369-female	2011	LG13	7.20	ss527789261	16.8	P<0.0001	11.8
Neochlorogenic acid	POP369- male	2012	LG11b	19.35	ss527789056	18.6	P<0.0001	10.32
Neochlorogenic acid	POP369- male	2012	LG17b	3.66	nullss475882243	10.3	P<0.005	9.8
Catechin	POP369-female	2011	LG17	55.66	ss527787854	12.4	P<0.0005	10.67
Catechin	POP369-female	2012	LG3	91.64	ss527788083	20.6	P<0.0001	16.84
Catechin	POP369-female	2012	LG17	55.93	ss527788581	12.6	P<0.0005	9.81
Catechin	POP369-male	2011	LG9b	4.92	nullss475881980	9.7	P<0.005	8.14
Catechin	POP369-male	2011	LG3	1.10	ss527789094	14.1	P<0.001	21.12
Catechin	POP369-male	2012	LG9	4.92	nullss475881980	9.7	P<0.005	13.56
Catechin	POP369-male	2012	LG3	1.10	ss527789094	14.7	P<0.0005	22.73
Catechin	POP369-male	2012	LG8	31.20	ss475876347	10.0	P<0.005	5.63
Epi-catechin	POP369-female	2011	LG14	2.52	nullss475879844	10.5	P<0.005	8.7
Epi-catechin	POP369-female	2011	LG15	5.00	ss527788028	10.3	P<0.005	10.45
Epi-catechin	POP369-female	2011	LG3	92.56	ss475876304	9.2	P<0.005	12.9

Trait	Parent	Year	LG	Position	SNP Marker	K value	Significance	Variance (%)
Epi-catechin	POP369-female	2012	LG3	91.64	ss527788083	22.9	P<0.0001	23.48
Epi-catechin	POP369-female	2012	LG14	8.80	ss527788968	8.0	P<0.005	8.84
Epi-catechin	POP369- male	2011	LG3	1.10	ss527789094	12.9	P<0.001	20.56
Epi-catechin	POP369- male	2012	LG3	4.88	nullss475877747	17.7	P<0.0001	17.21
Epi-catechin	POP369- male	2012	LG14	15.00	nullss475876400	8.1	P<0.005	8.48
Procyanidin B2	POP369-female	2011	LG15	5.00	ss527788028	11.6	P<0.001	12.92
Procyanidin B2	POP369-female	2011	LG14	2.52	nullss475879844	10.3	P<0.005	13.8
Procyanidin B2	POP369-female	2012	LG3	91.64	ss527788083	12.3	P<0.0005	13.15
Procyanidin B2	POP369-female	2012	LG14	4.93	ss527788030	8.2	P<0.005	8.4
Procyanidin B2	POP369- male	2012	LG3	24.63	nullss475876303	8.0	P<0.005	8.58
p-coumaryl quinic acid	POP369-female	2011	LG14	8.55	ss475877282	8.8	P<0.005	2.6
p-coumaryl quinic acid	POP369-female	2012	LG11	14.85	ss527788817	10.7	P<0.005	12.67
p-coumaryl quinic acid	POP369-female	2012	LG13	7.19	ss527789222	9.6	P<0.005	10.02
p-coumaryl quinic acid	POP369- male	2011	LG9	24.78	nullss475879328	9.6	P<0.005	9.79
p-coumaryl quinic acid	POP369- male	2011	LG14	15.00	nullss475876400	7.9	P<0.005	2.6
Isorhamnetin rutinoside	POP369-female	2011	LG3	84.05	nullss475881324	9.2	P<0.005	6.07
Isorhamnetin rutinoside	POP369-female	2012	LG17	58.61	ss527789419	12.3	P<0.0005	14.56
Isorhamnetin rutinoside	POP369- male	2012	LG7	12.17	ss527789815	13.1	P<0.0005	16.03
Isorhamnetin rutinoside	POP369- male	2012	LG9b	3.85	nullss475879274	11.9	P<0.001	13.81
Isorhamnetin galactoside/glucoside	POP369-female	2011	LG2	20.60	ss475881524	9.3	P<0.005	8.27
Isorhamnetin galactoside/glucoside	POP369-female	2012	LG9	20.25	nullss475881473	16.6	P<0.0001	15.28
Isorhamnetin galactoside/glucoside	POP369- male	2011	LG2	15.72	nullss475879045	10.1	P<0.005	7.93
Isorhamnetin galactoside/glucoside	POP369- male	2011	LG12	38.28	ss475880465	8.8	P<0.005	9.6
Quercetin arabinose	POP369- male	2011	LG5	26.44	ss527789573	17.2	P<0.0001	12.63
Quercetin arabinose	POP369- male	2011	LG2	114.50	ss475877452	11.8	P<0.001	9.12
Quercetin arabinose	POP369- male	2012	LG5	26.44	ss527789573	17.2	P<0.0001	17
Quercetin galactoside	POP369-male	2011	LG12	43.26	ss475876325	8.4	P<0.005	8.06

Trait	Parent	Year	LG	Position	SNP Marker	K value	Significance	Variance (%)
Quercetin galactoside	POP369-male	2012	LG3	1.10	ss527789094	17.8	P<0.0005	32.31
Quercetin galactoside	POP369-female	2012	LG9	20.25	nullss475881473	15.0	P<0.0005	10.32
Quercetin glucoside	POP369-female	2012	LG9	20.25	nullss475881473	10.5	P<0.005	12.33
Quercetin glucoside	POP369-male	2012	LG3	1.10	ss527789094	13.0	P<0.005	22.02
Quercetin glucoside	POP369-male	2012	LG16	16.29	nullss475878313	11.6	P<0.001	13.17
Quercetin rhamnoside	POP369- male	2012	LG13	0.00	nullss475882577	33.4	P<0.0001	33.75
Quercetin rhamnoside	POP369- male	2012	LG16	0.00	nullss475877116	9.1	P<0.005	6.79
Quercetin rhamnoside	POP369- male	2012	LG2	39.64	nullss475877063	9.0	P<0.005	4.2
Quercetin rutinoside	POP369- male	2012	LG16	16.29	nullss475878313	12.6	P<0.0005	3.89
Quercetin rutinoside	POP369- male	2012	LG7	16.79	ss475878863	12.1	P<0.005	11.22
Quercetin	POP369-female	2011	LG15	106.77	ss475881369	17.0	P<0.0001	15.27
Quercetin	POP369-female	2011	LG3	92.56	ss475876304	13.4	P<0.0005	14.73
Quercetin	POP369-female	2011	LG9	28.41	ss527787955	10.1	P<0.005	9.99
Quercetin	POP369-female	2012	LG11	56.91	ss475880309	9.7	P<0.005	9.01
Quercetin	POP369- male	2011	LG3	4.88	nullss475877747	13.2	P<0.0005	13.51
Quercetin	POP369- male	2011	LG13b	9.53	ss475881766	9.4	P<0.005	8.96
Comp_417.12(1)	POP369- male	2011	LG5	0.00	nullss475877663	20.6	P<0.0001	16.75
Comp_417.12(1)	POP369- male	2011	LG2	114.50	ss475877452	13.2	P<0.0005	9.5
Comp_417.12(1)	POP369- male	2012	LG5	0.00	nullss475877663	15.6	P<0.0001	13.36
Comp_417.12(2)	POP369- male	2011	LG5	26.44	ss527789573	20.7	P<0.0001	16.44
Comp_417.12(2)	POP369- male	2012	LG5	26.44	ss527789573	20.9	P<0.0001	20.6

Supplementary table 4.2: List of QTLs for fruit traits except FD for POP356. The Kruskal-Wallis (K) test was adopted to identify the QTLs as almost all traits were non normal in distribution, QTLs for each trait are given for each parent and for both years. SNPs are presented using the NCBI dbSNP accession number (ss #). Apple SNPs are represented with accession number starting with '4' while pear SNPs accessions start with '5'.

Trait	Parent	Year	LG	Position	SNP marker	K value	Significance	Variance (%)
Total soluble solids	POP356-female	2011	LG9	13.87	ss475882948	10.6	P<0.005	8.75
Total soluble solids	POP356- male	2011	LG10	20.55	ss475879783	15.5	P<0.0001	12.99
Firmness	POP356-female	2011	LG3b	7.85	ss527788732	10.6	P<0.005	7.39
Firmness	POP356-female	2011	LG3	0.00	ss475882105	9.5	P<0.005	10.88
Firmness	POP356- male	2011	LG17	3.38	ss475882022	13.9	P<0.0005	11.13
Firmness	POP356- male	2011	LG3b	46.79	ss475877642	7.9	P<0.005	5.08
Polyphenol oxidase	POP356-female	2011	LG6	27.44	ss527788560	9.6	P<0.005	9.78
Polyphenol oxidase	POP356-female	2011	LG2	41.72	ss475876969	9.1	P<0.005	10.9
Polyphenol oxidase	POP356-female	2011	LG17	1.64	ss475878591	8.5	P<0.005	7.15
Polyphenol oxidase	POP356- male	2011	LG2b	0.00	ss475876962	8.3	P<0.005	8.05
Ascorbic acid	POP356-female	2011	LG3	4.53	ss475877804	20.7	P<0.0001	16.05
Ascorbic acid	POP356- male	2011	LG3	7.29	ss475877810	17.3	P<0.0001	11.01
Ascorbic acid	POP356- male	2011	LG15	67.86	ss527789659	9.2	P<0.005	4.18
Chlorogenic acid	POP356-female	2011	LG9	9.59	ss475879580	17.4	P<0.0001	10.89
Chlorogenic acid	POP356-female	2011	LG17	0.00	ss475878373	15.2	P<0.0001	14.11
Chlorogenic acid	POP356-female	2011	LG11	62.38	ss475880068	9.6	P<0.005	8.96
Cryptochlorogenic acid	POP356-female	2011	LG17	0.00	ss475878373	18.2	P<0.0001	16.8
Cryptochlorogenic acid	POP356-female	2011	LG9	9.59	ss475879580	13.5	P<0.0005	8.16
Cryptochlorogenic acid	POP356-female	2011	LG11	62.38	ss475880068	9.6	P<0.005	9.62
Cryptochlorogenic acid	POP356- male	2011	LG9	54.42	ss527789259	8.1	P<0.005	7.75
Neochlorogenic acid	POP356-female	2011	LG17	0.00	ss475878373	9.9	P<0.005	10.3

Trait	Parent	Year	LG	Position	SNP marker	K value	Significance	Variance (%)
Neochlorogenic acid	POP356- male	2011	LG2c	16.26	ss527788654	10.6	P<0.005	6.63
catecchin	POP356-female	2011	LG10	41.25	ss475879480	15.4	P<0.0001	12.23
catecchin	POP356-female	2011	LG17	1.64	ss475878591	11.1	P<0.001	10.39
catecchin	POP356-female	2011	LG3	7.70	ss527789682	9.5	P<0.005	5.95
catecchin	POP356- male	2011	LG3	7.29	ss475877810	7.9	P<0.005	4.64
Epicatechin	POP356-female	2011	LG10	41.25	ss475879480	10.4	P<0.005	4.54
Epicatechin	POP356-female	2011	LG7	69.34	ss527789192	8.8	P<0.005	5.69
Epicatechin	POP356-female	2011	LG3	7.70	ss527789682	8.4	P<0.005	3.01
Epicatechin	POP356-female	2011	LG12	5.47	ss475880359	7.9	P<0.005	6.95
Epicatechin	POP356- male	2011	LG3	7.29	ss475877810	8.2	P<0.005	4.6
Epicatechin	POP356- male	2011	LG15	34.50	ss527789616	8.2	P<0.005	6.87
Procyanidin B2	POP356-female	2011	LG11	0.00	ss527788999	8.7	P<0.005	3.94
Procyanidin B2	POP356-female	2011	LG15	4.98	ss527789869	8.5	P<0.005	5.76
Procyanidin B2	POP356-female	2011	LG10	41.25	ss475879480	8.4	P<0.005	8.82
Procyanidin B2	POP356- male	2011	LG15	11.60	ss527787880	8.1	P<0.005	7.45
Isorhamnetin galactoside/glucoside	POP356-female	2011	LG5	14.94	ss527787941	12.0	P<0.005	6.4
Isorhamnetin galactoside/glucoside	POP356-female	2011	LG6	33.65	ss527788260	10.2	P<0.005	7.23
Isorhamnetin galactoside/glucoside	POP356- male	2011	LG5b	7.09	ss527787941	12.0	P<0.005	5.5
Isorhamnetin galactoside/glucoside	POP356- male	2011	LG6	0.00	ss475878577	8.8	P<0.005	3.39
Isorhamnetin rutinoside	POP356-female	2011	LG7	60.96	ss475876821	13.1	P<0.0005	12.69
Isorhamnetin rutinoside	POP356-female	2011	LG8b	3.65	ss527787780	12.5	P<0.005	11.05
Isorhamnetin rutinoside	POP356- male	2011	LG7	11.14	ss527789815	12.8	P<0.0005	12.18
Isorhamnetin rutinoside	POP356- male	2011	LG8	18.11	ss527787780	12.5	P<0.005	11.05
p-coumaryl quinic acid	POP356-female	2011	LG17	0.09	ss475878371	13.1	P<0.0005	33.19
p-coumaryl quinic acid	POP356-female	2011	LG9	18.53	ss527789332	11.6	P<0.001	2.34
Quercetin galactoside	POP356-female	2011	LG2	10.00	ss527789751	9.0	P<0.005	6.83
Quercetin galactoside	POP356-female	2011	LG5	10.99	ss475875943	8.1	P<0.005	2.45

Trait	Parent	Year	LG	Position	SNP marker	K value	Significance	Variance (%)
Quercetin galactoside	POP356- male	2011	LG2b	12.01	ss527789721	10.4	P<0.005	7.05
Quercetin galactoside	POP356- male	2011	LG5	53.71	ss475878398	9.9	P<0.005	5.15
Quercetin galactoside	POP356- male	2011	LG1	41.04	ss475880158	8.8	P<0.005	2.38
Quercetin glucoside	POP356-female	2011	LG2	10.00	ss527789751	10.3	P<0.005	9.25
Quercetin glucoside	POP356- male	2011	LG2b	12.01	ss527789721	10.3	P<0.005	9.18
Quercetin glucoside	POP356- male	2011	LG5	53.71	ss475878398	9.6	P<0.005	4.5
Quercetin arabinose	POP356-female	2011	LG5	0.00	ss475879840	27.6	P<0.0001	14.19
Quercetin arabinose	POP356- male	2011	LG5	75.13	ss475880255	35.2	P<0.0001	20.46
Quercetin arabinose	POP356- male	2011	LG14	0.00	ss475879459	9.8	P<0.005	9.89
Quercetin rhamnoside	POP356-female	2011	LG3b	37.28	ss475877507	11.2	P<0.005	5.02
Quercetin rhamnoside	POP356- male	2011	LG5	59.75	ss527788420	14.4	P<0.0005	6.67
Quercetin rhamnoside	POP356- male	2011	LG9	41.34	ss475883529	11.4	P<0.001	2.21
Quercetin rhamnoside	POP356- male	2011	LG3b	11.03	ss475877507	11.2	P<0.005	5.02
Quercetin rutinoside	POP356-female	2011	LG7	60.96	ss475876821	17.0	P<0.0001	10.96
Quercetin rutinoside	POP356-female	2011	LG12	24.10	ss475880191	14.4	P<0.001	12.1
Quercetin rutinoside	POP356-female	2011	LG2	9.00	ss475877341	10.0	P<0.005	3.68
Quercetin rutinoside	POP356- male	2011	LG7	0.00	ss475881589	15.9	P<0.0001	10.22
Quercetin rutinoside	POP356- male	2011	LG2	17.49	ss475883066	10.0	P<0.005	3.68
Quercetin	POP356-female	2011	LG1	20.25	ss475876950	12.8	P<0.005	8.67
Quercetin	POP356-female	2011	LG7	30.50	ss475878916	11.6	P<0.001	6.31
Quercetin	POP356-female	2011	LG12	42.55	ss527789730	8.1	P<0.005	5.33
Quercetin	POP356- male	2011	LG12	8.94	ss527788312	10.3	P<0.005	5.61
Comp_41712(1)	POP356-female	2011	LG5	0.00	ss475879840	40.5	P<0.0001	17.48
Comp_41712(1)	POP356- male	2011	LG5	75.13	ss475880255	45.6	P<0.0001	23.04
Comp_41712(2)	POP356-female	2011	LG5	0.00	ss475879840	39.9	P<0.0001	22.44
Comp_41712(2)	POP356- male	2011	LG5	75.13	ss475880255	44.1	P<0.0001	25.98

5 GENERAL DISCUSSION

This study was aimed to identify molecular markers that can be used for selection of new cultivars with low friction discolouration (FD) susceptibility in breeding programmes. New understanding of the physiological and genetic basis of FD in wide cross of genotypes has been developed from this study. This thesis offers a new insight in to genomic solutions of postharvest disorders of fruit trees using FD of pear as a model. This chapter presents lessons from this study with future suggestions (section 5.1) and future prospects (section 5.2).

5.1 Research learnings

5.1.1 Harvest maturity for genotypes from interspecific crosses

Working with breeding populations of fruit trees consisting of hundreds of different genotypes is generally challenging for achieving the conditions ideal for typical postharvest experiments. The genetic makeup of the pear samples is complex because of their interspecific origin (Figure 2.1). European pear requires low temperature conditioning for initiation of ripening whilst Asian pears generally ripen on the tree (Kingston, 1992). Fruit samples after harvest were stored for up to 3 months with later exposure to room temperature for at least 24 hours. It is likely that fruit samples with more Asian characteristics do not require long term exposure to chilling temperature (0-1°C) and were already over mature when assessed for FD. However, given that it would be impossible to establish harvest maturity indices for each genotype, there seems no better option than what was chosen here. Fruit were harvested on the basis of visible cues related to maturation: i.e. sequential harvests (whenever possible) commencing when fruit drop was first seen for a genotype, and selecting individual fruit showing a change in background colour from deep green to light green/yellow.

Given the complexities posed by interspecific populations for accurate maturity determination, crosses within species are recommended for studies of postharvest traits, especially in pear. Independent multiple harvests should be assessed for

complex quantitative traits that are known to be influenced by harvest date (maturity stage) and, as was done here, maturity-related data (e.g. soluble solids content, dry matter and firmness) should be captured to allow *post hoc* analysis of possible confounding influences from under- or over-maturity.

5.1.2 Do skin properties predispose fruit to FD?

Loss of compartmentation as a result of mechanical damage leads to the enzymatic oxidation of phenolics in damaged pear fruit. Since the skin appearance of different varieties can vary markedly, several authors have investigated whether some attributes of the skin may explain varietal differences in susceptibility (Amarante et al., 2001a; Palmer et al., 2008). Those studies have demonstrated that there can be significant variability in the skin of even a single commercial cultivar (Palmer et al., 2008) which may contribute to fruit-to-fruit variation in FD susceptibility. In current study, a total of 40 fruit (20 low and 20 high FD category) were tested for role of skin properties in FD susceptibility. No consistent differences were observed between the two groups of genotypes. This does not mean that the skin is unimportant to the development of FD; but it is clear that we do not yet have any particular skin attributes that appear to predispose the fruit surface to FD.

5.1.3 Role of phenolics as antioxidants in the incidence of FD

Phenolics are a wide spread group of secondary metabolites in almost every plant species and their quantitative distribution may vary from organ to organ within and between plants of the same species. Researchers have tried to correlate tissue browning to the amount of substrate (phenolics) or enzyme (PPO) activity of the fruit. However contradictory conclusions have been reached by different authors which illustrates the complexity of the browning mechanism. Interestingly, no preliminary literature is available regarding role of phenolics in FD susceptibility in Asian pears, with all published papers referring only to European pears (Meheriuk et al., 1994; Amiot et al., 1995; Bertolini and Trufelli, 2002; Hamauzu and Hanakawa, 2003; Burger et al., 2005). Although an overall weak negative correlation was identified in both populations between phenolic content and FD score, different trends were observed in individual genotypes. Phenolic compounds such as caffeic

esters and catechin act as good antioxidants as well as good substrates for browning processes. At relatively low concentrations they act as pro-oxidants for initiation of browning while at higher concentration they act as antioxidants (Fukumoto and Mazza, 2000).

Phenolics can prevent enzymatic browning by reacting with oxygen, with intermediate products by breaking the chain reaction or by acting as chelating agents and reducing Cu^{++} to Cu^{+} (Lindley, 1998; Li et al., 2011; Loannou, 2013). Phenolics are also known to interact directly with polyphenoloxidase (PPO) which can lead to inhibition of PPO activity (Le Bourvellec et al., 2003). Le Bourvellec et al. (2003) studied the inhibitory effect of caffeoylquinic acid, epicatechin and procyanidin oxidation products; they oxidized the mixture of caffeoylquinic acid and epicatechin by reacting with caffeoylquinic acid o-quinone to get oxidized products. Oxidized products from all compounds inhibited PPO activity and regenerated the original phenolic substrates. The related phenomenon of 'coupled oxidation' may occur in which the product (quinones) of the first step of oxidative browning further oxidizes other phenolics like flavanols which results in the regeneration of the original phenolics (Fig. 5.1, (Nicolas and Potus, 1994). Chlorogenic acid is reported to play a prominent role in the oxidative degradation of other phenolics with regeneration of chlorogenic acid itself. Reaction products resulting from chlorogenic acid oxidation are light to colourless in hue while quinones form darker compounds (Rouet-Mayer et al., 1990; Goupy et al., 1995).

Chlorogenic acid constituted the major phenolic in the studied genotypes. There was no clear relationship between chlorogenic acid and FD score in 2011 and 2012 while in 2013 a negative correlation of 0.46 ($P<0.05$) was observed. It can be speculated that in this study, phenolics actively participated as antioxidants during enzymatic browning to reverse the *o*-quinones back to original phenols as well as acting as substrates for PPO. The antioxidant effect may explain the overall weak negative correlation between phenolic concentration and FD across the wide range of genotypes in this study.

Ascorbic acid (AsA) was included in this study because of its antioxidant role. Quinones from oxidative reactions can react with ascorbic acid and result in

regeneration of initial phenols **(**Figure 5.1**)**. Browning can be stopped until ascorbic acid is consumed or depleted then formation of brown pigments will again take place (Rouet-Mayer et al., 1990). However, in this study there were no correlations between ascorbic acid concentration and FD or any other parameter. During long term storage, AsA can be oxidized to dehydroascorbic acid (DHA) (Mazurek and Pankiewicz, 2012). Only total ascorbic acid was analysed in this study. So there is a possibility that variable amounts of AsA were oxidized to DHA during storage in the various genotypes. This could be verified in future work.

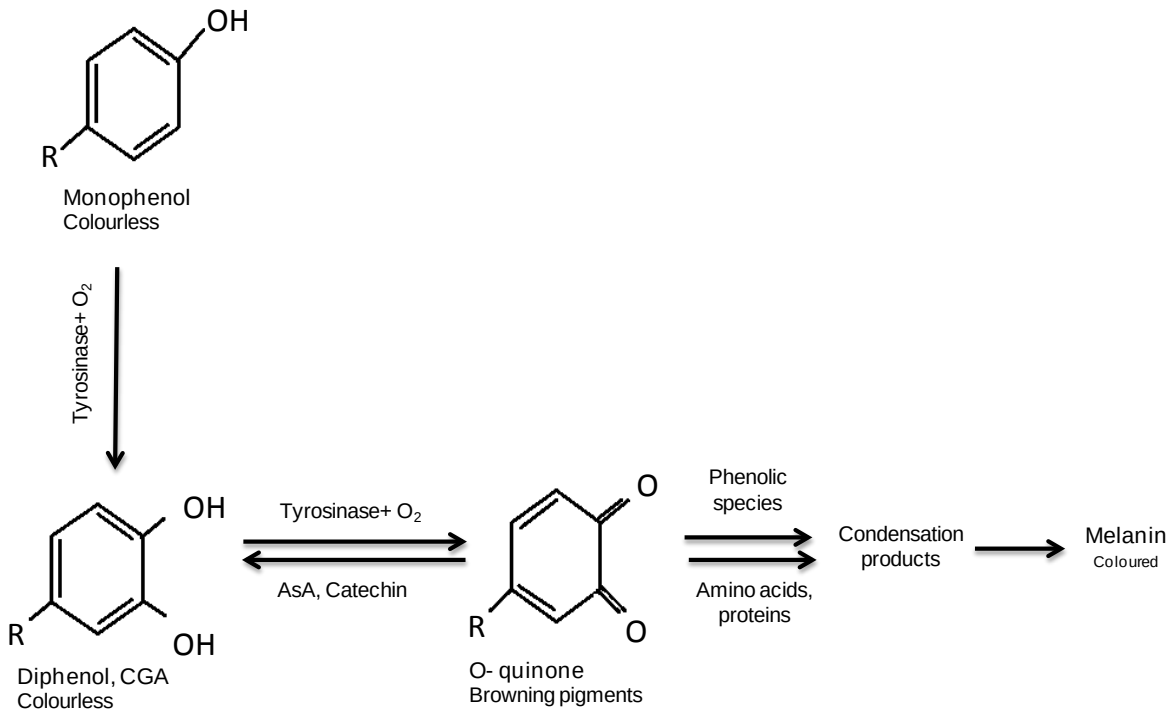

Figure 5.1: Enzymatic browning reaction catalysed by Tyrosinase (PPO) enzyme. Ortho-quinones reduced back to original phenols as a reaction of coupled oxidation or reducing agent. CGA: chlorogenic acid, AsA: Ascorbic acid.

Measurement of antioxidant activity is recommended to estimate the antioxidant effect of phenolics and AsA in context of enzymatic browning. Assays for

antioxidant activity like FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorbance capacity) are suggested due to their wide range of applicability (Cao et al., 1993; Benzie and Strain, 1996).

One final consideration relating to metabolite concentrations in this study is to note that metabolites were sampled 24 hours after FD initiation on each pear, using fruit skin from non-damaged parts of each fruit. It is possible that the FD assay could cause a systemic change in phenolic concentrations around undamaged portions of the fruit (Saltveit, 2000). One way to avoid this problem would be to take larger numbers of fruit for analysis (which would require older trees than were used here). So metabolite samples could be taken from undamaged fruit and FD assessments could be made on matching batches of fruit. Nevertheless, using the identical fruit for both FD and metabolite assessment still seems the best place to start.

5.1.4 Robustness of detected QTLs

A large number of QTLs for pear fruit quality traits including FD were identified. FD is a complex disorder and has been known to be influenced by genetic x environment (GxE) interaction as described in chapter 2. Besides these variations a number of QTLs were detected associated with FD of which two were stable across the years (2). Robustness of the QTLs can be tested by repeating the study in different seasons, at different growing environments and across the populations. In this study phenotypic data for POP369 were collected in two years (2011 and 2012) which provided confidence for QTL robustness in this population. QTLs from POP356 were considered real if they were reproducible across the populations. Practical implications of these stable QTLs are presented in section 5.3 in this chapter.

5.2 Future prospects

5.2.1 Breeding perspective of FD and other postharvest disorders

QTL mapping has been proposed as a means to unravel the genetic basis of complex quantitative traits like postharvest disorders that can eventually increase the efficiency of screening methods to select superior genotypes during the breeding

cycle. Major bottleneck in conventional breeding of fruit tree such as pear is their long juvenile period which means breeders have to maintain the progeny for some years before fruit quality traits can be evaluated. Marker assisted selection (MAS) is the simplest method where genotypes can be selected on the basis of presence and absence of marker information earlier in the breeding cycle which would be more efficient and cost effective. However, MAS is generally useful in case of monogenic traits controlled by a single major QTL while in the case of polygenic traits like postharvest disorders with high GxE interaction, it would be difficult to get high genetic gain even with a combination of several QTLs. Hence for breeding of postharvest disorders genomic selection offers more promises in terms of genetic gain.

This is the first reported QTL study of any postharvest disorder in pear, and no prior information for genetic study of this disorder is available in pear or in any other crop. As elucidated from the QTL study, FD is controlled by much small effect QTLs. Genomic selection (GS) seems a more plausible technique for selection of seedlings with low FD susceptibility. This technique is based the principle of prediction of genetic effect or phenotype of the individuals on the basis of their marker information instead of identification of underlying genes.

For genomic selection of FD, genotype and phenotype data collected from the 'training population' (POP369), pedigree information (Figure 2.1) and QTL information can be used to estimate genomic estimated breeding values (GEBVs). Only genotype data collected from validation populations can be collected and a prediction model developed from a "training population" can be used to predict the GEBVs for these non phenotyped individuals. Superior genotypes with higher GEBVs can be selected to use as parents for new crosses or for field evaluation as potential new cultivars (Bradbury et al., 2011). In either case it can shift the breeding resources for set of few elite seedlings compared to whole progeny of hundreds of seedlings.

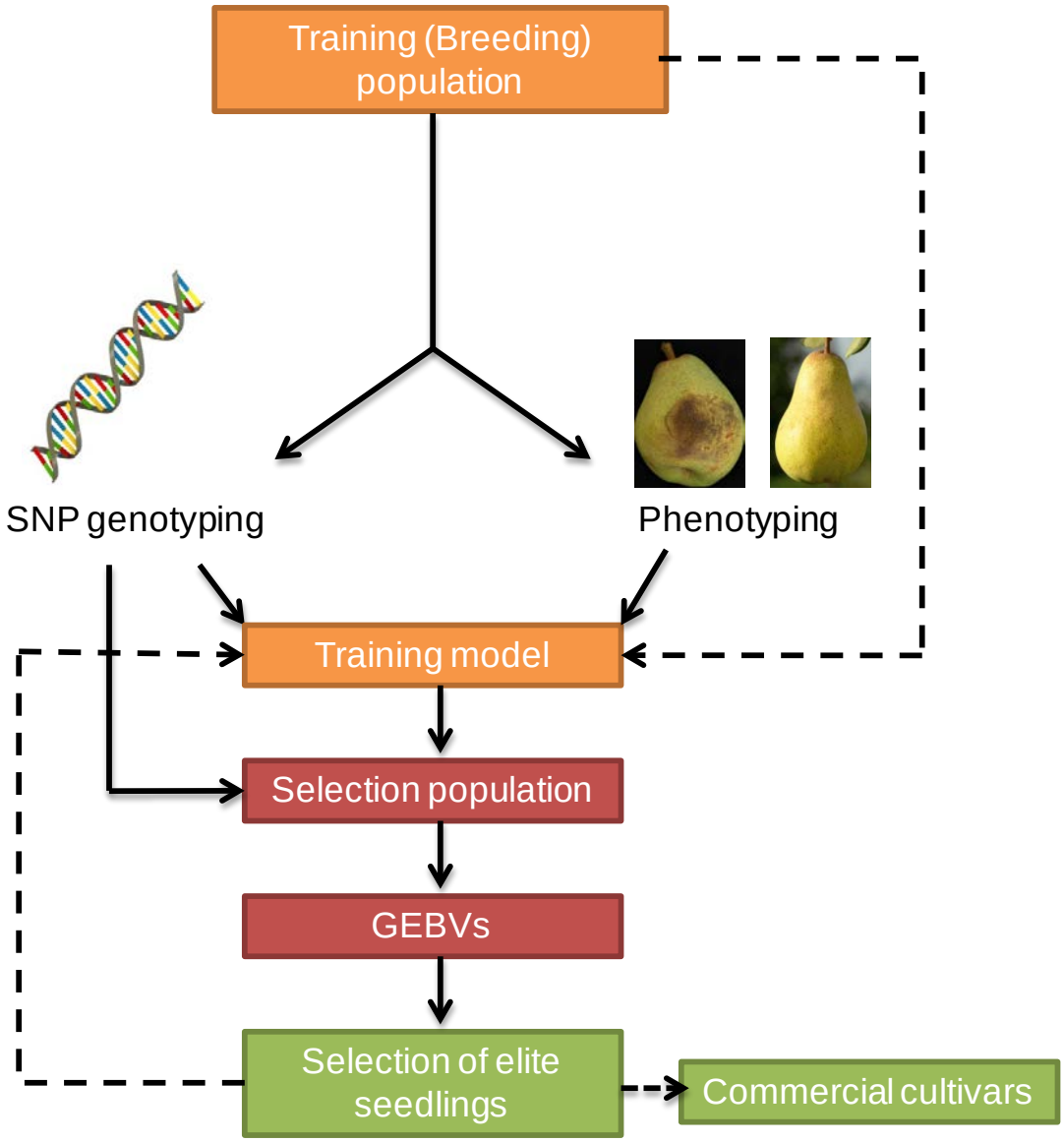

Figure 5.2: Genomic selection process for selection of low FD pear cultivars.

5.2.2 Candidate gene detection for stable QTLs

QTLs generally do not provide information about their molecular nature i.e. either they are associated with specific genes that regulate particular pathway underlying the trait of QTL or not. The identification of the candidate gene underlying a trait QTL can lead to additional levels of information. The process of narrowing down the QTL interval region to candidate genes through bioinformatics and molecular techniques has been explained by the schematic diagram in Figure 5.3 considering FD stable QTL as an example. A typical QTL region can have hundreds of positional candidate genes including genes negatively or positively influencing FD. Gene

filtration is performed on the basis of available information about their functionality. Genes of related functions have a propensity to cluster together in the genome. Hence there is a possibility to identify several genes and then validate further to check their involvement in underlying pathways. As a next step, individuals with extreme phenotype (individuals with “high” and “low” FD susceptibility) could be selected and analyzed for RNA sequence. This RNA sequence would be further aligned to reference genome sequence of ‘Bartlett’ to quantify differential RNA expression. A gene with related function (e.g. involved in phenolic or PPO metabolic pathways) positioned within the FD QTL interval and also showing higher expression for low or high FD would be regarded as the candidate gene of interest. However further validation studies by mapping the candidate gene to FD QTL or physiological analyses (measuring protein level, determining the enzyme activity) would be essential to confirm the potential role of candidate gene for disorder incidence or severity. Future studies can be extended to explore the function of tightly linked genes in the same genomic region which may follow the same pathway.

Recently sequenced Chinese (Wu et al., 2013) and European (Chagné et al., 2014) pear genomes are valuable tools to determine the region to filter the candidate genes for important physical and biochemical traits. Genomes of other related genera like apple (Velasco et al., 2010) and peach (Verde et al., 2013) are also available and can also be used to mine for candidate genes. The same approach is applicable across populations within a species (e.g. within European pears), across species within a genus (e.g. between European and Asian pears) and across genera within a family (e.g. between pear and apple in the Rosaceae).

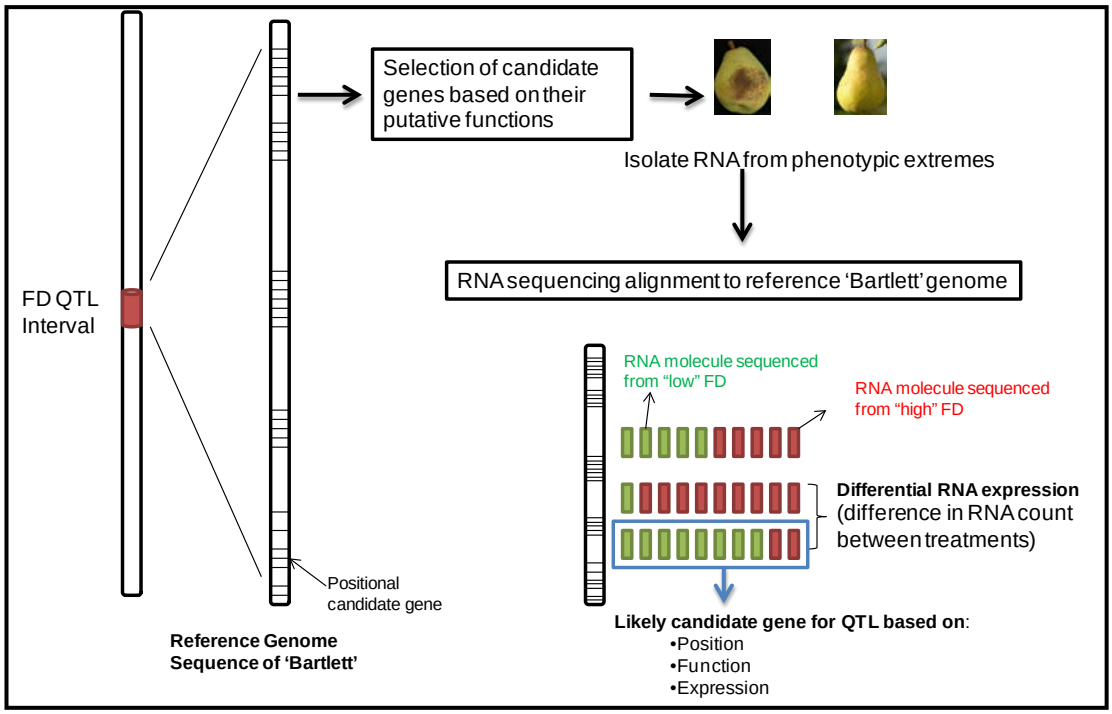

Figure 5.3: Pictorial representation of process of candidate gene detection from QTL.

5.2.3 Comparative genome mapping

Comparison of genera *Malus*, *Prunus* and *Fragaria* has revealed the high degree of macro- and micro- synteny within the Rosaceae family (Illa et al., 2011; Jung et al., 2012). Recent availability of whole genome sequence of European and Asian pears has also elucidated the conservation of gene blocks between apple and pear (Velasco et al., 2010; Wu et al., 2013; Chagné et al., 2014). Use of the first cross-genera (*Pyrus* and *Malus*) SNP microarray for genotyping of interspecific pear populations in this study has also confirmed the synteny between the genus *Malus* and *Pyrus* (Montanari et al., 2013). Comparative mapping can be used to detect and align the orthologous region of the genome which has been conserved through evolutionary time. This conservation postulates that a gene underlying certain trait in apple would also be controlling the same trait in pear. For example a scab-resistant gene, Rvp1, was mapped to LG2 of the European pear cultivar maps which is orthologous to the apple scab-resistant gene cluster on LG2 at same location indicative of functional synteny for scab resistance (Bouvier et al., 2012). The other example for orthologous genomic regions involved is identification of a stable flowering QTL at LG 5 of

European pear population while an apple flowering gene has been already mapped at the same location at LG5 (Knäbel et al. unpublished). Another QTL for *Cacopsylla pyricola* (pear psylla) is located at LG8 for interspecific pear population and a major gene for resistance to aphids located at same location at LG8 in apple (Montanari et al., unpublished).

In this study a QTL for chlorogenic acid has been detected on LG17 at the location where it has also been detected in apple. A candidate gene *HCT*/*HQT* (hydroxy cinnamate transferase/hydroxy quinate transferase) has been already identified on the same location at LG17 in apple (Chagné et al., 2012b). For POP369 this QTL is located on LG9 in both years and for both parents while it is already known that chromosome 9 and 17 are homologous (Velasco et al., 2010). With the availability of a draft genome sequence of apple it is possible to have sequence of a desired gene which facilitates the process of primer design. This primer can be used to estimate the effect of orthologous gene in pear progeny.

Phenolics may be good for human health (Kondratyuk and Pezzuto, 2004) but they are also regarded as risk factors for enzymatic browning (Amiot et al. 1995). This study has suggested that the reverse (negative correlation) may be true as negative associations between phenolic compounds and FD have been noticed fairly consistently from correlation (Table 2.4) and discriminant analysis results (Figure 2.11, 2.13 and 2.15). This means that using QTL to select pears with higher content of chlorogenic acid may be both desirable and achievable.

5.2.4 Metabolomic QTL detection

Metabolomics is the high throughput measurement of plant primary and secondary metabolites in a given biological system. Metabolome-wide association studies have gained importance in recent years in plant research both in basic and applied contexts. Metabolic mapping combines the genetic variation in the form of molecular markers along with metabolomic profiling to locate QTLs involved in regulation of the observed metabolite in metabolic network (Keurentjes et al., 2006). The metabolic QTLs can help to understand biochemical pathways and large scale

relationships among metabolic systems such as plant responses to biotic or abiotic stress, postharvest changes and nutritional value.

Metabolic QTLs have been used successfully in comprehensive understanding of genetic background of quantitative traits in crop plants (Keurentjes, 2009). For example glycine betaine concentration has been successfully used as a biomarker for tolerance to stress conditions and for quality determination in rice (Kusano et al., 2007).

Since little is known about variation of ripening in interspecific pears at the metabolic level, metabolic profiling at different harvest maturities could be used to monitor key changes at the metabolic level in different genotypes and correlate with corresponding variation in physiological behaviour (phenotype). Metabolic profiling together with high density SNP maps can be used to detect the mQTL which will help to establish genetically inferred links between metabolites. This QTL identification is an initial step to identify the candidate genes which can be further used in metabolic assisted breeding to select the seedlings with desired trait. This approach is applicable for other traits in pear and other fruit trees.

5.3 General conclusion

This study has demonstrated that despite the complexity of a postharvest disorder, it is achievable to evaluate the physiological, metabolite and genetic basis for underlying mechanisms. Similar approaches can be extended to understand the genetic basis of other physiological fruit disorders such as internal browning, soft scald and chilling injury.

Specifically these findings can assist fruit biologists, postharvest scientists and pear breeders to develop a better understanding of the genetic control of this highly challenging postharvest disorder. Application of strategies like genomic selection during breeding cycles may be a start for promising future for pear growers with a greater diversity of new FD free cultivars and offer a boost to the pear industry of New Zealand.

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APPENDIX

Appendix 1: Details from discriminant analysis (DA) for POP369 (2011, 2012) and POP356 (2011)

Step wise discriminant function analysis was performed to see what fruit trait (s) can discriminate between group of high, medium and low FD genotypes. Data was log transformed before conducting the DA.

Appendix 1A: POP369 (2011) discriminant function analysis (log-transformed data)

Input: Parameters studied

Friction discolouration
Total soluble solids
Firmness
Polyphenol oxidase
Ascorbic acid
Chlorogenic acid
Cryptochlorogenic acid
Neochlorogenic acid
Catechin
Epicatechin
Procyanidin B2
Isorhamnetin 3-galactoside
Isorhamnetin rutinoside
p-coumaryl quinic acid
Quercetin galactoside
Quercetin glucoside
Quercetin arabinoside
Quercetin rhamnoside
Quercetin rutinoside
Quercetin
Comp_417.12 (1)
Comp_417.12(2)

Output: Summary information for stepwise selection of variables

Forward selection

Selection criterion: Wilks' lambda

Best 10 variables:

Variable Criterion

Isorhamnetin rutinoside	0.90502
comp_417.12	0.85336
Quercetin	0.80515
Chlorogenic acid	0.76546
Quercetin rutinoside	0.72344
Isorhamnetin galactoside/glucoside	0.68629
Procyanidin B2	0.66013
AsA	0.63028
Quercetin glucoside	0.61675
comp_417.12(2)	0.60590

Message: Default seed for random number generator used with value 267647

Optimal variables selected

IRrut
comp_417.12
Quercetin
3CQA
Qrut
IRgal/glu
ProcyB2
AsA

Discriminant analysis

Latent vectors, roots, and trace from CVA

Vectors:

Scores	**Scores[1]**	**Scores[2]**
Iso rhamnetin rutinoside	3.440	1.107
comp_417.12	0.408	0.305
Quercetin	-1.234	0.567
Chlorogenic acid	1.600	2.972
Quercetin rutinoside	-1.090	0.779

Iso rhamnetin galact/glucoside	-1.420	-2.187
ProcyandinB2	0.676	-2.391
AsA	-1.245	1.218

Roots:

Scores

Scores[1] 0.4626

Scores[2] 0.0848

Scree Diagram for POP369 (2011)

No	Root	%%	Cum %	Scree Diagram (* represents 3%)
1	0.4626	845	845	85 **********
2	0.0848	155	1000	15 *****

Scale: 1 asterisk represents 3 units.

Canonical variate analysis

Significance tests for dimensionality greater than *k*

k	Chi-square	d.f.	pr.
0	53.31	16	<.001
1	9.40	7	0.225

Intergroup distances - Mahalanobis (D-squared)

< 2.1	0.0000		
2.1 - 5.1	1.8898	0.0000	
>= 5.1	2.0419	0.6101	0.0000
	< 2.1	2.1 - 5.1	>= 5.1

Table of counts for allocation of training units

FD_group3	< 2.1	2.1 - 5.1	>= 5.1	Total
< 2.1	36	3	3	42
2.1 - 5.1	11	17	9	37
>= 5.1	7	10	26	43
Total	54	30	38	122

Validation error rate

Using bootstrapping with 632 rule to calculate errors. Error: 45.45%

Percentage of each group allocated to groups

Decision	True group		
	< 2.1	2.1 - 5.1	>= 5.1
< 2.1	60.41	18.70	12.21
2.1 - 5.1	22.12	43.31	32.72
>= 5.1	17.48	37.99	55.07

Appendix 1B: POP36 (12) discriminant function analysis (log-transformed data)

Input: Parameters studied

Friction discolouration
Total soluble solids
Firmness
Polyphenol oxidase
Ascorbic acid
Chlorogenic acid
Cryptochlorogenic acid
Neochlorogenic acid
Catechin
Epicatechin
Procyanidin B2
Isorhamnetin 3-galactoside
Isorhamnetin rutinoside
p-coumaryl quinic acid
Quercetin galactoside
Quercetin glucoside
Quercetin arabinoside
Quercetin rhamnoside
Quercetin rutinoside
Quercetin

Comp_417.12 (1)
Comp_417.12(2)

Output: Summary information for stepwise selection of variables

Forward selection

Selection criterion: Wilks' lambda

Best 10 variables:

Variable	Criterion
PPO	0.92162
Quercetin	0.87602
Catechin	0.83770
Procyanidin B2	0.80683
TSS	0.77463
Quercetin rutinoside	0.75213
Firmness	0.73377
Quercetin rham noside	0.71741
Quercetin galactoside	0.70442
Quercetin glucoside	0.69104

Message: Default seed for random number generator used with value 527258

Optimal variables selected

PPO
Quercetin
Catechin
Procyanidin B2
TSS
Quercetin rutinoside

Discriminant analysis

Latent vectors, roots, and trace from CVA

Vectors:

Scores	Scores[1]	Scores[2]
PPO	-1.510	1.762
Quercetin	1.408	-1.859

Catechin	1.238	0.817
Procyanidin B2	-2.375	-0.389
TSS	7.821	5.623
Quercetin rutinoside	0.898	1.224

Roots:

Scores

Scores[1]	0.2919
Scores[2]	0.0292

Trace: 0.3210

Scree Diagram for POP369 (2012)

No	Root	%%	Cum %	Scree Diagram (* represents 3%)
1	0.2919	909	909	91 **********
2	0.0292		91	1000 9 ***

Scale: 1 asterisk represents 3 units.

Canonical variate analysis

Significance tests for dimensionality greater than *k*

k	Chi-square	d.f.	pr.
0	48.57	12	<.001
1	4.90	5	0.428

Intergroup distances - Mahalanobis (D-squared)

< 2.1	0.0000		
2.1 - 4.5	0.9889	0.0000	
>= 4.5	1.7178	0.3015	0.0000
	< 2.1	2.1 - 4.5	>= 4.5

Table of counts for allocation of training units

FD_group3	< 2.1	2.1 - 4.5	>= 4.5	Total
< 2.1	29	10	21	60

2.1 - 4.5	12	23	14	49
>= 4.5	4	14	49	67
Total	45	47	84	176

Validation error rate

Using bootstrapping with 632 rule to calculate errors, Error: 48.77%

Percentage of each group allocated to groups

Decision	True group		
	< 2.1	2.1 - 4.5	>= 4.5
< 2.1	59.40	24.29	23.39
2.1 - 4.5	28.42	41.78	24.65
>= 4.5	12.18	33.93	51.9

Appendix 1C: POP356 (2011) discriminant function analysis (log-transformed data)

Friction discolouration
Total soluble solids
Firmness
Polyphenol oxidase
Ascorbic acid
Chlorogenic acid
Cryptochlorogenic acid
Neochlorogenic acid
Catechin
Epicatechin
Procyanidin B2
Isorhamnetin 3-galactoside
Isorhamnetin rutinoside
p-coumaryl quinic acid
Quercetin galactoside
Quercetin glucoside
Quercetin arabinoside
Quercetin rhamnoside
Quercetin rutinoside
Quercetin
Comp_417.12 (1)
Comp_417.12(2)

Output: Summary information for stepwise selection of variables

Forward selection

Selection criterion: Wilks' lambda

Best 10 variables:

Variable	Criterion
Epicatechin	0.94397
Comp_417.12	0.92735
Quercetin galactoside	0.91386
Catechin	0.89848
Quercetin arabinose	0.88533
TSS	0.87193
Procyandin B2	0.86033
Chlorogenic acid	0.84005
PPO	0.83018
Quercetin	0.82600

Optimal variables selected

Epicatchin

Count of units with a complete set of variables

	Counts
FD_group3	
< 2.1	113
2.1 - 4.5	58
>= 4.5	35
Nobserved	206

Latent vectors, roots, and trace from CVA

Vectors:

Scores	Scores[1]
Epicatechin	1.070

Roots:

Scores
Scores[1] 0.05936

Trace: 0.05936

Canonical variate analysis

Significance tests for dimensionality greater than *k*

k	Chi-square	d.f.	pr.
0	11.71	2	0.003

Intergroup distances - Mahalanobis (D-squared)

< 2.1	0.00000		
2.1 - 4.5	0.25051	0.00000	
>= 4.5	0.21169	0.00163	0.00000
	< 2.1	2.1 - 4.5	>= 4.5

Table of counts for allocation of training units

FD_group3	< 2.1	2.1 - 4.5	>= 4.5	Total
< 2.1	84	34	19	137
2.1 - 4.5	15	17	10	42
>= 4.5	14	7	6	27
Total	113	58	35	206

Validation error rate

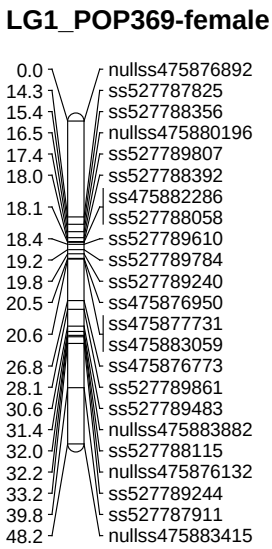
Using bootstrapping with 632 rule to calculate errors, Error: 49.33%

Percentage of each group allocated to groups

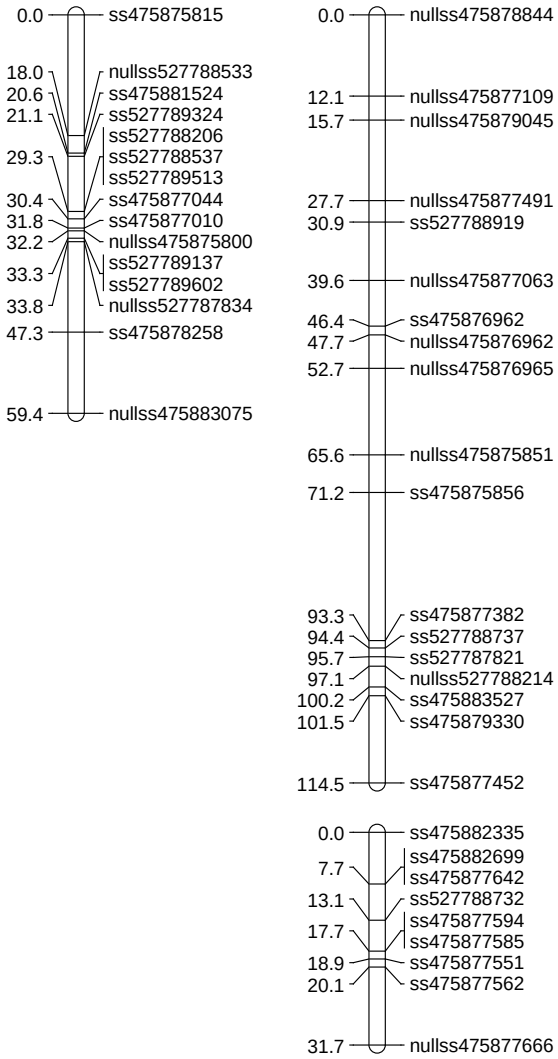
Decision	True group		
	< 2.1	2.1 - 4.5	>= 4.5
< 2.1	72.70	55.67	52.44
2.1 - 4.5	12.63	24.52	26.65
>= 4.5	14.68	19.81	20.90

Appendix 2A: Genetic linkage maps of male and female parents of POP369 used for QTL analysis.

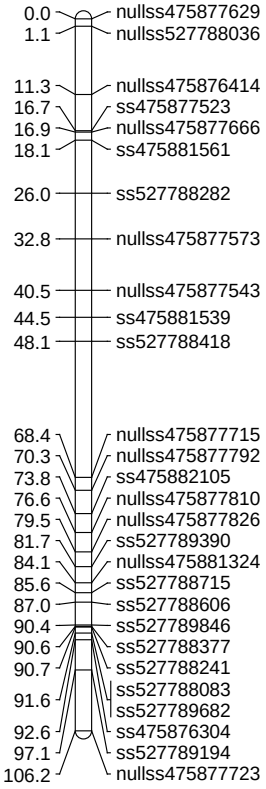
Maps for both parents for POP369 were reconstructed after filtering the maps presented in chapter 3. As the markers originally with segregation deviation from Mendelian ratio (3:1) were posing issues in statistical analysis.



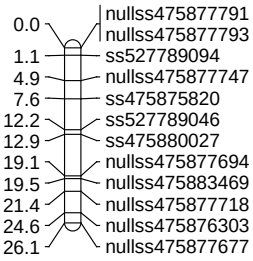
LG2_POP369-female LG2_POP369-male



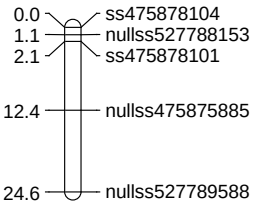
LG3_POP369-female



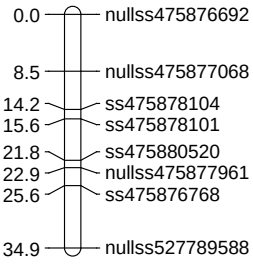
LG3_POP369-male



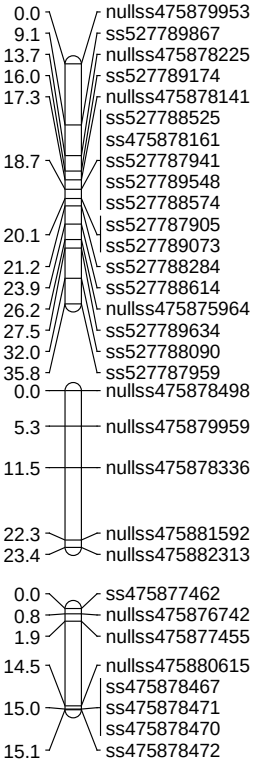
LG4_POP369-female



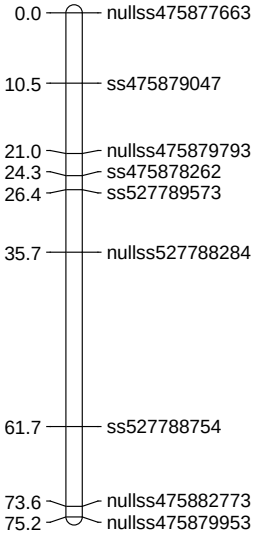
LG4_POP369-male



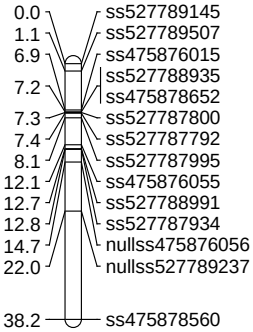
LG5_POP369-female



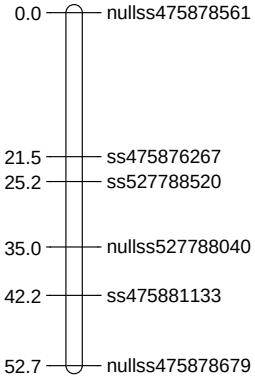
LG5_POP369-male



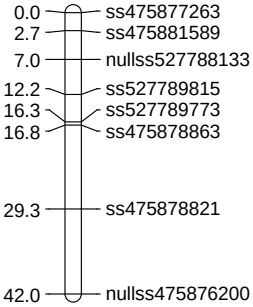
LG6_POP369-female



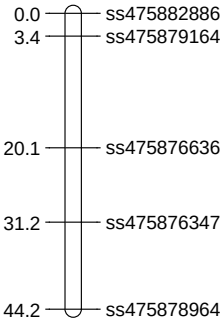
LG6_POP369-male



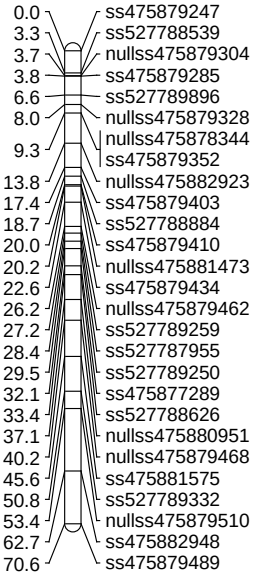
LG7_POP369-male



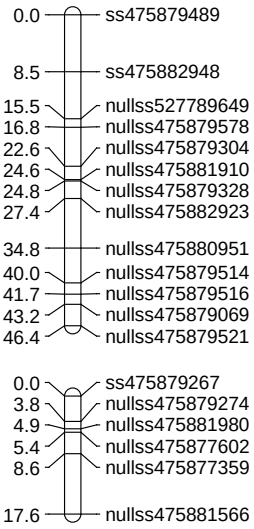
LG8_POP369-male



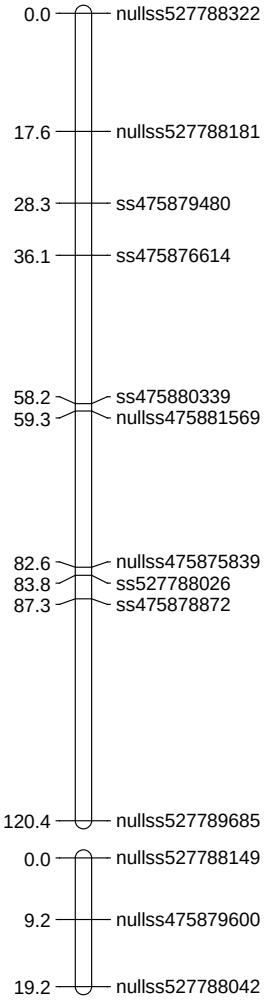
LG9_POP369-female



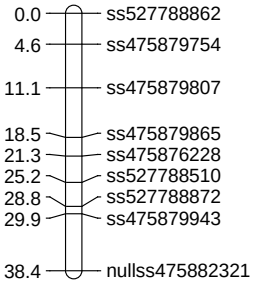
LG9_POP369-male



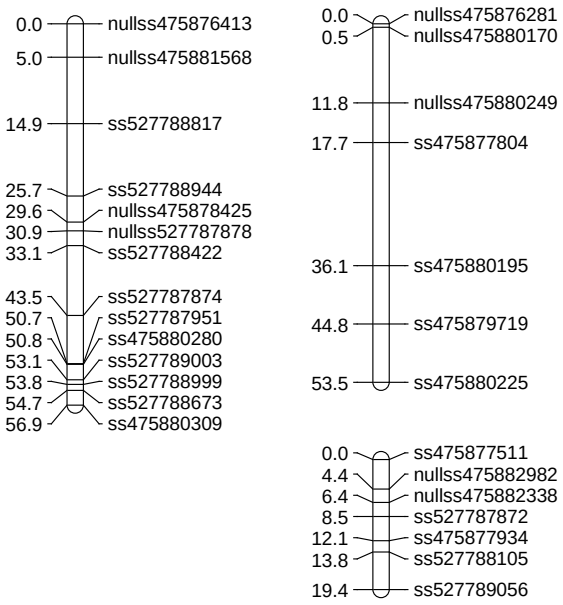
LG10_POP369-female



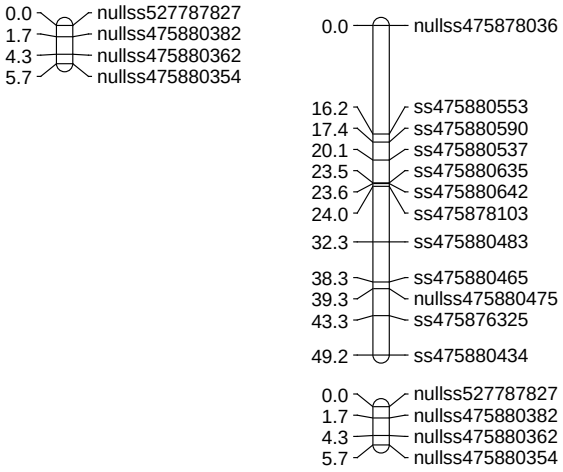
LG10_POP369-male



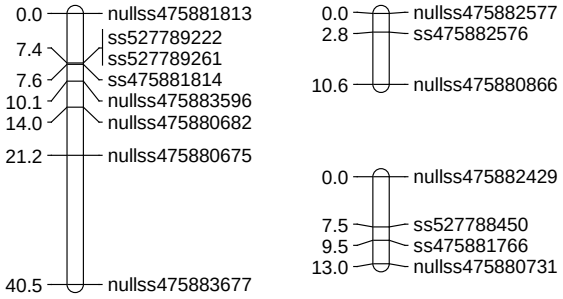
LG11_POP369-female **LG11_POP369-male**



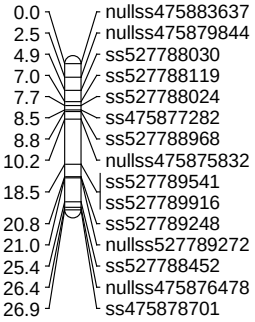
LG12_POP369-female **LG12_POP369-male**



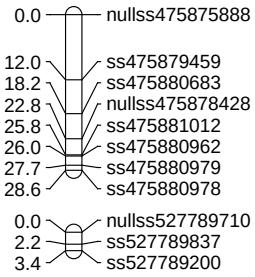
LG13_POP369-female **LG13_POP369-male**



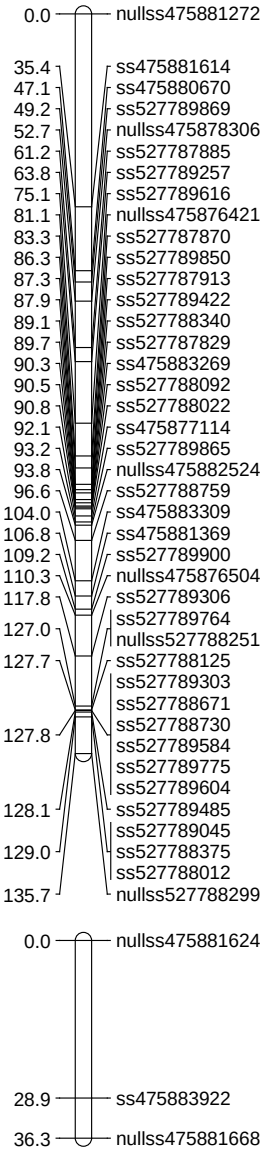
LG14_POP369-female



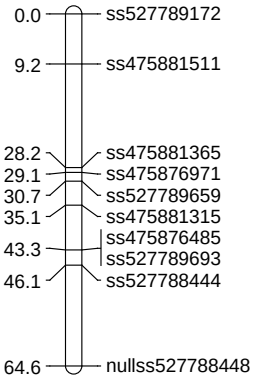
LG14_POP369-male



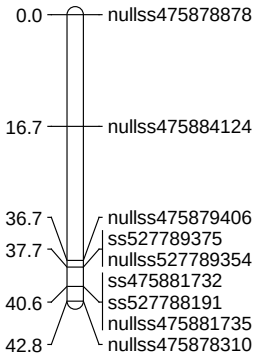
LG15_POP369-female



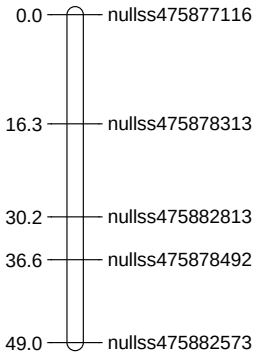
LG15_POP369-male



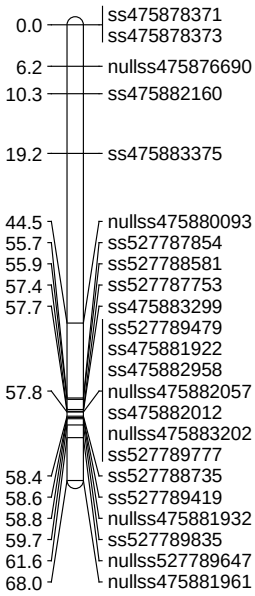
LG16_POP369-female



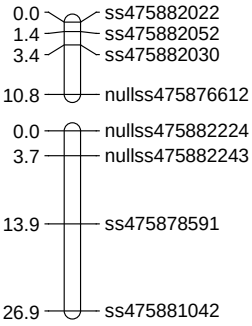
LG16_POP369-male



LG17_POP369-female

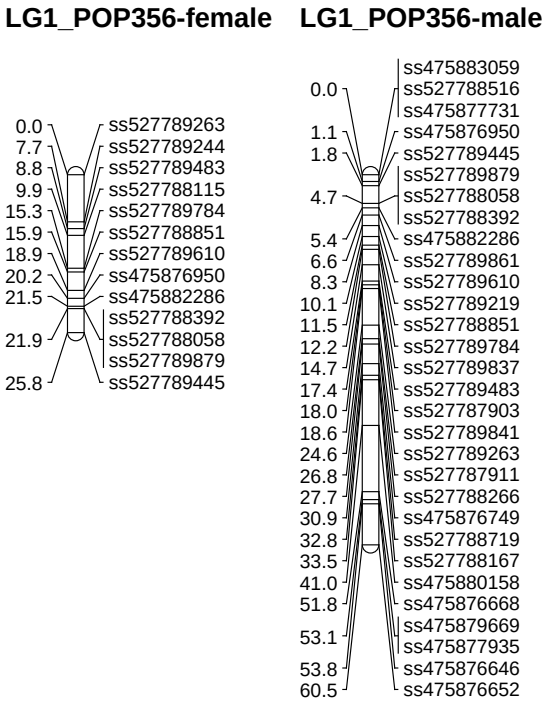


LG17_POP369-male

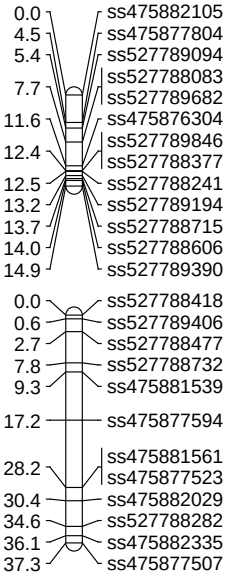


Appendix 2B: Genetic linkage maps of male and female parents of POP356 used for QTL analysis.

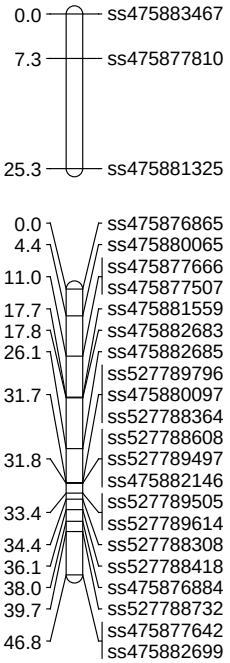
Maps for both parents for POP369 were reconstructed after filtering the maps presented in chapter 3. As the markers originally with segregation deviation from Mendelian ratio (3:1) were posing issues in statistical analysis.



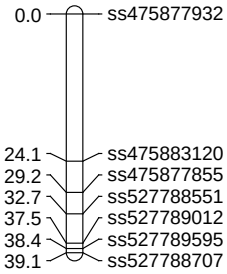
LG3_POP356-female



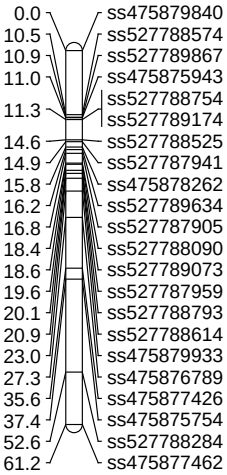
LG3_POP356-male



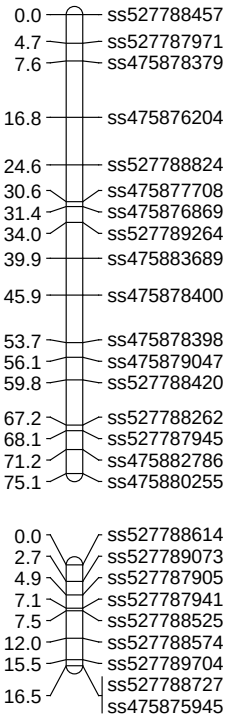
LG4_POP356-male



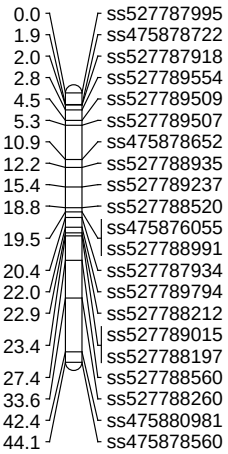
LG5_POP356-female



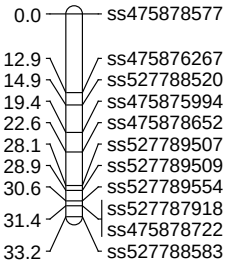
LG5_POP356-male



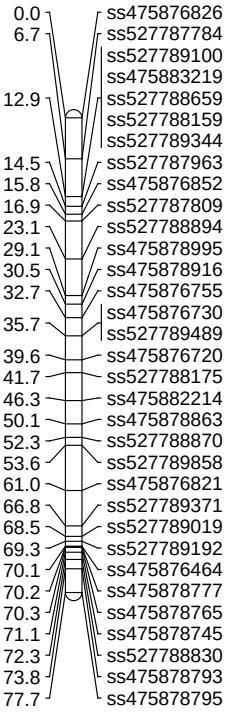
LG6_POP356-female



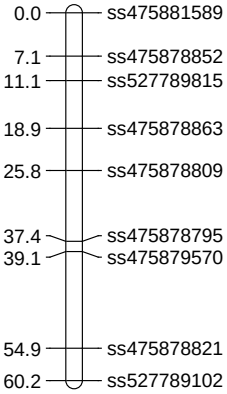
LG6_POP356-male



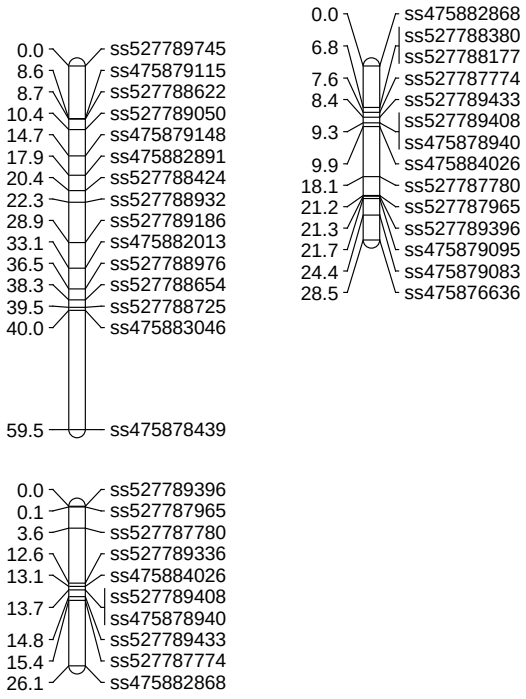
LG7_POP356-female



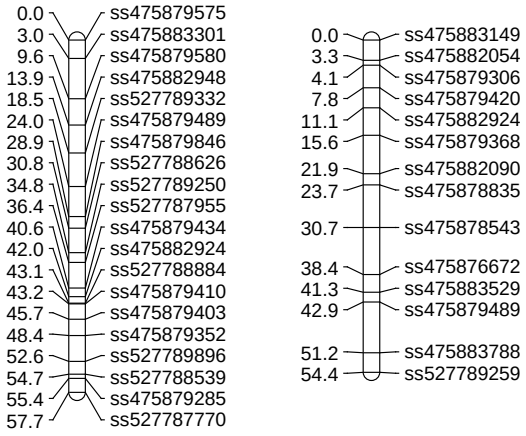
LG7_POP356-male



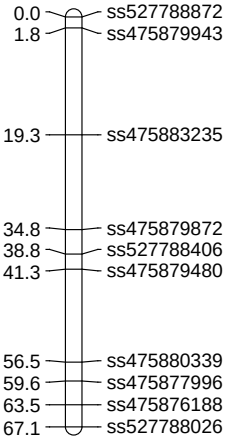
LG8_POP356-female **LG8_POP356-male**



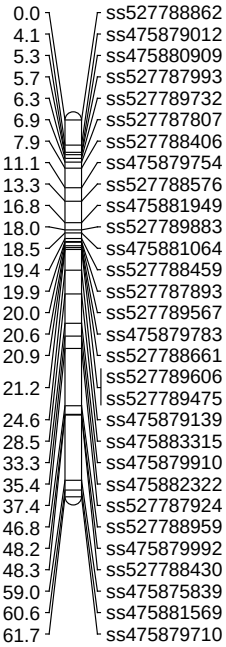
LG9_POP356-female **LG9_POP356-male**



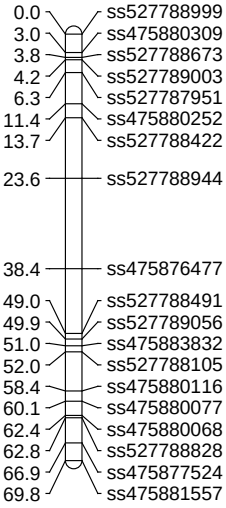
LG10_POP356-female



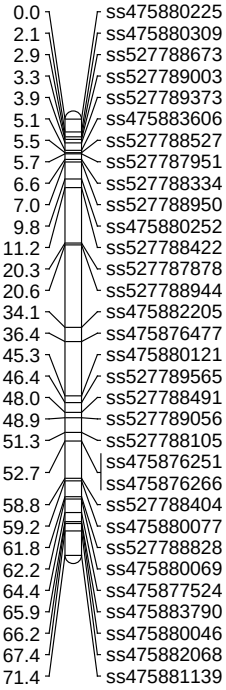
LG10_POP356-male



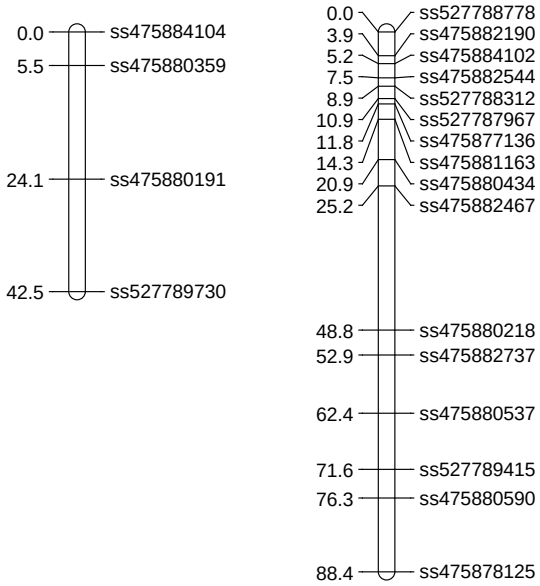
LG11_POP356-female



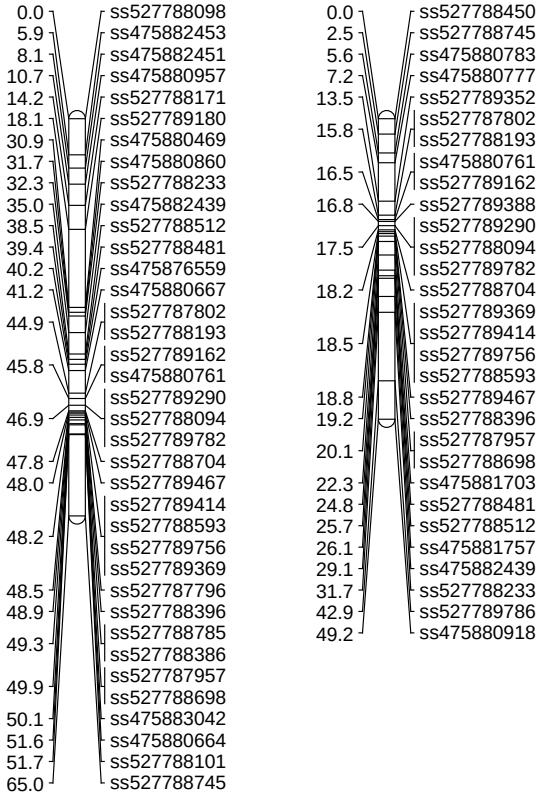
LG11_POP356-male



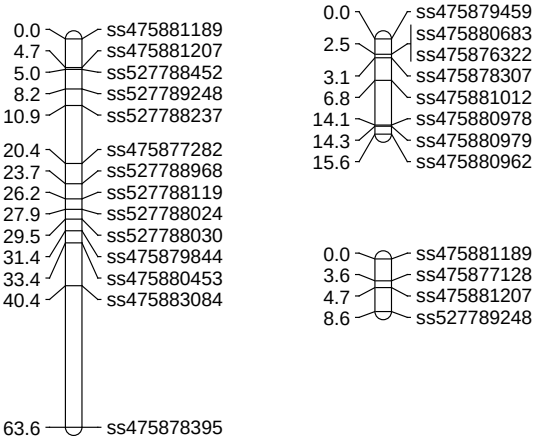
LG12_POP356-female **LG12_POP356-male**



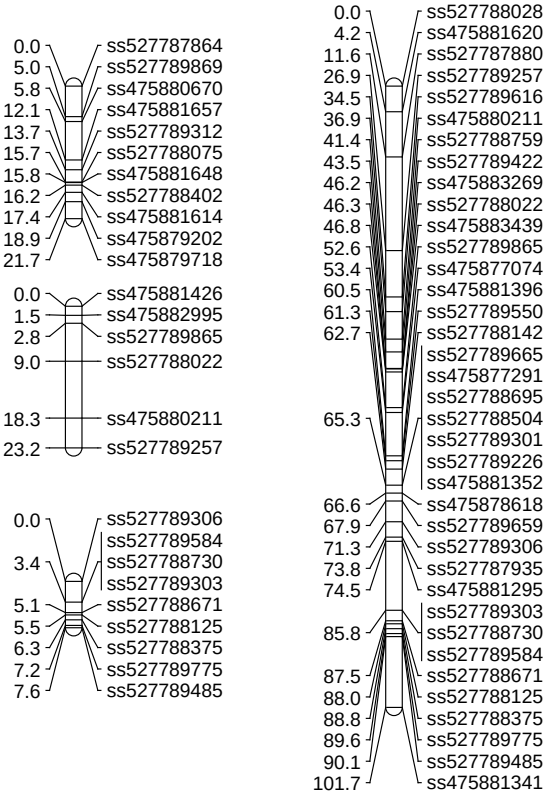
LG13_POP356-female **LG13_POP356-male**



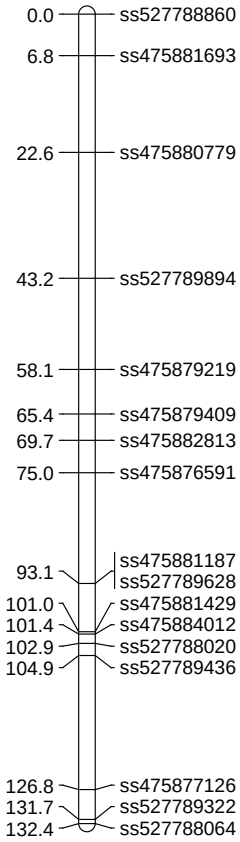
LG14_POP356-female **LG14_POP356-male**



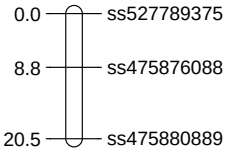
LG15_POP356-female **LG15_POP356-male**



LG16_POP356-male

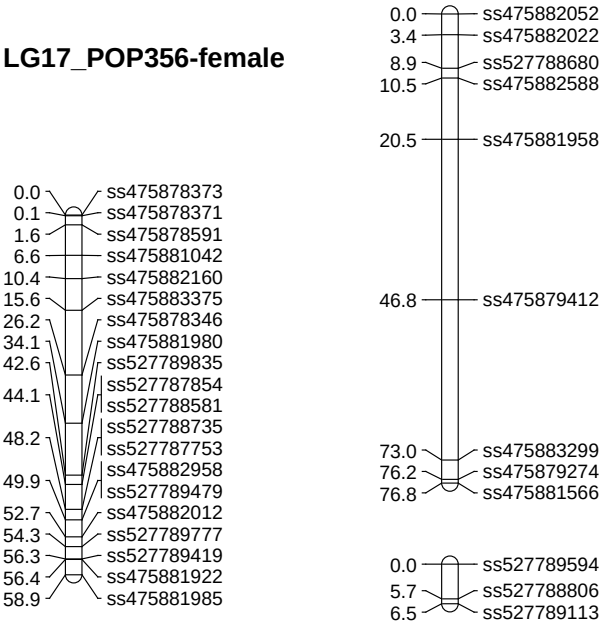


LG16_POP356-female



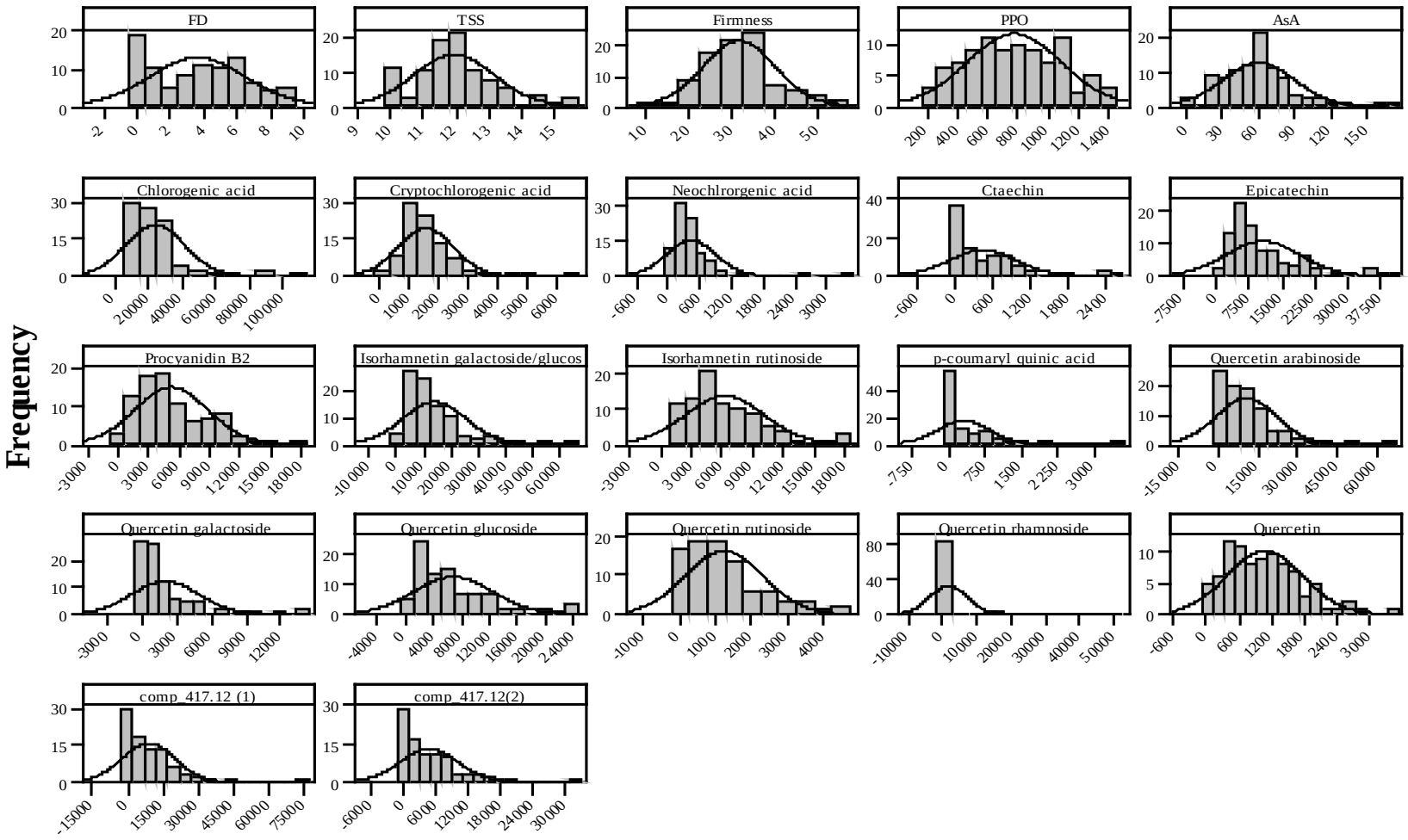
LG17_POP356-male

LG17_POP356-female

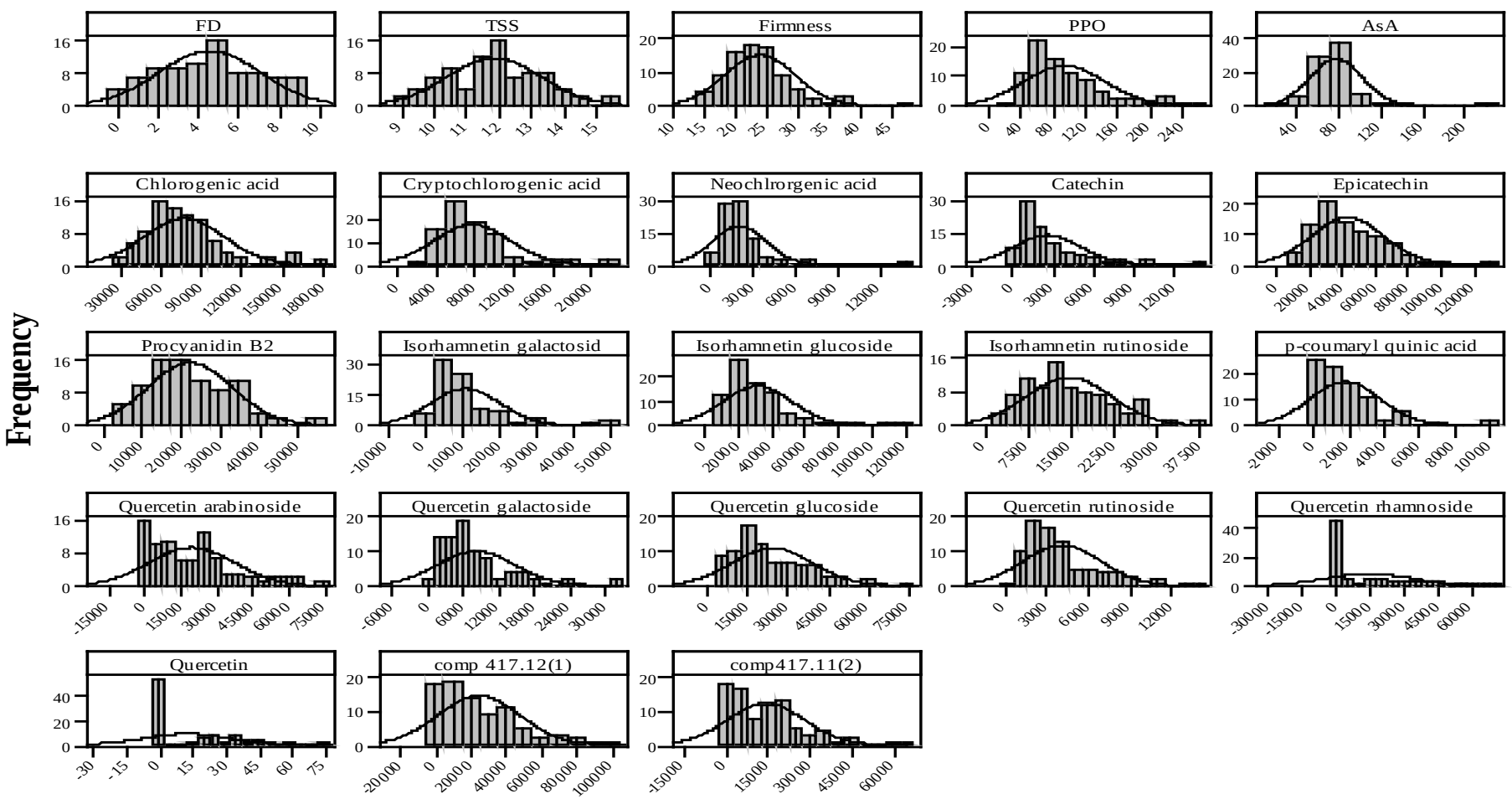


Appendix 3: Trait distribution in POP369_2011 (A), POP369_2012 (B) and POP356_2011 (C).

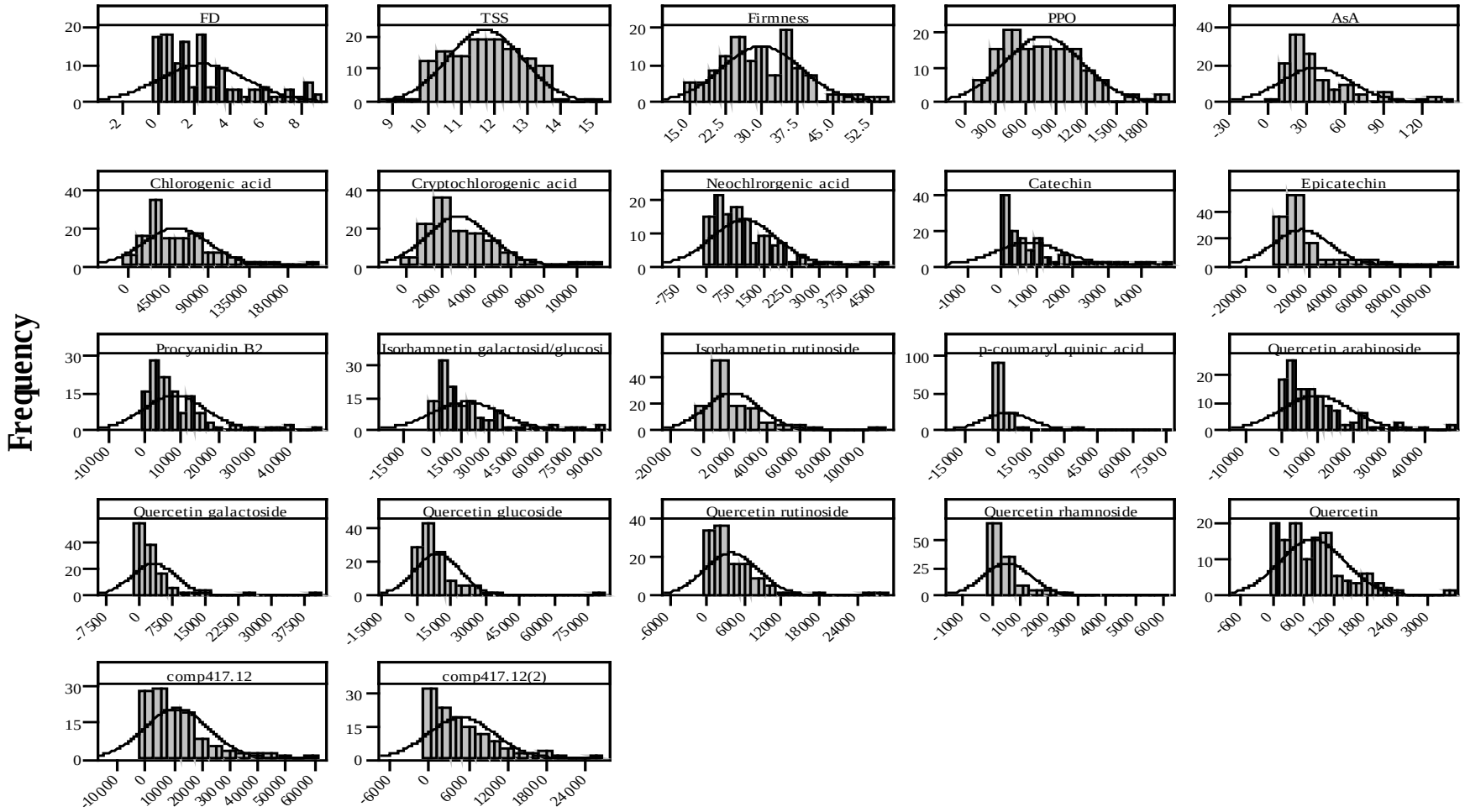
A



B


Frequency
FD
TSS
Firmness
PPO
AsA
Chlorogenic acid
Cryptochlorogenic acid
Neochlrorgenic acid
Catechin
Epicatechin
Procyanidin B2
Isorhamnetin galactosid
Isorhamnetin glucoside
Isorhamnetin rutinoside
p-coumaryl quinic acid
Quercetin arabinoside
Quercetin galactoside
Quercetin glucoside
Quercetin rutinoside
Quercetin rhamnoside
Quercetin
comp 417.12(1)
comp417.11(2)

C


Frequency
FD
TSS
Firmness
PPO
AsA
Chlorogenic acid
Cryptochlorogenic acid
Neochlrorgenic acid
Catechin
Epicatechin
Procyanidin B2
Isorhamnetin galactosid/glucosi
Isorhamnetin rutinoside
p-coumaryl quinic acid
Quercetin arabinoside
Quercetin galactoside
Quercetin glucoside
Quercetin rutinoside
Quercetin rhamnoside
Quercetin
comp417.12
comp417.12(2)