Development of microsatellite (SSR) marker multiplexes for future construction of a genetic linkage map for pear (*Pyrus communis* L.)

Hawwa Gabier

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A thesis submitted in partial fulfilment of the requirements for the degree of Masters of Science Biotechnology at the Faculty of Science, University of the Western Cape.

Supervisor: Marlene du Preez
November 2012

Abstract:

Development of microsatellite (SSR) marker multiplexes for future construction of a genetic linkage map for pear (*Pyrus communis* L).

Hawwa Gabier

Department of Biotechnology, Faculty of Science, University of the Western Cape, South Africa.

Recent advances in the field of plant genetics and application of molecular technologies has lead to greater understanding of various crop genomes and their organization.

The applications of these techniques include molecular markers which have been used to examine DNA variation within crop species. This allows for the creation of further genetic variation for new and favourable traits.

Molecular markers or DNA markers are short fragments of DNA that can be used to locate desirable genetic traits in the genome or show specific genetic differences.

The Maloideae subfamily includes fruit species such as pear. Pears (*Pyrus communis* L.) are large edible fruit that are grown in cool climates, native to coastal regions in Africa, Asia and Europe. The external appearance of this fruit plays a vital role on its rate of sale potential. Thus it is important for the appearances of the pear to meet the expectations of the consumer. External factors affecting the appearance of fruit, such as shape and colour, can have a large influence on the consumer's first impression and opinion of what the fruit may taste like (Jaeger and MacFie, *et al.,* 2001). The South African pear industry is the fourth largest in the fruit industry after apple, citrus and grape, exporting 3.8% to Europe (Ferrandi, *et al.,* 2005). Increase in production and export of the pear is dependant on the variety of cultivars with desired traits. New cultivars, especially ranges of new cultivars, with harvest dates from early

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to late in the season, can fill gaps in the marketing strategy of exporters and in the local markets (Human, *et al*., 2005)

Therefore, development of molecular markers allows for their possible use in maker-assisted selection and for the construction of a genetic linkage map thus leading to the location of favourable traits and ultimately the improvement of the quality of the pear.

In this study high throughput genomic DNA extractions were performed. The Cetyltrimethyl ammonium bromide (CTAB) method was employed as the results proved to be most promising. Furthermore the screenings of molecular markers were conducted in order to obtain DNA variation. Molecular markers were used to locate specific genetic differences. Multiplexing PCR was conducted using fluorescent primers for further screening and results proved to be useful as many variations could be observed.

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<u>**Key words:**</u>

Development of microsatellite (SSR) marker multiplexes for future construction of a genetic linkage map for pear (*Pyrus communis* .L).

Pear

Pyrus communis L.

Plant breeding

High throughput screening

Genetic variation

Microsatellite

Molecular markers

Multiplexing

Polymerase chain reaction

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Dedication:

I would like to dedicate this thesis to my parents (M. Fouad and Thuraya Gabier) who taught me to live, love and seek knowledge.

The life of the heart is knowledge; so preserve it,

the death of the heart is ignorance; so avoid it.

Declaration:

I declare that '**Development of microsatellite (SSR) marker multiplexes for future construction of a genetic linkage map for pear (*Pyrus communis* L.)** is my own work that has not been submitted for any degree or examination at any other University and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Hawwa Gabier

November 2012

Signed:…………………….

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Acknowledgements:

I begin in the name of God the Most Gracious the Most Merciful.

Through the hard work and dedication this research project would not have been possible, thus I would like to express my sincere gratitude to the following people and institutions:

The National Research Foundation (NRF) for providing financial support. To the Agricultural research council (ARC) for their assistance.

My supervisor, Ms Marlene du Preez, for her dedication, knowledge and guidance.

To Dr. M.Kashief Soeker for his assistance and guidance. Mahjoubeh Jalali safid Dashti for all the encouragement and motivation. Sana Chougle Surve for believing I can do anything. Zaida Dalvie for making me follow my dreams.

A heartfelt thank you to my parents (M. Fouad and Thuraya) and my two brothers (Abdul Hafieth and Abdul Hakam), my gratitude and appreciation to you for always standing by me.

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List of Abbreviations:

%	Percentage
A	Adenine
ABI	Applied Biosystems
AFLPs	Amplified fragment length polymorphisms
APS	Ammonium persulphate
B.C	Before Christ
BLASTN	Basic Local Alignment Search Tool Nucleotide
bp	Base pair
°C	Degree Celsius
CTAB	Cetyl trimethyl ammonium bromide
cv.	Cultivar
C	Cytosine
DArT	Diversity array technology
DFPT	Deciduous Fruit Producers Trust
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
E.U	European union
et al	'and others'
ft	Feet
FAO	Food and agriculture organization

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G	guanine
g	grams
HCl	Hydrochloric acid
IU	International unit
Kcal	Kilocalories
Kg	kilogram
L	litres
µg	microgram
ml	millilitre
mg	milligram
M	moles
PCR	polymerase chain reaction
pH	Potential of hydrogen
RFLP	restriction fragment length polymorphism
RAPD	random amplification of polymorphic DNA

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SSR	simple sequence repeats
SNP	single nucleotide polymorphism
T	thymine
TBE	Tris/Borate/EDTA
PVP	Polyvinylpyrrolidone
U.K	United Kingdom
USDA	national nutrient data base
v/v	volume/volume
w/v	weight/volume

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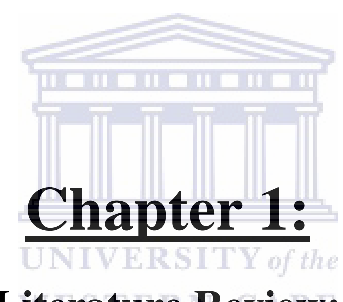

Chapter 1:

Literature Review:

Chapter 1:

Literature Review

1. Introduction:

1.1 Presentation of *Pyrus communis* L.

Pyrus comprises of over 20 species and belongs to the family of Rosaceae. *Pyrus* is native to areas such as Asia through to Japan and across Europe. Pear is regarded as one of the most important fruit crops. It has been cultivated in Europe and Asia for at least 2000 to 3000 years (Bell, 1990). According to the original distribution, *Pyrus* is divided into two native groups: Occidental and Oriental pear (Bailey, 1917). With regards to the occidental pears, *P.communis* is the most cultivated species and has been widely produced throughout Africa, Europe, North and South America (Bell, 1990). The Oriental pears consist of four most cultivated groups: *P. pyrifolia* (mainly in Japan and China) and another three Chinese species *P. ussuriensis*, *P. bretschneideri* and *P. sinkiangensis* (Bao *et al.*, 2007). Additionally, there are hybrids between species, for example, some of the perry pears from England and France are thought to be hybrids between *P. communis* and *P. nivalis*, and the major commercial pears in China are thought to be derived from *P. pyrifolia*, *P. ussuriensis* and *P. bretschneideri* (Bell, 1991).

Pears are rarely evergreen and are seen as deciduous, producing a medium sized tree with a narrow crown, although weeping forms as well as shrubby forms have been described. The tree bears a fruit that varies in shape from species to species. Some pears are described as having a round shape to the typical bulbous form of European pears. Cultivation of pears dates back thousands of years, thus selection of specific cultivars has occurred. Current world production of pears is almost 20 million tonnes, which comprises of 60% Asian and 40% European pears. China is by far the world's largest producer with 11.6 million tonnes, followed by Italy with 0.85 million tonnes and USA with 0.77 million tonnes (World Pear Review, 2006). Pears have

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many health benefits and are mostly consumed fresh. Smaller quantities are used for juices which can be further fermented to produce Perry.

The pear industry in South Africa is an important sector of the deciduous fruit industry. The main cultivars produced in South Africa are Packham's Triumph which is 29% of total production, Bon Chrétien not much less at 20%, Forelle at 24%, Early Bon Chrétien at 9%, Beurre Bosc at 4% and Doyenne du Comice at 3% (DFPT, 2008). Exportation of the fresh fruit for consumption totals at more than one third (38%) of the crop, 44% is processed and 18% is sold fresh locally (Ferrandi *et al.,* 2005).

1.1.1 Origin of *Pyrus*:

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This ancient fruit has been under cultivation both in Europe and Asia for a long time. The first records written about pear cultivation dates back to Greek writing around 1000 BC (Hedrick *et al*. 1921). Ancient Greek poet Homer praised pears as one of the 'gifts of God'. Three cultivars were described by Euphrates in Greece, and the Greek writers Cato and Pliny described 6 and 35 pear cultivars, respectively (Zohary and Hopf, 1988). On the Asian continent Sand pear (Japanese and Chinese species) has been domesticated as edible fruit and cultivated in Asia for more than 3000 years (Lombard and Westwood, 1987). China accounts for most of the world's Asian pear production with the *P. bretschneideri* cultivars 'Dong Shan Su Li', 'Ya Li' and 'Huang Hua Li' comprising the largest production area (Gemma, 2008). In later years pear varieties and culture advanced and by the 19th century 900 different varieties were described by the French pomologist Leroy. *Pyrus* was further classified into more than twenty primary diploid species distributed over Europe and Asia (Layne and Quamme, 1975) (Zohary and Hopf, 1988) and at least six naturally occurring inter-specific hybrids (Bell *et al.*, 1996). It was during the 18th and 19th centuries when the greatest advancement in pear improvement took

place, mainly in Belgium, France and England, with some pear cultivars selected at this time still widely cultivated today (Bell *et al*., 1996). The French and English settlers brought pears to the northern and southern American continents. A great diversity of germplasm was accumulated due to the use of other *Pyrus* species to breed for fireblight resistance and cold hardiness. (Watkins, 1976; Layne and Qamme, 1975). However, 87.7% of these cultivars were lost by 1980s, as recorded by a study cited by the Rural Advancement Fund International (Fowler and Mooney, 1990). Currently the world pear production depends on cultivar such as 'Abate Fetel', 'Williams', 'Beurre Anjou', 'Beurre Bosc', 'Comice', 'Conference', 'Dr. Jules Guyot', or 'Passa Crassana' which were selected in the 18th to 19th centuries. Recent and widely cultivated varieties such as 'Packham's Triumph' or 'Sensation Red Bartlett' have also derived from these progenitors. While 'Bartlett' is the most important cultivar of this species worldwide, 'Packham's Triumph' production is a close second in the Southern hemisphere growing regions (Palmer and Grills, 2008; Sanchez *et al.,* 2008) and 'Conference' is the dominant cultivar in Europe (Deckers and Schoofs, 2008). *Pyrus communis* L. (European pear) as previously mentioned belongs to the Maloideae subfamily of the Rosaceae. Other commercially important fruit in the same family include apple (*Malus pumila* Mill.), quince (*Cydonia oblonga* Mill.) and loquat (*Eriobotrya japonica).*

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1.1.2 Growth, structure and environmental aspects:

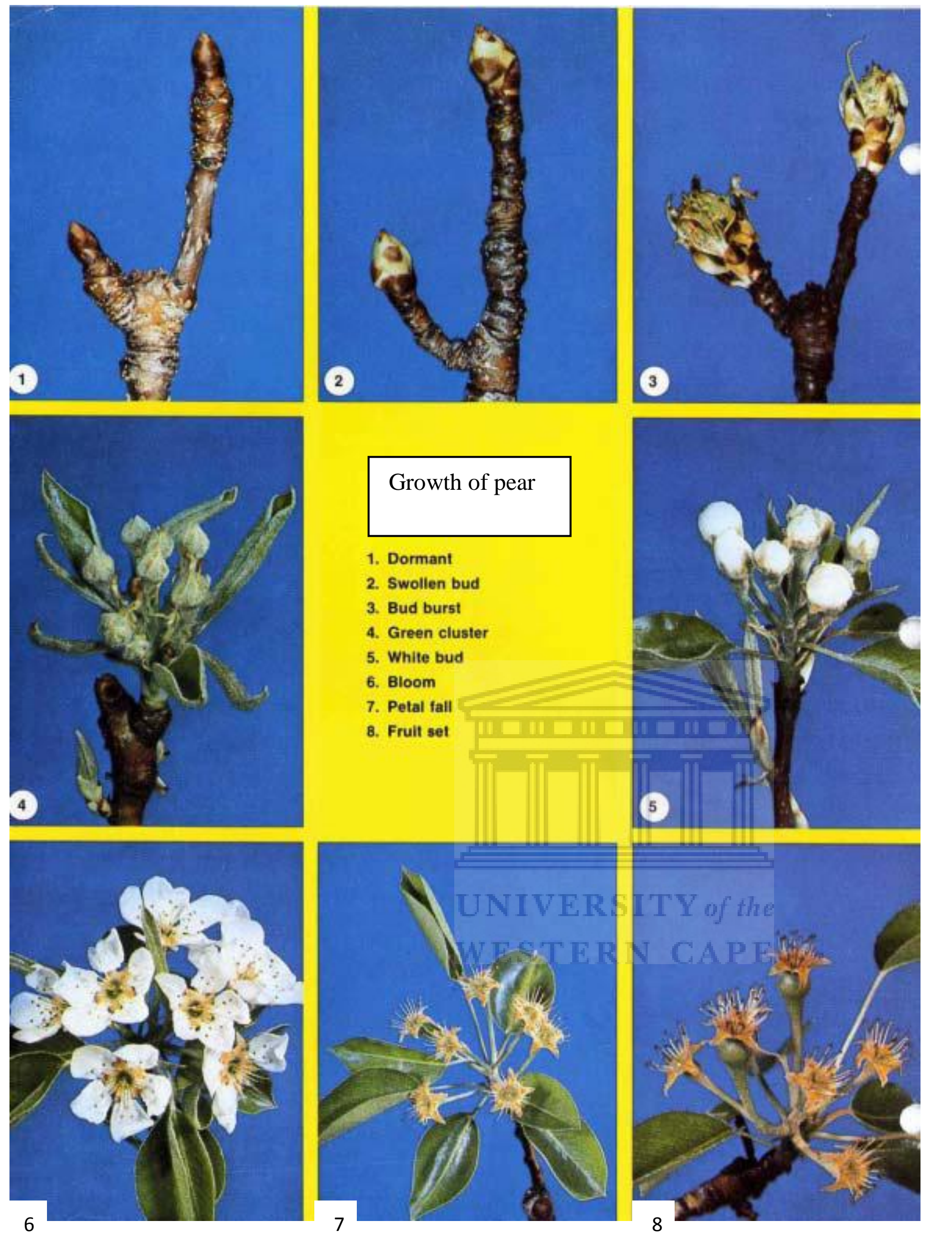

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Figure 1.1: Phenological growth stages of pear:
(1) Dormant, (2) swollen bud, (3) bud burst, (4) green cluster, (5) white bud, (6) bloom,
(7) petal fall, and (8) fruit set (Chapman P. J., Gertrude Catlin A., 1976).

Pears are the most tolerant of wet soil conditions compared to all the other deciduous fruit tree species. Pears are however perform best on deep, well-drained sites and are the most pest-ridden of all fruit trees, thus requiring numerous treatment sprays to rid them of pests. Pear

trees grow upright to very large height and require 5.4 meters spacing. Most pear trees require cross-pollination by another crop variety to produce a good crop set.

Pear trees are not as hardy as apple trees and require some winter chill to produce fruit. The fruit is picked when it has matured. Pears fruit has a range of skin colours such as brown, green, red and yellow. Pears with a red colouring, or blush, are not easily found in certain market places (Jaeger *et al*., 2003) although there is an increased demand for red coloured pears worldwide. The colour of the fruit results from the increase concentration and distribution of anthocynin pigments within the skin (Lancaster *et al*., 1994). There is a large variety of pears and its flavour varies with each cultivar. Pears have a distinctive flavour due to specific volatile organic compounds and have a juicy and firm texture. The aroma is influenced by factors such as genetic differences, pre-harvested factors, maturity at harvest, storage conditions, and fruit physiology. (Rapparini and Predieri, 2002).

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1.1.3 Nutritional composition:

The economic importance of edible Rosaceous is determined by the flavourful fruits which provide dietary choices for human health. It contains phytochemicals, such as flavonoids and other phenolic compounds, cyanogenic glucosides, phytoestrogens (Mazur *et al.,* 2000), and phenols that could potentially yield health and disease-fighting advantages (Macheix *et al.,* 1991; Swanson, 1998; Selmar, 1999).With the ever increasing population size, agricultural research has focused on increasing crop production to feed the growing world population. As malnutrition and infectious diseases, related to poor nutrition, remain highly prevalent, a shift towards identifying novel compounds with pharmacological action against these conditions. Using knowledge of genetics and bioinformatics will aid in the identification of such useful

compounds with the genes responsible for their production. The following table lists the nutritional value of pear per 100g.

Table 1.1: Health benefits of fresh pear (*Pyrus communis*) Fruit.

Compound	**Nutrient Value**
Energy	58 Kcal
Carbohydrates	13.81 g
Protein	0.38 g
Total Fat	0.12 g
Cholesterol	0 mg
Dietary Fiber	3.10 g
Vitamins	
Folates	7 µg
Niacin	0.157 mg
Pantothenic acid	0.048 mg
Pyridoxine	0.028 mg
Riboflavin	0.025 mg
Thiamin	0.012 mg
Vitamin A	23 IU
Vitamin C	4.2 mg
Vitamin E	0.12 mg
Vitamin K	4.5 µg
Electrolytes	
Sodium	1 mg
Potassium	119 mg
Minerals	
Calcium	9 mg
Copper	0.082 mg
Iron	0.17 mg
Magnesium	7 mg
Manganese	-
Phosphorus	11 mg
Zinc	0.10 mg
Phyto-nutrients	
Carotene-ß	12 µg
Crypto-xanthin-ß	2 µg
Lutein-zeaxanthin	45 µg

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Nutritive value per 100 g, (USDA National Nutrient data base).*IU= international unit (measures activity of vitamins. µg= microgram, mg = milligram.

According to research, pears were found to be less allergenic than many other fruits. Pears are low in salicylates and benzoates and are therefore recommended in exclusion diets for allergy sufferers (Gibson and Clancy, 1978). The fibre content of pears may reduce the risk of colon

polyps. This is due to the fact that pears are a good source of phosphorus, potassium, vitamin A, B1 B2 and C, folic acid, pectin and dietary fibres. Furthermore, it contains no cholesterol and are low in calories (Jiang, 1986).

1.1.4 Pear productions, economic influence and distribution:

Pears are ranked 2nd as the most important pome in the world, after apple. It is grown on all the continents of the world where China is the world's largest producers of pears (Table 1.2) with 72% of the commercially cultivated species *Pyrus* are native to Asia. Asia's production continues to increase since 1990, especially in China, India and Iran (Saito *et al*., 2005). United States accommodates about 1,500 clones, of which 40% of these are Asian pear varieties (Nee *et al*., 2002) According to Food and Agriculture Organization (FAO) of the United Nations report of 2006, the overall world production of pears reached 19.5 million metric tonnes in 2005.

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On the African continent South Africa is the leading pear distributor. In South Africa, the third largest exporter in the Southern Hemisphere (Human, 2007), the pear industry is the fourth largest industry, exceeded by apple, citrus and grape (White *et al.,* 2002). Although Africa is a small producer of *Pyrus communis*, the industry is very important to South Africa as the export has a great impact on the economy of the country. Export amounted to ±10 million cartons in 2003 (Ferrandi *et al.,* 2005) of which 38% went to European markets (Ferrandi *et al.*, 2005).

Table 1.2: Top ten pear producers in the world:

Country	Production (tonnes)	Footnote
People's Republic of China	12,625,000	F
Italy	840,516	O
United States	799,180	O
Spain	537,400	O
Argentina	520,000	F
South Korea	425,000	F
Turkey	349,420	O
Japan	325,000	F
South Africa	325,000	F
Netherlands	224,000	F
World	20,105,683	A

O = official figure, F = FAO estimate, * = Unofficial/Semi-official/mirror data, C = Calculated figure A = Aggregate (may include official, semi-official, or estimates);
(Source: Food And Agricultural Organization of United Nations: Economic And Social Department: The Statistical Division)

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1.1.5 Pear production in South Africa:

Hortgro Services, the substitute of the South African Deciduous Fruit Producers Trust (DFPT) have published the hard fruit export estimations from South Africa to the following destinations: The U.K., the EU and North America during the 2010 export season.

Table 1.3: Hard fruit exports from South Africa to UK, EU and North America (2010):

	Export 2008	Export 2009	Average last 2 years	Estimate 2010
Apples	26 965 627	26 614 742	26 790 184	26 551 147
Pears	13 153 619	14 447 466	13 800 542	14 574 958
Total	40 119 245	41 062 208	40 590 726	41 126 104

All volumes = 12.5 kg carton equivalent

As indicated in table 1.3 the apple export was expected to be somewhat smaller than in 2009 (-2% or -63,595 cartons), whilst the export of pears increased (+9% or +127,492 cartons). In total the South African export was expected to be in excess of 41 million boxes, an increase of +0, 2% or +63, 897 cartons compared to 2009.

Hortgro Services published a summary per pear variety in the following chart:

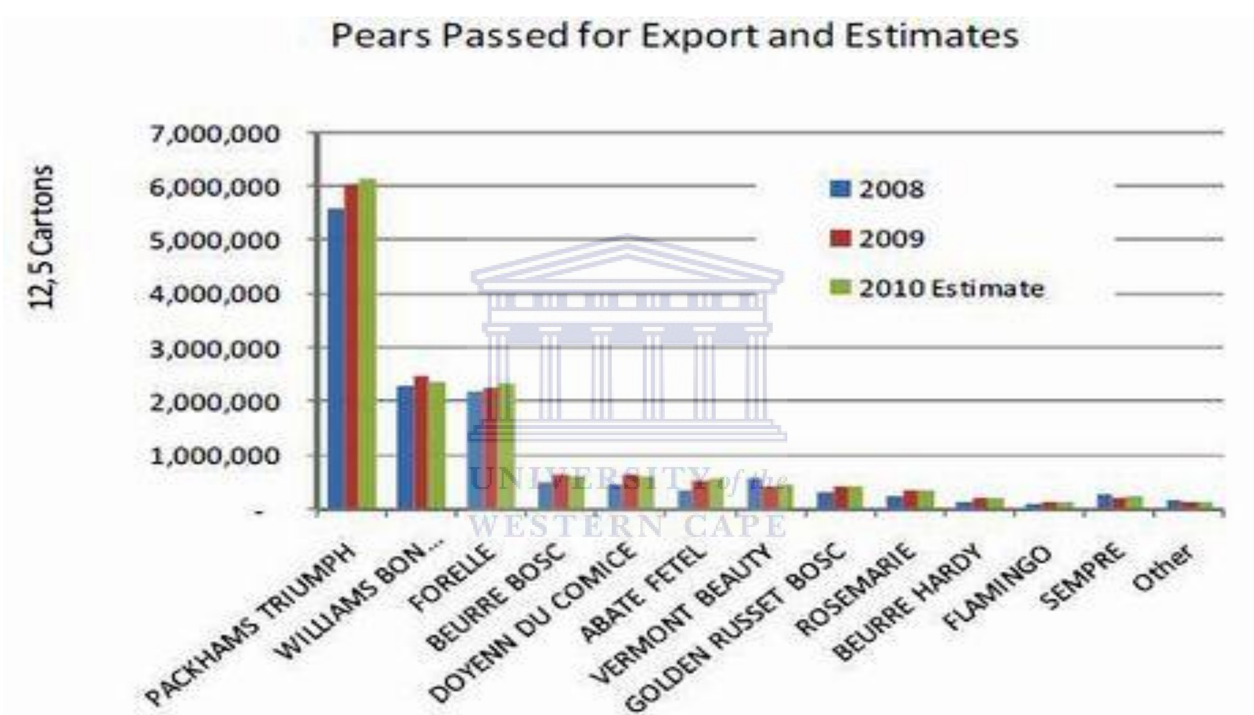

Figure 1.2: Pear export of three seasons, x-axis showing the pear variety per 12,5 cartons.

It is clear that an increase in export in 2010 was expected for most of the pear varieties, except the Williams/Bartlett (-4%), BeurreBosc (-6%), Comice (-1%), Golden Russet Bosc (-1%) and Beurre Hardy (-3%).

1.2 Plant breeding:

Plant breeding is aimed at improving the quality and diversity of agricultural crops. It leads to the production of new cultivars displaying one or several improved characters with regards to yield, disease resistance and/or quality (Chahal and Gosal, 2002). In essence it is the improvement of plants better adapted for human need. It is the calculated effort by plant breeders to nudge nature improving certain aspects of plants. Plant breeding can be defined as the art and science of changing the genetics of plant species in order to create improved genotypes/phenotypes for specifically defined purposes (Chahal and Gosal, 2002). The overall objective of plant breeding is to increase the marketability, while reducing the production cost (Hrazdina, 1994). This involves traditional breeding through controlled cross-pollination, or genetic engineering, followed by artificial selection of progeny (Tobutt *et al.*, 2000).

1.2.1 Resistance to disease:

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With the development of new pear varieties the presence of disease has become a major concern. One of the concerns is the susceptibility of pears to a number of diseases mostly caused by fungi. An important breeder's objective is the resistance of European pears against fungal disease such as scab (*Venturia pirina*), powdery mildew (*Podosphaera leucotricha*), brown spot (*Stemphylium vesicarium*) and the bacterial pathogen that causes fire blight (*Erwinia amylovova*). Scab, caused by the ascomycete fungus *Venturia pirina*, leads to severe damage on European pear varieties resulting in a loss of commercial value and requiring frequent use of fungicides (Bouvier, *et al.*, 2012). In Europe there is a reduction in pear growing areas manly due to the fire blight (Deckers and Schoofs, 2002). Asian pears experiences devastating effects from fungal disease such as scab (*Venturia nasicola*), rust *(Gymnosporangium asaticum*), and black spot (*Alternaria alternata*).

1.2.2 Appearance of fruit:

Pears generally have an attractive appearance to consumers. For example, the colour, shape and texture play an important role. External factors of the appearance of fruit, such as shape and colour, have a large influence on the consumer's first impression and opinion of what the fruit may taste like (Jaeger and MacFaie, 2001). There are wide variances in fruit colour that affects markets; yellow, green, and red pears, are attractive for Chinese consumers (Wei and Gao, 2002). The European consumers opt for a pear that is free of russet and resist bruising during harvesting, grading, and storage as well as ripening. Europeans prefer golden yellow and bright red blush. The red colouring of pears is a result of anthocyanin pigments (Francis, 1970). The general pattern of anthocyanin distribution in red pears is a non-pigmented epidermis and one or two non-pigmented hypodermal layers, which lay above two to five layers containing anthocyanin (Dayton, 1966).

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1.2.3 Insect resistance:

European pear production is faced with insects such as the native species that produces honeydew that allows a sooty fungus to grow on the pear surface. It results in damage to the tree. Codling moth (*Cydia pomonella L.*) is also a pest which affects pears. Pear breeders' main objective is to gain resistance against these pests especially in Europe and North America as it is the most damaging insects of apples and pears (Berry, 1998).

1.2.4. Traditional breeding:

Traditional breeding is the term used to explain the technique where parents with desired traits are cross fertilized to produce offspring that would contain the desirable traits from both parents. Traditional breeding has been applied to produce new varieties or lines with desirable

properties using deliberate interbreeding of closely or distantly related individuals (Janick *et al*., 1996). Traditional breeding can alter both simple and complex traits, at the same time losing varietal identity, while modern biotechnology techniques maintain the varietal identity but can only improve simple traits (Collard *et al.*, 2005). Seedlings that were cross pollinated may closely resemble their parents. However they are never identical to either parent thus losing the varietal identity. Modern biotechnology is thus greatly favoured due to the fact that molecular techniques are employed which allows molecular markers to monitor linkage during backcrossing generations.

1.2.5 Genetic engineering:

Genetic engineering, also known as recombinant DNA technology, can be described as the direct manipulation of the genes of an organism. It is a powerful technology that can be used to insert or block the expression of less desirable genes. This technology and its resulting products have always been highly regulated in the United States for example, and the scientific basis for this regulation is under constant review by the scientific community as well as by the relevant federal and state government agencies (McHughen, 2006). Thus, crops derived from genetic engineering technology receive a great amount of regulatory safety evaluations prior to commercial release. Genetic transformation may be useful for breeding pears that are resistant to plant disease. One such disease is fire blight (*Erwinia amylovora*) which is quite devastating to apple, pear and other Rosaceous species. Despite quarantine measures, the disease continues to spread throughout Western, Central and Southern Europe (Jock *et al*., 2002).

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1.3 Molecular markers:

1.3.1 Overview of molecular markers:

The advent of DNA marker technology has revolutionized the field of genetics (Cullis, 2002; Dodgson *et al.,* 1997; Rafalski and Tingey, 1993). For more than a century phenotypic markers and later isozyme markers dominated the field of classical genetics. The introduction of DNA-based markers during the second half of the 20th century changed the pace and precision of genetic analysis (Dodgson *et al.,* 1997). DNA-based markers have now led to the construction of whole genome linkage maps in many plant and animal genomes, a crucial step for several downstream applications such as gene cloning, genome analysis and marker-assisted selection of agricultural crops (Cullis, 2002; Dodgson *et al.,* 1997; Paterson, 1996).

DNA markers are also being increasingly used in genetic diagnostics, population studies, comparative genomics, pharmacogenomics, drug discovery and molecular evolution studies (Bennetzen, 2000; McCarthy and Hilfiker, 2000; Pfost *et al.,* 2000; Rafalski and Tingey, 1993). Molecular markers are used in the evaluation of genetic diversity and construction of physical as well as genetic maps. Mapping of linked markers helps in relating genetic distance to physical distance. The association between patterns of inheritance in mapping to genetic markers allows for the construction of genetic linkage maps. This aids in trying to illuminate the extinction of certain traits and improve diversity. Due to global warming many plant species have suffered, molecular markers can be used to assess plant response to climate change.

It is vital for the molecular marker to have some desirable properties. Depending on the type of study to be undertaken, a marker system can be identified that would fulfil at least a few of the characteristics (Weising *et al.,* 1995). These can be seen in the table below.

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Table 1.4: Desirable properties of molecular markers:

No.	Properties:	Features:
1.	Highly polymorphic nature	It is has to be polymorphic (polymorphism is measured for genetic studies).
2.	Co-dominant inheritance	For the determination of heterozygous and homozygous diploid organisms.
3.	Frequent occurrence in genome	Markers should be frequently distributed throughout the genome.
4.	Easy access (availability)	It should be easy, fast and inexpensive to detect.

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Various types of molecular markers are employed to determine DNA polymorphism and can be categorised as being markers that are hybridization based or polymerase chain reaction based. Hybridization based markers are visualised by hybridizing the restriction enzyme-digested DNA to a DNA fragment (labelled probe) of known origin or sequence. Polymerised chain reaction based markers include *in vitro* amplification of particular DNA sequence or loci with the aid of chosen oligonucleotide sequences (primers) as well as a thermo-stable DNA polymerase enzyme. This is followed by the separation of the amplified fragments by the use of electrophoresis and formation of a banding pattern. Polymerase chain reaction (PCR) is a versatile technique invented during the mid-1980s (Saiki *et al.* 1985). Primer sequences are chosen and allow base specific binding to the template in reverse orientation.

In summary revealing polymorphism at DNA level using molecular markers has played an important role in plant biotechnology for the past few decades. There are different types of markers that are based on biochemical, morphological and DNA characteristics. DNA based

markers are differentiated into two types, i.e. non-PCR based and PCR based markers. An example of a non-PCR based marker is RFLP while PCR based examples include RAPD, AFLP, SSR (microsatellites), and SNPs. The microsatellites DNA markers have also been widely used as they are co-dominant and easy to implement.

It is vital to have a clear concept of gene and genetic markers as well as their characteristics and purposes in order for the techniques to be used effectively.

1.3.2.1 Non PCR based techniques:

Molecular markers using restriction hybridization techniques were employed in the study of plant genetics. Molecular markers based on restriction-hybridization techniques were employed relatively early in the field of plant studies and combined the use of restriction endonucleases and the hybridization method (Southern, 1975).

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1.3.2.1 a. Restriction Fragment Length Polymorphism (RFLP):

This is a technique where organisms are differentiated by analysis of patterns which are derived from the cleavage of their DNA with restriction endonucleaes. DNA polymorphism is detected by hybridizing a chemically labelled DNA probe to the digested DNA which results in differential DNA fragment profiles. RFLP are highly polymorphic, co-dominantly inherited and highly reproducible. This technique allows for simultaneous screening numerous samples. This technique however is not widely used due to the fact that it is time consuming. It also involves radioactive reagents and large quantities of DNA that has to be of high quality.

RFLPs were widely used in gene mapping studies before PCR technology became available. This is because of their high genomic abundance and ample availability of different restriction enzymes and random distribution throughout the genome (Neale and Williams, 1991).

1.3.2.2 PCR based techniques:

1.3.2.2. a. Random Amplified Polymorphic DNA (RAPD):

This technique is based on enzymatic amplification of random DNA or target DNA segments. Polymorphism in DNA is detected by using a single primer of random nucleotide sequence. This technique employs a single primer which anneals to the genomic DNA at two different sites on the complementary strands of DNA template. A DNA product is formed using thermo cycling if these sites are in amplifiable range.

On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals (William *et al*., 1993). The main drawback of RAPDs is their low reproducibility (Schierwater and Ender, 1993). Therefore, the experiment needs to be optimized and standardized experimental procedures due to the sensitivity to the reaction conditions. High molecular weight DNA is required.

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1.3.2.2. b. Diversity arrays technology (DArT):

Diversity Arrays Technology (DArT) supports a genotyping platform for the entire genome which detects and scores hundreds of polymorphic loci. This technology was originally developed for rice (Jaccoud *et al*., 2001) and is now used successfully with other plants. DArT markers detect which of the markers or loci were transferred to the progeny and which contains the trait of interest.

The limitations of this technique are the fact that it includes many steps, i.e., preparing genomic representations for the target species, cloning, data management and analysis. Thus establishing a DArT system requires extensive investment in the laboratory as well as skilled manpower. DArT markers have only been used in a few species. Only a single independent

group has thus far successfully established the methodology to *Eucalyptus grandis* in South Africa (Lezar *et al*., 2004).

1.3.2.2. c. Amplified Fragment Length Polymorphism (AFLP):

AFLP is based on a selecting and amplifying a set of restriction fragments from a complex mixture of DNA fragments, which are obtained after the digestion of genomic DNA using restriction endonucleases. The samples are then analysed on a polyacrylamide gel where polymorphisms are detected with differences in fragment length. AFLP are dominant markers, and requires purified, high molecular weight DNA. The technique consists of four steps i.e., restriction of the DNA and ligation of oligonucleotides; this is followed by pre-selective amplification, selective amplification and gel analysis of amplified fragments.

1.3.2.2. d. Microsatellites:

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Microsatellites or simple sequence repeats (SSR) are ideal genetic markers used for detecting differences between and within species. The repeat units range from one to 13-mers in length and are usually repeated five to 20 times (Brown, 2006). It is useful in parentage determination, as well as for high throughput analysis via multiplexing with highly reproducible profiles. In nature, the most abundant SSR motif reported in plants is (AT), while (AC) is the most abundant in the human genome (Brown, 2006). Banding patterns are prevalent due to variations in the number of tandomly repeated units. SSR's are prevalent in the chloroplast as well as in mitochondrial genome forming a repetition of guanine and cytosine (GC). Nucleus encoded SSR's are bi-parental in inheritance and contain few loci with many alleles per locus. SSR's are genetic markers that are valuable due to the fact that they are transferable from one cultivar to another. They are easily and economically assayed by PCR (McCouch *et al.*, 1997; Adam-Blondon *et al.*, 2004), and they are co-dominant and can detect a high level of allelic

diversity. From the perspective of genetic linkage map construction, a marker on a locus with heterozygosity higher than 70% is commonly considered a highly informative marker, because the segregation can easily be monitored within a given population (Gupta *et al.*, 2003; Chagné *et al.*, 2004; Oraguzie *et al.*, 2005). Apple microsatellites were successfully mapped on pears, which belong to the same subfamily of Maloideae (Yamamoto *et al.*, 2001; Yamamoto *et al.*, 2002).

Aims and objectives of the study:

The increasing demands for the improvement of pear fruit quality has given rise to the research in microsatellites that are linked to a variety of fruit quality traits. This will lead to the identification of traits and the facilitation of marker-assisted selection of seedlings possessing such traits. South Africa is the third largest pear exporter in the Southern Hemisphere (Human, 2007), and in South Africa the pear industry is the fourth largest industry exceeded by apple, citrus and grape (White *et al.,* 2002). Thus, marker assisted selection is vital as it could aid in the improvement of South African pear cultivars. This study optimised the extraction of pear DNA, and it was proven to be a challenge as pear fruit contains many contaminants such as phenolic compounds. Furthermore, published primer sets were used and screened, and incorporated into a multiplex for whole population screening.

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To achieve these aims, the objectives below were formulated:

- The collection of leaves from each of the individual trees of ‘Bon Rouge’ (red skinned fruit) and ‘Packham’s Triumph’ (green skinned fruit), as well as from each seedling in the progeny.
- The extraction of genomic DNA from the leaves using CTAB method.

- To generate useful information from the results obtain from the parents, ‘Bon Rouge’ and ‘Packham’s Triumph’ using gel based identification.
- Test the SSR markers using polymerase chain reaction on the parents.
- High throughput screening on both parents and the offspring.

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Chapter 2:

Materials and Methods:

Chapter 2:

Materials and Methods:

2.1.1 Chemicals:

Chemical	Supplier
Acetone	Merck
Acetonitrile	Merck
Agarose D1 LE	Promega
Alkylamidosulphobetaine (ASB)-14	Bio-Rad
Ammonium bicarbonate	Merck
Ammonium persulphate (APS)	Merck
ß -Mercaptoethanol	Fermentas
Boric acid	Merck
3-[3-cholamidopropyl]-dimethyl-ammonio-1-Propane sulphonate (CHAPS)	Sigma
Chloroform	BDH chemicals
Ethylenediamine tetraacetic Acid (EDTA)	Merck
Ethanol	BDH chemicals
Formamide	Merck
Formic acid	Merck
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	Merck
Hydrochloric acid (HCl)	Merck
Iodoacetamide	Bio-Rad

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Iso–propyl alcohol	BDH chemicals
LIZ GS 500™	Applied Biosystems
Methanol	Merck
Megaplex PCR mix	Qiagen
Propan-2-ol	Merck
Proteinase K	Roche
RNase A	Roche
Sodium acetate	Merck
Sodium chloride (NaCl)	Merck
Sodium hydroxide (NaOH)	BDH chemicals
Sodium sulphite	Merck
Thiourea	Sigma
Trichloroacetic acid (TCA)	Merck
Tris (hydromethyl) aminomethane (Tris)	Merck
Trypsin	Promega
Urea	Merck
DNA ladders	Fermentas Life Sciences

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2.1.2 Buffers and Solutions:

1% agarose	1% agarose (w/v) in 1x TBE, 1 g agrose powder dissolved in 100 ml 1x TBE.

3% agarose	3% agarose (w/v) in 1x TBE, 3 g agrose powder dissolved in 100 ml 1x TBE.
80% Acetone	80% (v/v) acetone, in dH_20, 80 ml acetone, 20 ml dH_20.
2 x Cetyl trimethyl ammonium bromide (CTAB)	2% CTAB (2g), 28 ml of 5M NaCl, 10 ml of 1M Tris-HCl pH 8,4 ml of 0.5M EDTA, 1% PVP - 40 (1g), 200 µl of 2-mercaptoethanol.Volume made up to 100 ml with dH_20.
70% Ethanol	70% (v/v) absolute ethanol in dH_2O, 70 ml absolute ethanol, 30 ml dH_20.
Chloroform: isoamyl alcohol	25 (v/v) Tris-buffered, 24 (v/v) Chloroform, 1 (v/v) isoamyl alcohol, 24 ml of chloroform to1 ml isoamylalcohol (24:1) ratio.

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10x TBE	0.9M Tris, 0.89M Boric acid 0.032M EDTA, 108g Tris,7.44g EDTA, 55g Boric acid, dissolved in dH_20 and made up to 1 L.
1x TBE	Add 100 ml 10x TBE to 900ml of dH_20.
10 x TE Buffer	100 mM Tris-HCL (pH 7.5), 10mM EDTA, for 500ml dissolve 6g Tris and 1.86 g EDTA in 400ml dH_20 and adjust the pH to 7.5 with concentrated HCl. Volume made up to 500 ml and autoclaved.

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2.2 Plant material:

2.2.1 Pear population:

A pear population consisting of the two parents 'Bon Rouge' characterised by red skin and 'Packham's Triumph' by a green skin (figure 2.1) were grown on the experiment farm of the Agricutural Research Council Bien Donné, Simondium, Western Cape, South Africa. F1 population was'Bon Rouge' x 'Packham's Triumph' [(mutant of 'Bon Chretien') x ('Bon Chretien' x 'Uvedale St. Germain')] cross with a total of F1 population consisting of 187 seedlings.

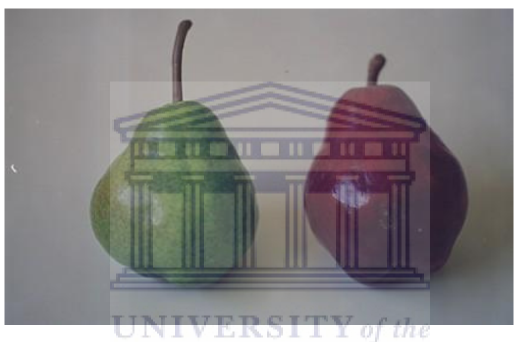

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Figure 2.1: 'Packham's Triumph' (green) and 'Bon Rouge' (red) fruit.

2.2.2 Leaf samples:

Leaves were harvested in 2011 from each of the individual trees of 'Bon Rouge' and 'Packham's Triumph', as well as from each seedling in the progeny. These leaves were collected in plastic bags, chilled on ice during transportation, and stored at -20 ºC.

Figure 2.2: Seedlings from the F1 population 'Bon Rouge' x 'Packham's Triumph' cross.

2.2.3 DNA extraction:

The CTAB (Hexadecyl trimethyl-ammonium bromide) method was employed for the extraction and isolation of DNA. CTAB (1 ml of 2 x) was warmed in a water bath until its temperature reached 65°C. The CTAB was removed from the water bath and 2 µl of ß-mercaptoethanol was added. Leaves from a seedling were placed in a mortar with liquid nitrogen and were ground to a fine powder using a pestle. A total weight of 100 mg of ground leaves were transfered to a 2 ml tube. It was allowed to cool to room temperature and 1 ml CTAB was added, and the sample gently homogenised. The homogenate was incubated at 65°C for 30 to 60 minutes in a water bath; the tubes were removed from the water bath and inverted intermittently. The tubes were centrifuged at 13,200 rpm for 30 minutes, after which the top layer (supernatant) was collected into an appropriate tube. At this point 3 layers were prevalent. This was followed by the addition of 10 µl of 10mg/ml proteinase K to the supernatant and incubation in a water bath for 37°C for 30 to 60 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the tube; this was vortexed briefly for 5 seconds and mixed gently for 10 minutes. The tube was centrifuged at 13,200 rpm for 10 minutes after which the top layer was collected and placed in an appropriate tube ensuring none of the interface was collected. At this point 340µl of ice-cold isopropanol was added to the mixture and inverted several times. DNA may be visible at this point. The tube was incubated overnight at -20 °C (or 2 hours at -20°C). After incubation the tube was centrifuged for 10-15 minutes at 13,200 rpm and the supernatant carefully discarded ensuring the pellet was still intact. The pellet was rinsed with 500 µl ice-cold 70% ethanol and centrifuged at 13,200 rpm for 10 minutes. The ethanol was removed from the tube with a pipette without disturbing the pellet. This step was repeated. The pellet was then air dried for 30 minutes and finally was resuspended in 50µl of 1x TE buffer and stored at - 20°C until further use.

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2.3 DNA quantification:

2.3.1 Nanodrop assay:

The DNA samples were quantified using a Nanodrop ™ spectrophotometer (GlobalSpec, Wilmington, DE, USA). After zeroing the Nanodrop, 2 µl of the extracted genomic DNA was loaded and the concentration recorded. The absorbance ratio at 260/280 for all the samples ranged from ~1.8 to ~1.9. Any values within this range indicate absence of contaminants such as salts or phenols in a sample (www.nanodrop.com/techsupport/nd-1000-users-manual.pdf).

2.3.2 Agarose gel electrophoresis:

The assessment of the quality of the DNA was carried out using 1% agrose gel. The gel was prepared by adding 1g of agrose to 100 ml of TBE buffer (90 mM Tris-HCl, 90 mM Boric acid [H_3BO_3] and 2.2 mM EDTA [pH 8.0]) and dissolved by using heat. The molten gel was cooled down to which 7µl of Gel Red stain was added. The molten gel was poured into a casting plate and allowed to solidify. A total of 8 µl of DNA sample and 3µl of loading buffer was mixed by pipetting and loaded into a well in the gel. The electrophoresis was conducted in 10x TBE at 10 V cm-1 for 45 min. The gel was then visualised with UV transillumination using the Gel-Doc UV Transilluminator (Bio-Rad, Hercules, California, USA).

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2.4. Microsatellite primers:

Published pear SSR primer pairs (Bassil and Postman, 2005) were used in this study. A total of 13 primers were used. Table 2.1 shows the SSR name, taxon, GenBank accession, repeat motif, SSR location, expected size and optimum temperature.

Table 2.1: List of microsatellite obtained from literature:

SSR Name	Taxon	GenBank Accession	Repeat motif	SSR location	Expected size	Optimum Tm
PYC-001	*P.communis*	AB084462	$(TA)_7$	3'UTR	341	66°C
PYC-002	*P.communis*	AJ504984	$(CT)_{10}$	-	165	64°C
PYC-003	*P.communis*	AJ504867	$(AT)_7$	-	256	66°C
PYC-004	*P.communis*	AJ504770	$(AT)_6$	-	170	64°C
PYC-005	*P.communis*	AJ504746	$(CT)_{11}$	-	167	58°C
PYC-006	*P.pyrifolia*	AB027617	$(TC)_5$	5'UTR	120	62°C
PYC-007	*P.pyrifolia*	AB027617	$(TCT)_7$ $(GT)_5$	Intron5	179	60°C
PYC-008	*P.communis*	AF386510	$(TG)_6$	5'UTR	314	64°C
PYC-009	*P.caucasic^{y}*	AF455809	$(CT)_{11}$	Intron2	302	64°C
PYC-010	*P.pyrifolia*	AB045710	$(GAA)_5(GAC)_5(GAA)_2$	5'UTR	200	62°C
PYC-011	*P.pyrifolia*	AF195224	$(AG)_7$	5'UTR	106	50°C
PYC-012	*P.communis*	S79358	$(ACA)_6$	CDS	180	66°C
PYC-013	*P.communis*	PC14009	$(TC)_{12}$ $TT(TC)_6$	5'UTR	118	62°C

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2.4.1 Optimisation of PCR conditions:

2.4.1.1 General PCR reaction:

For a 50µl PCR reaction, 25µl of Dream Taq was added to a PCR tube, as well as 15µl of DNA, 2µl of Primer (Reverse) and 2µl of Primer (Forward). The PCR mix was made up to 50µl with dH_20.

Optimum annealing temperatures were determined (Table 2.2) and used to amplify DNA from the parents 'Packham's Triumph' and 'Bon Rouge.'

Table 2.2: Representation of standardised PCR conditions:

Cycle	Step	Temp	Time
Cycle 1	Step 1	94°C	15 min
Cycle 2 (35x)	Step 2	94°C	30sec
	Step 3	Optimum tm(°C)	
	Step 4	72°C	1min
Cycle 3	Step 5	60°C	30 min
Cycle 4	Step 6	4°C	hold

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2.4.1.2 Agarose gel electrophoresis:

The assessment of the amplification of DNA was carried out using 3% agarose gels. The gel was prepared by adding 3 g of agarose to 100 ml of TBE buffer and dissolved by using heat. The molten gel was cooled down to which 7 µl of Gel Red Stain was added. The molten gel was poured into a casting plate and allowed to solidify. A total of 25 µl of sample was loaded

onto the gel. The electrophoresis was conducted in 10x TBE at 10V cm-1 for 45 minutes. The gel was then visualised with UV transillumination using the Gel-Doc UV Transilluminator (Bio-Rad, Hercules,California, USA).

2.5 Microsatellite multiplexing:

2.5.1 Multiplex primers:

The primers that were derived from the sequences closest to the SSR was fluorescently labelled using 6-FAM (blue), NED (black), VIC (green), PET (red) for detection using the ABI PRISM® 310 Genetic Analyzer. Primers that were polymorphic across the parental DNA were then incorporated into a multiplex.

Table 2.3: Showing SSR name, taxon, Genbank Accession, repeat motif location, expected size, optimum Tm, dye and its associated colour.

SSR Name	Taxon	GenBank accession	Repeat motif	SSR location	Expected Size	Optimum Tm	Dye	Colour
PYC 001	*P.communis*	AB084462	$(TA)_7$	3'UTR	341	66°C	6-FAM	Blue
PYC 008	*P.communis*	AF386510	$(TG)_6$	5'UTR	314	64°C	NED	Black
PYC009	*P.caucasica*	AF455809	$(CT)_{11}$	Intron 2	302	64°C	VIC	Green
PYC010a	*P.pyrifolia*	AB045710	$(TTTA)_4(TTA)_6$	Intron 6	200	64°C	PET	Red
PYC 012	*P.communis*	S79358	$(ACA)_6$	CDS	180	66°C	NED	Black
CN444542 SSR	*Malus x domestica*		GA		110-156		6-FAM	Blue

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2.5.2 PCR reaction for multiplex:

2.5.2.1 PCR reaction (5µl reaction):

For a 5 µl PCR reaction, 2.5 µl of Qiagen PCR mix into the 96 well plate along with 0.5 µl of dH20, 1 µl of primer mix and 1µl of DNA. The 20 µl primer mix was made up of 0.5 µl each (from a 10pmol/µl dilution), 0.5 µl of forward primer and 0.5 µl of reverse primer. The primers were made up to 20 µl with dH20.

2.5.2.2 PCR Conditions:

The 96-well plate was positioned on the ABI 2700 (Applied Biosystems) PCR machine and properly sealed. The standardised PCR conditions were as follows:

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PCR conditions for multiplexing as follows:

Cycle	Step	Temp (°C)	Time
Cycle 1	Step 1	94°C	15 min
Cycle 2 (40x)	Step 2	94°C	30sec
	Step 3	Optimum tm (°C)	
	Step 4	72°C	1min
Cycle 3	Step 5	60°C	30 min
Cycle 4	Step 6	4°C	hold

2.5.2.3 DNA dilution:

The DNA of the F1 population was diluted appropriately. The dilution ratio's of DNA are indicated in appendix A.

2.5.2.4 PCR product dilution for ABI:

PCR products were diluted by adding 1µl of PCR product to 19 µl of dH_20 in a 96 well plate. A volume of 1 µl was taken from this dilution and added to 10 µl of formamide containing 0.15 µl LIZ size standard.

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Chapter 3:

Results:

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Chapter 3:

Results:

3.1 Genomics:

3.1.1 Isolation of genomic DNA from pear leaves:

Genomic DNA was extracted and electrophoresed on a 3% agarose gel in order to assess the quality of the isolated DNA. Figure 3.1 shows the isolated genomic DNA of the parent plants,'Bon Rouge' and 'Packham's Triumph'. Both yielded high quality DNA for further analysis by PCR.

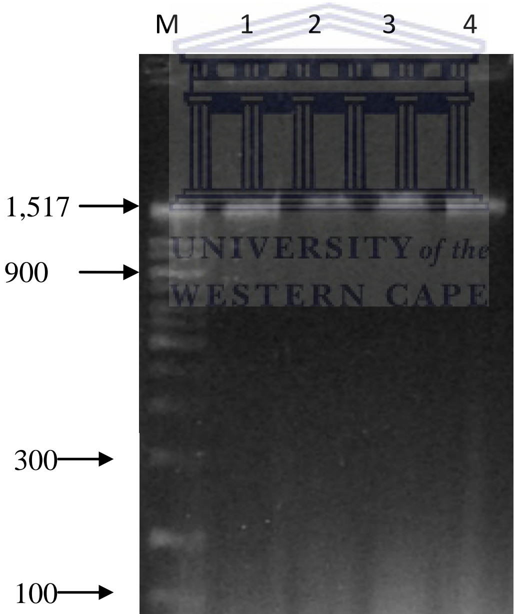

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Figure 3.1: Genomic DNA extracted from 'Packham's Triumph' and 'Bon Rouge', Lane 1 and 3: Packham's Triumph, Lane 2 and 4: 'Bon Rouge.'(M=100bp ladder,)

3.2 SSR analysis

3.2.1 SSR’s testing for polymorphism in parents:

PCR was conducted using primers (Table 2.1) and their products separated on a 3% agarose gel. This was to test for polymorphism in the parents. The PCR was run according to the annealing temperature of primers (Table 2.1).

The DNA was diluted to test for the optimum concentration with the tested primers.

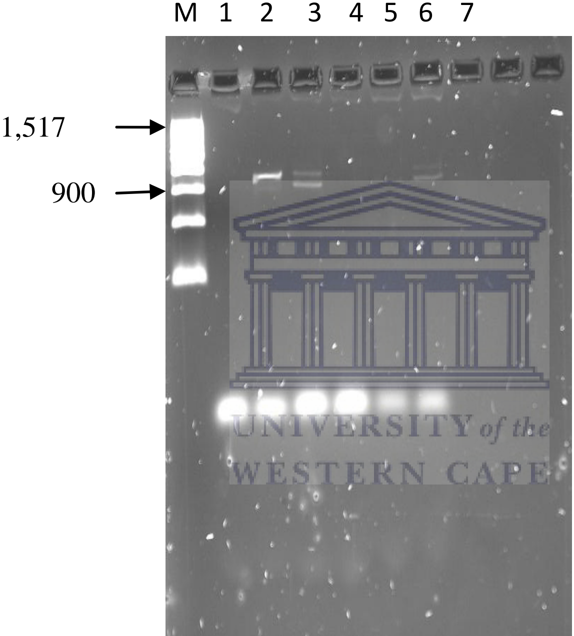

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Figure 3.2: Lane 1: ‘Packham’s Triumph’ 1:10 dilution, Lane 2: ‘Packham’s Triumph’ 1:100, Lane 3: ‘Packham’s Triumph’ 1:1000 dilution, Lane 4: ‘Bon Rouge’ 1:10, Lane 5: ‘Bon Rouge’1:100, Lane 6: ‘Bon Rouge’ 1:1000, Lane 7: negative control.

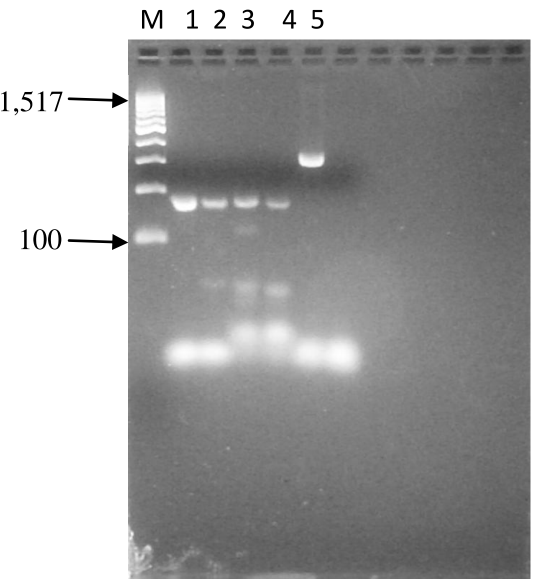

Figure 3.3: DNA amplicons from the parents 'Packham's Triumph' and 'Bon Rouge' using primers PYC-002, PYC-004, and PYC-008 with an annealing temperature of 64°C. M: molecular marker (100bp), Lane 1: 'Packham's Triumph' – PYC-002, Lane 2: 'Bon Rouge' – PYC-002, Lane 3: 'Packham's Triumph' – PYC-004, Lane 4: 'Bon Rouge' – PYC-004, Lane 5: 'Packham's Triumph' – PYC-008, Lane 6: 'Bon Rouge' – PYC-008 (PYC-008 was repeated, figure 3.4).

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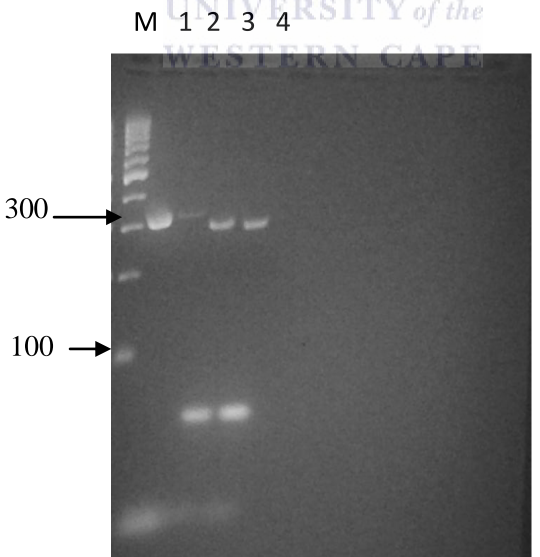

Figure 3.4: Parents 'Packham's Triumph' and 'Bon Rouge' using primers PYC-008 and PYC-009, with an annealing temperature of 64°C. M: molecular marker (100bp), Lane 1: 'Packham's Triumph' – PYC-008, Lane 2: 'Bon Rouge' – PYC-008, Lane 3: 'Packham's Triumph' – PYC-009, Lane 4: 'Bon Rouge' – PYC-009.

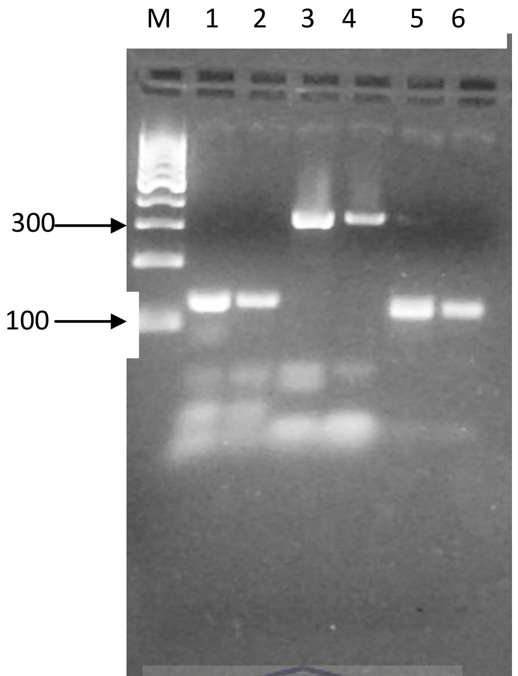

Figure 3.5: Parents 'Packham's Triumph' and 'Bon Rouge' with primers PYC-006, PYC-010ab and PYC-013 with an annealing temperature of 62°C. M: molecular marker (100bp), Lane 1: 'Packham's Triumph' – PYC-006, Lane 2: 'Bon Rouge' – PYC-006, Lane 3: 'Packham's Triumph' – PYC-010ab, Lane 4: 'Bon Rouge' – PYC-010ab, Lane 5: 'Packham's Triumph' – PYC-013, Lane 6: 'Bon Rouge' – PYC-013.

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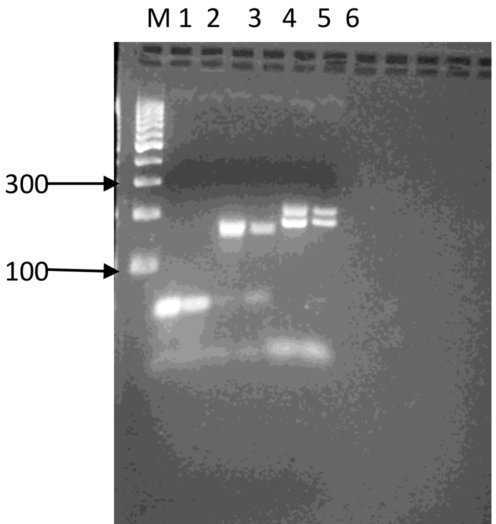

Figure 3.6: Parents 'Packham's Triumph' and 'Bon Rouge' using primers PYC-005, PYC-007, PYC-011 with an annealing temperature of 58 °C. M: molecular marker (100bp), Lane 1: 'Packham's Triumph' – PYC-005, Lane 2: 'Bon Rouge' – PYC-005, Lane 3: 'Packham's Triumph' – PYC-007, Lane 4: 'Bon Rouge' – PYC-007, Lane 5: 'Packham's Triumph' – PYC-011, Lane 6: 'Bon Rouge' – PYC-011.

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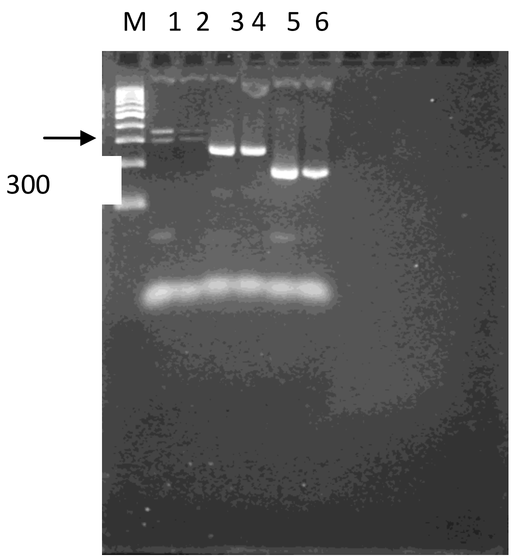

Figure 3.7: Parents 'Packham's Triumph' and 'Bon Rouge' using primers PYC-001, PYC-003 and PYC-012 with an annealing temperature of 66 °C. M: molecular marker (100bp), Lane 1: 'Packham's Triumph' – PYC-001, Lane 2: 'Bon Rouge' – PYC-001, Lane 3: 'Packham's

Triumph' – PYC-003, Lane 4: 'Bon Rouge' – PYC-003, Lane 5: 'Packham's Triumph' – PYC-012, Lane 6: 'Bon Rouge' – PYC-012.

3.3 Multiplexing and application to mapping population:

Microsatellites shown to be polymorphic were multiplexed. The table below shows a summary of primers used, their accession numbers, sequence, and the fluorescent label.

Table 3.1: Summary of primers:

SSR Name	Taxon	GenBank accession	Repeat motif	SSR location	Expected Size	Optimum Tm	Dye
PYC 001	*P.communis*	AB084462	(TA)7	3'UTR	341	66°C	6-FAM
PYC 008	*P.communis*	AF386510	(TG)6	5'UTR	314	64°C	NED
PYC009	*P.caucasica*	AF455809	(CT)11	Intron 2	302	64°C	VIC
PYC010a	*P.pyrifolia*	AB045710	(TTTA)4(TTA)6	Intron6	200	64°C	PET
PYC 012	*P.communis*	S79358	(ACA)6	CDS	180	66°C	NED Blue
CN444542 SSR	*Malus x domestica*		GA		110-156	64°C	6-FAM

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Table 3.2: Representing the scale, Primer name and primer sequence.

Scale	Primer /ProbeName	Primer/Probe Sequence (5'→ 3')
10pM	PYC001F	6-FAM CGGGATCAGACTACAAGATGTG
10pM	PYC001R	AGGCTCTAAGGAAGCCCAATAG
10pM	PYC008F	NED GTGCGATCCAATCCAAGAAG
10pM	PYC008R	GTTTCGAAAAGCAACCCAATCATATC
10pM	PYC009bF	VIC TACCTGGTTCACTACCCAATGC
10pM	PYC009bR	AATGCTACGAACTAAGCCCAAA
10pM	PYC010bF	PET CACATGGATACTTTGGACAAGC
10pM	PYC010bR	GTTTGGAGTGAAATCTAATCCTTGC

10pM	PYC012F	NED ATGCTAGACAGAGCGTGTCCTT
10pM	PYC012R	GGTCTCTTTCGGTGTAACTTGG
10pM	CN444542SSRF	6-FAM ATAAGCCAGGCCACCAAATC
10pM	CN444542SSRF	GTTTGCAGTGGATTGATGTTCC

Polymorphism was determined by using the ABI PRISM® 3130xl Genetic Analyzer, this method allowed for the analysis and size determination by using fluorescent labels which were attached to the primers (Table 3.1)

Seedlings 1-187 results can be found in appendix A on page 63.

The following group of figures are a representation of the electropherogram of the multiplex.

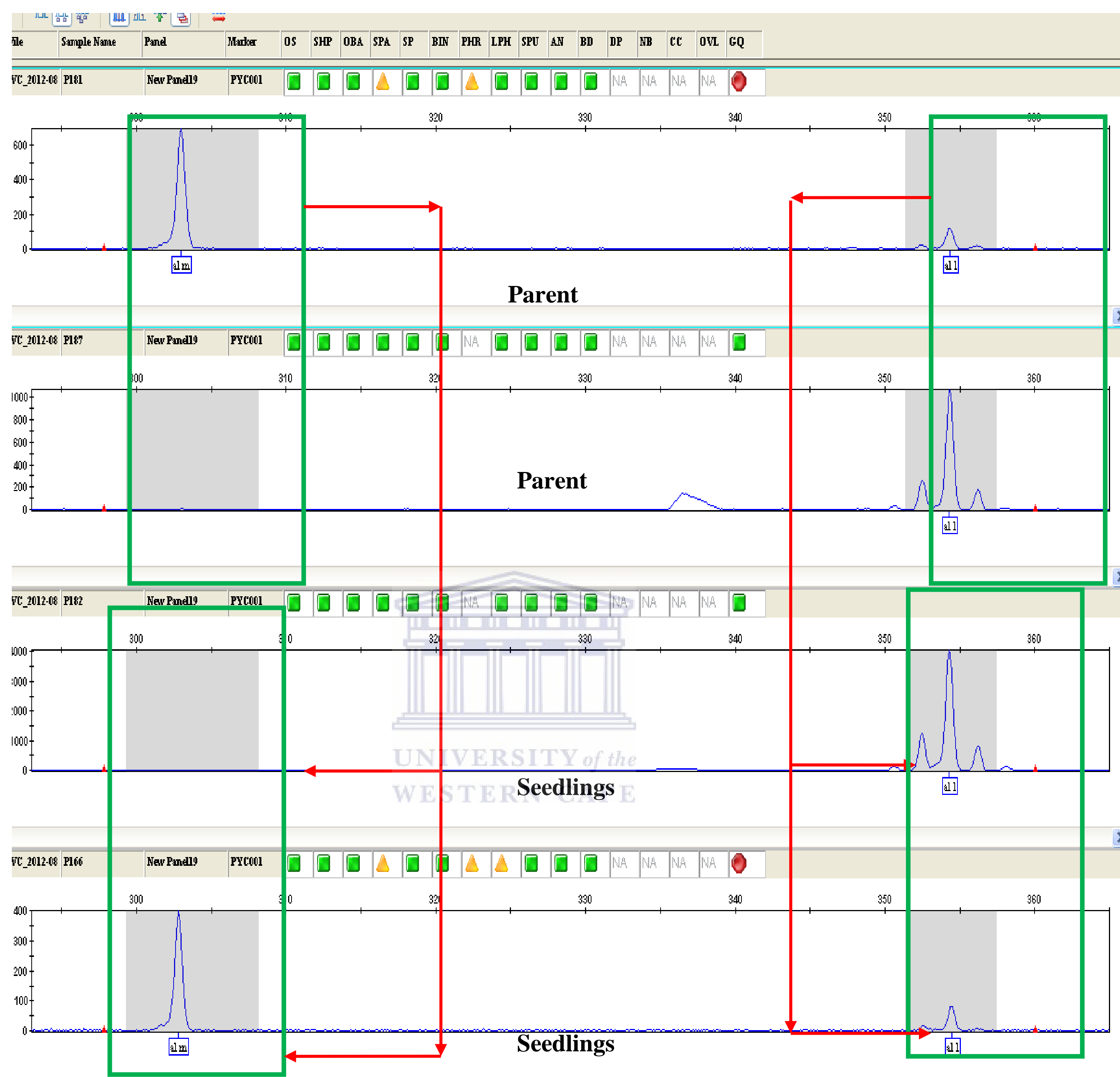

Figure 3.8: For the primer set PYC-001, an electropherogram of the multiplex on the parents and seedlings as seen on ABI PRISM® 3130xl Genetic Analyzer, when using GeneMapper 4.0™ software (Applied Biosystems, Foster City CA, USA.

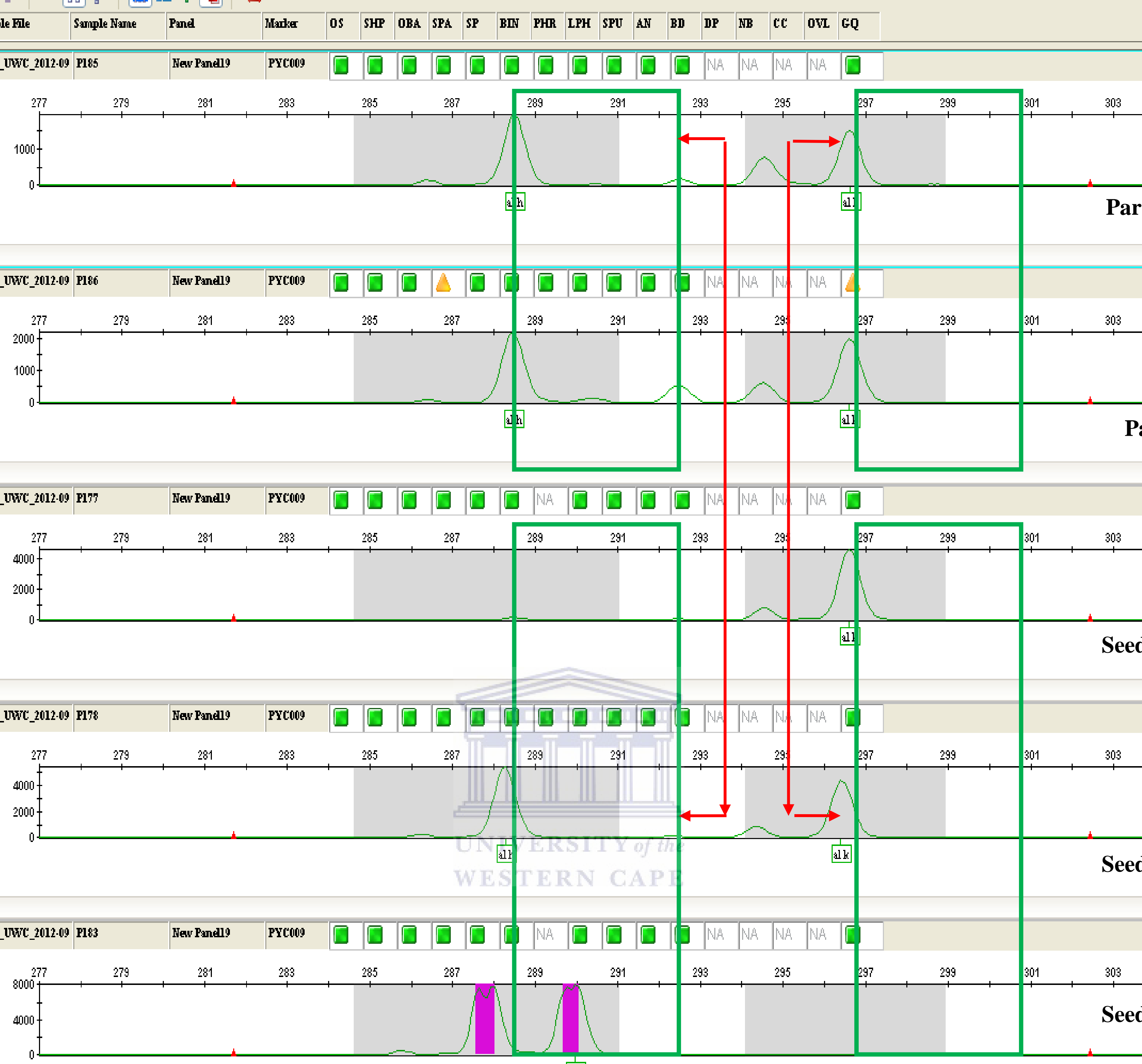

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Figure 3.9: For the primer set PYC-009, an electropherogram of the multiplex on the parents and seedlings as seen on ABI PRISM® 3130xl Genetic Analyzer, when using GeneMapper 4.0™ software (Applied Biosystems, Foster City CA, USA.

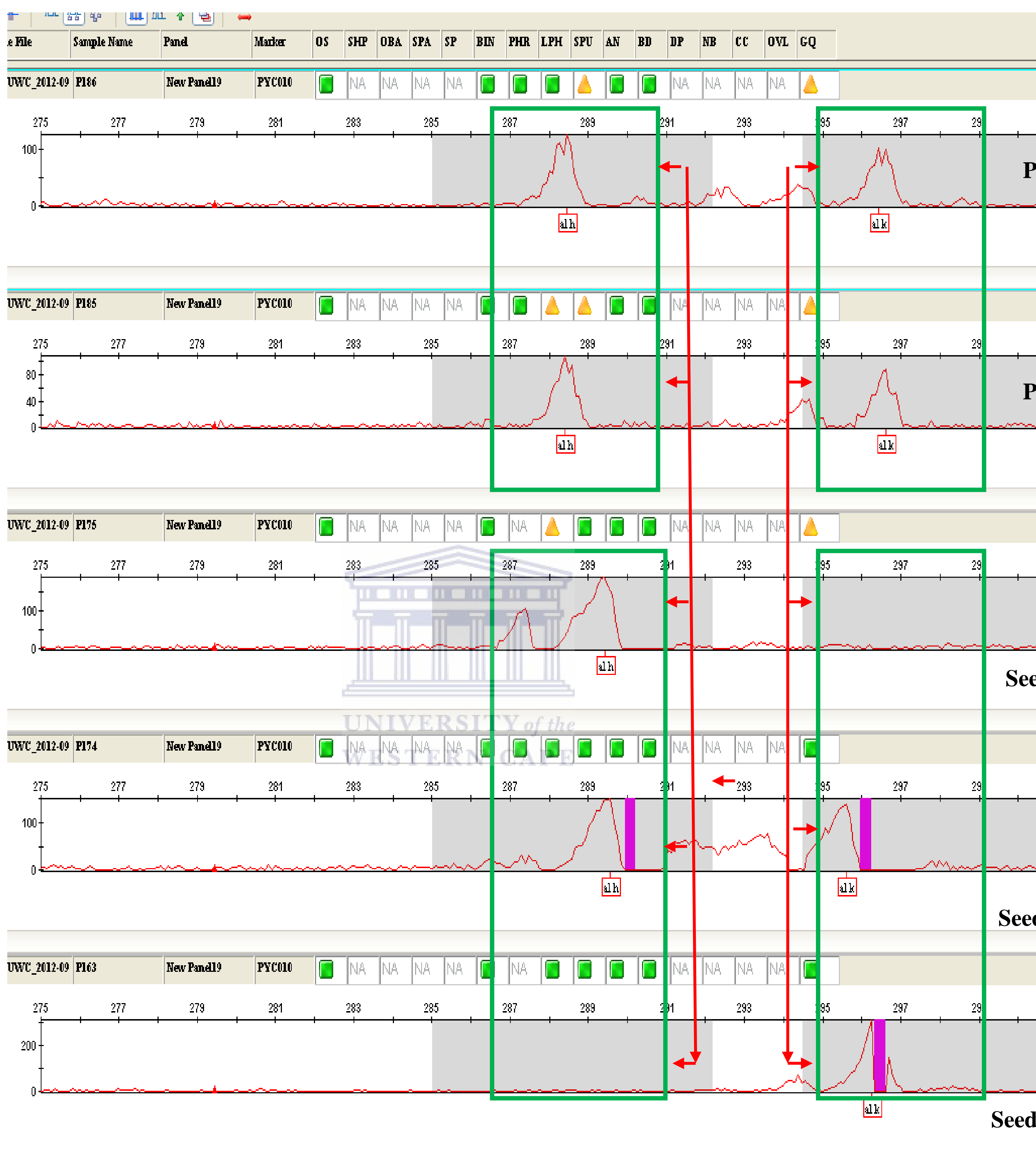

Figure 3.10: For the primer set PYC-010ab, an electropherogram of the multiplex on the parents and seedlings as seen on ABI PRISM® 3130xl Genetic Analyzer, when using GeneMapper 4.0™ software (Applied Biosystems, Foster City CA, USA.

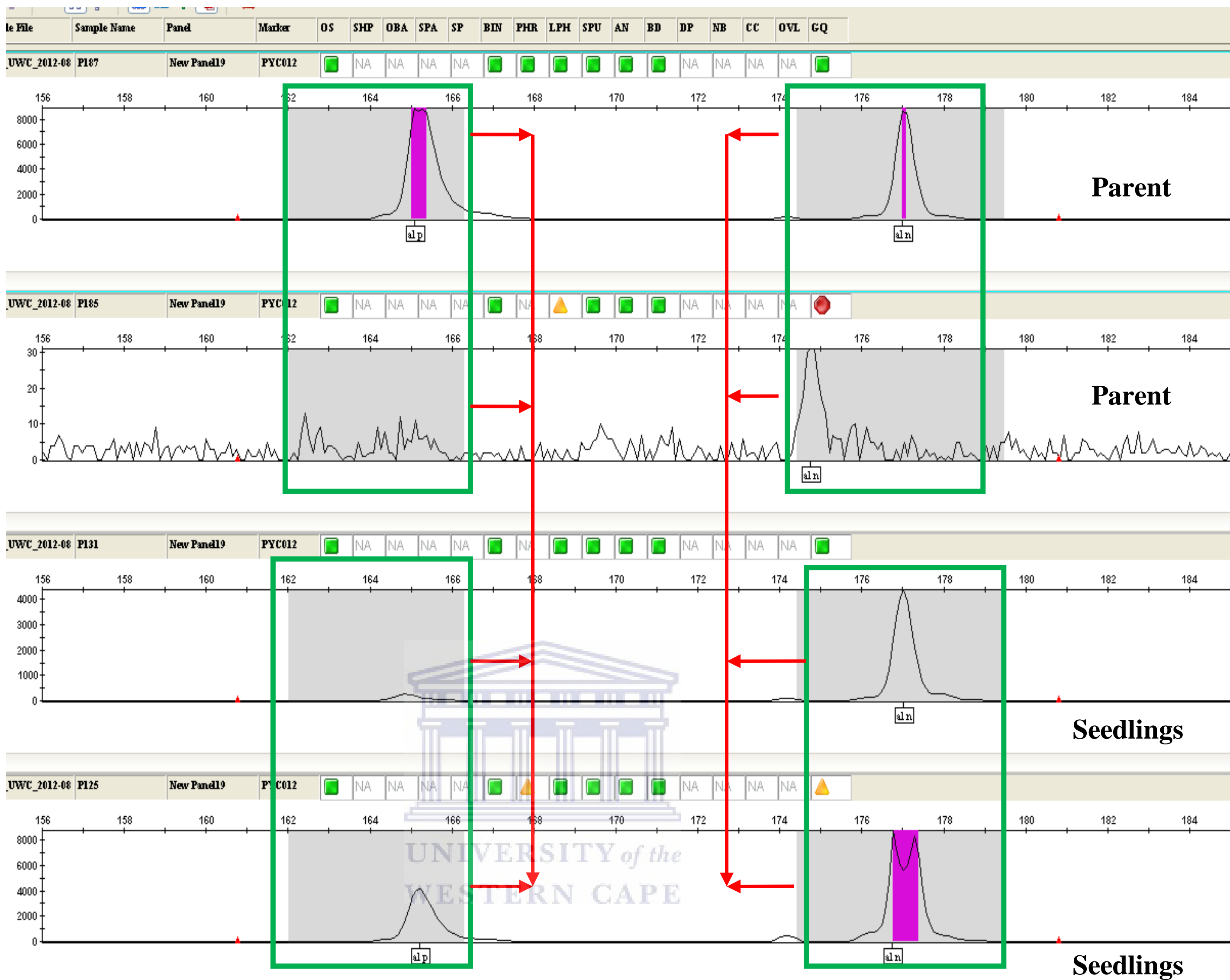

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Figure 3.11: For the primer set PYC-012, an electropherogram of the multiplex on the parents and seedlings as seen on ABI PRISM® 3130xl Genetic Analyzer, when using GeneMapper 4.0™ software (Applied Biosystems, Foster City CA, USA.

<u>Electropherogram key:</u>

ADO = Allele Display Overflow
OS = Offscale
AE = Allele Edit
LPH = Low Peak Height
SPU = Spectral Pull-Up
AN = Allele Number
BD = Broad Peak
OVL = Overlap
GQ = Genotype Quality

Chapter 4:

Discussion:

Chapter 4:

Discussion:

4.1 Discussion:

In order for breeding programs to be efficient as well as for breeding itself, it requires knowledge and understanding of the underlying genetic principals. The availability of high quality genetic linkage maps constructed with molecular markers such as SSR's enables the study of the whole genome structure and localization of genes of interest (Gupta *et al.*, 2003). SSR's are more reliable and informative markers allowing for selection based on genotyping of individuals. SSR's are co-dominant and reproducible and enables the alignment of genetic linkage maps obtained from different cultivars. This chapter discusses the successful extraction, optimisation and the development of a multiplex of SSR markers for high throughput screening of a population segregating for red fruit skin colour.

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4.1.1 Isolation of genomic DNA from pear leaves:

There are a variety of protocols for DNA extractions, the fundamentals remaining the same; the extraction needs to result in DNA being purified from cellular material and phenolic compounds in a manner that would prevent degradation. Thus a mechanical means of breaking down the cell wall and membranes to allow access to the nuclear material without degradation is required. However DNA extraction from plant tissue can vary depending on which material was used. Extraction from pear leaves posed to be difficult as the optimal concentration of DNA was not yielded. This was due to high level of phenolic compounds. Leaves, twigs, flowers, fruits and seeds of pear contain phenolic compounds (Friedrich, 1958a). After investigation, the CTAB method was thus the method of choice for extracting DNA from pear leaves.

An initial grinding stage with liquid nitrogen was performed, this allowed for the breakdown of the cell wall allowing access to DNA and the inactivation of harmful cellular enzymes. The DNA precipitated from the aqueous phase and washed thoroughly to remove contaminating particles. The CTAB method showed to give intact genomic DNA from pear leaves. Extractions from leaves that were harvested yielded high- quality DNA; this was confirmed on the Nanodrop spectrophotometer as the absorbance at 260 and 280 nm showed a ratio of ~ 1.8. Generally this is accepted as 'pure' for DNA. Further evaluation on an agrose gel was conducted. The concentration of the DNA samples ranged from 1576 ng/µl and 1837ng/µl for 'Packham's Triumph' and 1042ng/ µl and 1914ng/ µl for 'Bon Rough.' The DNA of both parents (figure 3.1) was resolved on a 1% agrose gel containing Gel Red dye. This was the stain of choice as the other stains were not sensitive for DNA detection. 'Bon Rouge' and 'Packham's Triumph' can be seen as a single intact band for each. Lane 1 contained 100bp molecular marker. It was found that 10ul of sample and 3ul of loading dye allowed for the visualization of bands, this was the minimum volume for visualisation. The lanes which followed contained the samples in the following order, Lane 2 and 4 containing an intact band of 'Packham's Triumph' DNA at 1,517 bp. Lanes 3 and 5 contained the sample 'Bon Rouge' with a fainter band, however present.

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4.2 Using primers to test for polymorphism in parents:

4.2.1 Testing for optimum concentrations of DNA:

Figure 3.2 shows the diluted DNA at 1:10, 1:100, 1:1000 concentrations. This was conducted prior to the test for polymorphism in parents in order to determine for the optimum concentration with primers. 'M' the abbreviation for molecular marker (100 bp). The DNA was diluted with pure dH_20 at different concentrations; i.e. 1:10 = 1µl of DNA with 9µl of dH_20. 1:100 = 1 µl of DNA with 99 µl of dH_20. 1:1000 = 1 µl of DNA with 999 µl of dH_20. Lane 1 containing 'Packham's Triumph' DNA sample diluted at 1:10 had no bands; however lane 2 and 3 shows the presence of bands at a concentration of 1:100 and 1:1000 respectively. 'Bon Rouge' was found to be more complex as faint bands were only

seen at concentration 1:1000 as seen in lane 6. Lane 7 contained the negative control, which is the primer mix without DNA.

4.2.2 SSR's testing for polymorphism in parents:

4.2.2.1 PCR conditions:

The PCR was conducted for each set of primers in a total reaction volume of 50 µl. The PCR conditions consisted of an initial denaturation at 94°C for 3min, followed by 35 cycles of denaturation at 94 °C for 40 seconds, annealing for 40 seconds at the optimum temperature for each primer set and an extension at 72 °C for 30 seconds. A final extension was conducted at 72°C for 30 min. The amplification products were then separated by electrophoresis on a 3% agrose gel, this included gel red stain. Finally Visualization on UV transilluminator was performed.

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A further primer set (CN44542SSR) was added. This primer set was not visualized on an agrose gel and interestingly did not show results when tested in multiplexing. It is thus important to confirm the validity of the primer sets by running it on agrose gels. This would save time used on multiplexing and also in an emphasized confirmation of the results in multiplexing. This ultimately reduces errors.

4.2.2.2 PCR Results:

Each band represents an allele. If one band is present, this means the sample is homozygous as it has two copies of the same allele. However if two bands are present then that sample is deemed heterozygous as it has two different alleles, i.e. It has two different -sized bands.

Optimum annealing temperatures were used to amplify DNA from 'Packham's Triumph' and 'Bon Rouge.' PYC-011 and PYC-010ab amplified DNA fragments were not the same as the expected sizes of 106 and 200 bp respectively (Table 2.1). Eight primer pairs amplified monomorphic DNA fragments based on 3% agarose gels: PYC-002, PYC-004, PYC-006, PYC-013, PYC-005, PYC-007 and PYC-011. The remaining primer sets; PYC-001, PYC-008, PYC-009, PYC-010ab and PYC-012, amplified polymorphic DNA fragments. As shown in figures 3.3, 3.4, 3.5, 3.6 and 3.7. Loci that appeared

polymorphic in this initial screening were then further analysed by high throughput screening which included the forward primers labelled fluorescently (Table 2.3).

4.3. Multiplexing PCR:

4.3.1 Microsatellite marker selection and allele frequency determination:

This technique is cost effective as it is the co-electrophoresis of multiple markers in each capillary. This technique makes use of fluorescent labelling which allows for colour and size as the distinguishing factor of independent loci. In this study a total of 187 pear samples, including the two parents, were screened with a multiplex of 4 fluorescently-labelled primers. These fluorescent labelled primers were 6-Fam (Blue), Ned (Black), VIC (Green) and PET (Red). Genemapper software (Applied Biosystems) was used to process the results in order to reveal the results of the electropherograms. The program automatically calibrates the size of the DNA. The genotyped files were manually scored to avoid errors. Segregation analysis found that some 'Bon Rouge' and 'Packham's Triumph' are homozygous for certain markers. Furthermore figures 3.8, 3.9, 3.10 and 3.11 reveals the fragments inherited from the parents. This graph shows an electropherograms, where the x-axis represents fragment base pair sizes (bp) and the y-axis the peak height. The number and size of the alleles of the parents detected by the agarose gels were compared. Primers set PYC-012 amplification sizes were similar to the expected sizes of the agarose gel at 180 bp and to the multiplex results. Primer sets; PYC-001, PYC-009 and PYC-010 was found to have fragments sizes were larger by a few bases than what was visualized on the agrose gel. However the sizes on the agrose gel were acceptably close to that of the multiplex with a size variation of a few bases.

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The results for the seedlings are given in the table (Appendix B-F).The presence of a particular SSR allele was given a score of ‘h’ and ‘k’ and its absence a score of ‘u’(Appendix B). In table (Appendix E) the presence of a particular SSR allele was given ‘m’ and ‘l’ and its absence a score of ‘u’.

The marker PYC0-001, PYC-009, and PYC-012, identified alleles in the seedlings showing similar sizes to the parents. Some seedlings showed no amplification. For the primer set PYC-009, 137 from 187 seedlings (72% of the population of seedlings) were polymorphic. PYC-010ab, 87 from the 187 seedlings (46% of the population of seedlings) were polymorphic. PYC-008 resulted in 120 seedlings showing polymorphism (64% of the population of seedlings).

The investigation of the primer sets on the 187 seedlings and parents ‘Packham’s Triumph’ and ‘Bon Rouge’ yielded the electrophrogram graphs presented in figures 3.8-3.9-3.10-3.11. These graphs illustrated successful genotyping by the GeneScan™ on the ABI PRISM® 3130xl Genetic Analyzer.

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Six SSR’s were further investigated; PYC-010ab was least informative in the seedlings while PYC-001, PYC-009 and PYC-012 were the most informative. CNN44552 SSR did not produce alleles thus it is valuable to test for polymorphism when testing SSR transferability between species.

Chapter 5:

Conclusion and future work:

Chapter 5:

Conclusion and future work:

The overall aim of this research project was to convert a set of microsatellite markers identified from the literature into two multiplex assays for high throughput screening on the population under study. Plant Breeding is one of the important sectors of agriculture for the development of new cultivars.

The progression towards a greater understanding in molecular techniques for genetic analysis has lead to a great understanding of the structure and activities of various crop genomes, and to use these techniques in the development of genetic linkage maps for use in the marker-assisted breeding of plants with desired traits. The key feature is to integrate large scale experimental data with molecular mechanisms. One of the challenges is the unavailability of well-defined molecular linkage maps.

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Towards achieving this aim, I have successfully converted a set of microsatellite markers identified from the literature into two multiplex assays for high throughput microsatellite screening on the population under study. Initially, a set of thirteen microsatellites were selected for incorporation into a multiplex assay, but after initial screening on the two parents of the population, only five were incorporated into multiplex assay. As a test case, I included a previously identified microsatellite CN444542SSR, into one of the multiplexes used to screen the population. This microsatellite could not be amplified in either of the two parents or the progeny. This indicates the need for the pre-screening of prospective microsatellites for multiplex incorporation in at least the parents of the family in the study.

Future work include using a larger set of molecular markers, especially SSR's as there is a low availability of these markers in pears. The use of larger numbers of markers would produce

tighter linkage of the separated groups, and saturate the linkage groups to obtain better genome coverage. This would aid the use of genetic linkage maps and breeding pears.

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Chapter 6:

References:

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<u>Chapter 7:</u>

<u>Appendices:</u>

Chapter 7:

Appendices:

Appendix A: DNA concentrations from seedling samples 1-96

Wells	Sample ID	OD260	Total DNA in 150ul (ng)	[ng/ul]	Rounded []	ul DNA	ul SABAX	Final []	Final volume
A1	PA001	0.0365	3011.3	20.075	20	8	19	6	27
B1	PA002	0.0147	1212.8	8.085	10	8	5	6	13
C1	PA003	0.0203	1674.8	11.165	10	8	5	6	13
D1	PA004	0.0156	1287	8.58	10	8	5	6	13
E1	PA005	0.0177	1460.3	9.735	10	8	5	6	13
F1	PA006	0.0437	3605.3	24.035	20	8	19	6	27
G1	PA007	0.0109	899.25	5.995	10	8	5	6	13
H1	PA008	0.0191	1575.8	10.505	10	8	5	6	13
A2	PA009	0.0169	1394.3	9.295	10	8	5	6	13
B2	PA010	0.0288	2376	15.84	20	8	19	6	27
C2	PA011	0.0104	858	5.72	10	8	5	6	13
D2	PA012	0.0199	1641.8	10.945	10	8	5	6	13
E2	PA013	0.0487	4017.8	26.785	30	8	32	6	40
F2	PA014	0.0613	5057.3	33.715	30	8	32	6	40
G2	PA015	0.0233	1922.3	12.815	10	8	5	6	13
H2	PA016	0.0196	1617	10.78	10	8	5	6	13
A3	PA017	0.0249	2054.3	13.695	10	8	5	6	13
B3	PA018	0.0155	1278.8	8.525	10	8	5	6	13
C3	PA019	0.0203	1674.8	11.165	10	8	5	6	13
D3	PA020	0.035	2887.5	19.25	20	8	19	6	27
E3	PA021	0.0467	3852.8	25.685	30	8	32	6	40
F3	PA022	0.0135	1113.8	7.425	10	8	5	6	13
G3	PA023	0.024	1980	13.2	10	8	5	6	13
H3	PA024	0.0247	2037.8	13.585	10	8	5	6	13
A4	PA025	0.0269	2219.3	14.795	10	8	5	6	13
B4	PA026	0.0162	1336.5	8.91	10	8	5	6	13
C4	PA027	0.0091	750.75	5.005	10	8	5	6	13
D4	PA028	0.0187	1542.8	10.285	10	8	5	6	13
E4	PA029	0.0129	1064.3	7.095	10	8	5	6	13
F4	PA030	0.033	2722.5	18.15	20	8	19	6	27
G4	PA031	0.0202	1666.5	11.11	10	8	5	6	13
H4	PA032	0.0215	1773.8	11.825	10	8	5	6	13
A5	PA033	0.0274	2260.5	15.07	20	8	19	6	27

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B5	PA034	0.0224	1848	12.32	10	8	5	6	13
C5	PA035	0.0234	1930.5	12.87	10	8	5	6	13
D5	PA036	0.0132	1089	7.26	10	8	5	6	13
E5	PA037	0.0294	2425.5	16.17	20	8	19	6	27
F5	PA038	0.0171	1410.8	9.405	10	8	5	6	13
G5	PA039	0.0097	800.25	5.335	10	8	5	6	13
H5	PA040	0.0322	2656.5	17.71	20	8	19	6	27
A6	PA041	0.0221	1823.3	12.155	10	8	5	6	13
B6	PA042	0.0179	1476.8	9.845	10	8	5	6	13
C6	PA043	0.0072	594	3.96	6	8	0	6	8
D6	PA044	0.0219	1806.8	12.045	10	8	5	6	13
E6	PA045	0.016	1320	8.8	10	8	5	6	13
F6	PA046	0.0145	1196.3	7.975	10	8	5	6	13
G6	PA047	0.0177	1460.3	9.735	10	8	5	6	13
H6	PA048	0.0354	2920.5	19.47	20	8	19	6	27
A7	PA049	0.0086	709.5	4.73	6	8	0	6	8
B7	PA050	0.016	1320	8.8	10	8	5	6	13
C7	PA051	0.0177	1460.3	9.735	10	8	5	6	13
D7	PA052	0.0122	1006.5	6.71	10	8	5	6	13
E7	PA053	0.028	2310	15.4	20	8	19	6	27
F7	PA054	0.0198	1633.5	10.89	10	8	5	6	13
G7	PA055	0.0104	858	5.72	10	8	5	6	13
H7	PA056	0.0323	2664.8	17.765	20	8	19	6	27
A8	PA057	0.0271	2235.8	14.905	10	8	5	6	13
B8	PA058	0.0204	1683	11.22	10	8	5	6	13
C8	PA059	0.0282	2326.5	15.51	20	8	19	6	27
D8	PA060	0.0306	2524.5	16.83	20	8	19	6	27
E8	PA061	0.0136	1122	7.48	10	8	5	6	13
F8	PA062	0.0192	1584	10.56	10	8	5	6	13
G8	PA063	0.022	1815	12.1	10	8	5	6	13
H8	PA064	0.0193	1592.3	10.615	10	8	5	6	13
A9	PA065	0.0293	2417.3	16.115	20	8	19	6	27
B9	PA066	0.011	907.5	6.05	10	8	5	6	13
C9	PA067	0.0167	1377.8	9.185	10	8	5	6	13
D9	PA068	0.0178	1468.5	9.79	10	8	5	6	13
E9	PA069	0.0298	2458.5	16.39	20	8	19	6	27
F9	PA070	0.0202	1666.5	11.11	10	8	5	6	13
G9	PA071	0.0151	1245.8	8.305	10	8	5	6	13
H9	PA072	0.0214	1765.5	11.77	10	8	5	6	13
A10	PA073	0.0245	2021.3	13.475	10	8	5	6	13
B10	PA074	0.014	1155	7.7	10	8	5	6	13
C10	PA075	0.0277	2285.3	15.235	20	8	19	6	27
D10	PA076	0.0312	2574	17.16	20	8	19	6	27
E10	PA077	0.0951	7845.8	52.305	50	8	59	6	67
F10	PA078	0.0384	3168	21.12	20	8	19	6	27
G10	PA079	0.0322	2656.5	17.71	20	8	19	6	27
H10	PA080	0.0288	2376	15.84	20	8	19	6	27
A11	PA081	0.0239	1971.8	13.145	10	8	5	6	13

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B11	PA082	0.0088	726	4.84	6	8	0	6	8
C11	PA083	0.0198	1633.5	10.89	10	8	5	6	13
D11	PA084	0.0376	3102	20.68	20	8	19	6	27
E11	PA085	0.0321	2648.3	17.655	20	8	19	6	27
F11	PA086	0.0466	3844.5	25.63	30	8	32	6	40
G11	PA087	0.0288	2376	15.84	20	8	19	6	27
H11	PA088	0.0337	2780.3	18.535	20	8	19	6	27
A12	PA089	0.0719	5931.8	39.545	40	8	45	6	53
B12	PA090	0.0175	1443.8	9.625	10	8	5	6	13
C12	PA091	0.0275	2268.8	15.125	20	8	19	6	27
D12	PA092	0.0618	5098.5	33.99	30	8	32	6	40
E12	PA093	0.022	1815	12.1	10	8	5	6	13
F12	PA094	0.0274	2260.5	15.07	20	8	19	6	27
G12	PA095	0.0276	2277	15.18	20	8	19	6	27
H12	PA096	0.0386	3184.5	21.23	20	8	19	6	27
A1	PA097	0.0294	2425.5	16.17	20	8	19	6	27
B1	PA098	0.0405	3341.3	22.275	20	8	19	6	27
C1	PA099	0.0384	3168	21.12	20	8	19	6	27
D1	PA100	0.0433	3572.3	23.815	20	8	19	6	27
E1	PA101	0.061	5032.5	33.55	30	8	32	6	40
F1	PA102	0.0149	1229.3	8.195	10	8	5	6	13
G1	PA103	0.0226	1864.5	12.43	10	8	5	6	13
H1	PA104	0.0431	3555.8	23.705	20	8	19	6	27
A2	PA105	0.0187	1542.8	10.285	10	8	5	6	13
B2	PA106	0.0233	1922.3	12.815	10	8	5	6	13
C2	PA107	0.0637	5255.3	35.035	40	8	45	6	53
D2	PA108	0.0392	3234	21.56	20	8	19	6	27
E2	PA109	0.0246	2029.5	13.53	10	8	5	6	13
F2	PA110	0.045	3712.5	24.75	20	8	19	6	27
G2	PA111	0.0443	3654.8	24.365	20	8	19	6	27
H2	PA112	0.0289	2384.3	15.895	20	8	19	6	27
A3	PA113	0.0545	4496.3	29.975	30	8	32	6	40
B3	PA114	0.0604	4983	33.22	30	8	32	6	40
C3	PA115	0.0481	3968.3	26.455	30	8	32	6	40
D3	PA116	0.0592	4884	32.56	30	8	32	6	40
E3	PA117	0.057	4702.5	31.35	30	8	32	6	40
F3	PA118	0.0264	2178	14.52	10	8	5	6	13
G3	PA119	0.0291	2400.8	16.005	20	8	19	6	27
H3	PA120	0.0297	2450.3	16.335	20	8	19	6	27
A4	PA121	0.0289	2384.3	15.895	20	8	19	6	27
B4	PA122	0.0711	5865.8	39.105	40	8	45	6	53
C4	PA123	0.0479	3951.8	26.345	30	8	32	6	40
D4	PA124	0.0628	5181	34.54	30	8	32	6	40
E4	PA125	0.0311	2565.8	17.105	20	8	19	6	27
F4	PA126	0.0557	4595.3	30.635	30	8	32	6	40
G4	PA127	0.0198	1633.5	10.89	10	8	5	6	13
H4	PA128	0.0288	2376	15.84	20	8	19	6	27
A5	PA129	0.0644	5313	35.42	40	8	45	6	53

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B5	PA130	0.0524	4323	28.82	30	8	32	6	40
C5	PA131	0.0542	4471.5	29.81	30	8	32	6	40
D5	PA132	0.0269	2219.3	14.795	10	8	5	6	13
E5	PA133	0.0379	3126.8	20.845	20	8	19	6	27
F5	PA134	0.0432	3564	23.76	20	8	19	6	27
G5	PA135	0.0286	2359.5	15.73	20	8	19	6	27
H5	PA136	0.0396	3267	21.78	20	8	19	6	27
A6	PA137	0.0425	3506.3	23.375	20	8	19	6	27
B6	PA138	0.0227	1872.8	12.485	10	8	5	6	13
C6	PA139	0.0248	2046	13.64	10	8	5	6	13
D6	PA140	0.0203	1674.8	11.165	10	8	5	6	13
E6	PA141	0.0465	3836.3	25.575	30	8	32	6	40
F6	PA142	0.0688	5676	37.84	40	8	45	6	53
G6	PA143	0.0294	2425.5	16.17	20	8	19	6	27
H6	PA144	0.06	4950	33	30	8	32	6	40
A7	PA145	0.1152	9504	63.36	60	8	72	6	80
B7	PA146	0.0345	2846.3	18.975	20	8	19	6	27
C7	PA147	0.2074	17111	114.07	110	8	139	6	147
D7	PA148	0.05	4125	27.5	30	8	32	6	40
E7	PA149	0.0437	3605.3	24.035	20	8	19	6	27
F7	PA150	0.041	3382.5	22.55	20	8	19	6	27
G7	PA151	0.0668	5511	36.74	40	8	45	6	53
H7	PA152	0.0391	3225.8	21.505	20	8	19	6	27
A8	PA153	0.0041	338.25	2.255	6	8	0	6	0
B8	PA154	0.0231	1905.8	12.705	10	8	5	6	13
C8	PA155	0.0353	2912.3	19.415	20	8	19	6	27
D8	PA156	0.0695	5733.8	38.225	40	8	45	6	53
E8	PA157	0.1483	12235	81.565	80	8	99	6	107
F8	PA158	0.0274	2260.5	15.07	20	8	19	6	27
G8	PA159	0.0211	1740.8	11.605	10	8	5	6	13
H8	PA160	0.0304	2508	16.72	20	8	19	6	27
A9	PA161	0.0317	2615.3	17.435	20	8	19	6	27
B9	PA162	0.0393	3242.3	21.615	20	8	19	6	27
C9	PA163	0.0293	2417.3	16.115	20	8	19	6	27
D9	PA164	0.0247	2037.8	13.585	10	8	5	6	13
E9	PA165	0.0358	2953.5	19.69	20	8	19	6	27
F9	PA166	0.0652	5379	35.86	40	8	45	6	53
G9	PA167	0.0365	3011.3	20.075	20	8	19	6	27
H9	PA168	0.0212	1749	11.66	10	8	5	6	13
A10	PA169	0.0155	1278.8	8.525	10	8	5	6	13
B10	PA170	0.0382	3151.5	21.01	20	8	19	6	27
C10	PA171	0.0603	4974.8	33.165	30	8	32	6	40
D10	PA172	0.0169	1394.3	9.295	10	8	5	6	13
E10	PA173	0.0363	2994.8	19.965	20	8	19	6	27
F10	PA174	0.0408	3366	22.44	20	8	19	6	27
G10	PA175	0.0341	2813.3	18.755	20	8	19	6	27
H10	PA176	0.038	3135	20.9	20	8	19	6	27
A11	PA177	0.047	3877.5	25.85	30	8	32	6	40

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B11	PA178	0.1689	13934	92.895	90	8	112	6	120
C11	PA179	0.0451	3720.8	24.805	20	8	19	6	27
D11	PA180	0.0796	6567	43.78	40	8	45	6	53
E11	PA181	0.0735	6063.8	40.425	40	8	45	6	53
F11	PA182	0.0263	2169.8	14.465	10	8	5	6	13
G11	PA183	0.0373	3077.3	20.515	20	8	19	6	27
H11	PA184	0.1371	11311	75.405	80	8	99	6	107
A12	PA185	0.4529	37364	249.095	250	8	325	6	333
B12	PA186	0.0234	1930.5	12.87	10	8	5	6	13
C12	PA187	0.0448	3696	24.64	20	8	19	6	27
D12									
E12									
F12									
G12									
H12									

Appendix B: Representation of primers sample name, marker, dye, alleles and sizes

Sample Name	Marker	Dye	Allele 1	Allele 2		Size 1	Size 2
P001	PYC008	Y	h	k	hk	288.62	320.13
P002	PYC008	Y	k	k	kk	321.43	
P003	PYC008	Y		u	u		
P004	PYC008	Y	h	k	hk	288.44	319.43
P005	PYC008	Y	h	k	hk	288.4	319.39
P006	PYC008	Y	k	k	kk	321.28	
P007	PYC008	Y	h	k	hk	288.04	319.49
P008	PYC008	Y	h	k	hk	288.33	319.36
P009	PYC008	Y	h	k	hk	288.6	320.2
P010	PYC008	Y	h	k	hk	288.78	319.99
P011	PYC008	Y	h	k	hk	288.48	319.92
P012	PYC008	Y	k	k	kk	319.86	
P013	PYC008	Y	h	k	hk	288.27	319.82
P014	PYC008	Y	h	k	hk	290.28	319.17
P015	PYC008	Y	h	k	hk	290.13	319.14
P016	PYC008	Y	h	k	hk	294.47	319.37
P017	PYC008	Y	k	k	kk	319.54	
P018	PYC008	Y	k	k	kk	319.62	
P019	PYC008	Y	h	k	hk	296.15	319.56
P020	PYC008	Y	h	k	hk	288.1	319.48
P021	PYC008	Y	k	k	kk	299.91	321.19
P022	PYC008	Y	k	k	kk	300	321.03
P023	PYC008	Y	h	k	hk	287.99	319.25
P024	PYC008	Y	h	k	hk	291.71	320.87
P025	PYC008	Y	h	k	hk	288.27	319.35
P026	PYC008	Y	h	k	hk	288.17	319.64
P027	PYC008	Y	h	h	hh	290.13	296.25

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P028	PYC008	Y	h	k	hk	288.12	319.42
P029	PYC008	Y	h	h	hh	292.08	300.17
P030	PYC008	Y	k	k	kk	320.87	
P031	PYC008	Y	h	k	hk	293.85	320.88
P032	PYC008	Y	h	k	hk	289.94	319.2
P033	PYC008	Y	h	k	hk	290.13	319.53
P034	PYC008	Y	h	h	hh	290.12	296.37
P035	PYC008	Y	k	k	kk	300.26	321.09
P036	PYC008	Y	h	k	hk	289.97	319.59
P037	PYC008	Y	h	k	hk	287.98	319.22
P038	PYC008	Y	k	k	kk	300.18	321.06
P039	PYC008	Y	h	k	hk	295.96	318.92
P040	PYC008	Y	h	k	hk	294.02	
P041	PYC008	Y		u	u		
P042	PYC008	Y		u	u		
P043	PYC008	Y	h	k	hk	297.35	
P044	PYC008	Y		u	u		
P045	PYC008	Y		u	u		
P046	PYC008	Y	h	k	hk	290.05	
P047	PYC008	Y	h	k	hk	296.65	
P048	PYC008	Y	h	k	hk	289.9	
P049	PYC008	Y	h	k	hk	288.1	319.8
P050	PYC008	Y	k	k	kk	319.6	
P051	PYC008	Y		u	u		
P052	PYC008	Y		u	u		
P053	PYC008	Y		u	u		
P054	PYC008	Y		u	u		
P055	PYC008	Y		u	u		
P056	PYC008	Y		u	u		
P057	PYC008	Y		u	u		
P058	PYC008	Y		u	u		
P059	PYC008	Y		u	u		
P060	PYC008	Y		u	u		
P061	PYC008	Y	h	k	hk	296.21	
P062	PYC008	Y		u	u		
P063	PYC008	Y		u	u		
P064	PYC008	Y	h	k	hk	287.85	
P065	PYC008	Y		u	u		
P066	PYC008	Y		u	u		
P067	PYC008	Y	h	k	hk	290.2	319.57
P068	PYC008	Y		u	u		
P069	PYC008	Y	h	k	hk	288.11	319.3
P070	PYC008	Y		u	u		
P071	PYC008	Y		u	u		
P072	PYC008	Y	h	k	hk	296.21	
P073	PYC008	Y	h	k	hk	290.3	
P074	PYC008	Y		u	u		
P075	PYC008	Y		u	u		

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P076	PYC008	Y		u	u		
P077	PYC008	Y		u	u		
P078	PYC008	Y		u	u		
P079	PYC008	Y		u	u		
P080	PYC008	Y	k	k	kk	318.97	
P081	PYC008	Y		u	u		
P082	PYC008	Y		u	u		
P083	PYC008	Y	k	k	kk	319.37	
P084	PYC008	Y		u	u		
P085	PYC008	Y		u	u		
P086	PYC008	Y	h	k	hk	296.24	319.07
P087	PYC008	Y	k	k	kk	318.94	
P088	PYC008	Y		u	u		
P089	PYC008	Y	k	k	kk	319.64	
P090	PYC008	Y		u	u		
P091	PYC008	Y		u	u		
P092	PYC008	Y	h	k	hk	296.33	319.31
P093	PYC008	Y	h	k	hk	288.07	
P094	PYC008	Y		u	u		
P095	PYC008	Y		u	u		
P096	PYC008	Y		u	u		
P097	PYC008	Y	k	k	kk	320.69	
P098	PYC008	Y	k	k	kk	320.49	
P099	PYC008	Y	u	k	uk	300.36	321.81
P100	PYC008	Y	h	k	hk	288.8	319.52
P101	PYC008	Y	h	k	hk	290.97	319.46
P102	PYC008	Y	h	k	hk	288.9	
P103	PYC008	Y	h	k	hk	288.37	
P104	PYC008	Y	h	k	hk	288.81	319.23
P105	PYC008	Y	h	k	hk	296.97	319.76
P106	PYC008	Y		u	u		
P107	PYC008	Y	u	k	uk	299.82	321.68
P108	PYC008	Y	h	k	hk	288.82	320.16
P109	PYC008	Y	h	k	hk	288.99	319.43
P110	PYC008	Y	h	k	hk	290.68	319.54
P111	PYC008	Y	h	k	hk	290.27	319.18
P112	PYC008	Y	h	k	hk	288.48	319.88
P113	PYC008	Y		u	u		
P114	PYC008	Y	h	k	hk	290.85	320.27
P115	PYC008	Y	h	k	hk	288.61	320.1
P116	PYC008	Y	u	k	uk	300.43	321.77
P117	PYC008	Y	h	k	hk	288.46	319.83
P118	PYC008	Y	h	k	hk	288.23	319.61
P119	PYC008	Y	h	k	hk	288.14	319.01
P120	PYC008	Y	h	k	hk	288.08	319.31
P121	PYC008	Y	h	k	hk	288.87	
P122	PYC008	Y	h	k	hk	290.75	320.22
P123	PYC008	Y	h	k	hk	290.69	320.1

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P124	PYC008	Y	h	k	hk	288.55	319.36
P125	PYC008	Y	h	k	hk	288.59	319.74
P126	PYC008	Y	h	k	hk	288.09	319.29
P127	PYC008	Y	h	k	hk	287.9	318.96
P128	PYC008	Y	h	k	hk	290.21	319.25
P129	PYC008	Y	k	k	kk	320.03	
P130	PYC008	Y	k	k	kk	320.06	
P131	PYC008	Y	u	u	uu	299.82	
P132	PYC008	Y	h	k	hk	290.53	320.04
P133	PYC008	Y	h	k	hk	290.44	319.73
P134	PYC008	Y	h	k	hk	288.44	319.46
P135	PYC008	Y	h	k	hk	288.14	319.1
P136	PYC008	Y	h	k	hk	288.21	319.36
P137	PYC008	Y	h	k	hk	288.51	
P138	PYC008	Y	h	k	hk	288.87	
P139	PYC008	Y	h	k	hk	288.72	319.42
P140	PYC008	Y	h	k	hk	290.81	319.96
P141	PYC008	Y	h	k	hk	296.82	319.53
P142	PYC008	Y	h	k	hk	288.11	319.36
P143	PYC008	Y	h	k	hk	289.95	318.97
P144	PYC008	Y	h	k	hk	290.1	319.26
P145	PYC008	Y	k	k	kk	320.08	
P146	PYC008	Y	h	k	hk	296.9	320.13
P147	PYC008	Y	k	k	kk	320.04	
P148	PYC008	Y	h	k	hk	288.45	320.01
P149	PYC008	Y	h	k	hk	288.34	319.93
P150	PYC008	Y	h	k	hk	290.18	319.62
P151	PYC008	Y	h	k	hk	288.06	319.31
P152	PYC008	Y	h	k	hk	288.06	319.46
P153	PYC008	Y	h	k	hk	296.93	320.32
P154	PYC008	Y	h	k	hk	296.74	320.1
P155	PYC008	Y	k	k	kk	320.03	
P156	PYC008	Y	h	k	hk	296.61	
P157	PYC008	Y		u	u		
P158	PYC008	Y	h	k	hk	290.27	319.14
P159	PYC008	Y	h	k	hk	290.01	319.02
P160	PYC008	Y	h	k	hk	292.08	320.87
P161	PYC008	Y	h	k	hk	288.58	320
P162	PYC008	Y	h	k	hk	296.53	319.89
P163	PYC008	Y	h	k	hk	296.42	319.78
P164	PYC008	Y	h	k	hk	295.97	319.37
P165	PYC008	Y		u	u		
P166	PYC008	Y	k	k	kk	319.32	
P167	PYC008	Y	h	k	hk	289.93	319.23
P168	PYC008	Y	k	k	kk	320.92	
P169	PYC008	Y	h	k	hk	288.63	320.09
P170	PYC008	Y	k	k	kk	320	
P171	PYC008	Y	h	k	hk	290.81	320.04

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P172	PYC008	Y	k	k	kk	319.9	
P173	PYC008	Y	h	k	hk	290.34	319.67
P174	PYC008	Y	h	k	hk	290.03	319.38
P175	PYC008	Y	h	k	hk	287.84	319.01
P176	PYC008	Y	h	k	hk	292.07	320.84
P177	PYC008	Y	h	k	hk	296.59	319.85
P178	PYC008	Y	k	k	kk	319.76	
P179	PYC008	Y	h	k	hk	288.18	319.75
P180	PYC008	Y	u	k	uk	300.18	321.26
P181	PYC008	Y	h	k	hk	288.47	319.57
P182	PYC008	Y	h	k	hk	290.07	319.34
P183	PYC008	Y	h	k	hk	289.97	319.04
P184	PYC008	Y	h	k	hk	287.89	319.54
P185	PYC008	Y	h	k	hk	288.68	320.08
P186	PYC008	Y	h	k	hk	288.73	319.92
P187	PYC008	Y	h	k	hk	290.4	319.84
X	PYC008	Y		u	u		
X	PYC008	Y		u	u		
X	PYC008	Y		u	u		
X	PYC008	Y	k	k	kk	319.28	
X	PYC008	Y		u	u		

Appendix C: Seedling results for PYC-009

P001	PYC009	G	h	k	hk	288.62	
P002	PYC009	G	k	k	kk	298	
P003	PYC009	G		u	u		
P004	PYC009	G	h	k	hk	288.7	296.77
P005	PYC009	G	h	k	hk	288.49	
P006	PYC009	G	k	k	kk	297.98	
P007	PYC009	G	h	k	hk	288.14	
P008	PYC009	G	h	k	hk	288.51	296.47
P009	PYC009	G	h	k	hk	288.69	
P010	PYC009	G	h	k	hk	288.51	296.7
P011	PYC009	G	h	k	hk	288.57	296.58
P012	PYC009	G	h	k	hk	290.48	296.5
P013	PYC009	G	h	k	hk	288.45	296.45
P014	PYC009	G	h	k	hk	290.19	296.33
P015	PYC009	G	h	k	hk	290.13	296.12
P016	PYC009	G	k	k	kk	296.2	
P017	PYC009	G	h	k	hk	290.34	296.42
P018	PYC009	G	h	k	hk	288.12	296.25
P019	PYC009	G	k	k	kk	296.34	
P020	PYC009	G	h	k	hk	288.19	296.27
P021	PYC009	G	k	k	kk	298.06	
P022	PYC009	G	k	k	kk	298.04	
P023	PYC009	G	h	k	hk	287.99	
P024	PYC009	G	h	k	hk	289.67	297.88

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P025	PYC009	G	h	k	hk	288.36	
P026	PYC009	G	h	k	hk	288.26	296.23
P027	PYC009	G	h	k	hk	290.22	296.34
P028	PYC009	G	h	k	hk	288.21	296.22
P029	PYC009	G	h	k	hk	289.78	297.98
P030	PYC009	G	k	k	kk	298.04	
P031	PYC009	G	k	k	kk	297.92	
P032	PYC009	G	h	k	hk	290.04	295.94
P033	PYC009	G	h	k	hk	288.16	
P034	PYC009	G	h	k	hk	290.12	296.19
P035	PYC009	G	k	k	kk	298	
P036	PYC009	G	h	k	hk	290.24	296.2
P037	PYC009	G	h	k	hk	287.98	296.14
P038	PYC009	G	k	k	kk	298.04	
P039	PYC009	G	k	k	kk	295.96	
P040	PYC009	G	k	k	kk	297.98	
P041	PYC009	G	h	k	hk	288.22	
P042	PYC009	G	k	k	kk	296.35	
P043	PYC009	G	h	k	hk	290.15	296.26
P044	PYC009	G	h	k	hk	288.11	
P045	PYC009	G	k	k	kk	296.11	
P046	PYC009	G	h	k	hk	289.87	295.85
P047	PYC009	G	h	k	hk	287.8	
P048	PYC009	G	h	k	hk	287.79	
P049	PYC009	G	h	k	hk	288.19	296.36
P050	PYC009	G	k	k	kk	296.22	
P051	PYC009	G	h	k	hk	290.14	296.2
P052	PYC009	G	k	k	kk	296.22	
P053	PYC009	G	k	k	kk	296.07	
P054	PYC009	G	h	k	hk	287.86	
P055	PYC009	G	h	k	hk	289.88	295.99
P056	PYC009	G	h	k	hk	289.94	296.01
P057	PYC009	G	h	k	hk	288.2	
P058	PYC009	G	h	k	hk	290.32	296.3
P059	PYC009	G	h	k	hk	288.18	296.3
P060	PYC009	G	h	k	hk	288.07	
P061	PYC009	G	k	k	kk	296.12	
P062	PYC009	G	h	h	hh	287.9	289.92
P063	PYC009	G	h	k	hk	289.64	295.74
P064	PYC009	G	h	k	hk	287.85	295.88
P065	PYC009	G		u	u		
P066	PYC009	G	h	k	hk	288.09	296.26
P067	PYC009	G	h	k	hk	290.11	296.22
P068	PYC009	G		u	u		
P069	PYC009	G	h	k	hk	288.02	
P070	PYC009	G		u	u		
P071	PYC009	G	h	k	hk	287.8	295.9
P072	PYC009	G	k	k	kk	296.12	

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P073	PYC009	G	h	k	hk	288.26	
P074	PYC009	G	h	k	hk	288.22	
P075	PYC009	G		u	u		
P076	PYC009	G	h	k	hk	288	
P077	PYC009	G	h	k	hk	290.05	296.05
P078	PYC009	G	h	k	hk	287.87	
P079	PYC009	G	k	k	kk	295.85	
P080	PYC009	G	h	k	hk	289.89	295.9
P081	PYC009	G	h	k	hk	290.3	296.37
P082	PYC009	G	h	k	hk	288.29	296.33
P083	PYC009	G	h	k	hk	288.11	296.24
P084	PYC009	G	h	k	hk	288.06	
P085	PYC009	G	h	k	hk	290.03	296.1
P086	PYC009	G	k	k	kk	295.97	
P087	PYC009	G	h	k	hk	287.88	
P088	PYC009	G	h	k	hk	289.97	296
P089	PYC009	G	h	k	hk	288.3	
P090	PYC009	G	h	k	hk	288.27	296.39
P091	PYC009	G	k	k	kk	296.3	
P092	PYC009	G	k	k	kk	296.15	
P093	PYC009	G	h	k	hk	287.98	
P094	PYC009	G	h	k	hk	287.77	
P095	PYC009	G	k	k	kk	295.86	
P096	PYC009	G	h	k	hk	287.86	295.89
P097	PYC009	G	h	k	hk	289.2	297.32
P098	PYC009	G	h	k	hk	289.11	297.14
P099	PYC009	G	k	k	kk	298.01	
P100	PYC009	G	h	k	hk	289.25	297.13
P101	PYC009	G	h	k	hk	289.01	297.14
P102	PYC009	G	h	k	hk	288.63	296.81
P103	PYC009	G	h	k	hk	288.46	296.55
P104	PYC009	G	h	k	hk	288.72	296.91
P105	PYC009	G	h	k	hk	289.18	297.41
P106	PYC009	G	h	k	hk	289.06	
P107	PYC009	G	k	k	kk	297.93	
P108	PYC009	G	h	k	hk	288.91	
P109	PYC009	G	h	k	hk	288.99	296.96
P110	PYC009	G	h	k	hk	288.67	
P111	PYC009	G	h	k	hk	288.42	
P112	PYC009	G	h	k	hk	288.67	
P113	PYC009	G		u	u		
P114	PYC009	G	h	k	hk	288.78	296.86
P115	PYC009	G	h	k	hk	288.7	
P116	PYC009	G	k	k	kk	298.06	
P117	PYC009	G	h	k	hk	288.55	296.57
P118	PYC009	G	h	k	hk	288.32	
P119	PYC009	G	h	k	hk	288.14	296.1
P120	PYC009	G	h	k	hk	288.17	

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P121	PYC009	G	h	k	hk	288.7	296.87
P122	PYC009	G	h	k	hk	290.84	296.86
P123	PYC009	G	h	k	hk	290.86	296.81
P124	PYC009	G	h	h	hh	288.73	290.72
P125	PYC009	G	h	k	hk	288.59	296.66
P126	PYC009	G	h	k	hk	288.27	296.33
P127	PYC009	G	h	k	hk	287.99	295.99
P128	PYC009	G	h	k	hk	290.3	296.25
P129	PYC009	G	k	k	kk	296.63	
P130	PYC009	G	h	k	hk	288.64	296.8
P131	PYC009	G	k	k	kk	298.02	
P132	PYC009	G	h	k	hk	290.53	296.64
P133	PYC009	G	h	k	hk	290.62	296.6
P134	PYC009	G	h	k	hk	288.08	
P135	PYC009	G	h	k	hk	288.14	296.26
P136	PYC009	G	h	k	hk	288.12	
P137	PYC009	G	h	k	hk	288.77	297.04
P138	PYC009	G	h	k	hk	288.61	296.75
P139	PYC009	G	h	k	hk	288.72	296.83
P140	PYC009	G	h	k	hk	290.54	296.64
P141	PYC009	G	k	k	kk	296.82	
P142	PYC009	G	h	k	hk	288.11	
P143	PYC009	G	h	k	hk	289.95	296.03
P144	PYC009	G	h	h	hh	288.29	290.28
P145	PYC009	G	h	k	hk	290.62	296.72
P146	PYC009	G	h	k	hk	288.5	296.64
P147	PYC009	G	k	k	kk	296.52	
P148	PYC009	G	h	h	hh	288.54	290.51
P149	PYC009	G	h	k	hk	288.43	296.5
P150	PYC009	G	h	k	hk	290.18	296.18
P151	PYC009	G	h	k	hk	287.87	296.05
P152	PYC009	G	h	k	hk	288.15	296.23
P153	PYC009	G	h	k	hk	288.62	296.76
P154	PYC009	G	k	k	kk	296.83	
P155	PYC009	G	h	k	hk	290.64	296.73
P156	PYC009	G	k	k	kk	296.61	
P157	PYC009	G		u	u		
P158	PYC009	G	h	k	hk	290.27	296.36
P159	PYC009	G	h	k	hk	289.92	295.93
P160	PYC009	G	h	k	hk	289.78	297.98
P161	PYC009	G	h	k	hk	288.49	296.61
P162	PYC009	G	k	k	kk	296.71	
P163	PYC009	G	k	k	kk	296.51	
P164	PYC009	G	k	k	kk	296.77	
P165	PYC009	G		u	u		
P166	PYC009	G	k	k	kk	296.17	
P167	PYC009	G	h	k	hk	290.11	296.12
P168	PYC009	G	k	k	kk	297.89	

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P169	PYC009	G	h	k	hk	288.54	
P170	PYC009	G	h	k	hk	288.48	
P171	PYC009	G	h	k	hk	288.49	
P172	PYC009	G	h	k	hk	288.36	296.42
P173	PYC009	G	h	k	hk	290.43	296.39
P174	PYC009	G	h	k	hk	290.21	296.25
P175	PYC009	G	h	k	hk	287.75	
P176	PYC009	G	h	k	hk	289.85	297.97
P177	PYC009	G	k	u	ku	296.59	
P178	PYC009	G	h	k	hk	288.29	296.39
P179	PYC009	G	h	k	hk	288.27	296.48
P180	PYC009	G	k	k	kk	297.99	
P181	PYC009	G	h	k	hk	288.2	296.31
P182	PYC009	G	h	k	hk	290.16	296.17
P183	PYC009	G	h	k	hk	289.97	
P184	PYC009	G	h	k	hk	287.98	296.14
P185	PYC009	G	h	k	hk	288.5	296.61
P186	PYC009	G	h	k	hk	288.46	296.6
P187	PYC009	G	h	k	hk	290.49	296.48
X	PYC009	G		u	u		
X	PYC009	G		u	u		
X	PYC009	G		u	u		
X	PYC009	G	h	u	hu	287.97	295.96
X	PYC009	G		u	u		

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Appendix D: Seedling results for PYC-010

P001	PYC010	R	h	h	hh	288.53	290.59
P002	PYC010	R	k	k	kk	297.91	
P003	PYC010	R		u	u		
P004	PYC010	R	h	k	hk	288.26	296.32
P005	PYC010	R	h	k	hk	290.01	
P006	PYC010	R	k	k	kk	297.71	
P007	PYC010	R	h	k	hk	289.7	
P008	PYC010	R	h	k	hk	287.79	295.93
P009	PYC010	R	h	h	hh	288.42	290.56
P010	PYC010	R	h	k	hk	288.42	296.52
P011	PYC010	R	h	k	hk	288.3	296.49
P012	PYC010	R	h	k	hk	290.03	295.96
P013	PYC010	R	h	k	hk	287.82	295.72
P014	PYC010	R	h	k	hk	289.73	295.78
P015	PYC010	R	h	k	hk	289.67	295.66
P016	PYC010	R	k	k	kk	296.38	
P017	PYC010	R	h	k	hk	290.25	296.33
P018	PYC010	R	h	k	hk	288.12	296.07
P019	PYC010	R	k	k	kk	295.97	

P020	PYC010	R	h	k	hk	287.64	296
P021	PYC010	R	k	k	kk	300.09	
P022	PYC010	R	k	k	kk	299.72	
P023	PYC010	R	h	h	hh	287.81	289.84
P024	PYC010	R	h	k	hk	291.24	299.45
P025	PYC010	R	h	h	hh	288.09	290.25
P026	PYC010	R	h	k	hk	287.99	296.23
P027	PYC010	R	h	k	hk	289.85	296.07
P028	PYC010	R	h	k	hk	287.67	295.77
P029	PYC010	R	h	k	hk	291.81	300
P030	PYC010	R	k	k	kk	300	
P031	PYC010	R	k	k	kk	299.43	
P032	PYC010	R	h	k	hk	289.76	295.38
P033	PYC010	R	h	k	hk	289.95	
P034	PYC010	R	h	k	hk	290.02	296.1
P035	PYC010	R	k	k	kk	300	
P036	PYC010	R	h	k	hk	289.79	295.75
P037	PYC010	R	h	k	hk	287.43	295.41
P038	PYC010	R	k	k	kk	297.85	
P039	PYC010	R	k	k	kk	295.5	
P040	PYC010	R	k	k	kk	299.45	
P041	PYC010	R		u	u		
P042	PYC010	R		u	u		
P043	PYC010	R		u	u		
P044	PYC010	R		u	u		
P045	PYC010	R		u	u		
P046	PYC010	R		u	u		
P047	PYC010	R		u	u		
P048	PYC010	R		u	u		
P049	PYC010	R		u	u		
P050	PYC010	R		u	u		
P051	PYC010	R		u	u		
P052	PYC010	R		u	u		
P053	PYC010	R		u	u		
P054	PYC010	R		u	u		
P055	PYC010	R		u	u		
P056	PYC010	R		u	u		
P057	PYC010	R		u	u		
P058	PYC010	R		u	u		
P059	PYC010	R		u	u		
P060	PYC010	R		u	u		
P061	PYC010	R		u	u		
P062	PYC010	R		u	u		
P063	PYC010	R		u	u		
P064	PYC010	R		u	u		
P065	PYC010	R		u	u		
P066	PYC010	R		u	u		
P067	PYC010	R	h	k	hk	290.02	296.13

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P068	PYC010	R		u	u		
P069	PYC010	R	h	k	hk	289.47	
P070	PYC010	R		u	u		
P071	PYC010	R		u	u		
P072	PYC010	R		u	u		
P073	PYC010	R	h	h	hh	288.26	290.21
P074	PYC010	R		u	u		
P075	PYC010	R		u	u		
P076	PYC010	R		u	u		
P077	PYC010	R		u	u		
P078	PYC010	R		u	u		
P079	PYC010	R		u	u		
P080	PYC010	R		u	u		
P081	PYC010	R		u	u		
P082	PYC010	R		u	u		
P083	PYC010	R		u	u		
P084	PYC010	R		u	u		
P085	PYC010	R		u	u		
P086	PYC010	R		u	u		
P087	PYC010	R		u	u		
P088	PYC010	R		u	u		
P089	PYC010	R		u	u		
P090	PYC010	R		u	u		
P091	PYC010	R		u	u		
P092	PYC010	R		u	u		
P093	PYC010	R		u	u		
P094	PYC010	R		u	u		
P095	PYC010	R		u	u		
P096	PYC010	R		u	u		
P097	PYC010	R	u	k	uk	291.16	297.23
P098	PYC010	R	h	k	hk	290.98	296.96
P099	PYC010	R	k	k	kk	300	
P100	PYC010	R	h	k	hk	288.98	
P101	PYC010	R	h	k	hk	290.44	296.6
P102	PYC010	R	h	k	hk	288.44	296.63
P103	PYC010	R		u	u		
P104	PYC010	R	h	k	hk	288.27	296.36
P105	PYC010	R	h	k	hk	289.1	297.15
P106	PYC010	R	k	k	kk	299.91	
P107	PYC010	R	k	k	kk	300	
P108	PYC010	R	h	k	hk	290.53	
P109	PYC010	R	h	k	hk	288.81	
P110	PYC010	R	h	k	hk	288.49	
P111	PYC010	R	h	k	hk	289.81	
P112	PYC010	R	h	k	hk	290.13	
P113	PYC010	R	k	k	kk	300.09	
P114	PYC010	R	h	k	hk	290.67	296.68
P115	PYC010	R	h	h	hh	288.43	290.51

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P116	PYC010	R	k	k	kk	300	
P117	PYC010	R	h	k	hk	287.91	296.03
P118	PYC010	R	h	k	hk	290.06	
P119	PYC010	R	h	k	hk	287.96	
P120	PYC010	R	h	k	hk	289.77	
P121	PYC010	R	h	k	hk	288.61	296.7
P122	PYC010	R	h	k	hk	290.58	296.86
P123	PYC010	R	h	k	hk	290.34	296.55
P124	PYC010	R	h	k	hk	290.2	
P125	PYC010	R	h	k	hk	288.42	296.05
P126	PYC010	R	h	k	hk	287.54	295.78
P127	PYC010	R	h	k	hk	287.34	295.53
P128	PYC010	R	h	k	hk	289.02	295.61
P129	PYC010	R	k	k	kk	296.78	
P130	PYC010	R	h	k	hk	288.55	296.8
P131	PYC010	R	k	k	kk	300	
P132	PYC010	R	h	k	hk	290.18	296.28
P133	PYC010	R	h	k	hk	290	296.07
P134	PYC010	R	h	k	hk	289.97	
P135	PYC010	R	h	k	hk	287.96	295.62
P136	PYC010	R	h	k	hk	289.65	
P137	PYC010	R	h	k	hk	288.69	296.69
P138	PYC010	R	h	k	hk	288.52	296.58
P139	PYC010	R	h	k	hk	288.64	296.48
P140	PYC010	R	h	k	hk	290.19	296.37
P141	PYC010	R	k	k	kk	296.46	
P142	PYC010	R	h	k	hk	289.65	
P143	PYC010	R	h	k	hk	289.59	295.57
P144	PYC010	R	h	k	hk	288.11	
P145	PYC010	R	k	k	kk	299.91	
P146	PYC010	R	h	k	hk	288.41	296.55
P147	PYC010	R	h	k	hk	288.32	296.34
P148	PYC010	R	h	k	hk	290.33	
P149	PYC010	R	h	k	hk	287.8	295.96
P150	PYC010	R	h	k	hk	290	296
P151	PYC010	R	h	k	hk	287.23	295.31
P152	PYC010	R	h	k	hk	287.44	295.6
P153	PYC010	R	h	k	hk	288.62	296.67
P154	PYC010	R	k	k	kk	296.56	
P155	PYC010	R	h	k	hk	290.46	296.64
P156	PYC010	R		u	u		
P157	PYC010	R		u	u		
P158	PYC010	R	h	k	hk	289.63	295.63
P159	PYC010	R	h	k	hk	289.46	295.47
P160	PYC010	R	h	k	hk	291.81	300
P161	PYC010	R	h	k	hk	288.49	296.7
P162	PYC010	R	k	k	kk	296.35	
P163	PYC010	R	k	k	kk	296.24	

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P164	PYC010	R	k	k	kk	296.51	
P165	PYC010	R		u	u		
P166	PYC010	R	k	k	kk	295.9	
P167	PYC010	R	h	k	hk	289.38	297.97
P168	PYC010	R	k	k	kk	299.45	
P169	PYC010	R	h	h	hh	288.46	290.57
P170	PYC010	R	h	h	hh	288.39	290.52
P171	PYC010	R	h	h	hh	288.31	290.27
P172	PYC010	R	h	k	hk	287.91	296.15
P173	PYC010	R	h	k	hk	289.71	295.75
P174	PYC010	R	h	k	hk	289.57	295.61
P175	PYC010	R	h	k	hk	289.42	
P176	PYC010	R	h	k	hk	291.88	299.45
P177	PYC010	R	k	k	kk	296.5	
P178	PYC010	R	h	k	hk	288.11	296.21
P179	PYC010	R	h	k	hk	288	296.3
P180	PYC010	R	k	k	kk	300	
P181	PYC010	R	h	k	hk	287.57	295.77
P182	PYC010	R	h	k	hk	289.8	295.9
P183	PYC010	R	h	u	hu	287.74	
P184	PYC010	R	h	k	hk	287.52	295.68
P185	PYC010	R	h	k	hk	288.41	296.61
P186	PYC010	R	h	k	hk	288.46	296.42
P187	PYC010	R	h	k	hk	290.23	296.48

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Appendix E: Seedling results for PYC-001

Sample Name	Marker	Dye	Allele 1	Allele 2	result	Size 1	Size 2
P001	PYC001	B	l	u	lu	353.9	
P002	PYC001	B	l	u	lu	353.91	
P003	PYC001	B		u	u		
P004	PYC001	B	m	l	ml	302.47	353.91
P005	PYC001	B		u	u		
P006	PYC001	B		u	u		
P007	PYC001	B	m	l	ml	303.21	354.23
P008	PYC001	B	m	l	ml	303.39	354.13
P009	PYC001	B	l	u	lu	353.99	
P010	PYC001	B	l	u	lu	353.92	
P011	PYC001	B	l	u	lu	353.87	
P012	PYC001	B	l	u	lu	353.96	
P013	PYC001	B		u	u		
P014	PYC001	B		u	u		
P015	PYC001	B		u	u		
P016	PYC001	B	l	u	lu	354.53	
P017	PYC001	B		u	u		
P018	PYC001	B		u	u		
P019	PYC001	B		u	u		

P020	PYC001	B	l	u	lu	353.89	
P021	PYC001	B	l	u	lu	354.04	
P022	PYC001	B	l	u	lu	354.15	
P023	PYC001	B	m	l	ml	302.88	354.25
P024	PYC001	B		u	u		
P025	PYC001	B	l	u	lu	353.93	
P026	PYC001	B	l	u	lu	354.03	
P027	PYC001	B	m	l	ml	303.24	354.1
P028	PYC001	B	m	l	ml	303.31	354.05
P029	PYC001	B	m	l	ml	303.03	354.03
P030	PYC001	B	l	u	lu	354.25	
P031	PYC001	B	l	u	lu	354.28	
P032	PYC001	B	m	l	ml	302.86	354.33
P033	PYC001	B	m	l	ml	303.47	353.95
P034	PYC001	B	l	u	lu	354.14	
P035	PYC001	B	l	u	lu	354	
P036	PYC001	B	m	l	ml	303.31	354.09
P037	PYC001	B	m	l	ml	303.27	354.11
P038	PYC001	B	m	l	ml	302.99	354.13
P039	PYC001	B	l	u	lu	354.3	
P040	PYC001	B	l	u	lu	354.29	
P041	PYC001	B	l	u	lu	353.98	
P042	PYC001	B		u	u		
P043	PYC001	B	l	u	lu	353.97	
P044	PYC001	B	l	u	lu	354.07	
P045	PYC001	B	l	u	lu	354.15	
P046	PYC001	B	l	u	lu	354.32	
P047	PYC001	B	l	u	lu	354.31	
P048	PYC001	B	l	u	lu	354.29	
P049	PYC001	B	l	u	lu	353.97	
P050	PYC001	B	m	l	ml	303.38	354.08
P051	PYC001	B	m	l	ml	303.23	354.06
P052	PYC001	B	m	l	ml	303.37	354.14
P053	PYC001	B	m	l	ml	303.24	354.08
P054	PYC001	B	m	l	ml	302.75	354.26
P055	PYC001	B	l	u	lu	354.24	
P056	PYC001	B	l	u	lu	354.22	
P057	PYC001	B	l	u	lu	353.94	
P058	PYC001	B	l	u	lu	354.09	
P059	PYC001	B	m	l	ml	303.31	354.02
P060	PYC001	B	m	l	ml	302.65	354.1
P061	PYC001	B	l	u	lu	354.11	
P062	PYC001	B	m	l	ml	302.94	354.28
P063	PYC001	B	l	u	lu	354.38	
P064	PYC001	B	m	l	ml	302.7	354.37
P065	PYC001	B	m	l	ml	303.37	354.12
P066	PYC001	B	l	u	lu	354.13	
P067	PYC001	B	m	l	ml	302.75	354.16

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P068	PYC001	B	l	u	lu	354.07	
P069	PYC001	B	l	u	lu	354.13	
P070	PYC001	B	l	u	lu	354.13	
P071	PYC001	B	l	u	lu	354.35	
P072	PYC001	B	u	u	u	340.73	
P073	PYC001	B	l	u	lu	353.97	
P074	PYC001	B	l	u	lu	354.1	
P075	PYC001	B	m	l	ml	303.28	353.96
P076	PYC001	B	l	u	lu	354.17	
P077	PYC001	B	l	u	lu	354.16	
P078	PYC001	B	m	l	ml	302.89	354.24
P079	PYC001	B	l	u	lu	354.33	
P080	PYC001	B	l	u	lu	354.22	
P081	PYC001	B	m	l	ml	303.39	354
P082	PYC001	B	m	l	ml	302.94	354.08
P083	PYC001	B	l	u	lu	354.07	
P084	PYC001	B	l	u	lu	354.07	
P085	PYC001	B	l	u	lu	354.09	
P086	PYC001	B	l	u	lu	354.18	
P087	PYC001	B	l	u	lu	354.33	
P088	PYC001	B	l	u	lu	354.19	
P089	PYC001	B	m	l	ml	303.5	353.94
P090	PYC001	B	l	u	lu	354.06	
P091	PYC001	B	l	u	lu	353.97	
P092	PYC001	B	l	u	lu	354.13	
P093	PYC001	B	l	u	lu	354.1	
P094	PYC001	B	l	u	lu	354.29	
P095	PYC001	B	l	u	lu	354.39	
P096	PYC001	B	l	u	lu	354.26	
P097	PYC001	B	l	u	lu	354.05	
P098	PYC001	B	l	u	lu	353.98	
P099	PYC001	B	m	l	ml	303.33	354.05
P100	PYC001	B	l	u	lu	355.78	
P101	PYC001	B	l	u	lu	354.08	
P102	PYC001	B	l	u	lu	354.16	
P103	PYC001	B	m	u	mu	302.85	349.01
P104	PYC001	B	l	u	lu	354.15	
P105	PYC001	B	l	u	lu	354	
P106	PYC001	B	m	l	ml	303.49	354.01
P107	PYC001	B	m	l	ml	303.37	354.07
P108	PYC001	B	l	u	lu	354.02	
P109	PYC001	B	l	u	lu	354.1	
P110	PYC001	B	l	u	lu	354.25	
P111	PYC001	B	l	u	lu	354.27	
P112	PYC001	B	m	l	ml	302.97	354.3
P113	PYC001	B	m	l	ml	303.34	354.08
P114	PYC001	B	l	u	lu	354.06	
P115	PYC001	B	l	u	lu	354.03	

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P116	PYC001	B	l	u	lu	351.36	
P117	PYC001	B	l	u	lu	354.07	
P118	PYC001	B		u	u		
P119	PYC001	B	l	u	lu	354.38	
P120	PYC001	B	l	u	lu	354.21	
P121	PYC001	B		u	u		
P122	PYC001	B	u	l	ul	345.99	354.13
P123	PYC001	B	l	u	lu	354.09	
P124	PYC001	B	m	u	mu	303.23	354.1
P125	PYC001	B	l	u	lu	354.09	
P126	PYC001	B	l	u	lu	354.24	
P127	PYC001	B	l	u	lu	354.34	
P128	PYC001	B	m	l	ml	302.84	354.4
P129	PYC001	B	l	u	lu	354.02	
P130	PYC001	B		u	u		
P131	PYC001	B	m	l	ml	303.31	354.08
P132	PYC001	B		u	u		
P133	PYC001	B		u	u		
P134	PYC001	B		u	u		
P135	PYC001	B		u	u		
P136	PYC001	B		u	u		
P137	PYC001	B		u	u		
P138	PYC001	B		u	u		
P139	PYC001	B		u	u		
P140	PYC001	B		u	u		
P141	PYC001	B		u	u		
P142	PYC001	B		u	u		
P143	PYC001	B		u	u		
P144	PYC001	B		u	u		
P145	PYC001	B		u	u		
P146	PYC001	B		u	u		
P147	PYC001	B		u	u		
P148	PYC001	B		u	u		
P149	PYC001	B		u	u		
P150	PYC001	B		u	u		
P151	PYC001	B		u	u		
P152	PYC001	B		u	u		
P153	PYC001	B		u	u		
P154	PYC001	B		u	u		
P155	PYC001	B		u	u		
P156	PYC001	B		u	u		
P157	PYC001	B		u	u		
P158	PYC001	B		u	u		
P159	PYC001	B		u	u		
P160	PYC001	B		u	u		
P161	PYC001	B		u	u		
P162	PYC001	B		u	u		
P163	PYC001	B		u	u		

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P164	PYC001	B		u	u		
P165	PYC001	B		u	u		
P166	PYC001	B	m	l	ml	302.8	354.44
P167	PYC001	B	l	u	lu	354.47	
P168	PYC001	B	l	u	lu	354.39	
P169	PYC001	B		u	lu		
P170	PYC001	B	l	u	lu	354.31	
P171	PYC001	B		u	u		
P172	PYC001	B	l	u	lu	354.26	
P173	PYC001	B	l	u	lu	354.34	
P174	PYC001	B	l	u	lu	354.43	
P175	PYC001	B		u	u		
P176	PYC001	B	l	u	lu	354.39	
P177	PYC001	B	l	l	ll	354.25	356.18
P178	PYC001	B		u	u		
P179	PYC001	B	l	u	lu	354.24	
P180	PYC001	B	l	l	ll	354.31	356.09
P181	PYC001	B	m	l	ml	303	354.33
P182	PYC001	B	l	u	lu	354.26	
P183	PYC001	B	l	u	lu	354.47	
P184	PYC001	B		u	u		
P185	PYC001	B		u	u		
P186	PYC001	B	l	u	lu	354.14	
P187	PYC001	B	l	u	lu	354.31	

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Appendix F: Seedling results for PYC-012

P001	PYC012	Y	p	n	pn	164.91	176.72
P002	PYC012	Y	p	n	pn	164.8	177.18
P003	PYC012	Y		u	u		
P004	PYC012	Y	p	n	pn	165.01	177.32
P005	PYC012	Y		u	u		
P006	PYC012	Y		u	u		
P007	PYC012	Y	p	n	pn	164.23	176.69
P008	PYC012	Y	p	n	pn	164.19	176.82
P009	PYC012	Y	p	n	pn	165.17	177.06
P010	PYC012	Y	p	n	pn	165.21	177.15
P011	PYC012	Y	p	n	pn	165.16	176.74
P012	PYC012	Y	p	n	pn	165.25	176.87
P013	PYC012	Y	p	n	pn	165.07	177.15
P014	PYC012	Y	n	u	nu	176.5	
P015	PYC012	Y		u	u		
P016	PYC012	Y	n	u	nu	176.46	
P017	PYC012	Y		u	u		
P018	PYC012	Y		u	u		
P019	PYC012	Y	n	u	nu	174.85	
P020	PYC012	Y	p	n	pn	164.82	176.73

P021	PYC012	Y	p	n	pn	164.59	177.23
P022	PYC012	Y	p	n	pn	164.35	177.08
P023	PYC012	Y	p	n	pn	164.32	177.24
P024	PYC012	Y	n	u	nu	175.74	
P025	PYC012	Y	p	n	pn	165.23	177.36
P026	PYC012	Y	p	n	pn	165.36	177.28
P027	PYC012	Y	p	n	pn	165.22	177.35
P028	PYC012	Y	p	n	pn	165.26	177.26
P029	PYC012	Y	p	n	pn	165.13	176.73
P030	PYC012	Y	p	n	pn	164.93	176.56
P031	PYC012	Y	p	n	pn	164.49	176.38
P032	PYC012	Y	p	n	pn	164.62	176.47
P033	PYC012	Y	p	n	pn	165.08	176.73
P034	PYC012	Y	n	u	nu	177.07	
P035	PYC012	Y	p	n	pn	164.75	176.66
P036	PYC012	Y	p	n	pn	164.76	177.28
P037	PYC012	Y	p	n	pn	164.57	177.27
P038	PYC012	Y	p	n	pn	164.33	176.7
P039	PYC012	Y	p	n	pn	164.36	177.29
P040	PYC012	Y	p	n	pn	164.33	177.27
P041	PYC012	Y	n	u	nu	176.97	
P042	PYC012	Y		u	u		
P043	PYC012	Y	p	n	pn	165.1	177.1
P044	PYC012	Y	p	n	pn	165.19	177.16
P045	PYC012	Y	p	n	pn	165.17	177.36
P046	PYC012	Y	p	n	pn	164.88	177.13
P047	PYC012	Y	p	n	pn	164.53	176.55
P048	PYC012	Y	p	n	pn	164.67	176.69
P049	PYC012	Y	p	n	pn	165.04	177.44
P050	PYC012	Y	p	n	pn	164.85	177.32
P051	PYC012	Y	p	n	pn	164.78	176.69
P052	PYC012	Y	n	u	nu	177.23	
P053	PYC012	Y	p	n	pn	164.53	177.29
P054	PYC012	Y	p	n	pn	164.38	176.7
P055	PYC012	Y	p	n	pn	164.32	177.23
P056	PYC012	Y	p	n	pn	164.24	176.69
P057	PYC012	Y	p	n	pn	165.13	177.24
P058	PYC012	Y	p	n	pn	165.19	176.76
P059	PYC012	Y	p	n	pn	165.04	176.53
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X	PYC012	Y	u	u	uu	180	

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Appendix G: Representing examples of BLAST results :

PYC 001 R

Pyrus communis PC-PG2 mRNA for polygalacturonase, complete cds

GenBank: AB084462.1

FASTA Graphics

Go to:

```
LOCUS       AB084462                1662 bp    mRNA    linear   PLN 03-OCT-
2008
DEFINITION  Pyrus communis PC-PG2 mRNA for polygalacturonase, complete cds.
ACCESSION   AB084462
VERSION     AB084462.1  GI:24475518
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KEYWORDS    .
SOURCE      Pyrus communis (pear)
  ORGANISM  Pyrus communis
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Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
eudicotyledons;
            rosids; fabids; Rosales; Rosaceae; Amygdaloideae; Maleae; Pyrus.
REFERENCE   1
  AUTHORS   Hiwasa,K., Kinugasa,Y., Amano,S., Hashimoto,A., Nakano,R.,
Inaba,A.
            and Kubo,Y.
  TITLE     Ethylene is required for both the initiation and progression of
            softening in pear (Pyrus communis L.) fruit
  JOURNAL   J. Exp. Bot. 54 (383), 771-779 (2003)
   PUBMED   12554720
REFERENCE   2  (bases 1 to 1662)
  AUTHORS   Hiwasa,K., Amano,S., Hashimoto,A., Nakano,R., Inaba,A. and
Kubo,Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAY-2002) Contact:Kyoko Hiwasa Okayama University,
ORIGIN
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PYC 007 a F

Pyrus pyrifolia gene for UDP-glucose pyrophosphorylase, complete cds
GenBank: AB027617.1

```
> dbj|AB027617.1|  Pyrus pyrifolia gene for UDP-glucose pyrophosphorylase,
complete
cds
Length=7647

 Score = 40.1 bits (20),  Expect = 0.068
 Identities = 20/20 (100%), Gaps = 0/20 (0%)
 Strand=Plus/Plus

Query  1     TGAAGTTCGAGATGGGTTGA  20
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Sbjct  1879  TGAAGTTCGAGATGGGTTGA  1898
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FASTA Graphics
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LOCUS       AB027617                7647 bp    DNA     linear   PLN 18-AUG-
2007
DEFINITION  Pyrus pyrifolia gene for UDP-glucose pyrophosphorylase, complete
            cds.
ACCESSION   AB027617
VERSION     AB027617.1  GI:8099154
KEYWORDS    UDP-glucose pyrophosphorylase.
SOURCE      Pyrus pyrifolia
  ORGANISM  Pyrus pyrifolia
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
eudicotyledons;
            rosids; fabids; Rosales; Rosaceae; Amygdaloideae; Maleae; Pyrus.
REFERENCE   1
  AUTHORS   Norioka,S., Kiyozumi,D. and Norioka,N.
  TITLE     Molecular cloning and nucleotide sequencing of a gene encoding
            UDP-glucose pyrophosphorylase of Japanese pear (Pyrus pyrifolia
            Nakai)
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 7647)
  AUTHORS   Norioka,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-MAY-1999) Naoko Norioka, Institute for Protein
            Research, Osaka University, Division of Protein Chemistry; 3-2
            Yamadaoka, Suita, Osaka 565-0871, Japan
            (E-mail:naoko@protein.osaka-u.ac.jp, Tel:81-6-879-8618,
            Fax:81-6-879-8619)
FEATURES             Location/Qualifiers
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4981 atcaatgtac ctagagcccg attccttcct gtgaaggcaa cttctgattt gcttcttgtc
5041 aaggtagcct tcttttcccg ctactagatc ttgaatcttt cacaatgact cgaggcttgt
5101 ctaatgcatt tatcttcttt tgcagtctga cctttataac gtaagagatg gcttcgttat
5161 ccggaacaaa ttgagaagag atccacaaaa tcctgttatt caattggggc ctgaatttaa
5221 gaaggtggat atttaatata gtgatgatct attgcctttt tctttatcgt tcctactacc
5281 tgatgataaa tgtgataatt ttctttccgt ataggttgac gatttcttga gtcgattcaa
5341 gtcaatcccc agcctccttg agtgtgatgg ccttcgggtt gatggtgaca tatggtttgg
5401 ttctggcatt aaactcaagg tacgcactta cagcaaaaat ttcaatcacc ggcttcttgt
5461 ctttcgatgt ttgtgtgaga tattgatatc taaattttgg ttgcttgtcg atggcataaa
5521 ttaaccgcgt cttatctccc ttactacaag actgcaacat cgattattaa ccatcccgtg
5581 ctcatttatc tcttttcatt tgtttcagaa acctttgaaa tcctgttgct gtgtttcgct
5641 gaacacatta tctactattg ttctgtatag tcttactaac gttgtctgcc gttgtttttt
5701 tacttaatgt aggggaacgt acattgctca gtaagttctg aagagatgtt aaacattcct
5761 gataacatca cgcttagaga ataggtaaaa caagaaaatg aatctagacc gtcgtatgtt
5821 acaaaatata ttgatggtca tcgctgtttt atacgtggta attgtgtact ctaactcttg
5881 gtgcaggata tcaatggctc tgaggatctc cacattacgc atcaagagag cgtgctaaag
5941 ttggagtcaa gggcacgatg gttgtgccgt gcttgtttac tccgatggcg aaataaaagc
6001 tttttgaatt tttgctgcac gagtttggta gagtttgcag gttaccatgt taagctctat
6061 ggtttttgaa ggaaggaata aaactttagt tttattttc tattttcatt ctcagttgtt
6121 tatttttcgt aacacagtgt tatgtatcat tgaagagaaa ttgtttttttc ccgtcttgca
6181 caaatttggt aatggaaaag gtgtgtgttt agcccaccgt tcatttttg tttttggta
6241 aacgctcacc attctttaat ccatgtataa atctcgagat ttgagcattg taatatagat
6301 gcacgacaag tttaatattt ttgcacctta cctatattaa taaaataaca gttcctaatc
6361 atacttttgc tgatgtttta agttatgtaa gcctgagtta tgttcactag ggttgataac
6421 tcaaagcgtt acttcaagat cggtgatcac ggctgtttca tataacttcg taaaagagag
6481 agtttgcttg tcaaagtcga acttggagcc ttctatctgt gtgtatgatt gaagacatgc
6541 gtaatggata ctccaagtcc acggtgagtt cgtctcttca accatgtaat taaatctaaa
6601 tgccaagtag tactgagtac atgcttgtca agctgatggg gaaaggccaa gtaaatgcaa
6661 tttctttgtt tgtgtgaatt gtttgttttt tgcttatccc taagaattat atagcggggc
6721 cgtatcattg atttgatctt agaaaaaatt atcattagca attatacaga gttgtagctc
6781 gagtagttaa aagcatttac tccacgttca aaattatgca cgattttcat ttatctcatc
6841 accaatattg ctttggagga aaataaaaac aaaatagcaa ccttagcaaa caaattttgg
6901 agataaaaag atctttcctt acaaaaatac cggttgatat atccaaatga actttcaaaa
6961 cgagtcttaa aacagtcaga aaattaaatt tcttagtctt atgagactag ttttgttcct
7021 agcatgtgtt aatccaagcc agaacatttg agagcaactc taattttttt aacggtggaa
7081 ctgataagag tatttacagt tgccactaac tccatgttca tctgctctga agttgtgggg
7141 ctggaaacca atatgtggaa agccactgta acaagtgaac cacattcatc cccatctca
7201 ggatgaccat cgccggatat caagaacccc gatgggagta tagctatgtt cgatgagtct
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7261 tccccactta ttgctacatt gagagtttgt atgtcaaccg gagcgtatat gataaaggat
7321 ccaagagggt cgatgtggca ctcttgaagc atcaacatgt tgtcctccgt cgggatgaaa
7381 ggctgctcaa tacacaaacc attaattcaa ttaaactaaa ctttgttgaa atgaaaattg
7441 ttaaaataaa catcataaga ctgaaatgtt ttacccgaat gatggatata tggttttcgg
7501 gatgagttcc acttgaaatg taggcaatct catgcactgg gattccatga gagagaacat
7561 cccactgaga gaaaaatcaa attaggaatt agtaaggtca cacttcaacc tttaattatt
7621 gataaatcaa gatataaacc tgaattc
```

PYC007 R

```
dbj|AB027617.1|  Pyrus pyrifolia gene for UDP-glucose pyrophosphorylase, complete
cds
Length=7647

 Score = 42.1 bits (21),  Expect = 0.034
 Identities = 21/21 (100%), Gaps = 0/21 (0%)
 Strand=Plus/Minus

Query  3     TTCATCAAAAGCAAGGGAACA  23
             |||||||||||||||||||||
Sbjct  2058  TTCATCAAAAGCAAGGGAACA  2038
```

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PYC 008

```
gb|AF386510.1|AF386510  Pyrus communis putative FKBP type peptidyl-prolyl cis-
trans isomerase
(DFKBP) gene, complete cds
Length=1468

 Score = 40.1 bits (20),  Expect = 0.068
 Identities = 20/20 (100%), Gaps = 0/20 (0%)
 Strand=Plus/Plus

Query  1   GTGCGATCCAATCCAAGAAG  20
           ||||||||||||||||||||
Sbjct  38  GTGCGATCCAATCCAAGAAG  57
```

Pyrus communis putative FKBP type peptidyl-prolyl cis-trans isomerase (DFKBP) gene, complete cds

GenBank: AF386510.1

FASTA Graphics

Go to:

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LOCUS       AF386510                1468 bp    DNA     linear   PLN 21-JAN-
2002
DEFINITION  Pyrus communis putative FKBP type peptidyl-prolyl cis-trans
            isomerase (DFKBP) gene, complete cds.
ACCESSION   AF386510
VERSION     AF386510.1  GI:18252320
KEYWORDS    .
SOURCE      Pyrus communis (pear)
  ORGANISM  Pyrus communis
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
eudicotyledons;
            rosids; fabids; Rosales; Rosaceae; Amygdaloideae; Maleae; Pyrus.
REFERENCE   1  (bases 1 to 1468)
  AUTHORS   El Sharkawy,I., Li,Z.G., Latche,A. and Lelievre,J.M.
  TITLE     Ripening related genes in pear (Pyrus communis)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1468)
  AUTHORS   El Sharkawy,I., Li,Z.G., Latche,A. and Lelievre,J.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-MAY-2001) Biologie Moleculaire et Physiologie de
la
            Maturation des Fruits, INP - ENSAT, Av. de l'Agrobiopole -
BP107,
            Castanet Tolosan Cedex 31326, France
FEATURES             Location/Qualifiers
ORIGIN
        1 cctaaaaatt gatacatcaa aaggtgtttt aaacggtgtg cgatccaatc caagaagctt
       61 tgtgcttgct catgtgtgtg tgtgtcgcgg ctttagatat gctgtttctc ttttttaagt
      121 tctaccgaaa tgcaatatca cagtattagt tctgttgctt ttcctacaag ttaggttcat
      181 cttctggaaa agggatgtcc gcagattacc aaggcgtttc ctgcatcaaa gttcaaggga
      241 tgcttatccg atgttctaat tcacaagtta ctataaatgc tacaaggtca agaatatgta
      301 aggatcagtt taatgtaatc aaaagaagag atatgattgg gttgcttttc ggagtttcaa
      361 gcgctgtgac aggcgcattt gaagctgagg gagctggtct gcccccagaa gagaagcctc
      421 gactgtgtga taattcttgc gagaaggagg taaaatgtat ggtgaggttg aattgatccc
      481 ttggctgcat tctatatgct atgggagttc agttgttaat tgttcacttc aaaactctag
      541 caggctgtaa aacttggatt ttactgatct ctcgatttat tctcgtaagt ccttgaaaat
      601 aatataaagt taaggaaata atatcttgat taaactctta gctcaaactg gaatgaacga
      661 agttacgttt tagggggaag ttttttttct gtagctcacc caaagaaaaa gttctactta
      721 ggtcttttag aatcagcttt aaatgatatt taaagtggtc ataccgattc ctttcttttg
      781 ggagttcagt gggtgtctgt cagtggcaaa tgccttttgt tgatatgatc tatcaattgt
      841 actggtaaat gatgtttgaa attgtcacta gttattgaag gtgcctatgg taactacaga
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 901 gtctggtttg cagtacaagg atattaaagt tggtcaaggc cccagtccac cagtcggttt
 961 tcaggtagca gcgaattatg tagccatggt tccatctgga caaatatttg acagttcatt
1021 ggagaagggt caagtttata tatttcgtgt tggctctggt caggtaattc caaagcggaa
1081 caactttcgt gggggaaaag gatgtttgct aaatatttgt aattcaaagt agaaaaggtg
1141 atcaagggac ttgatgaagg gatcttgtcc atgaaagtag gagggaagcg acgactctac
1201 atcccaggat cgttggcatt tcccaagggt ctcaattcag ctccgggaag accaagggtg
1261 gctccaagca gtccggttgt tttcgatgtg agcttggaat acgtaccagg ccttgacatt
1321 gaggaagagt aagagaatca gagtcctctc ttcttttatt tattttttct ttgtagttgt
1381 tgatcttttc tgaacacagg agcaacataa aagaatccat aatgtaagca cttgcactga
1441 atatgtcaat taattcaatt ttttcttc
```

PYC 009

```
 gb|AF455809.1|AF455809  Pyrus caucasica sorbitol 6-phosphate dehydrogenase
(S6PDH) gene,
partial cds
Length=1438

 Score = 44.1 bits (22),  Expect = 0.006
 Identities = 22/22 (100%), Gaps = 0/22 (0%)
 Strand=Plus/Plus

Query  1    TACCTGGTTCACTACCCAATGC  22
            ||||||||||||||||||||||
Sbjct  441  TACCTGGTTCACTACCCAATGC  462
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Pyrus caucasica sorbitol 6-phosphate dehydrogenase (S6PDH) gene, partial cds

GenBank: AF455809.1

FASTA Graphics

Go to:

```
LOCUS       AF455809                1438 bp    DNA     linear   PLN 27-DEC-
2001
DEFINITION  Pyrus caucasica sorbitol 6-phosphate dehydrogenase (S6PDH) gene,
            partial cds.
ACCESSION   AF455809
VERSION     AF455809.1  GI:17981606
KEYWORDS    .
SOURCE      Pyrus communis subsp. caucasica
  ORGANISM  Pyrus communis subsp. caucasica
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core
eudicotyledons;
            rosids; fabids; Rosales; Rosaceae; Amygdaloideae; Maleae; Pyrus.
REFERENCE   1  (bases 1 to 1438)
  AUTHORS   Bortiri,E., Oh,S.-H., Gao,F.-Y. and Potter,D.
  TITLE     Maximum likelihood and parsimony analyses of Prunus
```

```
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1438)
  AUTHORS   Bortiri,E., Oh,S.-H., Gao,F.-Y. and Potter,D.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-DEC-2001) Pomology, University of California-
Davis,
            One Shields Ave, Davis, CA 95616, USA
ORIGIN
        1 agctcattac aagagtgaag cagacgttgg ggaagcattt gcagaagctt ttaagactgg
       61 acttattaag agggaagaac ttttcattac caccaaggtc agttaattta tttacccttt
      121 ttcttttttt ttttgctact tttataaaat aatattggtg tgacaaattt aggctacgtc
      181 acaaaacgga cccgtgacaa ccaagtatta agtcgtcatt tataagagtc aaatttaaga
      241 ctcgtactta cgaatgaaca aaaatatatc aagactacaa tgcaaacagc gaaatattag
      301 agtttatctc atattttccg aagagactaa agtataattt tgttgttctt atattacaga
      361 tttggaattc agaccatggg catgtggtgg aggcctgtaa gaacagcctc gagaagcttc
      421 agttagatta tctggatctc tacctggttc actacccaat gcccacaagg cacaatggta
      481 cctacccctc tctctctctc tctctctctg ggggtagatt attggtcact caacgctgta
      541 acgttacata attataaata ggtaatttac aatgaattgg atttattttg cttgctgatg
      601 gtcacagcaa ttggtaaaac tgccagtctt ttgggcgagg ataaggtgtt ggacatcgat
      661 gtaacaattt cccttcaaca aacctgggag ggcatggaaa agaccgtcac tttgggctta
      721 gttcgtagca ttggtctcag gtacatccca tgagcattta tgctagactt tcatctttgt
      781 atccaaactc gattatctag aattcaccag tgaatgtcac taaccaaatg atcatcggct
      841 tccgttttgc agcaactacg acctctttct aactagagat tgcttggctt actccaaaat
      901 aaagcctgct gtgagccaat ttgaaaccca cccctatttc cagcgcgact ctctcgtcaa
      961 attctgcatc aaacacggcg ttcttcccac ggctcacacc cctctcggag gtgctgctgc
     1021 caacaaggat atgtttggtt ctgtttcacc tttggatgat ccaattctca atgtaagtct
     1081 tctctctttt agcgcgcaca ctgtatgaaa tacaactgga tcatagcgct agtaaaaata
     1141 caaccatact aatgattcac aaacgtatgc tcactcgagc gtttcatttt caggatgtgg
     1201 ctaagaaata cggaaagagc gtggcacaaa tctgtctgag gtggggaatt cagaggaaaa
     1261 cagcagtgat tccaaaatca tcgaaaattc agcgattgaa agagaatttg gtggttcttg
     1321 aattccagct gagcgatgaa gacatgcagc tcatctacag tatcgacagg aagtatcgta
     1381 ccagtctacc ttccaagact tggggcctta gacgtgtatg caagggcgaa ttcgttta
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