[1]GENETIC ANALYSIS OF RED PIGMENTATION IN 'BON ROUGE' PEARS (*Pyrus communis* L.).

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A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Science, University of the Western Cape

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ABSTRACT

Genetic Analysis of Red Pigmentation in Bon Rouge Pears (*Pyrus communis* L.)

Master of Science thesis, Department of Biotechnology, Faculty of Science, University of the Western Cape.

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European pear (*Pyrus communis* L.) is the third most important fruit in South Africa after citrus and apple. The Agricultural Research Council (ARC) breeding programme seeks to obtain a fully red coloured pear. Sports (mutants) with red skin and reddish leaves of various cultivars occur and some have been used in breeding programmes, where they transmit red colour as a single gene. The red trait in 'Max Red Bartlett', a mutant of 'Bartlett' (Synonym – 'Williams Bon Chretien'), was mapped in Italy to linkage group 4 (LG4). At ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa, 'Bon Rouge' pear, another red mutant of 'Williams Bon Chretien', was crossed with 'Packham's Triumph' generating an F1 population with a segregation of (54:71) red:green, approximating to a 1:1 ratio; indicating a simple Mendelian inheritance of the red trait. The aim of the study was to determine if the 'Bon Rouge' red colour trait maps to approximately the same position as the 'Max Red Bartlett' red colour trait on LG4, and if so, to identify SSR markers that are mapped closer to the red colour trait than were previously reported. The seven published pear and apple SSR markers mapped in the appropriate region of LG4 in pear and in apple maps were identified and screened in the parents and, where informative, were scored in 125 seedlings for co-segregation analysis. Single locus segregations were checked with JoinMap 4.1 and this program was also used to generate a genetic map for LG4 of the 'Bon Rouge' x 'Packham's Triumph' progeny using

the SSR markers and the red locus. Two linkage maps were constructed at a LOD threshold of 3 using the Kosambi mapping function, one each with the maximum likelihood and regression mapping algorithms. The genetic linkage map of LG4 of ‘Bon Rouge’ x ‘Packham’s Triumph’ consisted of seven SSR markers (2 from apple and 5 from pear). Markers CH01d03 and CH02c02b were mapped on the same position as the red trait in ‘Max Red Bartlett’ reported by Dondini *et al*. (2008) and four more markers were added. One of the newly mapped markers, NH011a has been found to be closely linked to the red trait, with an approximate distance of 4 cM. This marker can be used to indirectly select for the red gene in pear, for example to distinguish heterozygotes from homozygotes. This work sets the scene for further genetic studies on the red trait in pear breeding programmes.

DECLARATION

"I hereby declare that **Genetic Analysis of Red Pigmentation in Bon Rouge Pears (*Pyrus communis* L.)** is my own work and that, to the best of my knowledge, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma in any other university or institute of higher learning. All the sources used and cited have been duly indicated in text and acknowledged by complete references thereafter."

Sonwabo Booi November 2013

Signed:……

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I dedicate this thesis to my late-mother, Nomasomi Sylvia Mnini. The journey leading to completion of this thesis was filled with both joys and sorrows. I would like to especially thank the following people:

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ANS	Anthocyanidin synthase
ARC	Agricultural Research Council
ASAP	Allele-specific associated primers
bHLH	Basic helix loop helix
BR	‘Bon Rouge’
BR4(R)	Linkage Group 4 of ‘Bon Rouge’ using regression
BR4(ML)	Linkage Group 4 of ‘Bon Rouge’ using maximum likelihood
CHI	Chalcone isomerase
CHS	Chalcone synthase
cM	CentiMorgan
CR	Chilling requirement
DArT	Diversity array technology
DFR	Dihydroflavonol 4-reductase
DH	Double haploid
EST	Expressed sequence tag
F3’H	Flavanone 3-hydroxylase
g	Gram
G6P	Glucose 6-phosphate
hr	Hour
LG	Linkage group
LG4	Linkage group 4
LOD	Logarithm

LTR	Long terminal repeat
min	Minute
ML	Maximum likelihood method
MAS	Marker-assisted selection
mg	Milligram
ml	Millilitre
MRB	‘Max Red Bartlett’
MRB4	Linkage Group 4 of ‘Max Red Bartlett’
MYB	Myeloblastosis
NAD	Nicotinamide adenine dinucleotide
NAD-SDH	Nicotinamide adenine dinucleotide-sorbitol dehydrogenase
ng	Nanogram
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PDC	Pyruvate decarboxylase
PDH	Pyruvate dehydrogenase
PT	‘Packham’s Triumph’
QTL	Quantitative trait loci
RSA	Republic of South Africa
R	Regression method
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SCAR	Sequence characterised amplified region
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeat

TF	Transcription factor
TIR	Terminal inverted repeat
TSD	Target site duplication
UFGalT	UDP galactose: flavonoid 3-*O*-galactosyltransferase
ug	Microgram
ul	Microlitre
UV light	Ultra-violet light
USA	United States of America
w/v	weight per volume
v/v	volume per volume

CHAPTER 1: REVIEW OF LITERATURE

1.1 Botany and horticulture of pears

As one of the oldest fruit crops, pear has more than 3,000 years of cultivation history and is likely to have originated during the Tertiary period (65 million years ago) in southwestern China, Kazakhstan and Krygyzstan (Juniper *et al*., 2001). Pears are a member of the Rosaceae, or rose family, subfamily Maloideae (or Pomoideae) along with apple (*Malus pumila*), quince (*Cydonia oblonga*), loquat (*Eriobotrya japonica*) and chokeberry (*Aronia melanocarpa*). The subfamily Maloideae (x = 17) was long believed to be an allopolyploid that evolved from a hybridization between early members of the Spiraeoidae (x = 9) and the Prunoideae (x = 8) (Sax *et al*., 1933). More recently, it has been concluded that the Maloideae are a polyploid group member of the Spiraeoidae (Potter *et al*., 2007).

Cultivated pears are genetically diverse with more than 5,000 cultivars and accessions world-wide that can be divided into two major types, the European or Occidental pears (*P. communis* L.) and the Asiatic or Oriental pears, that includes Japanese pear (*Pyrus pyrifolia* Nakai), Chinese pear (*Pyrus bretschneideri* Rehd. and *Pyrus ussuriensis* Maxim.) (Fernandez-Fernandez *et al*., 2006). Species of *Pyrus* are generally diploid (2n = 34), although a few polyploid cultivars of European pear are known. Older cultivars of *P. communis* were of the firm, crisp type, while modern cultivars are softer and buttery. Pear, together with other pome fruits, is cultivated commercially in all temperate regions in more than 50 countries of the world (Bell *et al*., 1996). However, problems arise in warm regions and commercial cultivation there is more limited (Tromp *et al.*, 2005).

The genus Pyrus consists of 22 species originated in Europe, temperate regions in Asia and high-altitude locations in Africa, along with six natural interspecific hybrids. Among them, *P. communis* and *P. pyrifolia* are the most disseminated species, whereas *P. ussuriensis* and *P. xbretschneideri* are important for specific areas (Bell and Itai 2011). Genetic resources have not been fully explored due to the low morphological diversity, lack of differentiating characters among species and widespread crossability. There have been a few reports concerning the use of efficient molecular markers such as microsatellites for closely related genera in the Maloideae, *e.g. Sorbus* (Oddou-Muratorio *et al*., 2001) and *Pyrus* (Fernandez-Fernandez *et al*., 2006) to evaluate genetic diversity in particular.

1.2 Pear production and improvement in SoutH AfricA

South Africa is ranked seventh in the world in terms of European pear production, after Italy, USA, Argentina, Spain, India and Turkey, but is ranked fourth in terms of export after Argentina, Netherlands and Belgium. The pear industry is the third largest in the South African fruit industry after citrus and apples; it is very important in terms of size, fresh export and economic impact (Human, 2013). In 2012, it was reported that South Africa totalled 366,216 t from 11,561 ha of which 17% was distributed to the local fresh market, 43% was exported, 38% was canned and 2% was used for drying (Hortgro, 2012). Pears are planted in most of the deciduous fruit producing areas of South Africa, particularly in Ceres. Imported pear cultivars are usually poorly adapted to South African conditions, for example requiring more chilling units to break dormancy than are locally available.

According to a report released by Hortgro (2012), the most important cultivars in terms of number of trees in South Africa are: ‘Packham’s Triumph’ (green) at 29%; ‘Forelle’ (blushed) at 26%; ‘Bon Chretien’ (yellow) [synonym: ‘Williams’ or ‘Bartlett’] at 25%; and ‘Abate Fetel’ (green russetted) at 6%, of which ll of these pears are very old cultivars and the Agricultural Research Council (ARC), through its pear breeding programme, aims to develop new cultivars that will increase local consumption as well as increasing the export market for South African pears.

Blush pears are becoming popular with consumers, and may receive prices up to three times that of normal green/yellow types (Human, 2013). Red pears are on a down fall caused by the fading of the red colour towards harvest. Red and blushed pears acquire their red colour from anthocyanins present in their peel (Francis, 1970).

1.3 Anthocyanin

1.3.1 ANTHOCYANINS AND THEIR PURPOSE IN NATURE

In the literature, fruit skin colour has been frequently associated with the amount of anthocyanin and other antioxidants in fruit. Anthocyanins are water-soluble phenolic compounds and part of a large and widespread group of plant flavonoids. Anthocyanins are modified by glycosyl and aromatic or aliphatic acyl moieties, resulting in hundreds of anthocyanin molecules that differ in hue and stability. (Oren-Shamir, 2009). The anthocyanins responsible for the red skin colour of ‘Bon Rouge' and other fruits, belong to a class of flavonoids (Honda *et al.*, 2002). Other colour pigments that affect fruit colour are chlorophyll (green) and carotenoids (yellow) (Forkmann, 1993; Holton and Cornish, 1995). The accumulation of anthocyanins is also influenced by environmental stimuli, such as light, temperature and nutrition, as well as by genetic factors. Anthocyanins are also responsible for

the red colour in leaves, flowers and fruits. Saito and Yamazaki (2002) reported that anthocyanins are the main pigments in flowers and fruits and serve as visual signals to attract insects and animals for pollination and seed dispersal. Anthocyanins also play a role in photoprotection in autumn foliage and in the rapidly developing shoots of tropical trees.

Anthocyanin pigments accumulate in the epidermal cell vacuoles; their intensity and colour depends on external conditions, as well as on the microenvironment conditions in the vacuole (Harborne and Grayer, 1988). Unlike pigmentation in flowers and fruit, anthocyanin accumulation in leaves is affected by environmental stress. Since the pigments (chlorophyll, carotenoids) are the ones that absorb green/blue UV light, their accumulation possibly serves as an adaptive mechanism to protect plants from strong sunlight (Batschauer *et al*., 1996). Curry (1997) reported that low temperatures induced red skin colour development in many fruit crops, *e.g.*, apples, while Christie *et al*. (1994) showed that low temperatures also induced anthocyanin synthesis in vegetative tissues.

In apple, pear, and peach fruit, anthocyanin biosynthesis in the skin is enhanced by sunlight. However, anthocyanin biosynthesis and accumulation in blushed pears appears to be more complicated as the red skin of the fruit fades towards harvest and anthocyanin content decreases under strong light (Steyn *et al*., 2004). Evidence of the involvement of transcription factors such as MYB, basic helix loop helix (bHLH) or other transcription factors in anthocyanin biosynthesis has been reported to be lacking in pears and whether these genes are induced by sunlight remains unknown (Feng *et al*., 2010).

1.3.2 GENES INVOLVED IN ANTHOCYANIN BIOSYNTHESIS

The enzymes and genes involved in anthocyanin biosynthesis have been investigated extensively in petunia (*Petuia hybrida*), snapdragon (*Antirrhinum majus*) and maize as model plant species, resulting in the accumulation of knowledge regarding the biosynthetic pathway (Forkmann, 1993; Heller and Forkmann, 1988; Holton and Cornish, 1995). According to Saito and Yamazaki (2002), most genes for the biosynthetic enzymes have been isolated, and the biochemical reactions catalyzed by these enzymes in these model plants have been characterized. In addition, regulatory proteins and their genes were also isolated through analysis of genetic mutants, which exhibit altered flower colour. In most cases, these regulatory genes encoded transcription factors that control expression of the genes for biosynthetic enzymes (Mol *et al*., 1996; Mol *et al*., 1998; Winkel-Shirley, 2001).

Telias *et al*. (2011) stated that there are two categories of genes affecting the biosynthesis of anthocyanin. The first category encodes enzymes that are required for pigment biosynthesis. The second category is comprised of transcription factors, which are regulatory genes that influence the intensity and pattern of anthocyanin accumulation and control transcription of different biosynthetic genes.

Anthocyanin pigments are produced from phenylalanine via the flavonoid biosynthesis pathways involving at least seven key enzymes in pear. Most enzymes in the anthocyanin biosynthetic pathway in apple are well known (Fischer *et al*., 2007). The following are key enzymes involved in the production of anthocyanin that have already been identified: chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), chalcone isomerase (CHI), dihydroflavanol 4-reductase (DFR), anthocyanin synthase (ANS), and UDP glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) (Honda *et al.*, 2002). However, compared with apple and grape, few studies have been carried out to investigate the anthocyanin pathway in pear (Espley *et al*.,

2007; Kobayashi *et al*., 2002). Key genes controlling the formation of red skin are still unknown (Wu *et al*., 2013). Studies have indicated that some of the genes involved in the anthocyanin biosynthesis pathway may be regulated by a transcription factor of the MYB family, as in apples (Chagne *et al.,* 2007; Espley *et al.,* 2007). However, none of the transcription factors that regulate the expression of anthocyanin biosynthetic enzymes have been identified in pears.

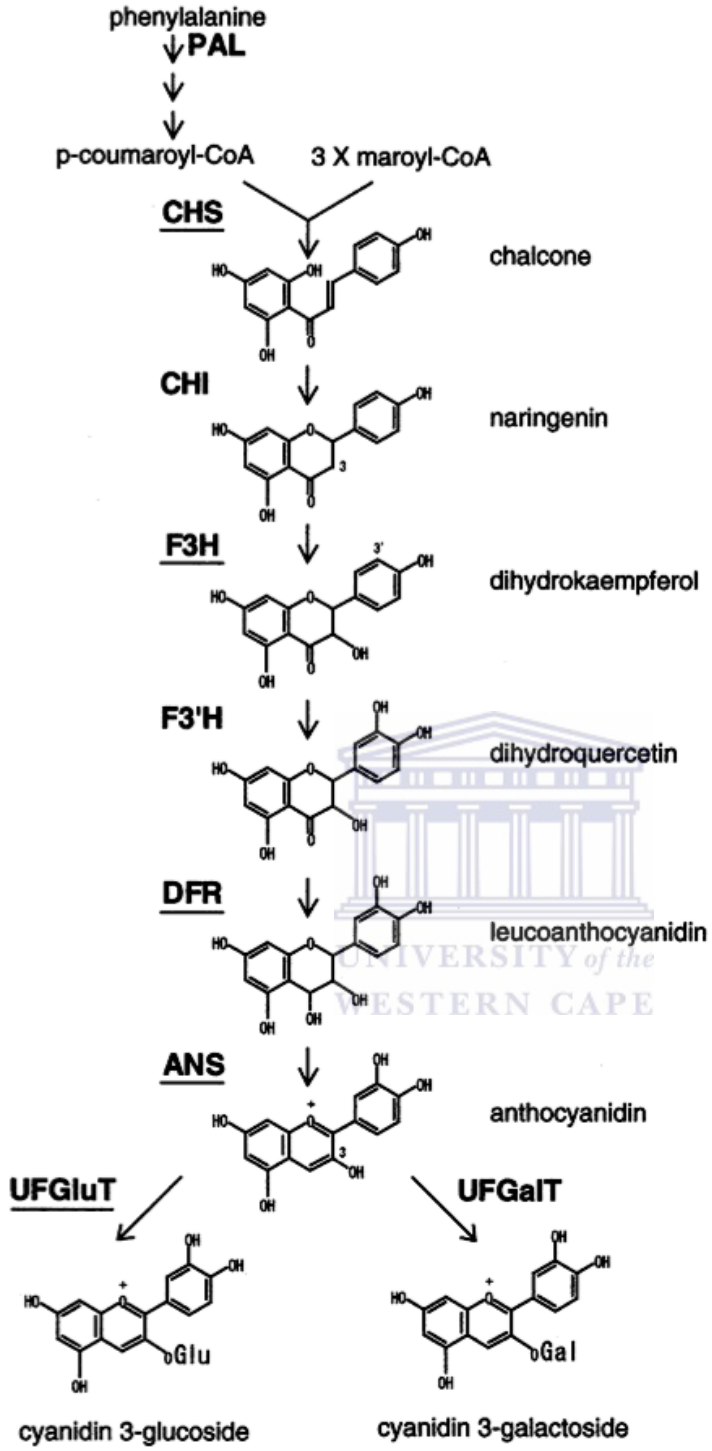


Figure: 1.1 Putative anthocyanin biosynthetic pathways in apple and pear skin, CHS = chalcone synthase, CHI = chalcone isomerase, F3H = flavanone 3-hydroxylase, F3'H = flavanoid 3'-hydroxylase, DFR = dihydroflavanol 4-reductase, ANS = anthocyanin synthase, UFGluT = UDP glucose:flavanoid 3-*O*-glucosyltransferase, UFGalT = UDP galactose:flavanoid 3-*O*-galactosyltransferase, glu = glucose, gal = galactose (Honda *et al*., 2002).

1.3.3 REGULATION OF RED COLORATION BY MYB TRANSCRIPTION FACTOR

1.3.3.1 Apple MYB transcription factor, MdMYB10

Three transcription factors (TFs), such as R2R3 MYB, bHLH (basic helix loop helix) and WD40 proteins, predominantly regulate genes in the anthocyanin biosynthesis pathway across all plant species reported to date, including apple. MYB TFs have been reported to play diverse functions in controlling pathways such as secondary metabolism, development, signal transduction, and disease resistance in plants. MYB TFs are classified by the number of highly conserved imperfect repeats in the DNA-binding domain, and consisting of either single or multiple repeats. Among these MYB TFs, the class of two repeats (R2R3) is deemed the largest, with 339 TFs reported in Arabidopsis and is associated with the anthocyanin biosynthesis pathway. Regulation of R2R3 MYB TFs can occur at different steps of the anthocyanin biosynthesis pathway. In perilla (*Perilla frutescens*), the R2R3 MYB TFs control transcription of all structural genes involved in anthocyanin biosynthesis (Vimolmangkang *et al.,* 2013). MYB transcription factors have been shown to induce anthocyanin accumulation in both heterologous (HET:tomato) and homologous (HOM:apple) systems, generating pigmented patches in transient assays in tobacco leaves and highly pigmented apple plants following stable transformation with constitutively expressed MdMYB10 (Chagne *et al*., 2007).

Takos *et al*., (2006) noted that the MdMYB10 regulatory gene activated promoters of structural genes in anthocyanin synthesis in transient assays and one allele of that gene segregated with skin colour in apple seedlings derived from cultivars with different skin colour. Chagne *et al*. (2007) mapped the MdMYB10 transcription factor to linkage group 9 in the apple progeny ‘Discovery’ x 91.136 B6-77, a red-flesh and red-leaf seedling from the open pollination of the red-flesh and red-leaf cultivar ‘Redfield’. The same authors mapped two

additional members of the MYB family to linkage groups 3 and 14, indicating that these genes are present in many copies at different loci in the apple genome (Chagne *et al.*, 2007; Takos *et al.*, 2006).

1.3.3.2 PEAR MYB TRANSCRIPTION FACTOR, PYMYB10

Anthocyanin is the main component of red skin colour in pears, and the degree of red coloration is determined by the content and composition of anthocyanins (Steyn *et al*., 2005). In pears, Cyanidin 3-galactoside and peonidin-3-galactoside were the two main anthocyanins responsible for red coloration in pear skins (Dayton, 1966; Dussi *et al*., 1995). A pear R2R3-MYB transcription factor gene, *PyMYB10*, was isolated from Asian pear (*Pyrus pyrifolia*) cultivar 'Aoguan' by Feng *et al*. (2010). After analyzing its sequence it was suggested that the *PyMYB10* gene was an ortholog of the *MdMYB10* gene, which regulated anthocyanin biosynthesis in red fleshed apple, 'Red Field'. A quantitative real-time PCR was conducted on pear which revealed that *PyMYB10* was expressed in pear skins, buds and young leaves, and that the level of transcription in buds was higher than in skin and young leaves.

The transcription of *PyMYB10* and genes of anthocyanin biosynthesis were more abundant in red-skinned pear cultivars compared to blushed cultivars. Feng *et al*. (2010) concluded that anthocyanin biosynthesis in pear was regulated by transcription factor *PyMYB10*. However, the research for the mutation of 'Williams' to 'Max Red Bartlett' revealed that *PyMYB10* was not directly responsible for red versus yellow colour, because the genes responsible for the colour difference were mapped to LG4, while *PyMYB10* was located at the terminal of LG9 (Pierantoni *et al*., 2007). These findings indicate that the anthocyanin biosynthesis pathway in *Pyrus* is more complicated than in apple (Wu *et al*., 2013). Therefore, further studies are needed to characterize the genetic basis of red colour in pear.

1.3.3.3 ANTHOCYANIN DEGRADATION

Anthocyanins often accumulate transienly, appearing and disappearing during plant development or with changes in environmental conditions. Anthocyanins are degraded in plants in two ways; during development and due to changes in environmental conditions (Oren-Shamir, 2009). Marias *et al*. (2001) reported that exposure to light for 144h at 37°C reduced the anthocyanin content of detached 'Cripps Pink' apples by more than half, but anthocyanin levels remained unchanged at the same temperature when fruit trees were shaded. Earlier, Dussi *et al.* (1995) conducted a study on 'Red Bartlett' pear trees where the trees were partially shaded for one month before harvest. The shading of the pears reduced the loss of anthocyanins and red colour occurring towards harvest in exposed fruit. Steyn *et al*. (2004) confirmed that high temperatures (30°C) accelerated the degradation of anthocyanins and the fading of red colour in detached fruit of 'Forelle' pears and in 'Royal Gala' apples. Light increased the rate of anthocyanin degradation and colour loss in 'Royal Gala'.

Anthocyanin degradation can be enzymatic, an example would be in the case of Cabernet Sauvignon grapes, anthocyanin degradation does not result in a dramatic change in colour as the pigments continue to be synthesized in parallel to their catabolism (Oren-Shamir, 2009). In this case, the degradation may be due to lower chemical stability of the pigments at elevated temperature.

Therefore, it is of importance to research the coloration mechanism with the aim of improving or changing the colour and anthocyanin content of fruits via breeding or gene engineering approaches.

1.4 Red skinned mutants in pear

In fruit crops the peel colour is an important factor of appearance that affects market price and consumer acceptance. Usually, brightly-coloured fruits are preferred by consumers. The colour of fruit skin in pears can be green, brown or red. There are several red cultivars which arose as mutants: *e.g.* 'Red Bartlett', 'Red Anjou', 'Starkrimson' (fully red sport of 'Clapp's Favourite'), 'Red Doyenné Du Comice', 'Cu miezul rosu', 'Max Red Bartlett', 'Rosi-Red Bartlett', 'Royal Red Hardy', 'Bon Rouge' (red sport of 'Williams Bon Chretien') (Braniste and Budan, 2009; Brown, 1966; Dondini *et al*., 2008; Francis, 1970; Jolly, 1993a; Lespinasse and Guerif 2011; Zielinski, 1963,).

The red colour in European pears originated from an orchard of 'Bartlett' pears, owned by A.D MacKelvie of Zillah in Washington, U.S.A. The orchard was planted in 1913, a bud mutation was noted from one of the 'Bartlett' trees, the mutation had red fruit and red leaves (Reimer, 1951). The mutation appeared as one small lateral branch approximately seven feet above the base of the tree. The remainder of the framework branch produced typical fruit above and below the mutation. The mutant was named 'Max Red Bartlett'. A controlled cross which involved 'Max Red Bartlett' and 'Winter Nelis', a variety with practically no red colouring, produced 27 seedlings with red leaves and 32 with green, suggesting a 1:1 segregation (X^2 = 0.4238; P = 0.50) and was attributed to the gene *C* for Cardinal Red (Reimer, 1951).

The red colour gene imported a deep red pigmentation to the epidermis of the leaf, stem and fruit, which persisted throughout the growing season (Brown, 1966). Zielinski (1963) presented further data that the variety 'Max Red Bartlett', carried the red colour gene in the heterozygous condition (*Cc*): in open pollinated seedlings of 'Max Red Bartlett', 43.7% of

the seedlings were red fruited and about 47% of the seedlings had red leaves. Bellini and Nin (2002) observed that 'Max Red Bartlett' reverted to its green parent phenotype, 'Williams Bon Chretien' in whole branches, or individual fruits or in certain sectors of fruit skin or its whole surface.

'Bon Rouge', a South African 'Williams Bon Chretien' bud mutant, , is a red skinned variety that was found by Mr Danie Marais on his farm Ongegund, Simondium as a bud mutation on a 'Williams Bon Chretien' tree. It was evaluated during the mid to late 1960's and was found to be a pear with good qualities (*e.g.* very good, distinctive flavor and a smooth, juicy texture). The 'Bon Rouge' tree is however also inclined to revert, producing fruit with little red to green colour (Jolly, 1993b). 'Bon Rouge' pears are characterized by red skinned fruit throughout the growing season and red leaves early in the season. Loss of red colour in leaves, and fruit skin colour at the ripening stage is duu to the disappearance of anthocyanins when synthesis falls below the rate of pigment turnover (Steyn et al., 2004).

According to Booi *et al.* (2005), 'Bon Rouge' pear trees show a high level of reversion to the parent phenotype that manifests as red and green sectors on leaves and fruit skin, or completely green leaves and fruit skin. An understanding of the genetic background of the red to green reversion in 'Bon Rouge' followed a 1.1 segregation pattern of the phenotypic trait in the F1 progeny of a cross between 'Bon Rouge' and 'Packham's Triumph' (Booi *et al.,* 2005; du Preez *et al.,* 2004). The phenomenon was seen either as branches of green fruits on a tree of red fruit, or as 'harlequin', fruits which were half-red and half-green. When 'Bon Rouge' was crossed with the green pear cultivar 'Packham's Triumph', the progeny segregated for anthocyanin production in leaves 23:25, indicating that a single locus controlled pigment production (Booi *et al*., 2005).

‘Royal Red Hardy’, is another red pear bud mutation, originated from ‘Beurre Hardy’ (Reimer, 1951). The mutant produced fruits entirely covered with dull red skin, and the young shoots, at least in the early stages of growth, also had reddish bark and leaves. The variety ‘Beurre Hardy’ itself produces greenish-yellow fruit with green leaves and bark. Another red type pear, with red fleshed fruit, red flower buds, red fruitlets, and a trace of red in the young shoots, ‘Sanguinole’ was crossed with ‘Conference’, a normal unpigmented flesh pear (Brown, 1966). From that cross a progeny of 40 seedlings were raised. Twenty three of the seedlings bore fruit with normal flesh colour and seventeen had red-fleshed fruits suggesting a 1:1 ratio (X^2 = 0.9; P = 0.3-0.5).

Dayton (1966) reported that pear variety ‘Starkrimson’, a mutation from ‘Clapps Favorite’, differed from all other red sports in having approximately 50% of cells in the outer hypodermal layer coloured with anthocyanin whereas other red sports had anthocyanin only in the second histogenic layer. Reimer (1951) also confirmed that crosses performed between ‘Max Red Bartlett’ strain and various other green cultivars produced about 50 % red seedlings; indicating a dominant control of this character.

Pear red sports have been used in various breeding programmes and various new skinned cultivars from controlled crosses have been released, ‘Red Doyenne du Comice’; ‘Red Hardy’; ‘Reimer Red’; ‘Starkrimson’; ‘Red D’ Anjou’ were examples of pear red sports used in breeding programmes (The Brooks and Olmo, third edition). The South African breeding programme and the IRTA Plant & Food Research programme have important breeding objectives for red colour development adapted for hot summer climates due to low chilling units required to break dormancy in those regions (Batlle *et al*., 2008).

The Agricultural Research Council (ARC), through its pear breeding programme at Infruitec-Nietvoorbij, aims to develop new cultivars of European pear – especially blushed types that will increase local consumption as well as increase the export market for South African pears. The ARC has already released several blushed cultivars: 'Rosemarie' in 1990 (Jolly *et al*., 1990), 'Flamingo' in 1993 (Jolly, 1993a) and, more recently, 'Cheeky' in 2009 (Human and von Mollendorff, 2009). Pears with red skin colour exist, but their supply is limited (Tao *et al*., 2004). So, in recent years, red skin pear (*Pyrus communis* L.) breeding and cultivation has become a dominant trend locally and the rest of the world (Wu *et al*., 2013).

1.5 Nature of mutation in 'Bon Rouge' pear

According to Flavell *et al.* (1994), there are two main classes of transposable elements according to whether their transposition intermediate is RNA or DNA, designated as retrotransposon (class 1) or DNA transposon (class 2), respectively. The class 1 has been reported to be the most abundant and wide-spread class of eukaryotic transposable elements, consisting of either a long terminal repeat (LTR) or non-LTR retrotransposons. Xu and Dooner (2005) grouped the DNA transposable elements into families based on sequence similarity of the homolog of the terminal inverted repeat (TIR) and the specific number of nucleotides comprising the target site duplication (TSD) caused by insertion of the element into host genomic DNA.

In pear, the reversion of red 'Bon Rouge' pear to its green 'Bon Chretien' phenotype or stripes characteristic of the red to green reversion in skin of mature 'Bon Rouge' pear fruit can be ascribed to response to stress, perhaps movement of transposable elements within the pear genome (du Preez *et al.* (2004). According to Telias *et al.* (2011), some apple (*Malus* ×

domestica Borkh.) varieties displayed attractive striping patterns and most apple cultivars (*e.g.* 'Royal Gala') produced fruit with a defined fruit pigment pattern, but in the case of 'Honeycrisp' apple, trees produced fruits of two different kinds: striped and blushed. The causes of this phenomenon were unknown. In their study, a conclusion was drawn that striped areas of ‘Honeycrisp’ and ‘Royal Gala’ were due to sectorial increases in anthocyanin concentrations.

Anthocyanin levels between red and green stripes could be explained by differential transcript accumulation of *MYB10*. Different levels of *MYB10* transcript in red versus green stripes were inversely associated with methylation levels in the promoter region. Although observed methylation differences were modest, trends were consistent across years and differences were statistically significant. It was evident that methylation may be associated with the presence of a (terminal-repeat retrotransposon in miniature) TRIM retrotransposon within the promoter region, but the presence of the TRIM element alone could not explain the phenotypic variability observed in 'Honeycrisp'. Therefore it was suggested that methylation in the *MYB10* promoter was more variable in 'Honeycrisp' than in 'Royal Gala', leading to more variable color patterns in the peel of this cultivar.

1.6 Molecular Markers

1.6.1 TYPES OF MOLECULAR MARKERS

DNA polymorphisms are allelic differences in the DNA sequence and are useful markers for the identification and characterization of plants and for developing genetic maps. It is useful to review briefly the main molecular markers available. The major nuclear markers include RFLPs, RAPDs, AFLPs, SNPs, DArTs and SSRs. In addition to these DNA markers, there are isozymes, which are protein markers based on multiple forms of an enzyme, which differ in electrophoretic mobility (Reish, 2000).

1.7 SSR technology

Microsatellites, or simple sequence repeats (SSRs) are stretches of DNA, *e.g.* $(TG)_n$ or $(AAT)_n$, consisting of tandemly repeating mono-, di-, tri-, tetra or pentanucleotide units, which are highly polymorphic, co-dominant inheritance and a large number of alleles per locus, abundant and fairly evenly distributed throughout the euchromatic part of genomes (Bruford and Wayne, 1993; Powell *et al*., 1996). Microsatellites have become the preferred genetic markers in animal and plant species not only because of their co-dominance and high degree of polymorphism but also because of their suitability for automation. Microsatellites make use of PCR, a single technique for which only a small amount of DNA is required and transferable among species in the same genus, even among genera in the same family (Yamamoto *et al*., 2001).

Technical problems such as PCR artifacts (stutter bands) complicate the automated scoring of microsatellite alleles. Stutter bands can appear along with the peak corresponding to the expected allele. This will cause uncertainty in the estimation of the allele size and also increase

the possibility of mistaking a heterozygote for a homozygote, if the two peaks are so close on the output from the automated machine that the ladders produced by the two alleles overlap. The problem can be overcome by running an internal standard (an allele of known size), plus a molecular size marker, along with the test sample. The standard will help to identify the "true" band among those amplified in the PCR.

Another problem that needs to be taken into account when using SSR loci is the presence of null alleles. Null alleles are not amplified and therefore are not scored at all on the automated machines; this can lead to underestimates of heterozygosity.

Some of the drawbacks about SSRs include the following: a lengthly and costly development phase and a relatively low throughput because of difficulties for automation and data management, especially when compared to SNPs. Hence the continued use of SSRs will depend on the possibility to overcome some of these limitations. Progresses in SSR development and genotyping have been made in several directions, suggesting that SSRs could remain relevant genetic markers, at least for specific applications. First, the emergence of next-generation sequencing technologies means that identifying SSRs has become cheaper and faster (Santana *et al.*, 2009). Second, multiplexing SSRs has become much easier. It can be accomplished through the co-amplification of multiple SSRs in a single PCR cocktail, a procedure called true multiplexing. Alternatively, PCR products from multiple amplification reactions can be combined in a single lane, a procedure called pseudo-multiplexing or poolplexing (Meudt and Clarke 2007). A combination of the two approaches is also possible. In true multiplex PCR, more than one target sequences are amplified by including more than one pair of primers in the reaction. Capillary electrophoresis equipments relying on automated laser-induced fluorescence DNA technology have facilitated the use of multiplexing. Loci with non-overlapping allele size ranges are labeled with the same fluorescent dye, whereas those

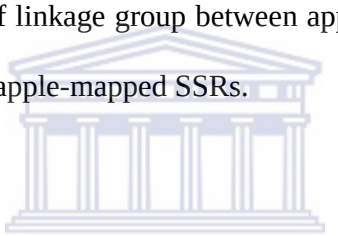
with overlapping allele size ranges are labeled with different dyes and resolved individually because of the different characteristic emission spectrum of each dye, hence considerably expanding multiplexing potential. In addition, one of the dyes is used as an in-lane size standard, improving the sizing precision of alleles. Multiplex PCR now forms the basis for many studies, on both diploid and polyploidy species, reducing very significantly the cost and time of genetic analyses (Jewell *et al.,* 2010).

1.7.1 SSR MARKERS AND THEIR APPLICATION IN POME FRUIT

DNA-based molecular markers have been used for cultivar and species identification in in pome fruit. Apple and pear are both classified in family Rosaceae. And it is well-known that apple SSRs can be successfully transferred to study the cultivar identification, parentage analysis, genetic diversity and map construction (Lu *et al.,* 2010).

A study was conducted where SSR markers were used to identify Chinese white pear cultivars. Nine SSR loci developed from apple and pear were used to identify 109 *Pyrus* accessions ncluding 92 loca Chines accessions of *P. bretschneideri*. The nine SSR loci revealed 129 alleles in 109 pear accessions and 114 alleles in 92 Chinese white pears. Among the 92 local Chinese accessions, 70 could be differentiated successfully except for 10 sets of synonymous or mutants.For the 92 accessions, the number of putative alleles per locus ranged from 7 to 18, with an average of 12.67; the average values of observed heterozygosity (Tian *et al.,* 2012). Also, SSR markers have been used to construct genetic linkage maps (Dondini *et al.,* 2008, Nishitani *et al*., 2009).

A comparative genome mapping between apple and pear maps using apple mapped SSR markers was conducted by Lu *et al.* (2010). Twenty nine apple-mapped SSRs were screened out to construct pear segment map of 'Mishirazi' (*P. pyrifolia* x *P. communis*) x 'Jinhua' (*P. bretschneideri*). The 'Jinhua' was primary taken as representative Chinese pear to compare genetic map of apple and European pear (*P. communis*), Japanese pear (*P. pyrifolia*) and Chinese pear (*P. bretschneideri*). The alignment of the species maps based on JoinMap analysis made it possible to verify the degree of synteny between the apple and pear genomes. The apple SSR loci showed the same order and corresponding distances in the apple and pear maps, suggesting the presence of highly conserved regions between the two genomes and that apple SSRs were more preferable to transfer and conserve to European pears tha Asian pears. The most conserved segments of linkage group between apple and pear were located in LG 9, LG 11 and LG 14 based on 101 apple-mapped SSRs.



1.8 Marker-Assisted Selection in Pome Fruit

Classical plant breeding has been practised for the past three thousand years. Classical plant breeders use phenotypic selection based on morphological characteristics to improve crop varieties. Often many cycles of breeding and backcrossing are needed in order to introduce desired characteristics from one parent into a genotype with suitable agronomic and quality characteristics (Tromp *et al.*, 2005).

Early flowering and fruiting is of paramount importance in the breeding of apples and pears. Breeding pome fruit is a difficult task mainly due to the lengthy juvenile phase and the very high level of heterozygosity of apple and pear genotype. The first consequence is that most of the valuable traits present in one parent cannot be inherited completely as a whole due to their heterozygosity (Liebhard *et al.*, 2002). The development of new techniques for the early

selection of seedlings carrying valuable traits has become a priority in pome fruit breeding. The development of molecular markers linked to important agronomic traits, whereby seedlings with desirable traits can be selected soon after germination on the basis of their marker genotype, also known as marker-assisted breeding has already made it possible to improve and to speed up selection procedures (Tartarini and Sansavini, 2003).

1.9 Pear and apple genome mapping for the red colour trait

A genetic linkage map represents the relative positions genetic markers on the chromosomes of a species. Linkage maps play a vital role in various fields of fundamental and applied genetic research, for instance Quantitative Trait Loci (QTL) analysis, marker-assisted breeding and map-based cloning of genes. Linkage maps enable studies of the genome structure, the localization of interesting genes, identification of quantitative trait loci (QTLs), and conduct of marker-assisted selection (MAS) and marker-assisted breeding (MAB) (Dondini *et al*., 2008). Construction of genetic linkage maps is a complex process that requires knowledge of genetics and statistics. It requires careful scrutiny of marker data before starting on the computation (Van Ooijen and Voorrips, 2001). The main aim of constructing linkage maps is to link molecular markers to each other and to genes governing traits of importance to breeders (*e.g.* red colour, scab resistance and powdery mildew).

Various apple molecular maps have been constructed for linking markers to traits of interest using the following crosses: ‘Rome Beauty’ x ‘White Angel’; ‘Wijcik’ x ‘NY75441-67’; ‘Wijcik’ x ‘NY75441-58’; ‘Prima’ x ‘Fiesta’, and ‘Fiesta’ x ‘Discovery’. Earlier maps were based on RAPD or RFLP markers. A detailed molecular map of apple based on more than one hundred SSRs published by Liebhard *et al*. (2002) has been widely used as a reference map.

Yamamoto *et al*. (2006) demonstrated that apple SSRs can be efficiently used in European and Asian pear, with the maps showing a fairly good degree of synteny between the species.

Genetic linkage maps have been constructed for various European pear cultivars based on EST-SSRs and AFLP markers: 'Bartlett' and 'La France' (Yamamoto *et al*., 2007; Yamamoto *et al*., 2013), 'Passe Crassane' and 'Harrow Sweet' (Dondini *et al*., 2004), 'Passe Crassane', 'Harrow Sweet', 'Abbe Fetel' and 'Max Red Bartlett' (Pierantoni *et al*., 2004, Dondini *et al*., 2008), 'Bartlett' and 'La France' (Nishitani *et al*., 2009). In addition, maps of Japanese pears were reported for 'Kinchaku' and 'Kousui' (Iketani *et al*., 2001), and 'Housui' (Nishitani *et al*., 2009; Yamamoto *et al*., 2002b, 2004). However, saturated high-density genetic linkage maps of pear have been constructed only for European pear cultivars 'Bartlett' and 'La France' (Yamamoto *et al*., 2007). More recently, Yamamoto *et al*. (2013) performed random shotgun sequences to obtain sequences from Japanese pear 'Housui' for use in developing molecular markers. Approximately 237 SSR markers were designed, with 128 loci added to at least one of three genetic linkage maps: for either European pears 'Bartlett' and 'La France', or Japanese pear 'Housui'.

A particularly important improvement was the construction of linkage map of 'Bartlett' using SSR markers from an interspecific cross between the European pear cultivar, 'Bartlett' and the Japanese pear cultivar, 'Housui', based on ESTs derived from cDNA libraries of Japanese pear 'Housui' (Hayashi *et al.*, 2002). Eighteen linkage groups from the map were identified that covered 949 cM, with an average distance of 4.2 cM between each pair of loci. The size of the linkage groups ranged from 10.7 cM to 90.3 cM. A linkage map of 'Housui' was also constructed; the map contained 17 linkage groups ranging from 91.3 cM to 14.5 cM and encompassed 926 cM (Hayashi *et al.*, 2002).

There has been an interest in mapping the red trait of ‘Max Red Bartlett’ in pear, which according to Reimer (1951) and Brown (1966) is controlled by a single dominant gene named *C*, the *Cardinal* red colour gene (Zielinski, 1963). Pigment analysis by du Preez and Rees (2008) confirmed the presence of cyanidin 3-galactoside in the red phenotype (MRB) compared to the green phenotype (‘Bartlett’).

Dondini *et al*. (2008) positioned the red skin colour gene in linkage group 4 of ‘Max Red Bartlett’. The red colour gene was flanked by two amplified fragment length polymorphism (AFLP) markers (E31M56-7, 13.5 cM; E33M48-5, 18.2 cM). The linkage group was 82.2 cM in length. This linkage group was identified by the presence of two apple SSR markers (CH01D03 and CH02C02b) that were mapped in apple and pear (Dondini *et al*. 2004; Liebhard *et al*. 2002; Pierantoni *et al*. 2004, 2007). SSR marker CH02c02b was 64 cM from the red colour gene, while the distance of marker CH01d03 was 19.4 CM from the red gene, making marker CH01d03 the closest SSR marker to the red colour gene to date (Dondini *et al*., 2008). The microsatellite markers mapped by Dondini *et al*. (2008) are not close enough to the red colour gene, therefore more microsatellite markers still need to be mapped in that linkage group to identify closer ones.

1.10 The objectives and rationale of the study

At the ARC, a progeny segregating for red leaves was raised from a cross whereby the maternal parent 'Bon Rouge' (fully red-skinned sport of 'Williams Bon Chretien') was hand pollinated at flowering time with the male pollen donor 'Packham's Triumph' (fully green-skinned pear). As mentioned earlier, 'Bon Rouge', a red-skinned mutant of 'Williams Bon Chretien' arose in South Africa. 'Williams Bon Chretien' itself arose in England in the 1770's and is also known as 'Bartlett'.

'Bon Rouge' pears are characterized by red skinned fruit throughout the growing season and red leaves early in the season. Loss of red colour in leaves, and fruit skin at the ripening stage is due to the disappearance of anthocyanins when synthesis falls below the rate of pigment turnover (Steyn et al., 2004). 'Packham's Triumph' pears are characterized by green skinned fruit and leaves throughout the growing season (Figure 1.2A). The red trait is unstable; this was observed on branches of 'Bon Rouge' pear trees planted in commercial orchards, whereby the colour reverted to the original 'Williams Bon Chretien' parent green phenotype or stripes on fruits at the ripening stage (Figure 1.2B) (du Preez *et al*., 2004).

Figure 1.2 (A) Mature red and green pears from the same 'Bon Rouge' pear tree and (B) stripes characteristic of the red to green reversion in skin of mature 'Bon Rouge' fruit.

'Packham's Triumph' originated in Australia in the 1890's from a cross between 'Williams Bon Chretien' and 'Uvedale's St. Germain'. 'Packham's Triumph' is a green fruited cultivar; it is the most popular eating pear variety in South Africa and was introduced in the country in 1922.

The purpose of this project was to map by means of microsatellite markers the red-trait segregating in ARC's 'Bon Rouge' x 'Packham's Triumph' progeny. In the light of the mapping of the 'Max Red Bartlett' red trait (Dondini *et al*., 2008), the particular objectives were:

- To determine if the red trait in 'Bon Rouge' locates to the same linkage group as the red trait in 'Max Red Bartlett', that is linkage group 4 (LG 4), and, if so,
- To identify SSR markers closer to the red trait than previously reported, to aid future genetic studies.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and consumables list

ABI 310 Polymer 4 (POP-4)	Applied Biosystems
Agarose D1 LE	Promega
Boric acid	Merck
Bromophenol blue	Sigma
Capillaries	Applied Biosystems
Chloroform	BDH
CTAB (N-acetyl-N-N-N-trimethyl ammonium bromide)	Saarchem
Deionised formamide	Applied Biosystems
dNTPs	Southern Cross Biotechnology
EDTA (Ethylene Diamine Tetra-acetic Acid)	Merck
Ethanol	Kimix
Ethidium bromide	Sigma
Glycerol	Merck
GeneScan® LIZ™-500 size standard	BDH
Isoamylalcohol	Merck
Isopropylalcohol	BDH
Mercaptoethanol	Roche
Proteinase K	Roche
Polyvinyl-pyrolidone 40 (PVP-40)	Sigma
POP-4	Applied Biosystems
RNase A	Roche

Sodium acetate	BDH
Sodium borohydride	Saarchem
Sodium chloride	Merck
Sodium hydroxide	BDH
Taq Polymerase	Qiagen
Tris (hydromethyl) aminomethane	Merck
Xylene cyanol	BDH

2.2 General stock solutions and buffers

Agarose loading buffer	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol in 30% (v/v) glycerol in deionised water
Chloroform-isoamylalcohol (CIA)	24:1 (v/v) % chloroform-isoamylalcohol
Ethanol (70%)	70% (v/v) absolute ethanol in deionised water
Proteinase K	20 mg/ml in deionised water
RNase A buffer	0.1 M sodium acetate, 0.3 mM EDTA, pH 4.8
RNase A (DNase free)	40 mg RNase A, 2 ml RNase A buffer
10 x TBE buffer	0.9 M Tris, 0.89 M boric acid, 0.032 M EDTA pH 8.3
10 x TE buffer	10 mM EDTA, 100 mM Tris-HCl, pH 8.0
0.8% agarose	0.8% (w/v) agarose in 1x TBE
1.5% agarose	1.5% (w/v) agarose in 1 x TBE
2 x CTAB	2% (w/v) CTAB, 1.4 M NaCl, 0.02 M EDTA, 2% (v/v) PVP-40, 0.2 ml stock solution of 0.5 M DTT, 0.1 M Tris-HCl, pH 8.0
PCR reagent kit	2 x PCR mix, 10 x PCR Primer mix, 20 ng/µl genomic DNA (template DNA), deionised water

2.3 Plant material collection

One hundred and twenty five seedlings of the two parents 'Bon Rouge' and 'Packham's Triumph', growing on their own roots were used for this study. Young healthy leaves were collected from the F_1 seedlings planted in the nursery at ARC Infruitec-Nietvoorbij, Bien Donné Experimental Farm [33˚.50'32.44" (S) / 18˚.58'40.22" (E)]. For the parents, leaves were collected from trees located in the pear germplasm collection situated on the same farm. Sample materials were bagged, frozen in liquid nitrogen, transported to the ARC Infruitec-Nietvoorbij Cultivar Development Laboratory and temporarily stored at -80˚C for future use.

2.4 DNA extraction

Genomic DNA was extracted from young fresh leaves using the CTAB method, essentially as described by Rogers and Bendich (1985), for species recalcitrant to DNA extraction as a consequence of polyphenolic components in the sample material. Approximately 1 g of fresh leaf tissue was placed in a mortar and a small amount of carborundum powder was added on top of the leaf material to assist in the process of grinding. Young leaves contain a high density of cells that have not expanded and so are rich in DNA. Approximately 2 ml of pre-warmed (60˚C) 2% CTAB extraction buffer was added and a pestle was used to grind the leaf material into a fine mixture. The amount of 2% CTAB extraction buffer added depends on the amount of leaf material that will be ground, but the amount added should be enough to give a slurry that flows freely. Approximately 1 ml of the suspension was transferred into a 2 ml Eppendorf tube and 5 µl of β-mercapto-ethanol was added and the mixture was mixed for a few minutes on a vortex. The suspensions were incubated at

62˚C for 1 hr with intermittent shaking to allow the release of cellular debris into the suspension. The specimens were allowed to cool at room temperature (25˚C) before centrifugation for 5 min at 10 000 x g. The supernatant was transferred to a new 2 ml Eppendorf tube to which 10 µl Proteinase K (10 µg/ml) was added and incubated at 37˚C in a water-bath for 30 min. Proteinase K is an endolytic protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. Equal volumes of chloroform: iso-amylalcohol (CIA) (24:1) were added three times to extract nucleic acids. The samples were inverted several times between each extraction step and centrifuged at 10 000 x g for 10-15 min at room temperature.

After centrifugation, the final aqueous phase was carefully transferred into a 1.5 ml clean Eppendorf tube. RNase A (10 mg/ml – DNase-free) was added and the samples were incubated at 37˚C (water-bath) for 1 hour. Equal volumes of CIA (24:1) were added and samples were inverted for 5 minutes before centrifugation at 10 000 x g for 10-15 min. The supernatant was transferred to a new 1.5 ml eppendorf tube and 2/3 volume ice-cold isopropanol was added to precipitate genomic DNA. Thread-like strands of genomic DNA started to form on some of the samples. The samples were inverted for 3 min and incubated at -20˚C overnight. The following day samples were centrifuged for 20 min at 13 200 x g. The supernatant was carefully discarded and 70% ethanol was added to remove any impurities in the DNA pellet which was now settling at the bottom of the 1.5 ml Eppendorf tube.

Subsequently, the pellet was completely dissolved in 200 µl volume of 0.3 M NaOAc and re-precipitated in 600 µl of ice-cold 100% ethanol and stored at -80˚C for 2 hrs. The precipitant was centrifuged at 10 000 x g for 15 min to pellet the genomic DNA. To

remove salts, the DNA pellet was washed twice with 80% ethanol and air-dried at room temperature. The pellet was dissolved in 50-100 µl 1 x TE buffer and stored at 4˚C. Extracted genomic DNA was later stored at -20˚C for future use.

2.5 Gel electrophoresis

DNA integrity of the extracted samples was established on a 0.8% agarose gel stained with ethidium bromide. Ten microliters volume of isolated stock DNA was mixed with 3 µl of loading buffer (0.25% bromophenol blue, 25% xylene cyanol). The samples were resolved on 0.8% agarose gel with 1 x TBE loading buffer at 10 V/cm for 1 hr and visualized under UV light and a photo of the gel was taken to check the intensity of the genomic DNA bands.

2.6 Genomic DNA quantification

To estimate DNA concentration by Absorbance at 260 nm, 2 µl of each of the stock DNA samples was placed in a NanoDrop® ND-1000 Spectrophotometer situated at Stellenbosch University (Department of Genetics, Central DNA Sequencing Facility) and the DNA concentration of each sample was measured. The UV-absorbance spectrum of DNA exhibits an absorbance maximum at 260 nm based on the aromatic ring structures of the DNA bases. This is the most convenient way to estimate DNA concentration and calculate yield, as long as the DNA preparation is relatively free of contaminants that absorb in the UV. Proteins, and residual phenol left from the isolation procedure, are typical contaminants that may lead to an overestimation of DNA concentration. After measurement of the DNA concentration, the samples were diluted to the desired

concentration, 15 ng/µl for SSR amplification, and the samples were electrophoresed on a 1.5% agarose gel to verify concentrations of each DNA sample.

2.7 Primer design, synthesis and labeling

Microsatellite primer sets for linkage group 4 (LG4) chosen from the pear literature were chosen especially on the basis of heterozygosity in 'Bartlett' (Celton *et al*., 2009; Fernandez-Fernandez *et al*., 2008; Guilford *et al*., 1997; Nishitani *et al*., 2009; Silfverberg-Dilworth *et al*., 2006; Yamamoto *et al*., 2007). The selected primer sets were commercially synthesized and fluorescently labelled at the 5' end with VIC™ (green), NED™ (yellow), PET™ (red) or 6-FAM™ (blue) fluorophore dyes (Applied Biosystems, South Africa).

Fluorescent labeling of primers makes it possible to distinguish fragment lengths accurately using automated fluorescence-based DNA detection systems, such as an ABI 3130 Genetic Analyzer. Also, fluorescent labelling of markers, using different dye colours, allows multiplexing markers of the same or similar size and is an inexpensive and efficient method of genotyping (Mansfield *et al*., 1994).

Eleven markers in four multiplexes were designed that contained two to three SSR markers per multiplex. The SSR marker pairs were labeled with different fluorescent dyes and amplified in the same PCR reaction per multiplex. To amplify genomic DNA, 10 µl PCR reactions were performed for a single reaction buffer according to manufacturer's instructions, where amplification multiplexes were amplified (co-amplification of multiple SSRs in a single PCR cocktail, a procedure called true multiplexing). The following PCR reaction was prepared: 5 µl of 2x PCR Mix, 1 µl of 10 x PCR Primer Mix, 2 µl of template

DNA (15 ng/µl) and 2 µl of dH_2O. A PCR temperature regime was set as follows: 95˚C for 15 min; 94˚C for 30 sec, 60˚C for 90 sec, 72˚C for 1 min, repeated for 40 cycles; followed by 60˚C for 30 min and 4˚C hold, with amplification performed in a 9700-Thermal Cycler (Applied Biosystems, Foster City CA, USA).

2.8 DNA product verification

Approximately 5 µl PCR product volumes were mixed with 3 µl loading dye and were electrophoresed on 1.5% agarose gels in 1x TBE at 60V for 4 hours to verify amplification. The gel was visualized under UV-light and a photo of the gel was taken to verify if there was amplification or not.

2.9 Sizing of SSRS on ABI PRISM® 3130XL Genetic Analyzer

Amplification products generated by PCR-amplification with fluorescently tagged primers were ran on an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, Foster City CA, USA), situated at Stellenbosch University (Department of Genetics, Central DNA Sequencing Facility) using the GeneScan™ 500 LIZ® size standard (PE Applied Biosystems, GeneMapper® Software Version 4.0, 2005). The DNA product separation was performed on the ABI 3130 Polymer (POP-4). Three microliters (3 µl) of PCR product and 10 µl of formamide and 0.3 µl of GeneScan™ 500 LIZ® standard were mixed together. The mixture was denatured at 95˚C for 10 min and chilled on ice for a few seconds. The manufacturer's protocol was used for the operation of the ABI PRISM® 3130xl Genetic Analyzer. Allele sizes were scored using the GeneMapper® Software Version 4.0.

2.10 SSR marker analysis

GeneMapper® Software Version 4.0 was used for the identification of polymorphic DNA products and the individual products were assigned as alleles of the appropriate marker loci. Allele binning was conducted according to the supplier's recommendations (GeneMapper® Software Version 4.0 Installation and Administration Guide, 2005). Segregation analysis was performed by referring to the different fragments obtained when screening each seedling with a specific marker. This allowed the allelic distribution in the progeny to be determined. Two alleles of a specific parent should segregate in the progeny in a 1:1 ratio if, *e.g.* one of the parents is heterozygous dominant (*Cc*) and the other parent is homozygous recessive (*cc*) for a particular trait, alternatively, if both parents are heterozygous dominant (*Cc*). Segregation types are that the positions of the alleles define their grand-parental origin. Segregation analysis is a manual process that relies on the comparison of parental allele sizes with seedling allele sizes. Filtering of alleles was performed manually to eliminate some 'false' alleles that the ABI PRISM 3130xl Genetic Analyzer had included and to include some true alleles that had been overlooked.

Preliminary scoring was checked manually and independently by a co-worker for any mistakes. Preliminary co-segregation analyses were performed using an X^2 test (chi-square) to indicate departure from random co-segregation. Scores were subsequently recorded for linkage analysis in JoinMap® 4.1.

2.11 Marker linkage analysis

LG4 is where the red color gene/s is located in pear. JoinMap® 4.1 was used to check single locus segregations and to construct a linkage map of the analysed markers (Van Ooijen, 2006). Two 'Bon Rouge' genetic linkage maps were constructed, one with regression and the other with maximum likelihood mapping algorithms. Mapping functions are formulas expressing the recombination fraction between two markers as a function of the map distance between them, or vice-versa, measured in centimorgans or Morgans. The two most commonly used transformations are Kosambi's and Haldane's mapping functions. Both mapping functions make it possible to convert recombination percentages into centiMorgan (cM) distances (Van Ooijen, 2006) but the Haldane's is a default mapping function. Independence LOD grouping parameter with a threshold of 3 and Kosambi's mapping function was used to generate the linkage groups in this study. MapChart® 2.1 was used to illustrate linkages of the LG4 markers of the two maps (Dondini *et al*., 2008).

It was possible to map red colour gene as a morphological marker in an SSR-enriched map of the cross 'Abbé Fétel' × 'Max Red Bartlett' (Pierantoni *et al*., 2004; 2007). Red colour gene mapped in linkage group 4, flanked by two amplified fragment length polymorphism (AFLP) markers (E31M56-7, 13.5 cM; E33M48-5, 18.2 cM). The linkage group was identified by the presence of two SSR markers (CH01d03; CH02c02b) that had been previously mapped in apple and pear (Pierantoni *et al*., 2004; 2007).

CHAPTER 3: RESULTS

3.1 Parent identification and cross pollination

A controlled cross was made successfully at Elgin Experimental Farm in October 2003, whereby the maternal parent 'Bon Rouge' (fully red-skinned sport of 'Bon Chretien') was hand pollinated at flowering time with the male pollen donor 'Packham's Triumph' (fully green-skinned pear). The cross was made by Mr Taaibos Human, Pear Breeder who works at the Agricultural Research Council, ARC Infruitec-Nietvoorbij, Bien Donné Experimental Farm, Simondium. The information for the two parents used is detailed in Table 3.1 (South African Deciduous Fruit Cultivars). A total of eight hundred progeny seedlings were originally raised of which one hundred and twenty five were used for this mapping study. This cross was selected to study the segregation of the red-versus-green colour phenotype with various apple and pear microsatellite markers (SSRs) of LG4 from different published pear maps. This was because in MRB, another red mutant, the red colour was shown to map to LG4 (Dondini *et al*., 2008).

Table 3.1. Description of the two pear cultivars, 'Bon Rouge' and 'Packham's Triumph', parents of the progeny in this study.

	Bon Rouge	**Packham's Triumph**
Origin	RSA, Bon Chretien mutation	Australia, (Bon Chretien x St Germain)
Chilling requirement	medium	medium-low
Vigour	moderate to strong	medium to strong
Growth habit	semi-upright	upright
Full bloom date	early October	middle to late September
Pollinators	Packham's Triumph, Beurre Bosc, Winter Nelis	Winter Nelis, Rosemarie, December
Bearing habit	spurs and shoots	spurs and shoots
Production	medium to high	high
Harvest date	middle January	middle February
Fruit mass	160 g	190 g
Fruit shape	oblong-ovate-pyriform	ovate-pyriform
Fruit skin colour	full red	green-yellow
Lenticels	conspicuous	conspicuous
Flesh colour	cream-white	cream-white
Taste	good	good, slightly sweet
Texture	soft	smooth
Storage ability	good	good

3.2 Fruit harvesting and seed extraction

Fruits from the hand pollination crosses made were harvested at the optimal ripening time at Elgin Experimental Farm from end-January to early-February. The harvested fruits were taken to Bien Donné Experimental Farm (Figure 3.1) and seeds were extracted in mid-February 2004, thoroughly washed in water and dried at room temperature (Mr. Taaibos Human, personal communication).

Figure 3.1. An aerial view of Bien Donné Experimental Farm (photo taken by Ms Hannel Ham of the ARC Infruitec-Nietvoorbij).

3.3 Seed stratification and growth

Since South African winters are not cold enough to meet the chilling requirements for seed germination, the seeds were stratified by storing them in plastic bags for three months at 4˚C in perlite dampened with a wide spectrum fungicide (Universal CAPTAB WP) (Figure 3.2). After stratification, seeds were sown in perlite in seedling trays and transplanted at the two-leaf stage into a growing medium containing fine bark and sand in the glasshouse at Bien Donné Experimental Farm. Normal watering and feeding of the seedlings with a balanced plant fertilizer was performed. After approximately three months, the seedlings were taken out of the glasshouse to the shade-net area to acclimatize. The seedlings were kept under the shade-net area from 2004-2012 for nine years and, during this nine year period were irrigated normally, weeded regularly and, when necessary, received fertilization (Taaibos Human, personal communication).

In January 2013, a subset of 125 progenies coded as progeny J5 was planted under field conditions in the nursery at Bien Donné Experimental Farm at 90 cm x 30 cm spacing to obtain better growth, in anticipation to collect graft-wood in August 2013 for propagation onto a standard rootstock to evaluate agronomic characters and to maintain the progenies for possible future genetic studies. The seedlings were tagged J5-1 to J5-125.

Figure 3.2. Stratification of seeds in the fridge at 4˚C in dampened perlite at Bien Donné Experimental Farm.

3.4 Phenotypic classification

Initially, within 90 days of germination, a total of 800 progenies were tagged and scored for leaf colour (Figure 3.3). Subsequently, as explained, 125 progenies from the cross were retained and tagged according to the visual colour of the leaves (red or green) in spring when the leaf colour difference was most pronounced. Later in the season (mid-January) the red colour tends to fade. Moreover, when seedlings are mature, it is difficult to differentiate the progenies as red-leaved seedlings can revert partially to green. Thus it is best to tagprogenies while they are still young.

The progeny of the cross ‘Bon Rouge’ x ‘Packham’s Triumph’ exhibit a segregation ratio for anthocyanin production in leaves of 54:71, which is approximately a 1:1 ratio for red-versus-green phenotype, indicating that a single gene locus controls anthocyanin pigment production in leaves. The chi-square test (X^2) values calculated for all 125 progenies are consistent with the hypothesis that the red colour is under the control of a single gene.

Figure 3.3. F1 seedlings from the cross ‘Bon Rouge’ x ‘Packham’s Triumph’ within 90 days of germination when the leaf colour difference was most pronounced, indicating a simple Mendelian inheritance.

3.5 GEnomic DNA isolation

Genomic DNA was extracted from ‘Bon Rouge’ x ‘Packham’s Triumph’ healthy leaves collected at the tips of shoots (Figure 3.4) was extracted successfully from the 125 seedlings obtained from the F1 progeny and from the two parents ‘Bon Rouge’ and ‘Packham’s Triumph' with the use of the CTAB method. The isolated genomic DNA was

further electrophoresed in a 0.8% agarose gel to verify presence and quality of the DNA extracted (Figure 3.5).

Figure 3.4. Leaves exhibiting segregation for colour, red or green within 'Bon Rouge' x 'Packham's Triumph' progeny.

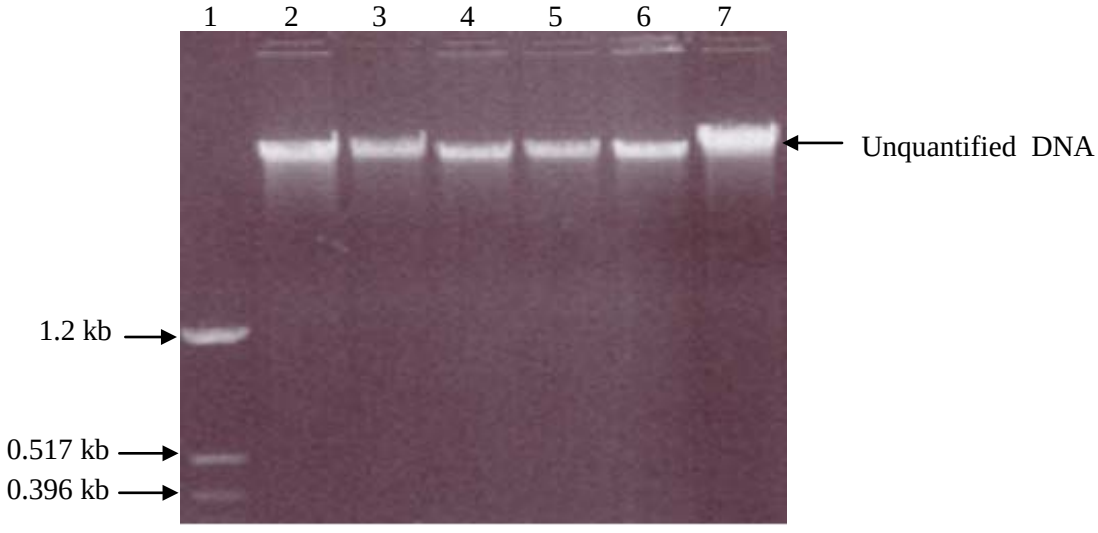


Figure 3.5. Unquantified genomic DNA extracts of two pear parents and four seedlings on a 0.8% agarose gel. Lane 1: molecular weight marker and Lanes 2-7: 'Bon Rouge', 'Packham's Triumph', J5-1, J5-3, J5-78 and J5-92, respectively.

3.6 DNA QUANTIFICATION

Before genomic DNA quantification, the concentration of unquantified genomic DNA had to be determined. Absorbance of pear genomic DNA of parents and all progeny individuals was conducted on the NanoDrop® 1000 Spectrophotometer and the actual concentrations of the extracted genomic DNA were recorded. Figure 3.6 shows absorbance readings of sample J42 on the NanoDrop® 1000 Spectrophotometer. An A260 = 1.0 indicates a DNA concentration = 50 ug/µl, assuming the DNA is pure. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of approximately 1.8 is generally accepted as pure for DNA; a ratio of approximately 2.0 is generally accepted as pure for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. A 260/230 ratio is used as a secondary measure of nucleic acid purity.

The 260/230 values for pure nucleic acid are often higher than the respective 260/280 values. Expected 260/280 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm. After quantification, concentration of unquantified genomic DNA for all the samples was adjusted to the required genomic DNA concentration of 15 ng/µl, which was confirmed on the 1.5% agarose gel as depicted in Figure 3.7.

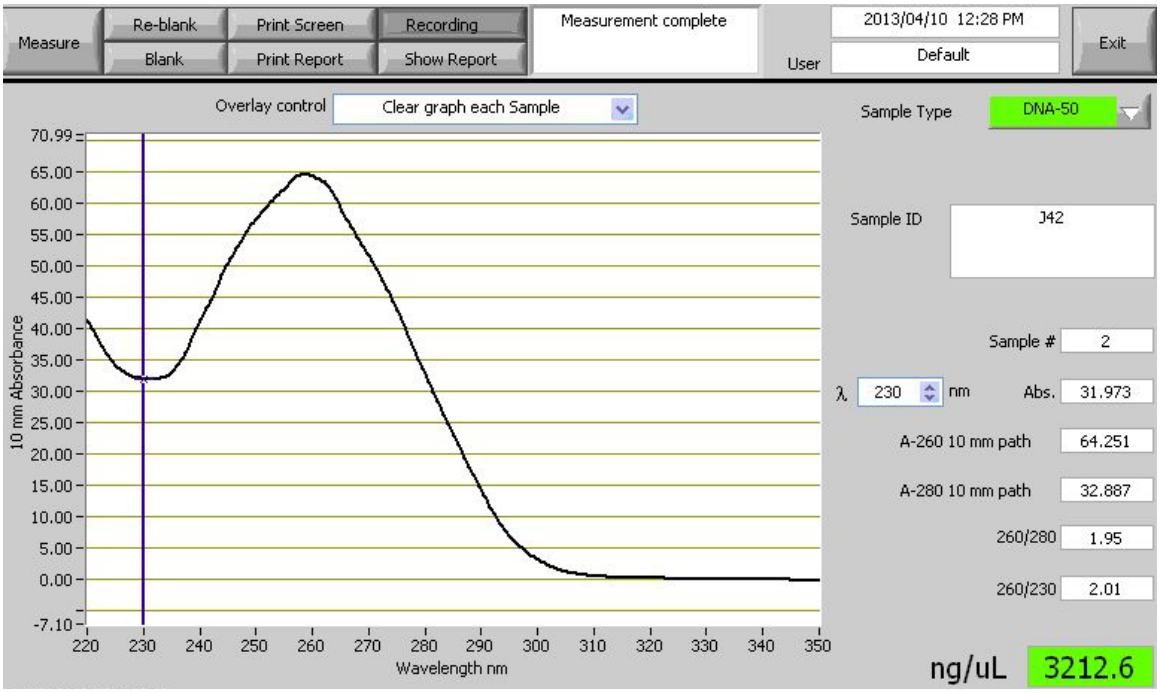


Figure 3.6. Absorbance readings of sample J42 on the NanoDrop® 1000 Spectrophotometer with a concentration of 3212.6 ng/µl.

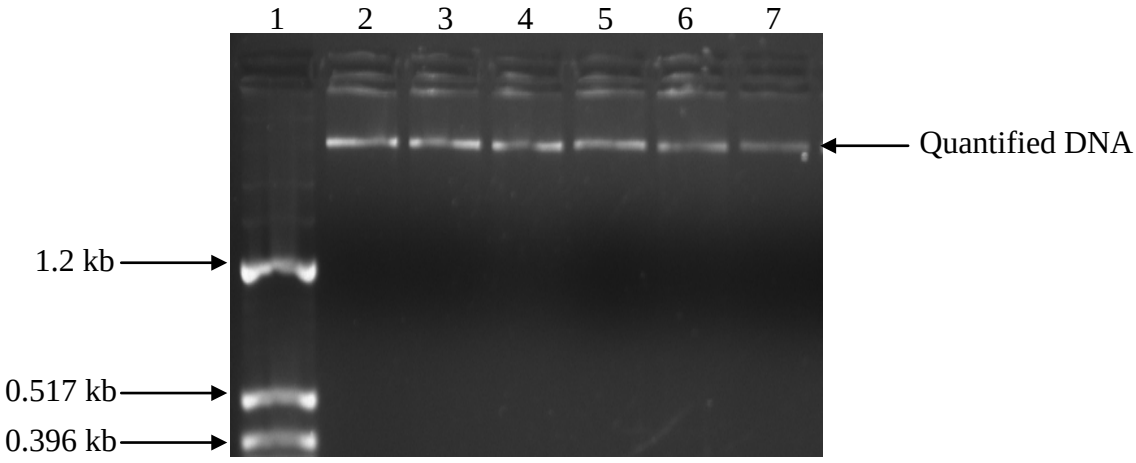


Figure 3.7. Quantified and equal concentration of 15 ng/µl genomic DNA extractions on a 1.5% agarose gel of the two pear parents and four progenies. Lane 1: molecular weight marker and Lanes 2-7: 'Bon Rouge', 'Packham's Triumph', J5-1, J5-3, J5-78 and J5-92, respectively.

3.7 Primer synthesis and labeling

A total of eleven microsatellite primer sets were initially selected from previous pear linkage maps of linkage group 4 (LG4), especially on the basis of heterozygosity in 'Bartlett' (Celton *et al*., 2008; Dondini *et al*., 2008; Nishitani *et al*., 2009; Yamamoto *et al*., 2007, 2013). The heterozygosity can be defined as the probability that an individual has two different alleles at a given locus. From the perspective of genetic linkage map construction, a locus with heterozygosity higher than 70% is considered a highly informative marker. The five segregation types encountered when working with an outbreeding species like pear is best explained by means of genotypic codes used by mapping software JoinMap (Van Ooijen, 2006). The Class 1 segregation involves those loci that are heterozygous in both parents and from a mapping perspective, are fully informative for both parental meiosis. Class 2 segregation includes those loci that, even though heterozygous in both parents, are heterozygous for the same two alleles, making the determination of the parental origin of alleles present in a heterozygous seedling impossible when viewing segregation from a single parent. These loci can be statistically be positioned on a genetic linkage map derived from the F1 population where both parental meiosis are viewed simultaneously. The last class, Class 3 segregation refers to those loci that are heterozygous in one parent on. The other parent might be homozygous for a corresponding allele or any other allele, thus resulting in the presence of either 2 or 3 alleles.

For mapping purposes, the allele from the homozygous parent is not informative. Although information from all 3 classes of loci can be used for the construction of a F1 genetic linkage map, only Class 1 and 3 can be used for the construction of parental genetic linkage map. It would be expected that the two alleles at any given locus will be equally

represented in the progeny with a segregation ratio of 1:1. Any deviation from this expected ratio results in segregation distortion, possibly due to sample error when working with a small population size, but also possibly through the occurrence of natural selection against a given allele or a genotype that it occurs in linkage disequilibrium with.

Details of the SSR primer sets screened in this study for mapping purposes are depicted in the Appendix Table A1. These primer sequences were commercially synthesized and fluorescently labelled at the 5' end with fluorophore dye colours (viz. 6-FAM™, VIC™, NED™ and PET™) by Life Technologies™.

3.8 PCR product verification

Prior to scoring of alleles of the two parents and the 125 seedlings from the 'Bon Rouge' x 'Packham's Triumph' on the ABI PRISM® 3130xl Genetic Analyzer, the PCR products of the two parents and the all 125 progenies amplified with all eleven microsatellite primer sets were electrophoresed on a 1% agarose gels to verify if there was amplification and the 1% agarose gel was stained with Ethidium Bromide to visualize the PCR products. Figure 3.8 shows PCR products of the two parents and six progenyon a 1% agarose gel amplified with SSR marker NH011a. The expected fragment sizes from this SSR marker was in the range of 166-172 base pairs.

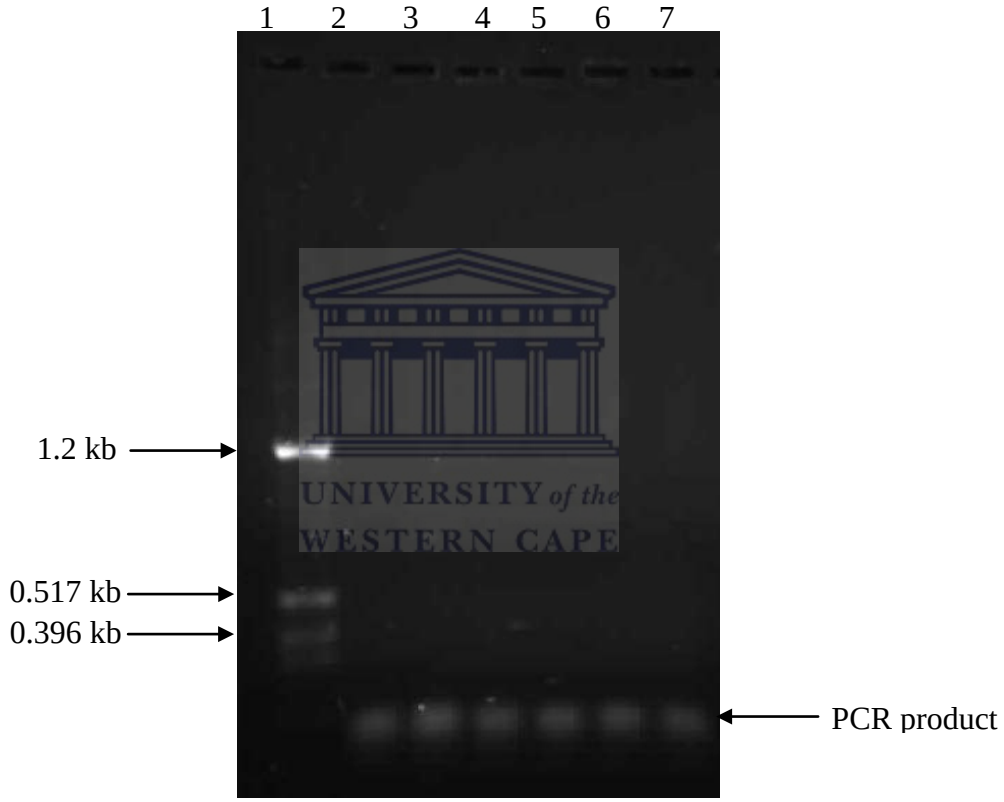


Figure 3.8. Verification of PCR amplification products of SSR marker NH011a resolved on a 1% agarose gel of the two pear parents and four progenies. Lane 1: molecular weight marker and Lanes 2-7: ‘Bon Rouge’, ‘Packham’s Triumph’, J5-1, J5-3, J5-78 and J5-92 respectively.

3.9 Mapping population screening with multiplexes

PCR amplification of the 125 progenies was done with 11 markers in four multiplexes that contained two to three SSR markers per multiplex (Table 3.3). Table 3.3 further shows the dye colour of each marker, the observed size range for each marker and the alleles sizes produced for each of the two parents used in this study, after PCR amplification with the Qiagen megaplex PCR kit. When multiplexes 2 and 3 were used to amplify the 125 seedlings and parents, markers CH01f02 and Hi08e04 failed to amplify and as a result there was no PCR product observed when few samples were randomly selected and electrophoresed on a 1% agarose gel. Microsatellite markers that showed good amplification products on a 1% agarose gels were further analyzed for allele scoring and genotyping.

Once PCR amplification was achieved, PCR products were further analyzed on the ABI PRISM® 3130xl Genetic Analyzer located at Stellenbosch University and sizing of the alleles was performed using GeneMapper® 4.0 software. The dataset was on average 90% complete. JoinMap® codes for the 'Bon Rouge' x 'Packham's Triumph' progeny were given on the basis of the segregation observed and the allele sizes of the two parents.

Table 3.2 Four multiplexes designed for the eleven SSR markers used in this study.

Multiplex 1	Primer number	Dye colour	Size (bp)	BR alleles	PT alleles	BR x PT JoinMap code
	NB141b	6-FAM™	66-140	122/124	122/136	<efxeg>
	NH011a	NED™	181	166/170	166/172	<efxeg>
Multiplex 2						
	CH02c02b	NED™	78-133	118/132	116/118	<efxeg>
	CH01f02	PET™	130-240	N/A	N/A	-
	NH008b	VIC™	205	162/176	166/176	<efxeg>
Multiplex 3						
	CH01d03	VIC™	120-160	133/150	131/133	<efxeg>
	Hi08e04	NED™	201-234	N/A	N/A	-
	NB127b	PET™	146-166	133/150	131/133	<efxeg>
Multiplex 4						
	CH04e02	NED™	110-170	-	-	-
	NZ05g08	PET™	115-147	-	-	-
	TsuENH019	VIC™	125	105/110	110/123	<efxeg>

Key: 'N/A' represents failure to amplify a product; '-' represents no JoinMap code selected

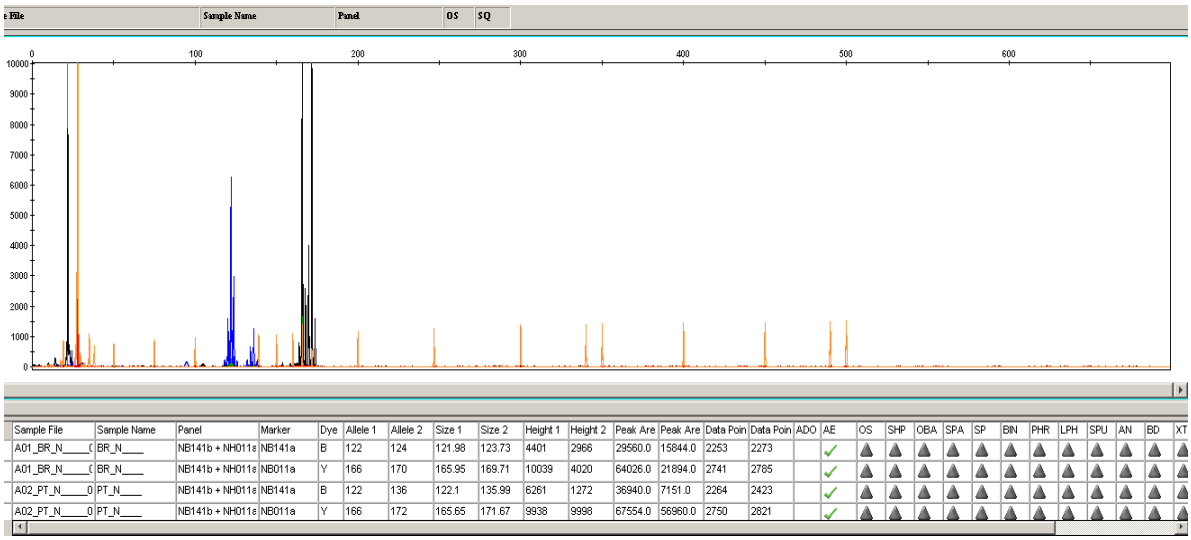
'Bon Rouge' and 'Packham's Triumph' always share an allele consistant with 'Packham's Triumph' being a seedling of 'Bon Chretien', as can be seen in Table 3.2 under origin of the pear cultivar 'Packham's Triumph'. In multiplex 3, SSR markers CH01d03 and NB127b produced the same alleles in both parents after PCR amplification. The same results were further evident when the PCR products were electrophoresed on the ABI PRIZM® 3130xl Genetic Analyzer (Figure 3.13 and Figure 3.14) on different fluorescent dyes. Genetic linkage map construction is far more complex in full-sib families of an outcrossing species than in species derived from pure lines: 1) markers may differ with regards to the number of segregating alleles (2, 3 or 4); 2) markers may not be

heterozygous in both parents; 3) markers that are heterozygous in both parents may be identical; 4) markers may have null alleles and 5) the linkage phase or grandparental origin, of markers are, in many cases, unknown.

Table 3.3 The seven SSR markers genotyped in 125 progeny seedlings and the segregation data for the red colour trait.

SSR marker	No. plants	Red leaved	Green leaved	Missing data
CH02c02b	125	53	69	3
CH01d03	125	52	60	13
NB127b	125	52	55	18
NB141b	125	49	71	5
NH011a	125	52	69	4
NH008b	125	52	66	7
TsuENH019	125	52	58	15

Markers CH01d03, NB127b and TsuENH019 had missing data of 16.35-22.5% (Table 3.3). A large proportion of missing data for a marker may indicate that the marker was difficult to score. In that case the quality of the remaining scores for that marker may also be disputed; such a marker should be excluded from the data (Van Ooijen, 2006). More than 30% of missing data can hamper the correct positioning of a marker on genetic maps; therefore the three SSR markers were included in the analysis as the missing data was less than 30%. The seven SSR markers were at this stage suitable for JoinMap® 4.1 analysis.



Sample File	Sample Name	Panel	Marker	Dye	Allele 1	Allele 2	Size 1	Size 2	Height 1	Height 2	Peak Are	Peak Are	Data Poin	Data Poin	ADO	AE	OS	SHP	OBA	SPA	SP	BIN	PHR	LPH	SPU	AN	BD	XT
A01_BR_N____(	BR_N____	NB141b + NH011a	NB141a	B	122	124	121.98	123.73	4401	2966	29560.0	15844.0	2253	2273		✓												
A01_BR_N____(	BR_N____	NB141b + NH011a	NB011a	Y	166	170	165.95	169.71	10039	4020	64026.0	21894.0	2741	2785		✓												
A02_PT_N____0	PT_N____	NB141b + NH011a	NB141a	B	122	136	122.1	135.99	6261	1272	36940.0	7151.0	2264	2423		✓												
A02_PT_N____0	PT_N____	NB141b + NH011a	NB011a	Y	166	172	165.65	171.67	9938	9998	67554.0	56960.0	2750	2821		✓												

Figure 3.9. Electropherogram showing raw data obtained after amplification of 'Bon Rouge' and 'Packham's Triumph' DNA with pear primer pairs NB141b (blue), NH011a (black) and a internal size standard LIZ™ (GeneScan™-500 LIZ™), (Orange), fluorescently labelled in a multiplex. Raw data are represented both graphically and in tabular form, with the sizing table listing the SSR markers used in the multiplex and allele sizes corresponding to the peaks.

The peaks shown in blue and black represent the amplified PCR products labeled with 6-FAM™ and NED™. The orange peaks represent the size standard LIZ™ (orange), (Figure 3.9). Figure 3.10 to Figure 3.16 shows sample output of both parents and four seedlings amplified using the seven successful SSR markers after analysis on the ABI PRISM® 3130xl Genetic Analyzer. The SSR markers produced different allele sizes in 'Bon Rouge' and 'Packham's Triumph' parents and the 125 progenies (Table 3.5).

SSR marker CH02c02b, fluorescently labelled with a yellow dye (NED™), produced allele sizes of 118 bp and 132 bp for 'Bon Rouge' and 116 bp and 118 bp for 'Packham's Triumph' (Figure 3.10). For this SSR marker the two parents shared a common allele of size 118 bp as expected in a backcross. Seedling J5-1 was homozygous for allele size 118 bp, which meant that this seedling inherited the allele 118 bp from both parents. Seedlings J5-3, J5-78 and J5-92 inherited different alleles from each parent and were heterozygous.

SSR marker NB011a, fluorescently labelled with a yellow dye (NED™), produced allele sizes of 166 bp and 170 bp for 'Bon Rouge' and 166 bp and 172 bp for 'Packham's Triumph' (Figure 3.11). Seedling J5-78 was homozygous for allele size 166 bp. Seedlings J5-1, J5-8 and J5-92 were heterozygous.

SSR marker NH008b, fluorescently labelled with a green dye (VIC™), produced allele sizes of 162 bp and 176 bp for 'Bon Rouge' and 166 bp and 176 bp for 'Packham's Triumph', (Figure 3.12). Seedling J5-8 was homozygous for allele size 176 bp. Seedlings J5-1, J5-78 and J5-86 were heterozygous, showing the segregation of alleles from parents to the seedlings after amplification by SSR marker NH008b. This marker is an apple marker, though some apple markers are transferable to other cultivars / populations of different species, the practical success of this transferability requires more intervention from the different users specific to their condition(s). Some primer pairs amplify more than two fragments per individual, SSR marker NH008b probably is a multilocus marker where the marker anneals to more than one site.

SSR marker CH01d03, fluorescently labelled with a green dye (VIC™), produced allele sizes of 133 bp and 150 bp for 'Bon Rouge' and 131 bp and 133 bp for 'Packham's Triumph' (Figure 3.13). Seedling J5-77 was homozygous for allele size 133 bp. Seedlings J5-1, J5-3 and J5-78 were heterozygous and the alleles segregated from parents to the seedlings after amplification by SSR marker CH01d03.

SSR marker NB127b, fluorescently labelled with a red dye (PET™), produced allele sizes of 133 bp and 150 bp for 'Bon Rouge' and 131 bp and 133 bp for 'Packham's Triumph'

(Figure 3.14). Seedling J5-71 was homozygous for allele size 133 bp. Seedlings J5-1, J5-3 and J5-59 were heterozygous.

SSR marker TsuENH019, fluorescently labelled with a green dye (VIC™), produced allele sizes of 105 bp and 110 bp for 'Bon Rouge' and 110 bp and 123 bp for 'Packham's Triumph', Figure 3.15. Seedling J5-75 was homozygous for allele size 110 bp. Seedlings J5-3, J5-7 and J5-92 were heterozygous.

SSR marker NB141b, fluorescently labelled with a blue dye (6-FAM™), produced allele sizes of 122 bp and 124 bp for 'Bon Rouge' and 122 bp and 136 bp for 'Packham's Triumph', Figure 3.16. Seedling J5-1 was homozygous for allele size 122 bp. Seedlings J5-3, J5-78 and J5-92 were heterozygous.

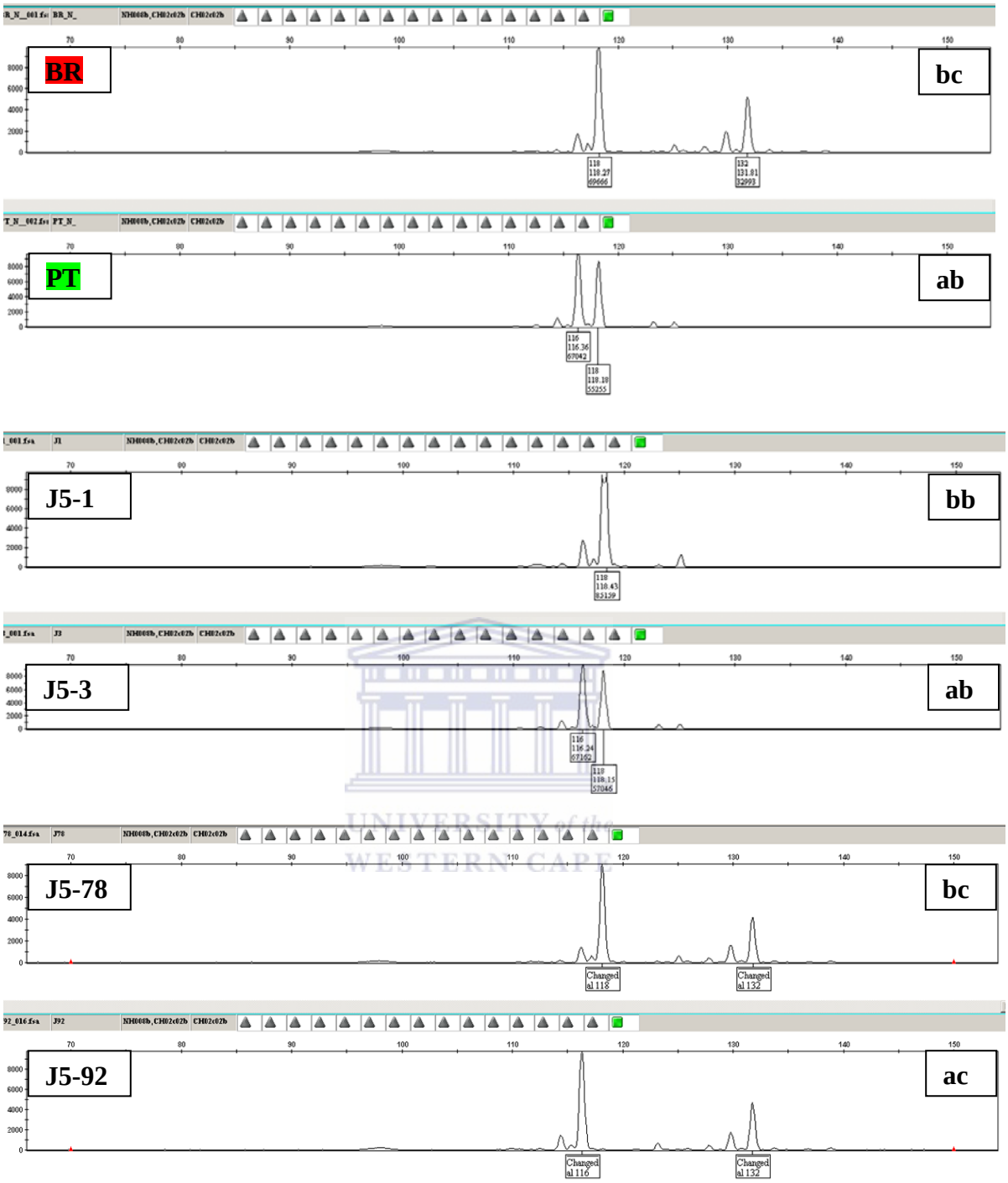


Figure 3.10. Electropherograms obtained after scoring of alleles with apple SSR marker CH02c02b fluorescently labelled with a yellow dye (NED™) on parents 'Bon Rouge' and 'Packham's Triumph' and seedlings J5-1, J5-3, J5-78 and J5-92.

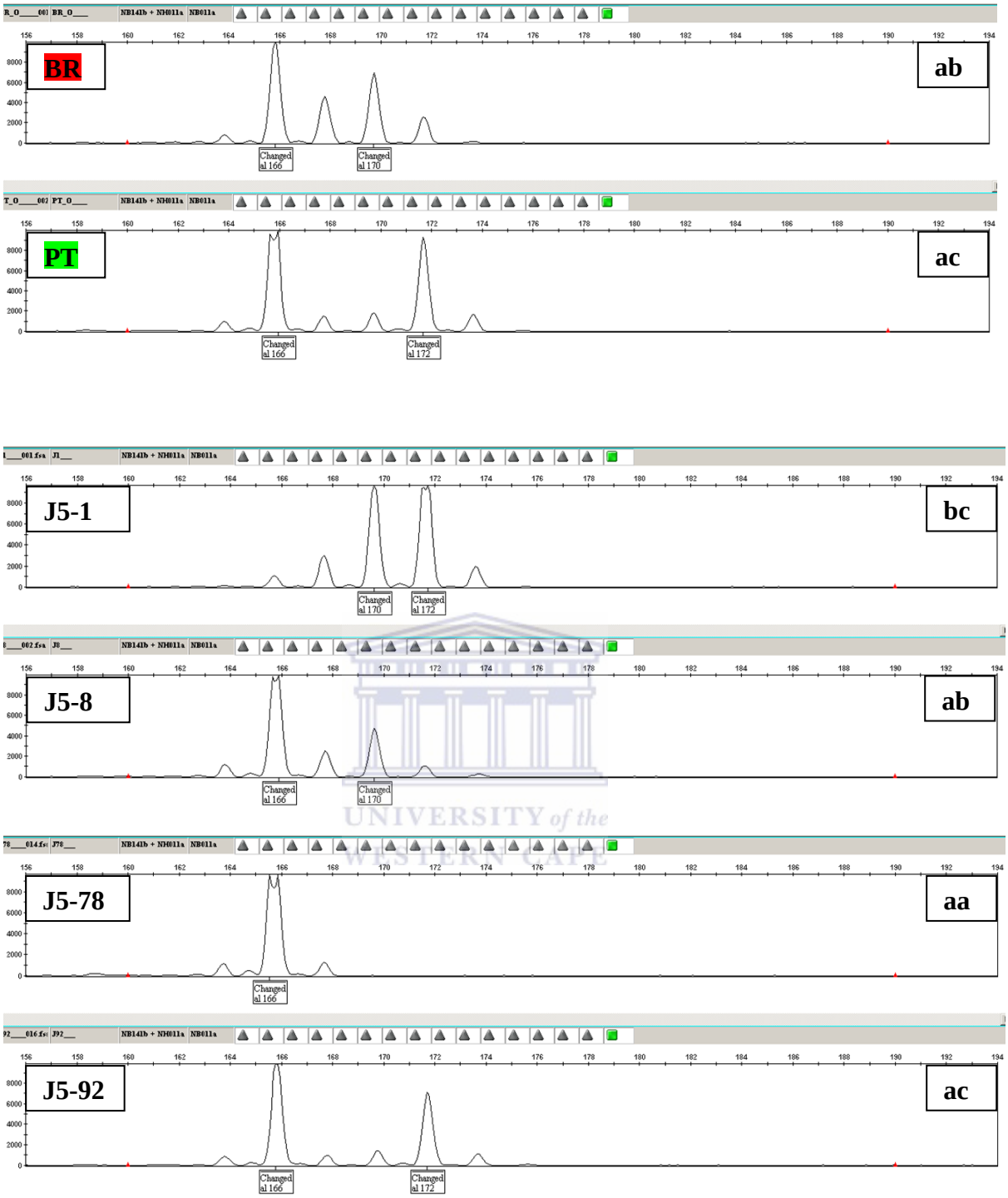


Figure 3.11. Electropherograms obtained after scoring of alleles with pear SSR marker NB011a fluorescently labelled with a yellow dye (NED™) on parents 'Bon Rouge' and 'Packham's Triumph' and seedlings J5-1, J5-3, J5-78 and J5-92.

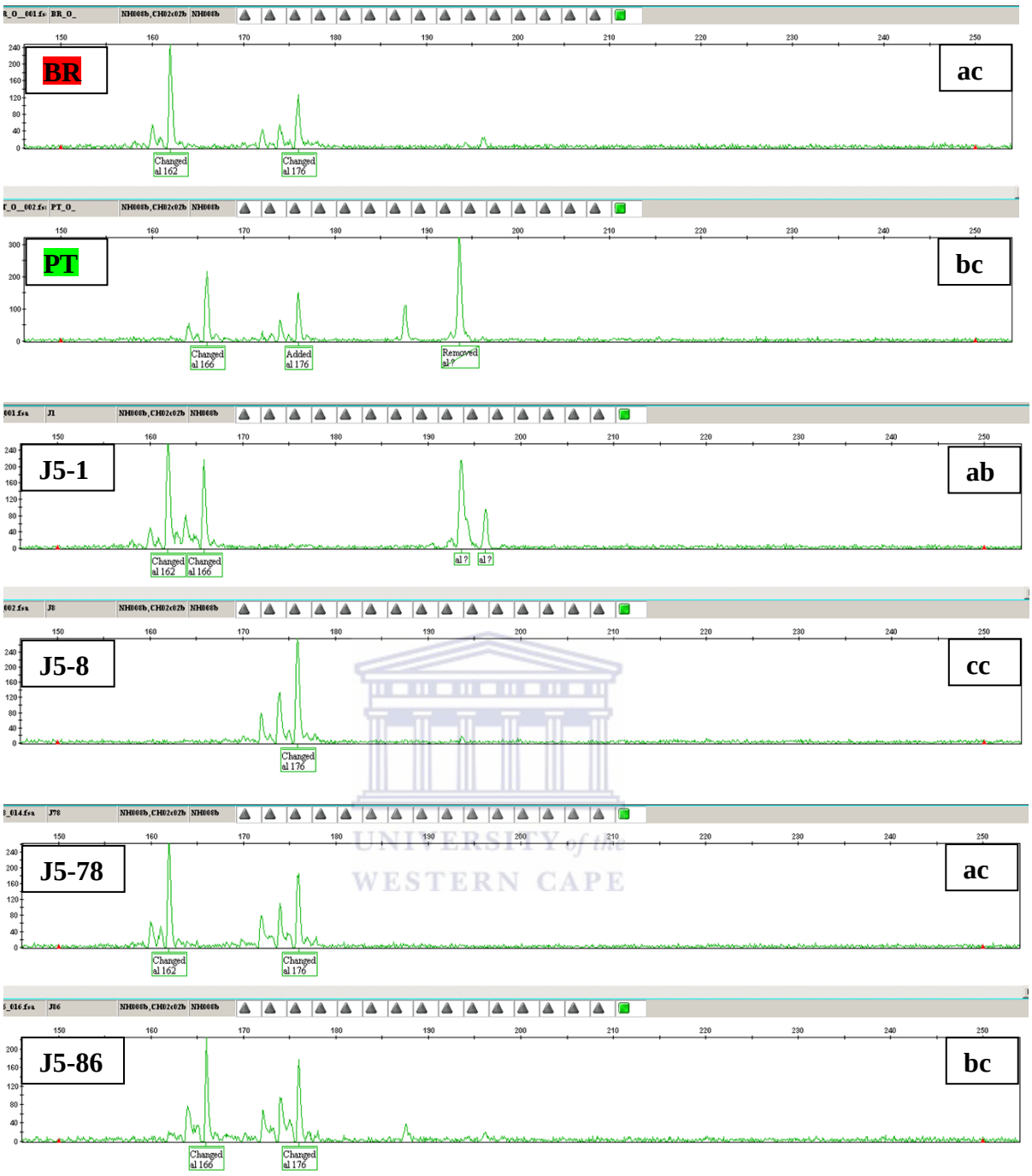


Figure 3.12. Electropherograms obtained after scoring of alleles with pear SSR marker NH008b fluorescently labelled with a green dye (VIC™) on parents 'Bon Rouge' and 'Packham's Triumph' and seedlings J5-1, J5-8, J5-78 and J5-86.

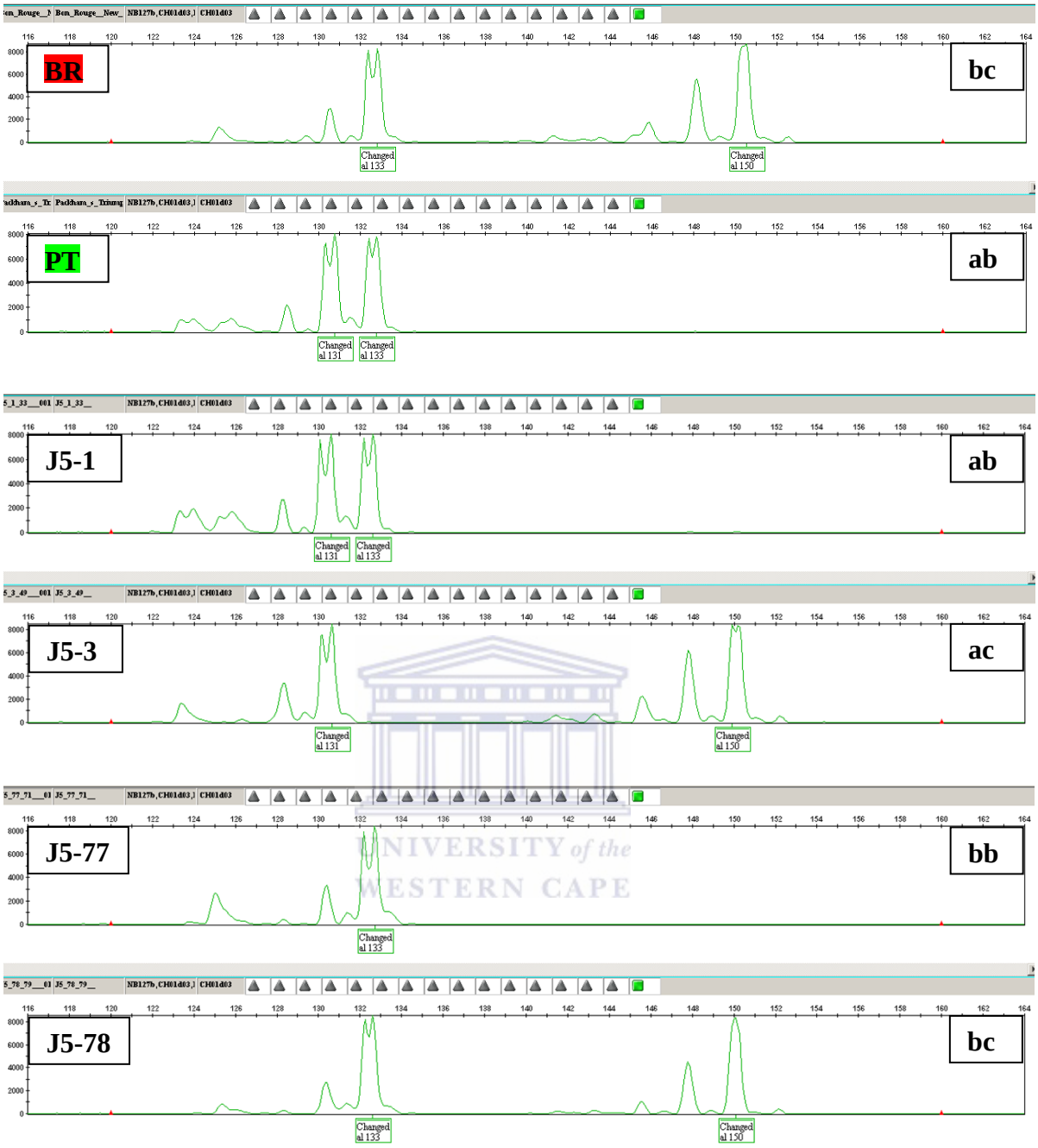


Figure 3.13. Electropherograms obtained after scoring of alleles with apple SSR marker CH01d03 fluorescently labelled with a green dye (VIC™) on parents 'Bon Rouge' and 'Packham's Triumph' and seedlings J5-1, J5-3, J5-77 and J5-78.

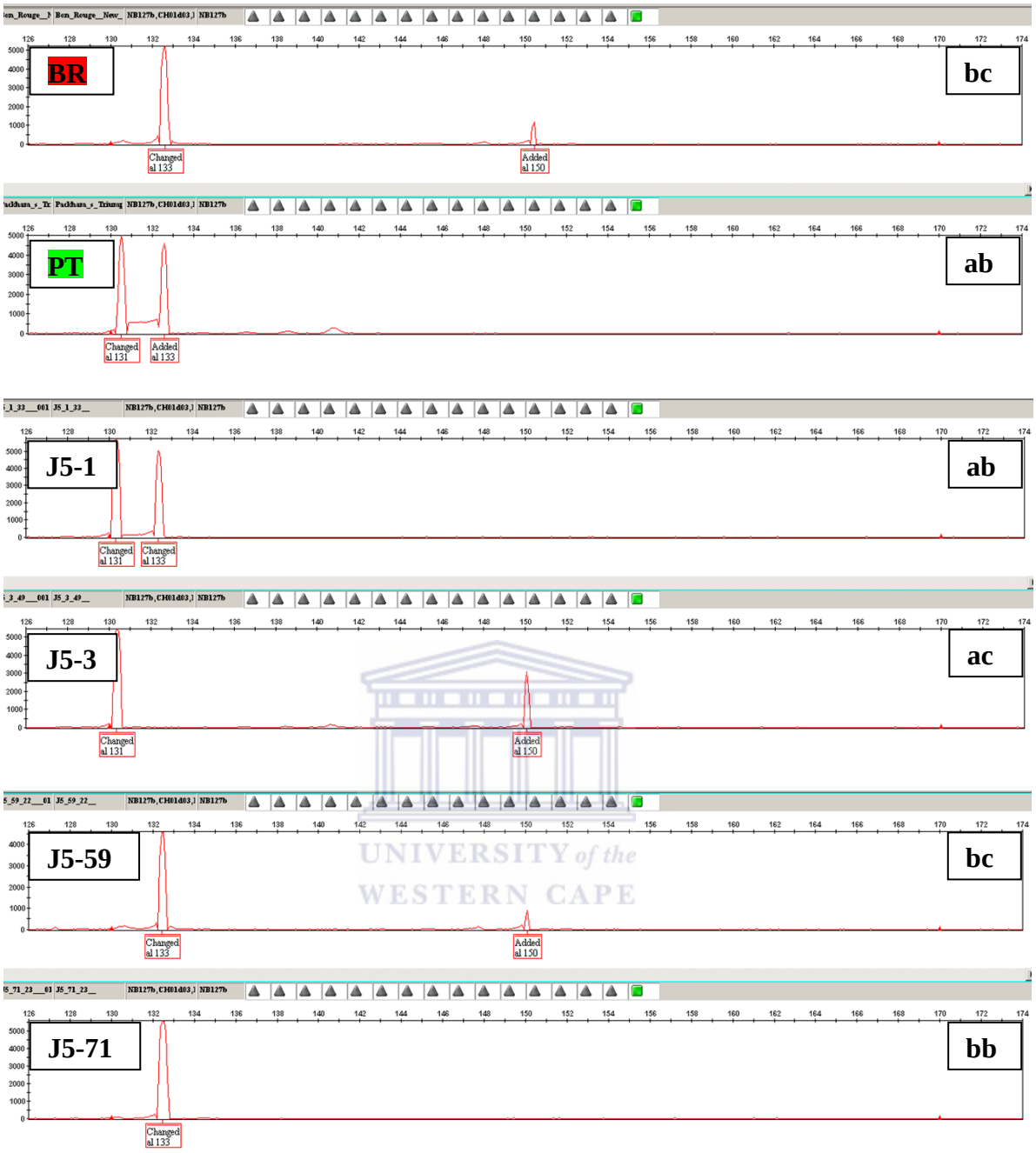


Figure 3.14. Electropherograms obtained after scoring of alleles with pear SSR marker NB127b fluorescently labelled with a red dye (PET™) on parents 'Bon Rouge' and 'Packham's Triumph' and seedlings J5-1, J5-3, J5-59 and J5-71.

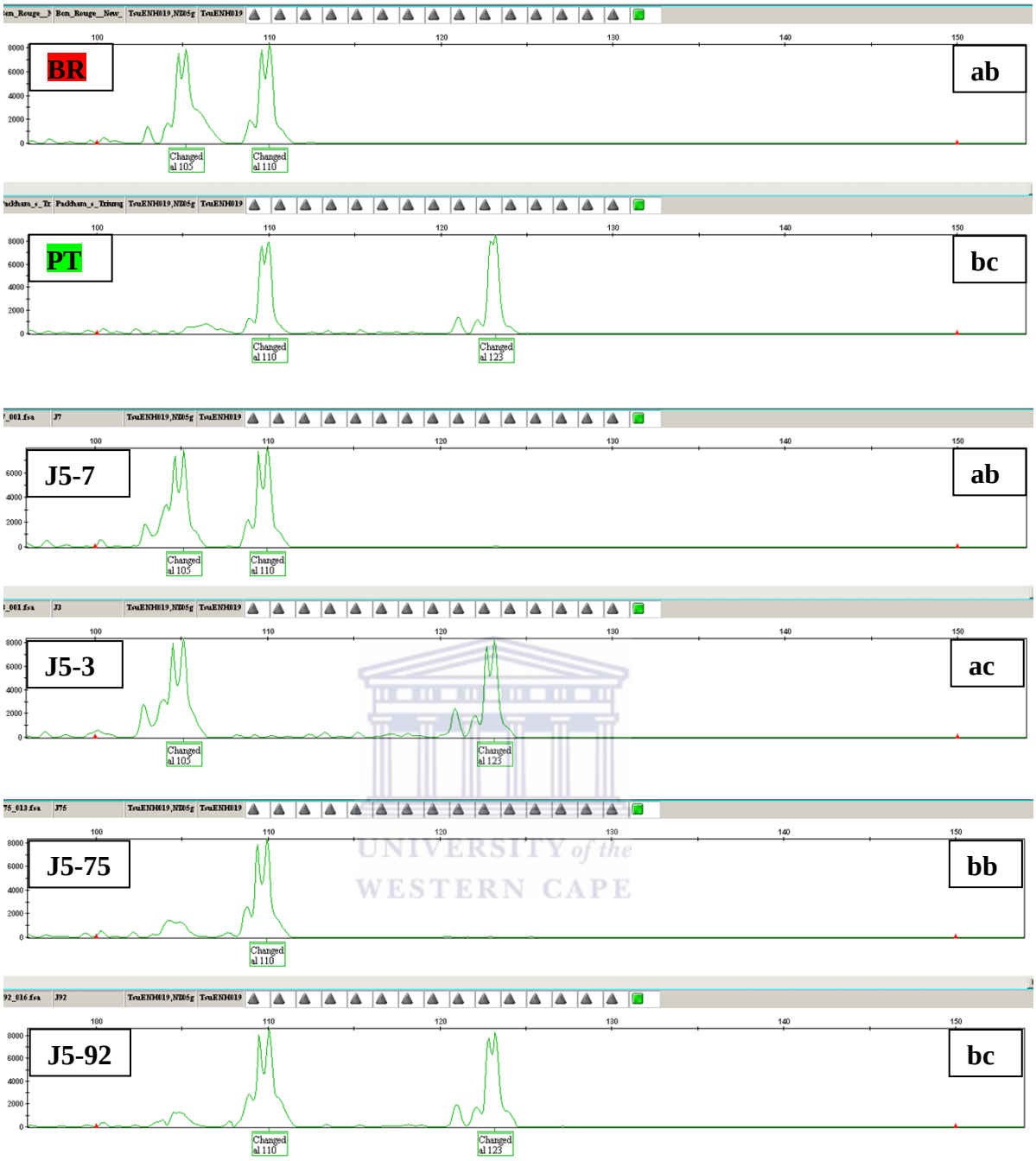


Figure 3.15. Electropherograms obtained after scoring of alleles with pear SSR marker TsuENH019 fluorescently labelled with a green dye (VIC™) on parents 'Bon Rouge' and 'Packham's Triumph' and seedlings J5-7, J5-3, J5-75 and J5-92.

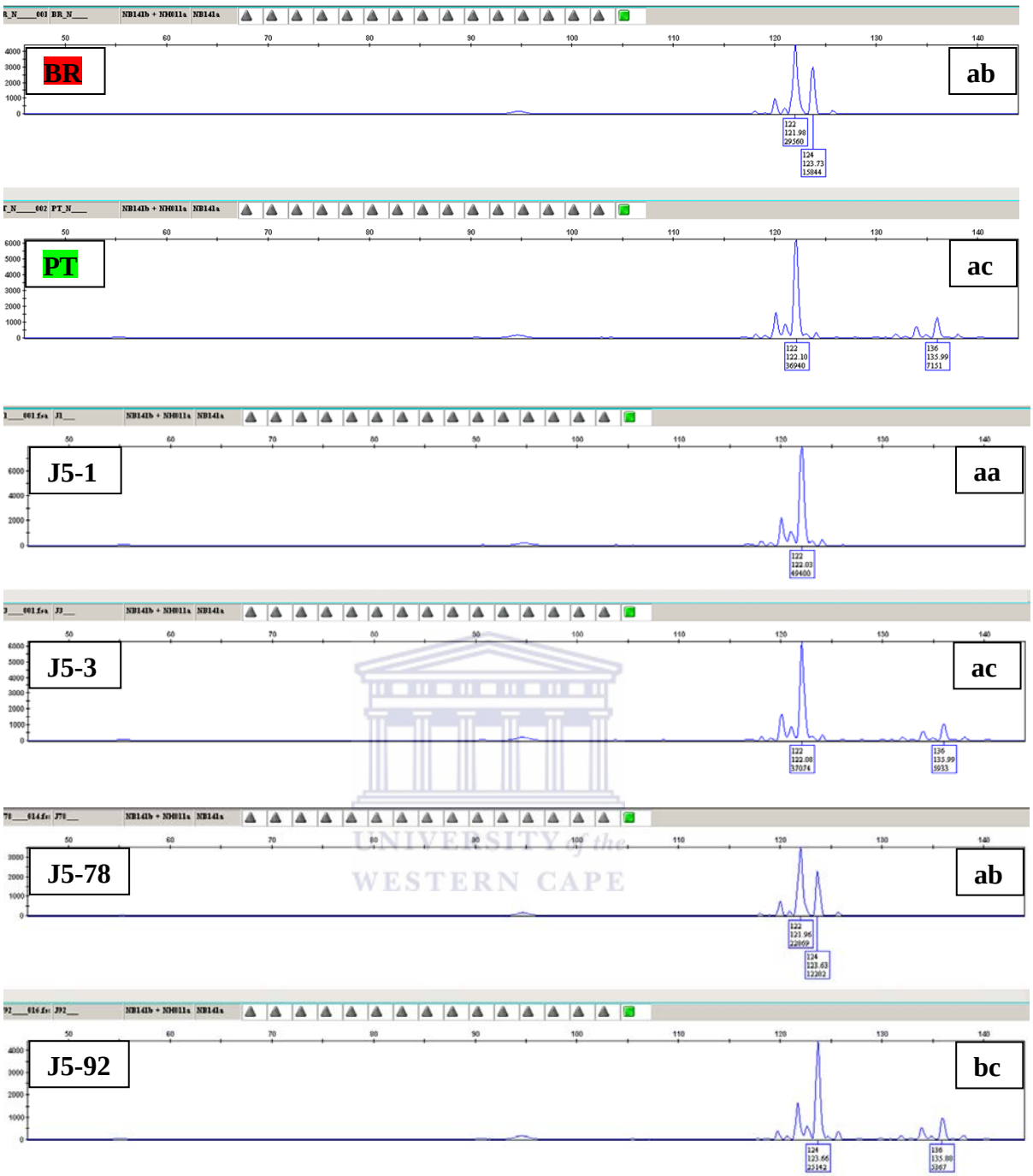


Figure 3.16. Electropherograms obtained after scoring of alleles with pear SSR marker NB141b fluorescently labelled with a blue dye (6-FAM™) on parents 'Bon Rouge' and 'Packham's Triumph' and seedlings J5-1, J5-3, J5-78 and J5-92.

Table 3.4. Allele sizes of parents 'Bon Rouge' and 'Packham's Triumph' for seven SSRs analyzed on the ABI PRISM® 3130xl Genetic Analyzer and scored with the use of GeneMapper® software, segregations observed and chi-square tests of each SSR locus.

SSR locus	PCR sizes (bp) (BR x PT)	Segregation	X^2
CH02c02b	118/132 x 116/118	bcxab	0.43
CH01d03	133/150 x 131/133	bcxab	0.12
NB127b	133/150 x 131/133	bcxab	0.22
NB141b	122/124 x 122/136	abxac	0.39
NH011a	166/170 x 166/172	abxac	0.06
NH008b	162/176 x 166/176	acxbc	0.40
TsuENH019	105/110 x 110/123	abxbc	0.13

Appendix Table A2 shows the amplification product sizes obtained on the ABI PRISM® 3130xl Genetic Analyzer after scoring with GeneMapper® 4.0 software for the seven markers applied on the 125 seedlings. The data were interpreted in terms of segregation and checked independently.

3.10 Genetic linkage map construction

JoinMap® 4.1 makes use of a letter coding system that represent different alleles, which is dependent on the type of segregation involved for a particular marker locus. Table 3.5 illustrates the letter coding system used in JoinMap® 4.1. These are five segregation types encountered when working with an outbreeding species like pear. They are defined, with always two characters left and two characters right of the x-character, in between two angle brackets; these characters left and right of the x define the alleles present in the first and second parent, respectively. From the segregation types it is easy to derive the possible genotypes that can be found in the offspring of the cross (Table 3.5).

Table 3.5. Segregation types and genotypes in outbreeder full-sib family (CP) for JoinMap®.

Class	Segregation type	Number of alleles	Parent 1	Parent 2	Genotype in the CP population	Expected ratio
1	<abxcd>	4	ab	cd	ac; ad; bc; bd	1:1:1:1
1	<efxeg>	4	ef	eg	ee; ef; eg; fg	1:1:1:1
2	<hkxhk>	2	hk	hk	hh; hk; kk	1:2:1
3	<nnxnp>	2 or 3	nn	np	nn; np	1:1
3	<lmxll>	2 or 3	lm	ll	lm; ll	1:1

Calculation of marker order and distances between markers were done separately. There was a point where some of the markers may have been excluded as their presence lead to 'insufficient linkage' between the markers within that group (as with SSR marker NH008b), or their incorporation in that LG resulted in a high 'mean chi-square value', indicating that a lot of double crossover events occur that is highly unlikely, as crossovers

are generally limited to one or two chromosome during meiosis. Regression mapping algorithm and Kosambi's mapping function were used for the determination of marker order within the group. Graphical representation of genetic linkage maps as well as the alignment of different maps was achieved with the use of MpaChart 2.2 (Voorrips, 2002).

As a preliminary exercise to locate the SSR markers on the map, recombination fractions of each marker with respect to the red locus were calculated and the results recorded in a tabular format (Table 3.6). The contingency X^2 values were also calculated as well as the recombination percentage at *P*-value 0.05. The recombination frequency between two loci depends on the distance between them. The closer the loci lie on the same chromosome, the more likely they will inherit together, and the recombination frequency, when studying a number of seedlings, will move closer to zero. A recombination frequency of zero does however not imply that the two loci are geographically next to each other on the genome, they might still be some distance apart, it merely is an indication that within the mapping population used, there was no recombination and it can be assumed that they lie close to each other on the genome. The chi-square test calculates if there are departures from random co-segregation that indicate linkage.

Table 3.6. Co-segregation of SSR markers with red locus in 'Bon Rouge' x 'Packham's Truimph' map.

Marker	X^2	***r* (%)**	***P*-value at 0.05**
CH02c02b x Red	14.01	43.08	2.89×10^{-3}
CH01d03 x Red	67.15	11.82	1.7387×10^{-14}
NB127b x Red	37.26	21.57	4.0558×10^{-8}
NB141b x Red	15.88	38.21	1.2013×10^{-3}
NH011a x Red	95.17	5.79	1.7008×10^{-20}
NH008b x Red	7.43	40.17	5.9457×10^{-2}
TsuENH019 x Red	58.96	14.55	9.786×10^{-13}

The 'Bon Rouge' genetic linkage maps were constructed using seven SSR marker loci with JoinMap® 4.1. Figure 3.17 shows two maps of LG4: 'Bon Rouge' constructed using regression mapping BR4(R), and 'Bon Rouge' constructed using maximum likelihood mapping BR4(ML). Both maps were constructed using independence LOD grouping parameter with a threshold of 3 and Kosambi's mapping function. In the regression and maximum likelihood the map lengths were 53.4 cM and 85.8 cM, respectively. The positioning of SSR markers on the maps for the two mapping algorithms (regression and maximum likelihood) are depicted in Table 3.7 and 3.8, respectively.

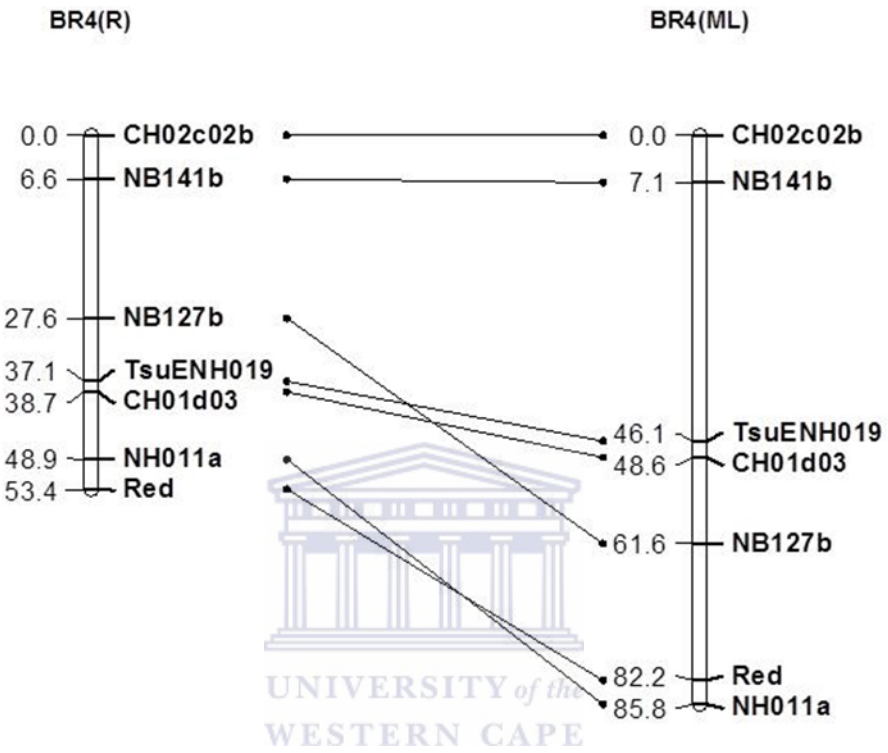


Figure 3.17. Genetic linkage maps of 'Bon Rouge' LG4 with regression BR4(R) and with maximum likelihood BR4(ML), constructed with JoinMap 4.1 and drawn using MapChart 2.2 (Voorrips, 2002). Genetic distances measured in (cM) are listed on the left side of each LG bar; locus names are listed on the right. Common SSR markers, including the *Red* gene on the genetic linkage maps are written in bold and similarities of markers between maps are indicated by cross over lines.

Table 3.7. SSR markers and red trait mapped in 'Bon Rouge' LG4, using regression mapping algorithm in JoinMap 4.1 and their map positions.

SSR Marker	LG Position	Distance (cM)
CH02c02b	4	0.0
CH01d03	4	38.7
NB127b	4	27.6
NB141b	4	6.6
NH011a	4	48.9
NH008b	-	-
TsuENH019	4	37.1
Red gene	4	53.4

Table 3.8. SSR markers and red trait mapped in 'Bon Rouge' LG4, using maximum likelihood mapping algorithm in JoinMap 4.1 and their map positions.

SSR Marker	LG Position	Distance (cM)
CH02c02b	4	0.0
CH01d03	4	48.6
NB127b	4	61.6
NB141b	4	7.1
NH011a	4	85.8
NH008b	-	-
TsuENH019	4	46.1
Red gene	4	82.2

Figure 3.18 shows two maps of LG4: 'Bon Rouge', constructed using regression mapping BR4(R) and 'Max Red Bartlett' (MRB4) from Dondini *et al.* (2008). The 'Bon Rouge' maps were constructed using JoinMap® 4.1 whereas JoinMap® 3.0 had been used to construct the 'Max Red Bartlett' map (MRB4). The 'Max Red Bartlett' map was constructed using a LOD score of 5.0 to group markers and Kosambi's mapping function (Dondini *et al*., 2008).

Concerning the map of 'Max Red Bartlett' (MRB4), constructed by Dondini *et al.* (2008), 90 progeny plants were used for the linkage analysis, with 45 plants with red fruits and 43 plants with non-red fruits and 2 plants without fruits; the segregation approximating to 1:1. The MRB4 linkage map was constructed using AFLP and SSR markers and was 82.2 cM in length. Note that in MRB4 constructed by Dondini *et al*. (2008), two AFLP markers (E31M56-7, 13.5 cM; E33M48-5, 18.2 cM) flanked the red trait (Figure 3.18). Only two SSR marker loci in LG4 of MRB4, CH01d03 and CH02c02b, were mapped, of which CH01d03 was closest to the red trait with a distance of 19.4 cM.

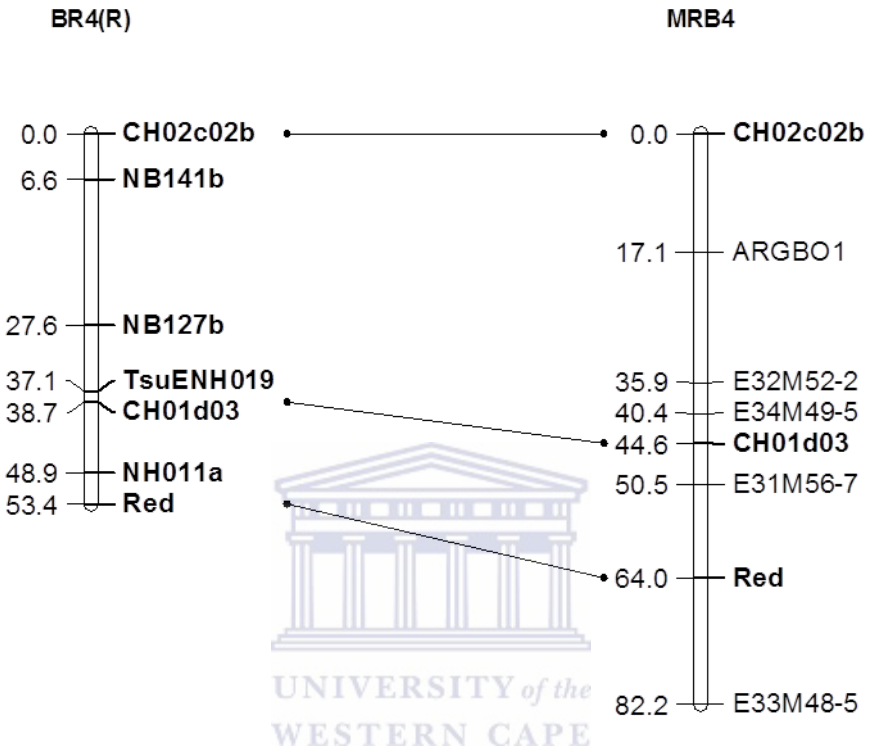


Figure 3.18. Genetic linkage maps of LG4 of 'Bon Rouge' with regression BR4(R), and 'Max Red Bartlett' (MRB4) from Dondini *et al.* (2008), drawn using MapChart 2.2 (Voorrips, 2002). Genetic distances measured in (cM) are listed on the left side of each LG bar; locus names are listed on the right. Common SSR markers, including the *red* gene on genetic linkage maps are written in bold (except NB141b, NB127b, TsuENH019 and NH011a, which were not mapped in MRB4 before by Dondini *et al.* 2008) and similarities of markers between maps are indicated by cross over lines.

The ‘Bon Rouge’ map (BR4(R)) was also compared with a map of LG4 of ‘Bartlett’ (Ba4) (Yamamoto *et al*., 2013) (Figure 3.19). JoinMap® 3.0 was used to construct the ‘Bartlett’ (Ba4) map with regression method using minimum LOD score of 7.0 and Kosambi’s mapping function. All the SSR markers in the ‘Bon Rouge’ map (BR4(R)) mapped almost exactly on the same position as in the ‘Bartlett’ map (Ba4).

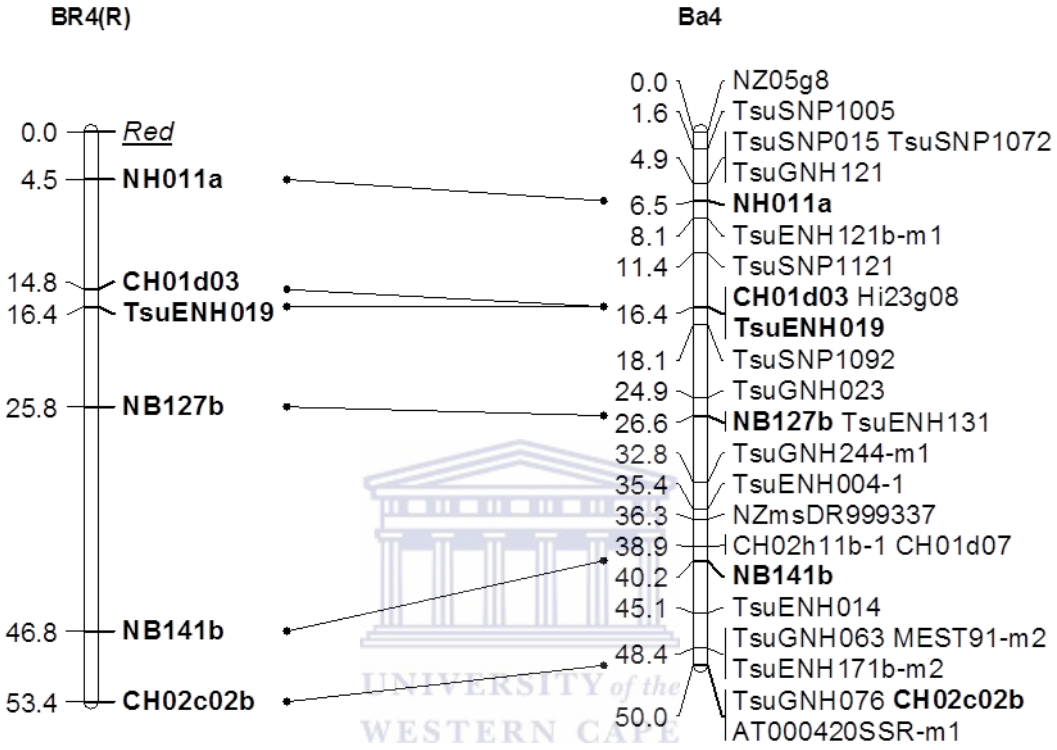


Figure 3.19. Genetic linkage maps of 'Bon Rouge' LG4 with regression BR4(R) and 'Bartlett' (Ba4) from Yamamoto *et al.* (2013) drawn using MapChart 2.2 (Voorrips, 2002). Genetic distances measured in (cM) are listed on the left side of each LG bar; locus names are listed on the right. Similarities of markers between maps are written in bold and by cross over lines.

CHAPTER 4: DISCUSSION AND CONCLUSION

The first important step to take when investigating the trait linked markers is to assemble the necessary germplasm and decide the trait to analyse. The trait of interest in this case was the red colour in 'Bon Rouge'. Making appropriate crosses to raise segregating progenies is important. Mapping populations commonly used in genomic research are generated by controlled crosses between parents, on the basis of variation at the phenotypic level for a given trait of interest (red colour trait in our case). When the parents used are heterozygous and outbreeding, as is the case with pear, genetic variations exists between the parents, which is essential in order to trace recombination events.

DNA analysis of individuals in any given mapping population involves the implementation of molecular markers in order to determine the genotypic code for each individual, at each locus. Progeny derived from a cross between two diploid, heterozygous parents may have up to four alleles segregating at any given locus and these alleles are expected to be passed onto the progeny in equal ratios. Once heterozygosity has been confirmed, the next step is to score the seedlings with polymorphic, informative markers. Analysis for co-segregation of the markers and the trait to detect linkages and estimate recombinations is essential to locate the trait on the genetic map.

It is interesting to note the high level of 'allele sharing' between 'Bon Rouge' and 'Packham's Triumph', caused by the fact that both parents share a common grandparent 'Bon Chretien', also known as 'William's Bon Chretien'. The inheritance of the red colour character in European pear and its map position in the mutant cultivar 'Max Red Bartlett' by Dondini *et al*. (2008) formed the background of this study to map the trait in ARC's 'Bon Rouge' x 'Packham's Triumph' progeny. The study demonstrated that the red trait in

‘Bon Rouge’ located to the same linkage group as the red-trait in ‘Max Red Bartlett’, that is linkage group 4 (LG4), and identified closer SSR marker to the red trait than previously reported, which will aid future genetic studies in pear breeding programmes.

Some SSR markers amplify more than two fragments per individual, as it has been observed in Figure 3.12 for the parent ‘Packham’s Triumph’ and progeny J5-1, where additional peaks were also amplified. The complexity of these multilocus markers depend largely on the number of loci amplified (two or more) as well as the difference in fragment sizes obtained from the two (or more loci). The ease with which these markers can be used will only become clear during segregation analysis of alleles in mapping populations in preparation for genetic linkage map construction. Amplification products smaller than the expected size could be ascribed to small deletions and non-specific primer annealing. The success of multiplexing depends on the principle that primers should have comparable annealing temperatures and that the primers sequences should not contain excessive regions of complementarity, which can lead to primers binding to each other rather than to the template DNA, leading to the formation of primer-dimers.

A total of four multiplexes were constructed in this study. The number of primer pairs comprising these multiplexes varied from two to three per multiplex. Certain primer sets within multiplex displayed weak amplification when analysed on the ABI PRISM® 3130xl Genetic Analyzer. Certain primer sets work better in a simplex reaction than in a multiplex. For such primer sets, increasing primer concentration or lowering the annealing temperature might improve results.

High quality primers are essential for successful multiplex amplification reactions. ‘Dye blobs’ can occur when fluorescent dyes are no longer linked to their respective primers. The migration of the free dye in the capillary during electrophoresis can interfere with the detection of true alleles present from other loci amplified in a multiplex. All the SSR markers in the multiplexes were chosen from published maps of LG4 in ‘Bartlett’ and other apple maps. Multiplexes save time as it would be more time consuming to score the markers individually on all the seedlings and they reduce the cost of sizing in the ABI sequencer. The failer of PCR amplification of some SSR containing regions might be ascribed by the presence of introns in genomic DNA.

Primers were grouped according to different dye colourson the multiplexes, as multiplexes comprising of the same colour make it difficult to score the allele sizes unless the size ranges are far apart. Therefore, it is important not to design primer sets with overlapping sizes as that would make it impossible to differentiate which alleles were amplified by which primer set.

A variety of computer software programs have been developed in the past to handle large numbers of segregating loci, in order to establish the best overall genetic map. Some of the most widely used programs include Mapmaker (Lincoln *et al*., 2003), GMendel and JoinMap. These software programs use the genetic principle that the position of, and distance between, loci on the genome is related to the frequency of recombination between the alleles of different loci. The distance between two genes is determined by their recombination fraction. The map-units are Morgans. One Morgan is the distance over which, on average, one crossover occurs per meiosis. When considering the mapping of

more than two points on the genetic map, it would be very handy if the distances on the map were additive. However, recombination fractions themselves are not additive.

JoinMap allows the use of the two mostly used mapping functions, Haldane's and Kosambi's mapping functions. The selected mapping function is used to translate recombination frequency into a map distance prior to the weighted least squares map estimations; the inverse function is used in the goodness-of-fit calculation and in the calculation of genotype probabilities. For linkage groups genotype data populations, one can choose between the regression mapping and the maximum likelihood mapping algorithms as calculation option. For pairwise data population groups or combined groups, only the regression mapping algorithm can be utilized. The regression mapping procedure according to Stam (1993), is a process of building a map by adding loci one by one, starting from the most informative pair of loci. For each added locus the best position is searched by comparing the goodness-of-fit of the calculated map for each tested position. When at the best position, the goodness-of-fit decreases too sharply or when the locus gives rise to negative distance estimates in the map, the locus is removed. Maximum likelihood (ML) mapping algorithm was developed after regression (L) mapping algorithm after seeing that there were more and higher density maps that needed to be mapped. Maximum likelihood algorithm uses a combination of several techniques to order loci and compute their mutual distances: these are simulated annealing, Gibbs sampling and spatial sampling. Gibbs sampling is used to estimate multipoint recombination frequencies that can be used to calculate the likelihoods. Simulated annealing searches for the order that has the maximum likelihood. Spatial sampling is a technique tha is needed to prevent getting trapped at local optima rather than arriving at the global optimum solution due to missing genotype information and genotype errors. Also the maximum likelihood mapping

algorithm appears to be sensitive to genotype errors and having many unknown genotypes in the dataset and also for dominance in repulsion (Van Ooijen *et al*., 2001). In terms of length, maximum likelihood mapping algorithm is expected to be the longer mapping algorithm due to the several techniques it uses to order loci and compute distances.

Linkage maps allow studies of the genome structure and the localization of genes of interest, and permit the identification of quantitative trait loci (QTLs) as a first step towards understanding the biological basis of complex traits. The steps involved in genomic map construction can be divided into five key steps. The first step would be to create a segregating mapping population; this is followed by single-locus analysis (determining the genotypes of all loci in all individuals), two-locus analysis (estimating the recombination frequencies between all pairs of loc), the establishment of linkage groups and marker ordering.

Genetic linkage maps were successfully constructed for the F1 mapping population under investigation. The steps followed during the construction of genetic linkage map led to the successful positioning of all segregating markers. Figure 3.17 shows genetic linkage maps of ‘Bon Rouge’ of LG4 with regression BR4(R) and with maximum likelihood BR4(ML) constructed with JoinMap 4.1 and drawn using MapChart 2.2. Looking at both regression and maximum likelihood mapping algorithms of ‘Bon Rouge’ linkage 4 maps (BR4) (Figure 3.17), the BR4(R) map was shorter than the BR4(ML) map. The reason for the differences in map lengths is due to mapping algorithms used to construct each map. Six marker loci were mapped in each case although the order of the loci on the maps differed. The differences were in the location of marker, NB127b and the red trait. On the BR4(R) map, NB127b was mapped between two SSR marker loci, NB141b and TsuENH019

whereas on the BR4(ML) map, it was mapped between CH01d03 and NH011a. In the BR4(R) map the red trait was mapped at the end of the LG and was linked to marker NH011a, with a mapping distance 4.5 cM. On the BR4(ML) map the red trait was flanked by two SSR markers, NB127b at 20.6 cM distance and NH011a at 3.6 cM distance.

The map of BR4(R) was compared to the map of (MRB4) which was constructed by Dondini *et al*. (2008) (Figure 3.18). Marker loci CH02c02b and CH01d03 mapped to approximately the same positions in the 'Bon Rouge' maps as they did in 'Max Red Bartlett' (Dondini *et al*., 2008). These two marker loci in 'Max Red Bartlett' were positioned at 0.0 cM and 44.6 cM, respectively, versus 0.0 cM and 38.7 cM in BR4(R) and 0.0 cM and 48.8 cM in BR4(ML). The closest SSR marker to the red trait gene in MRB4 was CH01d03, at a distance of 19.4 cM. Four new SSR markers were mapped in BR4(R) and BR4(ML), namely NB141b, NB127b, TsuENH019 and NH011a. The closest SSR marker to the red trait in the BR4 maps was NH011a at a distance of approximately 4 cM.

Also the BR4(R) map was compared to a very recent map of 'Bartlett' LG4 by Yamamoto *et al*. (2013). The orientation of the BR4(R) map was inverted for comparison to the Ba4 map (Figure 3.19). The Yamamoto *et al*. (2013) map included SSR markers TsuGNH and EST-SSR loci from apple, as well as SNP markers from pear (TsuSNP) but did not map the red trait. The SSR markers TsuGNH121 and NZ05g08 lie on the other side of the red trait when the Yamamoto *et al*. (2013) map is compared to the BR4(R) map. These should be scored in the 'Bon Rouge' x 'Packham's triumph' progeny as they may be useful markers in better defining the position of the red trait.

Although SSR markers CH01d03, Ch02c02b, NH011a, NB141b, NB127b and TsuENH019 mapped to LG4 in 'Bon Rouge' x 'Packham's Triumph', SSR marker NH008b did not map to the LG4. Fernandez-Fernandez *et al*. (2008) mapped the marker, NH008b in LG4 on an apple map but Yamamoto *et al*. (2013) did not map this marker. Presumably the locus amplified by this marker in apple is not homologous to that amplified in pear.

From this study it was clear that cross-species use of microsatellite primers is possible and that primers designed for apple can successfully be used to detect polymorphism,and ultimately for genetic map construction in pears. The results of this study with 'Bon Rouge' further indicated that the red trait gene mapped to the same position as the red trait in 'Max Red Bartlett' reported by Dondini *et al*. (2008). It would be important to investigate if the actual mutation in 'Bon Rouge' is similar to that in 'Max Red Bartlett'. In addition, marker NH011a has been found to be closely linked to the red trait gene, with an approximate distance of 4 cM. This marker can be used to indirectly select for the red trait gene in pear, for example to distinguish heterozygotes from homozygotes. Anthocyanin leaf pigmentation with a ratio 1:1 is controlled by a single locus. In this case, a heterozygote will express only the trait coded by the dominant allele, and the trait coded by the recessive allele will not be present. Therefore, this work sets the scene for further genetic studies on the red trait and its better utilization in the pear breeding programme.

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APPENDICES

Appendix Table A1. List of eleven microsatellite primer sets for LG4 that were used in this study, showing primer sequences, references and published map positions.

SSR locus	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size range (bp) in pear
CH02c02b	tgc atg cat gga aac gac (18)	tgg aaa aag tca cac tgc tcc (21)	125-150
CH01d03	cca ctt ggc aat gac tcc tc (20)	acc tta ccg cca atg tga ag (20)	120-150
CH04e02	ggc gat gac tac cag gaa aa (20)	atg tag cca agc cag cgt at (20)	110-170
CH01f02	acc aca tta gag cag ttg agg (21)	ctg gtt tgt ttt cct cca gc (20)	160-180
NB127b	*gtttctt* att aca agt tgg gga aaa gtt (28)	aac taa ttg att ctt ttt gct (21)	146-166
NB141b	cag aga aag aca gag gta gag aga a (25)	gga ttg atc gcc tta tgg ttg t (22)	122-136
NH011a	ggt tca cat aga gag aga gag (21)	ttt gcc gtt gga ccg agc (18)	166-172
NH008b	gga aaa gag aag gaa gaa gag aag g (25)	tga tag ggg cat ttc ggt aa (20)	162-176
Hi08e04	gca tgg tgg cct ttc taa g (19)	gtt tac cct ctg act caa ccc aac (24)	201-234
NZ05g08	cgg cca tcg att atc tta ctc tt (23)	gga tca atg cac tga aat aaa cg (23)	115-147
TsuENH019	ctc cgc tca ctc ctc aga agt tt (23)	*gtttctt* aat gct tga gtt ggt tga gct tc (30)	105-123

*Sequence reference = first author to develop the SSR locus

Appendix Table A2. GeneMapper® allele scores for parents 'Bon Rouge' and 'Packham's Triumph' and the 125 progeny scored using SSR markers CH01d03, Ch02c02b, NB127b, NB141b, NH008b, NH011a and TsuENH019.

	SSR Marker Loci						
Parents	**CH01d03**	**CH02c02b**	**NB127b**	**NB141b**	**NH008**	**NH011a**	**TsuENH019**
BR	133/150	118/132	133/150	122/124	162/176	166/170	105/110
PT	131/133	116/118	131/133	122/136	166/176	166/172	110/123
Individuals	**CH01d03**	**CH02c02b**	**NB127b**	**NB141b**	**NH008**	**NH011a**	**TsuENH019**
J5-1	131/133	118/118	131/133	122/122	162/166	170/172	110/123
J5-2	133/150	116/118	133/150	..	..	..	105/110
J5-3	131/150	116/118	131/150	122/136	162/166	170/172	105/123
J5-4	133/150	118/132	133/150	122/124	176/176	166/170	105/110
J5-5	133/150	118/132	133/150	122/124	176/176	166/170	105/110
J5-6	133/150	118/132	133/150	122/124	166/176	166/172	105/110
J5-7	133/150	118/132	133/150	122/124	176/176	166/170	105/110
J5-8	133/150	116/118	133/150	122/122	176/176	166/170	..
J5-9	131/150	118/132	131/133	122/124	176/176	166/172	105/123
J5-10	133/150	118/132	133/150	122/124	162/176	166/172	105/110
J5-11	133/150	118/132	133	122/124	162/176	166/170	105/110
J5-12	131/150	116/118	131/133	122/136	176/176	170/172	105/123
J5-13	131/150	118/132	131/150	122/124	162/166	166/172	105/123
J5-14	131/150	..	131/150	122/136	..	170/172	105/123
J5-15	131/150	118/132	131/150	122/124	176/176	170/172	105/123
J5-16	131/150	116/118	131/150	124/136	162/176	170/172	105/123
J5-17	133/150	118/132	133/150	122/124	176/176	166/170	105/110
J5-18	131/150	116/132	131/150	124/136	176/176	170/172	105/123
J5-19	131/150	116/132	131/150	124/136	176/176	170/172	105/123
J5-20	131/150	116/118	..	122/136	162/166	170/172	105/123
J5-21	131/150	118/132	131/133	122/124	176/176	170/172	105/123
J5-22	131/150	118/132	131/150	122/124	166/176	170/172	105/123
J5-23	131/150	116/132	131/133	124/136	176/176	170/172	105/123
J5-24	131/150	116/118	131/150	122/136	162/176	166/172	105/123
J5-25	133/150	118/132	133	122/124	162/176	166/170	105/110
J5-26	131/133	116/118	131/133	122/136	176/176	170/172	110/123
J5-27	131/150	118/132	131/133	124/136	166/176	170/172	105/123
J5-28	133/150	118/132	133/150	122/124	176/176	166/170	105/110
J5-29	..	118/132	133/150	..	162/176	170/172	..
J5-30	133/150	118/132	133/150	122/124	176/176	166/170	105/110
J5-31	131/150	116/132	131/133	124/136	176/176	170/172	105/123
J5-32	131/150	118/118	131/150	122/136	162/176	170/172	105/123
J5-33	133/150	118/132	133	122/124	162/176	166/170	105/110
J5-34	133/150	118/118	133	122/122	176/176	166/170	105/110
J5-35	133/150	118/118	133	122/124	162/166	166/170	105/110
J5-36	131/133	116/118	131/133	122/136	176/176	170/172	110/123
J5-37	131/150	118/118	131/150	122/122	176/176	170/172	105/123
J5-38	131/150	118/132	131/133	124/136	162/166	170/172	105/123
J5-39	133/150	116/118	133	122/136	162/166	166/170	105/110
J5-40	131/150	118/132	131/150	122/124	176/176	170/172	105/123
J5-41	133/150	116/118	133	122/136	176/176	166/170	105/123

J5-42	133/150	118/132	133	122/124	176/176	166/170	105/110
J5-43	133/150	118/132	133	122/124	162/176	166/170	105/110
J5-44	133/150	118/132	133	122/124	166/176	166/170	105/110
J5-45	131/150	118/132	131/150	122/124	176/176	170/172	105/123
J5-46	133/150	116/118	133/150	122/136	166/176	166/170	105/110
J5-47	131/150	116/118	131/150	122/136	162/166	170/172	105/123
J5-48	131/150	116/132	131/133	124/136	162/166	166/170	105/123
J5-49	133/150	118/132	133/150	122/124	176/176	166/170	105/110
J5-50	133/150	118/132	133/150	122/124	166/176	166/170	105/110
J5-51	133/150	118/132	133/150	122/124	166/176	166/170	105/110
J5-52	131/150	116/132	131/150	124/136	176/176	170/172	105/123
J5-53	133/150	118/132	133/150	122/124	162/176	166/170	..
J5-54	131/150	118/132	131/133	122/124	162/176	170/172	105/123
J5-55	..	118/118	..	122/122	162/176	166/166	..
J5-56	131/133	118/118	131/133	122/122	162/176	166/172	110/123
J5-57	131/133	116/118	131/133	122/136	176/176	166/172	110/123
J5-58	131/133	116/132	131/133	122/136	176/176	166/172	110/123
J5-59	133/150	116/132	133/150	124/136	166/176	166/166	105/110
J5-60	133/133	116/118	133	122/136	162/176	166/166	..
J5-61	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-62	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-63	131/133	116/118	131/133	122/136	166/176	166/172	110/123
J5-64	133/133	118/132	133	122/122	162/176	166/166	105/110
J5-65	133/150	116/132	133/150	124/136	166/176	166/166	105/110
J5-66	133/133	116/132	133	124/136	..	166/166	..
J5-67	131/133	116/132	131/133	124/136	176/176	166/172	110/123
J5-68	131/133	116/118	131/133	122/136	176/176	166/172	110/123
J5-69	131/133	116/132	131/133	124/136	162/166	166/172	110/123
J5-70	131/133	116/118	131/133	122/136	176/176	166/172	110/123
J5-71	133/133	118/132	133	122/122	162/176	166/166	110
J5-72	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-73	131/133	118/118	131/133	122/122	162/176	166/172	110/123
J5-74	131/133	116/132	131/133	124/136	176/176	166/172	110/123
J5-75	133/133	118/132	133	122/124	166/176	166/166	110
J5-76	131/133	116/118	131/133	122/136	162/166	166/172	110/123
J5-77	133/133	116/132	133	124/136	166/176	166/166	..
J5-78	133/150	118/132	133	122/124	162/176	166/166	105/110
J5-79	131/133	118/132	131/133	122/124	162/176	166/172	110/123
J5-80	131/133	118/118	131/133	122/122	176/176	166/172	110/123
J5-81	131/133	118/132	131/133	124/136	166/176	..	110/123
J5-82	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-83	133/133	118/132	133	122/124	162/176	166/166	..
J5-84	131/133	116/132	131/133	124/136	162/166	166/172	110/123
J5-85	..	118/118	..	122/122	176/176	166/166	..
J5-86	133/150	118/132	133/150	122/124	166/176	166/172	105/110
J5-87	131/133	116/118	131/133	122/136	162/176	166/172	..
J5-88	131/133	116/132	131/133	122/136	162/176	166/172	110/123
J5-89	..	118/132	..	122/124	162/166	166/172	..
J5-90	133/150	118/132	133/150	122/124	162/166	166/166	..
J5-91	..	116/118	..	122/136	162/176	166/166	..
J5-92	131/133	116/132	131/133	124/136	162/166	166/172	110/123
J5-93	..	118/118	..	122/122	162/166	..	..
J5-94	131/133	..	131/133	122/136	..	166/172	110/123
J5-95	131/133	118/118	131/133	122/122	..	166/172	110/123
J5-96	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-97	..	116/132	..	124/136	166/176	166/166	105/110
J5-98	..	116/132	..	124/136	162/166	166/166	105/110
J5-99	..	118/132	..	122/124	162/166	166/166	105/110
J5-100	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-101	131/133	118/118	..	122/122	176/176	166/172	110/123

J5-102	131/133	116/132	..	124/136	166/176	166/172	110/123
J5-103	131/133	116/132	131/133	124/136	176/176	166/172	105/123
J5-104	..	116/118	..	122/136	162/176	166/172	110/123
J5-105	131/150	116/132	131/133	124/136	162/166	170/172	105/123
J5-106	131/133	116/132	133	124/136	162/176	166/166	110/123
J5-107	133/133	118/118	..	122/122	176/176	166/172	110
J5-108	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-109	..	118/132	..	122/124	162/176	166/166	110
J5-110	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-111	133/150	118/132	..	122/124	176/176	166/172	105/123
J5-112	133/133	116/118	..	122/136	176/176	166/166	105/110
J5-113	131/133	116/118	131/133	122/136	176/176	166/172	110/123
J5-114	131/150	118/118	131/150	122/136	176/176	170/172	105/123
J5-115	133/150	116/132	133/150	124/136	166/176	166/166	..
J5-116	131/133	116/118	131/133	122/136	162/166	166/172	110/123
J5-117	131/133	116/118	131/133	122/136	162/166	166/172	110/123
J5-118	131/133	116/118	131/133	122/136	162/176	166/172	110/123
J5-119	131/133	118/118	131/133	122/122	162/166	166/172	110/123
J5-120	..	118/132	..	122/124	162/176	166/166	110
J5-121	133/150	118/118	133/150	122/124	176/176	166/172	105/110
J5-122	..	118/132	..	122/124	..	166/166	110
J5-123	131/133	116/118	131/133	122/136	162/166	166/166	110/123
J5-124	131/133	118/118	131/133	122/122	162/166	166/172	110/123
J5-125	131/133	118/132	131/133	124/136	166/176	166/172	110/123

Appendix Table A3. JoinMap® genotype codes for parents 'Bon Rouge' and 'Packham's Triumph' and the 125 progeny scored using SSR markers CH01d03, Ch02c02b, NB127b, NB141b, NH008b, NH011a and TsuENH019.

	SSR Marker Loci						
Parents	**CH01d03**	**CH02c02b**	**NB127b**	**NB141b**	**NH008**	**NH011a**	**TsuENH019**
BR	ef	ef	ef	ef	ef	ef	ef
PT	eg	eg	eg	eg	eg	eg	eg
Individuals	**CH01d03**	**CH02c02b**	**NB127b**	**NB141b**	**NH008**	**NH011a**	**TsuENH019**
J5-1	eg	ee	eg	ee	fg	fg	eg
J5-2	ef	eg	ef	..	..	..	ef
J5-3	fg	eg	fg	eg	fg	fg	fg
J5-4	ef	ef	ef	ef	ee	ef	ef
J5-5	ef	ef	ef	ef	ee	ef	ef
J5-6	ef	ef	ef	ef	eg	eg	ef
J5-7	ef	ef	ef	ef	ee	ef	ef
J5-8	ef	eg	ef	ee	ee	ef	..
J5-9	fg	ef	eg	ef	ee	eg	fg
J5-10	ef	ef	ef	ef	ef	eg	ef
J5-11	ef	ef	ee	ef	ef	ef	ef
J5-12	fg	eg	fg	eg	ee	fg	fg
J5-13	fg	ef	fg	ef	fg	eg	fg
J5-14	fg	..	fg	eg	..	fg	fg
J5-15	fg	ef	fg	ef	ee	fg	fg
J5-16	fg	eg	fg	fg	ef	fg	fg
J5-17	ef	ef	ef	ef	ee	ef	ef
J5-18	fg	fg	fg	fg	ee	fg	fg
J5-19	fg	fg	fg	fg	ee	fg	fg
J5-20	fg	eg	fg	eg	fg	fg	fg
J5-21	fg	ef	eg	ef	ee	fg	fg
J5-22	fg	ef	fg	ef	eg	fg	fg
J5-23	fg	fg	eg	fg	ee	fg	fg
J5-24	fg	eg	fg	eg	ef	eg	fg
J5-25	ef	ef	ee	ef	ef	ef	ef
J5-26	eg	eg	eg	eg	ee	fg	eg
J5-27	fg	ef	eg	fg	eg	fg	fg
J5-28	ef	ef	ef	ef	ee	ef	ef
J5-29	..	ef	..	eg	ef	fg	..
J5-30	ef	ef	ef	ef	ee	ef	ef
J5-31	fg	fg	eg	fg	ee	fg	fg
J5-32	fg	ee	fg	eg	ef	fg	fg
J5-33	ef	ef	ee	ef	ef	ef	ef
J5-34	ef	ee	ee	ee	ee	ef	ef
J5-35	ef	ee	ee	ef	fg	ef	ef
J5-36	eg	eg	eg	eg	ee	fg	eg
J5-37	fg	ee	fg	ee	ee	fg	fg
J5-38	fg	ef	eg	fg	fg	fg	fg
J5-39	ef	eg	ee	eg	fg	ef	ef
J5-40	fg	ef	fg	ef	ee	fg	fg
J5-41	ef	eg	ee	eg	ee	ef	fg
J5-42	ef	ef	ee	ef	ee	ef	ef
J5-43	ef	ef	ee	ef	ef	ef	ef
J5-44	ef	ef	ee	ef	eg	ef	ef

J5-45	fg	ef	fg	ef	ee	fg	fg
J5-46	ef	eg	ef	eg	eg	ef	ef
J5-47	fg	eg	fg	eg	fg	fg	fg
J5-48	fg	fg	eg	fg	fg	ef	fg
J5-49	ef	ef	ef	ef	ee	ef	ef
J5-50	ef	ef	ef	ef	eg	ef	ef
J5-51	ef	ef	ef	ef	eg	ef	ef
J5-52	fg	fg	fg	fg	ee	fg	fg
J5-53	ef	ef	ef	ef	ef	ef	..
J5-54	fg	ef	eg	ef	ef	fg	fg
J5-55	..	ee	..	ee	ef	ee	..
J5-56	eg	ee	eg	ee	ef	eg	eg
J5-57	eg	eg	eg	eg	ee	eg	eg
J5-58	eg	fg	eg	eg	ee	eg	eg
J5-59	ef	fg	ef	fg	eg	ee	ef
J5-60	ee	eg	ee	eg	ef	ee	..
J5-61	eg	eg	eg	eg	ef	eg	eg
J5-62	eg	eg	eg	eg	ef	eg	eg
J5-63	eg	eg	eg	eg	ef	eg	eg
J5-64	ee	ef	ee	ee	ef	ee	ef
J5-65	ef	fg	ef	fg	ef	ee	ef
J5-66	ee	fg	ee	fg	..	ee	..
J5-67	eg	fg	eg	fg	ee	eg	eg
J5-68	eg	eg	eg	eg	ee	eg	eg
J5-69	eg	fg	eg	fg	fg	eg	eg
J5-70	eg	eg	eg	eg	ee	eg	eg
J5-71	ee	ef	ee	ee	ef	ee	ee
J5-72	eg	eg	eg	eg	ef	eg	eg
J5-73	eg	ee	eg	ee	ef	eg	eg
J5-74	eg	fg	eg	fg	ee	eg	eg
J5-75	ee	ef	ee	ef	eg	ee	ee
J5-76	eg	eg	eg	eg	fg	eg	eg
J5-77	ee	fg	ee	fg	eg	ee	..
J5-78	ef	ef	ee	ef	ef	ee	ef
J5-79	eg	ef	eg	ef	ef	eg	eg
J5-80	eg	ee	eg	ee	ee	eg	eg
J5-81	eg	ef	eg	fg	eg	..	eg
J5-82	eg	eg	eg	eg	ef	eg	eg
J5-83	ee	ef	ee	ef	ef	ee	..
J5-84	eg	fg	eg	fg	fg	eg	eg
J5-85	..	ee	..	ee	ee	ee	..
J5-86	ef	ef	ef	ef	eg	eg	ef
J5-87	eg	eg	eg	eg	ef	eg	..
J5-88	eg	fg	eg	eg	ef	eg	eg
J5-89	..	ef	..	ef	fg	eg	..
J5-90	ef	ef	ef	ef	fg	ee	..
J5-91	..	eg	..	eg	ef	ee	..
J5-92	eg	fg	eg	fg	fg	eg	eg
J5-93	..	ee	..	ee	fg	..	..
J5-94	eg	..	eg	eg	..	eg	eg
J5-95	ee	ee	eg	ee	..	eg	eg
J5-96	eg	eg	eg	eg	ef	eg	eg
J5-97	..	fg	..	fg	eg	ee	ef
J5-98	..	fg	..	fg	fg	ee	ef
J5-99	..	ef	..	ef	fg	ee	ef
J5-100	eg	eg	eg	eg	ef	eg	eg
J5-101	eg	ee	..	ee	ee	eg	eg
J5-102	fg	fg	..	fg	eg	eg	eg
J5-103	eg	fg	eg	fg	ee	eg	fg
J5-104	..	eg	..	eg	ef	eg	eg

J5-105	fg	fg	eg	fg	fg	fg	fg
J5-106	eg	fg	ee	fg	ef	ee	eg
J5-107	eg	ee	..	ee	ee	eg	ee
J5-108	eg	eg	eg	eg	ef	eg	eg
J5-109	..	ef	..	ef	ef	ee	ee
J5-110	eg	eg	eg	eg	ef	eg	eg
J5-111	ef	ef	..	ef	ee	eg	fg
J5-112	eg	eg	..	eg	ee	ee	ef
J5-113	eg	eg	eg	eg	ee	eg	eg
J5-114	fg	ee	fg	eg	ee	fg	fg
J5-115	ef	fg	ef	fg	eg	ee	..
J5-116	eg	eg	eg	eg	fg	eg	eg
J5-117	eg	eg	eg	eg	fg	eg	eg
J5-118	eg	eg	eg	eg	ef	eg	eg
J5-119	eg	ee	eg	ee	fg	eg	eg
J5-120	..	ef	..	ef	ef	ee	ee
J5-121	ef	ee	ee	ef	ee	eg	ef
J5-122	..	ef	..	ef	..	ee	ee
J5-123	eg	eg	eg	eg	fg	ee	eg
J5-124	eg	ee	eg	ee	fg	eg	eg
J5-125	eg	ef	eg	fg	eg	eg	eg

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