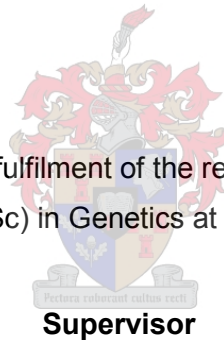


**MOLECULAR FINGERPRINTING AND MOLECULAR CHARACTERIZATION OF ARC'S
PEACH COLLECTION IN SOUTH AFRICA**

By

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Thesis presented in partial fulfilment of the requirements for the degree of
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Declaration

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Abstract

Peaches and nectarines are important deciduous fruits in South Africa, both belonging to the species *Prunus persica*. The Agricultural Research Council (ARC) at Infruitec-Nietvoorbij in the Western Cape is the primary source of peach cultivars in South Africa. The germplasm from which these cultivars are developed is maintained at Bien Donne Research Farm (Paarl, Western Cape) and includes the reference collection for the Department of Agriculture, Forestry and Fisheries (DAFF). The germplasm collection has only been phenotyped morphologically and could be prone to errors and duplications. This study had two aims; firstly it aimed to utilize molecular marker technology (*i.e.* microsatellites markers) to fingerprint the germplasm collection to facilitate authentication. Secondly, the study aimed at employing functional markers for two agronomic traits of economic interest *i.e.* the peach/nectarine trait (hairy fruit epidermis) and white/yellow flesh colour.

Nine reported polymorphic microsatellite markers were selected for the fingerprinting of 206 peach accessions, 20 almond accessions and seven hybrid accessions. One marker amplified multiple loci in both peaches and almonds while another marker did not amplify in either the almonds or the hybrids, and these were excluded. Therefore, the ARC peach accessions were successfully fingerprinted with eight microsatellite markers, and the almonds and hybrids with seven. Clustering analysis found fifty-eight accessions, including eighteen accession from the reference collection, were either misidentified or unresolved needing further molecular and morphological analysis. The accessions belonging to the reference collection are maintained by DAFF and were considered authentic prior to this study.

The germplasm was characterized for the peach/nectarine trait (hairy fruit epidermis) as controlled by the *MYB25* gene. It has been reported that a retrotransposon insertion in the third exon of the *MYB25* gene disrupts formation of epidermal hairs in nectarine. The marker indelG was developed and fluorescently labelled and used to detect the presence of the retrotransposon insertion (*g* allele) or its absence (*G* allele). Peaches were observed to have at least one *G* allele while nectarines were homozygous for the *g* allele. Seventy-five accessions were genotyped as homozygous *gg* (nectarine), 35 accessions were heterozygous *G/g* (peach) and 96 were homozygous *GG* (peach). The heterozygous peaches can be intercrossed to develop new nectarine cultivars from peaches. The *G* allele, indicative of hairy fruit epidermis, was found in the almonds and some hybrids. Follow up studies for the role of the *MYB25* gene in other *Prunus* species, especially in apricot (hairy), plum (glabrous) and cherry (glabrous), are recommended. The primers used in this study can be multiplexed with other primers and used for characterizing large number of samples at a relatively lower cost.

The germplasm collection was also genotyped for the *CCD4* gene that control the expression of white or yellow flesh colour. White flesh is the wildtype while yellow flesh results from loss of gene function through any of three mutations: a frameshift mutation at the TC microsatellite region, an A to T substitution (SNP) or a retrotransposon insertion. Three novel primer sets including fluorescently labelled primer pairs were designed to detect these mutations. The primer pair amplifying the TC microsatellite region (CCD4-SSR) in the *CCD4* gene identified the wild type allele, a frameshift mutant and a very rare reversion allele in the accessions. Overall, 25 accessions had the 122/122 bp genotype associated with white flesh, 138 accessions had the 124/124 bp genotype associated with yellow flesh colour, 42 accessions had the 122/124 bp genotype associated with the white flesh and one accession had the 124/128 bp genotype containing a reversion mutation associated with white flesh. The primer set amplifying the presence of the SNP (CCD-SNP) and its absence (CCD4-NoSNP) detected this SNP in 26 accessions, two of which were shown to be homozygous for the SNP mutation. The primer sets detecting the presence or absence of the retrotransposon (CCD4-Retro and CCD4-NoRetro) were not informative and the accessions could not be genotyped for this mutation. Therefore, the characterization of the flesh colour was incomplete and the deduced flesh colour are mostly tentative: with 33 accessions deduced as white flesh, 172 accessions as yellow flesh and 18 accessions as inconsistent and needing further follow up. Nevertheless, the partial genotypes and deduced phenotypes are useful and informative when designing of crosses in regard to flesh colour. The primers detecting the retrotransposon should be redesigned and used to complete flesh colour genotyping.

Overall, the microsatellite fingerprinting gave baseline data useful for future repropagation while molecular characterization for peach/nectarine and flesh colour will aid in the design of crosses with predictable outcomes. This study, therefore, lays a solid foundation for future molecular characterization and utilization in the ARC peach breeding programme.

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List of symbols and abbreviations

%	Percentage
°C	Degrees Celsius
<	Less than
>	Greater than
μM	Micromolar
3'	Three prime
5'	Five prime
A	Adenine
AFLP	Amplified Fragment Length Polymorphism
ARC	Agricultural Research Council
BC	Before Christ
Bp	Base pair
C	Cytosine
Cm	Centimetre
CTAB	Cetyltrimethylammonium Bromide
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic Acid
DUS	Distinctness, Uniformity and Stability
EDTA	Ethylene Diamine Tetra-acetate
G	Grams
G	Guanine
He	Expected heterozygosity
Ho	Observed heterozygosity
HWE	Hardy-Weinberg Equilibrium
Hz	Hertz
I	Shannon's information index
Kb	Kilobases
m/v	Mass per volume
MAS	Marker-Assisted Selection
Mbp	Mega base pairs
MgCl₂	Magnesium Chloride
Min	Minutes
ml	Millilitre
Mm	Millimetre
mM	Millimolar
Na	Number of alleles
NaOH	Sodium Hydroxide
Ng	Nanogram
pH	Concentration of Hydrogen ions in a solution
PIC	Polymorphic Information Content
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
T	Thymine
Taq	<i>Thermus aquaticus</i> DNA polymerase
TE	Tris-Ethylenediamine
Tm	Melting temperature
Tris-HCL	Tris-aminomethane Hydrochloric acid

UPGMA	Unweighted Pair Group Method with Arithmetic Average
UPOV	International Union for the Protection of New Varieties of Plants
V	Voltage
v/v	Volume per volume
Xg	Gravity
ZAR	South African Rand

CHAPTER 1: INTRODUCTION

1.1. BACKGROUND

Peaches and nectarines (*Prunus persica*) along with other members of the genus *Prunus* belong to the subfamily Prunoideae of the Rosaceae family (Bassi and Monet, 2008). Rosaceae is an important family that includes other fruit crops such as apple, strawberry, pear and raspberry. Peach, along with its close relative almond (*Prunus dulcis*), belongs to subgenus *Amygdalus*. The species of the genus *Prunus* are collectively and commonly referred to as “stone fruits” because the fruits, edible except in the case of almonds, have a large and hard endocarp containing the seed. Peaches rank second only to apples in terms of total consumption worldwide (USDA, 2012). Peach and nectarine are important deciduous fruit crops in South Africa (Hortgro, 2014), ranking fifth in terms of export value behind grapes, apples, pears and plums. South Africa itself is ranked as the seventh most important peach exporter in the world supplying approximately 2% of global peach exports. The peach industry in South Africa is worth ZAR 800 million annually and employs 10,000 people.

The peach industry in South Africa has benefited immensely from its cooperation with the Agricultural Research Council (ARC) and its peach breeding programme at Infruitec-Nietvoorbij. Cultivars from the ARC peach breeding programme are the foundation of the success of the peach industry in South Africa (Hortgro, 2014). The peach breeding programme at ARC, though relatively successful, faces a number of challenges regarding its germplasm collection. One challenge is that the germplasm has been primarily described using morphological traits. The use of such an approach may introduce errors in the germplasm due to the subjective nature of scoring. The recent advances of the marker technology there is an opportunity to fingerprint the accessions. An additional challenge concerns characterization with respect to various agronomic traits of economic interest. Though the phenotypes of each accession has been documented e.g. flesh colour or peach/nectarine trait, the genotypes cannot necessarily be deduced. This in turn makes the designing of the crosses challenging. The use of functional markers should solve this challenge and allow the breeder design appropriate crosses based on the genotypes of the accessions.

A set of nine commonly used genome-wide and polymorphic microsatellite markers were identified to generate fingerprints of the accessions in the peach collection (Cipriani *et al.*, 1999; Testolin *et al.*, 2000; Aranzana *et al.*, 2002b; Dirlewanger *et al.*, 2002). The fingerprints will provide the breeder with authenticated material for crosses and other aspects of the breeding programme. In addition, primers for certain agronomic traits were designed. These include fruit epidermis (peach vs. nectarine trait) and flesh colour (white vs. yellow). The hairiness of the fruit epidermis, which distinguishes peach from nectarine, is controlled by the gene for the transcription factor MYB25 (Vendramin *et al.*, 2014). Peach is dominant while

nectarine is recessive with the disruption in the third exon of the *MYB25* gene by a retrotransposon insertion. The white and yellow flesh colour in peach is controlled by a single gene for carotenoid cleavage deoxygenase (*CCD4*). The white flesh is the wild type and dominant, and yellow flesh is recessive and results from one or more of the three mutations in the *CCD4* gene (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013). Characterization of these traits will elucidate the genotypes of the accessions with regards to these traits and allow the breeders to design specific crosses. Thus, marker-assisted selection of parents, and subsequently of seedlings, can be implemented in the breeding programme.

1.2. AIMS AND OBJECTIVES

The first objective of the current study was to fingerprint the peach genetic resources in the ARC breeding programme with a set of commonly used polymorphic microsatellite markers. The second objective was to characterize the accessions for two functional agronomic traits (white/yellow flesh colour and the peach/nectarine trait).

The literature review on various aspects of this project is covered in Chapter 2. The fingerprinting with microsatellites is detailed in Chapter 3. The characterization of the functional genes, *MYB25* and *CCD4* are detailed in chapters 4 and 5, respectively. Final conclusions are presented in Chapter 6.

1.3. PROJECT FUNDING

This project was undertaken at the ARC Infruitec-Nietvoorbij and Stellenbosch University. The research was funded jointly by THRIP (The Technology and Human Resources for Industry Programme) and Hortgro Science on behalf of SASPA (South African Stone Fruit Producers' Association). A parliamentary grant was also allocated for the fingerprinting and molecular characterization of the germplasm at the ARC.

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CHAPTER 2: LITERATURE REVIEW

2.1. PEACHES

2.1.1. Peach botany and horticulture

Peach (*Prunus persica*) and its glabrous variant, nectarine, belong to the Prunoideae subfamily of the Rosaceae family (Bassi and Monet, 2008). The Rosaceae is a horticulturally important family with fruits such as apple and pear in the subfamily Maloideae and strawberry and raspberry in the Rosoideae. *Prunus* species, which also include almond (*P. dulcis*), apricot (*P. armeniaca*) and plum (*P. salicina*), are termed “stone fruits” because the fruits have a large and hard endocarp containing the seed.

The peach is a temperate deciduous fruit tree, which in the wild may grow to a height of about eight metres (Hesse, 1975; Bassi and Monet, 2008). The leaves are lanceolate and glabrous with serrate margins. The petiole is either eglandular or has glands that are either globose or reniform in shape. The flowers are generally pink, but white and red also occur, and may be showy or non-showy. The peach fruit itself is a typical drupe with an exocarp (skin), mesocarp (flesh) and endocarp (stone) enclosing the seed. The fruit is pubescent (peach) or glabrous (nectarine), beaked or round and freestone or clingstone. The mesocarp is white or yellow and may be more or less red around the pit. The flesh can soften drastically during ripening (melting) or remain relatively firm (non-melting) and the endocarp is deeply pitted, furrowed and very hard. The seed inside the endocarp is cotyledonous, and either sweet or bitter in taste (Bassi and Monet, 2008).

Although many *Prunus* species, including almonds, have a gametophytic incompatibility system, peach trees are self-compatible (Hesse, 1975; Bassi and Monet, 2008). Commercial peaches are commonly propagated clonally to preserve integrity of the cultivars but ‘landraces’ and some rootstocks are usually propagated by seed. Some rootstocks are also propagated by cuttings. The clonal propagation of scions usually involves grafting or budding onto rootstocks that are resistant to biotic or abiotic stresses.

Important centres of commercial peach production are found between latitudes 30° and 45° in both hemispheres (Scorza and Sherman, 1996; Janick and Paul, 2008). These regions have sufficient chilling hours (500-1,000) for peach to leaf out and blossom, and warm summers for ripening. However, some peach cultivars can also thrive in certain regions of the tropics and sub-tropics (Byrne *et al.*, 2000).

2.1.2. Peach history and distribution

Peach is reported as originating from China (De Candolle, 1885; Hedrick, 1917; Vavilov, 1951; Wang and Zhuang, 2001). It is generally considered indigenous to North West China, between the Tarim Basin and the northern slopes of the Kunlun Shan Mountains. However, another study (Zheng *et al.*, 2014) presented archaeological evidence that pointed to Eastern China along the Yangzi valley as the actual origin and centre of domestication. Cultivated since 1,000 BC, hundreds of peach cultivars have been documented (Huang *et al.*, 2008; Layne and Bassi, 2008). China is also a centre of origin of species considered ancestral to the modern peaches: the Tibetan and Gansu peach (*P. kansuensis* Rehd), the Mountain peach [*P. davidiana* (Carr) Franch], the Tibetan peach (*P. mira* Koehne), the Chinese wild peach (*P. consociiflora* Schneid) and *P. ferganensis* (Kost and Riab). Moreover, other variants *i.e.* doughnut peach (*P. persica* var. *platycarpa*) also originate from China.

The main routes of peach distribution from China to the West were across the Indian Ocean and the Silk Route through Persia, now Iran (Janick, 2003; Rieger, 2006; Janick and Paul, 2008; Bassi and Monet, 2008). Alexander the Great found peaches in Persia and introduced them to the Greeks (Hedrick, 1917) who, by 322 BC, had introduced the peach to the Romans who called the peach a 'persica', a Persian apple (erroneously describing the fruit as indigenous to Persia). The Romans introduced peach to the western parts of the empire including Spain, Italy and France. Spanish explorers are credited with bringing the peach to South America while French explorers brought the peach to the United States of America (USA).

Peaches were introduced to South Africa around 1665 by Jan van Riebeeck of the Dutch East India Company who also introduced other perennial crops and fruit trees to the Cape Colony (Pickstone, 1917; Aucamp, 1987). Peaches thrived in the Cape, so much so that many orchards were established and, by 1892, with the development of refrigerated shipping, peaches were being exported (Pickstone, 1917; Aucamp, 1987). The French Huguenots (a protestant sect) introduced good agricultural practices, which also aided the success of peaches in the Cape.

2.1.3. Global peach production and exports

The annual global peach output is estimated at around 19.4 million tons (USDA, 2012). The largest producers of commercial peaches worldwide are China, Italy, USA, Greece and Spain (FAO, 2012). China is by far the leading peach producer worldwide, contributing as much as half of the total world peach production. The European Union (EU) as a block is the major

exporter of peach worldwide, followed by USA, Chile and China. In Africa, significant producers are Egypt, Algeria and Tunisia with South Africa being the major exporter.

2.1.4. Peach production and export in South Africa

The South African stone fruit industry primarily focuses on plums, peaches and apricots. Plums are the highest in terms of export volumes followed by peaches (DAFF, 2015). The South African peach production is ranked 15th in the world (FAO, 2012). In terms of exports, South Africa is ranked 7th and supplies just 2% of the peaches worldwide (USDA, 2014). In Africa, the South African peach industry is the highest exporter on the continent despite being third (behind Egypt and Algeria) in terms of production.

The major regions of peach production in South Africa lie in the Western Cape. Other, smaller, production areas are in the Eastern Cape, Free State, Northern Province and Mpumalanga (DAFF, 2014). The Western Cape has a suitable Mediterranean climate characterized by hot-dry summers and cool-wet winters and has sheltered valleys between the mountainous regions. The South African peach industry is dominated by clingstone peaches (grown mainly for processing) with 5,690 hectares grown in the areas of Ceres, the Hex Valley, Klein Karoo, Langkloof East, Mpumalanga, Piketberg, Villiersdorp/Vyeboom, Wolseley/Tulbagh and Worcester (Hortgro Tree Census, 2014). Despite its predominance, only a limited number of clingstone cultivars are grown including 'Bonnigold', 'Cascade', 'Goudmyn', 'Kakamas', 'Keisie', 'Oom Sarel', 'Prof Malherbe', 'Prof Neethling', 'Sandvliet', 'Supreme', 'Western Sun' and 'Woltemade'. Freestone peaches are planted on a smaller scale (1,752 hectares) in Ceres, the Free State, Klein Karoo, Mpumalanga, Paarl, Piketberg and Wolseley/Tulbagh (Hortgro Tree Census, 2014). The cultivars include; 'Cederberg', 'Excellence', 'Fairtime', 'Nova Donna', 'San Pedro', 'Summer Sun', 'Sun Sweet', 'Temptation' and 'Witzenberg'.

The peach industry is valued at ZAR 800 million (Hortgro, 2014) and provides 10,000 jobs that in turn support approximately 42,000 people. The major importers of South African nectarines are the United Kingdom (UK), EU and the Middle East while for peaches the Middle East leads the UK and EU (DAFF, 2015). The export figures show that since the 2009/10 season, nectarine exports increased and peaked in 2011/12 and have since been in a decline while peach exports have been steadily climbing (DAFF, 2015).

The Agricultural Research Council's (ARC) breeding programme at Infruitec-Nietvoorbij is the primary source of peach cultivars for the South African peach industry (Hortgro, 2014). For instance, in the 2013/2014 season, 23% of the peach and 24% of the nectarine fresh exports and 100% of canning peaches were from cultivars developed by the ARC.

2.1.5. Nutritional value, consumption and uses

Peaches are wholesome and nutritious fruits (Rieger, 2006; Nutrition Data, 2007) with carbohydrates, fats, proteins, vitamins and minerals. All peaches contain vitamin B₃ (niacin) and vitamin C (ascorbic acid). Yellow fleshed peaches also have vitamin A (retinol) due to the presence of its precursors *i.e.* β -carotene and β -cryptoxanthin in the yellow mesocarp. Peaches are also rich in mineral elements such as potassium, copper, and manganese.

Peaches are usually consumed fresh, canned, dried or processed. The fruit can be turned into jams, juice, pulp for yoghurt, liquors and other products. Consumption preferences differ by region (Byrne *et al.*, 2012). Historically, Chinese and Asiatic consumers have long preferred white fleshed peaches, which are usually very sweet and have low acidity, while European and North American consumers favour yellow fleshed peaches, which are usually more acidic (Scorza *et al.*, 1985). However, more recently, with the development of many improved white and yellow flesh peach cultivars, preferences are not as distinct. Freestone peaches are preferred for fresh consumption and drying since they usually have the melting trait and removal of the stone is easy (Rieger, 2008). On the other hand, clingstones peaches (with the non-melting trait) are preferred for canning since they stay firm during processing and have good storing quality.

In some regions, the seeds are used to raise rootstocks while the endocarps can be used as a raw material for making charcoal or for surfacing paths (Yulin, 2002; Hu *et al.*, 2006). Moreover, in the Far East *i.e.* China and Japan, peaches have a significant spiritual and cultural value apart from the aesthetics of the peach blossoms.

2.1.6. Peach genetic resources

Genetic resources, popularly termed gene banks, are the raw material for traits of interest for crop improvement (CBD, 1993). These genetic resources provide breeders and geneticists with a wide genetic pool from which different traits can be introduced in the breeding programme. Peach genetic resources typically consist of collections of old cultivars, popular cultivars, sports (mutants) of cultivars and related wild species. Almond (*P. dulcis*), for instance, is a closely related species that can be hybridized with peach and/or used as a rootstock for peach scion cultivars. As with other fruit crops, peach gene banks are maintained as trees rather than seeds.

2.1.6.1. Genetic resources at the ARC

The ARC Bien Donne Research Farm (Paarl, Western Cape) has a collection of approximately 400 accessions (Pieterse, personal comm.). These genetic resources consist of: the national reference collection of peaches and almonds [belonging to the Department of Agriculture, Forestry and Fisheries (DAFF)], a rootstock collection (consisting of peaches, related *Prunus* species and hybrids) and a peach gene bank for the scion breeding programme.

2.1.7. Peach breeding programmes

There has been many documented peach breeding programmes developing peach cultivars all over the world (Fideghelli *et al.*, 2003). Okie (1998) described 700 peach and nectarine cultivars and the Brooks and Olmo Register of Fruit and Nut Varieties (ASHS, 1997) lists about 300 nectarine and 1,000 peach cultivars in North America alone. Since the 1990s, breeders globally released around 100 peach and nectarine cultivars per year (Della Strada *et al.*, 1996; Fideghelli *et al.*, 1998; Sansavini *et al.*, 2006). About 50% of all new peach and nectarine cultivars are developed in the USA and Europe with France and Italy producing about 30% of all cultivars (Fideghelli *et al.*, 2003). Other significant breeding programmes are in South Africa, Australia, China, Japan, Mexico and Brazil.

Breeding programmes have undergone major changes since the 1990s (Byrne *et al.*, 2005). The most significant change is the decrease in public funding for breeding programmes and increase in private breeding programmes. Other notable changes include: an emphasis on tree architecture to maximize fruit yield, concern about chemical use in orchards, attempts to expand peach growing areas to non-traditional areas, increased interest in the health benefits of fruits, the demand for better quality fruits and the need to improve post-harvest traits.

2.1.7.1. The ARC peach breeding programme

As mentioned earlier, the ARC peach breeding programme is the main source of the commercial peach cultivars in South Africa. The peach breeding programme started in 1937. It was established in a few rooms of the Stellenbosch-Elsenburg College of Agriculture of the University of Stellenbosch and was referred to as the Western Province Research Station (WPRS) (Olivier, 1960). The research station had three main permanent researchers, Dr. du Toit, Dr. Reynecke and Dr. Reinecke, and an East Malling consultant, Dr. Ronald Hatton (Kotze, 1987). An experimental farm, Bien Donne near Paarl, was purchased from the Rhodes Company for field experiments (Olivier, 1960). The research station later moved permanently from the University to the complex at the Reuben Nel Building (the Infruitec building) where it

is currently based. The WPRS worked closely with researchers from the University of Stellenbosch.

The WPRS established peaches and nectarines as one of the top priority fruits for research (Steyn, 1955). The breeding programme's main objective was to develop new cultivars that would replace poorly adapted and low quality imported peach cultivars (Wenzel *et al.*, 1975). The actual peach breeding programme was built on prior peach breeding work by H. Reinecke, who in 1932 identified and registered three cultivars: 'Maluti', 'Kakamas' and 'Early Dawn' (Wenzel *et al.*, 1975; ASHS, 1997). The breeding programme was divided into two groups: one focused on development of dessert cultivars while the other programme focused on canning cultivars; but both aimed at local adaptation (Steyn, 1955). The next generation of cultivars were superior selections as observed through field survey and testing (Black, 1952; Steyn, 1955). Open pollination of these cultivars with each other and crossing with foreign cultivars such as 'Goosen' led to new cultivars being developed. In the late 1950s, most of the pollination was controlled and done carefully by hand, and advanced techniques such as embryo rescue were already being practised (Pieterse, 2013). Currently the breeding programme is still based at Bien Donne and is divided into a scion cultivar breeding programme led by Mr. Werner Pieterse and a rootstock breeding programme led by Mr. Sonwabo Booï.

The breeding programme, though largely successful, has faced a number of challenges over the years (Pieterse, personal comm.). Concerning germplasm there are limited genetic resources, and a relatively small genetic pool from which new cultivars can be developed. Moreover, the germplasm collection in the breeding programmes have not been fingerprinted for authenticity or characterized with regards to most agronomical genes. The inadequacy of genetic resources can be addressed through obtaining novel cultivars, landraces, related wild species and hybrids from international breeding programmes; although the importation of scion wood is subject to very strict phytosanitary regulations. Regarding authentication, molecular markers such as microsatellite markers are readily available and relatively affordable. In terms of characterization, functional markers, tailored for specific agronomical traits are also available. Traits of interest, which can be characterized, include white/yellow flesh colour (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013) and the peach/nectarine trait (Vendramin *et al.*, 2014).

2.2. PEACH GENETICS

Peach is one of the most genetically well-characterized species in the Rosaceae (Bassi and Monet, 2008; Arus *et al.*, 2012). It is a model species for genomic studies of Rosaceae in

general and *Prunus* in particular. Peach is diploid with $2x=2n=16$ (Jelenkovic and Harrington, 1972) and a small genome size (220-230 Mbp) that has been widely mapped (Baird *et al.*, 1994; Meinke *et al.*, 1998; Quarta *et al.*, 1998; Zhebentyayeva *et al.*, 2008) and recently sequenced (Verde *et al.*, 2013). Peach and other *Prunus* genome information have been assembled into an accessible database along with other Rosaceae species online (www.rosaceae.org).

Peaches are self-compatible which permits inbreeding and reduces genetic diversity (Miller *et al.*, 1989). Moreover, the most common commercial cultivars have been developed from a limited collection of peach cultivars e.g. 'Chinese Cling', 'Belle of Georgia', 'J.H Hale' and 'Elberta' (Scorza *et al.*, 1985) resulting in a very narrow genetic base (Hesse, 1975; Scorza and Okie, 1990; Faust and Timon, 1995). In most American and European cultivars the nectarine trait originates from three main sources (Vendramin *et al.*, 2014): 'Quetta', discovered near the Quetta City in Pakistan in 1906, 'Goldmine' discovered in New Zealand in 1900 and 'Lippiatt' discovered in New Zealand in 1906.

2.3. MICROSATELLITE FINGERPRINTING

2.3.1. Microsatellites

The term 'microsatellite' was first used by Litt and Luty (1989) to describe a series of repeats in the genome that are one to six nucleotides long (Gupta *et al.*, 1996; Thiel *et al.*, 2003). Microsatellites are also known as Simple Sequence Repeats (SSRs) (Jacob *et al.*, 1991). The microsatellite repeats originate from errors during DNA replication, repair and recombination (Levinson and Gutman, 1987; Schlotterer and Tautz, 1992). Microsatellites are ubiquitous in the non-coding regions of the genome though they occur in coding regions as well (Tautz and Renz, 1984; Gupta *et al.*, 1996; Toth *et al.*, 2000).

2.3.2. Microsatellites as molecular markers

The widespread presence of microsatellites in the genome, their high level of polymorphism, codominant Mendelian inheritance, cross transferability and easy detection by PCR and electrophoresis methods makes these markers informative for various plant genetic studies (Morgante and Olivieri, 1993). Due to their codominant nature, (both alleles are detectable in a heterozygote), SSR markers are more informative in fingerprinting and parentage determination than other markers such as Random Amplified Polymorphic DNA (RAPDs) and Amplified Fragment Length Polymorphism (AFLPs) (He *et al.*, 2003; Lee *et al.*, 2004). Also, as SSRs are PCR-based, only small quantities of template DNA are needed (Kumar *et al.*, 2009; Wolko *et al.*, 2010). Microsatellite markers have flanking regions that are often highly

conserved in related species, which enables their cross species transferability (Huang *et al.*, 1998; Cipriani *et al.*, 1999; Sosinski *et al.*, 2000). Cross-specific amplification of microsatellite primers has been shown with *Prunus* and many other fruit and nut genera e.g. *Castanea*, *Juglans* and *Vitis* (Dirlewanger *et al.*, 2002). Furthermore, microsatellite markers can be fluorescently labelled and multiplexed with other markers resulting in cost reduction.

The development of microsatellite markers was initially an expensive, laborious and time consuming task (Zane *et al.*, 2002; Squirrell *et al.*, 2003; Thiel *et al.*, 2003); however, the availability of large collections of expressed sequence tags (EST) and genomic DNA from many species has made microsatellite mining easier. The gradual drop of sequencing costs has also made the use of microsatellites relatively affordable (Morgante *et al.*, 2002; Horn *et al.*, 2005; Luro *et al.*, 2008) so they are preferred for various genetic studies (Plaschke *et al.*, 1995; Rongwen *et al.*, 1995; Guilford *et al.*, 1997; Giovannini *et al.*, 2012).

2.3.3. Microsatellite markers in Rosaceae and *Prunus*

The first microsatellite markers developed in the family Rosaceae were in peaches (Cipriani *et al.*, 1999). Subsequently, numerous microsatellite markers have been developed in other members of Rosaceae e.g. apple (Guilford *et al.*, 1997; Hokanson *et al.*, 1998), black cherry (Downey and Lezzoni, 2000), almond (Testolin *et al.*, 2004; Mnejja *et al.*, 2005), apricot (Hagen *et al.*, 2004; Messina *et al.*, 2004), Japanese plum (Mnejja *et al.*, 2004) and cherry (Clarke and Tobutt, 2003; Vaughan and Russell, 2004).

2.3.4. Microsatellite fingerprinting of peach

Since the initial 17 microsatellite markers in *Prunus* were developed in peach (Cipriani *et al.*, 1999), many more microsatellite markers have been developed: 10 by Sosinski *et al.* (2000); 26 by Testolin *et al.* (2000); 35 by Aranzana *et al.* (2002b); 41 by Dirlewanger *et al.* (2002); 36 by Yamamoto *et al.* (2002); and 26 by Howad *et al.* (2005).

The first study to fingerprint peaches with microsatellite markers was that of Cipriani *et al.* (1999) who analysed 10 peach cultivars with 17 markers. This was followed by the fingerprinting of 50 cultivars with 26 markers by Testolin *et al.* (2000). These and other notable peach fingerprinting studies have been tabulated (Table 2.1).

Table 2.1. A summary of significant studies that fingerprinted peaches using microsatellite markers.

Study	Number of SSR markers	Number of samples
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Sosinski <i>et al.</i> (2000)	16	28
Dirlewanger <i>et al.</i> (2002)	36	27
Aranzana <i>et al.</i> (2003)	16	212
Marchese <i>et al.</i> (2005)	15	49
Rojas <i>et al.</i> (2008)	9	117
Giovannini <i>et al.</i> (2012)	16	26

Some laboratories have attempted to set up a standard panel of microsatellite markers for fingerprinting (Aranzana *et al.*, 2003; Rojas *et al.*, 2008; Wünsch, 2009); however, these panels have not been widely adopted.

Peach microsatellites primers may amplify microsatellites in related species including almond (Dirlewanger *et al.*, 2002; Ruthner *et al.*, 2006; Shiran *et al.*, 2007; Wünsch, 2009). Conversely numerous microsatellite markers have been developed in other *Prunus* species which can be used in peach e.g. black cherry (Downey and Lezzoni, 2000), almond (Testolin *et al.*, 2004; Mnejja *et al.*, 2005), apricot (Messina *et al.*, 2004; Hagen *et al.*, 2004), Japanese plum (Mnejja *et al.*, 2004) and cherry (Clarke and Tobutt, 2003; Vaughan and Russell, 2004).

2.4. AGRONOMIC TRAITS IN PEACH

2.4.1. Simple traits

First discovered by Gregor Mendel, simple traits are those controlled by a single gene (Bateson, 1902). These traits show discontinuous variation, and the gene has a dominant and a recessive allele. In heterozygotes, the dominant allele masks the expression of the recessive allele. Recessive alleles are only expressed when homozygous. Mutations in simple genes can introduce new phenotypes.

There are many simple traits in peach. With regards to the peach fruit, some simple traits include: white/yellow flesh (Connors, 1920), melting/non-melting texture (Bailey and French, 1949), peach/nectarine epidermis (Blake, 1932), freestone/clingstone type (Bailey and French, 1949), stony hard flesh (Yoshida, 1970), low acid (Monet, 1979) and sweet kernel (Werner and Cleller, 1997).

2.4.2. Molecular characterization of simple agronomical traits in peach fruit

Since a simple trait is controlled by a single gene, it is straightforward to develop functional markers that can characterize the alleles at the particular locus when the sequence basis for the variation is identified. Some simple traits of interest in peach that have been characterized

are the peach/nectarine trait (Vendramin *et al.*, 2014) and white and yellow flesh colour (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013).

The characterizing of these traits in peach accessions is important as knowledge of the genotypes of the accessions facilitates the designing of particular crosses to achieve specific breeding objectives.

2.5. MOLECULAR CHARACTERIZATION OF THE PEACH/NECTARINE TRAIT IN PEACH

2.5.1. Introduction to peach/nectarine trait

Peach and nectarine are two forms of peaches. The main difference is the presence of trichomes on the fruit epidermis of peach, which is “fuzzy”, which are absent from the nectarine, which is smooth (Blake, 1932). The trichomes are hair-like appendages that derive from differentiation of epidermal cells (Uphof, 1962). They play an important role in protecting plants against biotic and abiotic stresses.

2.5.2. Genetics of peach/nectarine trait

Early geneticists considered nectarine a recessive trait to peach (Bateson *et al.*, 1902). This view was confirmed by observations of some of the early peach breeders (Rivers, 1907). However, other breeders still suggested that nectarine was dominant to the peach (Burbank, 1920). Subsequent work concurred with the former conclusion and the locus *G* controlling this trait was proposed (Blake, 1932). The *G* locus has since been mapped in the distal part of linkage group 5 (Dirlewanger *et al.*, 2004; Le Dantec *et al.*, 2010; Cao *et al.*, 2016).

Trichome formation studies in *Arabidopsis* were the first to characterize some genes of interest; a member of the family of the *MYB* transcription factors was reported to control the expression of the trichomes (Opperheimer *et al.*, 1991; Wada *et al.*, 1997). In cotton, Machado *et al.* (2009) also identified a member of the *MYB* family of transcription factors as controlling trichome formation. Vendramin *et al.* (2014) identified *MYB25* as controlling this trait in peaches. In nectarine, the sequence is disrupted by an insertion, a 7 kb Ty1-copia retrotransposon, in the third exon (Fig. 2.1). This insertion is absent in the *MYB25* gene of peach in which trichome formation is not disrupted. Moreover, the *MYB25* gene in peaches is an ortholog of the relevant *MYB* gene in *Arabidopsis* and cotton.

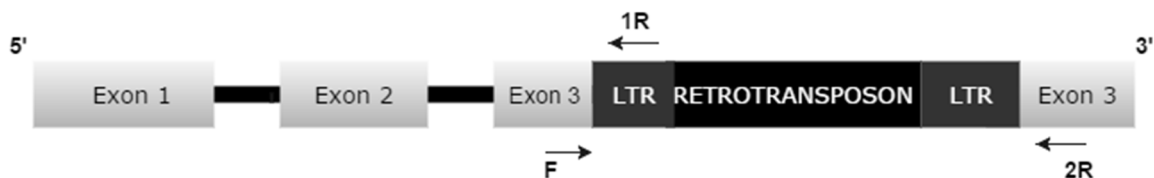


Fig. 2.1. The transcription factor *MYB25* gene in peach showing the 7 kb insertion in the third exon that results in the nectarine trait. The annealing positions for the primers (indelG-F, indelG-1R and indelG-2R) are shown with arrows. **Source:** Vendramin *et al.* (2014).

2.5.3. Molecular genotyping of the G locus

The discovery of the sequence differences between the two alleles allowed the designing of a primer set, indelG, which can detect the presence or absence of the retrotransposon insertion (Vendramin *et al.*, 2014) and thus genotype the G locus. This marker is in a three primer set consisting of a forward primer (indelG-F) and two reverse primers (indelG-1R and indelG-2R) (Fig. 2.1). The combination of indelG-F and indelG-1R detects the presence of a long terminal repeat of the retrotransposon insertion giving a band of 199 bp for the *g* allele. IndelG-F and indelG-2R detects the absence of the insertion giving a band of 941 bp for the *G* allele.

Vendramin *et al.* (2014) successfully genotyped 95 peach accessions with this primer set. However, the reverse primer (indelG-2R) detecting the *G* allele gives a large product (941 bp), that is suitable for visualization only on agarose gels. Designing a reverse primer amplifying the *G* allele with a product size less than 500 bp would allow the fluorescent labelling of the primers and more exact sizing of the amplicons with an automated sequencer.

2.6. MOLECULAR CHARACTERIZATION OF THE FLESH COLOUR IN PEACHES

2.6.1. Introduction to flesh colour

Peach flesh colour is either yellow or white, often with greater or lesser amounts of red pigmentation around the stone. The colour of the fruit flesh affects consumer preference and is thus an economically relevant trait (Gil *et al.*, 2002). In general, in China and Asiatic regions, consumers prefer white fleshed peaches, while in the USA and Europe, consumers prefer yellow fleshed peaches. Improved cultivars of both yellow and white fleshed peaches are actively sought (Williamson *et al.*, 2006).

The yellow flesh colour in peach is due to the accumulation of carotenoids (Morrison, 1990; Lancaster *et al.*, 1997). Carotenoids are a widely distributed group of naturally occurring pigments, usually red, orange or yellow in colour. These belong to the class of isoprenoid lipids and derive their colour from conjugated carbon-carbon double bonds, which have high absorption maxima (Rodriguez-Amaya, 2001) and functional groups attached to the carotenoid molecule. These tetraprenoid pigments are synthesized in chloroplasts and

chromoplasts (Hirschberg, 2001). Carotenoids along with anthocyanins are the main source of colouration in fruits and flowers important for attracting animals and insects for pollination and seed dispersal.

2.6.2. Genetics of flesh colour

Early work indicated that flesh colour in peach is a simple Mendelian trait and co-segregates with the hypanthium colour (Connors, 1920). The trait was reported to be controlled by the *Y* locus (*Y/y*) with white flesh colour dominant over the yellow flesh trait (Bailey and French, 1949; Faust and Timon, 1995). Bliss *et al.* (2002) pointed out that leaf colour at senescence also cosegregates with flesh colour and mapped the traits to a locus referred to as *LFCR* on linkage group 1. The *Y* locus was mapped to the same position as the *LFCR* locus in subsequent studies (Williamson *et al.*, 2004; Dirlwanger *et al.*, 2006; Martinez-Garcia *et al.*, 2013; Verde *et al.*, 2013; Cao *et al.*, 2016). Therefore, the *Y* locus is a pleiotropic locus controlling three traits *i.e.* flesh colour, hypanthium colour and leaf colour at senescence. Another trait, mid vein colour was also mapped to the *Y* locus (Ma *et al.*, 2013). Thus at the pleiotropic *Y* locus, the following states are dominant: white flesh, yellow senescent leaves, yellow hypanthium and white mid-vein. The recessive states are: yellow flesh, orange senescent leaves, orange hypanthium and yellow mid-vein.

The earliest study into the accumulation of carotenoids in various peach genotypes revealed marked differences between the levels of carotenoids in white and yellow fleshed peaches, with yellow fleshed peaches having significant levels of carotenoids (Morrison, 1990). In chrysanthemum, Ohmiya *et al.* (2006) identified a carotenoid degrading enzyme that affected coloration in the petal; carotenoid cleavage deoxygenase a (*CCDa*) was highly expressed in white chrysanthemum petals and significantly lower or absent in yellow chrysanthemum petals. The family of carotenoid cleavage deoxygenases (*CCDs*) generally catalyze the oxidative cleavage of yellow carotenoids resulting in two colourless apocarotenoids namely β -ionone and norisprenoids (Auldrige *et al.*, 2006). Brandi *et al.* (2011) observed a strong decrease in the expression of some *CCDs* in the yellow fleshed cultivar 'Red Haven' as compared to its mutant 'White Red Haven' and proposed that this enzyme was the potential cause of colour differences between white and yellow fleshed peaches. Three subsequent studies later confirmed that a gene from the *CCD* family, *CCD4*, controlled flesh colour in peaches (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013). White flesh was identified as the wild type and three independent mutation events, *i.e.* frame shift mutation, a single nucleotide polymorphism and a retrotransposon insertion, were responsible for the loss of gene function resulting in yellow flesh. In a recent study, Bai *et al.* (2015) knocked down the *CCD4* gene in white flesh peaches using virus induced gene silencing (VIGS) which resulted

in increased carotenoid (yellow) colouration in white fleshed peaches, further confirming the *CCD4* gene as a primary direct determinant of the flesh colour.

The wild type allele, which has a short microsatellite of seven TC repeats (TC_7), results in normal expression of this gene and white flesh colour. The three mutations that result in disruption of the gene function and result in yellow flesh colour (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013) are: an induced frame shift mutation at the microsatellite region in the *CCD4* gene due to an extra TC repeat (TC_8); a 6.2 kb long terminal repeat (LTR) retrotransposon insertion in the intron at the CATA site 38 bp before the 3' end; and an A to T substitution resulting in a single nucleotide polymorphism (SNP) at position 1520 of the coding sequence; introducing a premature stop codon. These mutations, if present in the homozygous condition, disrupt the expression of the *CCD4* gene resulting in accumulation of carotenoids causing the yellow flesh colour and associated traits.

The three mentioned studies (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013) employed different nomenclature for the wild type and the three mutant alleles but, for simplicity, *Y* refers to the wild type, y^1 to the microsatellite mutation, y^2 to the insertion of a retrotransposon and y^3 to the A/T substitution mutation (Fig. 2.2). A rare reversion mutation with ten TC microsatellites (TC_{10}), with restored function, has also been reported (Falchi *et al.*, 2013). The occurrence of at least two mutation events within an allele has also been reported in some cultivars *i.e.* TC_8 /SNP (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013).

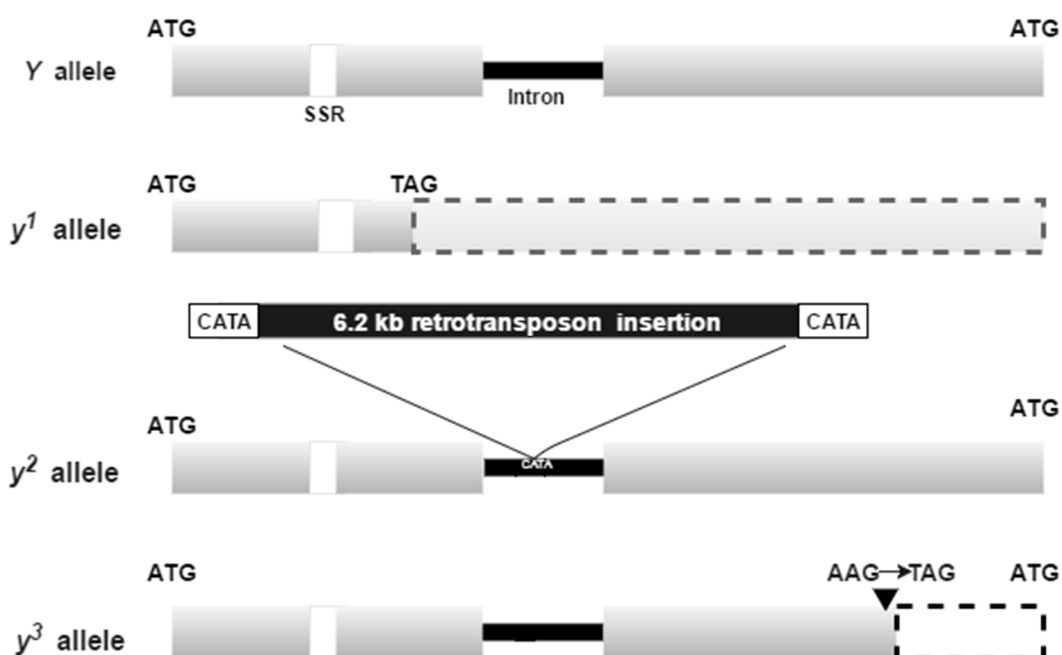


Fig. 2.2. *CCD4* gene in peach showing the wild type allele and three mutant alleles. The wild type *Y* allele has (TC)₇ repeats (white) and no other mutations within its exons (grey) or introns (black). Mutant allele *y*¹ has an extra TC repeat inducing a frameshift mutation; mutant allele *y*² has an LTR retrotransposon insertion in its intron disrupting gene function; mutant allele *y*³ has an A/T substitution. **Source:** Adami *et al.* (2013).

Interestingly, whereas all three mutations were reported in European cultivars (Adami *et al.*, 2013; Falchi *et al.*, 2013), no A/T substitution was observed in a study of Japanese cultivars (Fukamatsu *et al.*, 2013).

2.6.3. Molecular genotyping the *Y* locus

The three main studies (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013) genotyped various peach accessions for the *CCD4* gene. Adami *et al.* (2013) characterized 106 cultivars (59 yellow fleshed and 49 white fleshed), Falchi *et al.* (2013) characterized 35 cultivars (21 yellow fleshed and 14 white fleshed), and Fukamatsu *et al.* (2013) characterized 36 cultivars and 181 selections. The three studies used different primer sets and the large products observed were visualized on agarose gels. There is, therefore, an opportunity to develop a set of primers that detects the various mutations at the *Y* locus and that give amplicons with smaller products (< 500 bp), which can be fluorescently labelled and sized using an automated sequencer.

2.7. REFERENCES

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CHAPTER 3: MICROSATELLITE FINGERPRINTING OF THE ARC PEACH COLLECTION

3.1. INTRODUCTION

Prunus persica (peaches and nectarines) belongs to the subfamily Prunoideae of the family Rosaceae (Bassi and Monet, 2008). Peach and almond (*Prunus dulcis*) are closely related both being diploid ($2n=16$), belonging to the subgenus *Amygdalus* (Jelenkovic and Harrington, 1972) and are able to hybridize. Peach is self-compatible while almond is self-incompatible. Peach is one of the most genetically well-characterized species in the Rosaceae family (Bassi and Monet, 2008; Shulaev *et al.*, 2008; Arús *et al.*, 2012). Peach has a small genome size of 220-230 Mbp that has been widely mapped (Jung *et al.*, 2004; Horn *et al.*, 2005; Shulaev *et al.*, 2008), sequenced (Verde *et al.*, 2013) and is a model species for genomic studies of Rosaceae in general and *Prunus* in particular.

Peaches rank second only to apples in terms of global consumption volumes (USDA, 2012). The peach is South Africa's fifth most commercially significant deciduous fruit after grapes, apples, pears and plums (Hortgro, 2014). The commercial peach production area in South Africa is estimated at 9,800 hectares most of which are in the Western Cape. Peaches are grown for fresh consumption (dessert) and for processing (canning). The peach industry is estimated at ZAR 800 million, employing 10,000 people who in turn support 42,000 dependants. The Agricultural Research Council (ARC) is the major source of peach cultivars for the South African peach industry. The peach breeding programme at the ARC is based at Infruitec-Nietvoorbij in Stellenbosch with the germplasm collection maintained at Bien Donne Research Farm (Paarl, Western Cape). The peach genetic resources underpinning the breeding programme include a gene bank of scion cultivars and a rootstock collection that includes some ornamental peaches, interspecific hybrids and other related *Prunus* species. The breeding programme also maintains the national reference collection of peaches and almonds for the Department of Agriculture, Forestry and Fisheries (DAFF, 2014). The germplasm collection has only been described using morphological traits and the implementation of molecular marker fingerprinting technology would aid in the authentication of accessions, the elimination of errors and provide baseline information for future repropagation.

Microsatellite marker technology has become a standard and reliable method for fingerprinting germplasm around the world. The term "microsatellite" as first defined by Litt and Luty (1989) refers to tandem repeats of up to six nucleotides long that occur throughout the genome especially in non-coding regions (Gupta *et al.*, 1996; Thiel *et al.*, 2003). Microsatellites are regarded as useful molecular markers for a number of reasons (Morgante and Olivieri, 1993) including abundance in the genome, high levels of polymorphism, codominant inheritance, cross transferability and easy detection and sizing *i.e.* amplification using PCR. Their co-

dominant nature in particular allows the distinction of homozygous and heterozygous individuals at a given locus.

A large number of microsatellite markers have been developed in various laboratories and used to fingerprint peach accessions e.g. Cipriani *et al.* (1999); Testolin *et al.* (2000); Sosinski *et al.* (2000); Yamamoto *et al.* (2002); Aranzana *et al.* (2002b); Dirlewanger *et al.* (2002) and Howad *et al.* (2005); Marchese *et al.* (2005); Rojas *et al.* (2008); Wünsch (2009); Giovannini *et al.* (2012). Standardized panels of microsatellite markers have been proposed but not widely adopted (Aranzana *et al.*, 2003; Rojas *et al.*, 2006; Wünsch, 2009).

In this project, a set of nine microsatellite markers, which were developed in peach and which have been widely used in fingerprinting studies, were selected and used to fingerprint accessions in the ARC collection. Molecular fingerprints of the accessions will provide baseline data for cultivar verification and future repropagation. The verified cultivars can then be used with confidence for breeding and genetic studies.

3.2. MATERIALS AND METHODS

The protocols used for the sample collection and DNA extraction in this experimental chapter have been repeated in subsequent chapters for the sake of completeness only.

3.2.1. Plant material

Two hundred and six peach accessions, 20 almond accessions and seven *Prunus* hybrid accessions were fingerprinted in this study (Table 3.1). Eight of the peach accessions, seven of the almonds accessions and one of the hybrids were duplicates and are usually indicated with suffixes (1) and (2), except in the case of 'Tsukuba 4' accessions for which (5) and (6) were used. The accessions in the ARC collection are usually planted in sets of three trees, and three to five fresh young leaves were collected from the first tree of the set. The leaves were cut with a sterilized knife, bagged in a tagged polyethylene bag and put in a cooler box with ice. The collected leaves were stored in a -80°C freezer at the Infruitec-Nietvoorbij Cultivar Development Laboratory until DNA extraction.

Table 3.1. The accessions of peach and nectarine (206), almond (20) and the *Prunus* hybrids (7) from the ARC peach collection used for fingerprinting with microsatellite markers. BD10 Reference Collection, SV8C Gene bank, ZN7 Rootstock Collection, * ARC cultivar, CH complex hybrids

Accession	Crop	Location	Accession	Crop	Location
<i>Prunus persica</i>, Peach (P) or Nectarine (N)			Allgold	P	SV8C/2/21
2LA336	P	SV8C/1/25	Alpine*	N	BD10/22/31
Adriatica	P	SV8C/2/10	Annevesarrio	P	SV8C/1/46
Afri Rouge	P	BD10/15/4	April Glo	N	BD10/21/37
African Glo	N	BD10/23/16	ARC NE 1*	N	BD10/21/1
Afrisun*	P	BD10/15/7	ARC NE 2*	N	BD10/22/10

Accession	Crop	Location	Accession	Crop	Location
ARC NE 3*	N	BD10/23/40	Fantasia	N	BD10/22/13
ARC NE 4*	N	BD10/24/1	Fantasy*	P	BD10/19/37
ARC NE 5*	N	BD10/21/13	Fiesta Red	N	BD10/23/1
ARC NE 7*	N	BD10/24/37	Fire Rich	P	BD10/15/22
ARC NE 8*	N	BD10/22/4	Fire Sweet	N	BD10/24/28
ARC NE 9*	N	BD10/23/25	Flame Kist	N	BD10/25/ 28
ARC NE 10*	N	BD10/25/1	Flavor Crest	P	BD10/19/1
ARC NE 11*	N	BD10/25/25	Flavorine*	N	BD10/21/10
Arctic Rose	P	SV8C/1/22	Flavortop	N	BD10/22/16
Arctic Snow	N	BD10/24/10	Flordagold	P	BD10/18/43
Arctic Star	N	BD10/24/22	Flordaguard	P	ZN7/6/27
Arctic Sweet	N	BD10/24/16	FP-1	P	SV8C/1/53
Arm King	N	BD10/21/34	Golden Dawn	P	BD10/18/7
August Glo	N	BD10/23/34	Goud Myn*	P	BD10/17/22
August Pearl	P	SV8C/1/43	Guardian	P	ZN7/16/27
August Red	N	BD10/21/ 25	Gugliemina	P	SV8C/2/4
Autumn Crunch*	P	BD10/16/25	Hantam*	P	BD10/17/37
Autumn Gold	P	BD10/15/31	Honey Blush (1)*	P	BD10/16/43
Bella Donna*	N	BD10/25/4	Honey Blush (2)*	P	BD10/20/13
Bella Nova*	N	BD10- 23/37	Horizon*	N	BD10/22/1
Bella Rosa*	N	BD10/23/ 28	Impala	P	BD10/18/1
Big Top	N	BD10/22/19	Imperani*	P	BD10/19/22
Blaze Prince	P	SV8C/1/3	Impora*	P	BD10/19/31
Bokkeveld*	P	BD10/20/37	Jim Dandy	P	BD10/17/25
Bonnigold*	P	BD10/20/31	Jubilee	P	BD10/19/10
Britaney Lane	P	BD10/17/13	June Princess	P	SV8C/1/34
Cascade*	P	BD10/19/40	Kakamas (1)	P	BD10/16/19
Catherina	P	BD10/17/28	Kakamas (2)	P	ZN7/3/15
Cauresmillo (1)	P	ZN7/6/32	Kateru	P	ZN7/13/36
Cauresmillo (2)	P	ZN7/7/1	Keimoes	P	BD10/16/1
Cederberg*	P	BD10/17/19	Keisie*	P	BD10/17/40
Chuchu Picudo	P	ZN7/9/25	Klara	P	BD10/19/46
Cinderella*	P	BD10/20/1	Koks Laat	P	BD10/18/4
Classic*	P	BD10/18/40	Late Fair	N	BD10/24/ 40
Clococlan	P	BD10/19/4	Late Venus	N	BD10/22/7
Clondike White	P	BD10/14/4	LNR08A*	P	SV8C/2/22
Coconut Ice	P	SV8C/2/16	LNR08B*	P	SV8C/2/25
Corona	P	BD10/19/43	Lovell (1)	P	ZN7/9/31
Crimson Baby	P	SV8C/1/38	Lovell (2)	P	ZN7/9/36
Crimson Blaze*	N	BD10/21/7	Margaret's Pride*	N	BD10/21/43
Crimson Giant*	N	BD10/21/28	Maria Dolce	P	SV8C/1/40
Crimson Glo	N	BD10/23/10	May Glo	N	BD10/22/46
Culemborg*	P	BD10/16/40	May Kist	N	BD10/21/31
De Wet*	P	BD10/20/4	Monate*	P	BD10/19/25
December Princess	P	BD10/15/1	Mystic Magic	P	BD10/16/34
Desert Pearl*	P	BD10/15/10	Naledi	N	BD10/22/43
Desert Sun*	P	BD10/15/34	Nectar*	N	BD10/25/31
Diamond Ray	N	BD10/22/22	Nectaross	P	SV8C/2/13
Diamond Zee	N	BD10/23/31	Nemaguard 7	P	ZN7/4/3
Don Elite*	P	BD10/15/40	Nemared	P	ZN7/4/8
Donna Rosa*	N	BD10/25/7	Nemasun	P	ZN7/12/1
Donnarine*	N	BD10/24/4	Nova Donna*	P	BD10/20/25
Earli Blush*	P	BD10/15/28	Ohatsumomo	P	ZN7/5/20
Earli Gland	P	BD10/20/10	Oom Sarel*	P	BD10/17/16
Earli Gold*	P	BD10/20/28	Oribi*	P	BD10/16/13
Earli Rose	N	BD10/25/13	Orion (1)	P	BD10/16/37
Earli Sun	P	BD10/18/10	Orion (2)	P	SV8C/1/30
Early Glo*	N	BD10/22/37	Pe 9329	P	ZN7/5/24
Elandia*	P	BD10/15/46	Pintoo	P	SV8C/1/14
Elberta	P	BD10/19/34	Primrose*	N	BD10/25/16
Excellence*	P	BD10/17/10	Prita	N	BD10/24/7
Fairtime	P	BD10/16/16	Prof Malherbe*	P	BD10/18/25

Accession	Crop	Location
Prof Neethling*	P	BD10/17/43
Red Jewel	N	BD10/21/4
Red Velvet*	P	BD10/15/16
Regina Bianca	P	SV8C/2/1
Rich Lady	P	BD10/17/7
Robin White	P	SV8C/1/13
Rolees	P	BD10/16/10
Royal Gem*	N	BD10/22/40
Royal Glo	N	BD10/22/34
Ruby Prince	P	SV8C/1/31
Ruby Rose*	N	BD10/25/19
Ruby Sweet*	N	BD10/23/7
Safari	P	BD10/20/43
San Pedro	P	BD10/18/19
Sandvliet*	P	BD10/19/13
Sapo 778	P	ZN7/6/17
Scarlet*	P	BD10/18/22
September Free	P	SV8C/1/4
September Red	N	BD10/23/22
Siberian C1	P	ZN7/9/41
Siberian C2 (1)	P	ZN7/9/47
Siberian C2 (2)	P	ZN7/10/1
Silver Fire	N	BD10/24/25
Snow Crest	P	BD10/16/46
Snowwhite*	P	BD10/16/28
Sonette*	P	BD10/18/28
Southern Glo	N	BD10/23/13
Sparkle	N	BD10/21/40
Spring Baby	P	SV8C/1/20
Spring Crest	P	BD10/18/31
Stardust*	P	BD10/15/25
Stark Sunglo	N	BD10/24/34
Summer Early*	N	BD10/23/4
Summer Giant*	P	BD10/19/7
Summer Gold*	P	BD10/17/4
Summer Jewel	N	BD10/25/22
Summer Prince*	N	BD10/24/13
Summer Rich	P	BD10/15/19
Summertime*	P	BD10/17/1
Sun Burst*	N	BD10/23/19
Sun Crest	P	BD10/17/31
Sun Grand	N	BD10/24/46
Sun Raycer	N	BD10/21/16
Sun Sweet*	P	BD10/17/34
Sundry*	P	BD10/15/43
Sunectwentyone	N	BD10/21/22
Sunking	P	BD10/18/46
Sunlite	N	BD10/21/46
Sunray	P	BD10/20/22
Supec Fifteen	P	BD10/18/37
Supec Six	P	BD10/18/13
Super Rich	P	BD10/18/16
Supreme*	P	BD10/17/46
Sweet December	P	BD10/14/1
Sweet September	P	BD10/16/22
Tango*	N	BD10/25/34
Temptation*	P	BD10/20/46
Toscana	N	BD10/24/43
Transvalia*	P	BD10/20/19
Tsukuba 4 (5)	P	ZN7/4/23
Tsukuba 4 (6)	P	ZN7/4/18
Tsukuba 5	P	ZN7/6/22

Accession	Crop	Location
Uf Sun	P	BD10/15/37
UFO	P	BD10/15/13
Unico*	N	BD10/21/19
Walgant*	P	BD10/16/4
Waveren*	P	BD10/19/28
Western Cling*	P	BD10/18/34
Western Sun*	P	BD10/19/16
Witblom	P	ZN7/10/2
Zaigina	N	BD10/23/43

***Prunus dulcis*, Almond (A)**

Butte (1)	A	BD10/10/28
Butte (2)	A	ZN7/12/12
Carmel (1)	A	BD10/10/25
Carmel (2)	A	ZN7/12/22
El Fahem	A	BD10/10/1
Ferragnes (1)	A	BD10/10/4
Ferragnes (2)	A	ZN7/13/49
Feraster	A	ZN7/12/12
Ne Plus Ultra (1)	A	BD10/10/7
Ne Plus Ultra (2)	A	ZN7/14/1
Non Pareil (1)	A	BD10/10/10
Non Pareil (2)	A	ZN7/12/4
Padre	A	ZN7/13/39
Paper Shell	A	BD10/10/13
Peerless (1)	A	BD10/10/16
Peerless (2)	A	ZN7/13/54
Price (1)	A	BD10/10/31
Price (2)	A	ZN7/12/37
Sutter	A	BD10/10/34
Texas Mission	A	BD10/10/19

Peach x almond hybrids

Adarcias	P x A	ZN7/12/19
Adefuel	P x A	ZN7/13/44
GF 677	P x A	ZN7/3/7

Prunus hybrids

Atlas	CH	ZN7/1/1
Cadaman	CH	ZN7/1/6
Ferciana	CH	ZN7/2/4
Ferdor	CH	ZN7/2/6

3.2.2. DNA extraction

Genomic DNA was extracted from young leaves using a modified version of the CTAB method initially described by Doyle and Doyle (1990). A single leaf (~5 x 4 mm) was placed in a 2 µL Eppendorf tube with two ball bearings (Qiagen), one large and one small. Thereafter, 400 µL of pre-warmed 2% (m/v) CTAB and 4 µL of β-mercaptoethanol were added. The tube was then shaken for 30 seconds and placed in a prewarmed tissuelyser (Tissuelyser II, Qiagen) at a frequency of 30 Hz for 2 minutes for tissue degradation. The tube was subsequently incubated in a water bath at 60°C for 2 hours. After this incubation, 400 µL chloroform-isoamyl (24:1) was added to the tube and the contents mixed by inverting. This was followed by centrifugation (Labnet) at 15,000 xg for 10 minutes. The supernatant was placed into a new tube. This step was repeated twice after which 400 µL ice cold isopropanol was added and the tube was kept at -20°C overnight to allow precipitation to occur. The sample was centrifuged at 15,000 xg for 10 minutes. The isopropanol was gently decanted, 100 µL of 70% (v/v) ethanol added and the mixture centrifuged at 15,000 xg for 10 minutes to wash the pellet. The pellet was air dried in the fume hood at room temperature, and then dissolved in 50 µL TE buffer (10mM Tris-HCL-1mM EDTA) and stored in the freezer at -20°C.

The quality of the DNA was determined with a Biodrop spectrophotometer (Biochrom Ltd, Cambridge, UK). Calibration was done with 2 µL of dH₂O or TE buffer to set a baseline at 0.0 ng/µL and then 2 µL of each DNA sample was quantified and recorded. Dilutions were made to a concentration of 100 ng/µL and stored in a freezer at -20°C to reduce DNA degradation.

3.2.3. Primer selection and multiplex conditions

A panel of 13 microsatellite markers were selected for optimization. These markers were reported to be polymorphic and informative in peaches in previous studies (Cipriani *et al.*, 1999; Sosinski *et al.*, 2000; Testolin *et al.*, 2000; Aranzana *et al.*, 2002b; Dirlewanger *et al.*, 2002; Rojas *et al.*, 2008; Giovannini *et al.*, 2012). The cultivar 'Fairtime' was used to test amplification of the markers and determine optimal condition. Gradient PCRs were run to determine the optimal temperature for amplification. Amplicons were visualized on 0.8% (m/v) agarose gels stained with ethidium bromide. Four of the markers showed poor amplification and were not pursued further. The nine remaining markers showed good amplification and were adopted for use in this study. The forward primers of these markers were fluorescently labelled with the fluorophore dyes VIC (green), NED (yellow), PET (red) or 6-FAM (blue) (Applied Biosystems, South Africa) which enables the exact product size to be estimated using an automated sequencer. The fluorescent labelling of the markers also allows the running of more than one marker set in a reaction; a method usually referred to as 'multiplexing'

(Mansfield *et al.*, 1994). Multiplexing therefore allows the amplification a single DNA template with a mixture of markers in a single reaction, and in contrast to running each primer pair with the DNA template individually, reduces the overall cost of genotyping.

The nine selected markers (Table 3.2) were combined in three triplexes on the basis of product size as well as the type of fluorescent dye. Multiplex PCR was conducted in a final volume of 12 μL containing 0.5 μL of 100 ng/ μL DNA template, 6 μL Qiagen PCR mix, 4.3 μL RNase-free water and 1.2 μL primer mix (1x). PCR reactions were carried out in Gene Amp (Applied Biosystems) thermocyclers using the following conditions: an initial denaturation at 95°C for 15 minutes, followed by 25 cycles of 94°C for 30 seconds, 57°C for 90 seconds and 72°C for 1 minute, with a final extension at 60°C for 30 minutes.

Initially a subset of 10 peach accessions were tested with the three multiplexes. The amplicons were visualized on 0.8% agarose gels stained with ethidium bromide. Subsequently, amplicons were sent for sizing at the Central Analytic Facility (CAF) at the Department of Genetics, Stellenbosch University. The sizing was successful and product sizes were consistent with the literature. Full-scale genotyping of the ARC peach collection thus commenced.

Table 3.2. A panel of nine microsatellite markers selected for fingerprinting 206 peaches, 20 almonds and 7 *Prunus* hybrids in the ARC collection. LG = linkage group

Marker	LG	Forward Sequence	Reverse Sequence	Dye	Multiplex
¹ UDP96 005	1	gta acg ctcg ctac cac aaa	cac ccag ctc ata cac ctca	FAM	1
¹ UDP98 409	8	gct gat ggg ttt tat ggt ttt c	cgg act ctt atc ctc tat caa ca	VIC	1
² UDP98 412	6	agg gaa agt ttc tgc ac	gct gaa agt ttc tgc ac	PET	1
³ BPPCT 038	5	aat att gtt ggc ttc ttg catg	tga aag tga aac aag c	PET	2
⁴ CPPCT 006	6	aat taa ctc caa cag ctc ca	atg gtt gct taa ttc aat gg	VIC	2
² UDP98 022	1	ctag ttg tgc aca ctc acg c	gtc gca gga aca gta agc tc	FAM	2
³ BPPCT 001	2	aat tcc caa gga tgt gta tga g	cag gtg aat gag cca aag c	NED	3
³ BPPCT 007	3	aca ttg ctc gtc atc agc	cag att tct gaa gtt agc ggt a	VIC	3
⁴ CPPCT 044	2	atc tct ttg gcg tat caa gga	ggt ccc ata tca gct gaa cc	PET	3

¹Cipriani *et al.* (1999)

²Testolin *et al.* (2000)

³Dirlewanger *et al.* (2002)

⁴Aranzana *et al.* (2002b)

3.2.4. Sizing of microsatellite products

The automated sizing was done at CAF using ABI PRISM 3130 Genetic Analyzers. The process starts with the mixing of the amplicons with a size standard *e.g.* LIZ 500 (-250) that encompasses predicted size ranges. This mixture is then combined with a size standard buffer

that contains a denaturing agent and is run on the Genetic Analyzer. The amplicons, along with the size standard move through a capillary filled with polymer and are separated electrophoretically. During each electrophoresis run, the ABI Genetic Analyzer records the fluorescence intensity as a function of time and wavelength from regions on a charged coupled device (CCD) camera that correspond to different detection wavelength ranges. Primary data analysis by the ABI Genetic Analyzer involves applying a multicomponent matrix to the fluorescence intensity data to correct for spectral overlap between the dyes. When the correction is finished, the fluorescence intensities are colour coded and displayed as peaks in the electropherogram. The secondary analysis involves generating a sizing curve based on known size standards and the sizes of the unknown products can be determined (Applied Biosystems, 3130 Genetic Analyzer manual).

3.2.5. Microsatellite data analysis

Gene Mapper 5 software was used to label peaks and create appropriate bins according to the supplier's recommendations (Gene Mapper® Software Version 5.0 Installation and Administration Guide, 2005). A prominent peak was interpreted as an allele with two peaks expected for a heterozygote and one peak for a homozygote. The labelled alleles were checked to ensure that data were correct. The allelic plots from Gene Mapper 5 were printed and examined by a competent colleague to verify allelic scores. Data was captured in Excel (Microsoft, 2013) spreadsheets for further reference and usage.

3.2.6. Statistical analysis

Statistical analyses were done separately on the peaches, almonds and the hybrids. Micro-checker 2.2.3 software was first used to check data for misscoring due to stuttering, the presence of null alleles and allele dropout (Van Oosterhout *et al.*, 2004) and thus validate the data generated prior to further analysis. Genepop 4.3 software (Rousset, 2008) was used to examine the deviation from the Hardy-Weinberg Equilibrium using Markov exact tests (1,000 dememorization, 100 batches and 1,000 iterations/batch). GENALEX 6.501 software (Peakall and Smouse, 2012) was used to calculate some key genetic diversity statistics including N_a (number of alleles per locus), H_e (expected heterozygosity), H_o (observed heterozygosity) and I (Shannon's information index). The number of alleles per locus (N_a) is the total number of alleles amplified by a specific marker for all the samples. Observed heterozygosity (H_o) is the proportion of samples that are heterozygous compared to the total number of samples analyzed. The expected heterozygosity (H_e) is calculated using $H_e = 1 - \sum (p_i)^2$ as stipulated by Nei (1973) where p_i is the probability that two alleles from the same locus are different when chosen at random from a given population. Shannon's information index is calculated using the formula $I = -\sum (p_i) (\ln p_i)$. The informativeness of the markers was also tested by calculating

the polymorphic information content (PIC) using the formula, $PIC=1-\sum (p_{ij})^2$ in CERVUS 3.0.7 software (Kalinowski *et al.*, 2007).

The clustering pattern of accessions can be useful in identifying duplicates as well as possible misidentifications in the germplasm collection. A dendrogram constructed using genetic distance by the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) (Sneath and Sokal, 1973) was created in Mega 7 software using genetic distance matrices (Nei, 1973) generated in GENALEX software.

3.3. RESULTS

The molecular fingerprints of the 206 peach accessions were generated using eight microsatellite markers, and the fingerprints of the 20 almonds and the seven hybrids were generated with seven microsatellite markers (Table 3.3). In peach and almond, marker BPPCT 038 gave multiple alleles while marker UDP98 022 amplified very poorly in almonds and the hybrids. Therefore, BPPCT 038 was excluded from analysis of all accessions while UDP98 022 was excluded from the analyses of the almonds and the hybrids. One or two peaks (homozygous or heterozygous) were observed for each marker as expected in diploid individuals (Fig. 3.1).

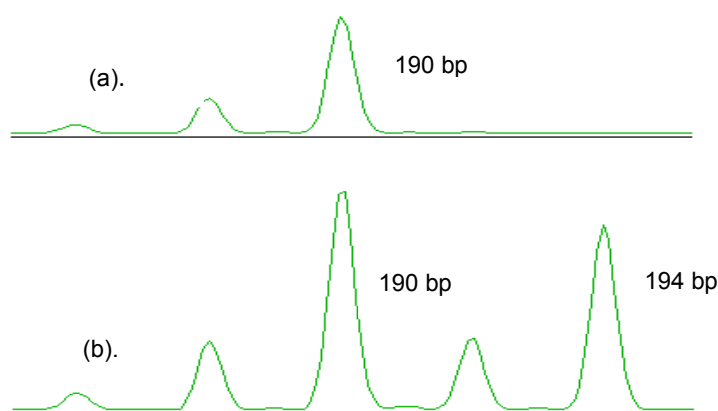


Fig. 3.1. Gene Mapper output showing the co-dominant nature of microsatellite markers as demonstrated in marker CPPCT 006 (a) homozygous 190/190 bp. (b) heterozygous 190/194

Table 3.3. Microsatellite genotypes for 206 peach accession in the ARC germplasm collection as fingerprinted with eight microsatellite markers.

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
<i>Prunus persica</i> (peaches and nectarines)								
2LA336	146/146	142/148	190/190	193/195	161/161	138/138	120/126	123/125
Adriatica	154/156	142/142	188/190	193/193	133/145	124/124	126/126	127/127
Afri Rouge	158/158	148/148	188/190	193/195	133/161	134/134	126/128	121/121
African Glo	158/158	140/140	190/190	193/195	161/161	134/134	120/120	123/123
Afrisun	158/158	144/148	178/190	193/195	161/161	134/138	120/120	123/129
Allgold	164/164	142/142	190/190	155/155	135/145	134/134	126/126	127/127
Alpine	158/158	140/142	190/190	195/195	161/161	134/134	120/126	123/123
Annevesarrio	154/158	142/148	190/198	171/171	143/143	134/134	126/142	109/121
April Glo	158/158	140/148	178/190	193/193	161/161	134/134	126/128	123/129
ARC NE 1	154/158	142/142	178/190	195/195	161/161	138/138	126/126	125/127
ARC NE 2	158/158	142/142	178/178	195/195	161/161	132/132	126/128	127/127
ARC NE 3	158/158	142/142	178/178	195/195	161/161	132/134	126/128	127/129
ARC NE 4	158/158	142/142	178/178	195/195	161/161	132/134	128/128	127/129
ARC NE 5	154/154	142/142	178/190	193/193	161/161	132/138	126/126	129/129
ARC NE 7	158/158	142/142	178/190	155/155	161/161	132/132	126/126	123/125
ARC NE 8	158/158	144/144	178/178	195/195	161/161	132/132	128/128	127/127
ARC NE 9	158/158	144/144	178/178	195/195	143/161	132/132	126/126	127/127
ARC NE 10	158/158	144/144	178/178	195/195	161/161	134/134	126/126	129/129
ARC NE 11	158/158	142/142	178/178	195/195	161/161	132/134	126/128	127/129
Arctic Rose	146/146	142/142	188/190	193/195	161/163	134/138	120/128	123/127
Arctic Snow	146/146	142/142	178/190	195/195	143/161	138/138	120/126	123/125
Arctic Star	154/158	148/148	190/190	193/195	161/163	132/134	120/120	123/123
Arctic Sweet	146/158	148/148	190/190	193/195	161/161	138/138	120/126	123/129
Arm King	154/158	144/144	178/188	193/193	161/161	134/134	126/126	127/127
August Glo	146/146	140/142	190/190	193/193	161/161	134/134	120/126	123/123

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
August Pearl	146/158	142/150	178/190	155/195	161/161	134/138	126/126	123/123
August Red	146/158	144/150	190/190	195/195	161/161	138/138	120/126	123/129
Autumn Crunch	154/158	142/142	190/190	189/195	143/161	134/134	126/126	125/129
Autumn Gold	158/158	142/148	178/190	193/195	143/161	134/138	126/126	123/125
Bella Donna	158/158	142/142	178/178	195/195	161/161	134/134	126/128	129/129
Bella Rosa	158/158	142/144	178/178	195/195	161/161	132/134	126/128	127/129
Big Top	146/158	140/148	188/190	193/195	161/161	138/138	126/150	123/129
Blaze Prince	128/158	140/150	188/190	193/195	133/161	136/138	126/128	121/129
Bokkeveld	154/158	142/142	188/190	193/195	143/161	134/138	126/126	123/129
Bonnigold	154/158	142/142	188/190	193/195	143/161	134/138	126/126	103/123
Britaney Lane	158/158	148/148	188/190	191/191	161/161	134/138	120/126	123/123
Cascade	128/154	140/142	178/190	193/193	161/161	124/134	126/126	123/125
Catherina	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Cauresmillo (1)	156/156	142/142	190/190	159/159	159/159	134/134	126/126	127/127
Cauresmillo (2)	156/156	142/142	190/190	159/159	159/159	134/134	126/126	127/127
Cederberg	146/152	144/148	188/190	193/195	143/161	134/138	120/126	129/129
Chuchu Picudo	152/152	142/142	190/190	159/159	145/145	134/134	126/126	123/123
Cinderella	158/158	146/148	190/190	193/193	161/161	134/134	126/126	127/129
Classic	154/156	142/148	188/190	193/193	143/143	134/138	126/126	123/123
Clococlan	158/158	148/148	188/188	193/193	161/161	138/138	126/126	123/129
Clondike White	158/158	148/148	178/178	193/195	159/161	138/138	120/126	129/129
Coconut Ice	146/158	140/152	188/190	193/195	133/133	134/134	126/126	121/121
Corona	160/160	142/142	190/190	195/195	161/161	134/134	126/126	129/129
Crimson Baby	158/158	142/150	190/190	193/195	161/161	138/138	120/126	123/129
Crimson Blaze	154/158	142/142	190/190	193/195	161/161	134/138	126/126	125/129
Crimson Giant	158/158	144/144	190/190	195/195	161/161	132/134	126/126	127/129
Crimson Glo	154/158	140/148	176/190	155/155	161/161	134/134	120/120	123/123
Culemborg	154/158	140/144	178/188	193/193	159/161	124/132	120/126	123/123
De Wet	158/160	140/148	190/190	195/195	143/161	134/134	126/126	123/129

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
December Princess	158/158	142/148	190/190	193/195	161/161	138/138	126/126	129/129
Desert Pearl	128/158	142/142	178/190	193/193	161/161	134/138	126/126	123/127
Desert Sun	146/154	142/148	190/190	189/195	143/143	134/134	126/126	123/125
Diamond Ray	158/158	142/142	190/190	195/195	161/161	138/138	126/126	129/129
Diamond Zee	154/154	150/150	190/190	195/195	145/155	138/138	124/124	117/125
Don Elite	154/158	142/142	190/190	195/195	159/161	134/134	126/128	127/127
Donna Rosa	158/160	142/148	190/190	195/195	161/161	134/134	120/120	123/123
Donnarine	146/158	142/148	190/190	193/193	161/161	134/138	120/126	123/127
Earli Blush	152/152	142/142	178/188	193/193	143/161	134/138	126/126	125/127
Earli Grand	158/158	144/148	190/190	195/195	161/161	134/134	126/126	123/129
Earli Gold	128/158	140/142	178/190	193/193	161/161	134/138	126/126	123/127
Earli Rose	158/160	142/148	190/190	191/191	161/161	134/134	120/126	117/123
Earli Sun	158/158	146/148	178/190	193/195	153/161	134/134	120/128	123/123
Early Glo	158/160	140/144	190/190	193/193	161/161	132/134	120/120	121/123
Elandia	152/158	142/142	178/178	189/189	161/161	132/134	128/128	127/129
Elberta	154/158	140/144	178/188	193/193	159/161	124/132	120/126	123/123
Excellence	158/158	144/148	190/190	193/195	143/161	134/138	120/126	129/129
Fairtime	146/158	142/148	178/190	193/195	161/161	138/138	118/124	121/129
Fantasia	158/158	142/148	190/190	195/195	161/161	132/138	120/126	123/129
Fantasy	128/160	142/142	178/190	189/189	161/161	124/134	126/126	123/125
Fiesta Red	158/160	142/142	190/190	155/155	161/161	134/134	120/120	123/123
Fire Rich	152/160	148/148	188/190	195/195	143/161	124/124	126/150	123/129
Fire Sweet	146/158	142/142	190/190	195/195	161/161	138/138	120/126	129/129
Flame Kist	146/146	148/148	190/190	193/195	161/161	138/138	120/120	123/123
Flavor Crest	150/158	142/148	178/178	195/195	143/143	138/138	126/126	129/129
Flavorine	154/158	142/148	190/190	195/195	161/161	134/138	126/126	127/129
Flavortop	158/158	142/148	190/190	195/195	161/161	132/138	120/126	129/129
Flordagold	158/158	148/148	190/190	193/195	161/161	134/134	126/126	127/129
Flordaguard	152/158	142/142	190/190	191/195	143/143	134/134	126/126	125/125

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
FP-1	152/154	142/142	190/190	189/189	143/143	134/134	126/126	123/125
Golden Dawn	158/160	148/148	188/190	193/195	161/161	138/138	126/126	123/123
Goud Myn	152/152	142/142	188/188	193/193	143/143	134/134	126/126	123/125
Guardian	160/160	142/142	190/190	193/193	143/143	124/124	124/126	121/129
Gugliemina	160/160	142/150	178/190	155/195	161/161	134/138	126/126	127/127
Hantam	154/158	144/148	178/190	189/195	143/161	134/138	126/128	123/129
Honey Blush (1)	158/158	140/148	190/190	195/195	143/161	134/134	126/126	123/129
Honey Blush (2)	154/154	140/142	190/190	193/193	161/161	134/134	126/126	123/123
Horizon	158/158	142/148	188/188	195/195	161/161	132/134	126/126	129/129
Impala	152/160	142/142	190/190	189/189	143/145	134/134	126/126	121/125
Imperani	152/158	142/148	190/190	189/195	161/161	134/134	126/126	125/129
Impora	150/154	148/148	190/190	189/189	161/161	134/134	126/126	125/125
Jim Dandy	166/166	140/150	188/190	173/195	133/161	134/134	126/128	121/121
Jubilee	158/158	148/148	190/190	195/195	161/161	138/138	126/128	129/129
June Princess	158/158	142/150	190/190	193/195	161/161	134/138	126/126	127/129
Kakamas (1)	152/152	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Kakamas (2)	154/154	142/142	190/190	189/189	141/141	134/134	124/124	125/125
Kateru	154/156	140/140	178/178	155/155	151/153	134/134	126/128	117/117
Keimoes	154/154	142/142	178/178	189/189	143/143	134/134	124/124	125/125
Keisie	154/156	142/142	190/190	189/193	143/143	134/134	126/126	123/123
Klara	158/160	142/148	190/190	189/189	143/161	134/134	126/126	125/127
Koks Laat	160/160	142/142	190/190	155/155	145/145	134/134	124/124	121/127
Late Fair	158/158	142/142	190/190	195/195	161/161	134/138	126/126	123/125
Late Venus (1)	146/146	142/148	190/190	193/193	161/161	138/138	120/126	123/125
Late Venus (2)	146/146	142/148	190/190	193/193	161/161	138/138	120/126	123/125
LNR08A	156/164	142/142	190/190	155/155	135/143	134/134	124/124	127/127
LNR08B	156/164	140/140	190/190	155/155	133/143	134/134	124/124	127/127
Lovell (1)	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Lovell (2)	154/154	142/142	190/190	155/177	145/145	136/136	126/126	123/123

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
Margaret's Pride	158/158	150/152	190/190	193/193	161/161	134/138	120/126	117/123
Maria Dolce	156/160	142/144	190/190	195/195	161/161	132/138	120/126	123/129
May Glo	158/158	140/140	190/190	155/193	161/161	134/134	120/120	123/127
May Kist	158/158	144/150	190/190	193/193	161/161	132/134	126/126	117/129
Monate	152/154	142/142	190/190	189/195	161/161	134/134	126/126	125/129
Mystic Magic	158/158	142/148	178/188	193/195	135/161	134/134	126/150	125/129
Naledi	158/158	142/148	190/190	193/193	161/161	134/134	120/120	123/123
Nectar	154/154	142/142	178/188	193/193	161/161	132/134	126/126	127/127
Nectaross	158/158	142/150	190/190	193/195	161/161	138/138	120/124	123/129
Nemaguard 7	166/166	142/142	188/190	173/173	141/141	136/136	128/128	129/129
Nemared	158/158	142/142	190/190	189/189	151/161	124/124	126/128	125/125
Nemasun	160/160	142/142	188/190	155/155	135/135	134/134	126/126	125/125
Nova Donna	154/154	148/148	190/190	193/193	161/161	134/138	126/126	123/123
Ohatsumomo	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Oom Sarel	152/152	142/142	188/190	193/193	143/143	134/134	126/126	123/123
Oribi	146/158	142/144	188/190	195/195	161/161	134/138	120/126	123/129
Orion (1)	156/160	142/144	178/188	193/193	135/161	132/134	120/126	123/123
Orion (2)	158/158	142/148	190/190	195/195	159/161	138/138	120/126	125/129
Pe 9329	154/158	142/142	190/190	189/195	143/161	134/134	126/126	125/129
Pintoo	128/158	140/150	178/190	193/195	133/161	136/138	126/128	121/129
Primrose	158/160	144/150	190/190	195/195	161/161	134/134	120/126	121/121
Prita	154/158	142/142	188/190	193/193	161/161	134/134	126/126	127/127
Prof Malherbe	152/154	142/142	190/190	189/193	143/143	124/134	126/126	123/125
Prof Neethling	154/154	142/142	190/190	189/193	143/143	134/134	126/126	125/125
Red Jewel	158/158	142/142	190/190	195/195	161/161	138/138	126/126	129/129
Red Velvet	158/158	142/142	190/190	195/195	159/161	134/134	126/128	127/127
Regina Bianca	158/162	148/148	188/188	195/195	143/161	124/138	126/126	129/129
Rich Lady	158/160	148/148	188/188	159/195	143/161	134/138	124/148	127/129
Robin White	152/152	142/148	178/178	169/195	143/143	138/138	126/128	131/131

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
Rolees	166/166	140/150	188/190	173/195	133/161	134/134	126/128	121/121
Royal Gem	146/158	142/142	190/190	193/193	161/161	134/134	120/126	123/127
Royal Glo	146/146	140/142	190/190	195/195	157/161	138/138	118/124	123/129
Ruby Prince	158/158	142/148	190/190	195/195	159/161	138/138	120/126	125/129
Ruby Rose	158/158	144/150	190/190	195/195	161/161	134/134	120/120	123/123
Ruby Sweet	158/158	140/142	190/190	155/155	151/161	134/134	120/120	123/127
Safari	158/158	148/148	188/190	193/193	161/161	138/138	126/126	123/129
San Pedro	158/158	142/148	178/178	193/193	161/161	134/134	126/126	123/129
Sandvliet	128/128	140/142	190/190	193/193	161/161	134/134	126/126	123/123
Sapo 778	150/158	140/154	188/190	155/193	125/143	136/138	128/150	123/129
Scarlet	158/158	144/148	178/190	155/161	161/161	134/138	120/126	123/127
September Free	158/158	142/142	178/190	155/195	161/161	132/132	126/126	129/129
September Red	146/158	142/148	190/190	193/195	161/161	138/138	120/120	125/129
Siberian C1	154/154	142/142	190/190	189/189	133/143	134/134	126/126	125/125
Siberian C2 (1)	154/154	142/142	190/190	189/189	141/141	134/134	126/126	125/125
Siberian C2 (2)	154/154	142/142	190/190	189/189	141/141	134/134	126/126	125/125
Silver Fire	158/160	140/148	190/190	193/193	161/163	134/138	120/120	123/127
Snow Crest	166/166	140/150	188/190	173/195	133/161	134/134	126/128	119/121
Snowwhite	154/158	140/142	178/190	193/193	161/161	132/134	120/126	123/127
Sonette	152/154	142/142	178/178	193/193	143/161	134/134	126/150	123/123
Southern Glo	158/158	140/140	190/190	193/195	161/161	134/134	120/120	123/123
Sparkle	156/158	142/148	190/190	195/195	161/161	132/134	120/120	123/129
Spring Baby	146/146	142/142	190/190	193/195	161/163	138/138	120/120	123/125
Spring Crest	152/158	142/148	178/188	195/195	143/161	134/138	126/126	123/129
Stardust	158/160	140/148	178/190	195/195	161/161	134/134	126/150	123/123
Stark Sunglo	146/166	140/150	188/190	173/195	133/161	134/134	126/128	121/121
Summer Early	158/158	144/150	188/188	195/195	161/161	134/134	126/126	117/127
Summer Giant	158/158	142/148	178/190	193/193	143/161	124/134	126/126	123/127
Summer Gold	154/156	142/142	188/190	193/193	143/143	134/134	126/126	123/123

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
Summer Jewel	146/146	142/148	190/190	195/195	161/161	134/138	126/126	123/129
Summer Prince	158/158	140/144	178/190	195/195	161/161	132/138	120/120	123/127
Summer Rich	158/158	142/148	178/188	193/193	143/161	124/134	126/150	125/127
Summertime	158/158	142/142	178/178	193/193	161/161	138/138	126/126	125/125
Sun Burst	158/158	140/148	190/190	155/155	161/161	134/134	120/126	123/127
Sun Crest	154/158	142/142	178/188	193/193	157/161	124/138	126/126	123/129
Sun Grand	158/158	142/148	190/190	195/195	161/161	132/138	120/126	123/129
Sun Raycer	152/158	142/148	190/190	195/195	161/161	134/134	126/126	117/127
Sun Sweet	158/158	144/148	190/190	195/195	161/161	134/134	126/126	123/129
Sundry	154/158	142/148	190/190	193/193	143/159	134/134	126/126	123/129
Sunectwentyone	154/158	142/142	188/190	191/191	161/161	134/134	126/126	127/127
Sunking	152/154	142/142	190/190	189/193	143/143	134/134	126/126	123/125
Sunlite	158/158	142/148	190/190	195/195	161/161	132/134	120/126	117/129
Sunray	152/158	142/148	178/188	195/195	143/161	134/138	126/126	123/129
Supec Fifteen	158/158	142/148	178/178	191/195	161/161	134/134	126/126	127/129
Supec Six	152/158	142/148	178/178	195/195	143/161	134/134	126/126	127/129
Super Rich	158/158	142/148	188/190	195/195	159/161	134/138	126/126	127/129
Supreme	154/156	142/142	178/178	189/193	143/161	124/134	126/126	125/125
Sweet December	154/158	142/142	188/188	161/161	161/161	138/138	150/150	123/123
Sweet September	158/158	148/148	190/190	193/195	161/161	138/138	120/126	123/129
Tango	158/158	140/148	178/178	195/195	161/161	134/134	126/126	117/127
Temptation	158/160	144/148	188/190	195/195	143/161	134/134	126/126	123/129
Toscana	158/160	140/144	190/190	155/155	161/161	134/134	120/120	123/127
Transvalia	128/154	142/148	190/190	193/193	153/161	134/138	126/126	117/117
Tsukuba 4 (5)	150/158	142/142	190/190	155/155	135/161	124/132	124/128	125/125
Tsukuba 4 (6)	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Tsukuba 5	152/158	142/142	190/190	191/195	143/43	134/134	126/126	125/125
Uf Sun	152/158	142/142	190/190	193/193	143/143	134/134	126/126	125/127
UFO	154/160	140/150	178/190	193/195	143/143	134/134	126/126	123/127

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
Unico	154/158	142/142	178/190	189/189	161/161	132/138	126/126	123/127
Walgant	152/152	142/142	190/190	189/189	143/143	134/134	126/126	123/125
Waveren	150/154	142/148	188/190	189/195	143/161	124/124	126/126	125/129
Western Cling	152/154	142/142	188/190	193/193	143/143	132/134	126/126	123/123
Western Sun	152/154	142/144	190/190	189/189	143/161	134/134	126/126	125/125
Witblom	168/168	148/148	188/190	155/155	143/143	132/132	126/126	123/123
Zaigina	146/146	142/148	190/190	195/195	161/161	132/134	120/126	123/129

Table 3.4. Molecular fingerprints of 20 almond accessions and seven hybrids from the ARC peach germplasm generated using seven microsatellite markers

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 409	UDP98 412
<i>Prunus dulcis</i> (almond)							
Butte (1)	158/158	134/150	176/186	167/181	143/149	140/140	111/127
Butte (2)	126/158	134/150	176/186	167/181	143/147	140/140	111/127
Carmel (1)	154/158	152/158	176/188	171/187	117/117	142/152	109/109
Carmel (2)	154/158	152/158	176/188	171/187	117/117	142/152	109/109
El Fahem	148/148	150/158	194/200	165/187	115/145	152/156	111/111
Ferragnes (1)	146/146	150/154	182/200	167/173	131/143	138/152	117/123
Ferragnes (2)	146/146	150/154	182/200	167/173	131/143	140/152	117/123
Ferraster	138/138	144/154	190/194	161/267	141/143	136/140	111/121
Ne Plus Ultra (1)	126/126	134/134	176/192	127/167	143/149	140/140	111/125
Ne Plus Ultra (2)	126/126	134/134	176/192	127/167	143/149	140/140	111/125
Non Pareil (1)	128/154	152/152	176/192	167/167	143/149	142/142	125/127
Non Pareil (2)	128/154	152/152	176/192	167/167	143/147	140/142	125/127
Padre	158/158	134/144	186/192	161/167	113/131	140/140	111/111
Peerless (1)	126/126	146/152	176/192	167/187	145/147	140/142	111/111
Peerless (2)	126/126	146/152	176/192	167/187	145/147	140/140	111/111
Price (1)	126/158	134/150	176/186	167/181	143/147	142/142	117/125

Price (2)	126/158	134/150	176/186	167/181	143/149	142/142	117/127
Sutter	154/158	134/150	186/192	167/167	131/145	140/140	117/125
Texas Mission (1)	158/158	134/152	188/198	167/181	133/149	134/140	111/117
Texas Mission (2)	156/158	134/150	186/198	167/181	133/149	134/140	111/117
Peach-Almond hybrids							
Adafuel	154/154	142/142	190/190	189/189	133/133	126/126	125/125
Adarcias	154/158	142/146	190/194	179/179	133/133	126/126	109/127
GF 677 (1)	138/160	144/146	190/194	155/167	131/131	128/152	127/127
GF 677 (2)	138/160	142/146	190/194	155/167	131/131	128/152	127/127
Prunus hybrids							
Atlas	128/168	142/150	188/190	193/195	133/161	126/128	121/129
Cadaman	132/160	124/142	184/190	171/195	115/155	126/128	113/125
Ferciana	152/164	136/142	196/196	191/191	155/155	132/132	109/109
Ferdor	130/130	124/124	198/198	171/171	133/161	136/136	115/115

The number of microsatellite repeats amplified by the various primers varied between the almonds and peaches. These specific alleles and other shared alleles were observed in both peaches and almonds (Table 3.5).

Table 3.5. The microsatellite alleles observed in the current study in 206 peaches and 20 almonds including common as well as private alleles.

Locus	Peach specific alleles	Almond specific alleles	Shared alleles
BPPCT 001	150, 152, 156, 160, 163, 166, 168	126, 138, 148	128, 146, 154, 158
BPPCT 007	140, 142, 148	134, 146, 154, 158	144, 150, 152
CPPCT 006	178, 190	182, 186, 192, 194, 198, 200	176, 188
CPPCT 044	155, 159, 161, 177, 189, 191, 193, 195	127, 165, 167, 181, 187, 201	171, 173
UDP96 005	125, 135, 151, 153, 155, 157, 159, 161 163	113, 115, 117, 131, 147, 149	133, 141, 143, 145
UDP98 409	118, 120, 124, 128, 150	134, 136, 138, 140, 152, 156	142
UDP98 412	103, 121, 129, 131	127	109, 117, 123, 125
UDP98 022	124, 132, 134, 136, 138	N*	N*

N* failed amplification

In peaches, the locus UDP98 005 was the most polymorphic with 13 alleles while both CCPCT 006 and UDP98 022 were the least polymorphic with four or five alleles, respectively (Table 3.4). As expected in a self-compatible crop, the deviation from the Hardy-Weinberg equilibrium in peaches is indicative of high levels of homozygosity and inbreeding. Observed homozygosity (H_o) ranged from 0.285 to 0.541 whereas the expected heterozygosity (H_e) ranged from 0.521 to 0.778. The Shannon's information index (I) ranged from 0.891 to 1.691 (Table 3.6). Regarding polymorphism as measured by the polymorphic information content (PIC), BPPCT 001 and UDP98 412 had the highest PIC scores of 0.837 and 0.834, while CCPCT 006 was the least polymorphic in this study with a score of 0.698.

Table 3.6. Number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content (PIC) and Shannon's information index (I) for the eight microsatellite markers used to fingerprint 206 peach accessions in the ARC collection

Locus	N_a	H_o	H_e	PIC	I
BPPCT 001	11	0.444	0.719	0.837	1.691
BPPCT 007	6	0.488	0.653	0.781	1.327
CCPCT 006	4	0.338	0.502	0.698	0.891
CPPCT 044	10	0.285	0.717	0.767	1.518
UDP96 005	13	0.324	0.551	0.747	1.28
UDP98 022	5	0.338	0.589	0.757	1.115
UDP98 409	7	0.343	0.521	0.719	1.061
UDP98 412	9	0.541	0.778	0.834	1.654

The clustering patterns of the peach accessions is shown as the UPGMA dendrogram and was useful in identifying duplicates, near misses and other clustering patterns (Fig. 3.1).

Some accessions were identified as identical in the dendrogram (Fig. 3.2) and matched at all loci (Table 3.7). Three nominal duplicates were observed as identical: 'Late Venus (1)' vs 'Late Venus (2)', 'Siberian C2 (1)' vs 'Siberian C2 (2)' and 'Cauresmillo (1)' vs 'Cauresmillo (2)' matched their namesakes. Eleven pairs of accessions, previously not known to be duplicates, were also identified (Table 3.7): 'Fantasia' vs 'Sun Grand', 'Orion (2)' vs 'Ruby Prince', 'Diamond Ray' vs 'Red Jewel', 'Earli Grand' vs 'Sun Sweet', 'Spring Crest' vs 'Sunray', 'ARC NE 3' vs 'ARC NE 11', 'Culemborg' vs 'Elberta', 'African Glo' vs 'Southern Glo', 'Jim Dandy' vs 'Rolees', 'Flordaguard' vs 'Tsukuba 5', 'Autumn Crunch' vs 'Pe 9329' and a quartet of 'Catherina' vs 'Lovel (1)' vs 'Ohatsumomo' vs 'Tsukuba 4 (6)'.

Table 3.7. Microsatellite genotypes of eleven pairs and a quartet of peach accessions that clustered as unexpected duplicates. Green match

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
<i>Prunus persica</i> (peaches and nectarines)								
Fantasia	158/158	142/148	190/190	195/195	161/161	132/138	120/126	123/129
Sun Grand	158/158	142/148	190/190	195/195	161/161	132/138	120/126	123/129
Orion (2)	158/158	142/148	190/190	195/195	159/161	138/138	120/126	125/129
Ruby Prince	158/158	142/148	190/190	195/195	159/161	138/138	120/126	125/129
Diamond Ray	158/158	142/142	190/190	195/195	161/161	138/138	126/126	129/129
Red Jewel	158/158	142/142	190/190	195/195	161/161	138/138	126/126	129/129
Sun Sweet	158/158	144/148	190/190	195/195	161/161	134/134	126/126	123/129
Earli Grand	158/158	144/148	190/190	195/195	161/161	134/134	126/126	123/129
Spring Crest	152/158	142/148	178/188	195/195	143/161	134/138	126/126	123/129
Sunray	152/158	142/148	178/188	195/195	143/161	134/138	126/126	123/129
ARC NE 3	158/158	142/142	178/178	195/195	161/161	132/134	126/128	127/129
ARC NE 11	158/158	142/142	178/178	195/195	161/161	132/134	126/128	127/129

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
Culemborg	154/158	140/144	178/188	193/193	159/161	124/132	120/126	123/123
Elberta	154/158	140/144	178/188	193/193	159/161	124/132	120/126	123/123
African Glo	158/158	140/140	190/190	193/195	161/161	134/134	120/120	123/123
Southern Glo	158/158	140/140	190/190	193/195	161/161	134/134	120/120	123/123
Jim Dandy	166/166	140/150	188/190	173/195	133/161	134/134	126/128	121/121
Rolees	166/166	140/150	188/190	173/195	133/161	134/134	126/128	121/121
Flordaguard	152/158	142/142	190/190	191/195	143/143	134/134	126/126	125/125
Tsukuba 5	152/158	142/142	190/190	191/195	143/143	134/134	126/126	125/125
Autumn Crunch	154/158	142/142	190/190	189/195	143/161	134/134	126/126	125/129
Pe 9329	154/158	142/142	190/190	189/195	143/161	134/134	126/126	125/129
Catherina	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Lovell (1)	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Ohatsumomo	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Tsukuba 4 (6)	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125

Other accessions that clustered very close to each other were termed 'near misses' and differed at two loci or less (Fig. 3.2; Table 3.8). These include: 'Kakamas' (1) vs 'Walgant'; 'Prof Malherbe' vs 'Sunking'; 'Blaze Prince' vs 'Pintoo'; 'Desert Pearl*' vs 'Earligold'; 'Bokkeveld' vs 'Bonnigold'; 'Cinderella' vs 'Flordaguard'; 'Clocaclan' vs 'Safari'; 'Don Elite' vs 'Red Velvet'; 'De Wet' vs 'Honey Blush (1)'; 'Arctic Sweet' vs 'Sweet September'; and 'Crimson Baby' vs 'Nectaross'.

Table 3.8. Genotype comparison of 22 accessions that clustered as near-misses and the differences at the various loci. Yellow variation

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
<i>Prunus persica</i> (peaches and nectarines)								
Kakamas (1)	152/152	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Walgant	152/152	142/142	190/190	189/189	143/143	134/134	126/126	123/125
Prof Malherbe	152/154	142/142	190/190	189/193	143/143	124/134	126/126	123/125
Sunking	152/154	142/142	190/190	189/193	143/143	134/134	126/126	123/125
Blaze Prince	128/158	140/150	188/190	193/195	133/161	136/138	126/128	121/129
Pintoo	128/158	140/150	178/190	193/195	133/161	136/138	126/128	121/129
Desert Pearl	128/158	142/142	178/190	193/193	161/161	134/138	126/126	123/127
Earli Gold	128/158	140/142	178/190	193/193	161/161	134/138	126/126	123/127
Bokkeveld	154/158	142/142	188/190	193/195	143/161	134/138	126/126	123/129
Bonnigold	154/158	142/142	188/190	193/195	143/161	134/138	126/126	103/123
Cinderella	158/158	146/148	190/190	193/193	161/161	134/134	126/126	127/129
Flordaguard	158/158	148/148	190/190	193/195	161/161	134/134	126/126	127/129
Clococlan	158/158	148/148	188/188	193/193	161/161	138/138	126/126	123/129
Safari	158/158	148/148	188/190	193/193	161/161	138/138	126/126	123/129
Don Elite	154/158	142/142	190/190	195/195	159/161	134/134	126/128	127/127
Red Velvet	158/158	142/142	190/190	195/195	159/161	134/134	126/128	127/127
De Wet	158/160	140/148	190/190	195/195	143/161	134/134	126/126	123/129
Honey Blush (1)	158/158	140/148	190/190	195/195	143/161	134/134	126/126	123/129
Arctic Sweet	146/158	148/148	190/190	193/195	161/161	138/138	120/126	123/129
Sweet September	158/158	148/148	190/190	193/195	161/161	138/138	120/126	123/129

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
Crimson Baby	158/158	142/150	190/190	193/195	161/161	138/138	120/126	123/129
Nectaross	158/158	142/150	190/190	193/195	161/161	138/138	120/124	123/129

Some accessions, which were nominal duplicates, clustered as different from their namesakes at some loci (Table 3.9a; Fig. 3.2). These include: 'Kakamas (1)' and (2)', 'Honey Blush (1)' and (2)', 'Lovell (1)' and (2)', 'Orion (1)' and (2)' and 'Tsukuba 4 (5)' and 4 (6)'.

Table 3.9a Five pairs of nominal duplicates that did not match their supposed duplicates and their genotypes comparison. Yellow Variation

Accession	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 022	UDP98 409	UDP98 412
<i>Prunus persica</i> (peaches and nectarines)								
Honey Blush (1)	158/158	140/148	190/190	195/195	143/161	134/134	126/126	123/129
Honey Blush (2)	154/154	140/142	190/190	193/193	161/161	134/134	126/126	123/123
Kakamas (1)	152/152	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Kakamas (2)	154/154	142/142	190/190	189/189	141/141	134/134	124/124	125/125
Lovell (1)	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125
Lovell (2)	154/154	142/142	190/190	155/177	145/145	136/136	126/126	123/123
Orion (1)	156/160	142/144	178/188	193/193	135/161	132/134	120/126	123/123
Orion (2)	158/158	142/148	190/190	195/195	159/161	138/138	120/126	125/129
Tsukuba 4 (5)	150/158	142/142	190/190	155/155	135/161	124/132	124/128	125/125
Tsukuba 4 (6)	154/154	142/142	190/190	189/189	143/143	134/134	126/126	125/125

The accessions 'Witblom', 'Kateru', 'Diamond Zee' and 'Nemaguard 7' did not cluster with the other peaches and their genotypes differed at most loci.

In almonds, UDP96 005 was the most polymorphic marker with 10 alleles and UDP98 412 and BPPCT 007 were the least with six alleles. The clustering patterns and genotype comparisons confirmed the seven nominal duplicates to be likely identical with mostly two loci or less differences (Fig. 3.4; Table 3.7).

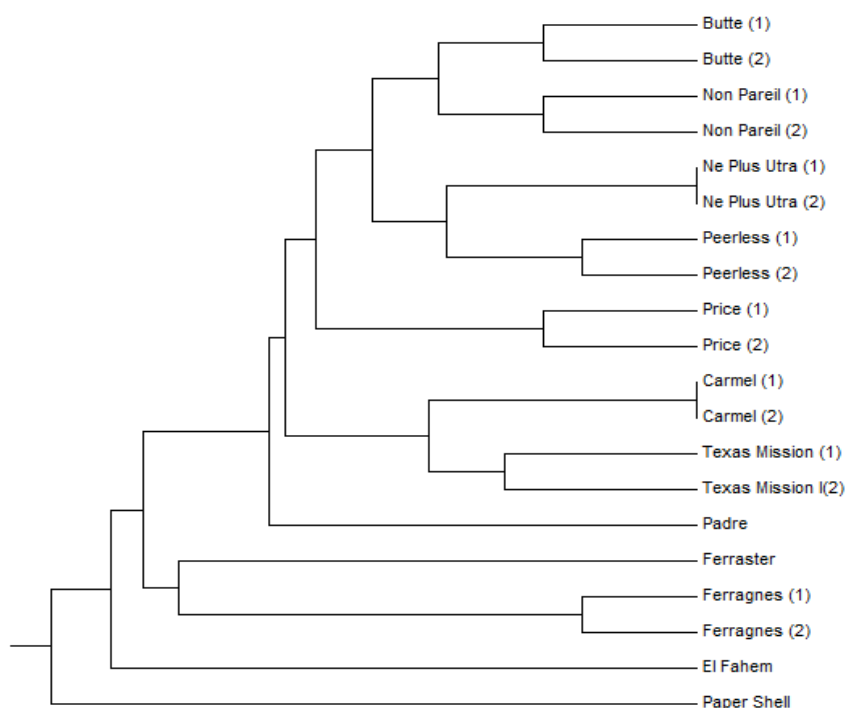


Fig. 3.3. UPGMA dendrogram of 20 almond accessions in the ARC collection genotyped with seven microsatellite markers used to identify likely duplicates and possible misidentifications

Table 3.9b. Comparison of the duplicates and likely duplicates of the almond accessions at seven microsatellite loci. (Green matching, Yellow variation)

ACCESSION	BPPCT 001	BPPCT 007	CCPCT 006	CPPCT 044	UDP96 005	UDP98 409	UDP98 412
Carmel (1)	154/158	152/158	176/188	171/187	117/117	142/152	109/109
Carmel (2)	154/158	152/158	176/188	171/187	117/117	142/152	109/109
Ne Plus Ultra (1)	126/126	134/134	176/192	127/167	143/149	140/140	111/125
Ne Plus Ultra (2)	126/126	134/134	176/193	127/167	143/149	140/140	111/125
Ferragnes (1)	146/146	150/154	182/200	167/173	131/143	138/152	117/123
Ferragnes (2)	146/146	150/154	182/200	167/173	131/143	140/152	117/123
Peerless (1)	126/126	146/152	176/192	167/187	145/147	140/142	111/111
Peerless (2)	126/126	146/152	176/192	167/187	145/147	140/140	111/111
Butte (1)	158/158	134/150	176/186	167/181	143/149	140/140	111/127
Butte (2)	126/158	134/150	176/186	167/181	143/147	140/140	111/127
Non Pareil (1)	128/154	152/152	176/192	167/167	143/149	142/142	125/127

Non Pareil (2)	128/154	152/152	176/192	167/167	143/147	140/142	125/127
Price (1)	126/158	134/150	176/186	167/181	143/147	142/142	117/125
Price (2)	126/158	134/150	176/186	167/181	143/149	142/142	117/127
Texas Mission (1)	158/158	134/152	188/198	167/181	133/149	134/140	111/117
Texas Mission (2)	156/158	134/150	186/198	167/181	133/149	134/140	111/117

The hybrids in this study were few and only 'GF 677' had a duplicate. The two 'GF 677' accessions had very similar genotypes and can be considered identical. The other two peach-almond hybrids ('Adarcias' and 'Adafuel') also clustered very close to each other.

3.4. DISCUSSION

3.4.1. Marker performance

In this study, eight microsatellite markers were used in fingerprinting 206 peaches, and seven microsatellite markers for fingerprinting 20 almonds and seven *Prunus* hybrids. These molecular data are now available for reference by the breeders.

In the peaches, the primer sets used in this study amplified successfully, except for primer BPPCT 038, which amplified multiple loci in peach (and also in the almond and hybrid accessions). Though used successfully in peach studies (Dirlewanger *et al.* 2002), other studies reported poor amplification as well as complex banding patterns for this marker (Rojas *et al.*, 2008; Dangl *et al.*, 2009; Turet-Sayar *et al.*, 2009). This may be due the primer not being very specific and amplifying other loci. The most polymorphic markers in this study (BPPCT 001 and UDP96 005) have been reported to be the most polymorphic by previous studies (Rojas *et al.*, 2008; Giovannini *et al.*, 2012). As expected in peach, a self-fertile species, high levels of homozygosity consistent with inbreeding were observed.

The most polymorphic marker in almonds was UDP98 005 and the least polymorphic marker were UDP98 412. The marker UDP98 022 amplified very poorly in almond and the hybrids suggesting likely sequence differences for the primer binding site and concurs with a previous study in Iranian almonds (Shiran *et al.*, 2007). This therefore suggests a lack of conservation at this locus with respect to the binding sites of the primers across peaches and almonds. Peach primers have been successfully used in fingerprinting almonds in other studies (Cipriani *et al.*, 1999; Shiran *et al.*, 2007; Zeinalabedini *et al.*, 2010, 2012). Therefore the seven markers used in this study can be used in fingerprinting almond accessions.

This study successfully used fluorescently labelled microsatellite markers for automated sizing of amplicons. This approach involves the use of one or more primer pairs with different fluorophores to simultaneously amplify a DNA template; a process termed multiplexing.

Multiplexing proved to be an effective method in the current study to reduce the overall costs of fingerprinting large numbers of samples.

3.4.2. Clustering of accessions

The UPGMA clustering of the accessions based on eight loci showed some interesting results. First, of the eight nominal duplicates, only three pairs appeared to be identical: 'Cauresmillo (1)' and (2)', 'Late Venus (1)' and (2) and 'Siberian C2 (1)' and (2)'. The other five supposed duplicates differed at least three loci, suggesting likely misidentification: 'Kakamas (1)' and (2)', 'Tsukuba 4 (5)' and (6)', 'Orion (1)' and (2)', 'Lovel (1)' and (2)', and 'Honey Blush (1)*' and (2)*'. One pair, 'Honey Blush (1)' and (2)', are from the reference collection and are supposedly to have been authenticated by DAFF. These should be replaced after further morphological and molecular checks. Since the accessions are grown in units of three trees, the other unsampled accessions should also be considered during these checks.

Some 26 nominally distinct accessions clustered as apparent duplicates in the dendrogram. These included 18 accessions from the reference collection: 'African Glo', 'Autumn Crunch*', 'ARC NE 3', 'ARC NE 11', 'Catherina', 'Culemborg', 'Diamond Ray', 'Earli Grand', 'Elberta', 'Fantasia', 'Jim Dandy', 'Red Jewel', 'Rolees', 'Spring Crest', 'Southern Glo', 'Sun Grand', 'Sun Sweet' and 'Sunray'. The other eight accessions were from the gene bank and the rootstock collection: 'Flordaguard', 'Lovell (1)', 'Ohatsumomo', 'Orion (2)', 'Pe 9329', 'Ruby Prince', 'Tsukuba 4 (6)' and 'Tsukuba 5'. These accession are either misidentified or could not be resolved using the primer set used in this study. Therefore an increase in the number of primers as well as morphological checks should be conducted prior to removal or replacement of accessions.

This study used a fewer markers than most peach cultivar survey studies; most of which used a minimum of 16 markers (Cipriani *et al.*, 1999; Testolin *et al.*, 2000; Sosinski *et al.*, 2000; Aranzana *et al.*, 2003; Dirlwanger *et al.*, 2002; Marchese *et al.*, 2005; Giovannini *et al.*, 2012). The increase in the number of markers would clarify some of the observed patterns by comparing the accessions at many loci thus increasing the likelihood of resolution. This approach will allow the distinctions between duplicates, near misses and conclusively identify misidentifications.

The clustering also showed that 22 accessions were 'near misses' and 17 of these were from the reference collection: 'Arctic Sweet', 'Bokkeveld', 'Bonnigold', 'Cinderella', 'Clococlan', 'Desert Pearl', 'Don Elite', 'De Wet', 'Earli Gold', 'Honey Blush (1)', 'Kakamas (1)', 'Prof Malherbe', 'Safari', 'Sunking', 'Sweet September', 'Red Velvet' and 'Walgant'. The other accessions were from the genebank and rootstock collection: 'Blaze Prince', 'Pintoo',

'Flordaguard', 'Crimson Baby' and 'Nectaross'. These observed clustering patterns are likely due to the inability of the marker set used in this study to resolve the genotypes. However it could also be that some of the accessions are closely related. Further checks using pedigree and morphological comparison (not done in this study due to time constraints) could help explain the observed patterns.

Some accessions clustered markedly distinctly from the other accessions; these include 'Witblom', 'Kateru', 'Diamond Zee' and 'Nemaguard 7'. 'Nemaguard 7' (rootstock), 'Witblom' (ornamental peach) and 'Kateru' (ornamental peach) are accessions that are mostly used for rootstock or ornamental purposes thus this distinctness is expected. On the other hand, 'Diamond Zee' is a scion cultivar and it's clustering further from the other scion cultivars and closer to the rootstocks and ornamental accessions suggests that it is either genetically distant from most of the accessions in the collection or misidentified and needing further morphological checks.

In the almond accessions, the seven markers discriminated the 20 accessions and duplicates were shown to be identical ('Carmel' and 'Ne Plus Ultra') or highly likely so (differed at two loci or less) ('Butte', 'Ferragnes', 'Non Pareil', 'Price' and 'Texas Mission'. The observation many near-misses than exact identical duplicates is likely due to mutations at the various loci. The hybrids were also successfully discriminated with the seven markers and the duplicates of 'GF 677' were shown to be identical.

3.4.3. Implications for the ARC breeding programme

This study has provided the ARC peach breeding programme with molecular fingerprints for the accessions in the collection. Prior to this study, only the materials in the reference collection were authenticated by staff of DAFF using phenotypic traits. The molecular fingerprints generated in this study will be useful when designing crosses, checking parentage and repropagating the germplasm collection. The findings in this study recommends the further verification of twenty-six accessions using molecular and morphological tests with view to replacing them with authenticated material. Eighteen of these accessions are from the reference collection that is maintained by DAFF and it is important to verify since there are supposed to be authentic. Another 22 accessions clustered so closely that further markers are needed to determine if they are either related or misidentified. Of these, 17 accessions belong to the reference collection which is supposed to be authentic. This study also observed that five nominal duplicates did not match their namesake suggesting likely misidentification and requiring follow up and replacement.

The molecular fingerprints from this study will be organised into an Excel sheet or online database that can be referenced by breeders when queries about accessions arise. The intention is to grant access to DAFF or industry bodies to help resolve issues of identity, parentage and breeders rights.

As it has been noted earlier, to clarify the molecular fingerprints of all the accessions there is the need for the addition of more microsatellite loci. An additional eight markers (to make a total of sixteen markers) should be used prior to taking action such as replacement. From consulting the literature the following eight additional markers are recommended for future studies: UDP98 008 (LG 3) (Cipriani *et al.*, 1999); EPPCU 1775 (LG 4) (Howad *et al.*, 2005); BPPCT 015 (LG 4) (Dirlewanger *et al.*, 2002); BPPCT 017 (LG 5) (Dirlewanger *et al.*, 2002); CPPCT 040 (LG 5) (Aranzana *et al.*, 2002b); CPPCT 022 (LG 7) (Aranzana *et al.*, 2002b); Pchms6 (LG 7) (Aranzana *et al.*, 2002b); BPPCT 008 (LG 8) (Dirlewanger *et al.*, 2002). These markers have been reported to be polymorphic in peaches and encompass all the linkage groups. The addition of these markers will also increase the number of markers to sixteen, which is on par with other cultivar surveys in peaches.

3.4.4. Implication for Department of Agriculture, Forestry and Fisheries (DAFF)

The Department of Agriculture, Fisheries and Forestry (DAFF) maintains a reference collection within the ARC collection for conducting distinction testing according to UPOV (International Union for the Protection of New Varieties of Plants) principles for Plant Breeder Rights. This study is therefore directly relevant to DAFF as well. The accessions in the reference collection have been fingerprinted with molecular markers for the first time. Moreover, fingerprints from other accessions not from that collection is also now available. The presence of this fingerprinting information will facilitate the verification of accessions for various purposes. Currently microsatellite markers cannot formally be used for distinctness testing as they are considered not completely reliable. However, the use of microsatellite markers in discriminating accessions would, if adopted, be complementary to the use of the UPOV guidelines for the description of new peach cultivars that only consider expressed morphological features. Eight microsatellite markers identified 18 accessions from the reference collection as likely misidentified and needing further verification and 17 accessions need analyses with additional markers to distinguish them further. An integrated approach that includes microsatellite markers, if adopted by DAFF, would be of great use in the peach industry in South Africa.

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CHAPTER 4: MOLECULAR CHARACTERIZATION OF THE PEACH/NECTARINE TRAIT IN THE ARC'S PEACH COLLECTION

4.1. INTRODUCTION

Peach (*Prunus persica*) and its glabrous variant, nectarine, belongs to the family Rosaceae, subgenus *Amygdalus*, along with almond (*Prunus dulcis*) (Bassi and Monet, 2008). Peach and almond are both diploid with $2n=16$ (Jelenkovic and Harrington, 1972) and can hybridize. Peach is self-compatible while almond is self-incompatible. Peach is the fifth most commercially significant deciduous fruit in South Africa with around 9,800 hectares planted, especially in the Western Cape (Hortgro, 2014). The peach industry generates about ZAR 800 million annually and employs 10,000 people. Unlike peach, the almond industry is almost non-existent with just one major commercial farm located between Montagu and Barrydale in the Western Cape (APCF, 2015).

The Agricultural Research Council (ARC) peach breeding programme is the primary source of fresh and canning peach cultivars grown by the South African peach industry (Hortgro, 2014). The breeding programme maintains its germplasm collection at Bien Donne Research Farm (Paarl, Western Cape). The collection includes peach accessions, almonds and some *Prunus* hybrids. The collection also includes the peach reference collection maintained for the Department of Agriculture, Forestry and Fisheries (DAFF).

Peach and nectarine are two forms of peach with the main difference being the presence of trichomes (hairs) on the fruit epidermis of peach and their absence in nectarine. Trichomes are hair-like appendages derived from differentiation of epidermal cells (Uphof, 1962). The trichomes on peach fruit are unicellular, non-glandular and can be seen on the ovary as early as four weeks before anthesis (Bassi and Monet, 2008). Therefore, peach and nectarine fruit can be distinguished at flowering time. Trichomes play an important role in protecting plants against various biotic and abiotic stresses. Almond fruit also have a hairy epidermis and no glabrous variants have been reported.

The earliest reference to nectarine as recessive to peach was by the geneticist Bateson (1902). Some prominent breeders of that era also observed the nectarine trait segregating as recessive to peach (Rivers, 1907). However, other breeders maintained that the peach was recessive to the nectarine (Burbank, 1920). Blake (1932) proposed that the nectarine was a glabrous mutant of peach at the locus *G* (*G/g*) for pubescence formation. The *G* locus was mapped much later to the distal end of linkage group 5 (Dirlewanger *et al.*, 2006; Le Dantec *et al.*, 2010; Verde *et al.*, 2013; Cao *et al.*, 2016).

Molecular studies into the genes controlling trichome formation were pioneered in *Arabidopsis thaliana* (Oppenheimer *et al.*, 1991; Wada *et al.*, 1997). In these two landmark studies, members of the *MYB* family of transcription factors were identified as responsible for controlling the formation of trichomes. Later, Machado *et al.* (2009) also identified a member of the *MYB* family as responsible for this trait in cotton. A study by Vendramin *et al.* (2014) using the candidate gene approach identified *MYB25*, a member of the *MYB* family, as responsible for the trichome formation in peach fruit. The *MYB25* gene is an ortholog of the relevant *MYB* gene in *Arabidopsis* and cotton. Expression profile analysis studies showed that transcripts of the *MYB25* gene were present in peaches but absent from nectarines. The sequencing of the *MYB25* gene in peach and nectarine showed a Ty1-copia retrotransposon insertion in the third exon of the *MYB25* gene in nectarine, which was absent in peach. The retrotransposon insertion was reported to disrupt gene function; resulting in the absence of trichomes.

A primer set called indelG was developed and successfully used to characterize the presence or absence of the retrotransposon at the *G* locus (Vendramin *et al.*, 2014). However, the product diagnostic for the dominant *G* allele (peach allele) is 941 bp and thus suitable for viewing with agarose or polyacrylamide gel electrophoresis rather than sizing on an automated sequencer. The diagnostic product size of the *g* allele (nectarine allele) is 197 bp.

The current study aims to characterize the accessions in the ARC's peach collection using a new primer set. This primer set, unlike the one previously published, is fluorescently labelled and amplifies small product sizes for both alleles (< 500 bp); therefore, sizing can be achieved using an automated sequencer. Moreover, this marker can be multiplexed with other markers reducing the overall cost of genotyping. The knowledge of whether the peaches in the ARC collection are homozygous (*G/G*) or heterozygous (*G/g*) is relevant to the breeders for the designing of crosses. For example, heterozygous peaches can be intercrossed to develop nectarine cultivars. For academic purposes only, some almonds and peach-almond hybrids were also included in the current study.

4.2. MATERIALS AND METHODS

The approaches to sample collection and DNA extraction used in this study are the same as those used in the first experimental chapter but are repeated here for completeness.

4.2.1. Plant material

Two hundred and six peach accessions (131 peaches and 75 nectarines), 20 almond accessions and seven hybrid accessions from the ARC's germplasm collection were used in

this study (Table 4.1). Eight peach and seven almond accessions had duplicate trees indicated accordingly with suffixes (1) and (2) except for 'Tsukuba 5' for which the suffixes (5) and (6) were used. Five of these nominal duplicates have been identified as likely erroneous from the fingerprinting chapter had have been indicated with a dagger (†). The accessions in the ARC germplasm collection are planted in sets of three trees, and only the first tree was sampled. Three to five fresh young leaves were cut with a sterilized knife, bagged in a tagged polyethylene bag and stored in a cooler box with ice. The collected leaves were stored in a -80°C freezer at Infruitec-Nietvoorbij Cultivar Development Laboratory until DNA extraction.

Table 4.1. Peach (131), nectarine (75), almond (20) and hybrid (7) accessions from the ARC peach collection genotyped for the peach/nectarine trait. BD10 Reference Collection, SV8C Gene bank, ZN7 Rootstock Collection; † fingerprinted (chapter 3) as likely erroneous, * ARC cultivar, CH complex hybrid.

Accession	Crop	Location	Accession	Crop	Location
<i>Prunus persica</i>, Peach (P) or nectarine (N)			Bella Nova*	N	BD10/23/37
2LA336	P	SV8C/1/25	Bella Rosa*	N	BD10/23/ 28
Adriatica	P	SV8C/2/10	Big Top	N	BD10/22/19
Afri Rouge	P	BD10/15/4	Blaze Prince	P	SV8C/1/3
African Glo	N	BD10/23/16	Bokkeveld*	P	BD10/20/37
Afrisun*	P	BD10/15/7	Bonnigold*	P	BD10/20/31
Allgold	P	SV8C/2/21	Britaney Lane	P	BD10/17/13
Alpine*	N	BD10/22/31	Cascade*	P	BD10/19/40
Annevesarrio	P	SV8C/1/46	Catherina	P	BD10/17/28
April Glo	N	BD10/21/37	Cauresmillo (1)	P	ZN7/6/32
ARC NE 1*	N	BD10/21/1	Cauresmillo (2)	P	ZN7/7/1
ARC NE 2*	N	BD10/22/10	Cederberg*	P	BD10/17/19
ARC NE 3*	N	BD10/23/40	Chuchu Picudo	P	ZN7/9/25
ARC NE 4*	N	BD10/24/1	Cinderella*	P	BD10/20/1
ARC NE 5*	N	BD10/21/13	Classic*	P	BD10/18/40
ARC NE 7*	N	BD10/24/37	Clococlan	P	BD10/19/4
ARC NE 8*	N	BD10/22/4	Clondike White	P	BD10/14/4
ARC NE 9*	N	BD10/23/25	Coconut Ice	P	SV8C/2/16
ARC NE 10*	N	BD10/25/1	Corona	P	BD10/19/43
ARC NE 11*	N	BD10/25/25	Crimson Baby	N	SV8C/1/38
Arctic Rose	N	SV8C/1/22	Crimson Blaze*	N	BD10/21/7
Arctic Snow	N	BD10/24/10	Crimson Giant*	N	BD10/21/28
Arctic Star	N	BD10/24/22	Crimson Glo	N	BD10/23/10
Arctic Sweet	N	BD10/24/16	Culemborg*	P	BD10/16/40
Arm King	N	BD10/21/34	De Wet*	P	BD10/20/4
August Glo	N	BD10/23/34	December Princess	P	BD10/15/1
August Pearl	N	SV8C/1/43	Desert Pearl*	P	BD10/15/10
August Red	N	BD10/21/ 25	Desert Sun*	P	BD10/15/34
Autumn Crunch*	P	BD10/16/25	Diamond Ray	N	BD10/22/22
Autumn Gold	P	BD10/15/31	Diamond Zee	N	BD10/23/31
Bella Donna*	N	BD10/25/4	Don Elite*	P	BD10/15/40

Accession	Crop	Location	Accession	Crop	Location
Donna Rosa*	N	BD10/25/7	Late Fair	N	BD10/24/ 40
Donnarine*	N	BD10/24/4	Late Venus	N	BD10/22/7
Earli Blush*	P	BD10/15/28	LNR08A*	P	SV8C/2/22
Earli Gland	P	BD10/20/10	LNR08B*	P	SV8C/2/25
Earli Gold*	P	BD10/20/28	Lovell†(1)	P	ZN7/9/31
Earli Rose	N	BD10/25/13	Lovell†(2)	P	ZN7/9/36
Earli Sun	P	BD10/18/10	Margaret's Pride*	N	BD10/21/43
Early Glo*	N	BD10/22/37	Maria Dolce	P	SV8C/1/40
Elandia*	P	BD10/15/46	May Glo	N	BD10/22/46
Elberta	P	BD10/19/34	May Kist	N	BD10/21/31
Excellence*	P	BD10/17/10	Monate*	P	BD10/19/25
Fairtime	P	BD10/16/16	Mystic Magic	P	BD10/16/34
Fantasia	N	BD10/22/13	Naledi	N	BD10/22/43
Fantasy*	P	BD10/19/37	Nectar*	N	BD10/25/31
Fiesta Red	N	BD10/23/1	Nectaross	N	SV8C/2/13
Fire Rich	P	BD10/15/22	Nemaguard 7	P	ZN7/4/3
Fire Sweet	N	BD10/24/28	Nemared	P	ZN7/4/8
Flame Kist	N	BD10/25/ 28	Nemasun	P	ZN7/12/1
Flavor Crest	P	BD10/19/1	Nova Donna*	P	BD10/20/25
Flavorine*	N	BD10/21/10	Ohatsumomo	P	ZN7/5/20
Flavortop	N	BD10/22/16	Oom Sarel*	P	BD10/17/16
Flordagold	P	BD10/18/43	Oribi*	P	BD10/16/13
Flordaguard	P	ZN7/6/27	Orion (1)	P	BD10/16/37
FP-1	P	SV8C/1/53	Orion† (2)	P	SV8C/1/30
Golden Dawn	P	BD10/18/7	Pe 9329	P	ZN7/5/24
Goud Myn*	P	BD10/17/22	Pintoo	P	SV8C/1/14
Guardian	P	ZN7/16/27	Primrose*	N	BD10/25/16
Gugliemina	P	SV8C/2/4	Prita	N	BD10/24/7
Hantam*	P	BD10/17/37	Prof Malherbe*	P	BD10/18/25
Honey Blush† (1)*	P	BD10/16/43	Prof Neethling*	P	BD10/17/43
Honey Blush† (2)*	P	BD10/20/13	Red Jewel	N	BD10/21/4
Horizon*	N	BD10/22/1	Red Velvet*	P	BD10/15/16
Impala	P	BD10/18/1	Regina Bianca	P	SV8C/2/1
Imperani*	P	BD10/19/22	Rich Lady	P	BD10/17/7
Impora*	P	BD10/19/31	Robin White	P	SV8C/1/13
Jim Dandy	P	BD10/17/25	Rolees	P	BD10/16/10
Jubilee	P	BD10/19/10	Royal Gem*	N	BD10/22/40
June Princess	N	SV8C/1/34	Royal Glo	N	BD10/22/34
Kakamas† (1)	P	BD10/16/19	Ruby Prince	P	SV8C/1/31
Kakamas† (2)	P	ZN7/3/15	Ruby Rose*	N	BD10/25/19
Kateru	P	ZN7/13/36	Ruby Sweet*	N	BD10/23/7
Keimoes	P	BD10/16/1	Safari	P	BD10/20/43
Keisie*	P	BD10/17/40	San Pedro	P	BD10/18/19
Klara	P	BD10/19/46	Sandvliet*	P	BD10/19/13
Koks Laat	P	BD10/18/4	Sapo 778	P	ZN7/6/17

Accession	Crop	Location	Accession	Crop	Location
Scarlet*	P	BD10/18/22	Tsukuba 4 (5) [†]	P	ZN7/4/23
September Free	P	SV8C/1/4	Tsukuba 4 [†] (6)	P	ZN7/4/18
September Red	N	BD10/23/22	Tsukuba 5	P	ZN7/6/22
Siberian C1	P	ZN7/9/41	Uf Sun	P	BD10/15/37
Siberian C2 (1)	P	ZN7/9/47	UFO	P	BD10/15/13
Siberian C2 (2)	P	ZN7/10/1	Unico*	N	BD10/21/19
Silver Fire	N	BD10/24/25	Walgant*	P	BD10/16/4
Snow Crest	P	BD10/16/46	Waveren*	P	BD10/19/28
Snowwhite*	P	BD10/16/28	Western Cling*	P	BD10/18/34
Sonette*	P	BD10/18/28	Western Sun*	P	BD10/19/16
Southern Glo	N	BD10/23/13	Witblom	P	ZN7/10/2
Sparkle	N	BD10/21/40	Zaigina	N	BD10/23/43
Spring Baby	P	SV8C/1/20	<i>Prunus dulcis</i> (Almond) A		
Spring Crest	P	BD10/18/31	Butte (1)	A	BD10/10/28
Stardust*	P	BD10/15/25	Butte (2)	A	ZN7/12/12
Stark Sunglo	N	BD10/24/34	Carmel (1)	A	BD10/10/25
Summer Early*	N	BD10/23/4	Carmel (2)	A	ZN7/12/22
Summer Giant*	P	BD10/19/7	El Fahem	A	BD10/10/1
Summer Gold*	P	BD10/17/4	Ferragnes (1)	A	BD10/10/4
Summer Jewel	N	BD10/25/22	Ferragnes (2)	A	ZN7/13/49
Summer Prince*	N	BD10/24/13	Feraster	A	ZN7/12/12
Summer Rich	P	BD10/15/19	Ne Plus Ultra (1)	A	BD10/10/7
Summertime*	P	BD10/17/1	Ne Plus Ultra (2)	A	ZN7/14/1
Sun Burst*	N	BD10/23/19	Non Pareil (1)	A	BD10/10/10
Sun Crest	P	BD10/17/31	Non Pareil (2)	A	ZN7/12/4
Sun Grand	N	BD10/24/46	Padre	A	ZN7/13/39
Sun Raycer	N	BD10/21/16	Paper Shell	A	BD10/10/13
Sun Sweet*	P	BD10/17/34	Peerless (1)	A	BD10/10/16
Sundry*	P	BD10/15/43	Peerless (2)	A	ZN7/13/54
Sunectwentyone	N	BD10/21/22	Price (1)	A	BD10/10/31
Sunking	P	BD10/18/46	Price (2)	A	ZN7/12/37
Sunlite	N	BD10/21/46	Sutter	A	BD10/10/34
Sunray	P	BD10/20/22	Texas Mission	A	BD10/10/19
Supec Fifteen	P	BD10/18/37	Peach-almond hybrids		
Supec Six	P	BD10/18/13	Adarcias	PxA	ZN7 12/19
Super Rich	P	BD10/18/16	Adefuel	PxA	ZN7/13/44
Supreme*	P	BD10/17/46	GF 677	PxA	ZN7/3/7
Sweet December	P	BD10/14/1	<i>Prunus</i> hybrids		
Sweet September	P	BD10/16/22	Atlas	CH	ZN7/1/1
Tango*	N	BD10/25/34	Cadaman	CH	ZN7/1/6
Temptation*	P	BD10/20/46	Ferciana	CH	ZN7/2/4
Toscana	N	BD10/24/43	Ferdor	CH	ZN7/2/6
Transvalia*	P	BD10/20/19			

4.2.2. DNA extraction

Genomic DNA was extracted from young leaves using a modified version of the CTAB method initially described by Doyle and Doyle (1990). A single leaf (~5 x 4 mm) was placed in a 2 µL Eppendorf tube with some ball bearings (Qiagen), one large and one small. Thereafter, 400 µL of pre-warmed 2% (m/v) CTAB and 4 µL of β-mercaptoethanol were added. The tube was then shaken for 30 seconds and placed in a prewarmed tissuelyser (Tissuelyser II, Qiagen) at a frequency of 30 Hz for 2 minutes for tissue degradation. The tube was subsequently incubated in a water bath at 60°C for 2 hours. After this incubation, 400 µL chloroform-isoamyl (24:1) was added to the tube and the contents mixed by inverting. This was followed by centrifugation (Labnet) at 15,000 xg for 10 minutes. The supernatant was placed into a new tube. This step was repeated twice after which 400 µL ice cold isopropanol was added and the tube was kept at -20°C overnight to allow precipitation to occur. The sample was centrifuged at 15,000 xg for 10 minutes. The isopropanol was gently decanted, 100 µL of 70% (v/v) ethanol added and the mixture centrifuged at 15,000 xg for 10 minutes to wash the pellet. The pellet was air dried in the fume hood at room temperature, and then dissolved in 50 µL TE buffer (10mM Tris-HCL-1mM EDTA) and stored in the freezer at -20°C.

The quality of the DNA was determined with a Biodrop spectrophotometer (Biochrom Ltd, Cambridge, UK). Calibration was done with 2 µL of dH₂O or TE buffer to set a baseline at 0.0 ng/µL and then 2 µL of each DNA sample was quantified and recorded. Dilutions were made to a concentration of 100 ng/µL and stored in a freezer at -20°C to reduce DNA degradation.

4.2.3. Primer selection and PCR conditions

A new primer (indelG-3R) was designed in-house at the ARC (Justin Lashbrooke, Post-doctoral fellow) to amplify a smaller region of the G allele with a product size < 500 bp. This primer formed part of a new primer set along with the previously published forward primer (indelG-F) and reverse primer (indelG-1R) (Vendramin *et al.*, 2014). The forward primer was fluorescently labelled with VIC for sizing with an automated sequencer (Fig. 4.1.; Table 4.2.).

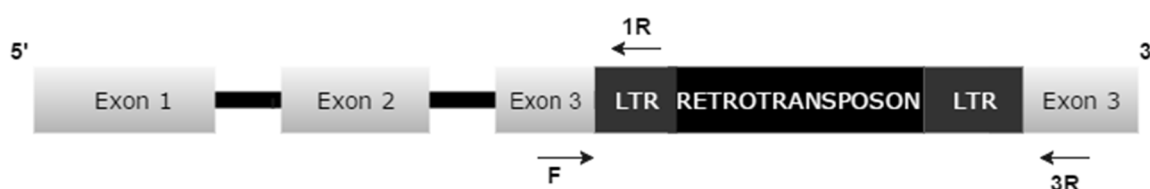


Fig. 4.1. Redesigned IndelG primer set, including new primer 3R, used to genotype the ARC germplasm collection for the *MYB25* gene.

Table 4.2. Sequences for the indelG primer set used to genotype the ARC peach germplasm collection for the *MYB25* gene controlling the peach/nectarine trait.

Primer	Forward Sequence	Reverse Sequences
IndelG-3R	ctt gca cct gag ttc gat tcc g (F)	ggcttcaatggcagaacaagg (1R) ctgatgggaagatgatcat (3R)

A subset of five peaches and five nectarines were run with the primer set using the following PCR protocols. PCRs were carried out in 12.5 μ L containing 100 ng of template DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 200 Mm of each dNTP, 0.2 mM of each primer and 0.5 U of *Taq* DNA polymerase. Amplifications were performed on a Gene Amp thermal cycler with the following temperature profile: initial denaturation at 95°C for 5 minutes, followed by further denaturation at 94°C for 30 seconds, annealing at 52°C for 45 seconds, and initial extension at 72°C for 45 seconds with final extension at 72°C for 10 minutes. Amplification was tested by running 4 μ L of each sample on 0.8% (m/v) agarose gels stained with ethidium bromide along with a 1kb DNA ladder (Thermo Scientific). Then amplicons were sent for automated sizing at the Central Analytic Facility (CAF) DNA sequencing unit at Stellenbosch University. The automated sizing was successful and the results of the test set were consistent with expected allele sizes. Subsequently genotyping the full set of accessions commenced.

4.2.4. Sizing of *MYB25* gene products

The automated sizing was done at CAF using ABI PRISM 3130 Genetic Analyzers in a process explained chapter 3 (Section 3.2.4).

4.2.5 Data analysis

Gene Mapper 5 Software was used to label peaks and create appropriate bins according to the supplier's recommendations (Gene Mapper® Software Version 5 Installation and Administration Guide, 2005). A prominent peak was interpreted as an allele, with two peaks expected for a heterozygote and one peak for homozygote. The plots from Gene Mapper 5 were printed and examined by a competent colleague to verify allelic scores.

4.3. RESULTS

The novel primer set for genotyping the *MYB25* gene for the peach/nectarine trait amplified two distinct amplicons, sized at 386 bp and at 199 bp when viewed as peaks with Gene Mapper 5 software. The 386 bp allele was observed in all peach accessions; either alone, presumably homozygous, or with the 199 bp allele. On the other hand, nectarines had only

the 199 bp peak. The 386 bp peak was therefore interpreted as indicating the dominant *G* allele for hairy fruit (peach) and the 199 bp peak, the recessive *g* allele for smooth fruit (nectarine). Three genotypes or allelic combinations were observed: *G/G* (386/386 bp), *G/g* (386/199 bp) and *g/g* (199/199 bp) (Fig. 4.2).

Of the 206 peach accessions, 75 were scored as 199/199 (*g/g*) and thus were deduced as nectarines, while 131 accessions were deduced as peach, of which 96 were homozygous (*G/G*) and 35 accessions were heterozygous (*G/g*) (Table 4.3). These deductions were consistent with the reported morphology.

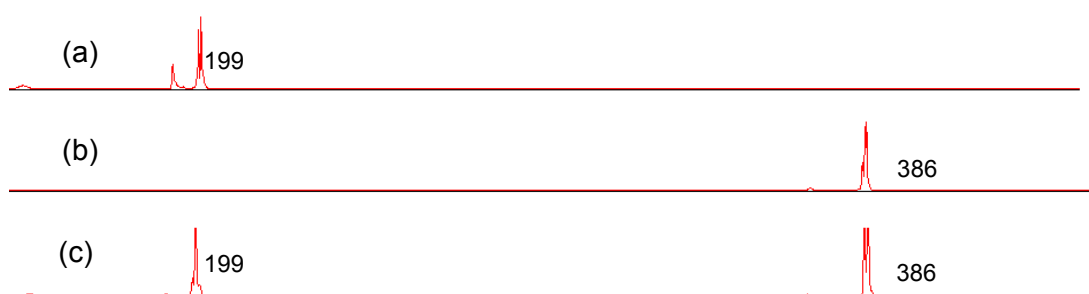


Fig. 4.2. Gene Mapper plot showing the three genotypes of the *MYB25* gene: (a). 199/199 (*g/g*) results in the nectarine phenotype; (b). 386/386 (*G/G*) results in the peach phenotype; (c). 199/386 (*G/g*) results in the peach phenotype.

The twenty almond accessions and three peach-almond hybrids were genotyped as *G/G* (386/386 bp). A further four *Prunus* hybrids, 'Atlas', 'Cadaman', 'Ferciana' and 'Ferdor', did not amplify successfully (Table 4.3.).

Table 4.3. The genotypes of peaches (206), almonds (20) and hybrids (7) from the ARC germplasm collection genotyped for the *MYB25* gene for hairy/smooth fruit epidermis. *G/G* (386/386 bp - Peach), *G/g* (199/386 bp - Peach), *g/g* (199/199 bp - Nectarine), n.a=no amplification, Obs=observed.

Accession	Genotype	Obs	Accession	Genotype	Obs
<i>Prunus persica</i>, peach (P) or nectarine (N)			ARC NE 3	<i>g/g</i>	N
2LA336	<i>G/G</i>	P	ARC NE 4	<i>g/g</i>	N
Adriatica	<i>G/G</i>	P	ARC NE 5	<i>g/g</i>	N
Afri Rouge	<i>G/G</i>	P	ARC NE 7	<i>g/g</i>	N
African Glo	<i>g/g</i>	N	ARC NE 8	<i>G/g</i>	P
Afrisun	<i>G/G</i>	P	ARC NE 9	<i>g/g</i>	N
Allgold	<i>G/g</i>	P	ARC NE 10	<i>g/g</i>	N
Alpine	<i>g/g</i>	N	ARC NE 11	<i>g/g</i>	N
Annevesarrio	<i>G/g</i>	P	Arctic Rose	<i>g/g</i>	N
April Glo	<i>g/g</i>	N	Arctic Snow	<i>g/g</i>	N
ARC NE 1	<i>g/g</i>	N	Arctic Star	<i>g/g</i>	N
ARC NE 2	<i>g/g</i>	N	Arctic Sweet	<i>g/g</i>	N

Accession	Genotype	Obs	Accession	Genotype	Obs
Arm King	<i>g/g</i>	N	Early Glo	<i>g/g</i>	N
August Glo	<i>g/g</i>	N	Elandia	<i>G/G</i>	P
August Pearl	<i>g/g</i>	N	Elberta	<i>G/G</i>	P
August Red	<i>g/g</i>	N	Excellence	<i>G/G</i>	P
Autumn Gold	<i>G/G</i>	P	Fairtime	<i>G/g</i>	P
Autumn Crunch	<i>G/G</i>	P	Fantasia	<i>g/g</i>	N
Bella Donna	<i>g/g</i>	N	Fantasy	<i>G/G</i>	P
Bella Nova	<i>g/g</i>	N	Fiesta Red	<i>g/g</i>	N
Bella Rosa	<i>g/g</i>	N	Fire Rich	<i>G/G</i>	P
Big Top	<i>g/g</i>	N	Fire Sweet	<i>g/g</i>	N
Blaze Prince	<i>G/G</i>	P	Flame Kist	<i>g/g</i>	N
Bokkeveld	<i>G/G</i>	P	Flavor Crest	<i>G/G</i>	P
Bonnigold	<i>G/G</i>	P	Flavorine	<i>g/g</i>	N
Britaney Lane	<i>G/G</i>	P	Flavortop	<i>g/g</i>	N
Cascade	<i>G/G</i>	P	Flordagold	<i>G/G</i>	P
Catherina	<i>G/g</i>	P	Flordaguard	<i>G/G</i>	P
Cauresmillo (1)	<i>G/g</i>	P	FP-1	<i>G/g</i>	P
Cauresmillo (2)	<i>G/g</i>	P	Golden Dawn	<i>G/G</i>	P
Cederberg	<i>G/G</i>	P	Goud Myn	<i>G/G</i>	P
Chuchu Picudo	<i>G/g</i>	P	Guardian	<i>G/G</i>	P
Cinderella	<i>G/G</i>	P	Guglielmina	<i>G/g</i>	P
Classic	<i>G/G</i>	P	Hantam	<i>G/G</i>	P
Clococlan	<i>G/G</i>	P	Honey Blush (1)	<i>G/G</i>	P
Clondike White	<i>G/g</i>	P	Honey Blush (2)	<i>G/G</i>	P
Coconut Ice	<i>G/G</i>	P	Horizon	<i>g/g</i>	N
Corona	<i>G/G</i>	P	Impala	<i>G/G</i>	P
Crimson Baby	<i>g/g</i>	N	Imperani	<i>G/G</i>	P
Crimson Glo	<i>g/g</i>	N	Impora	<i>G/G</i>	P
Crimson Blaze	<i>g/g</i>	N	Jim Dandy	<i>G/g</i>	P
Crimson Giant	<i>g/g</i>	N	Jubilee	<i>G/G</i>	P
Culemborg	<i>G/g</i>	P	June Princess	<i>g/g</i>	N
De Wet	<i>G/G</i>	P	Kakamas (1)	<i>G/G</i>	P
December Princess	<i>G/G</i>	P	Kakamas (2)	<i>G/G</i>	P
Desert Pearl	<i>G/g</i>	P	Kateru	<i>G/g</i>	P
Desert Sun	<i>G/g</i>	P	Keimoes	<i>G/G</i>	P
Diamond Ray	<i>g/g</i>	N	Keisie	<i>G/G</i>	P
Diamond Zee	<i>g/g</i>	N	Klara	<i>G/G</i>	P
Don Elite	<i>G/G</i>	P	Koks Laat	<i>G/G</i>	P
Donna Rosa	<i>g/g</i>	N	Late Fair	<i>g/g</i>	N
Donnarine	<i>g/g</i>	N	Late Venus	<i>g/g</i>	N
Earli Blush	<i>G/g</i>	P	LNR08A	<i>G/g</i>	P
Earli Gland	<i>G/G</i>	P	LNR08B	<i>G/g</i>	P
Earli Gold	<i>G/G</i>	P	Lovell (1)	<i>G/G</i>	P
Earli Rose	<i>g/g</i>	N	Lovell (2)	<i>G/G</i>	P
Earli Sun	<i>G/G</i>	P	Margaret's Pride	<i>g/g</i>	N
			Maria Dolce	<i>G/G</i>	P

Accession	Genotype	Obs	Accession	Genotype	Obs
May Glo	<i>g/g</i>	N	Sonette	<i>G/g</i>	P
May Kist	<i>g/g</i>	N	Southern Glo	<i>g/g</i>	N
Monate	<i>G/G</i>	P	Sparkle	<i>g/g</i>	N
Mystic Magic	<i>G/G</i>	P	Spring Baby	<i>G/G</i>	P
Naledi	<i>g/g</i>	N	Spring Crest	<i>G/G</i>	P
Nectar	<i>g/g</i>	N	Stardust	<i>G/G</i>	P
Nectaross	<i>g/g</i>	N	Stark Sunglo	<i>g/g</i>	N
Nemaguard 7	<i>G/G</i>	P	Summer Early	<i>g/g</i>	N
Nemared	<i>G/G</i>	P	Summer Giant	<i>G/g</i>	P
Nemasum	<i>G/g</i>	P	Summer Gold	<i>G/G</i>	P
Nova Donna	<i>G/G</i>	P	Summer Jewel	<i>g/g</i>	N
Ohatsumomo	<i>G/G</i>	P	Summer Prince	<i>g/g</i>	N
Oom Sarel	<i>G/g</i>	P	Summer Rich	<i>G/G</i>	P
Oribi	<i>G/G</i>	P	Summertime	<i>G/G</i>	P
Orion (1)	<i>G/G</i>	P	Sun Burst	<i>g/g</i>	N
Orion (2)	<i>G/G</i>	P	Sun Crest	<i>G/g</i>	P
Pe 9329	<i>G/g</i>	P	Sun Grand	<i>g/g</i>	N
Pintoo	<i>G/G</i>	P	Sun Raycer	<i>g/g</i>	N
Primrose	<i>g/g</i>	N	Sun Sweet	<i>G/G</i>	P
Prita	<i>g/g</i>	N	Sundry	<i>G/G</i>	P
Prof Malherbe	<i>G/G</i>	P	Sunectwentyone	<i>g/g</i>	N
Prof Neethling	<i>G/G</i>	P	Sunking	<i>G/G</i>	P
Red Jewel	<i>g/g</i>	N	Sunlite	<i>g/g</i>	N
Red Velvet	<i>G/G</i>	P	Sunray	<i>G/g</i>	P
Regina Bianca	<i>G/g</i>	P	Supec Fifteen	<i>G/G</i>	P
Rich Lady	<i>G/G</i>	P	Supec Six	<i>G/g</i>	P
Robin White	<i>G/G</i>	P	Super Rich	<i>G/G</i>	P
Rolees	<i>G/G</i>	P	Supreme	<i>G/G</i>	P
Royal Gem	<i>g/g</i>	N	Sweet December	<i>G/G</i>	P
Royal Glo	<i>g/g</i>	N	Sweet September	<i>G/G</i>	P
Ruby Prince	<i>G/G</i>	P	Tango	<i>g/g</i>	N
Ruby Rose	<i>g/g</i>	N	Temptation	<i>G/G</i>	P
Ruby Sweet	<i>g/g</i>	N	Toscana	<i>g/g</i>	N
Safari	<i>G/G</i>	P	Transvalia	<i>G/G</i>	P
San Pedro	<i>G/G</i>	P	Tsukuba 4 (5)	<i>G/g</i>	P
Sandvliet	<i>G/g</i>	P	Tsukuba 4 (6)	<i>G/g</i>	P
Sapo 778	<i>G/G</i>	P	Tsukuba 5	<i>G/g</i>	P
Scarlet	<i>G/G</i>	P	Uf Sun	<i>G/g</i>	P
September Free	<i>G/G</i>	P	UFO	<i>G/g</i>	P
September Red	<i>g/g</i>	N	Unico	<i>g/g</i>	N
Siberian C1	<i>G/G</i>	P	Walgant	<i>G/G</i>	P
Siberian C2 (1)	<i>G/G</i>	P	Waveren	<i>G/g</i>	P
Siberian C2 (2)	<i>G/G</i>	P	Western Cling	<i>G/G</i>	P
Silver Fire	<i>g/g</i>	N	Western Sun	<i>G/G</i>	P
Snow Crest	<i>G/G</i>	P	Witblom	<i>G/G</i>	P
Snowwhite	<i>g/g</i>	N	Zaigina	<i>g/g</i>	N

Accession	Genotype	Obs
<i>Prunus dulcis</i> (Almond)		
Butte (1)	G/G	
Butte (2)	G/G	
Carmel (1)	G/G	
Carmel (2)	G/G	
El Fahem	G/G	
Ferraster	G/G	
Ferragnes (1)	G/G	
Ferragnes (2)	G/G	
Ne Plus Ultra (1)	G/G	
Ne Plus Ultra (2)	G/G	
Non Pareil (1)	G/G	
Non Pareil (2)	G/G	
Padre	G/G	
Paper Shell	G/G	
Peerless (1)	G/G	
Peerless (2)	G/G	
Price (1)	G/G	
Price (2)	G/G	
Sutter	G/G	
Texas Mission	G/G	
Peach x almond hybrids		
Adarcias	G/G	
Adefuel	G/G	
GF 677	G/G	
<i>Prunus</i> hybrids		
Atlas	n.a	
Cadaman	n.a	
Ferciana	n.a	
Ferdor	n.a	

4.4. DISCUSSION

In this study 206 peaches, 20 almonds and seven hybrids from the ARC breeding collection were genotyped using a novel fluorescently labelled primer set that allows of the *G* allele (386 bp) and the *g* allele (199 bp) at the *MYB25* gene to be distinguished. The *G* allele was observed either in a homozygous or heterozygous condition in all accessions morphologically recorded as peaches, and the *g* allele was only observed in the homozygous state in those reported as nectarines. Previous studies have genotyped only seven of the 206 peaches included in the current study (Vendramin *et al.*, 2014; Rosbreed, 2015). These are: 'Big Top' (*g/g*), 'Earli Gold' (*G/G*), Crimson Glo (*g/g*), 'Fairtime' (*G/g*), 'Fantasia' (*g/g*), 'Nectaross' (*g/g*), and 'Siberian C2' (*G/G*). The genotypes for these accessions were consistent with the findings in the current study. Therefore, in the current study 199 peach accessions were genotyped for the first time for the *MYB25* gene.

The discovery of the *G* allele in almond accessions is consistent with almond having a hairy epidermis and an active *MYB25* gene. This also suggests that the ancestors of the peach and almond had an active *MYB25* gene and likely had hairy epidermises. Other *Prunus* species such as apricot (*P. armeniaca*), plum (*P. avium*) and cherry (*P. salicina*) could be candidates for further investigation of the role of the *MYB25* gene.

The nectarine trait is a loss of function mutation, a phenomenon that can be caused by various types of mutations. For instance, the yellow flesh colour trait in peach can result from three different mutations, which disrupt the normal functioning of the *CCD4* gene (Falchi *et al.*, 2013; Adami *et al.*, 2013; Fukamatsu *et al.*, 2013). In European peaches, the founders of the nectarine trait ('Goldmine', 'Lippiat' and 'Quetta') have not been genotyped (Vendramin *et al.*, 2014) though it would be of interest to investigate if all three have the same mutation. Furthermore, any landraces of nectarine from the centre of domestication, China, would also be useful in understanding the origin of the nectarine phenotype.

The failure of the indelG primers to amplify sequences in the some of the complex hybrids suggests that there may be changes at primer binding sites at the *G* locus in these species or that more optimization is required.

Fluorescently labelled primers have not been used previously to genotype the *MYB25* gene variants at the *G* locus. The previous study genotyping the *MYB25* gene in peaches used a set of markers that amplified a large product size for the *G* allele and were viewed by agarose gel electrophoresis (Vendramin *et al.*, 2014). Though successful, that approach was more laborious, time consuming and not suitable for genotyping large numbers of accessions. The primer set

used in the current study amplifies small product sizes that can be sized in an automated sequencer and viewed with Gene Mapper software resulting in data sets that are easy to handle. Since the primer set is fluorescently labelled, it can also be multiplexed with other markers such as microsatellite markers or other functional primers resulting in the reduction of overall costs.

These findings are of great use to the breeding programme at the ARC. The peach accessions have now been characterized as either homozygous or heterozygous for the peach/nectarine alleles. Heterozygous peaches provide an opportunity for the development of nectarine seedlings from peach parents thus introducing some favourable traits from peach to nectarine through intercrossing of heterozygous peaches.

4.5. CONCLUSION

This study has successfully used a new set of primers in amplifying the *MYB25* gene in peaches, and also almonds in the ARC germplasm collection. These primers are fluorescently labelled, amplify small product sizes and can be sized using an automated sequencer. This approach to genotyping is easier, faster, and less laborious than using the previously available primer set. This study has elucidated the genotypes of 96 homozygous peaches (*G/G*), 35 heterozygous peaches (*G/g*) and 75 nectarines (*g/g*). The 35 heterozygous peaches can be used to develop novel nectarine cultivars. This information will facilitate the designing of crosses by the breeders. The study also raises the question of the role of the *MYB25* gene in epidermal trichome hair formation in other *Prunus* species namely apricot, plum and cherry.

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**CHAPTER 5: MOLECULAR CHARACTERIZATION OF FLESH COLOUR IN THE ARC'S
PEACH GERMLASM**

5.1. INTRODUCTION

Prunus persica (peach and its glabrous variant, nectarine) belong to the family Rosaceae, subfamily Prunoideae, and subgenus *Amygdalus* along with almond (*P. dulcis*) (Bassi and Monet, 2008). Rosaceae is a horticulturally significant family that includes fruits like apple, pear, raspberry and strawberry. Peaches are the fifth most commercially significant deciduous fruit in South Africa after grapes, apples, pears and plums (Hortgro, 2014). With an estimated 9,800 hectares grown (mostly in the Western Cape), the South African peach industry is valued at approximately ZAR 800 million annually and employs 10,000 people who in turn support 42,000 dependants. The Agricultural Research Council's (ARC) peach breeding programme is the primary source of the commercial peach cultivars for the South African peach industry. The ARC's peach breeding programme maintains its germplasm of peach, almonds and hybrids at the Bien Donne Research Farm near Paarl in the Western Cape. The breeding programme also maintains a reference collection of peach and almond cultivars for the Department of Agriculture, Forestry and Fisheries (DAFF).

Peaches are usually consumed fresh or processed (canned or dried). The flesh colour of the peach is an important trait as it determines consumer preference (Gil *et al.*, 2002). Peaches can be white or yellow fleshed with often more or less red pigmentation around the stone. In China and the Far East, areas close to the centre of peach origin and domestication, consumers prefer white peaches while in the West (Europe and USA); consumers prefer yellow fleshed peaches (Williamson *et al.*, 2006). Almonds are consumed for the nuts and their mesocarp can generally be considered whitish.

Early reports indicated that flesh colour in peach is a simple Mendelian trait and co-segregates with the hypanthium colour (Connors, 1920). The trait was reported to be controlled by the Y locus with white flesh colour (*Y/-*) being dominant over the yellow flesh trait (*y/y*) (Bailey and French, 1949; Faust and Timon, 1995). Bliss *et al.* (2002) mapped white/yellow fruit flesh colour and yellow/orange senescent leaf colour to a *LFCR* locus which has been shown by subsequent studies to be the Y locus (Williamson *et al.*, 2004; Dirlewanger *et al.*, 2006; Martinez-Garcia *et al.*, 2013; Verde *et al.*, 2013; Cao *et al.*, 2016). A later study also showed the white/yellow leaf mid vein colour to be under the control of the Y locus (Ma *et al.*, 2013). Thus, the Y locus is a pleiotropic locus controlling colour of the fruit flesh, hypanthium, senescent leaf and leaf mid vein. The following states are dominant, white flesh, yellow senescent leaf, yellow hypanthium and white leaf mid vein whereas yellow flesh, orange senescent leaf, orange hypanthium and yellow leaf

mid vein are recessive. The gene for the carotenoid cleavage deoxygenase enzyme (*CCD4*) has been identified as controlling flesh colour in peaches in a number of recent studies (Brandi *et al.*, 2011; Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013; Ma *et al.*, 2013; Bai *et al.*, 2015). The white flesh peaches have one or two functional *CCD4* alleles, which results in expression of the *CCD4* enzyme that degrades carotenoids whereas yellow fleshed peaches are homozygous for the mutant alleles which do not code for a functional enzyme and allow the carotenoids to accumulate.

Three recessive mutations associated with the yellow flesh colour have been reported (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013). Firstly, there is a length polymorphism, a hypervariable (TC)_n microsatellite region located 47 nucleotides downstream of the start codon at which the wild type allele has seven TC microsatellite repeats while the mutant allele has an extra TC repeat (TC₈) that induces a frame shift mutation disrupting normal expression of the *CCD4* gene. In one case, ten TC repeats (TC₁₀) has been reported, which result in a reversion mutation restoring a functioning allele (Falchi *et al.*, 2013). The second mutation reported is an A/T substitution at position 1519 in the second exon resulting in a premature stop codon. This substitution introduces a single polymorphism (SNP) between alleles. The third mutation is an insertion of a long terminal repeat retrotransposon of 6,263 bp in the intron that disrupts the gene function. The nomenclature of the alleles has varied but the most common is: Y (the wild type allele), y^1 (the mutant allele with an extra repeat), y^2 (the mutant allele for the retrotransposon insertion) and y^3 (the mutant allele with the SNP). The various mutant alleles was observed to occur together in accessions in previous studies (Adam *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013). The variants in the *CCD4* gene could therefore be categorized as haplotypes and these are yet to be defined.

The sequencing of the alleles paved the way for PCR-based genotyping of the *CCD4* gene with various sets of primers (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013). Some of these primers have large amplicon sizes, which can easily be visualized on agarose gels. This approach is, however, not convenient for large datasets due to its laborious nature. In the current study, a novel multiplex of three fluorescently labelled primer sets giving small amplicon sizes of < 500 bp was used to genotype the ARC peach collection with respect to *CCD4*. Almond accessions (*Prunus dulcis*), generally considered as whitish flesh and some *Prunus* hybrids were also included. The genotypes will be of great use to breeders when designing crosses in the breeding programme.

5.2. MATERIALS AND METHODS

Some of the material and methods used in this study, especially those involving sample collection and DNA extraction, are similar to those described in Chapter 3 but repeated here for completeness.

5.2.1. Plant material

Two hundred and six peaches from the ARC's peach germplasm collection were used in this study together with 20 almonds and seven hybrid accessions (Table 5.1). Eight of the peach accessions, seven of the almond accessions and one of the hybrids were nominally duplicates and are indicated with suffixes (1) and (2), except for the Tsukuba 4 accessions where (5) and (6) were used. Five of these nominal duplicates have been identified as likely erroneous from the fingerprinting chapter had have been indicated with a dagger (†). Thirty-four of the accessions were known to be white fleshed while 172 accessions were known to be yellow fleshed from the breeding programme records. The accessions in the ARC collection are often planted in sets of three trees. Three to five fresh young leaves were collected from the first tree of the set with a sterilized knife, put in a tagged polyethylene bag and stored in a cooler box with ice. The collected leaves were stored in a freezer at -80°C at Infruitec-Nietvoorbij Cultivar Development Laboratory until DNA extraction.

Table 5.1. Peach and nectarine (206), almond (20) and *Prunus* hybrids (7) accessions from the ARC peach collection genotyped for the *CCD4* gene for flesh colour. BD10 Reference Collection, SV8C Gene bank, ZN7 Rootstock Collection, * ARC cultivar, † fingerprinted as likely erroneous, W white flesh, Y yellow flesh, RC recorded flesh colour.

Accession	RC	Location	Accession	RC	Location
<i>Prunus persica</i> (peaches and nectarines)			ARC NE 2*	Y	BD10/22/10
2LA336	Y	SV8C/1/25	ARC NE 3*	Y	BD10/23/40
Adriatica	Y	SV8C/2/10	ARC NE 4*	Y	BD10/24/1
Afri Rouge	Y	BD10/15/4	ARC NE 5*	Y	BD10/21/13
Afri Sun*	Y	BD10/15/7	ARC NE 7*	Y	BD10/24/37
African Glo	Y	BD10/23/16	ARC NE 8*	Y	BD10/22/4
Allgold	Y	SV8C/2/21	ARCNE 9*	Y	BD10/23/25
Alpine*	Y	BD10/22/31	ARC NE 10*	Y	BD10/25/1
Annevesarrio	W	SV8C/1/46	ARC NE 11*	Y	BD10/25/25
April Glo	Y	BD10/21/37	Arctic Rose	W	SV8C/1/22
ARC NE 1*	Y	BD10/21/1	Arctic Snow	W	BD10/24/10

Accession	RC	Location	Accession	RC	Location
Arctic Star	W	BD10/24/22	Donnarine*	Y	BD10/24/4
Arctic Sweet	W	BD10/24/16	Earli Blush*	Y	BD10/15/28
Arm King	Y	BD10/21/34	Earli Gland	Y	BD10/20/10
August Glo	Y	BD10/23/34	Earli Gold*	Y	BD10/20/28
August Pearl	Y	SV8C/1/43	Earli Rose	Y	BD10/25/13
August Red	Y	BD10/21/ 25	Earli Sun	Y	BD10/18/10
Autumn Crunch*	Y	BD10/16/25	Early Glo*	Y	BD10/22/37
Autumn Gold	Y	BD10/15/31	Elandia*	Y	BD10/15/46
Bella Donna*	Y	BD10/25/4	Elberta	Y	BD10/19/34
Bella Nova*	Y	BD10/23/37	Excellence*	Y	BD10/17/10
Bella Rosa	Y	BD10/23/ 28	Fairtime	Y	BD10/16/16
Big Top	Y	BD10/22/19	Fantasia	Y	BD10/22/13
Blaze Prince	W	SV8C/1/3	Fantasy*	Y	BD10/19/37
Bokkeveld*	Y	BD10/20/37	Fiesta Red	Y	BD10/23/1
Bonnigold*	Y	BD10/20/31	Fire Rich	Y	BD10/15/22
Britaney Lane	Y	BD10/17/13	Fire Sweet	Y	BD10/24/28
Cascade*	Y	BD10/19/40	Flame Kist	Y	BD10/25/ 28
Catherina	Y	BD10/17/28	Flavor Crest	Y	BD10/19/1
Cauresmillo (1)	W	ZN7/6/32	Flavorine*	Y	BD10/21/10
Cauresmillo (2)	W	ZN7/7/1	Flavortop	Y	BD10/22/16
Cederberg*	Y	BD10/17/19	Flordagold	Y	BD10/18/43
Chuchu Picudo	W	ZN7/9/25	Flordaguard	Y	ZN7/6/27
Cinderella*	Y	BD10/20/1	FP-1	Y	SV8C/1/53
Classic*	Y	BD10/18/40	Golden Dawn	Y	BD10/18/7
Clococlan	Y	BD10/19/4	Goud Myn*	Y	BD10/17/22
Clondike White	W	BD10/14/4	Guardian	W	ZN7/16/27
Coconut Ice	W	SV8C/2/16	Gugliemina	Y	SV8C/2/4
Corona	Y	BD10/19/43	Hantam*	Y	BD10/17/37
Crimson Baby	YF	SV8C/1/38	Honey Blush [†] (1)*	Y	BD10/16/43
Crimson Blaze*	Y	BD10/21/7	Honey Blush [†] (2)*	Y	BD10/20/13
Crimson Giant*	Y	BD10/21/28	Horizon*	Y	BD10/22/1
Crimson Glo	Y	BD10/23/10	Impala	Y	BD10/18/1
Culemborg*	W	BD10/16/40	Imperani*	Y	BD10/19/22
De Wet*	Y	BD10/20/4	Impora*	Y	BD10/19/31
December Princess	Y	BD10/15/1	Jim Dandy	Y	BD10/17/25
Desert Pearl*	Y	BD10/15/10	Jubilee	Y	BD10/19/10
Desert Sun*	Y	BD10/15/34	June Princess	Y	SV8C/1/34
Diamond Ray	Y	BD10/22/22	Kakamas (1)	Y	BD10/16/19
Diamond Zee	Y	BD10/23/31	Kakamas [†] (2)	Y	ZN7/3/15
Don Elite*	Y	BD10/15/40	Kateru	W	ZN7/13/36
Donna Rosa*	Y	BD10/25/7	Keimoes	Y	BD10/16/1
			Keisie*	Y	BD10/17/40

Accession	RC	Location	Accession	RC	Location
Klara	Y	BD10/19/46	Ruby Sweet*	Y	BD10/23/7
Koks Laat	Y	BD10/18/4	Safari	Y	BD10/20/43
Late Fair	Y	BD10/24/ 40	San Pedro	Y	BD10/18/19
Late Venus	Y	BD10/22/7	Sandvliet*	Y	BD10/19/13
LNR08A*	W	SV8C/2/22	Sapo778	Y	ZN7/6/17
LNR08B*	W	SV8C/2/25	Scarlet*	Y	BD10/18/22
Lovell† (1)	Y	ZN7/9/31	September Free	Y	SV8C/1/4
Lovell† (2)	Y	ZN7/9/36	September Red	Y	BD10/23/22
Margaret's Pride*	Y	BD10/21/43	Siberian C1	Y	ZN7/9/41
Maria Dolce	Y	SV8C/1/40	Siberian C2 (1)	Y	ZN7/9/47
May Glo	Y	BD10/22/46	Siberian C2 (2)	Y	ZN7/10/1
May Kist	Y	BD10/21/31	Silver Fire	W	BD10/24/25
Monate*	Y	BD10/19/25	Snow Crest	W	BD10/16/46
Mystic Magic	W	BD10/16/34	Snowwhite*	W	BD10/16/28
Naledi	Y	BD10/22/43	Sonette*	Y	BD10/18/28
Nectar*	Y	BD10/25/31	Southern Glo	Y	BD10/23/13
Nectaross	Y	SV8C/2/13	Sparkle	Y	BD10/21/40
Nemaguard 7	W	ZN7/4/3	Spring Baby	Y	SV8C/1/20
Nemared	W	ZN7/4/8	Spring Crest	Y	BD10/18/31
Nemasun	Y	ZN7/12/1	Star Dust*	Y	BD10/15/25
Nova Donna*	Y	BD10/20/25	Stark Sunglo	Y	BD10/24/34
Ohatsumomo	Y	ZN7/5/20	Summer Early*	Y	BD10/23/4
Oom Sarel*	Y	BD10/17/16	Summer Giant*	Y	BD10/19/7
Oribi*	Y	BD10/16/13	Summer Gold*	Y	BD10/17/4
Orion (1)	W	BD10/16/37	Summer Jewel	Y	BD10/25/22
Orion† (2)	W	SV8C/1/30	Summer Prince*	W	BD10/24/13
Pe 9329	Y	ZN7/5/24	Summer Rich	Y	BD10/15/19
Pintoo	W	SV8C/1/14	Summertime*	Y	BD10/17/1
Primrose*	Y	BD10/25/16	Sun Burst*	Y	BD10/23/19
Prita	W	BD10/24/7	Sun Crest	Y	BD10/17/31
Prof Malherbe*	Y	BD10/18/25	Sundry*	Y	BD10/15/43
Prof Neethling*	Y	BD10/17/43	Sun Grand	Y	BD10/24/46
Red Jewel	Y	BD10/21/4	Sun Raycer	Y	BD10/21/16
Red Velvet*	Y	BD10/15/16	Sun Sweet*	Y	BD10/17/34
Regina Bianca	W	SV8C/2/1	Sunec Twentyone	Y	BD10/21/22
Rich Lady	Y	BD10/17/7	Sunking	Y	BD10/18/46
Robin White	W	SV8C/1/13	Sunlite	Y	BD10/21/46
Rolees	Y	BD10/16/10	Sunray	Y	BD10/20/22
Royal Gem*	Y	BD10/22/40	Supec Fifteen	Y	BD10/18/37
Royal Glo	Y	BD10/22/34	Supec Six	Y	BD10/18/13
Ruby Prince	Y	SV8C/1/31	Super Rich	Y	BD10/18/16
Ruby Rose	Y	BD10/25/19	Supreme*	Y	BD10/17/46

Accession	RC	Location
Sweet December	Y	BD10/14/1
Sweet September	Y	BD10/16/22
Tango*	Y	BD10/25/34
Temptation*	Y	BD10/20/46
Toscana	Y	BD10/24/43
Transvalia*	Y	BD10/20/19
Tsukuba4† (5)	W	ZN7/4/23
Tsukuba4† (6)	W	ZN7/4/18
Tsukuba 5	W	ZN7/6/22
Uf Sun	Y	BD10/15/37
UFO	W	BD10/15/13
Unico*	Y	BD10/21/19
Walgant*	Y	BD10/16/4
Waveren*	Y	BD10/19/28
Western Cling*	Y	BD10/18/34
Western Sun*	Y	BD10/19/16
Witblom	W	ZN7/10/2
Zaigina	Y	BD10/23/43

***Prunus dulcis* (almonds)**

Butte (1)	BD10/10/28
Butte (2)	ZN7/12/12
Carmel (1)	BD10/10/25
Carmel (2)	ZN7/12/22
El Fahem	BD10/10/1

Accession	RC	Location
Ferragnes (1)		BD10/10/4
Ferragnes (2)		ZN7/13/49
Ferraster		ZN7/12/12
Ne Plus Ultra (1)		BD10/10/7
Ne Plus Ultra (2)		ZN7/14/1
Non Pareil (1)		BD10/10/10
Non Pareil (2)		ZN7/12/4
Padre		ZN7/13/39
Paper Shell		BD10/10/13
Peerless (1)		BD10/10/16
Peerless (2)		ZN7/13/54
Price (1)		BD10/10/31
Price (2)		ZN7/12/37
Sutter		BD10/10/34
Texas Mission		BD10/10/19

Peach x almond hybrids

Adarcias	ZN7/12/19
Adefuel	ZN7/13/44
GF 677	ZN7/3/7

Prunus hybrids

Atlas	ZN7/1/1
Cadaman	ZN7/1/6
Ferciana	ZN7/2/4
Ferdor	ZN7/2/6

5.2.2. DNA extraction

Genomic DNA was extracted from young leaves using a modified version of the CTAB method initially described by Doyle and Doyle (1990). A single leaf (~5 x 4 mm) was placed in a 2 µL Eppendorf tube with two ball bearings, one large and one small. Thereafter, 400 µL of pre-warmed 2% (m/v) CTAB and 4 µL of β-mercaptoethanol were added. The tube was then shaken for 30 seconds and placed in a prewarmed tissuelyser (Tissuelyser II, Qiagen) at a frequency of 30 Hz for 2 minutes for tissue degradation. The tube was subsequently incubated in a water bath at 60°C for 2 hours. After this incubation, 400 µL chloroform-isoamyl (24:1) was added to the tube and the contents mixed by inverting. This was followed by centrifugation (Labnet) at 15,000 xg for 10 minutes. The supernatant was placed into a new tube. This step was repeated twice after which 400 µL ice cold isopropanol was added and the tube was kept at -20°C overnight to allow precipitation to occur. The sample was centrifuged at 15,000 xg for 10 minutes. The isopropanol was gently decanted, 100 µL of 70% (v/v) ethanol added and the mixture centrifuged at 15,000 xg for 10 minutes to wash the pellet. The pellet was air dried in the fume hood at room temperature, and then dissolved in 50 µL TE buffer (10mM Tris-HCL-1mM EDTA) and stored in the freezer at -20°C.

The quality of the DNA was determined with a Biodrop spectrophotometer (Biochrom Ltd, Cambridge, UK). Calibration was done with 2 µL of dH₂O or TE buffer to set a baseline at 0.0 ng/µL and then 2 µL of each DNA sample was quantified and recorded. Dilutions were made to a concentration of 100 ng/µL and stored in a freezer at -20°C to reduce DNA degradation.

5.2.3. Primer selection and PCR conditions

Novel primers sets for the *CCD4* gene were designed in-house at the ARC (Justin Lashbrook, Post-doctoral fellow). These primers were designed to detect the three mutation events as well as the wild type allele (Table 5.2). The primers were also designed to give small product sizes < 500 bp and some were fluorescently labelled to allow automated sizing. The first fluorescently labelled primer pair (CCD4-SSR) flanked the microsatellite region and was designed to detect the (TC)_n length polymorphism. The second primer set was made up of two primer pairs. The first pair (CCD4-SNP) was fluorescently labelled and designed to detect the presence of the SNP event, amplifying a product of 297 bp when the SNP was present and which would not amplify when the SNP was absent. The second pair of primers (CCD4-NoSNP) was unlabelled and was designed to detect the absence of the SNP event amplifying a product of 297 bp when no SNP was present. This primer was designed to determine the copy number of the SNP (homozygous or heterozygous) in accessions where the SNP was detected. The third primer set was designed to detect the presence of a retrotransposon

insertion in the *CCD4* gene. Two fluorescently labelled primer pairs were designed. The first pair (CCD4-Retro) was designed to detect the presence of a retrotransposon by amplifying a product of 168 bp or failing to amplify in the absence thereof. The second primer pair (CCD4-NoRetro) was designed to amplify a product ~ 300 bp in the absence of the retrotransposon mutation, and was relevant for determining the copy number of the retrotransposon in the accessions in which it was detected.

The approach of this study for the detection of the SNP and retrotransposon mutation involved the use of the fluorescently labelled primer pairs to detect the presence of the mutations. The detection of amplification (peak) was to be interpreted as a presence of the mutation and no amplification was to be interpreted as absence of the mutation. Then the primer pairs detecting the absence of the retrotransposon and SNP would be run and visualised on gels primarily to determine the copy number of the mutation (heterozygous or homozygous conditions) in accessions where it has been detected.

A subset of 10 peaches with white flesh and 10 with yellow flesh was chosen for initial testing of the primers. A 13 μ L PCR reaction was made up of 0.6 μ L of 100 ng/ μ L of template DNA, 0.6 μ L of each primer, 6.25 multiplex mix (Qiagen) and 3.75 μ L of RNase free water. The PCR was run for 30 cycles under the following conditions: 95°C for 4 minutes, 94°C for 30 seconds, 45 seconds for 50°C, 72°C for 1 minute and a final extension at 72°C for 10 minutes. The amplicons were divided into two parts. One aliquot was used for testing amplification by electrophoresis on 0.8% agarose gel stained with ethidium bromide and the other aliquot was sent for automated sequencing at the Central Analytic Facility (CAF) at Stellenbosch University. Agarose gel electrophoresis was used to confirm amplification while automated sizing showed the various amplicons at the *CCD4* locus. When this testing was deemed successful, genotyping commenced on the rest of the accessions in the ARC collection.

Table 5.2. A panel of novel *CCD4* primer sets designed at the ARC for genotyping peach accessions with respect to white or yellow flesh colour.

Primer	Sequence	Dye	Mutation
CCD4-SSR (F)	cag tga agg gca ata cca g	FAM	CCD4 SSR mutation
CCD4-SSR (R)	tct aac aga gga aat gct gaa c		...
CCD4-SNP (F)	tgt cca atg tgg agc act t	VIC	SNP mutation
CCD4-SNP (R)	aca cta caa ctt gtt gag atc act t		...
CCD4-NoSNP (F)	tgt cca atg tgg agc acat	unlabelled	SNP mutation absent
CCD4-NoSNP (R)	atg gat gcc ttc tct tcc t		...
CCD4-Retro (F)	aat tac aca cta acc cca tgg	NED	CCD4 retrotransposon
CCD4-Retro (R)	agg att gta ttg gcc tgt tac		...
CCD4-NoRetro (F)	aat tac aca cta acc cca tgg	NED	CCD4 retrotransposon absent
CCD4-NoRetro (R)	ata agg gag atc aga ctc gc		...

5.2.4. Sizing of *CCD4* primer products

The automated sizing was done at CAF using ABI PRISM 3130 Genetic Analyzers in a process detailed in Chapter 3 (Section 3.2.4.)

5.2.5. *CCD4* data analysis

Gene Mapper 5 software was used to define product size. The allelic plots from Gene Mapper and the corresponding results from gel electrophoresis were checked by a competent colleague to verify the accuracy and interpretation of the allelic scores.

For the *CCD4*-SSR primer, prominent clusters of peaks were scored as alleles. Since peach is diploid, a single peak cluster was interpreted as indicating the homozygous condition while two peak cluster was interpreted as heterozygous. For the *CCD4*-SSR primer pair, a wild type allele (TC₇) and the variant (longer by 2 bps;TC₈) were expected and perhaps the rare reversion mutant; 6 bp longer (TC₁₀).

For the *CCD4*-SNP primer pair, a prominent peak was interpreted as presence of the SNP mutation and no peak as the absence of the SNP mutation. The accessions with the SNP mutation were further genotyped with *CCD4*-NoSNP to determine if the SNP was homozygous or heterozygous. The accession with one copy of the SNP mutation would amplify a product indicating the presence of at least one allele with no SNP while the accessions with two copies of the SNP did not amplify any product because the SNP was present in both alleles.

For the *CCD4*-Retro primers, a prominent peak would be interpreted as presence of the retrotransposon and absence of the peak as absence of the retrotransposon. The accessions with the retrotransposon would then be genotyped with the *CCD4*-NoRetro primer to determine the copy number of the mutation. The accessions with the retrotransposon in one allele (heterozygous) would amplify a product with this primer and those that had the retrotransposon present in both alleles, would not.

The product from the *CCD4*-SSR would act as an internal control in the *CCD4* multiplex and helped to avoid false negatives in the case of the SNP and retrotransposon mutations.

5.3. RESULTS

This study used three novel primer sets to detect the three mutations reported in the *CCD4* gene. Two of these primers sets, detecting the *CCD4* microsatellite and the *CCD4* SNP, successfully genotyped 206 peaches, 20 almonds and seven hybrids. The third novel primer

set detecting the presence of a retrotransposon insertion in the *CCD4* was uninformative in this study.

Firstly, the primer *CCD4*-SSR amplifying the polymorphic TC microsatellite region within the *CCD4* gene successfully amplified three unique products (peaks) of 122 bp, 124 bp and 128 bp in peach accessions (Fig. 5.1). The 122 bp was interpreted as the wild type (TC₇) since it was observed in the heterozygous or homozygous state in white fleshed accessions. The 124 bp peak was interpreted as the TC₈ mutant, which induces the frame shift mutation. Another product, of 128 bp, was interpreted as the rare reversion mutant (TC₁₀) only previously reported once (Falchi *et al.*, 2013). Overall, 25 accessions had the 122/122 bp genotype, 138 accessions had the 124/124 bp genotype, 42 had the 122/124 bp genotype and one accessions had the 124/128 bp genotype.

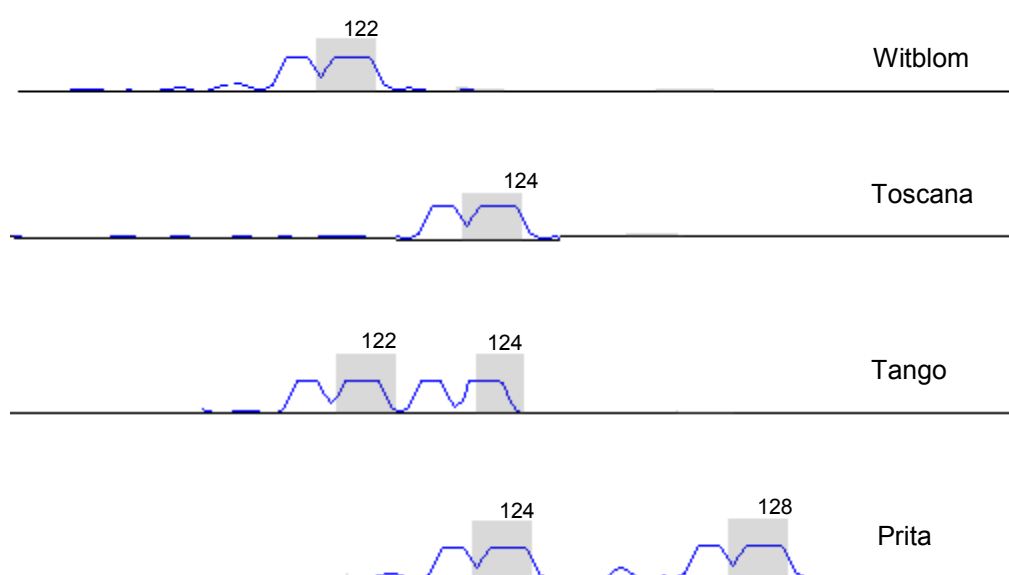


Fig. 5.1 Gene Mapper output showing the peak combinations observed in four peach accessions at the microsatellite region in the *CCD4* gene amplified by the *CCD4*-SSR primer set. 122 bp =TC₇, 124 bp=TC₈, 128 bp=TC₁₀.

Secondly, the primer *CCD4*-SNP that detected the presence of the SNP, successfully amplified in the peach collection. A prominent peak at 297 bp was interpreted as a SNP and the absence of this peak was interpreted as the absence of the SNP (Fig. 5.2). The product of the *CCD4* microsatellite acted as an internal control (Fig. 5.2). Overall, the SNP mutation was detected in 26 accessions.



Fig. 5.2. Gene Mapper output of two peach accessions amplified by primer CCD4-SNP (a) The peak (297 bp) indicating presence of the SNP at the *CCD4* gene (b) The absence of the peak indicating the absence of the SNP at the *CCD4* gene. The amplicons of microsatellite marker CCD4-SSR, which acted as an internal control, are also shown.

The unlabelled CCD4-NoSNP primer pair was only used in accessions that showed the presence of the SNP to determine the copy number of the SNP amplified product (297 bp). In the 26 accessions containing the SNP, amplification was only observed in 24 accessions and failed in two accessions. Thus the 24 accessions had at least a functional allele (heterozygous) while the two accessions had no functional allele (homozygous) because they had the SNP in both alleles (Fig. 5.3).



Fig. 5.3. A subset of ten accessions with the SNP mutation amplified with CCD4-NoSNP primer for the detection of the copy number for the SNP. From left: Scarlet, Snowwhite, Flordaguard, Summer Giant, Sunlite, Tango, Crimson Giant, June Princess, Don Elite and Earli Rose. Amplification of a band at ~ 300 bp means at least one wild type allele (heterozygous). Flordaguard and June Princess show no amplification and are considered to be homozygous for the SNP. Symbols + (band present) and – (band absent) have been added for clarity.

Thirdly, the primer set (CCD4-Retro) designed for detecting the presence of the retrotransposon proved to be uninformative; an unexpected product of 175 bp was seen in all accessions (Fig. 5.4).

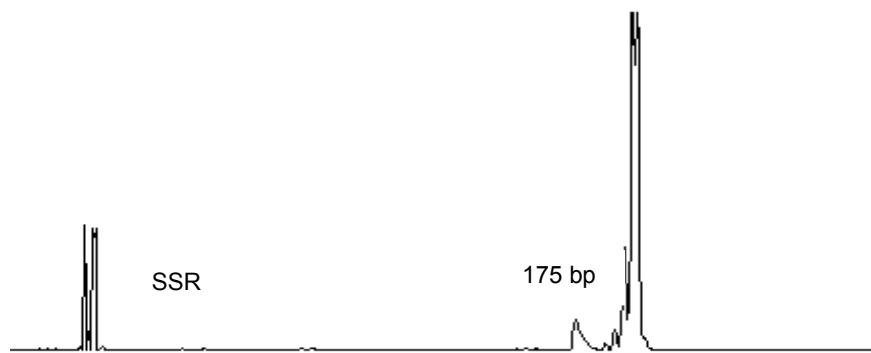


Fig. 5.4. Gene Mapper output for primer CCD4-Retro showing the unexpected 175 bp product observed in all accessions and therefore was uninformative. The CCD4-SSR product acted as an internal control.

The primer pair detecting the absence of the retrotransposon (CCD4-NoRetro); and relevant for detecting copy number of the retrotransposon in accessions where this retrotransposon was detected; was run on few samples for the sake of completeness and amplified products at ~ 300 bp (Fig 5.5). Since the primer pair detecting the retrotransposon was uninformative no data is reported for the retrotransposon event in this study.

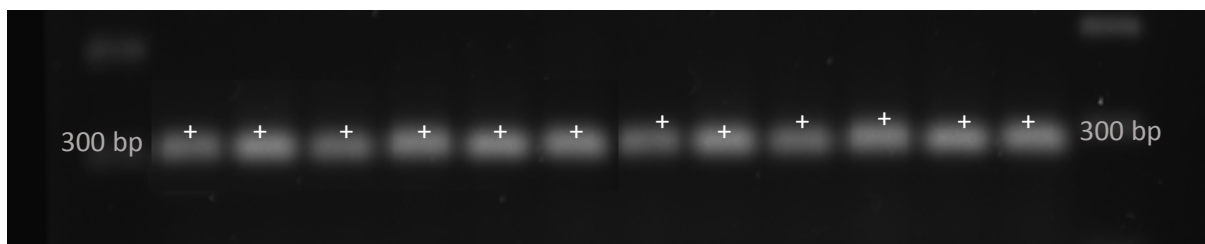


Fig. 5.5. Twelve accessions amplified with CCD4-NoRetro primer for the detection of the absence of the retrotransposon mutation. From left: ARC NE 1, Red Jewel, Crimson Blaze, ARC NE 5, Sun Raycer, Unico, Sunec Twentyone, August Red, ARC NE 2, Earli Grand, Honey Blush (1) and Transvalia. A band (+) indicates the absence of the retrotransposon insertion, and the absence of a band would have indicated presence of the retrotransposon. Symbols + (band present) and – (band absent) have been added for clarity.

The recorded flesh colour phenotypes for the accessions were obtained from the breeding programme records. Of these, 172 accessions were reported as yellow fleshed while 34 were reported as white fleshed. Using this information and the scores from the CCD4 TC microsatellite and SNP data, tentative deduced phenotypes were determined. Thirty-three of the thirty-four reported white flesh peaches could be deduced as likely white fleshed with one accessions being inconsistent with this expectation. Of the 172 reported yellow fleshed accessions, 155 accessions were deduced as likely yellow flesh and seventeen accessions were not consistent with the expected yellow flesh phenotype. Therefore, a total of eighteen accessions did not match their reported flesh colour.

Table 5.3. Genotypes of CCD4 microsatellite and SNP for 206 peach accessions, 20 almonds and seven hybrids for the ARC collection. RC recorded colour, MS CCD4 microsatellite score, A/A no SNP, A/T heterozygous for SNP, T/T homozygous for the SNP, DC deduced colour, Y yellow, W white, YF yellow flesh, WF, white flesh, * Inconsistent between reported and deduced flesh colour.

Accession	RC	MS	SNP	DC	Accession	RC	MS	SNP	DC
<i>Prunus persica</i> (peaches and nectarines)					Catherina	Y	124/124	A/A	YF
2LA336	Y	124/124	A/A	YF	Cauresmillo (1)	W	122/122	A/A	WF
Adriatica	Y	124/124	A/A	YF	Cauresmillo (2)	W	122/122	A/A	WF
Afri Rouge*	Y	122/124	A/A	WF	Cederberg	Y	124/124	A/A	YF
Afri Sun	Y	124/124	A/A	YF	Chuchu Picudo	W	122/122	A/A	WF
African Glo	Y	124/124	A/A	YF	Cinderella	Y	124/124	A/A	YF
Allgold*	Y	122/122	A/A	WF	Classic	Y	124/124	A/A	YF
Alpine	Y	122/124	A/T	YF	Clococlan	Y	124/124	A/A	YF
Annevesarrio	W	122/124	A/A	WF	Clondike White	W	122/124	T/A	WF
April Glo	Y	124/124	A/A	YF	Coconut Ice	W	122/124	A/A	WF
ARC NE 1	Y	124/124	A/A	YF	Corona	Y	124/124	A/A	YF
ARC NE 2	Y	124/124	A/A	YF	Crimson Baby	Y	124/124	A/A	YF
ARC NE 3	Y	124/124	A/A	YF	Crimson Blaze	Y	124/124	A/A	YF
ARC NE 4	Y	124/124	A/A	YF	Crimson Giant	Y	122/124	T/A	YF
ARC NE 5	Y	124/124	A/A	YF	Crimson Glo	Y	124/124	A/A	YF
ARC NE 7	Y	124/124	A/A	YF	Culemborg	W	122/122	T/A	WF
ARC NE 8	Y	124/124	A/A	YF	De Wet	Y	122/124	T/A	YF
ARCNE 9	Y	124/124	A/A	YF	December Princess	Y	124/124	A/A	YF
ARC NE 10	Y	124/124	A/A	YF	Desert Pearl	Y	124/124	A/A	YF
ARC NE 11	Y	124/124	A/A	YF	Desert Sun	Y	122/124	A/A	WF
Arctic Rose*	W	124/124	A/A	YF	Diamond Ray	Y	124/124	A/A	YF
Arctic Snow	W	122/124	A/A	WF	Diamond Zee	Y	124/124	A/A	YF
Arctic Star	W	122/124	A/A	WF	Don Elite	Y	122/124	T/A	YF
Arctic Sweet	W	122/124	A/A	WF	Donna Rosa	Y	124/124	A/A	YF
Arm King	Y	124/124	A/A	YF	Donnarine	Y	124/124	A/A	YF
August Glo*	Y	122/122	A/A	WF	Earli Blush	Y	124/124	A/A	YF
August Pearl	Y	124/124	A/A	YF	Earli Gland	Y	124/124	A/A	YF
August Red	Y	124/124	A/A	YF	Earli Gold	Y	124/124	A/A	YF
Autumn Crunch	Y	124/124	A/A	YF	Earli Rose	Y	122/124	T/A	YF
Autumn Gold*	Y	122/124	A/A	WF	Earli Sun	Y	124/124	A/A	YF
Bella Donna	Y	124/124	A/A	YF	Early Glo	Y	124/124	A/A	YF
Bella Nova	Y	124/124	A/A	YF	Elandia	Y	124/124	A/A	YF
Bella Rosa	Y	124/124	A/A	YF	Elberta*	Y	122/122	A/A	WF
Big Top	Y	124/124	A/A	YF	Excellence	Y	124/124	A/A	YF
Blaze Prince	W	122/124	A/A	WF	Fairtime	Y	124/124	A/A	YF
Bokkeveld	Y	124/124	A/A	YF	Fantasia	Y	124/124	A/A	YF
Bonnigold	Y	124/124	A/A	YF	Fantasy	Y	124/124	A/A	YF
Britaney Lane	Y	124/124	A/A	YF	Fiesta Red	Y	124/124	A/A	YF
Cascade	Y	124/124	A/A	YF	Fire Rich	Y	124/124	A/A	YF

Accession	RC	MS	SNP	DC	Accession	RC	MS	SNP	DC
Fire Sweet	Y	124/124	A/A	YF	Nemaguard 7	W	122/122	A/A	WF
Flame Kist	Y	124/124	A/A	YF	Nemared	W	122/122	A/A	WF
Flavor Crest	Y	124/124	A/A	YF	Nemasun*	Y	122/122	A/A	WF
Flavorine	Y	124/124	A/A	YF	Nova Donna	Y	124/124	A/A	YF
Flavortop	Y	124/124	A/A	YF	Ohatsumomo	Y	124/124	A/A	YF
Flordagold	Y	124/124	A/A	YF	Oom Sarel	Y	124/124	A/A	YF
Flordaguard	Y	122/122	T/T	YF	Oribi	Y	124/124	A/A	YF
FP-1	Y	124/124	A/A	YF	Orion (1)	W	122/122	A/A	WF
Golden Dawn	Y	124/124	A/A	YF	Orion (2)	W	122/122	A/A	WF
Goud Myn	Y	124/124	A/A	YF	Pe 9329	Y	124/124	A/A	YF
Guardian	W	122/122	A/A	WF	Pintoo	W	122/124	A/A	WF
Gugliemina	Y	124/124	A/A	YF	Primrose	Y	122/124	T/A	YF
Hantam	Y	124/124	A/A	YF	Prita	W	124/128	A/A	WF
Honey Blush (1)	Y	124/124	A/A	YF	Prof Malherbe	Y	124/124	A/A	YF
Honey Blush (2)*	Y	122/124	A/A	WF	Prof Neethling	Y	124/124	A/A	YF
Horizon*	Y	122/124	A/A	WF	Red Jewel	Y	124/124	A/A	YF
Impala	Y	124/124	A/A	YF	Red Velvet	Y	122/124	T/A	YF
Imperani	Y	124/124	A/A	YF	Regina Bianca	W	122/122	A/A	WF
Impora	Y	124/124	A/A	YF	Rich Lady	Y	124/124	A/A	YF
Jim Dandy*	Y	122/124	A/A	WF	Robin White	W	122/122	A/A	WF
Jubilee	Y	124/124	A/A	YF	Rolees*	Y	122/124	A/A	WF
June Princess	Y	122/124	T/T	YF	Royal Gem	Y	124/124	A/A	YF
Kakamas (1)	Y	124/124	A/A	YF	Royal Glo	Y	124/124	A/A	YF
Kakamas (2)	Y	124/124	A/A	YF	Ruby Prince	Y	124/124	A/A	YF
Kateru	W	122/122	A/A	WF	Ruby Rose	Y	122/124	T/A	YF
Keimoes	Y	124/124	A/A	YF	Ruby Sweet	Y	124/124	A/A	YF
Keisie	Y	124/124	A/A	YF	Safari	Y	124/124	A/A	YF
Klara	Y	124/124	A/A	YF	San Pedro	Y	124/124	A/A	YF
Koks Laat	Y	124/124	A/A	YF	Sandvliet	Y	124/124	A/A	YF
Late Fair	Y	124/124	A/A	YF	Sapo778	Y	124/124	A/A	YF
Late Venus	Y	124/124	A/A	YF	Scarlet	Y	124/124	T/A	YF
LNR08A	W	122/122	A/A	WF	September Free	Y	124/124	A/A	YF
LNR08B	W	122/122	A/A	WF	September Red*	Y	122/124	A/A	WF
Lovell (1)	Y	124/124	A/A	YF	Siberian C1	Y	124/124	A/A	YF
Lovell (2)*	Y	122/122	A/A	WF	Siberian C2 (1)	Y	124/124	A/A	YF
Margaret's Pride	Y	122/124	T/A	YF	Siberian C2 (2)	Y	124/124	A/A	YF
Maria Dolce*	Y	122/124	A/A	WF	Silver Fire	W	122/124	T/A	WF
May Glo	Y	124/124	A/A	YF	Snow Crest	W	122/124	T/A	WF
May Kist	Y	122/124	T/A	YF	Snowwhite	W	122/124	T/A	WF
Monate	Y	124/124	A/A	YF	Sonette	Y	124/124	A/A	YF
Mystic Magic	W	122/124	A/A	WF	Southern Glo	Y	124/124	A/A	YF
Naledi*	Y	122/124	A/A	WF	Sparkle	Y	124/124	A/A	YF
Nectar	Y	122/124	T/A	YF	Spring Baby	Y	124/124	A/A	YF
Nectaross	Y	124/124	A/A	YF	Spring Crest	Y	124/124	A/A	YF
					Star Dust	Y	124/124	A/A	YF

Accession	RC	MS	SNP	DC	Accession	RC	MS	SNP	DC
Stark Sunglo*	Y	122/124	A/A	WF	Carmel (1)	N/A	122/122	A/A	N/A
Summer Early	Y	122/124	T/A	YF	Carmel (2)	N/A	122/122	A/A	N/A
Summer Giant	Y	122/124	T/A	YF	El Fahem	N/A	122/122	A/A	N/A
Summer Gold	Y	124/124	A/A	YF	Ferragnes (1)	N/A	122/122	A/A	N/A
Summer Jewel	Y	124/124	A/A	YF	Ferragnes (2)	N/A	122/122	A/A	N/A
Summer Prince	W	122/124	A/A	WF	Ferraster	N/A	122/124	A/A	N/A
Summer Rich	Y	124/124	A/A	YF	Ne Plus Ultra (1)	N/A	122/122	A/A	N/A
Summertime	Y	124/124	A/A	YF	Ne Plus Ultra (2)	N/A	122/122	A/A	N/A
Sun Burst	Y	124/124	A/A	YF	Non Pareil (1)	N/A	122/122	A/A	N/A
Sun Crest	Y	124/124	A/A	YF	Non Pareil (2)	N/A	122/122	A/A	N/A
Sundry	Y	124/124	A/A	YF	Padre	N/A	122/122	A/A	N/A
Sun Grand	Y	124/124	A/A	YF	Paper Shell	N/A	122/122	A/A	N/A
Sun Raycer	Y	124/124	A/A	YF	Peerless (1)	N/A	122/122	A/A	N/A
Sun Sweet	Y	124/124	A/A	YF	Peerless (2)	N/A	122/122	A/A	N/A
Sunec Twentyone	Y	124/124	A/A	YF	Price (1)	N/A	122/122	A/A	N/A
Sunking	Y	124/124	A/A	YF	Price (2)	N/A	122/122	A/A	N/A
Sunlite	Y	122/124	T/A	YF	Sutter	N/A	122/122	A/A	N/A
Sunray	Y	124/124	A/A	YF	Texas Mission	N/A	122/122	A/A	N/A
Supec Fifteen	Y	124/124	A/A	YF	Peach x almond hybrids				
Supec Six	Y	122/124	T/A	YF	Adarcias	N/A	122/122	A/A	N/A
Super Rich	Y	124/124	A/A	YF	Adefuel	N/A	122/124	A/A	N/A
Supreme	Y	124/124	A/A	YF	GF 677	N/A	122/122	A/A	N/A
Sweet December	Y	124/124	A/A	YF	Prunus hybrids				
Sweet September*	Y	122/124	A/A	WF	Atlas	N/A	122/122	A/A	N/A
Tango	Y	122/124	T/A	YF	Cadaman	N/A	122/122	A/A	N/A
Temptation	Y	124/124	A/A	YF	Ferciana	N/A	122/122	A/A	N/A
Toscana	Y	124/124	A/A	YF	Ferdor	N/A	122/124	A/A	N/A
Transvalia	Y	124/124	A/A	YF					
Tsukuba4 (5)	W	122/122	A/A	WF					
Tsukuba4 (6)	W	122/122	T/A	WF					
Tsukuba 5	W	122/122	T/A	WF					
Uf Sun	Y	124/124	A/A	YF					
UFO	W	122/122	A/A	WF					
Unico	Y	124/124	A/A	YF					
Walgant	Y	124/124	A/A	YF					
Waveren	Y	124/124	A/A	YF					
Western Cling	Y	124/124	A/A	YF					
Western Sun	Y	124/124	A/A	YF					
Witblom	W	122/122	A/A	WF					
Zaigina	Y	124/124	A/A	YF					
Prunus dulcis (almonds)									
Butte (1)	N/A	122/122	A/A	N/A					
Butte (2)	N/A	122/122	A/A	N/A					

The SNP and the TC microsatellite mutations were also observed to occur together in this study. Twenty-one accessions with TC₇/TC₈, four accessions with TC₇/TC₇ and one accession with TC₈/TC₈ all also had the SNP mutation.

The study also included some 20 almonds and 7 *Prunus* hybrids for investigation purposes (Table 5.6). The almonds' mesocarp is whitish. The wildtype CCD4 microsatellite (122 bp) was observed in nearly all almonds and only once was a 124 bp mutant allele observed. In the hybrids, the wildtype microsatellite also showed predominantly the wildtype 122 bp allele and the 124 bp was only observed in one peach-almond hybrid ('GF 677') and complex hybrid ('Ferdor'). No SNP mutation was observed in either the almonds or the hybrids.

5.4. DISCUSSION

This study genotyped 206 peaches, 20 almonds and seven hybrids belonging to the ARC germplasm collection for the *CCD4* gene responsible for the flesh colour using new primer sets. The duplex of CCD4-SSR and CCD4-SNP successfully genotyped the peach accessions in this study while the CCD4 primer set for detecting the retrotransposon mutation was uninformative. Most of these primers detecting the presence of the mutations were fluorescently labelled and amplified products < 500 bp suitable for automated sizing.

This study detected length polymorphism at the TC microsatellite region of the *CCD4* gene that was first reported in previous studies (Adami *et al.*, 2013; Falchi *et al.*, 2013; Fukamatsu *et al.*, 2013). The wild type allele (TC₇) was identified as the 122 bp amplicon and its mutant as the 124 bp (TC₈) amplicon. The TC₇ allele results in white flesh while the TC₈ allele causes a frameshift mutation; resulting in yellow flesh. Overall, 139 accessions had the TC microsatellite mutation. Moreover, in this study the very rare TC microsatellite mutation with 10 TC repeats (TC₁₀) was found in 'Prita'. This mutation results in the restoration of normal gene function resulting in white flesh. This mutation has only been reported once previously, in a white fleshed peach cultivar 'Silver King', the seedling of a yellow fleshed cultivar 'Arm King' (Falchi *et al.*, 2013). Interestingly, 'Prita' is also a seedling of 'Arm King'. 'Arm King' itself, has no TC₁₀ mutation (Falchi *et al.*, 2013). The source of the mutation in both 'Silver King' and 'Prita' is unknown since the pollen parent of both these cultivars is unknown. It could be interesting to investigate whether 'Prita' and 'Silver King' could have been mixed up during the unconventional introduction of some foreign cultivars to South Africa. Since 'Silver King' is not available in the ARC collection, DNA should be acquired from elsewhere to make comparisons.

The *CCD4* microsatellite primer pair also amplified the TC microsatellite regions in *CCD4* gene in almonds. Only the wild type TC₇ allele was detected with the exception of 'Ferraster' in which the TC₈ allele was also observed. The predominance of the wild type TC₇ in almonds suggests that the common ancestor of peaches and almonds was likely white fleshed with an active *CCD4* gene. Detection of the TC₈ allele in the almond accession 'Ferraster' further suggests that the TC microsatellite region in the *CCD4* gene may have mutated independently in almond. 'Ferraster' itself is the progeny of crosses between 'Cristomorto' (Italian cultivar) and 'Ardechoise' (French cultivar) and it could be of interest to check the genotypes of these parent cultivars. The literature review does not indicate any reported yellow fleshed almond cultivars.

The *CCD4* microsatellite mutant allele (TC₈) was also detected in a peach-almond hybrid ('GF 677') and complex *Prunus* hybrid ('Ferdor') along with the wild type allele. The TC₈ in 'GF 677' may have arisen from the peach parent or the almond and cannot be independently confirmed. The detection of the TC₈ in the complex *Prunus* hybrid 'Ferdor' may suggest the need for a follow up study into the role of *CCD4* gene or its orthologs in other *Prunus* species. For instance, the carotenoid influenced colours (orange and yellow) can also be observed in apricots while in cherry and plum show flesh colour is whitish.

The study also detected the SNP mutation in 26 peach accessions. The SNP was predominantly heterozygous except for two accessions ('Flordaguard' and 'June Princess') where it was present in the homozygous state. Thus, the SNP mutation occurred fewer times than the TC microsatellite mutation, and this is consistent with other studies (Adami *et al.*, 2013; Falchi *et al.*, 2013).

The SNP and the TC microsatellite mutations were also observed together in this study. Twenty-one accessions with TC₇/TC₈, four accessions with TC₇/TC₇ and one accession with TC₈/TC₈ had the SNP mutation.

The SNP occurred either in the wild type (TC₇) or the mutant (TC₈) allele. For instance, 'Crimson Giant' (yellow fleshed) and 'Clondike White' (white fleshed) have the same TC₇/TC₈ genotype and are heterozygous for the SNP. In 'Crimson Giant', the SNP is presumably in the TC₇ while in 'Clondike White' the SNP is presumably in TC₈. Another example is the white flesh cultivar 'Robin White' (TC₇/TC₇) with no SNP mutation and the yellow fleshed cultivar 'Flordaguard' (TC₇/TC₇) which is homozygous for the SNP (T/T). The SNP mutation in 'Flordaguard' presumably occurs in both alleles thus resulting in yellow flesh despite having the TC₇ wild type that typically results in a white flesh colour.

The SNP mutation was not detected in the almonds or the hybrids and may be a more recent mutation that occurred after the speciation of peaches and almonds.

The primer pair designed to amplify the retrotransposon insertion in the *CCD4* gene amplified a uniform product that was of an unexpected length. This uniform product suggests that the primer pair amplified a different region that is common in all accessions and unrelated to the *CCD4* gene. This primer pair must therefore be redesigned before it can be useful to genotype the retrotransposon. The lack of the retrotransposon data makes the characterizations in this study, tentative.

The presence of the three independent mutation events at the *CCD4* gene may indicate the need to consider them as haplotypes. The nomenclature of the haplotypes have not be done previously and considering all the known variants the following twelve haplotypes are proposed by the current study (Table 5.7). However, some of these haplotypes are likely theoretical due to the probability of multiple mutations occurring simultaneously in an allele. The description of the haplotypes provides an opportunity for the development of markers that can genotype the various mutations and genotype the combinations in a few reactions. The absence of retrotransposon data prevents this study from allocating the haplotypes.

Table 5.7. Twelve haplotypes for the *CCD4* gene for the three mutations. SSR CCD4-SSR, SNP CCD4-SNP, Retro CCD4-Retrotransposon, TC₇ wild type, TC₈ mutant, AA no SNP, AT heterozygous for SNP, TT homozygous for SNP, NN no retrotransposon, RN heterozygous for retrotransposon, RR homozygous for retrotransposon, FC flesh colour phenotype, WF white flesh, YF yellow flesh.

Haplotype	SSR	SNP	Retro	FC	Haplotype	SSR	SNP	Retro	FC
Haplotype 1	TC ₇	A	N	WF	Haplotype 7	TC ₈	T	N	YF
Haplotype 2	TC ₇	A	R	WF or YF	Haplotype 8	TC ₈	T	R	YF
Haplotype 3	TC ₇	T	N	WF or YF	Haplotype 9	TC ₁₀	A	N	WF
Haplotype 4	TC ₇	T	R	WF or YF	Haplotype 10	TC ₁₀	A	R	WF or YF
Haplotype 5	TC ₈	A	N	YF	Haplotype 11	TC ₁₀	T	N	WF or YF
Haplotype 6	TC ₈	A	R	YF	Haplotype 12	TC ₁₀	T	R	WF or YF

The eighteen accessions that were shown to be inconsistent with expected flesh colour were followed up with the peach breeder using leaf senescent colour in the orchard, and the observations of these accessions seemed to match the phenotypes deduced from the CCD4-SSR and CCD4-SNP genotypes. However, the observations could not be conclusive as senescent leaf colour observations were tricky and inconclusive at the time of the

observations were conducted. Therefore, further follow up has been recommended for the breeder during the coming fruit season.

The knowledge of the genotypes of various alleles and mutations in the *CCD4* gene is of great importance to the breeder. This information is relevant in the designing of the crosses to develop peach progenies with yellow or white flesh. Therefore, it is important that fluorescently labelled primers for detecting the retrotransposon be redesigned and used to complete the genotyping of the *CCD4* gene in the ARC accessions.

5.5. CONCLUSION

The current study genotyped the *CCD4* gene with regards to the microsatellite and the SNP alleles in 206 peaches, 20 almonds and seven hybrids. The data generated are of use to the breeders in designing crosses for developing cultivars of specific flesh colour.

The genotypes in this study form a foundation for further flesh colour studies in the accessions belonging to the ARC collection. Of interest to the breeder is the level of whiteness in the flesh; some accessions have white flesh colour whereas others are 'creamy' which affects the perception of quality from consumers. Building on this work, this phenomenon could be investigated. It is possible that the *CCD4* gene or its ortholog also control flesh colour in other *Prunus* fruit such as apricots, cherry and plums. Therefore, the role of the *CCD4* gene in other *Prunus* species should be investigated as a follow up to this study.

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CHAPTER 6: GENERAL DISCUSSION AND FUTURE CONSIDERATIONS

6.1. INTRODUCTION

Peaches (*Prunus persica*) are an economically significant deciduous fruit in South Africa along with apples, pear, grapes and plums. A ZAR 800 million industry, the South African peach industry supports 10,000 jobs. The industry must continue to plant cultivars that meet the consumers' ever-changing preferences and that can adapt to the phenomenon of climate change.

The breeding programme at the Agricultural Research Council (ARC), now over 40 years old, has been the leader in the peach cultivar development and the peaches exported from South Africa are predominantly of cultivars developed at the ARC. The programme maintains collections of peaches, related *Prunus* species and hybrids in the germplasm collection at Bien Donne Research Farm (Paarl, Western Cape). This germplasm collection, which is the raw material for the new cultivars, has been characterized only using morphological features. Nowadays, reliable and cost-effective microsatellites are available for fingerprinting accessions to ascertain trueness of type and eliminate misidentifications. Moreover, functional markers can be used to determine cultivar genotypes for various traits that affect fruit usage and consumer preference. These traits include, but are not limited to, flesh colour, flesh texture, and hairiness of the fruit epidermis. The information obtained from genotyping the germplasm for such traits is useful for breeders when designing crosses.

This study represents the first time molecular markers were used to fingerprint the ARC peach collection and characterize it with respect to hairy epidermis (peach or nectarine) and flesh colour (white or yellow).

6.2. MICROSATELLITE FINGERPRINTING OF THE PEACH GERmplasm

This study used nine microsatellite markers in three triplexes to fingerprint the germplasm collection. One primer was not investigated further as it amplified multiple alleles; thus eight markers successfully fingerprinted 206 peach accessions. However, the study also identified 58 accessions including 18 from the reference collection that could be duplicated, closely related or misidentified. There is the need for further follow up on these accessions for example by adding more primers to increase the number of loci compared as well as morphological comparisons. This approach will definitively elucidate the observations and action can be taken to resolve the findings.

The fingerprinting of almond and hybrids was also successful although one of the markers used in the peaches failed to amplify successfully in almonds and hybrids so that seven microsatellite markers were used to fingerprint the 20 almonds and seven hybrids. Overall, the markers used in this study were selected from the literature where they were reported as

polymorphic and spread throughout various linkage groups in the genome. Unlike other deciduous fruits such as apples and pears, there is no standard set of primers used by all the laboratories in peaches. The multiplexing of these primers proved to be cost effective in fingerprinting of the germplasm.

The molecular tools for fingerprinting germplasm change as new technologies emerge and it is therefore necessary to keep up with developments to remain competitive. Two alternatives to fingerprinting with microsatellites include single nucleotide polymorphism (SNP) and next generation sequencing (NGS). SNP genotyping differentiates individual on the basis of single nucleotide differences. The most common SNP technique used for genotyping is the SNP chip or microarrays. SNP markers used for genotyping have the following advantages: presence of SNPs in coding area of DNA that directly affect protein function, high throughput genetic analysis (more than microsatellites) and stable inheritance at a locus. The next generation sequencing (NGS) technology enables the reading of all the sequences in an individual's genome (Varshney *et al.*, 2009; Metzker *et al.*, 2010). This process generates massive amounts of sequence data. The use of sequence reads to genotype accessions is commonly referred to as genotyping by sequencing (GBS), an approach which can discriminate accessions correctly, identify alleles and resolve duplicate accessions (Egan *et al.*, 2012). There are various sequencing techniques that are continuously being improved and increasingly getting cheaper so much so that there is a race to develop a very low cost effective genome sequencing methods (Hayden, 2014; Christensen *et al.*, 2015). Prior to NGS, only a few genomes of model organism were available for genetic research but now new genomes are being published frequently and sequences are available for studying various traits (Smith, 2016). Though this technology is already being used, a number of disadvantages exist (Varshney *et al.*, 2009). The most prominent disadvantage include the extremely large data sets generated by sequencing which in turn require advanced computational and statistical tools which may not be readily available to a common breeder.

6.3. GENOTYPING THE PEACH/NECTARINE TRAITS IN THE ARC'S PEACH COLLECTION

The study successfully genotyped the *MYB25* gene responsible for hairy or glabrous epidermis in 206 peaches using a novel indelG primer set amplifying the third exon of the gene and giving products small enough to be detected on the automated sequencer. The primer detected the absence of a retrotransposon (*G* allele) or presence of the retrotransposon (*g* allele). All the peaches were homozygous, *GG*, or heterozygous, *Gg*; while nectarines were homozygous *gg*. Of interest, is the 35 accessions genotyped as heterozygous (*Gg*) since they can be crossed to develop new nectarine cultivars from peach cultivars. This study is the first

time fluorescently labelled *MYB25* primers have been used to characterize peaches and nectarine genotypes using an automated sequencer. In a previous study (Vendramin *et al.*, 2014) the primers amplified large amplicons that were visualised on agarose gel, an approach that was laborious, imprecise and not suitable for large number of samples.

The primer set also amplified the *G* allele in almonds; thus indicating the absence of the retrotransposon in the third exon of the *MYB25* gene in almonds consistent with the presence of hairs of the almond fruit epidermis. The presence of *G* allele in almond suggests that the common ancestor of the peach and almond were likely hairy with an active *MYB25* gene. This finding also poses the question as to the role of the *MYB25* gene in other *Prunus* species such as apricot (hairy), plum (glabrous) and cherry (glabrous). A follow up study is highly recommended.

6.4. GENOTYPING FLESH COLOUR IN THE ARC'S PEACH COLLECTION

This study used a novel multiplex of primer sets to detect the various genotypes of the *CCD4* gene at the *Y* locus for the flesh colour. The primer pair amplifying the microsatellite region in the *CCD4* gene successfully characterized the wild type allele (TC₇), frameshift mutant (TC₈) and a rare reversion mutant (TC₁₀). The reversion mutant was observed in only one accession ('Prita') while the other alleles were common. The primer set amplifying the A to T substitution (SNP) successfully detected the mutation in 26 accessions and two accessions of these were confirmed to be homozygous for the SNP (mention again TT for example). The primer set detecting the presence of the retrotransposon was not informative. Therefore, the characterization of the flesh colour is based on the *CCD4* microsatellite and the SNP, an approach which is fairly informative, but the deduced phenotypes should be viewed as tentative until the retrotransposon data is generated with newly designed primers. This study also showed that almonds have an active *CCD4* gene and the wild type TC₇ allele was observed frequently while the mutant TC₈ allele occurred just once. This is significant because it may suggest that the common ancestor to the peach and almond may have been white fleshed with an active *CCD4* gene. It can also be inferred that the *CCD4* microsatellite region may be an active region for mutations. The SNP was not detected in any almonds suggesting that it may be a more recent mutation. The results in the few hybrids used in this study is consistent with the above conclusions and ask the question of the role of *CCD4* gene in other *Prunus* species *i.e.* apricot, plum and cherry. Therefore, an investigation into the *CCD4* gene in these species is highly recommended. The data generated for the *CCD4* gene, although tentative, is still relevant for the designing of crosses.

6.5. CURATION OF THE ARC PEACH GENE BANK

The fingerprinting of the peach collection for the ARC is the first step in implementing some aspects of molecular breeding. The fingerprints generated in this study will be incorporated into a database that can be used by the breeders and the South African peach industry. Fingerprinting information is useful for authentication and for checking parentages and when repropagating. Verification with fingerprints is cheaper than verifying the new trees phenotypically over several years. The fingerprints can also supplement data gathered in accord with the UPOV guidelines in matters of breeder's rights if such an approach is adopted by the Department of Agriculture, Forestry and Fisheries (DAFF). Thirty-one accessions, including nineteen accessions from the reference collection, need to be followed up, as they could be misidentified and were unresolved with the primer sets used in this study. An increase in the number of primers combined with the comparison of morphological traits of these accessions should clarify the observations. This could not be done in this study due to time constraints but should be undertaken in the next growing season, in spring for floral traits and summer for fruit traits.

The characterization of agronomic traits *i.e.* peach/nectarine trait and flesh colour and the identification of the heterozygotes will be useful for guiding the breeder during selection of parents. Breeders can use the data from this study to design specific crosses with respect to these traits.

The sequences responsible for the various other agronomic traits have been determined and in the future primers can be designed to genotype accessions in the ARC breeding programme. One of these is *CAD1* (constitutively activated cell death 1) which determines the flat vs round shape in peach (Cao *et al.*, 2016). Flat peach is dominant over round peach. An *A/T* polymorphism in the fifth intron of the *CAD1* gene is associated with this trait with *A* (flat) genotypes dominant over *T* genotypes (round); thus *A/A* and *A/T* results in flat peaches and *T/T* resulting in round peach. Another trait of interest is the maturing date (MD) which is also a simple gene trait (Pirone *et al.*, 2013). A recent study identified the gene for the NAC transcription factor *ppa008301m* as the determinant. The sequencing of this gene showed that the allelic variant for the early maturing accessions had an extra 9 bp insertion in the exon of the gene which was clearly absent in all the late maturing allelic variants. Primers for genotyping have been developed and can be acquired. These primers give large products and can only be visualised on agarose gels. There is therefore an opportunity to redesign these primers to obtain smaller amplicons suitable for fluorescent labelling. The genotyping of accessions for MD is of use to breeders as they can develop cultivars with varying maturing times thus take advantage of the global warming as well as global markets.

6.6. MARKER ASSISTED SELECTION OF SEEDLINGS

Since the new primers for the various traits in this study have genotyped the various accessions successfully, marker assisted selection of seedlings can be implemented on a small scale. Using the primer sets, the seedlings can be genotyped and those that inherited the alleles indicating the desired traits can be kept and the others discarded. This study lays the foundation upon which other traits can be investigated using a similar approach and in the near future seedlings or parents of new cultivars can be screened for various traits. This approach would save the resources including money by focusing on only the accessions with desired traits as determined by markers.

6.7. LIMITATIONS TO THE STUDY

The fingerprinting of the accessions had two main limitations. Firstly, one of the reportedly polymorphic markers amplified multiple loci and another amplified poorly in almonds and hybrids and had to be discarded. This led to fingerprinting of the accessions with only eight markers for the peach collection, and seven for the almonds and hybrids. This limited number of markers may be the cause of the failure to distinguish some of the accessions. Most published studies in peach used about fifteen or more markers to resolve the genotypes. Therefore, more markers have been identified and are recommended for further fingerprinting as a follow up to this study. Secondly, there is no standard microsatellite marker panel for fingerprinting peaches, in contrast to apples and pears where standard sets exist. This makes the comparison of data from different laboratories difficult.

In terms of characterization of the traits, there was a limitation in genotyping the retrotransposon mutation in the *CCD4* gene. The primer set for detecting the mutation amplified a uniform product in all products, which was interpreted as unrelated to the expected results. Therefore, the accessions were characterized only in terms of the *CCD4* microsatellite and SNP mutation, resulting in deduced phenotypes that are mostly tentative until the retrotransposon data is generated. Therefore, this primer set should be redesigned and the data set completed.

6.8. FUTURE CONSIDERATIONS

This current study provides a framework for future work in the peach collection. Extracted DNA is available for further characterization. The approaches of characterizing accessions with various primers can be applied to other traits such as fruit shape (Cao *et al.*, 2016) and maturing date (Pirona *et al.*, 2013) and for stone adhesion (Peace *et al.*, 2005, 2007; Gu *et*

al., 2016). The characterization of agronomic traits in the germplasm will be useful in the effective utilization of the available accessions.

Since germplasm is the raw material for every breeding programme, the fingerprinting and genotyping of accessions represents the foundation of an exemplary breeding programme. Authenticated material is important for the breeding programme and the peach fruit industry. The fingerprinting approach could be used in determining Plant Breeder Rights and could be used to supplement the characterization of new cultivars using UPOV guidelines as currently done by DAFF.

The assembled genotypes for the flesh colour and peach/nectarine trait provides parents that are characterized and available for designing crosses to give predictable results. The use of the fluorescently labelled markers and their multiplexing is shown to be a cost effective and reliable method for genotyping. The data generated will be organized into a database that is accessible to breeders. Therefore, this study is an example for similar studies involving peach germplasm.

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