Fingerprinting and Molecular Characterisation of ARC's Apricot and Plum Collection

by

Thembeka Amanda Nyawo

Submitted in partial fulfilment of the requirements for the degree

Master of Science in Genetics

Department of Genetics

Faculty of Science

Stellenbosch University

Stellenbosch

Supervisor:

Prof R Roodt-Wilding

Department of Genetics, Stellenbosch University, South Africa

Co-supervisors:

Mr KR Tobutt* and Dr ED Louw**

*Agricultural Research Council (Infruitec-Nietvoorbij), Stellenbosch, South Africa

**Department of Horticultural Science, Stellenbosch University, South Africa

December 2017

Declaration

By submitting this thesis electronically, I Thembeka Amanda Nyawo, hereby declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2017

Acknowledgements

I am very grateful to God for the opportunity to further my studies, the learning experiences and the wisdom. Without Him by my side all the way, I would not even be alive.

I would like to express gratitude to my supervisors Prof Rouvay Roodt-Wilding and Dr Esmé Louw for the guidance, constructive criticism and patience. Thank you for assisting me with various aspects of my work.

To Ken Tobutt, thank you for not just being a supervisor but for also being a caring mentor. I have learnt a lot from being under your supervision and thank you for your teachings that will be useful throughout my career.

I would also like to thank the ARC-Infruitec Nietvoorbij staff for welcoming me into their lab and always being happy to advise on work related challenges.

Thank you to Carl Hortsmann and Khashief Soeker for your willingness to assist with lab work and advice.

To my parents and siblings, thank you for supporting me in every way possible. I would not have come this far without you.

Thank you to the Bien Donne family for the memories and support, you made my stay enjoyable.

Abstract:

FINGERPRINTING AND MOLECULAR CHARACTERISATION OF THE ARC APRICOT AND PLUM COLLECTIONS

In South Africa apricot (*Prunus armeniaca*) and plum (*Prunus salicina*) production forms part of the economically important stone fruit industry, which is mainly situated in the Western Cape, Northern Cape and the Eastern Cape. Cultivars of main importance to the industry are primarily supplied by the Cultivar Development Division of the Agricultural Research Council Infruitec-Nietvoorbij. The ARC produces new and improved apricots and plums through the breeding of cultivars and selections maintained in the stone fruit germplasms held at Bien Donne experimental farm in the Western Cape. Visual inspection of the gene banks has revealed mislabelling/mis-identification of cultivars and inefficient record keeping of the genetic information of the available cultivars. It is therefore essential to fingerprint and characterise the gene banks on a molecular level, in order to confirm trueness to type of the cultivars and to confirm parentages.

A set of microsatellite primers designed from peach were used for fingerprinting 106 apricot and 95 plum accessions. Ten (in apricot) and eight (in plum) of the microsatellite primers were grouped into four multiplexes and were successfully used to determine the fingerprints. The obtained data was used as a starting point for comparing fingerprints of apricot and plum cultivars. In apricot, all reported parentages were confirmed to be true; however in plum one accession was found not to be related to the reported parents. Trueness to type was determined by evaluating the genetic relationship using UPGMA cluster analysis, where by four apricot cultivars were identified as false.

The self-incompatibility genotypes of the apricot and plum collections were evaluated through the first and second intron amplification of the *S-RNase* gene using consensus primers. Furthermore, allele-specific *SFB* primers were used to distinguish self-compatible cultivars. In apricot, 14 PCR products were amplified corresponding to 14 previously published *S*-alleles. In plum, amplification of nine *S*-alleles was observed. Self-compatible apricots displaying the *Sc* allele were confirmed in 70 accessions. The self-compatibility *S*-allele (*Se*) in plum was identified in 39 accessions. Two cultivars were also observed that were self-compatible but which did not have the Se allele; indicating the possibility of another source of the self-compatibility phenotype.

The findings of this study, which confirmed trueness to type as well as parentages of the cultivars, provides confidence for the breeders when planning crosses. The molecular fingerprints identified in this study also have the potential of being used as a database for cultivar comparison. In terms of the

SI genotypes identified, the findings provide some level of certainty for the commercial farmers to expect good yield and provides information which assists in orchard planning, provided that they plant cultivars with different SI genotypes or self-compatible cultivars.

List of Abbreviations

%	Percentage
°C	Degrees Celsius
µl	Microlitre
µM	Micromolar
3’	Three Prime
5’	Five Prime
A	Adenine
AFLP	Amplified Fragment Length Polymorphism
ARC	Agricultural Research Council
bp	Base pair
C	Cytosine
CTAB	Cetyltrimethylammonium Bromide
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetra-acetate
g	Gram
G	Guanine
GSI	Gametophytic Self-Incompatibility
ha	hectares
He	Expected heterozygosity
Ho	Observed heterozygosity
HWE	Hardy-Weinberg Equilibrium
I	Shannon’s information index
ISSR	Inter-Simple Sequence Repeats
kb	Kilo-bases
MAS	Marker-Assisted Selection
min	Minutes

ml	Millilitre
mM	Millimolar
m/v	Mass per volume
Na	Number of alleles
PCR	Polymerase Chain Reaction
RAPDs	Randomly Amplified Polymorphic DNA
SAPO	South African Plant improvement Organisation
SI	Self-Incompatibility
SC	Self-Compatibility
SSR	Simple Sequence Repeats
ssp	Subspecies
T	Thymine

Table of Contents

List of Tables

List of Figures

Fig. 2.2. The structural representation of the *S-RNase* gene in *Prunus*, including the signal peptide (SP), the first and second intron, the five conserved regions (C1, C2, C3, C4 and C5) and the rosaceous hyper-variable region (HV) (Sonneveld *et al*., 2003). The arrows indicate primer binding sights for the two introns and the exons are represented by the blue lines.

Fig. 2.3. Structure of *SFB* gene in *Prunus* indicating the conserved F-box motif at the 5' end, two variable (V1, V2) and two hyper-variable (HVa , HVb) regions and an intron positioned in the 5' region (Ikeda *et al*., 2004; Nunes *et al*., 2006).

Fig. 3.1a. Preliminary MICRO-CHECKER results for the marker UDP98022 scored I diploid apricot, indicating excess homozygotes and evidence of possible null alleles. Similar results were observed for markers UDP98409; UDP98412; BPPCT025; BPPCT007 and UDP96005.

Fig. 3.1b. MICRO-CHECKER output for the marker UDP98412 scored in diploid apricot, indicating no evidence of mis-scoring, no allele drop-out and no null alleles. Similar results were observed for markers CPPCT044; CPDCT045; BPPCT001 and CPPCT006.

Fig. 3.2. Dendrogram obtained by UPGMA cluster analysis in MEGA, indicating similarities among ARC apricot cultivars and selections.

Fig. 3.3. MICRO-CHECKER output for the marker UDP98412 scored in diploid plums, indicating no evidence of mis-scoring, no allele drop-out and no null alleles.

Fig. 3.4. Dendrogram constructed from UPGMA cluster analysis output of the microsatellite data from the ARC plum accessions, showing the relationships between the accessions.

Fig. 4.1. Non-scale schematic structure of the two genes in the *S*-locus*, S-RNase* and *SFB*. Indicated are the annealing sites of consensus *S-RNase* and *SFB* primers, together with Signal Peptide (SP), intron regions, conserved regions (C1-C5) and hyper-variable regions (HVR) in the *S-RNase* gene; and the intron region, variable region (V1;V2) and hyper-variable region (HVa; HVb) in the *SFB* gene (Adapted from Halasz *et al*., 2010).

Fig 4.2. GENEMAPPER output of first intron products amplified using the SRC primers, for the ARC reference collection cultivar 'Bulida' (*S2Sc*). Similar output was observed in other accessions.

Fig 4.3. Second intron products from apricot reference collection loaded as (from the left): ladder (L), 1-Grandir (*S19 S(d)*), 2- Supergold (*Sc*), 3- Soldonne (*S2Sc*), 4- Bulida (*S2Sc*), 5- Ladisun (*S2Sc*), 6- Paletyn (*Sc*), 7- Cape bebeco (*S6Sc*), 8- Charisma (*S7Sc*), 9- Peeka (*Sc*), 10- Royal (*S1Sc*), 11- Alpha (*S1S2*), 12- Suapriseven (*S(d)Sc*), 13- Suaprieight (*S(d)Sc*), 14- Suaprinine (*S1S(d)*), 15- Atricot (*S2Sc*).

Fig. 5.1 Second intron products, amplified using the EM-PC primers. DNAs of plum accessions from the reference collection arranged as: 16-Sensation (*SeSb*), 17- Crocodile Dundee (*SkSh*), 18-Fortune (*ScSb*), 19- Harry Pickstone (*SkSb*), 20- Ruby Red (*Sc*), 21- Suplum 25 (*Sb*), 22- Reubennel (*SgSb*), 23- Lady West (*ScSb*), 24- LadyRed (*ShSb*), 25- Suplum 11 (*SeSh*), 26- Laetitia (*ShSe*), 27-Red Gold (*SkSh*), 28- Songold (*SkSh*), 29-Lamoon (*ShSc*), 30- Ruby Star (*SkSe*).

Chapter 1:
General introduction

1.1 Background

Apricots and plums are two of the most economically important stone fruit crops in the genus *Prunus*, within the family Rosaceae. Stone fruit are distributed across the world and thrive under Mediterranean climates. In South Africa, the province responsible for the highest stone fruit production is the Western Cape, followed by the Northern Cape and the Eastern Cape (Hortgro, 2016). The majority of apricot and plum produce is dedicated to the export industry; therefore, it is crucial to remain competitive.

In South Africa, apricot and plum production is supported by breeding programmes, such as those of the Agricultural Research Council (ARC) stone fruit breeding programme, which provides producers with various options for good export quality cultivars. South African breeding objectives include production of cultivars that are adapted to South African growth conditions, early and late ripening cultivars suitable for the export market and attractive fruit. Maintaining genetic diversity within a breeding programme is an important aspect in order to ensure continuous effectiveness of the programme. Genetic variation amongst genetic resource collections can be maintained through sharing of information and resources. The characterisation and accurate documentation of genetic variation within stone fruit germplasms are essential for management and provides useful information to beeders (Aranzana *et al*., 2012).

Historically, variation in genetic resources was described through visual inspection of morphological characteristics. These, however, have certain limitations, including lack of variation amongst the studied genotypes and variation induced by the environment (Nybom *et al*., 2014). For example, fruit crops can adapt to specific microclimates and exhibit significantly different phenotypes when moved from one location to another (Krichen *et al*., 2006). According to De Vicente *et al*. (2005), molecular characterisation offers enhanced diversity detection (including gene and genotype detection) that traditional methods do not offer. In addition DNA characterisation offers improved detection in comparison to other methods such as isozymes. This is because molecular methods identify variation in genotypes, *i.e.*, on the ultimate level of variation offered by the DNA sequences of cultivars and are unaffected by changes in the environment.

The development of DNA marker technology has become an essential tool for the molecular characterisation of plant species and has improved the effectiveness of plant breeding programmes (Rafalski and Scott, 1993). Among other widely used PCR-based techniques, simple sequence repeats (SSRs, also known as microsatellites) have been described as the preferred DNA marker for the assessment of genetic diversity within plant species due to their highly polymorphic nature, abundance and co-dominant inheritance (Joshi and Albertse, 2013).

In addition to applications such as genetic diversity studies, cultivar identification and linkage mapping, DNA markers are also utilised in the molecular characterisation of biological systems in

plants, such as the self-incompatibility (SI) system in angiosperms. Self-incompatibility is defined as the inability of a fertile seed plant to produce a zygote after self-pollination (De Nettancourt, 1977). It is a strategy used by angiosperms to promote outcrossing to maintain genetic diversity and avoid inbreeding depression. Much progress has been made in understanding the molecular genetics of SI using combined molecular approaches, and the relevant genes in *Prunus* have been reported (Charlesworth, 2010).

Self-incompatible cultivars require another cultivar with a similar flowering period and in close proximity to act as pollinators and are dependant on bees to transport the pollen (Folta and Gardiner, 2009). Knowledge of self-incompatibility genotypes provides useful information to breeders and growers for the selection of pollen donors and orchard planning (Nashima *et al*., 2015). Self-compatibility is therefore a desirable trait in many fruit and nut crops due to the ease of self-fertilisation as it allows orchards of single cultivars to be planted (Kaothien-Nakayama *et al*., 2010).

1.2 Aim and objectives

In this study, the aim was to fingerprint the ARC's apricot and plum collection at a molecular level, using a set of internationally recognised microsatellite primers and to identify self-(in)compatibility genotypes of the apricot and plum accessions.

Objective 1 was to fingerprint the accession and, where possible, confirm trueness to type of international cultivars and to verify parentage of South African cultivars and selections by comparing their microsatellite profiles using a set of 16 internationally recognised SSR primers designed in peach.

Objective 2 was to identify self-(in)compatibility genotypes of cultivars by amplifying the alleles in the first and second intron of the *S-RNase* gene found at the self-(in)compatibility locus, which will provide useful information to breeders when planning crosses (Bester *et al*., 2013).

1.3 Description of chapters

Chapter 2 is a literature review, which gives an outline of the genetic background of apricot and plum breeding, concentrating on the findings reported by others on the application of molecular and evolutionary techniques in apricot and plum breeding.

Chapter 3 is an experimental chapter that focuses on the application of microsatellites to fingerprinting apricot and plum accessions of the ARC's germplasm bank.

Chapter 4 is an experimental chapter that focuses on the genotyping of apricots from the ARC's germplasm collection with regards to the SI locus.

Chapter 5 is an experimental chapter that focuses on the genotyping of plum accessions from the ARC's plum germplasm collection with regards to the SI locus.

Chapter 6 provides a summary, discussion and general conclusion of the significance of the findings from this study.

1.4 References

Aranzana M.J., Barreneche T. and Arus P. (2012): Diversity analysis. In: Kole C. and Abbott A.G. Genetics, genomics and breeding of stone fruits. CRC, Clemson SC, pp 55-75.

Bester C., Tobutt K.R., Mansvelt E.L., Blomerus M.L. and Jolly N. (2013): The value and impact of the ARC Infruitec-Nietvoorbij gene banks. Acta Horticulturae, 1007: 950-980.

Charlesworth D. (2010): Self (in)compatibility. Biology Reports, 2: 1-6.

De Nettancourt D. (1977): Incompatibility in angiosperms. Monographs on Theoretical and Applied Genetics. Vol 3. Springer, New York, pp 1-24.

De Vicente M.C., Guzman F. A., Engels J. and Ramanatha-Rao V. (2005): Genetic characterisation and its use in decision making for the conservation of crop germplasm. In: Ruane J. (ed), The role of biotechnology in exploring and protecting agricultural genetic resources. FAO, Rome, Italy pp 121-128.

Folta K.M. and Gardiner S.E. (2009): Genomics-based opportunities in apricot. In: Folta K.M. and Gardiner S.E. (ed), Genetics and genomics of Rosaceae. Springer, pp 315-330.

Hortgro (2016): Key deciduous fruit statistics: http://hortgro.co.za/wp-content/uploads/2017/06/HORTGRO-Key-Deciduous-Fruit-Statistics-2016.pdf (accessed: 21/08/2017).

Joshi S.V. and Albertse E.H. (2013): Development of a DNA fingerprinting database and cultivar identification in sugarcane using a genetic analyser. Proceedings of South African Sugarcane Technologists Association, 86: 200-212.

Kaothien-Nakayama P., Isogai A. and Takayama S. (2010): Self-incompatibility systems in flowering plants. Plant Development Biology-Biotechnology Perspectives, 3: 459-485.

Krichen L., Mnejja M., Arus P., Marrakchi M. and Trifi-Farah N. (2006): Use of microsatellite polymorphisms to develop an identification key for Tunisian apricots. Genetic Resources and Crop Evolution, 53: 1699-1706.

Nashima K., Terakami S., Nishio S., Kunihisa M., Nishitani C., Saito, T., and Yamamoto T. (2015): *S*-genotype identification based on allele-specific PCR in Japanese pear. Breeding Science, 65: 208-215.

Nybom H., Weising K. and Rotter B. (2014): DNA fingerprinting in botany: past, present, future. Investigative Genetics, 5: 1-35.

Rafalski J.A. and Scott T. (1993): Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. Trends in Genetics, 9: 275-280.

Chapter 2

Literature Review:

Apricot and plum breeding from a genetic point of view

2.1 Introduction

South Africa's diverse weather and climatic conditions enable the country to cultivate and produce a variety of fruits. The country is known globally as a producer and exporter of citrus, subtropical and deciduous fruits. The deciduous fruit industry in South Africa is well established, and consists of mainly pome fruit (apples and pears), and stone fruit (apricots, peaches, nectarines and plums) as well as table grapes (Potelwa *et al*., 2014). The South African industry is however faced with the challenge of remaining internationally competitive in terms of producing good quality cultivars. Currently, the total planted area for deciduous fruit in South Africa amounts to 79 748 hectares, with a total of 2 225 producers, employing 109 791 labourers (Hortgro, 2016). The Western Cape has the largest production area in the country, representing 75% of the total area planted. The Northern Cape is the second largest production area representing 15% of the total area followed by the Eastern Cape (8%) (Hortgro, 2016).

2.2 Stone fruit botany

Stone fruits are soft-fleshed temperate fruits known for their delectable flavours. They are a good natural source of vitamins and minerals, and there is an increasing interest in the potential value of phenolics that possess antioxidant properties and can be used as nutraceuticals (Potter, 2012). Stone fruit trees can be large trees or shrubs with typically showy 5-merous flowers with a single carpel that matures into a drupe. A drupe is characterised by an exocarp, or skin; and mesocarp, or flesh that surrounds a hard shell (endocarp or stone) with a single seed inside. The leaves are simple, alternate, usually lanceolate, unlobed and the flowers are usually white to pink, sometimes red, with five petals and five sepals (Cullen *et al*., 2014).

Stone fruits are members of the genus *Prunus* and includes apricot (*P. armeniaca* L.), European plum (*P. domestica* L.), Japanese plum (*P. salicina* Lindl.), peach and nectarine (*P. persica* (L.) Batch.), sour cherry (*P. cerasus* L.), sweet cherry (*P. avium* L.) and the almond (*P. dulcis* Miller) (Srinivasan *et al*., 2005). *Prunus* is traditionally placed within the rose family, Rosaceae, as a subfamily, the Amygdaloideae (or Prunoideae), but sometimes placed in its own family, the Prunaceae (or Amygdalaceae) (Potter *et al*., 2007). Tree crops belonging to the *Prunus* genus originated in Europe, Central Asia and China; and were spread through vegetative propagation thoughout the world (Janick, 2005; Verde *et al*., 2013).

2.3 Apricot

Apricots (*P. armeniaca)* are golden orange fruits that are often tinged red (commonly referred to as blush) on the surface when exposed to sunlight. The fruit are not too juicy but smooth and sweet, although some may be woolly and/or acidic. Exceptional fruit quality requires the right balance of sugar and acids as well as strong apricot aroma (Considine and Considine, 1982). The importance of other characteristics of fruit quality depends on their intended use, *e.g.* fruit size and colour are important for fresh market apricots whereas firm flesh is desirable for canning and for the fresh market. Apricots generally have a high sugar content making them suitable for processing (Gurrieri *et al*., 2001). They can be used as dried or canned fruit, and in jams or compotes, as well as juice or nectar. Specific cultivars are suitable for specific types of processing, *e.g.* the cultivars 'Soldonne' and 'Ladisun' are of good drying quality, whereas 'Royal Blenheim' is used for juice, freezing and drying (Horstmann, personal communication).

Apricots are a good source of vitamin A (carotene) and vitamin C (ascorbic acid) (Girish *et al*., 2011). The surface of the fruit can be glaborous (smooth) or pubescent (velvety) and the endocarp is generally freestone (the flesh is detached from the seed allowing easy removal) rather than clingstone (flesh firmly attached to the seed) (Eiermann, 2012). The apricot tree grows to 8-12 m tall and has increased cold hardiness and resistance to temperature change during winter compared to peach. The leaves are ovate with a round base and pointed leaf apex. The flowers have five white to pinkish petals produced in spring before the leaves emerge and act to attract pollinators more effectively (Eiermann, 2012).

2.3.1 Apricot production

The global apricot industry is well developed. According to Food and Agriculture Organisation Statistics (FAOSTAT) (2013) the total world apricot production for 2012 was 4 038 520 tons; however production fluctuates considerably from year to year. Globally the largest apricot producing countries are Turkey (795 768 tons) and Iran (460 000 tons) (FAOSTAT, 2013). More than 55% of the world's apricot production is restricted to countries with Mediterranean climates such as Turkey, Spain, Italy, France and Greece (FAOSTAT, 2013).

In South Africa, apricots are the third most economically important stone fruit crop after peach and plum (Potelwa *et al*., 2014). The majority of South African apricots are processed before being exported to various markets (Hortgro, 2016. In 2016, a total area of 2 838 ha was planted under apricots and this crop contributed 4% of the total area planted to deciduous fruits (79 748 ha). A total of 40 642 tons of apricots were produced during the 2015/2016 season. Of these, 74% were processed, approximately 14% was dried, while 8% and 4% were exported and sold on the local markets, respectively (Hortgro, 2016).

2.3.2 Apricot species

The most common species of apricot is *P. armeniaca* (Zaurov *et al*., 2013) that originated from China (Manchuria) from where it reached Europe via Asia through Armenia (Maghuly *et al.,* 2006). Other closely related species with similar fruit are: *P. brigantina* (Briancon apricot) native to the French alps; *P. dasycarpa* (purple apricot) native to the former USSR; *P. mandshurica* (Manchurian apricot) native to Manchuria and Korea; *P. mume* (Japanese apricot) native to Japan and China; and *P. sibirica* (Siberian apricot) native to eastern Siberia, Manchuria and Northern China (Layne *et al.,* 1996; Bortiri *et al*., 2001). Most species are diploid (2*n*=2*x*=16) (Arumaganathan and Earle, 1991) and most commercial cultivars are self-fertile, but several are self-incompatible (Halasz *et al*., 2010).

In general, apricot cultivars are severely restricted in their ecological adaptation (Mehlenbacher *et al*., 1991). In an attempt to classify apricot cultivars according to their adaptability in different ecological regions, Kostina collected apricots from several geographical regions and established collections (Kostina, 1969). From the cultivated apricots, four major eco-geographical groups were distinguished: Central Asian; Irano-Caucasian; European and Dzhungar-Zailij. The apricots in each of the eco-geographical groups showed differences in predominating types of trees and fruit (Layne *et al*., 1996).

2.4 Plums

Within the genus *Prunus,* plums constitute the most numerous and diverse group of fruit tree species. More than 6 000 varieties of plum, belonging to more than 20 species, which differ in phenotypic variation, geographical origin, chromosome number and climatic demands, are under cultivation (Blazek, 2007). The immense variety of plums, the global distribution of the fruit and its adaptability to varying conditions make them important for future development. One of the distinctive types of plums are prunes, also commonly referred to as dried plums. Both fresh and dried plums possess laxative effects amongs other health benefits; this property is conferred by their richness in fibre (Lever *et al*., 2014).

2.4.1 Plum production

Of the pome and stone fruit crops, plums are second to apples in terms of planted area, with over 2.1 million ha cultivated worldwide (Gomez-Plaza and Ledbetter, 2010). Global plum production has increased dramatically during the last decade, with the majority of the increase coming from new Asian orchards. China, with six million tons, and Romania, with 400 000 tons, were the world's leading producers in the 2013/2014 season (Potelwa *et al*., 2014). South Africa's plum production was 79 364

tons in the 2015/2016 season, with the gratest share (74%) of plum production destined for the export market, while the remainder is consumed locally as fresh produce (23%) and with the lowest production share dedicated to processing (3%). The Western Cape is the main producer of plums in South Africa. In 2016, the three main plum production areas were the Klein Karoo (1 526 ha), Paarl (973 ha) and Wolseley / Tulbagh (508 ha). The total area planted within South Africa is 5 093 ha for plums and 264 ha for prune trees (Hortgro, 2016).

2.4.2 Plum species

The geographic distribution of both wild and cultivated plums spreads throughout the northern temperate regions; other species are primarily found in Asia, Europe and America (Topp *et al.*, 2012). Rehder (1954) surveyed plum species, and the findings were essential in the characterisation of many plum species grouped into three groups: European, Asian and American species. The cultivated European plum, *P. domestica* L., is the most important species in Europe. Asian species include *P. salicina* Lindl. (Japanese plum), and *P. simonii* Carriere (Simon or apricot plum). There are at least five American species: *P. americana* Marshall (a common wild plum), *P. nigra* Ait. (Canada plum), *P. angustifolia* Marshall (Chickasaw plum), *P. hortulana* L. H. Bailey (Hortulana plum) and *P. munsoniana* W. Wight & Hedrick (wild goose plum). Commercially, the European plum and Japanese plum types, including hybrids with other diploid plum species, are of importance (Milosevic *et al*., 2013).

2.4.3 European plum

Prunus domestica (*2n=6x*=48) is a hexaploid that is well adapted to cooler regions, generally freestone (endocarp free from the mesocarp) and used for both processing (drying and canning) and fresh market. The genetic origin of European plum remains a controversial issue. Flory (1947) suggested that *P. domestica* originated as a hybrid between *P. cerasifera* Ehrh., a diploid, and *P. spinosa* L., a tetraploid, followed by chromosome doubling of the triploid hybrid.

Prunus domestica has been divided into three different subspecies: *insititia* (L.) Pior, *italica* Borkh and *oeconomica* Borkh (Johansson and Olden, 1962). Based on morphological characteristics, *P. domestica* ssp. *insititia* is considered by some authorities as the separate species *P. insititia* L. (Nassi *et al*., 2003). The trees of this subspecies are easily distinguishable from those of true *P*. *domestica*, in that they are dwarf and compact and have smaller and nearly ovate leaves. The fruit of *P. domestica* ssp. *insititia* are smaller in size and nearly round in form, varying from sweet to sour and with colours ranging from yellow to purple. Various commercial rootstocks belong to *P. domestica* ssp. *insititia*.

Within *P. domestica* ssp. *italica*, the group of 'Reine Claude' or 'Green Gage' is the best known. The group is characterised by more or less round fruit, which has a very slight suture and skin colour varies between green, yellow or slightly red (Blazek, 2007). The flesh is sweet, tender (semi-firm) and juicy. The tree has a round crown, dark coloured bark, and the shoots are thick with 'persistent' pubescence. The leaves are large, broad, and more or less wrinkled.

Prunus domestica ssp. *oeconomica*, is the most common type of European plum. Typically the fruit is elongated or oval, with one side being straighter than the other and the flesh is greenish-yellow or golden, firm, often of very good quality, and freestone (Blazek, 2007). The tree is usually large, upright and spreading. The leaves are elliptical with pubescence on the upper surface.

Dried European plums are known as prunes. While all prunes are plums, not all plums can be dried into prunes. The high sugar content in European plum makes them suitable for drying. Most commercial plum varieties are self-fertile and do not need pollinator cultivars, but bees can be used to improve seed set (Norton and Krueger, 2007).

2.4.4 Japanese plum

Prunus salicina originated in China, where it has been cultivated for several thousand years (Ramming and Cociu, 1991). The trees are especially revered in China for their gnarled branches, profuse early flowering, and fragrance; with the painting of plum trees being a specialised art form (Janick, 2005). It is also a very ancient crop in Japan and Korea (Mnejja *et al.*, 2004). The fruit is mainly used for fresh market. In most Japanese plums the stone is firmly attached to the flesh (clingstone). From the hybridisation of Japanese plums with American plums, numerous Japanese-American hybrid plum trees have been developed that produce very large and good quality plums that are more resistant to pests and are more cold hardy than the European plums (Okie and Ramming, 1999).

The tree is rather small, with the straight branches having a tendency to form spurs throughout their length (Ramming and Cociu, 1991). The leaves are mostly oblong and often reddish in spring. Trees flower early and usually heavily. Fruits are varied, mostly large and firm, being characterised by a yellow base colour overlaid by various shades of red and purple (Blazek, 2007).

2.5 Plum and apricot interspecific hybrids

Interspecific hybridisation between related species is a method for increasing the genetic diversity and the genetic resources available for crop improvement. Interspecific hybridisation between *Prunus* species occurs naturally in the wild and has resulted in the development of novel fruit types which are

commercially recognised (Srinivasan *et al*., 2005). Plum-apricot hybridisation is generally successful between diploids (Anderson and Weir, 1967). The problem of hybrid sterility and poor seed set due to cytological differences between species however limits the scope of interspecific hybridisation in sexually reproducing plants. The primary aim of hybridisation is to meet market demand for high quality fruit and add variety to the fruit market.

Hybrids of related *Prunus* species were introduced more than hundred years ago, when Luther Burbank developed the first plum-apricot hybrid. Since then more crosses between plums and apricots, and recently between plums and peaches have been developed (Eiermann, 2012). The first plum-apricot cross developed was the plumcot. Plumcots are interspecific hybrids of Japanese plums and apricots (Frecon and Ward, 2012). Plumcots resemble their plum parents in appearance, with smooth skin and a slightly sweet taste (Hill and Perry, 2010). The trees are usually self-fertile. Pluots were developed by Floyd Zaiger and Zaiger Genetics, and “Pluot” is a registered trademark of Zaiger Genetics. Pluots are later generation plum-apricot hybrids that show more plum than apricot characteristics (Hill and Perry, 2010). These hybrids require a pollinator, either another pluot or Japanese plum (Eiermann, 2012). Aprium varieties were also developed in the late 1980s by Floyd Zaiger and Zaiger Genetics (Frecon and Ward, 2012). Apriums are complex plum-apricot hybrids that show more apricot traits; genetically and morphologically they are often one quarter plum and three quarter apricot (Eiermann, 2012). The small yellow fruit ripens relatively early, in spring or early summer. The presence of a pollinator (pluot or apricot) enhances the chances of fruit set (Eiermann, 2012).

2.6 Genetic resources and breeding programmes for apricots and plums

With a few exceptions, South Africa’s agriculture is based on introduced species and the sustainability of the industry is dependent upon continued access to the broader gene pool located elsewhere in the world (Moss, 1994). The diversity of South Africa’s genetic resources and competitiveness of South Africa’s market can be maintained through the introduction of new varieties.

The effectiveness of breeding programmes is dependent on the availability of variable plant material collected from different sources. The germplasm collections may consist of, amongst others, imported cultivars, land race (locally adapted) cultivars and seedlings from open pollination in the wild. For this reason, accurate identification of germplasm accessions and maintenance of good records are of great importance so that the breeders can confidently use them. The strategy used by most fruit breeding programmes is based on morphological observations. This approach has been successful in producing most of the varieties currently available on the market. However, the disadvantage of this approach is that it is time consuming and costly (Meneses and Orellana, 2013) and may contain mis-identifications. Therefore, the development of molecular markers for early identification and

fingerprinting of accessions may be useful to improve the effectiveness of breeding programmes (Wünsch and Hormaza, 2002).

2.6.1 Apricot and plum breeding objectives

In most apricot and plum breeding programmes, the principal objective is the development of fruits that can be grown successfully in a particular locality and that can be marketed profitably (Okie and Ramming, 1999). Trees must be productive and must be resistant or tolerant to factors that impair productivity, *e.g.* hardiness in northern regions, low chilling requirements for buds in southern regions and resistance to diseases such as brown rot and plum pox potyvirus (PPV) (Okie and Ramming, 1999). A marketable fruit must have an attractive appearance, adequate size and firmness and acceptable flavour and texture.

In South Africa, one of the recognised plum breeding programmes is conducted at ARC (Agricultural Research Council) Infruitec-Nietvoorbij in Stellenbosch in the Western Cape. The programme focuses on breeding *P. salicina* (although there are also other species in the plum gene bank), and some of the plum breeding programmes' goals include developing large fruited plums with attractive skin colour, cultivars with early harvest and late harvest, as well as environmental adaptability. Storage ability (up to four weeks) is crucial for exporting the fruit by sea freight. Breeding of low chill plums is also a priority as achieving the moderate chill requirement (450 to 1200 Infruitec chilling units) of current cultivars might become problematic due to climate change. Other international plum breeding programmes include: California - which focuses on developing cultivars with large and firm fruit suitable for long distance shipping (CTFA, 1996); Asia - breeding for resistance to plant diseases such as rust and red leaf blotch (Li, 1993); and Australia - focus on producing large sized, early ripening, high quality fruit suitable for export to Asia (Topp and Russell, 1989).

Apricot breeding is done by numerous researchers worldwide where environmental conditions are suitable for growth. Countries such as Italy (Pennone, 1999), New Zealand (Hofstee *et al*., 1999), the Slovak Republic (Benedikova, 2006) and many others have active apricot breeding programmes. One of the challenges faced in apricot breeding is self-incompatibility or self-sterility of the cultivars; these cultivars require pollinators to set commercial crops.

2.7 ARC apricot and plum breeding programmes

Many studies for deciduous fruits, vines and wines are conducted at ARC Infruitec-Nietvoorbij located in Stellenbosch in the Western Cape, South Africa. Its Cultivar Development Division has built up a substantial collection of germplasm to support its active breeding programmes. The ARC's apricot

and plum breeding programme supports the fresh fruit and processing industry and strives to produce cultivars that are well adapted to South African weather conditions, need minimal pesticides to control pests and disease, have good productivity and attractive fruit.

The stone fruit germplasm collections are planted in Bien Donne Experimental Farm, located in Grootdrankenstein in the Western Cape. The apricot and plum gene banks comprise of 106 apricot and 40 plum cultivars, accessions and rootstocks. The origins of these cultivars include the USA, South Africa, China, France and Italy for apricots (mostly *P. armeniaca* and a few *P. mume*) and USA, France and Sweden for plums (*P. cerasifera*, *P. salicina, P. domestica, P. insititia* and some plum hybrids).

2.8 Molecular markers

Molecular or genetic markers include proteins and DNA that can be found at specific locations of the genome (Priyono and Putranto, 2014). They are used to 'tag' or 'flag' the position of a particular characteristic or a particular gene. In a genetic cross, genetic markers will typically remain linked with the characteristics of interest. Thus, individuals can be selected in which the genetic marker is present, since the desired trait is indicated by the marker. Therefore, DNA markers represent the most significant advance in breeding, and constitute the most important application of molecular biology to plant breeding (Grover and Shama, 2014).

Historically various molecular analysis techniques, mainly Randomly Amplified Polymorphic DNA (RAPDs) and Amplified Fragment Length Polymorphisms (AFLPs), successively contributed to the identification of stone fruit germplasm, and characterisation of its genetic diversity (Esmenjaud and Srinivasan, 2012). Amongst PCR (Polymerase Chain Reaction) based techniques, Simple Sequence Repeats (SSRs) are currently the preferred technique for fingerprinting cultivars in different plant species that are important in the fruit industry (Arismendi *et al*., 2012). SSRs (also known as microsatellites) consist of motifs of one to six nucleotides in length that are repeated several times (Kelkar *et al*., 2010). As a result of the high mutation rates, SSRs are highly polymorphic *i.e.* different individuals tend to exhibit variation manifested as differences in repeat number (Guichoux *et al*., 2011). These markers are also co-dominant, meaning that the banding patterns of homozygotes can be clearly distinguished from the traces of heterozygotes (Ouborg *et al*., 1999). However, the limitation of the development of SSR primers is that the process is costly and time-consuming.

2.8.1 Application of SSRs among Prunus *species*

In *Prunus,* SSRs have been extensively used for the fingerprinting of germplasm, *e.g.* peach (Marchese *et al.*, 2005), apricot (Campoy *et al*., 2010), almond (Kadkhodaei *et al*., 2010), cherry (Lacis *et al*., 2009) and plum (Wünsch, 2009). Hormaza (2002) was the first to use SSRs in molecular

characterisation and similarity relationships among a collection of 48 apricot genotypes, originating from diverse geographic areas, using 37 primer pairs recovered in different species of *Prunus*. More recently, Klabunde *et al.,* (2014) did DNA fingerprinting of Japanese plum cultivars. In that study, 47 Japanese plum genotypes were determined using eight SSR markers. Uses of SSRs, other than fingerprinting, include parentage analyses, genetic structure analyses and genetic mapping (Ellegren, 2004; Mittal and Dubey, 2009; Jones and Wang, 2010).

2.8.2 Transferability of SSRs amongst related species

In *Prunus*, many SSR primers have been published and the same primers are often used across different species. Hormaza (2002) used SSR primers developed in different species of *Prunus* to identify and characterise the genotypes of 48 apricot cultivars and establish their genetic variation. The results of the study revealed that SSR marker developed in other *Prunus* species could be used for fingerprinting related species such as apricot, *i.e.* evidence of SSR cross-species transferability. The ability of SSRs to be transferred among related species is made possible by the highly conserved flanking regions on which the primers are designed. The significant conservation of the genomes of different *Prunus* species has been reported by comparative mapping studies. Colinearity of the genomes of diploid *Prunus* species was illustrated by comparison of the anchor marker position on the *Prunus* reference map with those on 13 other maps constructed with a subset of 562 markers from a Prunus reference map (Dirlewanger et al., 2004). Consequently, SSRs, due to their transferability across species and their convenience to use compared to AFLPs, became the preferred markers for cultivar identification (Gupta and Varshney, 2000).

The transferability within the family Rosaceae was studied in more detail by Mnejja *et al*. (2010) who investigated 17 genomic microsatellite primer pairs from *Prunus* in a set of eight cultivars from each of nine Rosaceae species (almond, peach, apricot, Japanese plum, European plum, cherry, apple, pear and strawberry). In the study, they found that most *Prunus* primer pairs (83.6%) amplified bands of the expected size range in other surveyed *Prunus* species. Several sets of SSRs have been widely used for fingerprinting stone fruit within the *Prunus* genus. Dirlewanger *et al.* (2002) reported on 41 SSR primer pairs (BPPCT) from a CT-enriched genomic library of the peach cultivar ‘Merrill O’ Henry’. All primer pairs gave amplification products with peach and 33, with cherry. They also tested cross-species amplification between *Prunus* species: sweet cherry, sour cherry, European plum, almond, apricot and Myrobalan plum. The transferibility of microsatellites of peach to other *Prunus* species was very high in all tested *Prunus* species, confirming reports by Cipriani *et al*. (1999) of up to 88% transferability among *Prunus* species. Aranzana *et al.* (2002) reported on the development and variability analysis of SSRs markers in peach. Thirty-five SSRs (CPPCT) were isolated from a genomic DNA library enriched with AG/CT repeats developed from the peach cultivar ‘Merrill

O'Henry'. Testolin *et al.* (2000) isolated and sequenced 26 microsatellites (UDP) from two genomic libraries of peach cultivar 'Redhaven', enriched for AC/GT and AG/CT, respectively. The above mentioned microsatellite primers were tested on other *Prunus* species and were highly transferable in apricot (Hormaza, 2002); plum (Decroocq *et al*., 2004; Mnejja *et al*., 2004); almond (Martinez-Gomez *et al*., 2003) and cherry (Cantini *et al*., 2001). Expressed Sequence Tag SSRs (EST-SSRs) have been reported to have a higher degree of tranferabiltiy across related species, compared to genomic-SSRs, because EST-SSRs possess conserved sequences among homolous genes and they originate from the transcribed regions in genomes (Wu *et al*., 2014). The study by Wu *et al*., 2014 indicated that 95.3% EST-SSR markers were more transferable to nine other Paeonia species.

2.9 Self-(in)compatibility

De Nettancourt (1977) suggested that in flowering plants, the most common evolutionary strategy that prevents self-fertilisation and promotes outcrossing is known as self-incompatibility (SI). According to Surbanovski *et al*. (2007) "The genetic control, attributed to the single multi-allelic *S*-locus, was first explained in *Nicotiana* (Solanaceae; East and Mangelsdorf, 1925) and later demonstrated in many rosaceous species including *P. avium* (sweet cherry; Crane and Lawrence, 1929), *P. dulcis* (almond; Gagnard, 1956), *Malus pumila* (apple; Kobel *et al*., 1939) and *Pyrus serotina* (Japanese pear; Terani *et al*., 1946)".

2.9.1 The gametophytic self-(in)compatibility trait

In several families, including Rosaceae and Solanaceae, the SI system is gametophytic. In gametophytic self-incompatibility (GSI), the SI phenotype of the pollen is determined by its own gametophytic haploid genotype. This is the more common type of SI, and is also found in Papaveraceae (Franklin *et al*., 1995). The self-(in)compatibility behaviour in the genus *Nicotiana* was studied by East and Mangelsdorf (1925), who revealed that the hereditary behaviour of self-(in)compatibility is carried on a single *S*-locus. As a result of later findings that the *S*-locus is a multigene complex, the term "haplotype" has been adopted to denote variants of the locus, and the term "allele" is used to denote variants of a given polymorphic gene at the *S*-locus (McCubbin and Kao, 2000). The allelic series of the *S*-locus is denoted by different letters or numbers.

The incompatibility response is determined by two genes found at this locus, one expressed in the stylar tissue and the other in the pollen tissue (details of the two genes to follow) (Romero *et al*., 2004). In GSI, pollen-tube growth is arrested in the style when the haploid pollen *S*-allele matches

either of the two *S*-alleles of the diploid pistil alleles (East and Mangelsdorf, 1925). If two different cultivars have identical *S*-genotypes, they are mutually self-incompatible (SI), in other words they are cross- or inter-incompatible, resulting in infertility. When a pollen grain bears an *S*-allele different from those of the pistil, the cross will be compatible and there should be seed set. Semi-compatibility occurs if the pollinator shares one allele with the pistil so that half of the pollen genotypes are different and half are the same; as a result, there may be reduced seed set (Fig. 2.1).

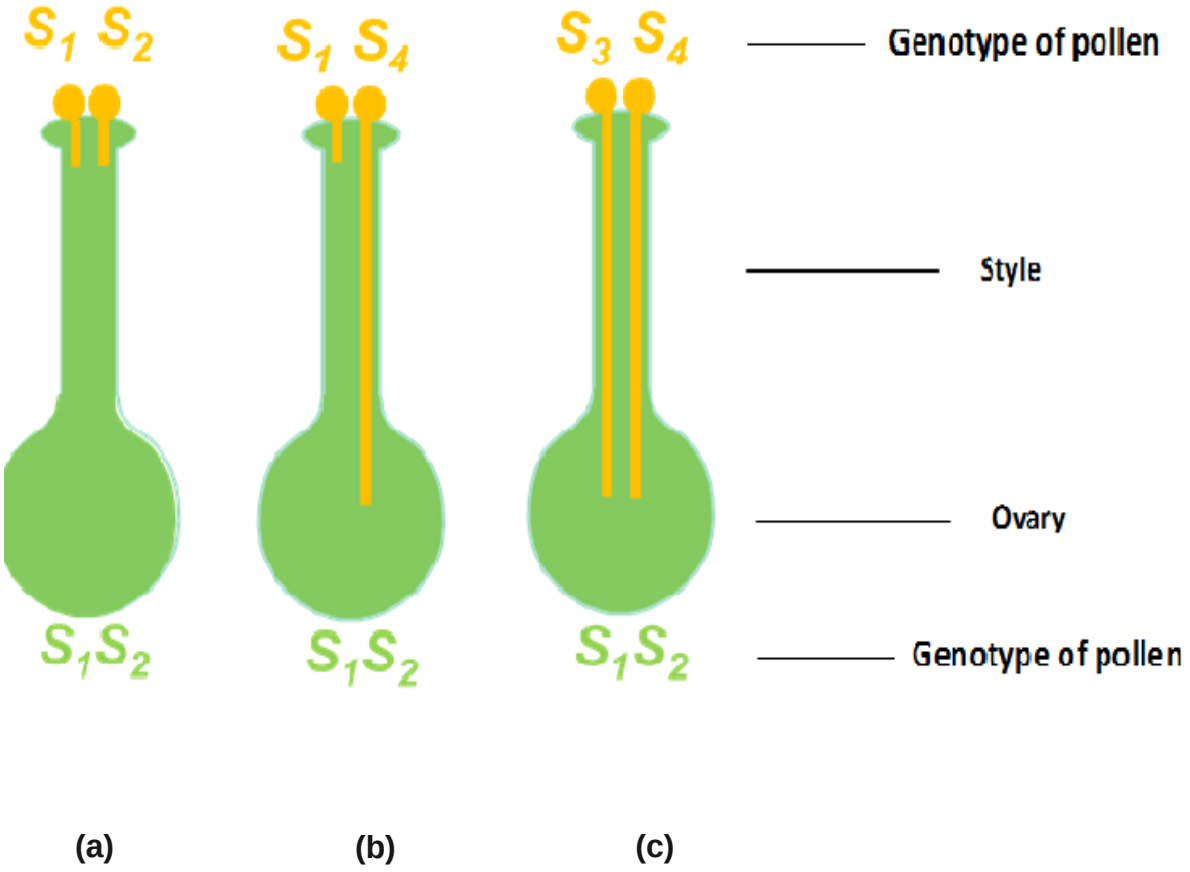


Fig. 2.1. Diagrammatic representation of gametophytic incompatibility relationships in *Prunus*, (a) incompatible, (b) semi-incompatibility and (c) fully compatible. Observation of pollen tube development in the style based on interaction of genotypes of pistil and genotypes of the pollen (*S* indicating the genotypes).

2.9.2 Self-(in)compatibility in apricot and plum

From cross-pollination tests, East and Mangelsdorf (1925) suggested that cultivars with the same combinations (genotypes) can be grouped into (in)compatibility groups in which cultivars within the same group are cross-sterile with each other, but are cross-fertile with cultivars of other groups. In apricot cross-(in)compatibility between cultivars was studied by Szabo and Nyeki (1991), and the first

group of cross-incompatible apricot cultivars was reported to consist of four large fruited Hungarian cultivars ('Cegledi Orias', 'Ligeti Orias', 'Szegedi Mammuti' and 'Nagykorosi Orias'). Egea *et al*. (1991) also reported cross-incompatibility between two important Spanish cultivars ('Monoqui Fino' and 'Moniqui Borde'). It was also noted that cultivars that form an inter-incompatibility group are often genetically related (Egea and Burgos, 1996).

With the use of molecular methods, Vilanova *et al*. (2005) reported on the PCR-based identification of eight known self-(in)compatibility alleles of apricot, and in combination with previously reported *S*-genotypes, one self-(in)compatibility group (I) and one universal donor group (O) containing unique *S*-genotypes and self-compatible cultivars (SC) was proposed (Table 2.1).

Table 2.1. Genotypes of apricot cultivars, determined by non-equilibrium pH gradient electrofocusing of stylar proteins (Burgos *et al*., 1998) and PCR amplification of *S-RNase* alleles (Alburquerque *et al*., 2002; Hancock *et al*., 2008), grouped into proposed self-incompatibility groups (SI = self-incompatible, O = universal donors, SC = self-compatible).

Cultivar	Genotype	Group	Reference
Goldrich	*S1S2*	SI	Egea and Burgos, 1996; Burgos *et al.,* 1998
Hagrand	*S1S2*	SI	Hancock *et al.,* 2008
Lambertin-1	*S1S2*	SI	Hancock *et al.,* 2008
Sunglo	*S2S3*	O	Burgos *et al.,* 1998
Harcot	*S1S4*	O	Burgos *et al.,* 1998
Moniqui	*S2S6*	O	Burgos *et al.,* 1998
Canino	*S2Sc*	O (SC)	Alburquerque *et al.,* 2002
Colorao	*S5Sc*	O (SC)	Burgos *et al.,* 1998
Beliana	*S7Sc*	O (SC)	Alburquerque *et al.,* 2002
Currot	*S7Sc*	O (SC)	Alburquerque *et al.,* 2002

2.9.3 The S-RNase gene in Prunus

The *S*-locus is composed of two genetically linked fragments, referred to as the stylar *S* (*S-RNase*) and pollen *S (SFbox)* (Surbanovski *et al*., 2007). In *Prunus,* the incompatibility phenotype of the style is determined by a ribonuclease called *S-RNase* (Bošković and Tobutt, 1996). Molecular and transgenic analyses have shown that pollen rejection by the pistil is mediated by the *S-RNase* (Lee *et al.,* 1994). As the ribonuclease activity of *S-RNase* is essential for the rejection of incompatible pollen tubes (Huang *et al.,* 1996), incompatible pollen tubes are degraded by the *S-RNase* which is known to have cytotoxic effects. In *Prunus*, the constituents of the *S-RNase* gene include five highly conserved regions (C1 to C5) and a hyper-variable region (HVR in Rosaceae), located between C2

and C3 (Ishimizu *et al*., 1998; Sonneveld *et al*., 2003) (Fig. 2.2). The HVR regions are believed to be important for *S*-allele recognition and for initiating the self-incompatibility response (Ishimizu *et al*., 1998).

There are two introns, one is located between C2 and C3, and an additional intron, unique to *Prunus,* is located between the signal peptide and C1 (Yamane *et al*., 2001; Beppu *et al*., 2002). The two introns are amplified by PCR primers that anneal in the flanking regions and differ in size. The first intron between the signal peptide and C1 is smaller than the second intron located between C2 and C3. Therefore, different sizes of the distinctive alleles are observed (Gharesheikhbayat, 2010).

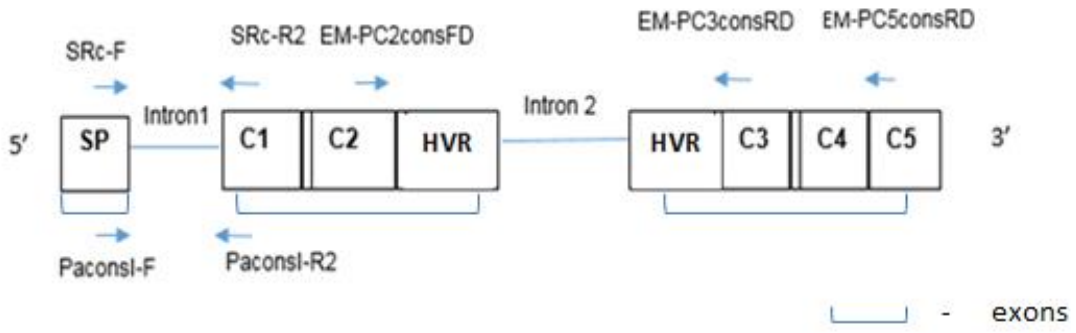


Fig. 2.2. The structural representation of the *S-RNase* gene in *Prunus*, including the signal peptide (SP), the first and second intron, the five conserved regions (C1, C2, C3, C4 and C5) and the rosaceous hyper-variable region (HVR) (Sonneveld *et al*., 2003). The arrows indicate primer binding sights for the two introns and the exons are represented by the blue lines.

2.9.4 S-haplotype-specific F-box (SFB) gene

The specificity of the pollen in both plums and apricots is determined by the product of the F-box gene *SFB* (Sijacic *et al*., 2004). Entani *et al*. (2003) reported the identification of the pollen *S*-determinant gene in Japanese apricot. The group investigated the genomic structure of the *S*-locus region of the *S*1- and *S*7-haplotypes of *P. mume* (Japanese apricot), and identified 13 genes around the *S-RNase* gene. Among them, only one F-box gene, termed SLF (*S*-Locus F-box), fulfilled the conditions for an *S*-determinant gene: (i) together with the *S-RNase* gene, it is located within the highly divergent genomic region of the *S*-locus, and (ii) it exhibits *S*-haplotype specific diversity among three analysed *S*-halotypes (Entani *et al.,* 2003). However the mechanism of action of the *SFB* gene is not well

understood. The alignment of amino acid sequences of *SFB*s of these *Prunus* species (Kao and Tsukamoto, 2004; Ushijima *et al*., 2004) highlighted the presence of two hyper-variable regions (HVa and HVb) (Fig. 2.3). Gharesheikhbayat (2010) suggested that the absence of both HVa and HVb regions in two self-compatible haplotypes, one of cherry and one of Japanese apricot, encoded partial loss-of-function mutations in *SLF/SFB* (Ushijima *et al*., 2004). This information further supports that the *SLF/SFB* is the pollen S-determinant.

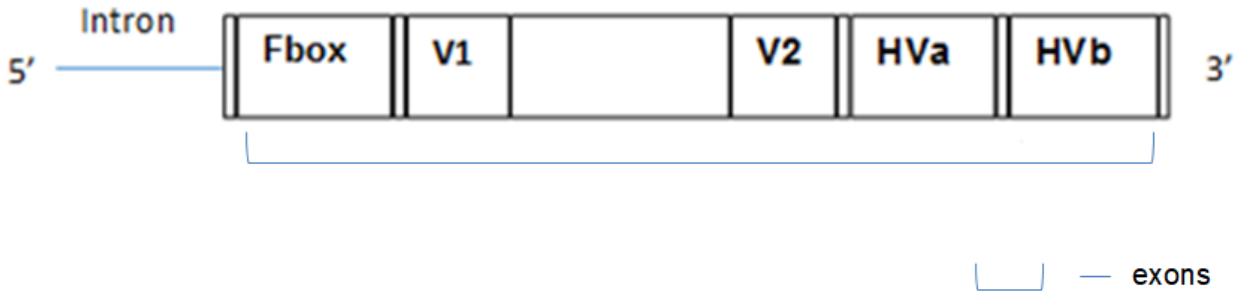


Fig. 2.3. Structure of *SFB* gene in *Prunus* indicating the conserved F-box motif at the 5' end, two variable (V1, V2) and two hyper-variable (HVa , HVb) regions and an intron positioned in the 5' region (Ikeda *et al*., 2004; Nunes *et al*., 2006).

2.9.5 Traditional genotyping of the S-locus in Prunus

Traditionally, (in)compatibility is determined by monitoring fruit set after controlled pollination under field conditions. The disadvantage of this method is that fruit set is influenced by weather conditions and poor set may be due to unfavourable weather rather than incompatibility. The second approach used is the observation of pollen tube growth in the pistil using fluorescence microscopy after controlled pollination. It enables more reliable conclusions compared to the field tests (Viti *et al.*, 1997; Milatovic *et al*., 2013) but is laborious.

2.9.6 Molecular genotyping of the S-locus in Prunus

In addition to these biological methods, two molecular methods have recently been used to determine (in)compatibility relationships in *Prunus*. One is the detection of stylar ribonucleases (*S-RNases*) (Burgos *et al*., 1998; Alburquerque *et al*., 2002; Halasz *et al*., 2005) based on molecular methods developed in cherry by Bošković and Tobutt (1996). In the study by Bošković and Tobutt (1996), Isozyme analysis was used to detect stylar ribonucleases. Stylar extacts were separated using Iso-

electric focusing (IEF) and stained to reveal bands corresponding to *S*-alleles. This method was used as a more rapid approach for *S*-genotyping, in contrast to the more timely method of conducting a series of crosses and assessing pollen growth or fruit set. Although fruitset studies are inexpensive, they can be affected by environmentl and physiological conditions and are sometimes poor in distinguishing compatible and incompatible genotypes (Tromp and Borsboom, 1994). In the case of the ribonucleous isozyme analysis, it is not clear when Rnase activity is directly associated with *S*-allele products. The other is DNA amplification and identification by PCR analysis (Sutherland *et al*., 2004b; Halasz *et al*., 2005).

In the PCR detection method, consensus primers based on conserved regions, flanking both the first and second intron, were developed which distinguish *S*-alleles on the basis of the size of the PCR product, so that *S*-genotypes can be deduced from amplification patterns (Ghareshеikhbayat, 2010). Tao *et al.* (1999) used sequence information of sweet cherry regions *S*2, *S*3 and *S*6 to design three consensus primers: Pru-C2, Pru-C4R and Pru-C5, which amplify the second intron. Another consensus primer, PCE-R, was designed from the C3 region of sweet cherry and combined with Pru-C2 to *S*-genotype sour cherry cultivars (Yamane *et al.* 2001). Although initially designed and used in cherry, these primers were subsequently applied to *S*-genotype cultivars of other related *Prunus* species *e.g.* almond (Ushijima *et al*., 1998), Japanese plum (Beppu *et al*., 2002) and apricot (Vilanova *et al.,* 2005).

There were however some limitations with the Tao *et al*. (1999) primers with the detection of *S*-alleles in a wider range of plant material. Consequently, Sutherland *et al*. (2004a) designed degenerate consensus primers from conserved regions of 27 *S-RNase* sequences available from *Prunus* species. These primers (EM-PC2consFD, EM-PC3consFD and EM-PC5consRD) flank the second intron, and tested in previously genotyped sweet cherry, almond and apricot cultivars, were informative in a range of species, detecting alleles in all cultivars tested.

Subsequently Ortega *et al.* (2005) reporting on almond, designed fluorescently labelled primers (PaConsI-F and PaConsII-R) to improve discrimination between alleles of similar but not identical sizes. These fluorescent primers are informative in the analysis of the first intron amplification products on an automated sequencer.

2.9.7 S-genotyping in apricot

Self-incompatibility is common in apricot cultivars in Central and Iranian-Caucasian geographic groups. In contrast, cultivars of the European group were traditionally considered self-compatible (Kostina, 1970). However the number of known self-incompatible apricot cultivars of the European group has increased rapidly over the last two decades. Szabo and Nyeki (1991) reported self-

incompatibility in nine cultivars, Paydas *et al*. (2006) in 37 cultivars and hybrids and Milatovic and Nikolic (2007) in 14 cultivars.

In Spain, Vilanova *et al*. (2005) reported seven *S-RNase* second intron alleles in apricot (*S1-S7*), with product sizes ranging from 448 bp to 2 260 bp, and one self-compatibility (*Sc*) allele of 2 800 bp, using the primers designed by Tao *et al*. (1999). Donoso *et al*. (2009) later used the consensus primers (EM-PC2consFD and EM-PC3consRD) (Sutherland *et al.,* 2004a) to identify *S*-alleles in apricots and amplification products corresponded with those presented by Sutherland *et al.* (2004a), who indicated that this primer combination provides amplification of the six alleles, *S1* to *S6* (245 bp to 2 000 bp), as well as the *Sc* allele (2 800 bp). Halasz *et al*. (2005) identified nine new putative alleles (*S8* to *S16*) in Hungarian apricot cultivars using non-equilibrium pH gradient electro-focusing (NEpHGE). In 2008, *S*-genotypes of 16 Chinese apricot cultivars were determined using the EM-PC2consFD and EM-PC3consRD primers, and the results were confirmed by cross-pollination tests. Twelve *S-RNase* alleles were designated *S*9 to *S*20 (Zhang *et al*., 2008). Using the same primer set, Halasz *et al*. (2013) also detected ten previously described alleles, namely *S2*, *S3*, *S6*, *S7*, *S8*, *S9*, *S11*, *S12*, *S13* and *S19* with product sizes ranging between 310 bp to 1 980 bp. The product sizes reported for S1 to S30 as well as the Sc allele are given in table 2.2. Wu *et al*. (2009) also identified eight *S*-alleles (*S23* to *S30*) with product sizes ranging between 453 bp to 1 453 bp. Reports of apricot *S-RNase*-genotypes have been documented (Table 2.3).

Table 2.2. Polymerase chain reaction product sizes of *S*-alleles reported for the second intron in apricot, amplified using primers EM-PC2consFD and EM-PC3consRD (Sutherland *et al.,* 2004a; Halasz *et al*., 2005; Halasz *et al*., 2013); PruC2 and PruC4R; AS1II and AmyC5R (Zhang *et al*., 2008) and PruC2, PCE-R and Amy-C5 (Wu *et al*., 2009).

S-alleles	**size (bp)**	**Reference**
S1	2 000	Sutherland *et al.,* 2004a
S2	850	Sutherland *et al*., 2004a
S3	245	Sutherland *et al*., 2004a
S4	310	Sutherland *et al*., 2004a
S5	1 200	Sutherland *et al*., 2004a
S6	1 300	Sutherland *et al*., 2004a
S7	820	Halasz *et al.,* 2013
S8	2 800	Halasz *et al.,* 2013
S9	500	Halasz *et al*., 2013
S10	943	Halasz *et al*., 2005
S11	1 700	Halasz *et al.,* 2013
S12	370	Halasz *et al.,* 2013
S13	1 250	Halasz *et al.,* 2013
S14	492	Zhang *et al*., 2008
S15	1 004	Halasz *et al*., 2005
S16	2 874	Halasz *et al.,* 2005

S17	487	Zhang *et al*., 2008
S18	1 337	Zhang *et al*., 2008
S19	1 980	Halasz *et al*., 2013
S20	1 934	Zhang *et al*., 2008
S23	672	Wu *et al*., 2009
S24	588	Wu *et al*., 2009
S25	994	Wu *et al*., 2009
S26	453	Wu *et al*., 2009
S27	397	Wu *et al*., 2009
S28	1 353	Wu *et al*., 2009
S29	452	Wu *et al*., 2009
S30	1 123	Wu *et al*., 2009
Self-compatible (*Sc*)	2 800	Sutherland *et al*., 2004a

Table 2.3. *S*-genotypes of apricot cultivars, and self-(in)compatibility status as determined by traditional methods such as controlled pollination tests and observation of pollen tube growth using fluorescent microscopy, as well as by molecular methods *e.g. S-RNase* PCR amplification.

Cultivar	S-genotypes	Self-(in)compatibility	Reference
Adilcevaz 1	*S8S19*	SI	Halasz *et al*., 2010
Adilcevaz 3	*S13S19*	SI	Halasz *et al*., 2010
Adilcevaz 5	*S9S13*	SI	Halasz *et al*., 2010
Agerik	*S7S13*	SI	Halasz *et al*., 2010
Akcadag Gunay	*S3S9*	SI	Halasz *et al*., 2010
Alioglu 49	*S8S12*	SI	Halasz *et al*., 2010
Alyanak	*S2S8*	SI	Halasz *et al*., 2010
Artivin P.A	*S2S7*	SI	Halasz *et al*., 2010
Aurora	*Sx-(2 585)bp*	SI	Burgos *et al*., 1998
Bebeco	*S6Sc*		Donoso *et al*., 2009
Beliana	*S7Sc*	SC	Vilanova *et al*., 2005
Bergeron	*ScSy*	SC	Szabo and Nyeki, 1991
Bulida	*S2*	S-	Donoso *et al*., 2009
Canakkale	*ScSc*	SC	Halasz *et al*., 2010
Canino	*S2Sc*	SC	Vilanova *et al*., 2005
Canino Tardio	*S1Sc*	S-	Donoso *et al*., 2009
Cataloglu	*S6S9*	SI	Halasz *et al*., 2010
Cegledi Orias	*S8S9*	SI	Halasz *et al.,* 2005
Cekirge 52	*S9S20*	SI	Halasz *et al*., 2010
Cigli	*S7S19*	SI	Halasz *et al.,* 2010
Cologlu	*S8S9*	SI	Halasz *et al.,* 2010
Colorao	*S5Sc*	SC	Vilanova *et al.,* 2005
Currot	*ScSc*	SC	Vilanova *et al.,* 2005
Dortyol 2	*S9S?*	SI	Halasz *et al.,* 2010
Dortyol 4	*S2S19*	SI	Halasz *et al.,* 2010
Ethembey	*ScS8*	SC	Halasz *et al.,* 2010
Farmingdale	*S1S2*	S-	Donoso *et al.,* 2009
Gec Aprikoz	*S6S11*	SI	Halasz *et al.,* 2010
Ginesta	*ScSc*	SC	Vilanova *et al*., 2005
Gitano	*S5S6*	SI	Sutherland *et al*., 2004a
Goldrich	*S1S2*	SI	Zuriaga *et al.,* 2013
Gonci Magyar Kajszi	*Sc*	SC	Halasz *et al.,* 2005
Grandir	*ScSc*	SC	Donoso *et al.,* 2009
Guz Aprikozu	*S6S?*	SI	Halasz *et al.,* 2010
Haci Haliloglu	*S9S13*	SI	Halasz *et al.,* 2010
Hacikiz	*S6S8*	SI	Halasz *et al*., 2010
Harcot	*S1S4*	SI	Sutherland *et al*., 2004a
Hargrand	*S1S2*	SI	Burgos *et al*., 1998
Harmat	*S10S11*	S-	Halasz *et al.,* 2005
Hasanbey	*S2S9*	SI	Halasz *et al.,* 2010
Hybrid 8	*S13S14*	S-	Halasz *et al*., 2005
Imrahor	*S3S?*	SI	Halasz *et al.,* 2010
Iri Bitirgen	*S2S6*	SI	Halasz *et al*., 2010
Ismailaga	*S9S11*	SI	Halasz *et al.,* 2010
Kabaasi	*S9S13*	SI	Halasz *et al*., 2010
Kadioglu	*S8S9*	SI	Halasz *et al*., 2010
Kamelya	*S9S13*	SI	Halasz *et al*., 2010

Karacabey	*ScS2*	SC	Halasz *et al.,* 2010
Katy	*S1S2*	SI	Zuriaga *et al.,* 2013
Kayisi Erigi	*S11S?*	SI	Halasz *et al*., 2010
Kayseri P.A	*S3S8*	SI	Halasz *et al.,* 2010
Kech-pshar	*S15 Sz(660)bp*	S-	Halasz *et al*., 2005
Konservinyi Pozdini	*ScSy*	SC	Halasz *et al.,* 2005
Korai Zamatos	*S12S13*	S-	Halasz *et al.,* 2005
Krimskyi Medunec	*S8*	SI	Halasz *et al*., 2005
Kurukabuk	*S7S9*	SI	Halasz *et al*., 2010
Lambertine	*S1S2*	SI	Sutherland *et al*., 2004a
Levent	*S6S19*	SI	Halasz *et al*., 2010
Ligeti Orias	*S8S9*	SI	Halasz *et al*., 2005
Lorna	*S2*	S-	Donoso *et al*., 2009
Mahmudun Erigi	*S13S?*	SI	Halasz *et al.,* 2010
Mandulakaszi	*ScSy*	SC	Halasz *et al.,* 2005
Marculesti	*Sc*	S-	Halasz *et al*., 2005
Mari de Cenad	*Sc*	SC	Halasz *et al.,* 2005
Mektep	*ScS8*	SC	Halasz *et al*., 2010
Mimaia	*ScSy*	SC	Halasz *et al*., 2005
Modesto	*ScS13*	SC	Halasz *et al*., 2005
Moniqui	*S2S6*	SI	Vilanova *et al*., 2005
Nikitskyi	*Sc*	SC	Halasz *et al.,* 2005
Ninfa	*S3*	S-	Donoso *et al*., 2009
NJA-8	*Sc or S8*	S-	Halasz *et al.,* 2005
No 1 Zerdali	*S6S12*	SI	Halasz *et al*., 2010
No 2 Zerdali	*S9S13*	SI	Halasz *et al.,* 2010
Orange Red	*S6Sx*	S-	Donoso *et al*., 2009
Ordubat	*S7S12*	SI	Halasz *et al*., 2010
Ozal	*S6S9*	SI	Halasz *et al*., 2010
Palau	*ScSc*	SC	Vilanova *et al.,* 2005
Palsteyn	*ScSc*	S-	Donoso *et al*., 2009
Pasa Mismisi	*ScS8*	SC	Halasz *et al*., 2010
Patterson	*ScSc*	S-	Donoso *et al*., 2009
Pelese di Giovanniello	*S1S2*	S-	Donoso *et al*., 2009
Perla	*S2*	S-	Donoso *et al*., 2009
Pisana	*S2Sc*	S-	Donoso *et al.,* 2009
Rial Fino	*S6Sc*	SC	Sutherland *et al*., 2004a
Royal	*Sx-690bp*	S-	Donoso *et al*., 2009
Sakit 1	*S6S9*	SI	Halasz *et al*., 2010
Sakit 3	*S3S19*	SI	Halasz *et al*., 2010
Sam	*ScS2*	SC	Halasz *et al*., 2010
Sebbiyiki	*S2S19*	SI	Halasz *et al*., 2010
Seftalioglu	*S8S9*	SI	Halasz *et al.,* 2010
Sekerpare	*S3S6*	SI	Halasz *et al*., 2010
Shalakh (Aprikoz)	*S11S13*	SI	Halasz *et al*., 2010
Soganci	*S6S9*	SI	Halasz *et al*., 2010
Sulmona	*Sc*	SC	Halasz *et al*., 2005
Sunglo	*S2S3*	SI	Sutherland *et al*., 2004a
Tilton	*ScSc*	S-	Donoso *et al.,* 2009
Tokaloglu Izmir	*S3S19*	SI	Halasz *et al.,* 2010
Turfanda Izmir	*S7S8*	SI	Halasz *et al.,* 2010
Veecot	*S2*	S-	Donoso *et al*., 2009
Venus	*Sc*	SC	Halasz *et al*., 2005
Voski	*S11S13*	S-	Halasz *et al.,* 2005
X1 Zerdali	*S6S12*	SI	Halasz *et al.,* 2010

X2 Zerdali	*S7S12*	SI	Halasz *et al*., 2010
X3 Zerdali	*S9S20*	SI	Halasz *et al*., 2010
Yegen	*S8S11*	SI	Halasz *et al*., 2010
Yerli Izmir	*ScS7*	SC	Halasz *et al.,* 2010
Zaposdolje	*Sc or S8*	S-	Halasz *et al*., 2005
Zard	*S16*	S-	Halasz *et al.,* 2005
Ziraat Okulu	*S2S8*	SI	Halasz *et al.,* 2010

SI = self-incompatible, SC = self-compatible, S- = unknown genotypes.

The self-compatibility of Sc was suggested by Vilanova *et al.* (2005) to be due to a pollen-part mutation of the S8 haplotype with a 358 bp insertion in the *SFB*c gene, which causes a frame-shift in translation that produces a non-functional truncated protein (Yamane and Tao, 2009). Halasz *et al.* (2010) also reported that differentiation of SI and SC coding regions could not be achieved in the analysis of the *S-RNase*, but could be distinguished in the *SFB* gene. Hence a new primer was designed (AprSFBC8), used in combination with the reverse primer PaconsIR, to distinguish between cultivars with *S*c and *S*8 sizes. The *SFB*c allele has a product size of approximately 500 bp, while genotypes carrying the *SFB*8 allele showed a product size of approximately 150 bp.

Mutations in non *S*-locus factors have also been associated with self-compatibility in sweet cherry (Wünsch and Hormaza, 2004), almonds (Fernandez *et al.*, 2009) and diploid strawberry (Bošković *et al.,* 2010). Genetic evidence of factors unlinked to the *S*-locus required for GSI, also called modifier genes, was previously observed in Solanaceae. In apricot, the cultivar 'Canino' (*S2Sc*) was found to contain two different mutations conferring SC, an insertion in the *SFBc* gene that produces a truncated *SFBc* protein, as mentioned above and a mutation in a modifier gene (*m*) unlinked to the *S*-locus, both independently causing the loss of pollen-*S* function (Zuriaga *et al.,* 2012).

In apricot, the North American cultivar 'Katy' and Spanish local cultivar 'Canino' have been reported to have pollen-part mutations (PPMs) conferring SC by putatively affecting modifiers. Both PPMs were mapped at the *M*- and *M*'- loci (Zuriaga et al., 2012). Recently, a list of 67 cultivars or accessions were genotyped for the the *S*-and *M*- locus (Munoz-Sanz et al., 2017). In the study up to 20 *S*-alelles were detected and haplotype analysis performed by genotyping and determining linkage-phases of 7 SSR markers, showed that the *m* and *m*' PPMs are linked to the *m*o-halpotype associated with SC.

2.9.8 Self-(in)compatibility in plum

Most diploid species of plum are self-incompatible while hexaploid European plums may be self-incompatible or self-compatible (Hegedus and Halasz, 2006). According to Sutherland *et al*. (2004b), early controlled self- and cross-pollination trials in plum were used to identify cultivars as fully self-compatible, semi self-compatible or self-incompatible (Rawes, 1921).

The first *S*-genotype (*SaSb*) of a Japanese plum cultivar ('Sordum') was reported by Yamane *et al*. (1999). The banding patterns were obtained with an *S-RNase* gene-specific primer set, Pru-C2 and PCE-R, which amplify across the second intron (Tao *et al*., 1999) giving bands ranging between 470 bp to 1 540 bp. In addition, Beppu *et al*. (2002) determined the S-genotypes of 17 Japanese plum cultivars (Table 2.4). Five novel *S-RNase* alleles (*Sj* to *Sn*) were identified with the same primer set, with the product sizes ranging from 320 bp to 1 600 bp. Later, Guerra *et al*. (2012) also reported on Japanese plum second intron amplification products using the PaconsI-F and PaconsI-R2 primers that were assigned to *So* to *Ss* with product sizes ranging between 580 bp to 1 304 bp.

In recent studies, Halasz *et al.* (2007) identified SI alleles in commercially important Japanese plum and pluot cultivars. They used the consensus primers designed by Sutherland *et al.* (2004a) to amplify the second intron and used the fluorescently labelled forward primer (PaConsI-F), in combination with the reverse primer EM-PC1consRD, to amplify the first intron (Sonneveld *et al*., 2003; Ortega *et al*., 2005). PCR products for the second intron gave bands ranging from 350 bp to 1 550 bp and first intron products showed 295 bp to 388 bp bands. These authors also used allele-specific primers, designed by Sapir *et al*. (2004), for *S3* to *S6* alleles and these primers resulted in successful amplification that corresponded with previously reported allele sizes. However, no *S1* alleles could be detected using S1 allele-specific primers.

Hegedus and Halasz (2006) noted the common *S*-genotype (*ScSe*) in the self-compatible cultivars, 'Santa Rosa', 'Late Santa Rosa' and 'Beauty' and suggested that the *Se*-haplotype is responsible for the self-compatibility trait. In self- and cross-pollination experiments between cultivars with the *ScSe* genotypes, the resultant seedlings segregated into two classes, *ScSe* and *SeSe* (Beppu *et al.*, 2005), suggesting that seedlings inherited only the *Se*-haplotype from the male parent, confirming that the *Se*-haplotype is responsible for self-compatibility. However, Guerra and Rodrigo, (2015) reported that self-compatibility was also associated with other S-haplotypes: *Sb* (Guerra *et al*., 2009; Beppu *et al*., 2010), *Sg* (Beppu *et al*., 2012) and *Ss* (Beppu *et al*., 2012). Moreover, numerous Japanese plum cultivars displaying the *Se* or *Sb* alleles have been described as self-incompatible. Further investigation of this phenomenon is therefore necessary to increase understanding of the basis of self-compatibility in Japanese plum.

To date, 19 incompatibility alleles (*S*-alleles) have been reported (*Sa* to *Ss*) and Sapir *et al.* (2004) cloned five *S*-alleles from three commercially important Japanese plum cultivars and labelled them with the numeric codes, *S1, S3* to *S6* (Halasz *et al.*, 2007). The *S3* allele was shown to correspond to *Sk* in the alphabetic nomenclature, *S4* to *Sc*, *S5* to *Se* and *S6* to *Sf*.

Table 2.4. Product sizes of the *S-RNase* alleles in Japanese plum, determined by PCR amplification across the second intron using Pru-C2, PCE-R, and Pru-T2 primers (Beppu *et al*., 2002; Beppu *et al*., 2003; Guerra *et al*., 2009).

S-alleles	Size (bp)	Reference
Sa	470	Beppu *et al.*, 2002
Sb	1 540	Beppu *et al*., 2002
*Sc */S4*	1 170	Beppu *et al*., 2002
Sd	1 290	Beppu *et al*., 2002
*Se */S5*	1 420	Beppu *et al*., 2002
Sf/S6	1 120	Beppu *et al*., 2002
Sg	1 230	Beppu *et al*., 2002
Sh	520	Beppu *et al*., 2002
Si	410	Beppu *et al*., 2002
Sj	1 660	Beppu *et al.*, 2003
Sk/S3	360	Beppu *et al.*, 2003
Sl	870	Beppu *et al.*, 2003
Sm	320	Beppu *et al.*, 2003
Sn	640	Beppu *et al.*, 2003
So	1 304	Guerra *et al*., 2009
Sp	600	Guerra *et al*., 2009
Sq	1 270	Guerra *et al*., 2009
Sr	760	Guerra *et al*., 2009
Ss	580	Guerra *et al*., 2009

Note: * self-compatible alleles

In Japanese plum self-(in)compatibility groups were proposed by Halasz and co-workers (2007). In table 2.5 below, the *S*-genotypes of plum cultivars and their self-(in)compatibility groups are listed. According to Halasz *et al*. (2007), the cultivars 'Black Amber', 'October Sun', 'TC Sun', and 'Super Giant' are known to share the *SbSc* genotype, which was confirmed by test crosses. These cultivars belong to the widest incompatibility group (*SbSc*) currently known in Japanese plum.

Table 2.5. Previously reported *S-RNase*-genotypes, and self-(in)compatibility groups of Japanese plum cultivars identified by PCR method and evaluation of pollen tube growth, confirmed by cross-pollination tests.

Cultivar	S-genotypes	Group	Reference
Abandacia	*SfSh*	SI	Beppu *et al*., 2003
Angeleno	*ScSh*	SI	Halasz *et al*., 2007
Bakemonosumomo	*SbSi*	SI	Beppu *et al.*, 2002
Beauty	*ScSe*	SC	Beppu *et al*., 2002
Black Amber	*SbSc*	SI	Halasz *et al*., 2007
Black Diamond	*Se*	SC	Sapir *et al*., 2004
Bonnie	*SgSh*	SI	Beppu *et al*., 2003
Botan	*SaSm*	SI	Beppu *et al*., 2003
Burmosa	*SaSb*	SI	Beppu *et al*., 2003
Combination	*SgSl*	SI	Beppu *et al*., 2003
Flavor Grenade	*SbSc*	SI	Halasz *et al*., 2007
Flavor King	*SbSe*	SC	Halasz *et al.*, 2007
Formosa	*SbSd*	SI	Beppu *et al*., 2002

Friar	*ShSk*	SI	Halasz *et al*., 2007
Frontier	*SbSf*	SI	Beppu *et al.,* 2003
Gran Colle	*SbSf*	SI	Beppu *et al.,* 2003
Green Sun	*ScSh*	SI	Halasz *et al*., 2007
Harypickstone	*SbSk*	SI	Beppu *et al*., 2003
Honey Rosa	*SbSg*	SI	Beppu *et al.,* 2003
Kasahara Hatankyou	*SbSi*	SI	Beppu *et al*., 2003
Kelsey	*SfSh*	SI	Beppu *et al*., 2002
Kelsey Paulista	*SfSh*	SI	Beppu *et al*., 2003
Lantz	*SbSl*	SI	Beppu *et al*., 2003
Laroda	*SbSc*	SI	Beppu *et al*., 2002
Late Santa Rosa	*ScSe*	SC	Beppu *et al.,* 2002
October Sun	*SbSc*	SI	Halasz *et al*., 2007
Oishinakata	*SbSc*	SI	Beppu *et al*., 2002
Oishiwasesumomo	*ScSd*	SI	Beppu *et al*., 2002
Queen Anne	*SbSh*	SI	Beppu *et al*., 2002
Queen Rosa	*ScSh*	SI	Beppu *et al.,* 2003
Red Beauty	*SaSb*	SI	Beppu *et al*., 2002
Rio	*SaSe*	SC	Beppu *et al.,* 2002
Royal-Zee	*ScSe*	SC	Sapir *et al*., 2004
Santa Rosa	*ScSe*	SC	Beppu *et al.,* 2002
Shiro	*Sf*	SI	Halasz *et al*., 2007
Simka	*SeSk*	SC	Beppu *et al*., 2003
Sordum	*SaSb*	SI	Yamane *et al*., 1999
Starkgold	*SgSk*	SI	Beppu *et al*., 2003
Summer Queen	*ScSf*	SI	Beppu *et al*., 2003
Super Giant	*SbSc*	SI	Halasz *et al*., 2007
Sweet Autumn	*ScSe*	SC	Halasz *et al.,* 2007
Taiyo	*SbSc*	SI	Beppu *et al.,* 2002
TC Sun	*SbSc*	SI	Halasz *et al*., 2007
Tecumseh	*SfSj*	SI	Beppu *et al*., 2003
Terada	*SaSf*	SI	Beppu *et al*., 2002
Verna Deliciuos	*SbSf*	SI	Beppu *et al.,* 2003
White plum	*SfSg*	SI	Beppu *et al*., 2002
Wickson	*SkSf*	SI	Sapir *et al*., 2004
Yonemono	*SbSh*	SI	Beppu *et al*., 2002
Younai	*SfSh*	SI	Zhang *et al*., 2008

SI = self-incompatible, SC = self-compatible

Several Japanese plum-type cultivars and selections (Table 2.6) from different plant collections, nurseries and commercial orchards of different origin were analysed previously by several researchers. These cultivars could be assigned to 27 self-incompatibility groups (including group O with unique genotypes) and one self-compatibility group, with the groups containing between two to 29 cultivars.

Table 2.6. (In)compatibility groups (I to XXVI) and *S*-genotypes of Japanese plum cultivars, updated from Guerra and Rodrigo (2015) by including accessions genotyped in the current study, which are underlined.

Group I *SaSb*	
606	Guerra *et al*., 2009
Angelo	Jun *et al*., 2007
Armstrong	Jun *et al*., 2007
Burmosa	Beppu *et al*., 2003
Late Soldam	Jun *et al*., 2007
Mammoth	Jun *et al*., 2007
Red Beaut	Beppu *et al*., 2002

Soldam	Jun *et al*., 2007
Sordum	Yamane *et al*., 1999

Group II *SbSc*	
3556	Guerra *et al*., 2012
3726	Guerra *et al*., 2012
Benikayama	Jun *et al*., 2007
Beniryozen	Jun *et al*., 2007
Black Beaut	Guerra *et al*., 2009
Blackamber	Halasz *et al*., 2007
Delbartazur	Guerra *et al*., 2009
Early Sun	Guerra *et al*., 2012
Flavor Granade	Halasz *et al*., 2007
Fortune	Guerra *et al*., 2009
Golden Globe[a]	Guerra *et al*., 2009
Golden Plum[a]	Guerra *et al*., 2009
Golden Plumza	Guerra *et al*., 2009
Green Sun[a]	Guerra *et al*., 2009
Gulfrose	Sapir *et al*., 2008
Honey Red	Jun *et al*., 2007
Jupiter	Jun *et al*., 2007
Laroda	Beppu *et al*., 2002
Natuotome	Jun *et al*., 2007
October Sun	Halasz *et al*., 2007
Oishinakata	Beppu *et al*., 2002
Purple Queen	Jun *et al*., 2007
SGPR3318	Guerra *et al*., 2012
SGPR3726	Guerra *et al*., 2012
Sugared	Jun *et al*., 2007
Super Giant	Halasz *et al*., 2007
Taiyo	Beppu *et al*., 2002
TC Sun	Halasz *et al*., 2007
Wase Taiyo	Jun *et al*., 2007

Group III *SbSf*	
AU Amber	Guerra *et al*., 2012
AU Road Side	Guerra *et al*., 2012
Emarald	Sapir *et al*., 2008
Frontier	Beppu *et al*., 2003
Golden Globe[a]	Guerra *et al*., 2009
Gran Colle	Beppu *et al*., 2003
Verna Delicious	Beppu *et al*., 2003

Group IV *SbSh*	
Akihime,	Jun *et al*., 2007
Betty Ann	Sapir *et al*., 2008
Black Gem	Sapir *et al*., 2008
Blue Knight	Sapir *et al*., 2008
E137	Guerra *et al*., 2012
E316	Guerra *et al*., 2012
Eldorado	Guerra *et al*., 2009
Freedom	Guerra *et al*., 2009
Friar[a]	Guerra *et al*., 2009
Heibaoshi	Zhang *et al*., 2007
Hiromi Red	Guerra *et al*., 2009
Larry Ann	Guerra *et al*., 2009
Nubiana[a]	Guerra *et al*., 2009
PR34	Guerra *et al*., 2012
Qiuji	Zhang *et al*., 2007
Queen Ann,	Beppu *et al*., 2002
Scarlet	Jun *et al*., 2007
Songria 10	Guerra *et al*., 2009
Yonemomo	Beppu *et al*., 2002

Group V *SbSi*	
Bakemonosumomo	Beppu *et al*., 2002
Kasahara Hatankyou	Beppu *et al*., 2003

Group VI *SfSh*	
Abundance[a]	Beppu *et al*., 2003
Black Ruby	Guerra *et al*., 2012
Kelsey Paulista	Beppu *et al*., 2003
Kelsey[a]	Beppu *et al*., 2003
Mariposa	Guerra *et al*., 2012
Younai	Zhang *et al*., 2007

Group VII *ScSh*	
Queen Rosa	Beppu *et al*., 2003
30-an-71	Guerra *et al*., 2009
Angelenex	Guerra *et al*., 2009
Gaia	Guerra *et al*., 2009
Ruby Crunch	Guerra *et al*., 2009
Sweet August	Guerra *et al*., 2011
3485	Guerra *et al*., 2012
Royal Diamond	Guerra *et al*., 2012
Ruby Queen	Guerra *et al*., 2012
Green Sun[a]	Halasz *et al*., 2007
Angeleno	Sapir *et al*., 2004
Nvgelei	Zhang *et al*., 2007

Group VIII *SeSh*	
26-bd-10	Guerra *et al*., 2009
3517	Guerra *et al*., 2012
3530	Guerra *et al*., 2012
3575	Guerra *et al*., 2012
Autumn Pride	Sapir *et al*., 2008
Black Diamond	Sapir *et al*., 2004
Black Gold	Guerra *et al*., 2009
Black Late	Guerra *et al*., 2009
Diamex	Guerra *et al*., 2009
Earliqueen	Guerra *et al*., 2009
Extremagold	Guerra *et al*., 2009
Huangjiazuanshi	Zhang *et al*., 2007
John W	Guerra *et al*., 2009
Kaiqinman	Kitashiba *et al*., 2008
PR9	Guerra *et al*., 2012
Showtime	Guerra *et al*., 2009
Souvenir	Guerra *et al*., 2009

Group IX *SfSg*	
Golden Japan	Guerra *et al*., 2009
Jinshali	Zhang *et al*., 2007
Manchurian	Jun *et al*., 2007
Shiro	Sapir *et al*., 2008
White Plum	Beppu *et al*., 2002

Group X *ShSk*	
31-sg-6	Guerra *et al*., 2009
3611	Guerra *et al*., 2009
3989	Guerra *et al*., 2009
Elephant Heart	Jun *et al*., 2007
Explorer	Jun *et al*., 2007
Friar[a]	Halasz *et al*., 2007
Golden Plum[a]	Guerra *et al*., 2009
Howard Sun	Guerra *et al*., 2009
Redgold	Zhang *et al*., 2007
Songold	Guerra *et al*., 2009
Xiguali	Zhang *et al*., 2008
3527	Guerra *et al*., 2012
AU Rosa	Guerra *et al*., 2012

Group XI *ScSe*	
Autumn Giant	Guerra *et al*., 2009
Black Splendor	Guerra *et al*., 2009
Casselman (syn Kesselman)	Sapir *et al*., 2008
Champion	Guerra *et al*., 2011
E326	Guerra *et al*., 2012
Meiguili	Zhang *et al*., 2007
Royal Garnet	Guerra *et al*., 2009
Royal Zee	Sapir *et al*., 2004
Roysum	Sapir *et al*., 2008
Sweet Autumn	Halasz *et al*., 2007
Sybarite	Guerra *et al*., 2011

Group XII *SbSe*	
ARC PR-1	This study
ARC PR-4	This study
Black Jewell	Guerra *et al*., 2011
Durado	Sapir *et al*., 2008
Extreme	This study
Flavor Fall	This study
Flavor King	H(07), This study
Flavor Rich	This study
Freya	Guerra *et al*., 2012
Murietta	Sapir *et al*., 2008
Pioneer	G(09), This study
Satin Gold	This study
Saphire	Guerra *et al*., 2009
Sensation	This study
SGPR551	Guerra *et al*., 2012
Siekiguchiwase	Jun et al., 2007
Sparkly	Guerra *et al*., 2012
Suplum 28	This study
Tomar	Sapir *et al*., 2008

Group XIII *SeSf*	
Black Star	Guerra *et al*., 2009
Morris	Guerra *et al*., 2012
Primetime	Guerra *et al*., 2009

Group XIV *SaSc*	
87–91	Guerra *et al*., 2009
Crimson Glo	Guerra *et al*., 2009
White Queen	Jun *et al*., 2007

Group XV *SgSh*	
30-AN-7	Guerra *et al*., 2009
Bonnie	Beppu *et al*., 2003
Ruby Sweet	Guerra *et al*., 2012

Group XVI *SfSk*	
Kelsey[a]	Guerra *et al*., 2009
Weikeshum	Zhang *et al*., 2007
Wickson	Sapir *et al*. (2004)

Group XVII *SbSo*	
Ambra	Guerra *et al*., 2009
Olinda	Guerra *et al*., 2009

Group XVIII *SaSf*	
Ozarkpremier	Zhang *et al*., 2007
Terada	Beppu *et al*., 2002

Group XIX *SbSd*	
3442	Guerra *et al*., 2012
Formosa	Beppu *et al*., 2003
Harry Pickstone	Beppu *et al*., 2003

Group XX *SbSk*	
Furongli	Zhang *et al*., 2007
Gaixiandali	Zhang *et al*., 2007
Homeking Delicious	Jun *et al*., 2007
Paruru	Jun *et al*., 2007
Shiho	Jun *et al*., 2007

Group XXI *SeSk*	
Newyorker	Sapir *et al*., 2008
Simon	Guerra *et al*., 2012

Group XXII *SaSe*	
Dolly	Sapir *et al*., 2008
Riou	Jun *et al*., 2007

Group XXIII *ScSd*	
Oishiwasesumomo	Beppu *et al*., 2002

Jumbo Oishiwase	Jun *et al*., 2007
Plum Inoue	Jun *et al*., 2007
Royal Oishiwase	Jun *et al*., 2007
Select Oishiwase	Jun *et al*., 2007
Wasegekko	Jun *et al*., 2007

Group XXIV *SeS11*	
Lihesihao	Zhang *et al*., 2008
Qiuli	Zhang *et al*., 2008

Group XXV *S15S16*	
Kuandiandali	Zhang *et al*., 2008
Pingguoli	Zhang *et al*., 2008

Group XXVI S*21S22*	
Zhenzhuli	Zhang *et al*., 2008
Zhuxueli	Zhang *et al*., 2008

Group O	
3458 (*Se*-)	Guerra *et al*., 2012
95-03 (*S27Sa*)	Zhang *et al*., 2008
96-08 (*S24S25S26*)	Zhang *et al*., 2008
Abundance*(*SaSk*)	Guerra *et al*., 2009
Biocherrie (*SeSg*)	Jun *et al*., 2007
Botan (*SaSm*)	Beppu *et al*., 2003
Byron Gold (*ShSr*)	Guerra *et al*., 2012
Cocheco (*Sa*-)	Jun *et al*., 2007
Combination (*SgSl*)	Beppu *et al*., 2003
Dameigui (*S23S24S17*)	Zhang *et al*., 2008
Danchengxingmei (*S18S20*)	Zhang *et al*., 2008
Dazhili (*S10*-)	Kitashiba *et al*., 2008
Fugongqingpili (*S11S7*)	Zhang *et al*., 2008
Gaia (*ScSh*)	Guerra *et al*., 2012
Huang Pi Li (*SfS7*),	Zhang *et al*., 2007
Joana Red (*SrSs*)	Guerra *et al*., 2009
Kiyou=Kiyo (*SbScSf*)	Jun *et al*., 2007
Lantz (*SbSl*)	Beppu *et al*., 2003
Mali (*S10S14*),	Zhang *et al*., 2007
Meiguodeli (*S11S20*)	Zhang *et al*., 2008
Mitard (*SqSf*)	Guerra *et al*., 2009
October Red (*ShSp*)	Guerra *et al*., 2009
Qianping (*S10Si*)	Zhang *et al*., 2008
Red Heart (*ScSo*)	Guerra *et al*., 2009
Shiro (*Sf*-)	Halasz *et al*., 2007
Songria 15 (*SaSh*)	Guerra *et al*., 2009
Starkgold (*SgSk*)	Beppu *et al*., 2003
Summer Queen (*ScSf*)	Beppu *et al*., 2003
Superior (*SaSn*)	Beppu *et al*., 2003
Tecumseh (*SfSj*)	Beppu *et al*., 2003
Xiangjiabli (*S7Sf*)	Zhang *et al*., 2007
Xinjiangsanhao (*S17S19*),	Zhang *et al*., 2008
Yingtaoli (*S11S8*)	Zhang *et al*., 2008
Zuili (*S7Sh*)	Zhang *et al*., 2008

Group SC	
Beauty (*ScSe*)	Beppu *et al*., 2002
Casselman (*ScSe*)	Beppu *et al*., 2002
Honey Rosa (*SbSg*)	Beppu *et al*., 2003
Karari (*SbSt*)	Beppu *et al*., 2012
Laetitia (*SeSh*)	Guerra *et al*., 2009
Late Santa Rosa (*ScSe*)	Beppu *et al*., 2002
Methley (*SbSg*)	Jun *et al*., 2007
Nubiana (*SbSc*)	Guerra *et al*., 2009
Red Rosa (*ScSe*)	Sapir *et al*., 2008
Rio (*SaSe*)	Beppu *et al*., 2002
Rubirosa (*ScSe*)	Guerra *et al*., 2011
Santa Rosa (*ScSe*)	Guerra *et al*., 2009
Simka (*SeSk*)	Beppu *et al*., 2003
Zanzi Sun (*SbSc*)	Guerra *et al*., 2009

a Cultivars described with several different S-genotypes. (Guerra *et al*., 2009; Guerra *et al*., 2012)

2.10 References

Alburquerque N., Egea J., Perez-Tornero O. and Burgos L. (2002): Genotyping apricot cultivars for self (in)compatibility by means of RNases associated with *S*-alleles. Plant Breeding, 121: 343-347.

Anderson E.T. and Weir T.S. (1967): *Prunus* hybrids, selections and cultivars at the University of Minnesota fruit breeding farm. Minnesota Agricultural Experiment Station Bulletin, 252: 6-52.

Aranzana M.J., Garcia-Mas J., Carbo J. and Arus P. (2002): Development and variability analysis of microsatellite markers in peach. Plant Breeding, 121: 87-92.

Arismendi M.J., Hinrichsen P., Almanda R., Pimentel P., Pinto M. and Sagredo B. (2012): Characterisation of genetic diversity of stone fruit rootstocks used in Chile by means of microsatellite markers. Journal of American Society of Horticultural Science, 137: 302-310.

Arumaganathan K. and Earle E. (1991): Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter, 9: 208-218.

Benedikova D. (2006): Apricot breeding in Slovak Republic for fruit quality and resistance to disease. Acta Horticulturae, 701: 355-358.

Beppu K., Komatsu N., Yamane H., Yaegaki H., Yamaguchi M., Tao R., Kataoka I. (2005): Se-haplotype confers self-compatibility in Japanese plum (Prunus salicina Lindl.). Journal of Horticultural Science and Biotechnology, 80: 760-764.

Beppu K., Kumai M., Yamane H., Tao R. and Kataok I. (2012): Molecular and genetic analysis of the S-haplotype of the self-compatible Japanese plum (Prunus salicina Lindl.) 'Methley'. Journal of Horticultural Science and Biotechnology, 87: 493-498.

Beppu K., Syoyase K., Yamane H., Tao R. and Kataoka I. (2010): Inheritance of self-compatibility conferred by the Se-haplotype of Japanese plum and development of Se-RNase gene-specific primers. Journal of Horticutural Science and Biotechnology, 85, 215-218.

Beppu K., Takemoto Y., Yamane H., Yaegaki H., Yamaguchi M., Kataoka I. and Tao R. (2003): Determination of *S*-haplotypes of Japanese plum (*Prunus salicina* Lindl.) cultivars by PCR and cross pollination tests. Journal of Horticultural Science and Biotechnology, 78: 315-318.

Beppu K., Yamane H., Yaegaki H., Yamagachi M., Kataoka L. and Tao R. (2002): Diversity of *S-RNase* genes and S-haplotypes in Japanese plum (*Prunus salicina* Lindl.). Journal of Horticultural Science and Biotechnology, 77: 658-664.

Blazek J. (2007): A survey of the genetic resources used in plum breeding. Acta Horticulturae, 734: 31-45.

Bortiri E., Oh S., Jiang J., Baggett, S., Granger A., Weeks C., Buckingham, M., Potter D. and Parfitt D.E. (2001): Phylogeny and systematics of *Prunus* (Rosaceae) as determined by sequence analysis of ITS and the chloroplast trnL-trnF spacer DNA. Systematic Botany, 26: 797-807.

Bošković R. and Tobutt K.R. (1996): Correlation of stylar ribonuclear zymograms with in-compatibility alleles in sweet cherry. Euphytica, 90: 245-250.

Bošković R., Sargent D.J. and Tobutt K.R. (2010): Genetic evidence that two independent *S*-loci control *RNase*-based self-incompatibility in diploid strawberry. Journal of Experimental Botany, 61: 755-763.

Burgos L., Perez-Tornero O., Ballester J. and Olmose E. (1998): Detection and inheritance of stylar ribonucleases associated with incompatibility alleles in apricot. Sexual Plant Reproduction, 11: 153-158.

Campoy J.A., Martinez-Gomez P., Ruiz D., Rees J. and Celton J.M. (2010): Developing microsatellite multiplex and megaplex PCR systems for high-throughput characterisation of breeding progenies and linkage maps spanning the apricot (*Prunus armeniaca* L.) genome. Plant Molecular Biology Reporter, 28: 560-568.

California Tree Fruit Agreement (CTFA) (1996): Annual report: California Tree Fruit Agreement, Sacramento, California.

Cantini C., Iezzoni A.F., Lamboy W.F., Boritzki M. and Struss D. (2001): DNA fingerprinting of tetraploid cherry germplasm using SSRs. Journal of American Society of Horticultural Science, 126: 205-209.

Cipriani G., Lot G., Huang W.G., Marrazzo M.T., Peterlunger E. and Testolin E. (1999): AC/GT and AG/CT microsatellite repeats in peach (*Prunus persica* (L.) Batsch): Isolation, characterisation and cross-species amplification in *Prunus.* Theoretical and Applied Genetics, 99: 65-72.

Considine D.M. and Considine G.D. (1982): Foods and food production encyclopedia. Van Nostrand Reinhold, New York, pp 83-91.

Crane M.B. and Lawrence W.J.C. (1929): Genetical and cytological aspects of incompatibility and sterility in cultivated fruits. Journal of Pomology and Horticultural Science, 7: 276-301.

Cullen J., Knees S.G. and Cube H.S. (2014): The European Garden Flora, 2nd ed. Cambridge University Press, Cambridge, pp 1-5.

De Nettancourt D. (1977): Incompatibility in Angiosperms. Monographs on Theoretical and Applied Genetics. Vol 3. Springer, New York, pp 1-24.

Decroocq V., Hagen L., Fave M-G., Eyquard J.P. and Pierronnet A. (2004): Microsatellite markers in the hexaploid *Prunus domestica* species and parentage lineage of three European plum cultivars using nuclear and chloroplast simple-sequence repeat. *Molecular Breeding*, 13: 135-142.

Dirlewanger E., Cosson P., Tavaud M., Aranzana M.J., Poizat C., Zanetto A., Arus P. and Laigret F. (2002): Development of microsatellite markers in peach (*Prunus persica* (L.) Batsch) and their use in genetic marker analysis in peach and sweet cherry (*Prunus avium* L.). Theoretical and Applied Genetics, 105: 127-138.

Dirlewanger E., Graziano E., Joobeur T., Garriaga-Caldere F., Cosson P., Howad and Arus P. (2004): Comparative mapping and marker-assisted selection in Rosaceae fruit crops. Processings of the National Academy of Science of the United States of America, 101: 9891-9896.

Donoso J.M., Aros D., Meneses C. and Infante R. (2009): Identification of *S*-alleles associated with self-incompatibility in apricot (*Prunus armeniaca* L.) using molecular markers. Journal of Food, Agriculture and Environment, 7: 270-273.

Dosba F., Bernhard R. and Zanetto A. (1994): Importance des resources genetiques des *Prunus.* Comptes-rendure Academie d' Agriculture de France, 80: 45-57.

East E.M. and Mangelsdorf A.J. (1925): A new interpretation of the hereditary behaviour of self-sterile plants. Proceedings of the National Academy of Science of the United States of America, 11: 166-171.

Egea J. and Burgos L. (1996): Detecting cross-incompatibility of three North American cultivars and establishing the first incompatibility group in apricot. Journal of the American Society for Horticultural Science, 121: 1002-1005.

Egea J., Garcia J.E., Egea L. and Berenguer T. (1991): Self-incompatibility in apricot cultivars. Acta Horticulturae, 293: 285-293.

Eiermann C. (2012): Fruit Trees in Small Spaces: Abundant Harvest from Your Own Backyard, Timber Press, London, pp 63-85.

Ellegren H. (2004): Microsatellites: simple sequences with complex evolution. Nature Reviews Genetics, 5: 435-445.

Entani T., Iwano M., Shiba H., Che F., Isogla A. and Takayana S. (2003): Comparative analysis of the self-incompatible (*S*-) locus region of *Prunus mume*: identification of a pollen expressed F-Box gene with allelic diversity. Genes to Cells, 8: 203-213.

Esmenjaud D. and Srinivasan C. (2012): Molecular breeding, In: Genetics, Genomics and Breeding of Stone Fruits. Kole C. and Abbott A.G. (eds). CRC, Clemson, pp 158-211.

FAOSTAT (2013): Food and Agriculture Organization of the United Nations. FAOSTAT statistics Database, http://faostat.fao.org/page/collections (accessed: 24/08/2014)

Fernandez A., Hanada T., Alonso J.M., Yamane H. and Tao R. (2009): A modifier locus affecting the expression of the *S-RNase* gene could be the cause of breakdown of self-incompatibility in almond. Sexual Plant Reproduction, 22: 179-186.

Flory W. (1947): Crossing relationships among hybrid and specific plum varieties, and among the several *Prunus* species which are involved. American Journal of Botany, 34: 330-335.

Franklin F.C.H., Lawrence M.J. and Franklin-Tong V.E. (1995): Cell and molecular biology of self-incompatibility in flowering plants. International Review of Cytology, 158: 1-64.

Frecon J.L. and Ward D.L. (2012): Testing and evaluation of plum and plum hybrid cultivars. Fruit Notes, 77: 12-22.

Gagnard J.M. (1956): Recherches surles caracteres systematiques et sur les phenomenes de sterilite chez les varities d' amandiers cultivees en Algerie. Annales de Institut Agricole (Algerie), 8: 1-163.

Gharesheikhbayat R. (2010): Self-incompatibility in apricot (*Prunus armeniaca*); New achievement and molecular aspects of *S*-locus allele segregation (published doctoral dissertation). Alma Mater Studiorum – University of Bologna, pp 3-16.

Girish K., Tsering S., Richa A. Yadav A. and Shashi B.S. (2011): Antioxidant capacity and Phenolic content of Apricot (Prunus armeniaca L.) kernel as a function of genotype. Plant Foods for Human Nutrition of Genotype, 66: 376-383.

Gomez-Plaza E. and Ledbetter C. (2010): The flavour of plums. In: Handbook of fruit and vegetable flavors. Hui Y.H. (ed). John Wiley & Sons, Hoboken, NJ, USA, pp 415-430.

Grover A. & Sharma P.C. (2014): Development and use of molecular markers: past and present. Critical Reviews in Biotechnology, 1-13.

Guerra F., Audergon J., Albagna G. and Reich M. (2001): Soluble sugars and carboxylic acids in ripe apricot fruit as parameters for distinguishing different cultivars. Euphytica, 11: 183-189.

Guerra M.E. and Rodrigo J. (2015): Japanese plum pollination: A review. Scientia Horticulturae, 197: 674-684.

Guerra M.E., Rodrigo J., López-Corrales M., Wünsch, A. (2009): *S-RNase* genotyping and incompatibility group assignment by PCR and pollination experiments in Japanese plum. Plant Breeding, 128: 304-311.

Guerra M.E., Lopez-Corrales M. and Wünsch A. (2012): Improved S-genotyping and new incompatibility groups in Japanese plum. Euphytica, 186: 445-452.

Guichoux F., Lagache L., Wagner S., Chaumeil P., Leger P., Lepais O., Lepottevin C., Malausa T., Revardel E., Salin F. and Petit R.J. (2011): Current trends in microsatellite genotyping. Molecular Ecology Resources, 11: 591-611.

Gupta P.K. and Varshney R.K. (2000): The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. Euphytica 113: 163-185.

Halasz J., Hegedus A., Herman R., Stefenovits-Banyai E. and Peryc A. (2005): New self-incompatibility alleles in apricot (*Prunus armeniaca* L.) revealed by stylar ribonuclease assay, an S-PCR analysis. Euphytica, 145: 57-66.

Halasz J., Hegedus J., Szabo Z., Nyeki J. and Pedryc A. (2007): DNA-based S-genotyping of Japanese plum and pluot cultivars to clarify incompatibility relationships. HortScience, 42: 46-50.

Halasz J., Hegedus A., Szikriszt B., Ercisli S., Orhan E. and Unlu H.M. (2013): The *S*-genotyping of wild-grown apricots reveals only self-incompatible accessions in the Erzincan region of Turkey. Turkish Journal of Biology, 37: 733-740.

Halasz J., Pedryc A., Ercisli S., Yilmaz K. U. and Hegedus A. (2010): *S*-genotyping supports the genetic relationship between Turkish and Hungarian apricot germplasm. Journal of American Society of Horticultural Science, 135: 410-417.

Hancock J.F., Scorza R. and Lobos G.A. (2008): Peaches. In: Temperate Fruit Crop Breeding: Germplasm to Genomics. Hancock J.F. (ed) Springer, Berlin, pp 265-298.

Hegedus A. and Halasz J. (2006): Self-incompatibility in plums (*Prunus salicina* Lindl, *Prunus cerasifera* Ehrh. and *Prunus domestica* L.): A mini review. International Journal of Horticultural Science, 12: 137-140.

Hill L. and Perry L. (2010): The fruit gardener's bible: A complete guide to growing fruits and nuts in the home garden. Storey Publishing, North Adams, MA, USA, pp 170.

Hofstee M.E., Malone M. and Howard C. (1999): Apricot breeding in New Zealand. Acta Horticulturae, 488: 171-173.

Hormaza J.I. (2002): Molecular characterisation and similarity relationships among apricot (*Prunus armeniaca* L.) genotypes using simple sequence repeats. Theoretical and Applied Genetics, 104: 321-328.

Hortgro (2016): Key deciduous fruit statistics: http://hortgro.co.za/wp-content/uploads/2017/06/HORTGRO-Key-Deciduous-Fruit-Statistics-2016.pdf (accessed: 21/08/2017).

Huang S., Lee H.S., Karunanandaa B. and Kao T.H. (1996): Ribonuclease activity of *Petunia inflata* S protein is essential for rejection of self-pollen. Plant Cell, 6: 1021-1028.

Ikeda K., Igic B., Ushijima K., Yamane H., Hauck N.R., Nakano R., Sassa H., Iezzoni A.F., Kohn J.R. and Tao R. (2004): Primary structural features of the S-haplotype-specific F-box protein, *SFB*, in *Prunus*. Sexual Plant Reproduction, 16: 235-243.

Ishimizu T., Endo T., Yamaguchi-Kabata Y., Nakamura K.T., Sakiyama F. and Norioka S. (1998): Identification of regions in which positive selection may operate in S-RNase of Rosaceae: implication for S-allele recognition sites in *S-RNase*. FEBS Letters, 440: 337-342.

Janick J. (2005): The origin of fruits, fruit growing and fruit breeding. Plant Breeding Review, 20: 255-320.

Johansson E. and Olden E.J. (1962): Damsons, plums gages, myrobalans. In: Handbuchder Pflanzenziichtung Kappert H. and Rudorf W. (eds) Paul Parey, Berlin, pp 602- 607.

Jones O.R. and Wang J. (2010): Colony: a program for parentage and relationship inference from multilocus genotype data. Molecular Ecology Resources, 10: 551-555.

Jun J., Kwon J.H., Chung K.H., (2007): Identification of self-incompatibility genotypes in Japanese plum (*Prunus salicina* Lindl.) by polymerase chain reaction and cross-pollination tests. Horticulture, Environment and Biotechnolgy, 48: 228–234.

Kadkhodaei S., Shahnazari M., Nekouei M.K., Ghasemi M., Etminani H., Imani A. and Ariff A.B. (2010): A comparative study of morphological and molecular diversity analysis among cultivated almonds (*Prunus dulcis*). Australian Journal of Crop Science, 5: 82-91.

Kao T.H. and Tsukamoto T. (2004): The molecular and genetic bases of S-RNase-based self (in)compatibility. Plant Cell, 16: 72-83.

Kelkar Y.D. Strubczewski N., Hile S.E., Chiaromonte F., Eckert K.A. and Makova K.D. (2010): What is a microsatellite: a computational and experimental definition based upon repeat mutational behaviour at A/T and GT/AC repeats. Genome Biology Evolution, 2: 620-635.

Kitashiba H., Zhang S.L., Wu J., Shirasawa K. and Nishio T. (2008): *S* genotyping and *S* screening utilizing *SFB* gene polymorphism in Japanese plum and sweet cherry by dot-blot analysis. Molecular Breeding, 21: 339–349.

Klabunde G.H.F., Dalbo M.A. and Dorani R.O. (2014): DNA fingerprinting of Japanese plum (*Prunus salicina*) cultivars based on microsatellite markers. Crop Breeding and Applied Biotechnology, 14: 139-145.

Kobel F., Steineyger P. and Anliker J. (1939): Weiter Untersuchungen uber die Befruchutungsverhaltnisse der Apfel – und Birnsorten. Landwirtschaftliches Jarhbuch de Schweiz, 53: 169-191.

Kostina F.K. (1969): The use of varietal resources of apricots for breeding. Trudy Gosudarstvennogo Nikitskogo botanicheskogo Sada, 40: 45-63.

Kostina F.K. (1970): Issledovaniya po samoopyleninju abrikosa (Investigations on the apricot self-pollination). Trudy Gosudarstvennogo Nikitskogo botanicheskogo Sada, 45: 7-17.

Lacis G., Rashal I., Ruisa S., Trajkovski V., Iezzoni A.F. (2009): Assessment of genetic diversity of Latvian and Swedish sweet cherry (*Prunus avium* L.) genetic resources collections by using SSR (microsatellite) markers. Scientia Horticulturae, 121: 451-457.

Layne R.E.C., Bailey C.H. and Hough L.F. (1996): Apricots. In: Fruit breeding: Tree and Tropical Fruits. Janick J. and Moore J.N. (eds). John Wiley, New York, pp 79-111.

Lee H.S., Huang S. and Hao T.H. (1994): *S* protein control rejection of incompatible pollen in *Petunia inflata.* Nature, 367: 560-563.

Lever E., Cole J., Scott S. M., Emery P. W. and Whelan K. (2014): Systematic review: the effect of punes on gastrointestinal function. Alimentary Pharmacology and Therapeutics 40: 1-9.

Li F. (1993): Promising hardy plum selections. China Fruits, 1: 1-2.

Maghuly F., Borroto-Fernandez E., Zegler R., Marschall K., Katinger H. and Laimer M. (2006): Genetic differentiation of apricot (*Prunus armeniaca* L.) cultivars with SSR markers. European Journal of Horticultural Science, 71: 129-134.

Marchese A., Tobutt K.R. and Caruso T. (2005): Molecular characterisation of Sicilian *Prunus persica* cultivars using microsatellites. Journal of Horticultural Science and Biotechnology, 80: 121-129.

Martinez-Gomez P., Arulsekar S., Potter D. and Gradziel T.M. (2003): An extended interspecific gene pool available to peach and almond breeding as characterised using simple sequence repeat (SSR) markers. Euphytica, 131: 313-322.

McCubbin A.G. and Kao T.H. (2000): Molecular recognition and response in pollen and pistil interactions. Annual Review of Cell and Development Biology, 16: 333-364.

Mehlenbacher S.A., Cociu V. and Hough L.F. (1991): Apricot (*Prunus*). In: Genetic Resources of Temperate Fruit and Nut Crops. Moore J.N. and Ballington J.R. (eds). ISHS Acta Horticulturae, 290: 65-107.

Meneses C. and Orellana A. (2013): Using genomics to improve fruit quality. Biological Research, 46: 347-352.

Milatovic D. and Nikolic D. (2007): Analysis of self-(in)compatibility in apricot cultivars using fluorescence microscopy. Journal of Horticultural Science and Biotechnology, 82: 170-174.

Milatovic D., Nikolic D. and Krška B. (2013): Testing of self-(in)compatibility in apricot cultivars from European breeding programs. Horticultural Science (Prague), 40: 65-71.

Milosevic T., Milosevic N. and Glisic I. (2013): Agronomic properties and nutritionl status of plum trees (Prunus domestica L.) influenced by different cultivars. Journal of soil science and plant nutrition, 13: 706-714.

Mittal N. and Dubey A. (2009): Microsatellite markers: A new practice of DNA based markers in molecular genetics. Pharmacognosy Reviews, 3: 235-246.

Mnejja M., Garcia-Mas J., Howad W., Badenes M.L. and Arus P. (2004): Simple Sequence Repeat (SSR) markers of Japanese plum (*Prunus salicina* Lindl.) are highly polymorphic and transferable to peach and almond. Molecular Ecology, 4: 163-166.

Mnejja M., Garcia-Mas J., Audergon J.M. and Arus P. (2010): *Prunus* microsatellite marker transferability across rosaceous crops. Tree Genetics and Genomes, 6: 689-700.

Moss H. (1994): A corporate plant genetic resources strategy for the Agricultural Research Council. Stellenbosch, South Africa, pp 30-33.

Munoz-Snaz J. V., Zuriaga E., Lopez I., Badenes M.L. and Romero C. (2017): Self-(in)compatibility in apricot germplasm is controlled by two major loci, S and M. BMC Plant Biology, 17: 1-16.

Nassi M.O., Ruffa E., Me G., Lepori G. and Radicati L. (2003): A contribution to the systematics of a Piedmontese plum ecotype. Plant Breeding, 122: 532-535.

Norton M.V. and Krueger W.H. (2007): Growing prunes (dried plums) in California: An overview. Agriculture and Natural Resources, Oakland, pp 1-7.

Nunes M.D.S., Santos R.A.M., Ferreira S.M., Vieira J., and Vieira C.P. (2006). Variability patterns and positively selected sites at the gametophytic self-incompatibility pollen *SFB* gene in a wild self-incompatible *Prunus spinosa* (Rosaceae) population. New Phytologist, 172: 577–587.

Okie W.R. and Ramming D.W. (1999): Plum breeding world-wide. HortTechnology, 9: 162-176.

Ortega E., Sutherland B.G., Dicenta F., Bošković R. and Tobutt K.R. (2005): Determination of incompatibility genotypes in almond using first and second intron consensus primers: detection of new *S*-alleles and correction of reported *S*-genotypes. Plant Breeding, 124: 188-196.

Ouborg N.J., Piquet Y. and Van Groenendael J.M. (1999): Population genetics, molecular markers and the study of dispersal in plants. Journal of Ecology, 87: 551-568.

Paydas S., Eti S., Gulcan R., Derin K. and Yilmaz K.U. (2006): In vitro investigation on pollen quality, production and self-incompatibility of varieties in Malatya, Turkey. Acta Horticulturae, 701: 75-80.

Pennone F. (1999): Promising apricot selections obtained by the selection of Caserta of the Instituto Sperimentale per la Frutticoltura of Rome. Acta Horticulturae, 488: 191-196.

Potelwa Y., Moobi M. and Ntombela S. (2014): South African fruit trade flow. National Agricultural Marketing Council. Issue no 15, pp 1-13.

Potter D. (2012): Basic information on the stone fruit crops. In: Genetics, Genomics and Breeding of Stone Fruits. Kole C. and Abbott A.G. (eds) CRC, Clemson SC, USA, pp 1-21.

Potter D., Eriksson T., Evan K.C., Oh S. Smedmark J.E.E., Morgan D.R., Kerr M., Robertson K.R., Arsenault M., Dickinson T.A. and Campbell C.S. (2007): Phylogeny and classification of Rosaceae. Plant Systematics and Evolution, 266: 5-43.

Priyono and Putranto R. A. (2014): Molecular markers and their application forDNA fingerprinting and genetic diversity studies in Coffea species. MenaraPerkebunan, 82, 39–50.

Ramming D.W and Cociu V. (1991): Plums (*Prunus*). In: Genetic resources of temperate fruits and nuts. Moore J.N and Ballington J. (eds) Acta Horticulturae, 290: 233-287.

Rawes A.N. (1921): Pollination in orchards (IV).Self-fertility and self-sterility in plums. Journals of the Royal Horticulture Society, 39: 353-356.

Rehder A. (1954): Manual of Cultivated Trees and Shrubs. Macmillan, New York. Pp. 930.

Romero C., Vilanova S., Burgos L., Martinez-Calvo J., Vicente M., Llacer G. and Badenes M.L. (2004): Analysis of the *S*-locus structure in *Prunus armeniaca* L.: Identification of S-haplotypes *S-RNase* and F-box genes. Plant Molecular Biology, 56: 145-157.

Sapir G., Stern R.A., Eisikowitch D. and Goldway M. (2004): Cloning of four new Japanese plum *S*-alleles and determination of the compatibility between cultivars by PCR analysis. Journal of Horticultural Science and Biotechnology, 79: 223-227.

Sapir G., Stern R.A., Shafir S. and Goldway M. (2008): S-RNase based-genotyping of Japanese plum (*Prunus salicina* Lindl.) and its application on the assortment of cultivar-couples in the orchard. Scientia Horticulturae, 118: 8-13.

Sijacic P., Wang X., Skirpan A.L., Wang Y., Dowd P.E., McCuddin A.G., Huang S. and Kao T. (2004): Identification of pollen determinants of *S-RNase*-mediated self-incompatibility. Nature, 429: 302-305.

Sonneveld T., Tobutt K.R. and Robbins T.P. (2003): Allele-specific PCR detection of sweet cherry self-incompatibility alleles *S*1 to *S*6 using consensus and allele-specific primers. Theoretical and Applied Genetics, 107: 1059-1070.

Srinivasan C., Padilla I.M.G. and Scorza R. (2005): *Prunus* spp. Almond, apricot, nectarine, peach and plum. In: Biotechnology of Fruit and Nut Crops. Litz R.E. (ed). CABI, Wallingford, UK, 29: 514-540.

Surbanovski N., Tobutt K.R., Konstantinovic M., Maksimovic V., Sargent D.J., Stevanovic V., Ortega E. and Bošković R.I. (2007): Self-incompatibility of *Prunus tenella* and evidence that reproductively isolated species of *Prunus* have different *SFB* alleles coupled with an identical S-*RNase* allele. The Plant Journal, 50: 723-734.

Sutherland B.G., Robbins T.P. and Tobutt K.R. (2004a): Primers amplifying a range of *Prunus S*-alleles. Plant Breeding 123: 582- 584.

Sutherland B.G., Tobutt K.R. and Robbins T.P. (2004b): Molecular genetics of self-incompatibility in plums. Acta Horticulturae, 663: 557-562.

Szabo Z. and Nyeki J. (1991): Blossoming, fructification and combination of apricot varieties. Acta Horticulturae, 293: 295-302.

Tao R., Yamane H., Sugiura A., Marayama H., Sassa H. and Mori H. (1999): Molecular typing of *S*-alleles through identification, characterisation and cDNA cloning for *S-RNases* in sweet cherry. Journal of the American Society of Horticultural Science, 124: 224-233.

Terami H., Torikata H. and Shimazu Y. (1946): Analysis of the sterility factors existing in varieties of the Japanese pear (Pyrus serotina Rehd. var. culta Rehd). Student Horticulture Instistute of Kyoto University, 3: 267–272.

Testolin R., Marrazzo T., Cipriani G., Quarta R., Verde I., Dettori M.T., Pancaldi M. and Sansavini S. (2000): Microsatellite DNA in peach (*Prunus persica* (L.) Batsch) and its use in fingerprinting and testing the origin of cultivars. Genome, 43: 512-520.

Topp B.L. and Russell D.M. (1989): Breeding early ripening Japanese plums. Acta Horticulturae, 240: 27-30.

Topp B.L., Russell D.M., Neumuller M., Dalbo M.A. and Lui W. (2012): Plum. In: Fruit Breeding: Handbook of Plant Breeding. Badenes M.L. and Byrne D.H. (eds), vol 8: *571-621.*

Tromp J. and Borsboom, (1994): The effect of autumn and spring temperature on fruit set and on the effective pollination period in apple and pear. Scientia Horticulturae, 60, 23-30.

Ushijima K., Sassa H., Tao R., Yamane H., Dandekar A.M., Gradziel T.M. and Hirano H. (1998): Cloning and characterisation of cDNA encoding *S-RNases* from almond

(*Prunus dulcis*): primary structural features and sequence diversity of the *S-RNases* in Rosaceae. Molecular Genomics and Genetics, 260: 261-268.

Ushijima K., Yamane H., Watari A., Kakehi E. and Ikeda K. (2004): The S-haplotype-specific F-box gene, *SFB*, is defective in self-compatible haplotypes of *P. avium* and *P. mume*. Plant Journal, 39: 573-586.

Verde *et al*. (2013): The high quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. Nature Genetics, 45: 487-494.

Vilanova S., Badenes M.L., Burgos L., Martinez-Clavo J. and Llacer G. (2005): Self-compatibility of two apricot selections is associated with two pollen-part mutations of different nature. Plant Physiology, 42: 629-641.

Viti R., Monteone P. and Guerriero R. (1997): Incompatibility in apricot (*Prunus armeniaca* L.): Methodological considerations. Journal of Horticultural Sciences, 72: 961-970.

Wu J., Cai C. F., Cheng F. Y., Cui H. L. & Zhou H. (2014): Characterisation and development of EST-SSR markers in tree peony using transcriptome sequences. Molecular Breeding, 34: 1853–1866.

Wu J., Gu C., Zhang S.L., Zhang S.J., Wu H.Q., and Heng W. (2009): Identification of S-haplotype-specific *S-RNase* and *SFB* alleles in native Chinese apricot (*Prunus armeniaca* L.). Journal of Horticultural Science and Biotechnology, 84: 645-652.

Wünsch A. (2009): Cross-transferability polymorphic SSR loci in *Prunus* species. Scientia Horticulturae, 120: 348-352.

Wünsch A. and Hormaza J.I. (2002): Cultivar identification and genetic fingerprinting of temperate fruit tree species using DNA markers. Euphytica, 125: 59-67.

Wünsch A. and Hormaza J.I. (2004): Genetic and molecular analysis in Cristobalina sweet cherry, a spontaneous self-compatible mutant. Sexual Plant Reproduction, 17: 203-210.

Yamane H. and Tao R. (2009): Molecular basis of self-(in)compatibility and current status of S-genotypes in Rosaceous fruit trees. Journal of Japanese Society of Horticultural Science, 78: 137-157.

Yamane H., Tao R. and Sugiura A. (1999): Identification and cDNA cloning for *S-RNases* in self-incompatible Japanese plum (*Prunus salicina* Lindl. cv. Sordum). Plant Biotechnology, 16: 389-396.

Yamane H., Tao R., Sugiura A., Hauck R. and Iezzoni A.F. (2001): Identification and characterisation of *S-RNase* in tetraploid sour cherry. Journal of the American Society of Horticultural Science, 126: 661-667.

Zaurov D.E, Molnar T.J., Eisenman S.W., Ford T. M., Mavlynova R.F., Capik J.M. Funk C.R. and Goffreda J.C. (2013): Genetic Resources of apricots (Prunus armeniaca L.) in Central Asia. Hortscience, 48: 681-691.

Zhang S.L., Huang S.X., Kitashiba H. and Nishio T. (2007): Identification of S-haplotype-specific F-box gene in Japanese plum (*Prunus salicina* Lindl.). Sexual Plant Reproduction, 20: 1–8.

Zhang L., Chen X., Chen X., Zhang C., Liu X., Ci Z., Zhang H., Wu C. and Liu C. (2008): Identification of self-incompatibility (*S*) genotypes of Chinese apricot cultivars. Euphytica, 160: 241-248.

Zuriaga E., Molina L., Badenes M.L. and Romero C. (2012): Physical mapping of a pollen modifier locus controlling self-incompatibility in apricot and synteny analysis within the Rosaceae. Plant Molecular Biology, 79: 229-242.

Zuriaga E., Munoz-Sanz J.V., Molina L., Gisbert A.D., Badenes M.L. and Romero C. (2013): An *S*-locus independent pollen factor confers self-compatibility in 'Katy' apricot. PLoS ONE, 8: 1-14.

Chapter 3:

Fingerprinting of ARC apricot and plum collection using microsatellite markers

3.1 Introduction

South Africa is known internationally as a producer and exporter of high quality deciduous fruit. The South African stone fruit industry (including plums, apricots, nectarines and peaches) directs 48% of their production towards the export industry (Hortgro, 2016). Approximately 66% of the total South African 2013/2014 apricot exports was destined for the European and Russian markets. Similarly, 68% of the plums were exported to Europe and Russia; 20% to the Middle East and 9% to the Far East and Asia (Potelwa *et al*., 2014).

In the 2013/2014 season, the leading apricot cultivars exported were ‘Bulida’, ‘Soldonne’ and ‘Imperial/Palsteyn’ and ‘Charisma’. Cultivars important in the plum export industry include

'Laetitia', 'Songold' and 'Suplumsix' (Hortgro, 2016). With the exception of 'Charisma', all the above mentioned cultivars are Agricultural Research Council (ARC) bred cultivars. According to Bester *et al*. (2013), more than 75% of the plum industry in South Africa is based on ARC bred cultivars. Similarly, more than 98% of the apricot canning, drying and dessert industries are based on ARC bred varieties. This makes the ARC a major contributor to the South African apricot and plum production and export industry. To remain competitive, it is essential to breed cultivars that are not only of good quality but can withstand a changing climate.

The success of the ARC apricot and plum breeding programme is dependent on the quality and variability of the plant genetic resources. The germplasm established in collaboration with DAFF (Department of Agriculture, Forestry and Fisheries) at Bien Donne in Groot Drakenstein, Western Cape, South Africa, serves as a reference orchard for all stone fruit cultivars grown commercially in South Africa (traditional, current and new) (Bester *et al*., 2013). The apricot collection is mostly composed of *Prunus armeniaca* and a few *P*. *mume* species. The plum collection is mostly composed of the Japanese plum *P. salicina* but also other species including the European plum *P. domestica*, *P. instititia* and *P. cerasifera.* The ARC also breeds some apricot and plum interspecific hybrids, which have become of great interest to the stone fruit industry, due to their importance in increasing fruit quality and diversity in the stone fruit industry.

Evaluation of the ARC apricot and plum collections have revealed some inconsistencies with the labelling of accessions, which makes the authenticity of the accessions questionable and limits the confidence of use in the breeding programme. Mis-identification and mislabelling of the cultivars seems to be the major challenge, therefore verification of trueness to type of cultivars and confirmation of parentage of South African cultivars and selections is necessary for effective use in the breeding programme.

Traditionally cultivar identification and characterisation was done through phenotypic observation; a technique that has been surpassed by more reliable DNA-based molecular marker technologies. DNA markers represent a relatively cost effective and robust method for the identification of plant material and are not influenced by the environment; contrary to morphological identification (Mathias *et al*., 2007). Other benefits include high precision genetic information from minimal amounts of tissue, independent of plant growth stage and simultaneous analysis of samples (Arismend *et al*., 2012). One of the most popular PCR-based markers are microsatellites, also known as simple sequence repeats (SSRs). These markers are regarded as the best DNA markers for fingerprinting because they are highly polymorphic, abundant, randomly distributed in the genome and display co-dominant inheritance (Gupta *et al*., 1996; Wünsch and Hormaza, 2002).

Microsatellites are also highly transferable amongst species of the same genus. In the case of *Prunus*, primers flanking microsatellites were cloned and sequenced in peach and were useful for genetic studies in related species such as apricot (Hormaza, 2002), almond (Martinez-Gomez *et al*., 2003) and cherry (Cantini *et al*., 2001). Due to the transferability of microsatellite primers, in the current study a set of primers developed in peach were used in an effort to limit costs by using the same primers as a similar study conducted by Kwalimba (personal communication).

In the current study the objective is to use a set of microsatellite primers developed in peach (Cipriani *et al*., 1999; Testolin *et al*., 2000; Aranzana *et al*., 2002; Dirlewanger *et al*., 2002; Mnejja *et al*., 2004) to fingerprint the apricot and plum collection of the ARC Infruitec-Nietvoorbij. The data will be used to confirm parentage and trueness to type, and to develop a set of reference data for use in future breeding and genetic studies.

3.2 Materials and Methods

Some sections of the materials and methods are similar across the three experimental chapters of this thesis but have been included in each chapter for completeness.

3.2.1 Plant material

The plant material is grown at Bien Donne Experimental Farm located in Groot Drakenstein, Western Cape, S.A. It consists of 106 apricot and 39 plum accessions from the gene bank (SV8A), 15 apricot and 57 plum cultivars from the stone fruit reference collection (BD10), eight plum rootstocks (ZN7) and six plum cultivars from the plum crossing collection (ZN5) (Table 3.1). The apricot gene bank and reference collections comprise of 59 cultivars and 49 selections of *P. armeniaca*, as well as three accessions of *P. mume* (two cultivars and one *P. mume* x *P. armeniaca* hybrid) (Table 3.2). The plum collections include 61 cultivars and nine selections of *P. salicina*; two cultivars of *P. instititia*; three cultivars of *P. domestica*; seven cultivars of *P. cerasifera* and 17 interspecific hybrids. Generally, the plant material was supplied by the South African Plant improvement Organisation (SAPO). The trees were planted in sets of threes but in this study only one tree was sampled (usually the first). Occasionally there were two accessions of the same name that were designated 1 and 2 if they were in the same orchard; these trees were considered to be the same and were only planted separately if there were more than three trees supplied by SAPO. Twelve of the apricot and two of the plum cultivars were grown in more than one plot. Two apricot and five plum accessions were duplicated in the gene banks and 12 apricot and six plum accessions were

duplicated in different plots. The selection LE 3241 showed significant variation amongst the trees, therefore leaves were collected from both trees and were designated a and b.

Table 3.1. Apricot accessions grown at ARC Bien Donne Experimental Farm, used for fingerprinting in the current study. Plant material was collected from the gene bank (plot SV8A), reference collection (plot BD10) and rootstock collection (plot ZN7). R/T represents row and tree number.

Cultivar / Selection	Orchard	R/T
***P. armeniaca* cultivars**		
Alpha	BD10	03/13
Alpha	SV8A	03/15
Anshu	SV8A	05/05
Autumn Glo	SV8A	10/43
Barracca	SV8A	03/23
Bergarouge	SV8A	15/24
Bulida	BD10	02/04
Cape Bebeco	BD10	03/01
Cape Bebeco	SV8A	01/11
Castleton	SV8A	08/38
Charisma	BD10	03/04
Charisma*	SV8A	12/17
Comedie (1)	SV8A	12/30
Comedie (2)	SV8A	15/15
Early Baidy	SV8A	06/25
Fantasme Avikour	SV8A	10/28
Frater	SV8A	07/22
Giada	SV8A	15/11
Glada	SV8A	14/35
Goldrich	SV8A	15/20
Goldstrike	SV8A	06/10
Grandir	BD10	01/01
Guillia	SV8A	13/46
Heiwa	SV8A	06/37
Icapl	SV8A	02/18
Ivresse	SV8A	14/13
Junshiro Los	SV8A	02/29
Kathy	SV8A	06/29
Ken Psar	SV8A	02/27
Ladisun*	SV8A	01/26
Ladisun	BD10	02/07
Malan Royal*	SV8A	09/01
Malice	SV8A	09/37
Moorpark	SV8A	06/34
Moorpark nuwe	SV8A	05/29
Nja-19	SV8A	03/13
Olimp	SV8A	05/34
Palsteyn	BD10	02/10
Palsteyn*	SV8A	11/27
Peeka	BD10	03/07
Peeka*	SV8A	11/35
Pu Sha Shin	SV8A	07/02
Real de Timola	SV8A	09/04
Rouge de Fournes	SV8A	10/34
Royal	BD10	03/10
Royal	SV8A	12/44
Royal Blenheim	SV8A	09/07
Royal FR	SV8A	07/27
Sancastresse	SV8A	04/26
Satungsky	SV8A	05/20
Satunska	SV8A	14/21
Shimizugou	SV8A	14/19
Soldonne	BD10	02/01
Soldonné*	SV8A	01/26
Soledane	SV8A	15/28
Suaprieight	BD10	04/04
Suaprieight	SV8A	10/37
Suaprinine	BD10	04/07
Suapriseven	BD10	04/01
Suapriseven	SV8A	11/04
Suapritwo	SV8A	10/01
Supergold	BD10	01/04
Supergold*	SV8A	01/04
T.B. Therma	SV8A	12/26
Travatt	SV8A	05/08
Trigems	SV8A	12/01
Vesna	SV8A	14/16
Zinostojky	SV8A	04/28
***P. armeniaca* selections**		
1002A	SV8A	03/04
1002B	SV8A	02/33

Cultivar / Selection	Orchard	R/T
1003A	SV8A	05/37
1004B	SV8A	02/35
1005A	SV8A	04/32
1006B	SV8A	02/22
1007A	SV8A	02/11
1007B	SV8A	03/25
1008A	SV8A	04/37
1012A	SV8A	04/01
1013B	SV8A	06/07
1017B	SV8A	07/07
1018B	SV8A	04/07
1020A	SV8A	06/32
1020B	SV8A	07/41
1023B	SV8A	06/04
1027B	SV8A	07/28
1028Ba	SV8A	04/34
1028Bb	SV8A	08/02
1030A	SV8A	06/01
1031A	SV8A	07/35
1031B	SV8A	03/07
1031B	SV8A	04/06
1032B	SV8A	05/23
1037A	SV8A	03/32
1038A	SV8A	04/14
3B-9-25*	SV8A	10/31
4A/3/36*	SV8A	09/42
4A/3/39*	SV8A	11/01
8A-10-14*	SV8A	10/13
12D-10-15*	SV8A	10/26
12D-31-5*	SV8A	10/20
12D-39-5*	SV8A	10/08
15-ED-103	SV8A	02/14
118-LE-133	SV8A	10/04
20C-24-20*	SV8A	14/04
77-LA-579	SV8A	09/32
AP93-9*	SV8A	09/26
AP94-10*	SV8A	09/28
AP94-34*	SV8A	09/34
AP95-56*	SV8A	11/07
Clone 0.11	SV8A	15/10
Dof 1-A-586	SV8A	14/46
LE 3241 (a)	SV8A	15/17
LE 3241 (b)	SV8A	15/18
Ng3	SV8A	08/05
Ng5	SV8A	03/28
Nja-19	SV8A	03/13
"Unknown"	SV8A	03/34
S5A- 2- 4	SV8A	10/40
***P. mume* cultivar**		
Gessekai	SV8A	15/08
***P. mume* selection**		
Mume Bungo	SV8A	03/35
Hybrid		
Bungo	SV8A	08/37

Hybrid *(P. mume* x *P. armeniaca);* * ARC developed cultivars / selections; “Unknown”- Unknown cultivar resembling rootstock

Table 3.2. Plum and plum hybrid accessions grown at ARC Bien Donne Experimental Farm, used for fingerprinting in the current study. Plant material was collected from the gene bank (plot SV8A), reference collection (plot BD10) and rootstock collection (plot ZN7). R/T represents row and tree number.

Cultivar / Selection	Orchard	R/T	Cultivar / Selection	Orchard	R/T
***P. salicina* cultivars**			Laroda	SV8B	01/46
ARC PR-1*	BD10	28/40	Mac Verma	SV8B	02/26
ARC PR-2*	BD10	29/46	Maridon	ZN7	06/12
ARC PR-3*	BD10	30/07	Methley	BD10	28/10
ARC PR-4*	BD10	28/01	Mirell	BD10	30/46
African Delight*	ZN5A	04/52	Pioneer*	BD10	28/04
Autunm Sun	BD10	30/31	Pioneer	ZN5A	04/02
Betty Anne	BD10	30/04	Purple King	SV8B	02/29
Black Amber	SV8B	02/01	Purple Majesty	BD10	28/22
Black Egg	BD10	28/43	Red Gold*	BD10	29/31
Bruce (I)	ZN7	05/10	Reubennel*	BD10	29/16
Bruce (II)	ZN7	13/31	Ruby Prince*	BD10	31/07
Casselman	BD10	29/43	Ruby Red*	BD10	29/10
Celebration* (1)	BD10	30/01	Ruby Star*	BD10	29/40
Celebration (2)	SV8B	02/16	Ruby Sun*	BD10	31/01
Crocodile Dundee	BD10	29/01	Santa Rosa	BD10	28/19
Explorer	SV8B	02/04	Sapphire*	BD10	28/13
Extreme	BD10	28/07	Sapphire	ZN5A	03/01
Flavor Fall	BD10	31/28	Satin Gold*	BD10	31/10
Flavor King	BD10	31/31	Sensation*	BD10	28/46
Flavor Rich	BD10	31/34	Simka	BD10	31/13
Fortune	BD10	29/04	Solar Eclipse*	BD10	30/34
Fortune	ZN5A	04/07	Songold*	BD10	29/34
Gaviota	BD10	28/25	Southern Belle	BD10	30/16
GF43	ZN7	03/02	Souvenir*	BD10	28/28
Golden Finger	BD10	30/40	Souvenir	ZN5A	04/07
Golden King	BD10	30/37	Sun Kiss*	BD10	28/34
Golden Kiss*	BD10	28/31	Sundew*	BD10	28/37
Golden Kiss	ZN5A		Sunset	BD10	31/16
Green Red	BD10	30/43	Suplum 6	BD10	30/13
Harry Pickstone*	BD10	29/07	Suplum 11	BD10	29/25
Hiromi Red	BD10	28/16	Suplum 25	BD10	29/13
Jesobai	ZN7	15/01	Suplum 28	BD10	31/19
Lady Red*	BD10	29/22	Sweet Aroma	BD10	31/22
Lady West*	BD10	29/19	Winner	BD10	31/25
Laetitia*	BD10	29/28	17-10-212*	SV8B	02/23
Laetitia	ZN5A	03/26	17-32-118*	SV8B	01/37
Lamoon	BD10	29/37	4A-8-13*	SV8B	02/13
Late Lamoon	BD10	30/10	4C-8-20*	SV8B	02/10

Cultivar / Selection	Orchard	R/T	Cultivar / Selection	Orchard	R/T
***P. insititia* cultivars**			**Hybrids**		
Adesoto (1)	SV8B	02/38	By 69-1637P *	SV8B	03/04
Adesoto (2)	SV8B	03/10	Fereley Jaspi *	SV8B	03/16
Pixy (1)	SV8B	02/45	Fereley	SV8B	02/19
Pixy (2)	SV8B	02/41	Ferlenain †	SV8B	02/42
			S5A-25-5 ‡ *	SV8B	01/16
***P. domestica* cultivars**			S5A-26-11 ‡ *	SV8B	01/22
Eruni	SV8B	03/13	S5A-26-13 ‡ *	SV8B	01/04
Jubileum	SV8B	02/07	S5A-26-28 ‡ *	SV8B	01/10
Oneida	SV8B	01/43	S5A-26-30 ‡ *	SV8B	01/19
			S5A-26-35 ‡ *	SV8B	01/01
***P. cerasifera* cultivars**			S5A-33-25 ‡ *	SV8B	01/28
Adara	SV8B	01/40	S5A-34-25 ‡ *	SV8B	01/13
Ademir (1)	SV8B	03/01	S5A-34-28 ‡ *	SV8B	01/31
Ademir (2)	SV8B	02/32	S5A-34-37 ‡ *	SV8B	01/25
Erfdeel	BD10	30/19	S5A-34-37 ‡ *	SV8B	01/25
French Prune	BD10	30/25	S5A-35-34‡ *	SV8B	01/34
P. marianna (1)	SV8B	02/48	Sel 6-64	SV8B	03/07
P. marianna (2)	SV8B	02/35			
P. marianna (I)	ZN7	06/02			
P. marianna (II)	ZN7	06/07			
Prune d'Agen	BD10	30/22			
V D Merwe	BD10	30/28			

* ARC developed cultivars / selections; † ((*P.besseyi* x unknown parent) x F2.0); ‡ (*P. salicina* x *P. armeniaca*; § (*P. salicina* x *P. spinosa*)

3.2.2 DNA extraction

For the extraction of genomic DNA, young leaves were collected into labelled bags and stored in a -20ºC freezer. DNA was extracted according to the cetyltrimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990), with slight modifications. Three leaf discs were put in a 2 ml Eppendorf tube with three ball bearings of 3 mm in size or one ball bearing of 8 mm in size; 400 µl of CTAB extraction buffer (heated at 65ºC) and 4 µl β-Mercaptoethanol was added to each tube in a fume hood and samples were initially mixed by inversion. The leaves were homogenised using a Tissuelyser II ball mill (Qiagen) at 30 Hz for 4 min; samples that were not completely homogenised were run for an additional 2 min. This was followed by incubation in a 60ºC water-bath for 1 hour, with inversion every 10 to 20 min. After the removal of the ball bearings with a stainless steel magnet, 400 µl of chloroform-isoamyl alcohol (24:1 ratio) was added, mixed by inverting and centrifuged at 13 500 rpm for 15 min using a centrifuge (Labnet). The top aqueous phase was aliquoted into a new 2 ml Eppendorf tube, 400 µl of chloroform was again added and the samples were then centrifuged

at 13 500 rpm for 10 min. The top aqueous phase was transferred into a new tube and precipitated with 320 µl cold isopropanol (Merck) overnight in a refrigerator. After overnight incubation, the samples were centrifuged for 15 min at 13 500 rpm and the supernatant was discarded. The pellet was finally washed with 70% (v/v) cold ethanol. The ethanol was discarded and the pellet was dried for 45 min, re-suspended in TE (1X) buffer and stored in a refrigerator until required.

The quality and quantity of DNA was determined with a BioDrop ND-1000 spectrophotometer (Thermo Scientific) according to the manufacturer's instructions. Samples that showed poor quality and quantity were re-extracted. Good quality DNA was diluted to a concentration of 30 ng/µl; the samples were subsequently stored at -20°C until further use to minimise degradation of the DNA.

3.2.3 Primer selection and multiplex design

Microsatellite primers were selected from an internationally recognised set of primers used for fingerprinting species within *Prunus* (Table 3.3). Initially a set of 16 primers were selected, and optimisation PCRs were conducted on a set of apricot and plum cultivars to observe amplification. Three of the markers however showed poor amplification and were eliminated from the set. The efficiency of the remaining 13 primers was then evaluated based on reports of previous application in apricot and plum fingerprinting. Four of the selected markers, indicated with an asterisk, were previously identified as 'universal' markers (transferable between various *Prunus* species), namely: BPPCT007 and CPPCT006 (Wünsch, 2009) CPDCT045 and BPPCT038 (Mnejja *et al*., 2010).

Table 3.3. Thirteen internationally recognised microsatellite primer pairs selected to fingerprint apricots and plums. Forward and reverse sequence and fluorescent dye of labelled forward primer are indicated.

Primer	Forward	Reverse	Dye	Reference
BPPCT001	aattcccaaaggatgtgtatgag	caggtgaatgagccaaagc	NED	Dirlewanger *et al*., 2002
BPPCT007 *	tcattgctcgtcatcagc	cagatttctgaagttagcggta	VIC	Dirlewanger *et al*., 2002
BPPCT017	ttaagagtttgtgatgggaacc	aagcataatttagcataaccaagc	HEX	Dirlewanger *et al*., 2002
BPPCT025	tcctgcgtagaagaaggtagc	cgacataaagtccaaatggc	PET	Dirlewanger *et al*., 2002
BPPCT038 *	tatattgttggcttcttgcatg	tgaaagtaaacaatggaagc	PET	Dirlewanger *et al*., 2002
CPPCT006 *	aattaactccaacagctcca	atggttgcttaattcaatgg	VIC	Aranzana *et al*., 2002
CPPCT022	caattagctagagagaattattg	gacaagaagcaagtagtttg	HEX	Aranzana *et al*., 2002
CPPCT044	ttctctttggcgtatcaagga	ggtcccatatcagctgaacc	PET	GDR (http//www.bioinfo.wsu.edu)
CPDCT045 *	tgtggatcaagaaagagaacca	aggtgtgcttgcacatgttt	NED	Mnejja *et al*., 2005

UDP96005	gtaacgctcgctaccacaaa	cacccagctcatacacctca	FAM	Cipriani *et al*., 1999
UDP98022	ctagttgtgcacactcacgc	gtcgcaggaacagtaagctc	FAM	Testolin *et al*., 2000
UPD98409	gctgatgggttttatggttttc	cggactcttatcctctatcaaca	VIC	Cipriani *et al*., 1999
UDP98412	agggaaagtttctgctgcac	gctgaagacgacgatgacga	PET	Testolin *et al*., 2000

* Universal markers according to Mnejja et al., (2010)

Prior to designing multiplexes, optimisation gradient temperature PCRs of the SSR loci were conducted to identify optimum annealing temperature. The PCR reagents and conditions were set up in accordance with work previously published in stone fruit fingerprinting studies that used the same SSR markers (Aranzana *et al*., 2002; Dirlewanger *et al*., 2002). The 13 primers were then grouped into four multiplexes, paying attention to the dye label of the forward primer and size of the amplification products. Primers that had the same annealing temperature were grouped together while primers with the same dye were placed in different multiplexes (Table 3.4).

Table 3.4. Microsatellite multiplexes (markers and volumes) used in the apricot and plum fingerprinting study.

	Markers	Dye	Approximate product size (bp)	Annealing temperature	Concentration (µM) per 20µl
Multiplex 1	UDP98412	PET	129	52°C	0.2
	UDP98409	VIC	129		0.2
	UDP98022	FAM	139		0.25
Multiplex 2	BPPCT025	PET	197	53°C	0.2
	CPPCT006	VIC	190		0.2
	UDP96005	FAM	155		0.25
Multiplex 3	CPPCT044	PET	175	52°C	0.2
	BPPCT001	NED	159		0.2
	CPPCT022	HEX	240		0.25
Multiplex 4	CPDCT045	NED	142	53.5°C	0.2
	BPPCT007	VIC	149		0.25
	BPPCT017	HEX	174		0.25
	BPPCT038	PET	135		0.25

3.2.4 Microsatellite genotyping

Preparation of each multiplex PCR was done in ice, with a total volume of 20 µl in each PCR tube (Axygen). Aliquots of 11.9 µl of PCR multiplex mix (Qiagen), 3.75 µl of RNase-free water, 1.5 µl of DNA and primers (Table 3.4) prepared from 10mM stock concentration were added. The PCR were carried out in GeneAmp (Applied Biosystems) and G-storm (G-storm direct) thermal cyclers with the following PCR parameters: an initial denaturing step at 94°C for 15 min, followed by 35 cycles of 30 s at 94°C, 90 s of designated annealing temperature for each multiplex (described in Table 3.4) and 60 s at 72°C, with a final extension step of 30 min at 60°C. To confirm amplification, a subset of PCR products was resolved by 1% (m/v) agarose (Conda Laboratory) gel electrophoresis at 70V for 45 min, in a 1X TBE buffer (Tris, Boric acid, EDTA) and a 1 kb Plus ladder (Thermo Scientific). Upon verification, the full set of amplification products were separated by capillary electrophoresis on a 3130 DNA capillary analyser (Applied Biosystems) at the Central Analytical Facility's DNA sequencing unit at Stellenbosch University, and sizing of peaks was estimated in comparison with the internal standard, GS500(-250)LIZ (Applied Biosystems). GENEMAPPER software version 5.0 (Applied Biosystems) was used to visualise the peaks and score the alleles. Data were checked by an overseer. Single peaks were identified as indicating homozygosity and where more than two peaks were observed; possible polyploidy or polysomy was deduced.

3.2.5 Statistical analysis

To validate the collated data generated from GENEMAPPER, MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al*., 2004) was used to examine the possibility of false-scoring of alleles due to allele drop-out, stuttering and presence of null alleles, prior to genetic data analysis.

For the purpose of calculating the genetic diversity statistics, the entire set of accessions (from the gene bank and from the reference collection) excluding false and polyploid accessions (as identified by allele comparison of GENEMAPPER allele scores) was used. No separate analysis of interspecific hybrids and polyploid accessions was conducted. For each microsatellite locus, GENALEX software version 6.501 (Peakall and Smouse, 2010) was used to calculate the number of alleles per locus (Na), and estimate the expected genetic heterozygosity (He), observed heterozygosity (Ho) and Shannon's information index (I). The number of alleles indicates the direct count of alleles amplified by a marker in all the accessions tested. Expected heterozygosity (He) for each marker is calculated using the formula: $He=1-\Sigma(pi)^2$ (Wei, 1973), where *pi* is the probability that two alleles from the same locus are different when chosen at random from a given population. Observed heterozygosity (Ho) is the proportion of samples that are heterozygous and is obtained by dividing the number

of heterozygous samples by the total number of samples evaluated. Shannon's information index, *I=ΣpiInpi*, provides an unbiased measure of allelic diversity per locus, using *pi* (the probability that two alleles from the same locus are different when chosen at random from a given population), with *In,* the natural log, and Σ, the sum of the calculation. The polymorphic information index of the markers used was calculated using the formula $PIC=\Sigma(pi)^2$ in CERVUS version 3.07 (Kalinowski *et al*., 2007).

Genetic differentiation among the studied apricot cultivars was calculated using a pairwise genetic test on an individual-by-individual (*N*x*N*) basis in GENALEX. Deviation of markers from Hardy-Weinberg Equilibrium (HWE) was tested using Markov chain exact tests (1000 dememorisation, 100 batches and 1000 iterations per batch), computed with GENEPOP version 4.6.9 (Rousset, 2008). The genetic distance matrix was converted to a Molecular Evolutionary Genetics Analysis (MEGA) input file from which a dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster method in MEGA version 6, using default settings (Tamura *et al*., 2013).

3.3 Results

3.3.1 Apricot

The molecular fingerprints of the apricot accessions were obtained from 10 microsatellite markers. The great majority of the apricot accessions belonged to *P. armeniaca.* Data for the three accessions belonging to *P. mume* are not presented because in this section the focus is on *P. armeniaca* accessions. Statistical analysis included in this study assumed diploidy, therefore in the occasional cases of accessions that showed more than two peaks for some markers, they were excluded from the analysis.

3.3.1.1 Marker performance

Initially 13 microsatellite primer pairs were tested for fingerprinting the apricot accessions. However, only 10 markers (BPPCT001, BPPCT007, UDP98409, BPPCT025, CPPCT006, CPPCT044, CPDCT045, UDP98412, UDP98022 and UDP98005) successfully amplified in the 106 and 15 (Reference Collection) apricot DNA samples. The other three markers (BPPCT025; BPPCT038 and CPPCT22) were excluded because they resulted in difficulty when scoring fingerprints due to stuttering and background pull-up.

3.3.1.2 Statistical analysis

Prior to further analysis, preliminary MICRO-CHECKER analysis detected that six of the 10 markers used in the current study (BPPCT007, BPPCT025, UDP98409, UDP98412, UDP98022 and UDP96005), presented the possibility of null alleles, suggested by the apparent excess homozygotes (Fig. 3.1a). There was, however, no indication of scoring errors as a result of stuttering or allele drop-out. The remaining five markers showed no evidence of mis-scoring due to the presence of null alleles, stuttering or allele drop-out (Fig. 3.1b). Regardless, all tested markers were retained in the statistical analysis.

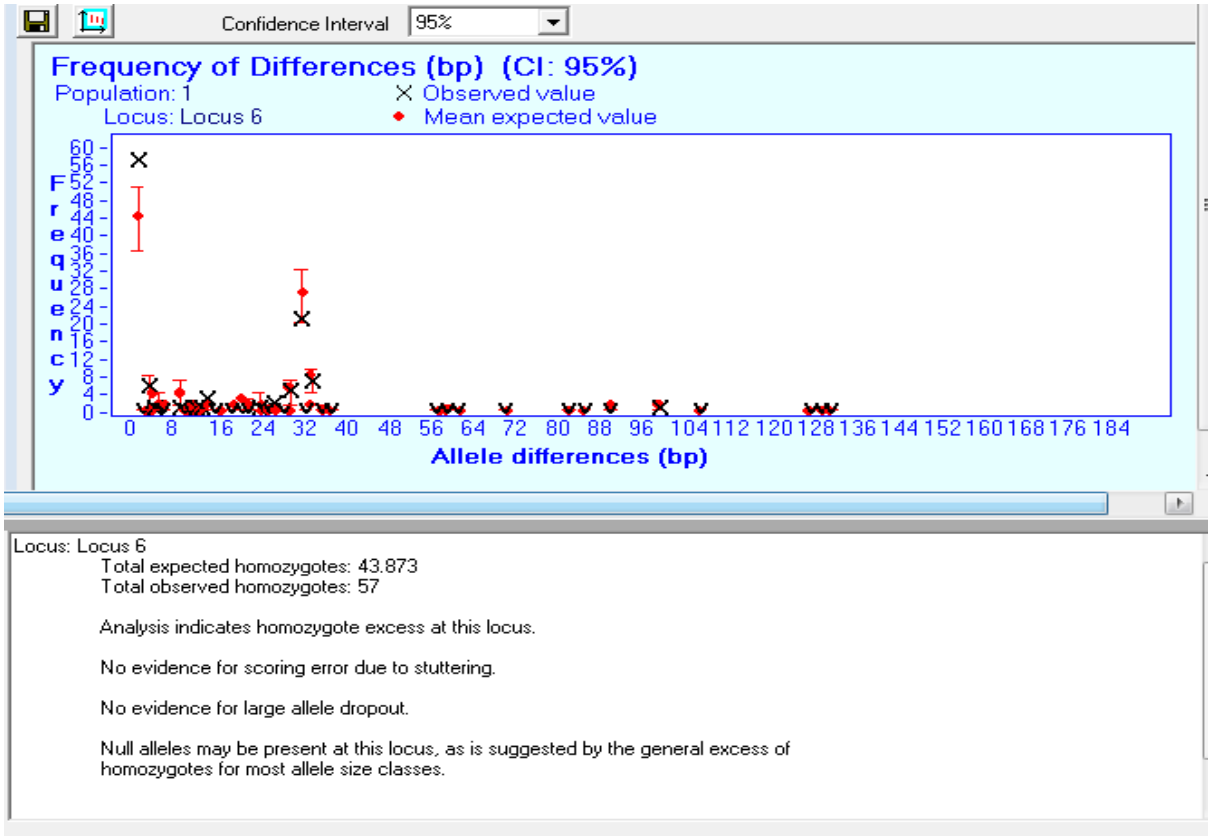
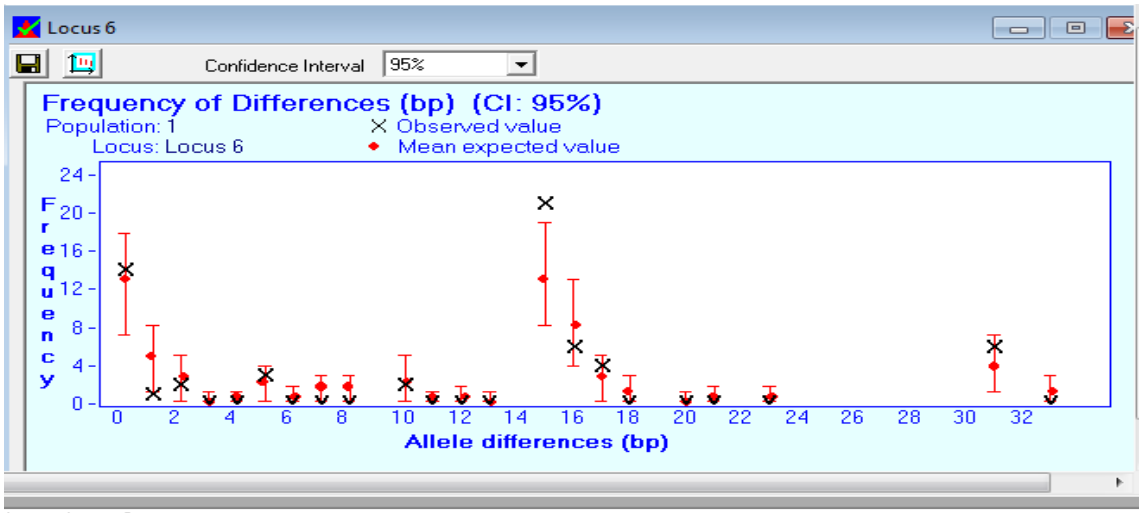


Fig. 3.1a. Preliminary MICRO-CHECKER results for the marker UDP98022 scored in diploid apricot, indicating excess of homozygotes and thus evidence of possible null alleles. Similar results were observed for markers BPPCT025, BPPCT007, UDP98409, UDP98412 and UDP96005.



Locus: Locus 6
Total expected homozygotes: 13.245
Total observed homozygotes: 14

No evidence for scoring error due to stuttering.

No evidence for large allele dropout.

No evidence for null alleles.

Fig. 3.1b. MICRO-CHECKER output for the marker UDP98412 scored in diploid apricot, indicating no evidence of mis-scoring, no allele drop-out and no null alleles. Similar results were observed for markers BPPCT001; CPPCT044; CPDCT045 and CPPCT006.

The majority of the accessions tested showed amplification of two peaks for each locus and, as explained, accessions showeing one peak, was considered to be homozygous for that locus. Considering the entire set (excluding non-diploid accessions) of apricot accessions, the number of alleles per locus ranged from five in the case of markers BPPCT001 and CPPCT044, to 15, in the case of UDP98409 (Table 3.5).

Table 3.5. Number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC) and Shannon's information index (I) detected using 10 microsatellite markers for the accessions of *P. armeniaca* from the apricot gene bank and apricot reference collection (RC).

Locus	Na	Ho	He	PIC	I
UDP98409	15	0.710	0.815	0.790	2.034
UDP98412	13	0.673	0.782	0.746	1.734
UDP98022	11	0.421	0.759	0.719	1.690
BPPCT025	8	0.393	0.487	0.467	1.121
CPPCT006	13	0.607	0.672	0.649	1.632
UDP98005	13	0.477	0.594	0.558	1.314
CPPCT044	5	0.449	0.498	0.430	0.871

BPPCT001	5	0.383	0.530	0.447	0.919
CPDCT045	14	0.579	0.655	0.618	1.490
BPPCT007	14	0.570	0.803	0.778	1.990
Mean	11.000	0.525	0.656	0.6202	1.479

The expected heterozygosity ranged from 0.487, in the case of marker BPPCT025, to 0.815, for UDP98409. The observed heterozygosity ranged from 0.393, for marker BPPCT025, to 0.710 in the case of marker UDP98409. The mean expected heterozygosity and observed heterozygosity was 0.656 and 0.525, respectively. The PIC value ranged from 0.467 in the case of marker BPPCT025, to 0.790 for UDP98409. The mean PIC value was 0.6202 for the entire apricot population analysed. Shannon's information index was the lowest for marker CPPCT044 with a value of 0.871 and was the highest for marker UDP98409 with a value of 2.034. The mean value of Shannon's information index for the whole apricot population was 1.479. For all markers tested in the *P. armeniaca* cultivars and selections, significant deviation from Hardy-Weinberg Equilibruim was observed (data not shown).

3.3.1.3 Identification of fingerprints amplified using microsatellite primers

For the purpose of this study, the 10 microsatellite markers were useful to unambiguously identify SSR genotypes of 107 apricot accessions. Genotypes showing a single amplified fragment were considered as homozygous for that particular locus. Some accessions listed in table 3.1 showed complex amplification patterns, which resulted in difficult scoring of alleles; therefor the data for those accessions was not presented. Some markers showed an additional third peak for some accessions and were therefore excluded from the statistical analysis which assumes diploidy (Table 3.6).

Table 3.6. Genotypes of 107 diploid apricot accessions from the ARC Bien Donne Experimental Farm revealed by 10 microsatellite markers. Triploid accessions are excluded from the table, accessions marked as RC are from the reference collection (BD10) and duplicated accessions are marked as 1 and 2.

Cultivars	UDP98409	UDP98412	UDP98022	BPPCT025	CPPCT006	UDP98005	CPPCT044	BPPCT001	CPDCT045	BPPCT007
Alpha (RC)	132	88	118 / 132	151 / 163	198 / 200	110 / 141	149 / 151	116 / 118	130	135 / 148
Alpha	132	115	162	163	200	106	149 / 152	116 / 118	130 / 136	148 / 168
Anshu	145 / 147	110	162	153	200	118 / 131	149	116 / 120	130 / 134	144 / 160
Atricot (RC)	132 / 153	108 / 115	118	163	200	110	149 / 151	116 / 118	126 / 130	148 / 154
Autumn Glo	132 / 161	110	118 / 162	153/163	196 / 200	110	149 / 151	116	130 / 136	148 / 155
Barracca	132	108	118 / 132	163	200	110	151	116 / 118	130	147 / 153
Bergarouge	132 / 140	108 / 119	118	151 /163	200 / 204	110 / 118 /141	149 / 151	116 / 118	126 / 130	135 / 148 / 155 / 158
Bulida (RC)	153	108 / 115	118	163	198 / 200	110	149 / 151	116 / 118	126 / 130	148 / 154
Cape Bebeco	132 / 161	88 / 108	118	151 / 163	196 / 200	128 / 141	151	116	130	135 / 148
Castleton	132	88 / 110	118 / 132	153/163	200	110 / 143	151	116 / 118	130 / 136	135
Charisma (RC)	132	88	132	163	200	110	151	116	126	146
Charisma	132	88	132	162/163	200	110	151	116	126	146
Comedie (1)	132 / 163	115	132	151/163	200	110	151	116	130	148 / 155
Comedie (2)	132 / 163	115	132	151/163	200	110 / 118	151	116	126 / 130	148 / 155
EarlyBaidy	136	88	118 / 136	151/163	198 / 200	110	151	116 / 118	126 / 130	153
Fantasme avikour	161 / 163	119	118	163	188 / 200	110	151	116	130	155
Frater	161	100 / 108	162	161/163	178 / 200	110	149 / 188	118 / 138	130 / 151	135
Giada	153 / 161	110 / 115	118 / 162	163	200	110	149 / 151	116	130 / 136	155
Goldrich	132 / 136	110 / 115	132 / 162	153/163	200	118	151	116 / 118	126 / 136	168
Goldstrike	132 / 136	114 / 121	132 / 136	153/163	198 / 200	110	149 / 151	118	130 / 136	148 / 168
Guillia	132 / 161	108 / 110	118 / 162	153/163	200	110	149 / 151	116 / 118	130 / 136	148 / 155
Heiwa	139 / 142	94 / 110	162	153/163	200 / 204	118 / 131	149 / 151	116	136 / 146	135
Icapl	132 / 161	110 / 115	162	151/163	196 / 200	110	151	116 / 118	124 / 136	158

Cultivars	UDP98409	UDP98412	UDP98022	BPPCT025	CPPCT006	UDP98005	CPPCT044	BPPCT001	CPDCT045	BPPCT007
Ivresse	149	98 / 108	126 / 149	167	172 / 190	110	149 / 168	138	132 / 147	127 / 182
Junshiro Los	123 / 149	100	123 / 162	153/160	180 / 200	118 / 130	151	116	134 / 145	144
Kathy	132 / 161	88 / 108	118	163	196 / 200	110 / 141	149 / 151	116 / 118	130	135 / 148
Ken Psar	140 / 145	100 / 108	162	158/163	176 / 188	115 / 141	149 / 151	120	124 / 130	148 / 158
Ladisun (RC)	132 / 153	98 / 115	118 / 132	163	200	110	149 / 151	116 / 118	126 / 130	148 / 154
Ladisun	132 / 153	108 / 115	118	163	200	110	149 / 151	116 / 118	126 / 130	148 / 155
Malan Royal	132 / 161	88 / 108	118 / 132	163	196 / 200	110 / 139	149 / 151	116 / 118	130	135 / 148
Malice	153 / 163	88 / 119	118	160	187 / 200	110	151	116	130	146 / 155
Moorpark	132 / 161	108 / 115	118 / 132	163	200	110 / 141	151	116 / 118	130	135
Moorpark nuwe	132	108 / 115	132	153/163	200	141	151	116	130	135 / 148
Nja-19	132	108 / 115	132	163	200	141 / 143	149 / 152	116 / 118	130	148
Olimp	122 / 165	108 / 115	118 / 122	151/163	190 / 198	128 / 130	149 / 151	116	126 / 130	127 / 148
Palsteyn (RC)	132 / 161	88 / 110	118 / 162	163	196 / 200	110 / 143	151	116	126 / 136	148
Palsteyn	132 / 161	88 / 110	118 / 162	163	196 / 200	110 / 143	149 / 151	116	126 / 136	148
Peeka (RC)	153 / 161	88 / 108	118	163	196 / 200	110	149 / 151	116	126 / 130	148 / 155
Peeka	153 / 161	88 / 108	118	162/163	196 / 200	110	151	116	130	148 / 155
Pu sha shin	140 / 155	94 / 110	140 / 162	163	196	114 / 118 / 131	151	116 / 120	124	155 / 156
Real de Timola	132 / 161	108 / 115	118 / 132	162/163	196 / 200	110 / 139	151	116	130	135 / 153
Rouge de Fournes	136 / 142	110 / 115	132	151/153	194 / 200	110	151	116	126 / 136	135
Royal (RC)	132 / 161	88 / 108	118 / 132	163	196 / 200	110 / 141	149	118	130	135
Royal	132 / 161	88 / 108	118 / 132	163	196 / 200	110 / 141	149 / 151	116 / 118	130	135 / 148
Royal Blenheim	132 / 161	108	118 / 132	162/163	196 / 200	110	149 / 151	118	130	135 / 148
Royal FR	132 / 161	88 / 108	118 / 132	162/163	196 / 200	110 / 139	149 / 151	116 / 118	130	135 / 148
Sancastresse	132 / 140	108 / 115	118 / 162	153/163	198 / 200	110	149 / 151	116 / 118	130 / 134	148
Satungsky	132 / 161	88 / 108	118	163	196 / 200	141	149 / 151	116	130	135
Satunska	149	98 / 108	126 / 149	167	172 / 190	110	149 / 168	138	132 / 147	127 / 182
Shimizugou	149	98 / 108	126 / 149	167	172 / 190	110	149 / 168	138	132 / 147	127 / 158

Cultivars	UDP98409	UDP98412	UDP98022	BPPCT025	CPPCT006	UDP98005	CPPCT044	BPPCT001	CPDCT045	BPPCT007
Soldonné (RC)	136 / 153	88 / 115	118	153/163	198 / 200	110 / 141	151	116 / 118	126 / 130	148 / 155
Soldonné	153	108 / 115	118	163	198 / 200	110	149 / 151	116 / 118	126 / 130	148 / 155
Suaprieight RC)	161	108 / 110	162	153/163	200	110	151	116	130	158
Suaprieight	161	108 / 110	162	153/163	200	110	151	116	130	135 / 158
Suaprinine (RC)	149 / 161	88 / 110	162	163	196 / 200	110	149 / 151	116 / 118	130 / 136	158
Suapriseven (RC)	155 / 161	88 / 110	162	163	196 / 200	110	152	116 / 118	130 / 136	158
Suapriseven	155 / 161	88 / 110	162	163	196 / 200	110	151	116 / 118	130 / 136	158
Suapritwo	155 / 161	88 / 108	162	163	196 / 200	110	149 / 151	116 / 118	130 / 136	135 / 158
Supergold (RC)	153 / 161	88	118 / 162	163	200	110	151	116	126 / 136	148
Supergold	153 / 161	88	118 / 162	163	200	110	151	116	126 / 136	148
T.B.Therma	132 / 161	108	132	163	190 / 194	110 / 141	151	116 / 118	126 / 130	148 / 155
Travatt	132 / 161	108 / 115	118 / 132	163	200	110 / 141	151	116 / 118	130	127 / 135
Trigems	132	88 / 110	132 / 162	153 / 163	200	110	151	116	139	150
Vesna	149	98 / 108	126 / 149	167	172 / 190	110	149 / 168	138	132 / 147	127 / 182
Zinostojky	122 / 153	98 / 119	122 / 126	153 / 158	190 / 194	128 / 141	149 / 151	116 / 118	124 / 130	127 / 144

Selections	UDP98409	UDP98412	UDP98022	BPPCT025	CPPCT006	UDP98005	CPPCT044	BPPCT001	CPDCT045	BPPCT007
1002A	132 / 161	115 / 119	122	158 / 163	190 / 200	130 / 141	151	116	130	148 / 155
1002B	122 / 165	108	122	163 / 188	188	130 / 141	151	118	124 / 136	135
1003A	132 / 155	88	118	163 / 188	188 / 200	110 / 141	151	118	130	148 / 155
1004B	153 / 155	108	118	163 / 188	188 / 194	110 / 141	151	118	126 / 130	148
1005A	132 / 132	108	132	163	200	141	151	116	130	146 / 148
1006B	161 / 163	88 / 119	118	158 / 163	188 / 190	110 / 141	151	116	150 / 160	150 / 160
1007A	132 / 161	102 / 108	118	163	190 / 200 / 204	110 / 141	149 / 151	118	130	148
1007B	163 / 165	119	163	151 / 163	190 / 198	110	149 / 151	118	126 / 136	135 / 148
1008A	122 / 153	94 / 108	122	163	176 / 194	141 / 143	151	116	136	148 / 150
1012A	122 / 153	108 / 115	162	163	176 / 188	110 / 141	151	118	126 / 130	135 / 148

Cultivars	UDP98409	UDP98412	UDP98022	BPPCT025	CPPCT006	UDP98005	CPPCT044	BPPCT001	CPDCT045	BPPCT007
1013B	136 / 153	108	136	163	176 / 188	110	151	116	130	148
1017B	153	108	153	160 / 161	174	139 / 142	151	118	130	135 / 148
1018B	153 / 165	88 / 108	153	163	176 / 188	110 / 141	152	118	130	135
1020A	153	115	118	151 / 163	200	110 / 141	151	116 / 118	126	147 / 153
1020B	122 / 153	108 / 115	118	162 / 163	176 / 188	110 / 139	149	116	130 / 136	148
1023B	153	108	153	163	176	141 / 143	151	116 / 118	136	135 / 155
1027B	132 / 161	88 / 108	132	163	196 / 200	110 / 141	149 / 151	116 / 118	130	135
1028Ba	132 / 155	88	118	163	188 / 200	141	149 / 151	118	129 / 139	148 / 155
1028Bb	132	88	118	162 / 163	196 / 200	139	149 / 151	118	130	135
1030A	155	88	118	163	188	110	151	118	130	153
1031A	161 / 161	88	118	163	194 / 200	110 / 141	149 / 151	116	130	148
1031B	136 / 153	102 / 108	136	163	176 / 188	110 / 141	151	116	130	146 / 148
1032B	122 / 153	108 / 115	118 / 122	163	176 / 188	110 / 141	149 / 151	116	130 / 136	148
1037A	132 / 153	88 / 108	118	163	188 / 200	110 / 141	149 / 151	118	130	148 / 155
1038A	132 / 155	88	118 / 162	163	188 / 200	110 / 141	151	118	124 / 130	148 / 155
3B-9-25	132 / 161	88 / 110	118 / 162	163	196 / 200	110 / 143	151	116	126 / 136	148
4A-3-36	163	108	163	163	194	110	149	116	130	135
4A-3-39	132	108	132	162 / 163	200	110	149	116	130	168
8A-10-14	153 / 161	88 / 115	118 / 162	163	200	141 / 143	149 / 151	116 / 118	130 / 136	155
12D-10-15	132 / 153	88 / 110	132 / 162	163	196 / 200	110 / 143	151	116	126 / 136	148
12D-31-5	132 / 153	110 / 115	118 / 132	163	200	110 / 143	149 / 151	116 / 118	126 / 130	148
12D-39-5	132 / 163	108 / 110	118 / 162	163	200	110 / 143	149 / 151	116 / 118	130 / 136	148
15-ED-103	132 / 140	88 / 110	132	163	200 / 204	110	151	116	130	135 / 155
118-LE-133	132 / 155	88 / 110	132 / 162	162 / 163	200	118	151	116 / 118	136	127 / 158
20C-24-20	132 / 153	88 / 108	118 / 132	162 / 163	200	110	151	116 / 118	126	148 / 154
77-LA-579	132 / 140	108 / 115	118 / 162	162	200 / 204	141 / 143	151	116	130 / 136	135
AP93-9	132	110 / 115	132	153	200	142	149 / 151	116	130	135 / 148 /158

Cultivars	UDP98409	UDP98412	UDP98022	BPPCT025	CPPCT006	UDP98005	CPPCT044	BPPCT001	CPDCT045	BPPCT007
AP94-10	132 / 161	88	162	163	200	110	151	116	126 / 136	146 / 148
AP94-34	161	88	118	162 / 163	200	110 / 139	149 / 151	116	126 / 136	148 / 155
AP95-56	132 / 153	88 / 108	118 / 162	163	200	110	149 / 151	116	130 / 136	135 / 148
Clone 0.11	140 / 145	100 / 108	162	158 / 163	176 / 188	115 / 141	149 / 151	120	124 / 130	148 / 158
Dof-1A586	153 / 161	110 / 115	118 / 162	163	200	141	149 / 151	116	126 / 136	135 / 148
LE 3241 (a)	140 / 163	104 / 108	118 / 162	153	190 / 196	118 / 141	151	116	130 / 134	155 / 158
LE 3241 (b)	161	88 / 108	161	163	196 / 200	110	151	116	130	148
Ng3	132	108	132	151 / 163	198	110 / 141	149 / 151	116	130	148
Ng5	132 / 155	108 / 110	132 / 162	151 / 153	196 / 200	110	151	116 / 118	124 / 130	158
Nja-19	132	108 / 115	132	163	200	200	149 / 152	116 / 118	130	148
"Unknown"	132 / 161	88	118	163	196 / 200	110 / 141	149 / 151	116 / 118	130	135 / 148
S5A-2-4	132 / 153	110 / 115	118 / 162	162 / 163	200	110	151 / 151	116	130 / 136	148

3.3.1.4 Parentage verification

In the case of 15 accessions, for which the parentage had previously been reported by an internal ARC source (Horstmann, personal communication), the fingerprints of the progeny cultivars/selections were compared with those of the reported parents (Table 3.7, 3.8).

Table 3.7. Reported parentages of 15 apricot accessions from the ARC gene bank (Horstmann, personal communication).

Progeny	Parent 1	Parent 2
Supergold	(Royal x Bulida)	Alpha
Soldonne	(Royal x Bulida)	Alpha
Goldstrke	Goldrich	(Earlini x Blenril)
Malan Royal	Seedling of Royal	Unknown
AP94-10	10-G-200	Supergold
AP94-34	15-26-37	Supergold
12D-39-5	Palsteyn	4-11-79
8A-10-14	Palsteyn	15-9-11
12D-31-5	Palsteyn	Unknown
12D-10-15	Palsteyn	Castleton
3B-9-25	Palsteyn	Unknown
S5A-2-4	4-10-62	Soldonne
AP95-56	Veecot	Supergold
Palsteyn	Peeka	Verdun
Peeka	Royal	Bulida

Table 3.8. Microsatellite fingerprints, amplified using 10 microsatellite markers, of 15 accessions indicating inheritance of one or two alleles from reported parents.

Cultivar/ Selection	UDP98 409	UDP98 412	UDP98 022	BPPCT 025	CPPCT 006	UDP98 005	CPPCT 044	BPPCT 001	CPDCT 045	BPPCT 007
Supergold RC *	153,161	88,88	118,162	163,163	200,200	110,110	151,151	116,116	126,136	148,148
Supergold *	153,161	88,88	118,162	163,163	200,200	110,110	151,151	116,116	126,136	148,148
Soldonné *	153,153	108,115	118,118	163,163	198,200	110,110	149,151	116,118	126,130	148,155
Royal †	132,161	88,108	118,132	163,163	196,200	110,141	149,151	116,118	130,130	135,148
Bulida RC ‡	153,153	108,115	118,118	163,163	198,200	110,110	149,151	116,118	126,130	148,154
Goldstrike *	132,136	114,121	132,136	153,163	198,200	110,110	149,151	118,118	130,136	148,168
Goldrich †	132,136	110,115	132,162	153,163	200,200	118,118	151,151	116,118	126,136	168,168
Malan Royal *	132,161	88,108	118,132	163,163	196,200	110,139	149,151	116,118	130,130	135,148
Royal †	132,161	88,108	118,132	163,163	196,200	110,141	149,151	116,118	130,130	135,148
AP95-56 *	132,153	88,108	118,162	163,163	200,200	110,110	149,151	116,116	130,136	135,148
AP94-10 *	132,161	88,88	162,162	163,163	200,200	110,110	151,151	116,116	126,136	146,148

AP94-34 *	161,161	88,88	118,118	162,163	200,200	110,110	149,151	116,116	126,136	148,155
Supergold RC †	153,161	88,88	118,162	163,163	200,200	110,110	151,151	116,116	126,136	148,148
8A-10-14 *	153,161	88,115	118,162	163,163	200,200	141,143	149,151	116,118	130,136	155,155
3B-9-25 *	132,161	88,110	118,162	163,163	196,200	110,143	151,151	116,116	126,136	148,148
12D-10-15 *	132,153	88,110	132,162	163,163	196,200	110,143	151,151	116,116	126,136	148,148
12D-31-5 *	132,153	110,115	118,132	163,163	200,200	110,143	149,151	116,118	126,130	148,155
Palsteyn RC †	132,161	88,110	118,162	163,163	196,200	110,143	151,151	116,116	126,136	148,148
S5A-2-4 *	132,153	110,115	118,162	162,163	200,200	110,110	151,151	116,116	130,136	148,148
Soldonné RC †	136,153	88,115	118,118	153,163	198,200	110,141	151,151	116,118	126,130	148,155
Soldonné †	153,153	108,115	118,118	163,163	198,200	110,110	149,151	116,118	126,130	148,155
Palsteyn *	132,161	88,110	118,162	163,163	196,200	110,143	149,151	116,116	126,136	148,148
Peeka RC †	153,161	88,108	118,118	163,163	196,200	110,110	149,151	116,116	126,130	148,155
Peeka †	153,161	88,108	118,118	162,163	196,200	110,110	151,151	116,116	130,130	148,155
Peeka *	153,161	88,108	118,118	163,163	196,200	110,110	151,151	116,116	130,130	148,155
Bulida RC †	153,153	108,115	118,118	163,163	198,200	110,110	149,151	116,118	126,130	148,154
Royal §	132,161	88,108	118,132	163,163	196,200	110,141	149,151	116,118	130,130	135,148

* indicates the progeny accession. † indicates the first parental accession. ‡ indicates the possible grand-parents. § indicates second parental accession. RC indicates cultivars from the reference collection. Color coordination represents amplification of same alleles between parents and progeny accessions.

3.3.1.5 Genetic relationships and clustering of ARC apricot cultivars and selections

Cluster analysis of the apricot accessions proved to be useful in facilitating the detection of possible mis-identification/mislabelling, identifying similarities between cultivars that were thought to be different and clarifying the species to which some cultivars belong. The dendrogram (Fig. 3.2) revealed that the cultivars ‘Travatt’ (59); ‘Sancastresse’ (44) and ‘Heiwa’ (20) were highly similar or identical and the cultivar ‘Satungsky’ (45) was very close to these three cultivars. It was evident that these four accessions clustered separately from the other accessions, indicating the possibility that they actually belong to a different species of apricot. Another possibility is that they may be rootstocks. Additionally, other accessions that were closely related also clustered together. For example two ‘Soldonne’ cultivars that were planted in different plots, one in the gene bank (gene bank) and the other in the reference collection (RC). Other sets of accessions such as [‘Palsteyn‘(32); 1037 (84)] and [AP94-34; ‘Junshiro Los’ (23)] also clustered together.

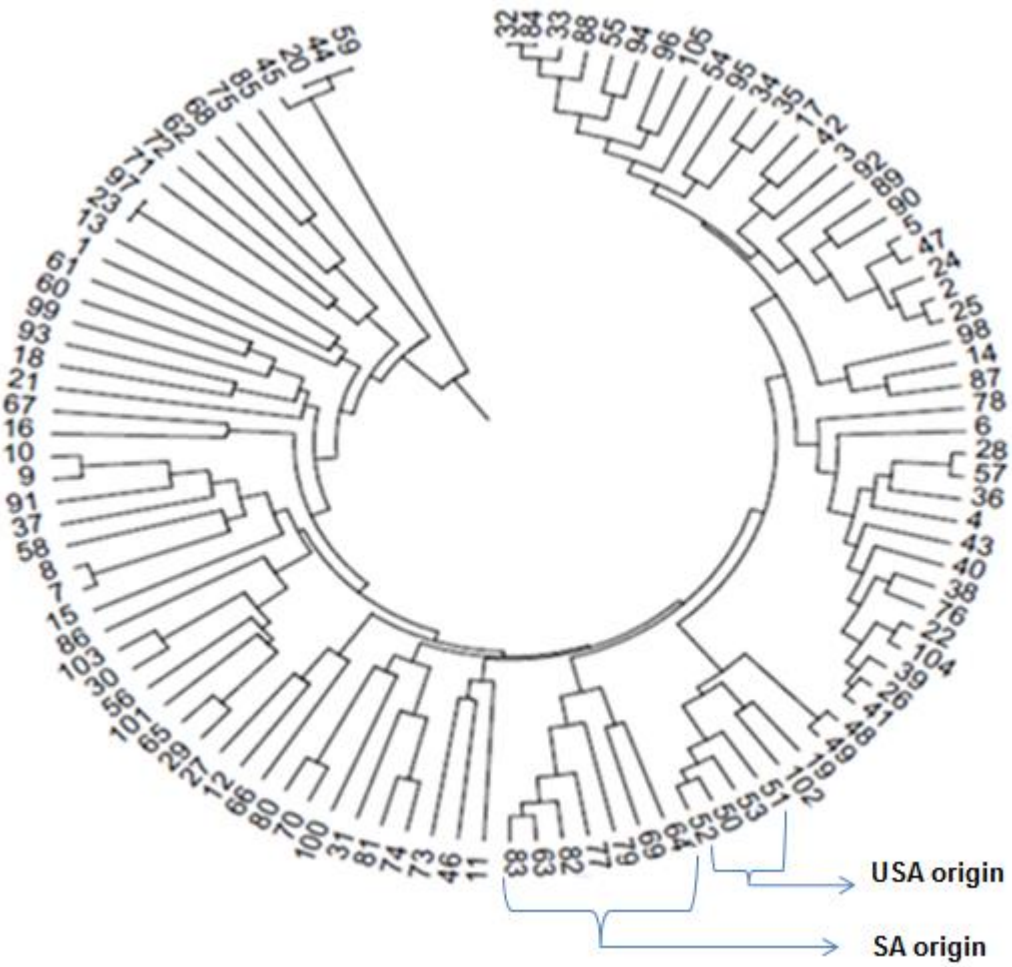


Fig. 3.2. Dendrogram obtained by UPGMA cluster analysis in MEGA, indicating similarities among ARC apricot cultivars and selections.

1-Alpha RC	22-Ivresse	43-Royal FR	65-1003A	87-4A 3 36
2-Alpha	23-Junshiro Los	44-Sancastresse	66-1004B	88-4A 3 39
3-Anshu	24-Kathy	45-Satungsky	67-1005A	89-8A 10 14
4-Atricot RC	25-Ken Psar	46-Satunska	68-1006B	90-12D 10 15
5-Autumn Glo	26-Ladisun RC	47-Shimizugou	69-1007B	91-12D 31 5
6-Barracca	27-Ladisun	48-Soldonné RC	70-1008A	92-12D 39 5
7-Bulida RC	28-Malan Royal	49-Soldonné	71-1012A	93-15 ED 103
8-Castleton	29-Malice	50-Suaprieight RC	72-1013B	94-20C 24 20
9-Charisma RC	30-Moorpark	51-Suaprieight	73-1017B	95-77 LA 579
10-Charisma	31-Moorpark nuwe	52-Suaprinine RC	74-1018B	96-AP94 10
11-Comedie 1	32-Nja-19	53-Suapriseven RC	75-1020A	97-AP94 34
12-Comedie 2	33-Olimp	54-Suapriseven	76-1020B	98-AP95 56
13-Early Baidy	34-Palsteyn RC	55-Suapritwo	77-1023B	99-Clone 0.11
14-Fantasme	35-Palsteyn	56-Supergold RC	78-1027B	100-Dof 1 A 586
Avikour	36-Peeka RC	57-Supergold	79-1028B (a)	101-LE 3241 (a)
15-Frater	37-Peeka	58-T.B. Therma	80-1028B (b)	102-LE3241 (b)
16-Giada	38-Real de Timola	59-Travatt	81-1030A	103-Ng3
17-Goldrich	39-Rouge de Fournes	60-Trigems	82-1031B 2	104-Ng5
18-Goldstrike	40-Royal RC	61-Vesna	83-1032B	105-Nja-19
19-Guillia	41-Royal	62-Zinostojky	84-1037A	106-Unknown
20-Heiwa	42-Royal Blenheim	63-1002A	85-1038A	107-S5A 2 4
21-Icapl		64-1002B	86-3B 9 25	

3.3.2 Plum

The molecular fingerprints of the apricot accessions were obtained from eight microsatellite markers. The great majority of the plum accessions belonged to *P. salicina*, however the data for other species (*P. cerasifera*, *P. domestica* and *P. insititia*) is not presented due to the complex patterns observed. Statistical analysis included in this study only assumed diploidy, therefore in the occasional cases of accessions that showed more than two peaks for some markers, they were excluded from the analysis.

3.3.2.1 Marker performance

Of the 13 markers tested, eight markers (UDP98409, UDP98412, BPPCT025, CPPCT006, CPPCT044, BPPCT001, CPDCT045 and BPPCT007) were useful in amplifying products in the full set of plum (*P. salicina*) accessions giving easy to score patterns. The markers primarily showed two peaks for the *P. salicina* accessions.

3.3.2.2 Statistical analysis

According to the MICRO-CHECKER analysis, one marker (UDP98409) showed the possibility of the presence of null alleles as suggested by an excess of homozygotes for most loci. This marker was, however, not excluded from further analysis as there was no observed evidence of scoring errors due to stuttering or large allele drop-out. The remaining seven markers revealed no evidence of null alleles and scoring errors; thus, the data were considered valid for further statistical analysis (Fig. 3.3).

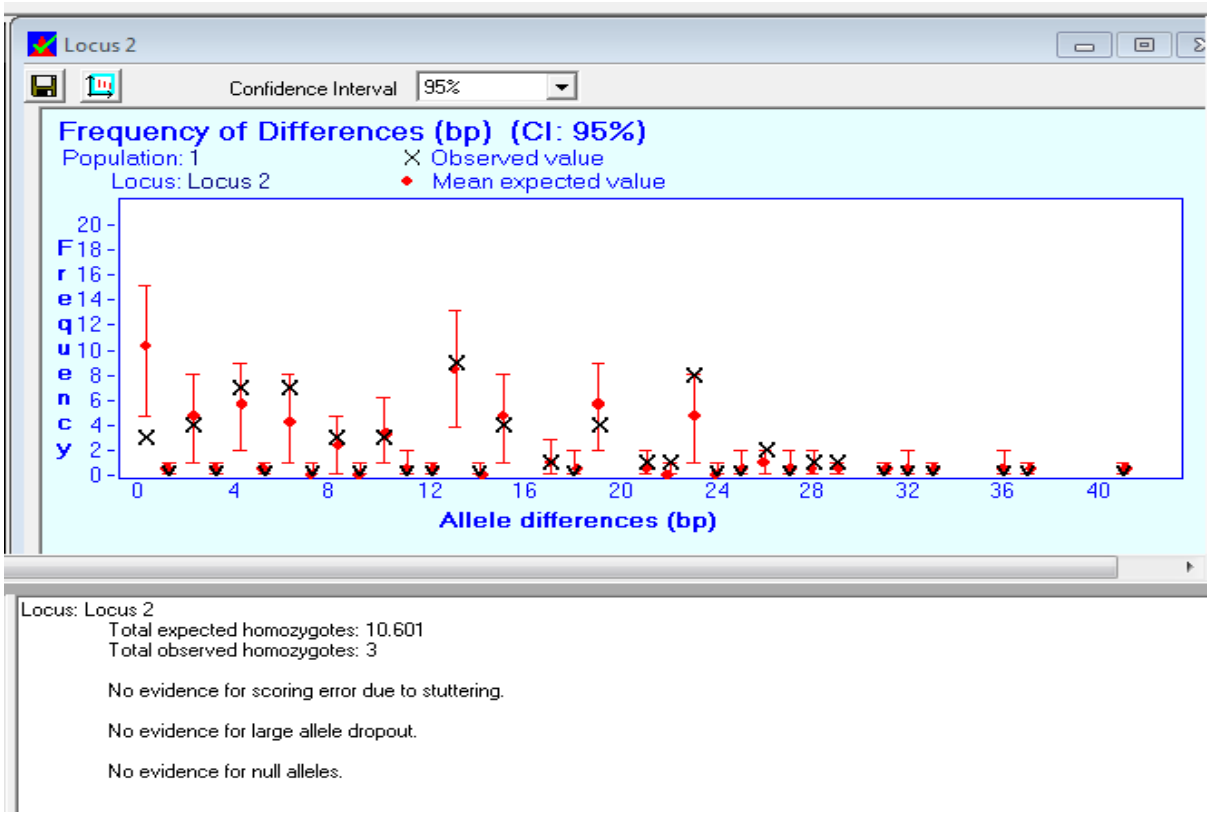


Fig. 3.3. MICRO-CHECKER output for the marker UDP98412 scored in diploid plums, indicating no evidence of mis-scoring, no allele drop-out and no null alleles.

In each locus, there were from one to five peaks per accession. Accessions showing one peak were considered to the homozygous and accessions showing multiple peaks were excluded from further analysis. In all there were 72 alleles (Table 3.9) ranging from four (CPPCT044) to 13 (UDP98412) alleles per locus, with a mean value of 9.0 alleles per locus.

Table 3.9. Number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC) and Shannon's information index (I) identified using eight microsatellite loci for the plum accessions from the ARC gene bank and reference collection (RC).

Locus	Na	Ho	He	PIC	I
BPPCT001	8	0.763	0.782	0.744	1.700
BPPCT007	12	0.915	0.842	0.825	2.076
BPPCT025	10	0.847	0.823	0.792	1.876
CPDCT045	8	0.712	0.643	0.574	1.255

CPPCT006	8	0.746	0.667	0.625	1.412
CPPCT044	4	0.424	0.412	0.369	0.763
UDP98409	9	0.441	0.620	0.590	1.392
UDP98412	13	0.949	0.827	0.797	1.942
Mean	9.000	0.725	0.703	0.6645	1.552

For the entire diploid plum population, the mean value of Ho was 0.725. The lowest value of Ho was 0.441 for marker UDP98409 and the highest Ho was 0.949 for marker UDP98412. Expected heterozygosity was the lowest for marker CPPCT044 with a value of 0.412 and the highest value of 0.842 was observed for marker BPPCT007. The polymorphic information index ranged from 0.369 (CPPCT044) to 0.797 (UDP98412), with a mean value of 0.6645. Shannon's information index was the lowest for marker CPPCT044 (0.763) and highest for marker BPPCT007 (2.076), with mean value of 1.552. All markers analysed in diploid plum deviated significantly from the HWE.

3.3.2.3 Identification of microsatellite fingerprints

The molecular fingerprints of 95 plum accessions from the ARC gene bank and reference collection were obtained from eight microsatellite primers. Most primers produced a maximum of two peaks in each genotype in accordance with the diploid *P. salicina* species. However, in the case of the cultivars 'Betty Anne' and 'Mac Verma', which were supposedly diploid, a third allele was present in three markers (Table 3.10). Some accessions listed in table 3.2 showed complex amplification patterns, which resulted in difficult scoringof alleles; therefor the data for those accessions was not presented.

Table 3.10. Allele sizes of eight microsatellite loci, showing unambiguous discrimination of plums accessions from the ARC gene bank and reference collection.

Name	UDP98409	UDP98412	BPPCT025	CPPCT006	CPPCT044	BPPCT001	CPDCT045	BPPCT007
ARC PR-1	142	112 / 125	163 / 190	197	147	124 / 139	145 / 147	119 / 144
ARC PR-2	142	112 / 127	157 / 190	197	147	140	145	119 / 133
ARC PR-3	142	127 / 135	157 / 190	197 / 211	147	124 / 139	145 / 147	119 / 133
ARC PR-4	136 / 142	125	174 / 190	188 / 211	147	124 / 139	144 / 147	134 / 148
Autumn Sun	134 / 142	112	165 / 190	197	147	139	145	119 / 144
Betty Anne	136 / 142	112 / 131 / 135	163 / 174 / 190	188 / 197 / 211	147/154	140 / 143	145 / 147	119 / 133
Black Amber	142	112 / 125	163 / 190	197	147	124/ 139	147	119 / 141
Black Egg	134 / 142	125 / 135	190	197 / 211	147	124 /155	145 / 147	144 / 155
Casselman	138 / 142	112 / 135	163 / 174	195 / 197	147	124 / 139	145 / 147	133 / 141
Celebration (1)	142	125 / 127	157 / 190	188 / 197	147/168	139 / 155	145	119 / 131
Celebration (2)	142	125 / 127	157 / 190	188 / 197	147/168	140 / 155	143 / 145	119 / 131
Crocodile Dundee	136 / 142	112 / 135	165 / 190	188 / 211	147/168	135 / 140	145 / 147	119
Explorer	122 / 130	127 / 135	174	188	147/154	139	143 / 147	119
Extreme	138 / 142	125 / 131	155 / 174	177 / 211	147	124 / 139	144 / 147	134 / 155
Flavor Fall	130	112 / 125	163 / 174	188 / 197	147/168	124 / 139	143 / 145	119 / 133
Flavor King	130	112 / 131	163 / 174	188 / 211	147	122 / 139	124 / 147	119 / 148
Flavor Rich	138 / 142	125 / 131	163 / 174	197	147	124 / 139	145	119 /141
Fortune	130	112 / 125	163 / 192	188 / 197	147	124 / 134	145	131
Golden King	142	127 / 131	157 / 163	197	147	124 / 155	143 / 145	144 / 155
Golden Kiss	142	127 / 131	157 / 163	197	147	124 / 155	145	144 / 155
Green Red	136 / 142	127 / 131	155 / 157	208 / 211	147/168	134 / 139	145	119 / 141
Harry Pickstone	134 / 142	112 / 125	165 / 190	197 / 208	154/168	124 / 139	143 / 145	119 / 133
Hiromi Red	142	125 / 135	157 / 163	188 / 197	154/168	124 / 139	124 / 147	131 / 155
Lady Red	142	112 / 135	155 / 165	197 / 208	147	140	145	144 / 155
Lady West	142	112 / 125	163 / 174	195 / 197	147	139	143 / 147	119 / 155
Laetitia	134 / 142	112 / 135	163 / 190	197 / 211	147	124 / 139	145 / 147	133 / 144

Name	UDP98409	UDP98412	BPPCT025	CPPCT006	CPPCT044	BPPCT001	CPDCT045	BPPCT007
Lamoon	142	112 / 135	163 / 190	197	147	124 / 139	147	119 / 155
Late Lamoon	134 / 142	112 / 133	163 / 190	197 / 208	147	124	145 / 147	119 / 134
Loroda	134 / 142	112 / 125	163 / 190	197 / 211	147	122 / 124	134 / 147	148 / 155
Mac Verma	128 / 136 / 138	113 / 125	157 / 174 / 184	177 / 188 / 197	147 / 168	122 / 139	145 / 147	119 / 144 / 148
Methley	138 / 142	99 / 127	163 / 174	177 / 197	147 / 168	140	143	119 / 144
Mirell	142	112 / 135	163 / 190	197	147	124 / 139	145 / 147	119 / 155
Pioneer	138 / 142	125 / 131	155 / 174	177 / 211	147	124 / 139	144 / 147	134 / 155
Purple King	142	113 / 127 / 133	157 / 184 / 190	188 / 197	147 / 168	122 / 155	145 / 147	119 / 144 / 148
Purple Majesty	142	123 / 131	155 / 190	188 / 197	147	124	145 / 147	133 / 155
Red Gold	134 / 145	112 / 127	157 / 165	188 / 197	147 / 168	139 / 155	145	119 / 144
Reubennel	138 / 142	108 / 125	157 / 174	177 / 197	154 / 168	139 / 140	143 / 147	119 / 133
Ruby Prince	142	125 / 131	155 / 190	197 / 211	147 / 154	124 / 139	145 / 147	120 / 134
Ruby Red	134 / 142	112 / 127	163 / 190	197 / 211	147	124 / 139	145 / 147	120 / 134
Ruby Star	142	106 / 112	163	197	147	140	145	119 / 133
Ruby Sun	136 / 142	112 / 125	163 / 190	188 / 197	147	124 / 140	145 / 147	140 / 148
Santa Rosa	142	112 / 131	155 / 163	197	147	122 / 124	145 / 147	133 / 155
Sapphire	136 / 142	125 / 131	155 / 190	188 / 197	147	124 / 139	147	148 / 155
Satin Gold	134 / 142	112 / 125	157 / 190	197 / 211	147 / 168	139 / 155	145	119 / 144
Sensation	142	125 / 131	155 / 174	188 / 197	147	124 / 139	145 / 147	119 / 155
Simka	163	112 / 131	163	177 / 197	147 / 168	122 / 139	145 / 147	133 / 148
Songold	134 / 142	112 / 127	157 / 165	188 / 197	147 / 168	139 / 155	145	119 / 144
Southern belle	136 / 142	127 / 131	163	177 / 188	147 / 154	140	145 / 147	199 / 137
Souvenir 2	142	127 / 131	155 / 157	197	147 / 168	124 / 155	145 / 147	144 / 155
Sun Kiss	142	112 / 135	163 / 190	188 / 211	147	122 / 139	145 / 147	140
Sundew	134 / 142	125 / 135	190	197 / 211	147	124 / 155	145 / 147	144 / 155
Sunset	142	112	163	197	147	124 / 139	145 / 147	119 / 155
Suplum 11	142	131 / 135	155 / 190	197 / 211	147 / 168	134 / 139	145 / 147	119 / 141
Suplum 25	136 / 142	94 / 123	157 / 192	197 / 211	147 / 168	124 / 139	145 / 147	148 / 155
Suplum 28	130	108 / 131	155 / 157	188 / 197	147	122	145 / 147	131

Name	UDP98409	UDP98412	BPPCT025	CPPCT006	CPPCT044	BPPCT001	CPDCT045	BPPCT007
Suplum 6	130	112 / 135	163 / 190	188 / 197	147 / 168	124 / 134	145 / 147	141 / 155
Sweet Aroma	142	112 / 135	155 / 163	197 / 211	147 / 168	134	145 / 147	119 / 155
Winner	130	112 / 131	155 / 163	188 / 197	147 / 168	124 / 134	145 / 147	119 / 155
17-10-212	134 / 142	112	163	197	147	122 / 139	126 / 147	119 / 155
17-32-118	134 / 142	125 / 127	157 / 190	188 / 197	147 / 168	124 / 115	145 / 147	144 / 155
4A-8-13	134 / 142	112 / 125	165 / 190	197 / 208	147	137 / 139	145	199 / 144
4C-8-20	134	108 / 112	157	188 / 197	147 / 154	139 / 155	145	141 / 144
P. insititia								
Adesoto (1)	149 / 159	99 / 104	167	172 / 189	149 / 168	137 / 157	132 / 137 / 147	127 / 129 / 158
Adesoto (2)	126 / 132 / 149	92/96/104/110/119	156/165/170/172	186/188/190/198	156 / 168 / 170	139 / 142	126 / 128	125/131/135/139/141
Pixy (1)	149 / 159	99 / 104	167	172 / 189	151 / 168	137 / 157	132 / 137 / 147	127 / 129 / 158
Pixy (2)	149 / 159	96 / 104 / 110	153/156/160/172	170/184/186/189/195/218	149 / 168	124/137/139/142	120/122/126/128/134	125/131/139/141
P. domestica								
Eruni	126 / 142	96/104/112/121	154/162/166/172/182	191 / 215 / 218	158/161/168/172/177	124/144/150/155	126/128/134/136138	125/127/137/144/150
Jubileum	142 / 145 / 147	94/96/110/135	156 / 161	186 / 194	160 / 166	139 / 148	126 / 128 / 134	125/131/137/141/144/150
Oneida	122 / 149	96/101/106/113	153/164/172/184	191 / 195	160 / 168 / 170	139 / 142 / 146	122/124/126/128	127/131/135/141/148
P. Marianna (1)	126 / 147	96 / 99 / 108	160 / 167 / 182	172/189/191/193	149 / 154 / 168	137 / 139 / 157	128 / 132 / 137	125/127/141/144/158
P. Marianna (2)	126 / 147	99 / 108	160/165/167/182	172/189/191/193	149 / 154 / 168	136/137/139/157	128 / 132 / 137	125/127/141/144/158
P. cerasifera								
Adara	128 / 136	112 / 125	163 / 177	191 / 193	158 / 160	132 / 142	122 / 140	133 / 137
Ademir (1)	132	99 / 125	167 / 188	180 / 202	168 / 172	132	140 / 143	133 / 144
Ademir (2)	132	99 / 125	167 / 188	180 / 202	168 / 172	132	140 / 143	133 / 144
Erfdeel	122 / 126 / 145	96 / 104	161/165/170/172/182	188/190/195/197	156/158/166/170	124/137/139/142/144	126 / 128	125/131/139/150
French Prune	122 / 126 / 145	96 / 104	161/165/170/172/182	188/190/195/197	156/158/166/170	124/137/139/142/144	126 / 128	125/131/139/150
Prune d' agen	122 / 126 / 145	96 / 104	161/165/170/172/182	188/190/195/199	156/158/166/170	124/137/139/142/144	126 / 128	125/131/139/150
V D Merwe	122/ 126 / 145	96 / 104	161/165/170/172/182	188/190/195/197	156/158/166/170	124/137/139/142/144	126 / 128	125/131/139/150
Hybrids								
By 69-1637P	155	110 / 135	157 / 174	195 / 197	151 / 168	116 / 139	136 / 143	131 / 158
Fereley Jaspi	122	99 / 125	163 / 174	180 / 189 / 197	147 / 156 / 160	137 / 139 / 148	120 / 122 / 143	119 / 127 / 144

Name	UDP98409	UDP98412	BPPCT025	CPPCT006	CPPCT044	BPPCT001	CPDCT045	BPPCT007
Ferely	122	99	163 / 174	180 / 189 / 197	147 / 156 / 160	137 / 139	120 / 122 / 143	119 / 127 / 144
Ferlenain (P2038)	126 / 142	96	160 / 179	189 / 194	151 / 154 / 174	142 / 159	126 / 143	106 / 133 / 169
S5A-25-5	132	89 / 125	163 / 190	188 / 201	147 / 151	116	130 / 145	133 / 148
S5A-25-20	161	89 / 125	163 / 174	197	147 / 151	116	126 / 145	133 / 155
S5A-26-11	149	99 / 108 / 125	167 / 174	172 / 177 / 189	149 / 168	137 / 157	132 / 143	119 / 127 / 158
S5A-26-13	161	89 / 125	163 / 174	197	147 / 151	116	126 / 145	133 / 155
S5A-26-28	161	108 / 125	163 / 174	197	151 / 168	116 / 134	130 / 145	133 / 148
S5A-26-30	161	89 / 123	151 / 190	197 / 201	147 / 151	116 / 134	126 / 145	133 / 135
S5A-26-35	153	108 / 125	163 / 174	167 / 201	147 / 151	116	130 / 145	133 / 148
S5A-33-25	153	89 / 125	163 / 174	197 / 201	151 / 168	116	130 / 145	133 / 148
S5A-34-25	153	89 / 123	163 / 190	188 / 201	151 / 168	116 / 134	126 / 145	133 / 155
S5A-34-28	159 / 161	89 / 123	163 / 190	197 / 201	147 / 151	116	136 / 145	133 / 148
S5A-34-37	161	108 / 123	163 / 190	197	147 / 151	116 / 155	130 / 145	133 / 148
S5A-35-34	153	89 / 125	163 / 174	197 / 201	151 / 168	116	130 / 145	133 / 148
Sel 6-64	149	99 / 108 / 127	167 / 184	172 / 188	149 / 168	122 / 137 / 157	132 / 147	119 / 127 / 158

3.3.2.4 Parentage verification

The parentage of 17 accessions were compared with the reported parents (Table 3.11), which were also fingerprinted in this study. These accessions included interspecific hybrids S5A ('Songold' x 'Red Beauty'), 'Loroda' ('Gaviota' x 'Santa Rosa'), 4A-8-13 ('Songold' O.P.), 'Celebration' ('Songold' O.P.) and 17-10-212 ('Laroda' O.P.). The progenies shared one or both of the parental alleles at most loci. However, in the case of the markers UDP98412 and CPPCT006 (Table 3.11), allele sizes of some apricot and plum hybrid progenies had a one base pair shift from the parental cultivars, *e.g.* 89 bp and 201 bp, whereas the apricot parent ('Supergold') showed a size of 88 bp and 200 bp, respectively. It was also noted that S5A-26-11 did not show any correspondence with the reported parent, indicating that the reported parentage of S5A-26-11 may be incorrect.

Table 3.11. Reported parentages of 17 accessions from the ARC gene bank (Horstmann, personal communication).

Progeny	Parent 1	Parent 2
S5A-25- 5	Red Beauty	Supergold
S5A-26-13	Red Beauty	Supergold
S5A-25-20	Red Beauty	Supergold
S5A-26-28	Red Beauty	Supergold
S5A-26-35	Red Beauty	Supergold
S5A-26-30	Red Beauty	Supergold
S5A-26-11	Red Beauty	Supergold
S5A-34-25	Red Beauty	Supergold
S5A-33-25	Red Beauty	Supergold
S5A-34-28	Red Beauty	Supergold
S5A-34-37	Red Beauty	Supergold
S5A-35-34	Red Beauty	Supergold
Laroda	Santa Rosa	Gaviota
4A-8-13	Songold	
Celebration	Songold	
17-10-212	Laroda	

Table 3.12. Comparison of fingerprints of 17 plum accessions with reported parentages. All but one accession (S5A-26-11) showed fingerprints consistent with reported parentages.

Name	UDP98409	UDP98412	BPPCT025	CPPCT006	CPPCT044	BPPCT001	CPDCT045	BPPCT007
S5A-25-5*	132	89,125	163,190	188,201	147,151	116	130,145	133,148
S5A-25-20*	161	89,125	163,174	197	147,151	116	126,145	133,155
S5A-26-11*	149	99,108,125	167,174	172,177,189	149,168	137,157	132,143	119,127,158
S5A-26-13*	161	89,125	163,174	197	147,151	116	126,145	133,155
S5A-26-28*	161	108,125	163,174	197	151,168	116,134	130,145	133,148
S5A-26-30*	161	89,123	151,190	197,201	147,151	116,134	126,145	133,135
S5A-26-35*	153	108,125	163,174	167,201	147,151	116	130,145	133,148

S5A-33-25*	153	89,125	163,174	197,201	151,168	116	130,145	133,148
S5A-34-25*	153	89,123	163,190	188,201	151,168	116,134	126,145	133,155
S5A-34-28*	159/161	89,123	163,190	197,201	147,151	116	136,145	133,148
S5A-34-37*	161	108,123	163,190	197	147,151	116,155	130,145	133,148
S5A-35-34*	153	89,125	163,174	197,201	151,168	116	130,145	133,148
Supergold †	153,161	88,88	163,163	200,200	151,151	116,116	126,136	148,148
Laroda*	134,142	112,125	163,190	197,211	147	122,124	134,147	148,155
Santa Rosa†	142	112,131	155,163	197	147	122,124	145,147	133,155
Gaviota§	122	108,125,129,131	,157,163	177,197,211	145	116,124	138,143,147	119,133,144,148,155
4A-8-13*	134,142	112,125	165,190	197,208	147	137,139	145	199,144
Songold†	134,142	112,127	157,165	188,197	147,168	139,155	145	119,144
Celebration*	142	125,127	157,190	188,197	147,168	139,155	145	119,131
Celebration*	142	125,127	157,190	188,197	147,168	140,155	143/145	119,131
Songold†	134,142	112,127	157,165	188,197	147,168	139,155	145	119,144
17-10-212*	134,142	112	163	197	147	122,139	126,147	119,155
Laroda†	134,142	112,125	163,190	197,211	147	122,124	134,147	148,155

* indicates the progeny accession. † indicates the first parental accession. § Indicates second parental accession. Colour coordination represents amplification of same alleles between parents and progeny accessions

3.3.2.5 Cluster analysis

To identify possible duplicates among ARC plum accessions, a dendrogram was produced using UPGMA cluster analysis from eight microsatellite loci (Fig. 3.4). Two cultivars clustered separately, namely 'Ademir' (1) and (2), indicating that the two cultivars belonged to a different species, *i.e. P. cerasifera*. All other accessions grouped together; indicating that they were indeed from the same species, *i.e. P. salicina*. A number of accessions showed a very close relationship, for example 'Songold' and 'Redgold'; 'Golden King' and 'Golden Kiss'; 'Celebration' (1) and (2) as well as 'Lamoon' and 'Mirell'. The dendrogram was also useful in identifying cultivars that were identical. Cultivars 'Extreme' and 'Pioneer' were identical when analysed with the current selectin of markers, as were 'Black Egg' and 'Sundew'.

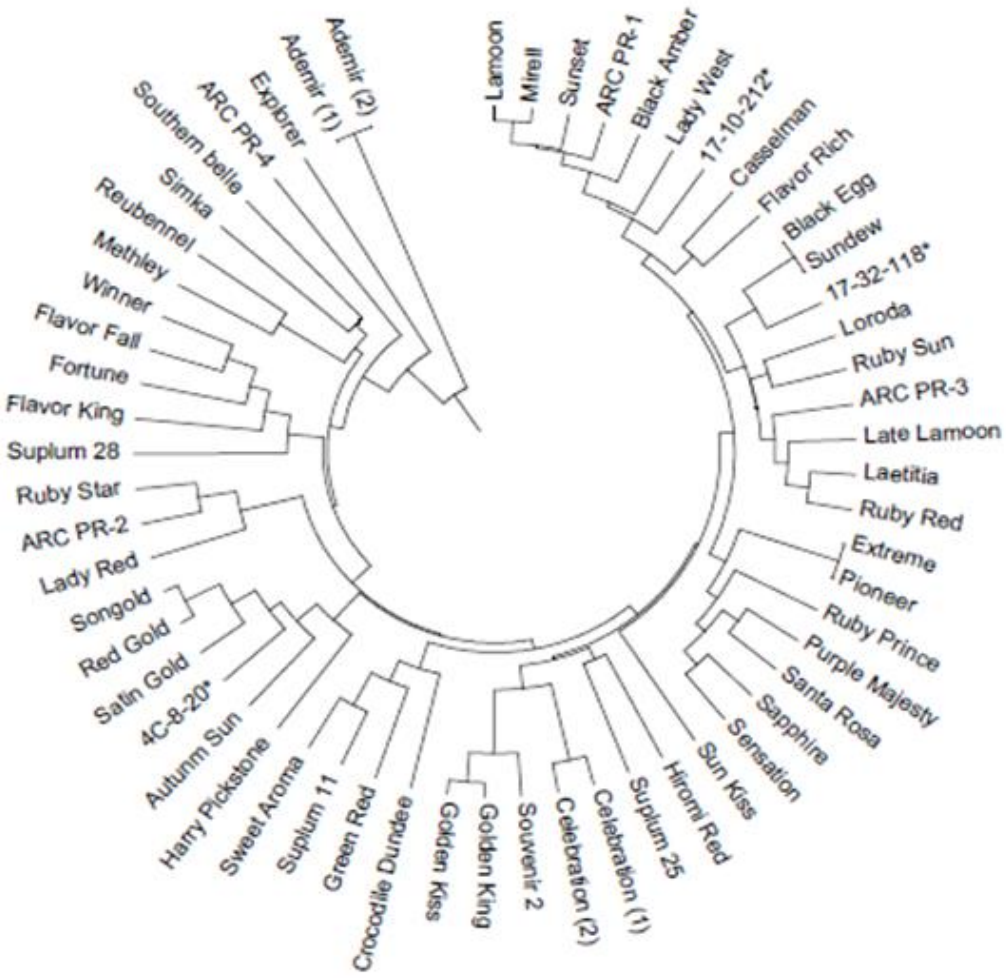


Fig. 3.4. Dendrogram constructed from UPGMA cluster analysis output of the microsatellite data from the ARC plum accessions, showing the relationships between the accessions.

3.4 Discussion

The microsatellites developed from peach proved useful for fingerprinting and verifying parentage of ARC apricot and plum gene bank collections. As suggested by Hormaza (2002), the automated analysis proved to be useful as it provides a means to resolve allelic variation in relation with product sizes at a fine scale. The results confirmed the cross-species transferability of the microsatellite primers that were initially designed in peach but proved to be effective in apricot and plum (Dirlewanger *et al*., 2002). The size range of the markers observed in the current study was similar to that reported in other studies in related species

(Aranzana *et al*., 2002; Dirlewanger *et al*., 2002; Hormaza, 2002; Krichen *et al*., 2006) (Table 3.12).

Table 3.13. Comparison of microsatellite product size ranges, showing transferability or primers between peach and apricot cultivars

Marker	Previously reported product range in peach (bp)	Product range in apricot observed in current study (bp)	Product range in plum observed in current study (bp)	Reference
BPPCT001	125 - 195	124 - 155	122 - 155	Dirlewanger *et al*., 2002
BPPCT007	119 - 149	119 - 144	119 - 144	Dirlewanger *et al*., 2002
BPPCT025	147 - 159	157 - 190	155 - 190	Dirlewanger *et al*., 2002
CPPCT006	175 - 201	177 - 211	127 - 211	Aranzana *et al*., 2002
CPPCT044	145 - 175	147 - 180	147 - 180	Aranzana *et al*., 2002
CPDCT045	128 - 140	124 - 147	120 - 147	Krichen *et al*., 2006
UDP98409	122 - 164	122 - 163	122 - 161	Hormaza *et al*., 2002
UDP98412	90 - 130	94 - 135	99 - 135	Hormaza *et al*., 2002

3.4.1 Apricot

3.4.1.1 Marker efficiency

The markers that were excluded from fingerprinting (CPPCT022, BPPCT017 and BPPCT038) gave poor amplification with additional non-allelic products that made scorring difficult. As other studies showed successful use of some of the markers that were unsuccessful in the current study (Sanchez-Perez *et al*., 2005), the non-satisfactory performance of these markers may be due to manufacturing errors (Ellis *et al*., 2011). Nevertheless, most markers showed good amplification confirming the cross-species transferability of markers originally designed in peach (Dirlewanger *et al*., 2002). The transferability of the markers suggests a high level of synteny within the *Prunus* genus (Aranzana *et al*., 2002). Similar results from other studies suggest that the homology among *Prunus* species partly explains the low level of breeding barriers to interspecific gene introgression and highlights the opportunity for successful gene transfer between closely related species, which is a major priority in most breeding programmes (Sanchez-Perez *et al*., 2005).

All the markers assayed in this study were polymorphic, with number of alleles ranging from five to 15. In the current study, the UDP markers had alleles ranging between 11 and 15 whereas Hormaza (2002) and Sanchez et al., (2005) reported product sizes from two to seven

alleles in apricot. The product size ranges of these markers were similar with the previously published sizes (Hormaza, 2002). This difference may result from the wide variety of cultivars from different origins that led to the introduction of new alleles being present in the ARC collections. The same results were observed in a study by Zhebentyayeva *et al*. (2003), also assaying genetic variation within an apricot germplasm using some of the UDP markers designed from peach, where higher levels of polymorphism (between two to 13 alleles) were evident. Such high polymorphism was observed due to the diverse nature of the tested germplasm, consisting of 74 apricot cultivars.

3.4.1.2 Diversity statistics

Diversity statistics were calculated for all 10 markers including markers that showed the presence of null alleles and possible scoring errors due to stuttering.

In the current study, the observed heterozygosity ranged from 0.383 to 0.710 per marker, which is similar to the findings reported by Bouguirba *et al*. (2010) on the genotyping of 82 apricot accessions from Tunisian gene pools using 24 microsatellites, where the observed heterozygosity ranged from 0.312 to 0.762. Similar patterns were observed for the expected heterozygosity, with values observed in this study being higher than findings reported by Bouguirba *et al*. (2010). This observation may be due to the fact that in the Bouguirba *et al*., (2010) study, apricot cultivars were sampled from the northern, central and southern regions of Tunisia. On the contrary the ARC apricot germplasm fingerprinted in the current study is comprised of cultivars from different countries including France, USA, Spain, Sweden and South Africa, hence the higher heterozygosity resulting from new alleles introduced from the different origins. The higher values of heterozygosity observed in the current study may also result from cross-hybridisation and selection in breeding programmes, which enhances heterozygosity within the collection (Martin *et al*., 2011).

3.4.1.3 Parentage investigation

The investigation of parentage confirmed the identity of 15 apricot accessions whose proposed parental cultivars were present in the accessions fingerprinted in the current study. All the accessions (with proposed parentages) that were tested showed consistent patterns with their reported parentages with at least one allele from each parent. Accessions that matched the reported parentage were regarded as true to type. The only inconsistencies observed were in the cultivars ‘Goldstrike’ and ‘Goldrich’ for marker UDP98005, but considering that only one

parent was fingerprinted in this study, it is possible that the progeny inherited the other allele from the second parent that was not fingerprinted in the current study.

3.4.1.4 Cluster analysis

The cluster analysis of apricot cultivars and selections are shown in Fig. 3.2, accessions clustered to some extent on the basis of origin, possibly attributed to the origins of the cultivars in the ARC collections. For example the selections developed by ARC1002B (64), 1007B (69), 1028Ba (79), 1023B (77), 1031B (82), 1002A (63) and 1032B (83) clustered closely together highlighting the close relatedness of these accessions. Likewise some cultivars originating from USA, namely 'Suapriseven' (53), 'Suaprieight' (50 & 51) and 'Suaprinine' (52) (Fig. 3.2) clustered closely. Other previous pedigree studies of apricot cultivars also showed clustering of cultivars according to their geographic origins (Badenes *et al*., 1998; Zhebentyayeva *et al*., 2003).

The accessions 'T. B. Therma' (59), 'Sancastresse' (44), 'Heiwa' (20) and 'Satungsky' (45) clustered separately from the other accessions; possibly indicating that they were not the same species as the other accessions. Field observation suggested that these four accessions were highly similar to one another and indeed showed morphological characteristics, such as leaves and tree architecture, different fromother accessions. This suggested that the trees growing were falsely identified. Additionally the microsatellite alleles of the cultivars were checked, perculiar alleles were observed thus most likely indicating that they were false.

The cluster analysis was also useful in identifying similarities between cultivars that were initially considered different. The results indicated genetic similarities of three sets of accessions, ['Junshiro Los' (23) and AP9434 (97)]; ['T. B. Therma' (59), 'Sancastresse' (44) and 'Heiwa' (20)] and [Nja-19 (32) and 1037A (84)]. These accessions showed identical or near identical microsatellite patterns.

3.4.2 Plum

3.4.2.1 Marker efficiency

In the current study, it was possible to identify the molecular fingerprints of 95 plum cultivars and selections. The product sizes of the microsatellite amplicons varied from the previously reported product ranges (Cipriani *et al*., 1999; Testolin *et al*., 2000; Aranzana *et al*., 2002; Dirlewanger *et al*., 2002; Mnejja *et al*., 2005). For example, marker BPPCT025 with an expected product range of 147 -159 bp in peach, showed a slightly different range of 153 -190

bp when used to fingerprint the plum accessions in the present study. The size ranges obtained in the current study were in line with results by Wang *et al*. (2015) in a fingerprinting study on a collection of beach plums, using a set of microsatellite primers designed from peach where different product ranges were also observed.

3.4.2.2 Parentage analysis

When the reported parentages (Horstmann, personal communication) of four plum accessions and 12 apricot and plum interspecific hybrids were tested by comparing microsatellite patterns of parents and offspring, in most cases there were scored alleles for each locus. However, in the case of the hybrid S5A-26-11, a third allele was present for the markers UDP98412, CPPCT006 and BPPCT007 and there were no matching fingerprints with the reported parent in any of the markers used. These results suggest that S5A-26-11 may be a triploid and with parentage different from that of other hybrids. Arguably the presence of a third peak in three markers may be due to duplications of those microsatellite loci (Zhang and Rosenberg, 2007), however, as the third peaks were only observed in S5A-26-11; it is likely this hybrid is diploid. The cultivar 'Gaviota' also had more than two peaks, despite being considered to be diploid (Carrasco *et al*., 2012). Despite the presence of additional peaks, it was evident that the cultivar 'Laroda' contained at least one of the alleles from its proposed parent 'Gaviota'.

In the case of markers UDP98412 and CPPCT006, the allele scores of the 12 apricot and plum hybrid selections varied by 1 bp from their proposed parentage. The one bp difference was only observed in the apricot and plum hybrid progenies. This may result from allelic variation due to instability in replication of the two alleles (88 bp and 200 bp) in the two loci (UDP98412 and CPPCT006), which results in subsequent incorrect amplification of microsatellites (Viguera *et al*., 2001).

Microsatellites were useful in confirming the parentage of plum cultivars and selections in the current study, thus the accessions that matched the reported parentages were regarded as true to type.

3.4.2.3 Polyploidy

In this study, 13 of the 95 accessions amplified between three to five alleles for some of the markers. The cultivars 'Betty Anne', 'Mac Verma' and 'Purple King' were initially presumed to be diploid. However, three or four peaks were observed in some of the markers, including UDP98 409, BPPCT025, CPPCT006 and BPPCT007; in each case indicating that these

cultivars may be triploid. However, triploid *Prunus* are expected to be sterile. Among the accessions from the gene bank and the Reference Collection there were known polyploid cultivars belonging to *P. Marianna, P. cerasifera*, *P. domestica* and *P. insititia*. The multiple alleles observed in the current confirmed their polyploid natureas previously reported by Weinberger (1975) and Bouhadida *et al*. (2009).

Polyploidy is sometimes associated with infertility (Dermen, 1965). According to Ranney (2006), infertility can arise in polyploids because of the presence of more than two homologous chromosomes often resulting in spurious pairing between multiple chromosomes, unpaired chromosomes and gametes with unbalanced chromosome numbers Vorsa and Johnson-Cicalese (2012). Although some polyploids are infertile, others are quite fertile (Vorsa and Johnson-Cicalese, 2012). Futhermore polyploids with odd numbers of chromosome sets are generally sterile. However, to clarify this, it is necessary to conduct controlled crosses among observed polyploid cultivars and observe pollen germination. As triploids produce aneuploid pollen and ovules of low fertility (Dermen, 1965), this information will be useful to breeders when selecting parental cultivars for the crossing programme.

3.4.2.4 Cluster analysis

The clustering of accessions was useful in identifying two pairs of accessions (‘Black Egg’ and ‘Sundew’; ‘Extreme’ and ‘Pioneer’) known to be different cultivars, but possibly identical. Knowledge of the parent cultivars of these accessions would have allowed the genotypes to be checked for consistency with parental patterns to confirm if they are true to name. A study by Sehic *et al*. (2015) in which the genetic diversity of the Nordic plum germplasm was investigated using nine microsatellites identified various synonyms.

As expected ‘Celebration’ 1 and 2 clustered very closely together indicating that they were true to name. Cultivars with similar names, *i.e.* ‘Songold’, ‘Red Gold’ and Satin Gold’; ‘Golden King’ and ‘Golden Kiss’ also clustered close to one another, indicating their similar origin. Tshabalala (2015) reported that the ‘Redgold’ was released in 1979 as an improvement of ‘Songold’ that the ARC originally released in 1972.

The detected molecular differences between the accessions indicate a high level of diversity within the tested population, as demonstrated by the high level of sub-clustering of the accessions. Klabunde *et al*. (2014), in a study of 47 Japanese plum cultivars genotyped with eight microsatellite markers to distinguish and characterise the cultivars, reported similar observations. Similarly, Gouta *et al*. (2010) in a study on the genetic diversity and relatedness of a Tunisisan almond germplasm found 10 genomic microsatellite markers to be sufficient to

investigate the genetic diversity within a collection of 82 local almond cultivars. The eight microsatellite markers used in the current study were therefore sufficient to identify genetic variation amongst the tested cultivars.

All the markers tested for HWE deviated from HWE. This observation may be due to the systematic propagation of the germplasm material. The accessions were from a collection of clonally propagated material rather than a natural population.

3.5 Conclusion

In the present study, genotyping the ARC apricot and plum collections using a set of previously published peach microsatellite markers proved to be useful in establishing 'baseline' data that will be useful when repropagating the collection. In some cases it was possible to check trueness to name and verify parentages. The trueness to type investigation revealed that of the 107 apricot accessions tested, four cultivars were most likely falsely identified. The partial parentages of 15 apricot and 17 plum accessions were confirmed with only one false parentage identified in plum. Arrangement of data with Microsoft Excel was useful to compare fingerprints and using the UPGMA cluster analysis method was convenient in identifying similarities and genetic relationships between the accessions. The results are useful in the development of a set of microsatellite fingerprints for a majority of apricot and plum cultivars grown in South Africa. These can be used for comparing fingerprints between laboratories, provided that the same SSRs are used and reference cultivars are included for calibration, and provide useful information within the breeding programme. The accession records will be updated with this identification data which will bring the South African collections in line with good international practice.

3.6 References

Aranzana M.J., Garcia-Mas J., Carbo J. and Arus P. (2002): Development and variability analysis of microsatellite markers in peach. Plant Breeding, 121: 87-92.

Arismendi M.J., Hinrichsen P., Almanda R., Pimentel P., Pinto M. and Sagredo B. (2012): Characterisation of genetic diversity of stone fruit rootstocks used in Chile by means

of microsatellite markers. Journal of American Society of Horticultural Science, 137: 302-310.

Badanes M.L., Martinez-Calvo J. and Llacer G. (1998): Analysis of apricot germplasm from the European ecogeographical group. Euphytica, 102: 93-99.

Bester C., Tobutt K.R., Mansvelt E.L., Blomerus M.L. and Jolly N. (2013): The value and impact of the ARC Infruitec-Nietvoorbij gene banks. Acta Horticulturae, 1007: 950-980.

Bouhadida M., Casas A.M., Gonzalo M.J. Arus P., Moreno M.A. and Gogorcena Y. (2009): Molecular characterisation and genetic diversity of *Prunus* rootstocks, Scientia Horticulturae, 120: 237-245.

Bouguirba H., Khadari B., Krichen L., Trifi-Farah N., Santoni S. and Audergon J.M. (2010): Grafting versus seed propagated apricot population, two main gene polls in Tunisia evidenced by SSR markers and model-based Bayesian clustering. Genetica, 138: 1023-1032.

Cantini C., Iezzoni A.F., Lamboy W.F., Boritzki M. and Struss D. (2001): DNA fingerprinting of tetraploid cherry germplasm using SSRs. Journal of American Society of Horticultural Science, 126: 205-209.

Carrasco B., Diaz C., Moya M., Gebauer M. and Garcia-Gonzalez R. (2012): Genetic Characterisation of Japanese plum cultivars (*Prunus salicina*) using SSR and ISSR molecular markers. Plant Breeding, Genetic and Genetic Resources, 39: 533-543.

Cipriani G., Lot G., Huang W.G., Marrazzo M.T., Peterlunger E. and Testolin E. (1999): AC/GT and AG/CT microsatellite repeats in peach (*Prunus persica* (L.) Batsch): Isolation, characterisation and cross-species amplification in *Prunus.* Theoretical and Applied Genetics, 99: 65-72.

Choi C., Tao R. and Anderson R. (2002): Identification of self-incompatibility alleles and pollen incompatibility grops in sweet cherry by PCR based *S*-allele typing and controlled pollination. Euphytica, 123: 9-20.

Decroocq V., Hogel S., Fave M.G., Eyquart J.P. and Pierronnet A. (2004): Microsatellite markers in the hexaploid *Prunus domestica* apecies and using nuclear and chloroplast simple-sequence repeats. Molecular Breeding, 13: 135-142.

Dermen H. (1965): Colchiploidy and histological imbalance in triploid apple and pear. American Journal of Botany, 52: 353-359.

Dirlewanger E., Cosson P., Tavaud M., Aranzana M.J., Poizat C., Zanetto A., Arus P. and Laigret F. (2002): Development of microsatellite markers in peach (*Prunus persica* (L) Batsch.) and their use in genetic marker analysis in peach and sweet cherry (*Prunus avium* L.). Theoretical and Applied Genetics, 105: 127-138.

Doyle J. J. and Doyle J. L. (1990): Isolation of plant DNA from fresh tissue. Focus, 12, 13-15.

Ellis J. S. et al. (2011): Microsatellite standardization and evaluation of genotyping error in a large multi-partner research programme for conservation of Atlantic salmon (*Salmo Salar* L.). Genetica, 139: 353–367.

Gouta H., Ksia E., Buhner T., Moreno M.A., Zrrouk M., Mliki A. and Gogorcena Y. (2010): Assessment of genetic diversity and relatedness among Tunisian almond germplasm using SSR markers. Hereditas, 147: 283-292.

Grover A. and Sharma P.C. (2014): Development and use of molecular markers past and present. Critical Reviews in Biotechnology, 1-13.

Gupta P.K., Balyan H.S., Sharma P.C. and Ramesh B. (1996): Microsatellites in plants: a new class of molecular markers. Current Science, 70: 45-54.

Hormaza J.I. (2002): Molecular characterisation and similarity relationships among apricot (*Prunus armeniaca* L.) genotypes using simple sequence repeats. Theoretical and Applied Genetics, 104: 321-328.

Hortgro (2016): Key deciduous fruit statistics (2016): http://hortgro.co.za/wp-content/uploads/2017/06/HORTGRO-Key-Deciduous-Fruit-Statistics-2016.pdf (accessed: 21/08/2017).

Kalinowski S.T., Taper M.L. and Marshall T.C. (2007): Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16:1099-1106.

Klabunde G.H.F., Dablo M.A. and Nodari R.O. (2014): DNA fingerprinting of Japanese plum (*Prunus salicina*) cultivars based on microsatellite markers. Crop Breeding and Applied Biotechnology, 14: 139-145.

Martin C., Herrero M. and Hormaza J.I. (2011): Molecular characterisation of apricot germplasm from an old Stone collection. PLoS ONE 6: e23979.

Martinez-Gomez P., Arulsekar S., Potter D. and Gradziel T.M. (2003): An extended interspecific gene pool available to peach and almond breeding as characterised using simple sequence repeat (SSR) markers. Euphytica, 131: 313-322.

Mathias M., Sagredo B. and Kalazich J. (2007): Use of SSR markers to identify potato germplasm in the INTA Chile breeding programme. Chilean Journal of Agricultural Research, 67: 3-15.

Mnejja M., Garcia-Mas J., Howad W., Badenes M.L. and Arus P. (2004): Simple Sequence Repeat (SSR) markers of Japanese plum (*Prunus salicina* Lindl.) are highly polymorphic and transferable to peach and almond. Molecular Ecology, 4: 163-166.

Peakall R. and Smouse P.E. (2010): GENALEX 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537-2539.

Priyano and Putranto R.A. (2014): Molecular markers and their application for DNA fingerprinting and genetic diversity studies in Coffea species. Menara Perkebunan, 82: 39-50.

Potelwa Y., Moobi M. and Ntombela S. (2014): National Agricultural Marketing Council. South African Fruit Trade Flow, 15: 1-13.

Ranney T.G. (2006): Polyploidy: From evolution to new plant development. Combined Proceedings International Plant Propagators Society, 56: 137-142.

Rousset F. (2008): Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Molecular Ecology Resources 8:103-106.

Salanti G., Amountza G., Ntzani E.E. and Ioannidis J.P.A. (2005): Hardy-Weinberg equilibrium in genetic association studies: an empirical evaluation of reporting, deviations and power. European Journal of Human Genetics, 13: 840-848.

Sanchez-Perez R., Ruiz D., Dicenta F., Egae J. and Martinez-Gomez P. (2005): Application of simple sequence repeats (SSR) markers in apricot breeding: Molecular characterisation, protection and genetic relationships. Scientia Horticulturae, 103: 305-315.

Sehic J., Nybom H., Hjeltnes S.H. and Gasi F. (2015): DNA marker-assisted identification of *Prunus* accessions. Acta Horticulturae, 1101: 153-158.

Tamura K., Stecher G., Peterso D., Filipski A. and Kumar S. (2013): MEGA 6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30: 2725-2729.

Testolin R., Marrazzo T., Cipriani G., Quarta R., Verde I., Dettori M.T., Pancaldi M. and Sansavini S. (2000): Microsatellite DNA in peach (*Prunus persica* (L.) Batsch) and its use in fingerprinting and testing the origin of cultivars. Genome, 43: 512-520.

Tshabalala P.M. (2015): Estimating the economic rate of return to plum research investments in South Africa. www.arc.agric.za (accessed 23/03/2017)

Van Oosterhout C., Hutchinson W.F., Wills D.P.M and Shipley P. (2004): MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4: 535-538.

Viguera E., Canceill D. and Ehrlich S.D. (2001): Replication spillage involves DNA polymerase pausing and dissociation. The EMBO Journal, 20: 2587-2595.

Vorsa N and Johnson-Cicalese (2012): American Cranberry. In: Badenes M.L. and Byrne D.H. (eds), Fruit breeding. Springer, New York, pp 191-224.

Wang X.M., Wu W.L., Zhang C.H., Zhang Y.P., Li W.L. and Huang T. (2015): Analysis of the genetic diversity of beach plums by simple sequence repeat markers. Genetic and Molecular Research, 14: 9693-9702.

Weinberger J.H. (1975): Plums. In: Janick J. and Moore J.N. (eds), Advances in fruit breeding. Purdue University Press, West Lafayette, IN, pp. 336-347.

Wünsch A. and Hormaza J.I. (2002): Cultivar identification and genetic fingerprinting of temperate fruit tree species using DNA markers. Euphytica, 125: 56-67.

Zhang K. and Rosenberg N. A. (2007): On the genealogy of a duplicated microsatellite. Genetics, 177: 2109-2122.

Zhebentyayeva T.N., Reighard G.L., Gorina V.M. and Abbott A.G. (2003): Microsatellite (SSR) analysis for assessment of genetic variability in apricot. Theoratical and Applied Genetics, 106: 435-444.

Chapter 4:

Molecular characterisation of the self-incompatibility locus in apricots within the ARC's germplasm collection

Some sections of chapter 4 and 5 are similar, especially in the introduction and materials and methods but have been included in both chapters for completeness.

4.1. Introduction

Apricot (*P. armeniaca*) is a stone fruit belonging to the genus *Prunus* in the family Rosaceae. In South Africa, it is often grown for the processing industry. Some cultivars, including

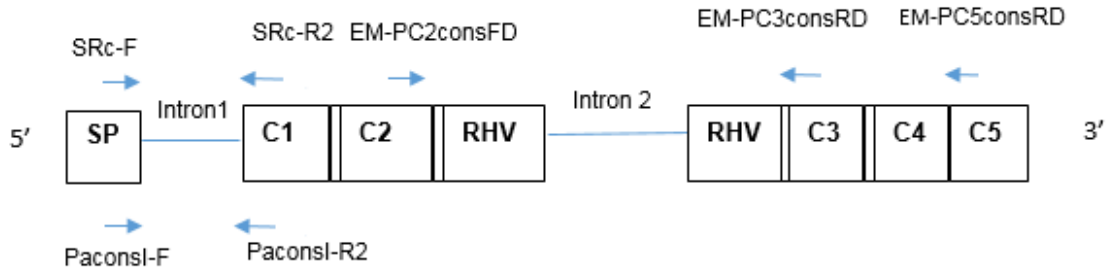
traditional South African cultivars, are self-compatible and can be grown conveniently in single cultivar orchards whereas others are self-incompatible and need to be planted with appropriate pollinator cultivars to set fruit. Knowledge of self-(in)compatibility relationships of apricot cultivars is therefore important to growers when planning an orchard and selecting cross-pollinators. This information is also useful to plant breeders designing breeding programmes.

Self-incompatibility (SI) in the Rosaceae is gametophytic (De Nettancourt, 2001) and is regulated by a single multi-allelic *S*-locus comprising at least two genes, one expressed in the style and one in the pollen. In the gametophytic incompatibility (GSI) system, incompatibility occurs when the *S*-allele of a pollen grain (haploid) coincides with either of the *S*-alleles of the style (diploid) (East and Mangelsdorf, 1925). In such a case the pollen tube growth is terminated in the style making fertilisation impossible. Two cultivars may be cross-incompatible, semi-compatible or fully compatible depending on whether they share two, one or zero alleles. Self-compatibility (SC) can result from mutations in the stylar or pollen parts of the locus so that there is no recognition or other loss of function, though there is also evidence of some non-*S* mutations that confer self-compatibility.

In *Prunus,* given the importance of the (in)compatibility system in breeding and production, understanding the mechanism of action of GSI, from a molecular genetic point of view, has received much attention. *Prunus* species have been reported to have at least two genes at the *S*-locus, one encoding a stylar glycoprotein with ribonuclease activity (*S-RNase*) (Bošković and Tobutt, 1996) and the other a cognate *S*-haplotype-specific-Fbox gene (*SFB*/*SLF*) (Entani *et al*., 2003; Ushijima *et al*., 2003; Romero *et al*., 2004). The *S-RNase* gene is expressed in the style and comprises a signal peptide (SP), five highly conserved regions (C1 to C5), a hyper-variable region (HVR) located between C2 and C3, and two introns (Sassa *et al*., 1997; Ushijima *et al*., 2003). Characterisation of *S-RNase* genotypes is often based on these two introns, which exhibit allele-specific length polymorphisms. The first intron, unique to *Prunus*, lies between the sequence responsible for secretion signal peptide and C1 region and the second intron is located between the C2 and C3 region in the HVR (Fig. 4.1) (Yamane *et al*., 2001). The second gene encodes the pollen *S*-haplotype-specific F-box gene (*SFB*/*SLF*) (Entani *et al*., 2003; Romero *et al*., 2004). The *SFB*/*SLF* gene is expressed in the pollen and contains one intron at the 5' end, an F-box motif and two hyper-variable regions, HVa and HVb at the C-terminus (Ikeda *et al*., 2004; Nunes *et al*., 2006) (Fig. 4.1). Romero *et al*. (2004) sequenced the *S*-locus region in three different apricot *S*-haplotypes and showed that the *S-RNase* and *SFB* genes were in very close proximity (with a physical distance of 2.9kb) allowing them to be inherited as a unit (Roalson and McCubbin, 2003). Several *S-RNase* and *SFB* protein gene pairs have been determined from *Prunus*: *P. salicina* (Beppu *et al*., 2003), *P.*

dulcis (Tao *et al*., 1997), *P. cerasus* (Yamane *et al*., 2001), *P. mume* (Entani *et al*., 2003) and *P. armeniaca* (Romero *et al*., 2004).

S-RNase gene



SFB/SLF gene

Fig. 4.1. Non-scale schematic structure of the two genes in the *S*-locus, *S-RNase* and *SFB*. Indicated are the annealing sites of consensus *S-RNase* and *SFB* primers, together with Signal Peptide (SP), intron regions, conserved regions (C1-C5) and hyper-variable regions (HVR) in the *S-RNase* gene; and the intron region, variable region (V1;V2) and hyper-variable region (HVa; HVb) in the *SFB* gene (Adapted from Halasz *et al*., 2010).

Initial studies concerned with *S*-genotyping focused on amplification of the second intron. The *S-RNase* gene-specific primer sets (Pru-C2, Pru-C4R and Pru-C5), amplifying the second intron, were designed from conserved regions of the *S-RNase* gene (Tao *et al*., 1999). The primers were useful for the *S*-genotyping of sour cherry (Yamane *et al*., 2003); Japanese apricot (Tao *et al*., 2000; Yaegaki *et al*., 2001) and Japanese plum (Beppu *et al*., 2002).

However these primers, designed from a small set of allele sequences from just one or two species, failed to amplify all alleles (Sutherland *et al*., 2004a). Sutherland *et al.* (2004a) hence designed degenerate consensus primers, flanking the second intron, from conserved regions of 27 *S-RNase* gene sequences from five *Prunus* species, which proved to be more broadly applicable.

Regarding the first intron, amplification products were small and therefore difficult to discriminate on agarose gel electrophoresis. Ortega *et al*. (2005) reported a more precise approach to determine and confirm SI genotypes in almond, through PCR amplification of the first intron alleles using a fluorescently labelled forward primer (PaconsI-F), designed from cherry, used in combination with a reverse primer (EM-PC1consRD) designed from 22 published sequences of *Prunus S-RNases*. Allele sizes of the first intron region were generally under 500 bp. Sonneveld *et al*. (2006) similarly identified the first intron *S*-alleles of sweet cherry cultivars using the same fluorescently labelled forward primer (PaconsI-F) in combination with a different reverse primer, PaconsI-R2. In apricot, first intron amplification has been done using a forward primer (SRc-F) (Romero *et al*. 2004), designed from the conserved regions of three different apricot *S*-alleles. Kodad *et al*. (2013) added a fluorescent label to the forward primer and successfully used it in combination with a reverse primer (SRc-R) designed by Vilanova *et al*. (2005) to identify first intron *S*-alleles in 55 Moroccan apricot cultivars.

It should be noted that in apricot, a 2 800 bp product of the second intron region is assigned to the *S8* allele, or haplotype, and to *Sc*, which is associated with self-compatibility (Alburquerque *et al*., 2002). According to Halasz *et al*. (2010), the *Sc* haplotype, which confers self-compatibility, results from a pollen part mutant of the self-incompatible *S8* haplotype with a 358 bp insertion in the *SFB* gene. Distinguishing between the self-compatible (*Sc*) and self-incompatible (*S8*) alleles can be accomplished through PCR amplification of the *SFB* allele, using allele-specific primers (*SFB*c/8) that result in products of approximately 500 bp in the case of the self-compatible (*Sc*) allele and 150 bp in the case of *S8* (Halasz *et al*., 2010).

In recent years, much progress has been made in identification via PCR of *S*-genotypes in apricot, often using primers developed in other *Prunus* species. Up to 30 self-incompatibility alleles (*S1* to *S30*) have been identified in apricot (Sutherland *et al*., 2004a; Zhang *et al*., 2008; Wu *et al*., 2009; Halasz *et al*., 2013). Self-compatibility (SC) is associated with a mutation on the *S8*-haplotype; thus conferring SC. In 2012, Halasz and co-workers reported on S-genotyping in a range of stone fruit, including 120 apricots using primers designed to amplify the first intron (Sonneveld *et al*., 2003) and the second intron (Sutherland *et al*., 2004a). Using

the Romero *et al*. (2004) and Sutherland *et al*. (2004a) primers to amplify the first and second intron, respectively, Kodad *et al*. (2013) identified S-genotypes of 55 Moroccan apricot (*P. armeniaca*) cultivars.

Little is known about the self-(in)compatibility genotypes of apricots grown in South Africa. The Agricultural Research Council (ARC) Institute at Infruitec-Nietvoorbij, located in the Western Cape, conducts apricot and plum breeding programmes, which supplies the industries. It holds a gene bank of accessions for use in breeding and the National reference collection, which contains most of the cultivars grown commercially in South Africa. There is a need to identify the self-(in)compatibility genotypes of the cultivars that are used in the breeding programme and/or are released to growers. In this study, the objective was to use appropriate fluorescently labelled primers for the first intron (Romero *et al*., 2004; Vilanova *et al*., 2005; Sonneveld *et al*., 2006), and for the second intron, non-labelled primers (Sutherland *et al*., 2004a) to determine the S-genotypes of accessions of the ARC apricot collections. This information will be useful to breeders when planning crosses and to growers planning orchards.

4.2 Materials and methods

4.2.1 Plant material

The plant material is grown at the ARC's Bien Donne Experimental Farm located in Groot Drakenstein, Western Cape, South Africa. It consists of 106 apricot accessions from the gene bank (SV8A) and 15 apricot from the stone fruit reference collection (BD10) (Table 3.2; Chapter 3). The 108 accessions, nearly all belonging to *P. armeniaca*, trees had generally been supplied by the South African Plant improvement Organisation (SAPO). The same plant material was also used in the fingerprinting chapter. The accessions and their locations are recorded in Table 3.1. The accessions were planted in sets of three trees, and if they were morphologically uniform, just one was sampled, usually the first. Occasionally there were two accessions of the same cultivar. These were distinguished as 1 and 2 if they were in the different orchards, as was the case with 12 accessions. However, if they were planted in the same orchard they were considered to be the same accessions and designated A and B.

4.2.2 DNA extraction

For the extraction of genomic DNA, young leaves of the accessions were collected in spring and stored in a -80^{o} freezer, until used. DNA was extracted using the cetyltrimethyl ammonium

bromide (CTAB) method described by Doyle and Doyle (1990), with slight modifications. Three leaf discs were put in a 2 ml Eppendorf tube with 3 ball bearings of 3 mm size, 400 µl of CTAB extraction buffer (heated to 65°C) and 4 µl β-Mercapto-ethanol (Sigma Aldrich) was added to each tube within a fume hood. Samples were initially mixed by inversion. The leaves were homogenised in a Tissuelyser II ball mill (Qiagen) at 30Hz for 4 min; leaves that were not completely homogenised were run for an additional 2 min. This step was followed by incubation of the tubes in a 60°C water-bath for 1 hour, with inversion every 10 to 20 min. After the removal of the ball bearings with a magnet, 400 µl of chloroform-isoamyl alcohol (24:1 ratio) was added, mixed by inverting and centrifuged at 13 500 rpm for 15 min using a centrifuge (Labnet). The top aqueous phase was aliquoted into a new 2 ml Eppendorf tube, 400 µl of chloroform-isoamyl alcohol (24:1 ratio) was again added and the samples were then centrifuged at 13 500 rpm for 10 min. The top aqueous phase was transferred a final time into a new Eppendorf tube and precipitated with 320 µl cold isopropanol overnight in a refrigerator. After overnight incubation, the samples were centrifuged for 15 min at 13 500 rpm and the supernatant was discarded; the pellet was then washed with 70% cold ethanol. The ethanol was removed and the pellet was dried for 45 min, re-suspended in TE buffer and left in the refrigerator until required.

The quality and quantity of DNA was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) according to the manufacturer's instructions. If samples showed poor quality and quantity, extractions were repeated. Good quality DNA (with A260/A280 ratio between 1.8 and 2.0) was then diluted to a concentration of approximately 20-30 ng/µl and the samples were stored at -20°C until further use to minimise degradation of the DNA.

4.2.3 Primers amplifying the S-RNase *gene and* SFBSc/8 *allele of the S-locus*

Pairs of consensus primers flanking the first or second intron of the *S-RNase* gene, as well as allele-specific primers for the *SFBSc/8* allele, were used in this study (Table 4.1; Fig. 4.1).

To amplify the first intron, the consensus forward primer PaConsI-F (Sonneveld *et al*., 2003) designed from the region coding for the signal peptide region and labelled with a HEX fluorescent dye, was used in combination with reverse primer PaConsI-R2 (Sonneveld *et al*., 2006) designed from the C1 region of the DNA sequences of cherry *S-RNase* alleles. Reports of application of these primers in apricot is minimal; therefore to confirm efficiency and correspondence, another primer set previously used for first intron amplification in apricot (SRc-F and SRc-R), and also fluorescently labelled (Romero *et al*., 2004; Vilanova *et al*., 2005, respectively) was used.

To amplify the second intron, the consensus primers, amplifying the second intron of the *S-RNase* gene, designed from the second (C2), third (C3) and fifth (C5) conserved regions of 27 *S-RNase* allele sequences of five *Prunus* species (Sutherland *et al*., 2004a) were used in the following two combinations: EM-PC2consFD + EM-PC3consRD; and EM-PC2consFD + EM-PC5consRD. However, the primer combination, amplifying the second intron, designed from the conserved regions C2 + C5 (EM-PC2cons-FD + EM-PC5consRD) displayed poorer amplification in most apricot accessions, showing non-specific amplification and in some case no amplification; therefore the results for this primer pair are not reported.

Additionally, to distinguish between the *Sc* and *S8* alleles, which have identical *S-RNase* haplotypes but differ with respect to the *SFB*, a fluorescently labelled primer pair specific for *SFB*c/*SFB*8 (AprFBC8-F + AprFBC8-R) (Halasz *et al*., 2010) was used.

Table 4.1. Sequences of primers, designed previously to amplify first or second introns of the *Prunus S-RNase* gene, and to distinguish SFBs of *Sc* and *S8.*

Primer	Target region	Sequence	Ta^{o}	Reference
PaConsI-F	1st intron	5'-(c/a)ct tgt tct tg(c/g) ttt (t/c)gc ttt ctt c-3'	54	Sonneveld *et al*., 2003
PaConsI-R2	1st intron	5'-gcc att gtt gca caa att ga-3'	54	Sonneveld *et al*., 2006
SRc-F	1st intron	5'-ctc gct ttc ctt gtt ctt gc-3'	54	Romero *et al*., 2004
SRc-R	1st intron	5'-ggc cat tgt tgc aca aat tg-3'	54	Vilanova *et al*., 2005
EM-PC2consFD	2nd intron	5'-tca cma tyc atg gcc tat gg-3'	58	Sutherland *et al*., 2004a
EM-PC3consRD	2nd intron	5'-aws trc crf gyt tgt tcc att c-3'	58	Sutherland *et al*., 2004a
EM-PC5consRD	2nd intron	5'-caa aat acc act tca tgt aac ar-3'	58	Sutherland *et al*., 2004a
AprFBC8-F	*SFBSc/S8*	5'-cat gga aaa agc tga ctt atg g-3'	55	Halasz *et al*., 2010
AprFBC8-R	*SFBSc/S8*	5'-gcc tct aat gtc atc tac tct tag-3	55	Halasz *et al*., 2010

Ta^{o} – annealing temperature

4.2.4 S-RNase *genotyping*

4.2.4.1 PCR amplification of first intron

PCR amplification was conducted following a procedure described by Sonneveld *et al*. (2003). Genomic DNA of approximately 20-30 ng/µl (2 µl) was used in a 25 µl reaction, which contained 5 µl of 1X PCR buffer (Promega), a final concentration of 2.5 mM $MgCl_2$ (2.5 µl), 0.2 mM dNTPs (0.5 µl), 0.2 µM of each of the two primers (0.5 µl), 0.625 U of *Taq* DNA polymerase (0.125 µl) (Promega) and 13.875 µl of dH_2O. PCR amplification was carried out in the following conditions: 2 min at 94^{o}C, followed by 35 cycles of 1 min at 94^{o}C, 1 min at 54^{o}C and 1 min at 72^{o}C, and a 5 min final extension at 72^{o}C. The PCR was set up in ice, then

transferred to a GENEAMP (Applied Biosystems) or a G-storm (G-Storm Direct) thermal cycler.

The first subset of PCR products was resolved by 1.5% (m/v) agarose gel electrophoresis (Conda laboratory) at 80 V for 90 min. To verify amplification, the products were electrophoresed with 2 µl of 6X loading dye (Fermentas) against a 1 kb ladder (Fermentas) in a 1% (m/v) TBE buffer (Tris-Boric Acid-EDTA). Upon verification, the full set of the first intron amplification products, all smaller than 500 bp, were separated by capillary electrophoresis on a 3130 DNA capillary analyser (Applied Biosystems) at the Central Analytical Facility's DNA sequencing unit at Stellenbosch University. Sizes of peaks were estimated in comparison with the internal standard GS500(-250)LIZ (Applied Biosystems) and data were captured in Excel. GENEMAPPER software version 5.0 (Applied Biosystems) was used to visualise the peaks and score the alleles. Data were checked and allele scores were compared and verified with previously reported allele sizes to compile provisional genotypes (Table 4.2).

4.2.4.2 PCR amplification of second intron

To amplify the second intron, a protocol described by Sutherland *et al*. (2004a) was followed. In a 20 µl volume the following were added: 20-30 ng/µl DNA (2 µl), 4 µl of 1X PCR buffer, 2 mM $MgCl_2$ (2 µl), 0.2 mM dNTPs (0.4 µl), 2 µl of Q solution, 0.5 U of *Taq* DNA polymerase (0.14 µl), 0.3 µM of each of the two primers (0.6 µl) and 8.25 µl of dH_2O. PCR conditions were set up as follows: initial denaturation for 2 min at 94°C and 35 cycles of 94°C for 10 s, 58°C for 2 min and 68°C for 10 s, with an increment of 10 s per cycle on the extension step after the 10^{th} cycle. The PCR was set up in ice and centrifuged before running the samples on a GENEAMP thermal cycler.

Amplified products were resolved using gel electrophoresis on a 1.5% TBE buffer agarose gel at 70 V for 2 h and stained using ethidium bromide. Usually this separation technique is useful for the separation of products larger than 500 bp that cannot be analysed with the standard set-up on an 3130 DNA capillary analyser. Provisional identification of bands was done by comparison to previously published *S*-allele product sizes using a 1 kb plus ladder (Fermentas) (Table 4.1).

4.2.4.3 PCR amplification of *SFBSc/S8* alleles

To distinguish between the *Sc* and *S8* alleles, primers amplifying the *SFBc/8* allele were used for accessions showing the *Sc*/*S8* peak and band. The protocol described by Halasz *et al.* (2010) was followed: 30 ng/µl of genomic DNA (2 µl) was used for PCR amplification in a 25 µl reaction volume. Each (25 µl) reaction contained 2.5 µl of Tris-HCl containing KCl (10nm)

(pH 8.3) (Thermo Scientific), 1.5 mM of $MgCl_2$ (1.5 µl), 10 mM of dNTP's (0.5 µl), 0.4 µM of each of the two primers (1 µl), 0.625 U of *Taq* DNA polymerase (0.125 µl) and 15.25 µl of dH_2O. The PCR amplification was carried out with the following temperature profile: an initial denaturation of 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 1.5 min and 72°C for 2 min, and a final extension of 72°C for 5 min. The PCR was prepared on ice and run on a GENEAMP thermal cycler.

A subset of PCR products were first separated on 2% TBE agarose gel (stained with ethidium bromide) at 100 V for 2 h. The labelling of the forward primer with a PET dye allowed more precise identification of allele sizes when analysed by capillary electrophoresis. Upon confirmation of amplification products, the selected subset of amplification products were run on a 3130 DNA capillary analyser at the Central Analytical Facility's DNA sequencing unit at Stellenbosch University. Scoring of peaks was estimated in comparison with the internal size standard, GS500(-250)LIZ. GENEMAPPER software version 5.0 was used to visualise the peaks and score the alleles. The data were checked by an overseer to ensure accuracy.

4.2.4.4 Reference *S*-alleles

A number of studies have used primers amplifying the first or second introns to determine the *S*-genotypes of apricots. The reported sizes previously published of known *S*-alleles were used as references to facilitate the correlation of data, *i.e.* bands and peaks (Table 4.2). First intron amplification has previously been reported using SRc-F + SRc-R by Halasz *et al*. (2010; 2013) in apricots. The primers PaconsI-F + PaconsI-R2, recently used to amplify the first intron in cherry (Sonneveld *et al*., 2006), has not previously been used in apricot. Therefore, identification and correlation of first intron amplification products using these primers were based on observation of patterns when combining information from the SRc (first intron) and EM-PC (second intron) primers (Table 4.2). Second intron amplification in apricot has previously been carried out with several primer pairs: EM-PC2cons-FD + EM-PC3consRD (Sutherland *et al*., 2004a; Halasz *et al*., 2013; Kodad *et al*., 2013); PruC2-F + PruC4-R and PruC2-F + PruC5-R (Zhang *et al*., 2008); and Pru-C2-F, PCE-R + Amy-C5-R (Wu *et al*., 2009).

Table 4.2. Previously reported first and second intron amplification product sizes (~30) of apricot *S*-alleles, used as reference alleles for scoring of amplification products in the current study.

	1st intron (bp) (Fluorescently detected)			2nd intron product sizes (bp) (EM-PC2cons-FD+EM-PC3consRD) (detected using gel electrophoresis)			2nd intron (bp) (PruC2+PruC4R; AS1II+AmyC5R)	2nd intron (bp) (PruC2, PCE-R + Amy-C5)
***S*-allele**	**Halasz *et al*., 2010; 2013 (SRc-F+SRc-R)**	**Current study (SRc-F+SRc-R)**	**Current study (PaconsI-F+PaconsI-R2)**	**Sutherland *et al*., 2004a**	**Halasz *et al*., 2013**	**Kodad *et al*., 2013**	**Zhang *et al*., 2008**	**Wu *et al*., 2009**
Sc/8	355	355	347	2800	2800	2800	-	-
S1	-	413	404	2000	-	-	-	-
S2 *	332	332	na	850!	900	950!	-	-
S3	269	na	na	245‡	310‡	-	-	-
S4 *	-	324	na	310‡	-	-	-	-
S5	-	238	229	1200	-	-	-	-
S6 §	424	na	415	1300	1300	1300	-	-
S7	401	402	393	-	820!	750!	-	-
S8	see above	na	na	-	-	-	-	-
S9	203*	203	194	-	500!	-	658!	-
S10	-	na	na	-	-	-	266	-
S11	305	305	295	-	1700†	1500†	464†	-
S12	262*	na	na	-	-	-	360	-
S13	379	379	370	-	1250†	1350†	401†	-
S14	-	na	na	-	-	-	492	-
S15	-	na	na	-	-	-	469	-
S16	-	na	na	-	-	-	481	-
S17	-	na	na	-	-	-	487	-
S18	-	398	389	-	-	-	1337	-

S19 *	334	337	na	-	-	-	546	-
S20	222	na	na	-	500	-	1934	-
S21	-	na	na	-	-	-	-	-
S23	-	na	na	-	-	-	-	672
S24	-	na	na	-	-	-	-	588
S25	-	na	na	-	-	-	-	994
S26	-	na	na	-	-	-	-	453
S27	-	na	na	-	-	-	-	397
S28	-	Na	na	-	-	-	-	1353
S29	-	Na	na	-	-	-	-	452
S30	-	Na	na	-	-	-	-	1123
Sx	343	Na	na	-	-	-	-	-
Sy	236	Na	na	-	-	-	-	-

* Interpretation of *S*-alleles amplified with SRc-F&R primers and not PaconsI-F + R2 in current study. † significant variation of reported allele sizes. ‡ inconsistent scoring of alleles: Sutherland *et al*. (2004a) S3~245, S4~310; Halasz *et al*. (2013) scored S3~310. § Interpretation of *S*-alleles amplified with PaconsI-F&R but not SRc-F&R in current study. ! Minor discrepancies – allele sizes vary between publications with ~50bp difference. na: no amplification obtained; -: no amplification reported

4.2.4.5 Apricot sequence alignments

In an attempt to investigate primer binding regions, published sequences of apricot *S-RNase* alleles were aligned. Conserved (C1-C5) and hyper-variable regions, deduced from amino acid sequences, were identified using the CLUSTAL OMEGA online version method (using standard parameters).

Published nucleic acid sequences of apricot *S*-alleles were obtained from NCBI BLAST (www.ncbi.nlm.nih.gov), in refence to Gharesheikhbayat (2010). The following bioinformatics programs were applied to conduct sequence alignments:

The DNA sequences of the *S*-alleles obtained from NCBI contained intron regions, which made alignment difficult. Thus, the sequences were first converted to mRNA sequences to remove the introns, using the SPIDEY tool on NCBI: http://www.ncbi.nlm.nih.gov/spidey/. The mRNA sequences were further translated to protein sequence using the EXPASY online software: http://web.expasy.org/translate/. Sequence alignment analysis for protein sequences was conducted on CLUSTALX: http://www.ebi.ac.uk/Tools/msa/clustalo/.

4.3 Results

4.3.1 S-genotype verification

In general, the primer combinations used for the second intron amplified one or two primary bands (see appendix A). In some cases, shadow bands were observed but only the two main bands were taken into account. Unexpectedly, more than two bands were observed in the apricot accessions ‘Bergarouge’ and ‘Soledane’.

S-alleles were scored initially on the basis of correlation with previously published second intron *S*-alleles and the first intron *S*-alleles were then deduced from second intron products. However for data presentation, first intron data is presented before second intron data.

The *S*-genotypes of the nine apricot for which genotypes have previously been reported (Table 4.3), and were compared to calibrate scoring of alleles. Amongst the ARC accessions, *S*-alleles of five cultivars matched at least one of the reported allele and four apricot cultivars were known to be self-compatible (Table 4.3). The observed genotypes were not completely consistent with reported *S*-genotypes; some of the cultivars showed one of the reported alleles, some amplified completely different alleles and other cultivars had the expected / reported genotypes. The cultivars that were known to be self-compatible all showed amplification of the *Sc/S8* allele, which confirms their self-compatibility phenotype (Table 4.4).

Table 4.3. Comparison of a set of ARC apricot cultivars with previously reported *S*-genotypes.

Apricot cultivars	**Reported genotype**	**Reference**	**Observed genotype**
Bulida	*S2*	Donoso *et al*., 2009	*S2Sc*
Cape Bebeco	*S6Sc*	Donoso *et al*., 2009	*S6Sc*
Goldrich	*S1S2*	Zuriaga *et al*., 2013	*S2Sc*
Kathy	*S1S2*	Zuriaga *et al*., 2013	*S1Sc*
Palsteyn	*Sc*	Donoso *et al*., 2009	*ScSc*
SC apricot cultivars			**Observed genotype**
Peeka	Self-compatible	Horstmann, pc	*ScS19*
Royal	Self-compatible	Horstmann, pc	*S1Sc*
Royal (RC)	Self-compatible	Horstmann, pc	*S19Sc*
Soldonne	Self-compatible	Horstmann, pc	*S2Sc*

pc - personal communication

4.3.2 Identification of S-genotypes in apricot

Identification of *S*-genotypes was attempted in 69 cultivars and 50 selections of apricot (*P. armeniaca*). Amplification of the second intron alleles using the primers EM-PC2cons-FD + EM-PC3cons-RD and EM-PC2cons5-RD was generally successful, although its efficiency was variable. The primer combination designed from the C2 + C3 conserved regions (EM-PC2cons-FD + EM-PC3cons-RD) showed good amplification in apricots. PCR products with sizes not corresponding to those reported by references in table 4.2 were designated alphabetically. Amplification of first intron products with the two primer pairs did not always correspond. Some alleles were only amplified with one of the two primer pair; some were amplified with both primer sets and in some cases neither of the primer pairs. The SRc primers designed in apricot (Romero *et al*., 2004; Vilanova *et al*., 2005) proved to be useful in identifying 11 *S*-alleles. The additional primer pair (PaconsI-FD + PaconsI-RD), previously used in almond (Ortega *et al*., 2006), was used for the first time to amplify first intron products in apricot . Amplification products observed with this primer pair also provided useful information, up to 10 *S*-alleles, despite not being used previously for first intron amplification in apricot.

Table 4.4. Apricot accessions *S*-genotyped in this study, through PCR amplification of the first and second introns of the *S-RNase* gene, using a set of consensus primers as well as allele-specific primers. Identified *S*-alleles correlated with previously reported *S*-alleles according to band sizes (bp).

Accessions	**1st intron products (bp)**	**1st intron products (bp)**	**2nd intron products (bp)**	**SFB product in accessions showing *Sc/S8***	**Deduced S-genotypes**
Selections	**SRc-F+SRc-R**	**Pacons-F+ Pacons-F R2**	**EM-PC2consFD+ EM-PC3consRD**	**AprSFBSc/8**	
1002A	332, 355	347	900, 2 800	510	*S2Sc*
1002B	203, 355	194, 347	500, 2 800	150	*S9S8*
1003A	355	347	2 800	510	*Sc*
1004B	355, 402	347	820, 2 800	510	*S7Sc*
1005A	355	347	2 800	510	*Sc*
1006B	355	347	2 800	150	*S8*
1007A	355	347	2 800	510	*Sc*
1007B	379	370	1 350, 2 000		*S13(S1)*
1008A	238	229, 415	1 200, 1 300		*S5S6*
1012A	203, 332	194	500, 900		*S9S2*
1013B	402	415	820, 1 300		*S7S6*
1017B	203, 305	295	500, 1 700		*S9S11*
1018B	332	415	900, 1 300		*S2S6*
1020A	332, 355	347	900, 2 800	510	*S2Sc*
1020B †	n.a	n.a	900, 1 300		*(S2S6)*
1023B	203	n.a	500, 1 300		*S9(S6)*
1027B	355	347	2 000, 2 800	510	*(S1)Sc*
1028Ba	355, 391	347	2 800	510	*Sc*
1028Bb †	n.a	n.a	2 000, 1 300		*(S1S6)*
1030A	355	347	2 800	510	*Sc*
1031A	n.a	415	2 000		*(S1)*
1031B	355	347	2 800	510	*Sc*
1031B	402	415	820, 1 300		*S7S6*
1032B	332, 355	347	900, 2 800	510	*S2Sc*
1037A	355	347	2 800	510	*Sc*
1038A	355	347	2 800	510	*Sc*
118-LE-133	413	404	2 000, 690		*S1(S(a))*
12D-10-15	332,377,402	393	900, 2 200		*S2S(d)*
12D-31- 5	332, 402	393	900, 2 200		*S2S(d)*
12D-39- 5	402	393	820		*S7*
15-ED-103	355	347	2 800	510	*Sc*
20A-8-1	355	347	2 000, 2 800	510	*(S1)Sc*
20C-24-20	355, 402	347	820, 2 800	510	*S7Sc*
3B- 9-25	355, 402	347	2 200, 2 800		*S(d)Sc*
4A/3/36	n.a	415	1 300		*S6*
4A/3/39	n.a	415	2 000, 1 300		*(S1)S6*

77-LA-579	332, 355	347	900, 2 800	510	*S2Sc*
8A-10-14	332, 355	347	900, 2 800	510	*S2Sc*
AP93-9 *	332, 402	347	900, 2 200		*S2S(d)*
AP94-10 *	355	347	2 000, 2 800	510	*(S1)Sc*
AP94-34 *	355	347	2 000, 2 800	510	*(S1)Sc*
AP95-56 *	402	n.a	1 300, 2 200		*(S6)S(d)*
Clone 0.11	324, 332	322	310, 690		*S4(S(a))*
Dof 1-A-586	332, 402	393	900, 2 200		*S2S(d)*
LE 3241 (a)	n.a	347,415	2 800	510	*Sc*
LE 3241 (b)	n.a	347	820, 1 350		*(S7S13)*
NG3	270, 355	347	260, 2 800	510	*S10Sc*
Ng5	402	393, 415	1 300, 2 200		*S6S(d)*
Nja-19	332, 355	347	900, 2 800	510	*S2Sc*
S5A- 2- 4	332, 402	393	900		*S2*
Cultivars					
Alpha	402	393	2 200		*S1*
Alpha (RC)	332		900, 2 000		*S2(S1)*
Anshu	371		490, 1 300		*S14(S6)*
Atricot (RC)	332, 355	347	900, 2 800	510	*S2Sc*
Autumn Glo	355		2 200,2 800	510	*(S(d))Sc*
Barracca	355	347	2 800	510	*Sc*
Bergarouge	332, 355	347	900, 1 300, 2 200, 2 800	510	*S2(S6)Sc*
Bulida (RC)	332, 355		900, 2 800	510	*S2Sc*
Cape Bebeco	355	347, 415	1 300, 2 800	510	*S6Sc*
Cape Bebeco (RC)	355		1 300, 2 800	510	*(S6)Sc*
Castleton	332, 402	393	900, 2 200		*S2S(d)*
Charisma *	355, 402	347	820, 2 800	510	*S7Sc*
Charisma (RC)	355, 398		820, 2 800	510	*(S7)Sc*
Comedie (a)	332, 355	347	900, 2 800	510	*S2Sc*
Comedie (b)	332, 355	347	900, 2 800	510	*S2Sc*
Early Baidy	355	347	2 800	510	*Sc*
Fantasme avikour	355	347	2 800	510	*Sc*
Frater	391	n.a	1 300, 1 350		*(S6S13)*
Gessekai	355	347	2 000, 2 800	510	*(S1)Sc*
Giada	332,402	393	900		*S2*
Glada (RS)	381	207,370	510, 2 200		*(S(c)S(d))*
Goldrich	332, 402	393	900, 2 200		*(S(d)S2*
Goldstrike	332, 413	404	690, 900		*(S(a))S2*
Grandir (RC)	337,402	393	1 980, 2 200		*S19S(d)*
Guillia (Giulia)	355, 402	347	2 800	510	*Sc*
Heiwa	376, 413	367	1 500, 2 000		*S(b)S1*
Icapl	332,402	393	900		*S2(S(d))*
Ivresse	381	207, 370	510, 2 200		*(S(c)S(d))*
Junshiro Los	371,413	362	490, 2 000		*S14S1*

Kathy	355	347	2 000, 2 800	510	*(S1)Sc*
Ken Psar	324,332	322	310, 690		*S4(S(a))*
Ladisun *	332,355	347	900, 2 800	510	*S2Sc*
Ladisun (RC)	332, 355	347	900, 2 800	510	*S2Sc*
Malan Royal *	355	347	2 000, 2 800	510	*(S1)Sc*
Malice	355, 402	347	820, 2 800	510	*S7Sc*
Moorpark	n.a	347	900, 2 800	510	*(S2)Sc*
Moorpark nuwe	332	n.a	900, 1 300		*S2(S6)*
Olimp	305	212,295	1 700, 2 700		*S11S(e)*
Palsteyn *	355	347, 393	2 800, 2 200	510	*ScS(d)*
Palsteyn (RC)	355	n.a	2 800	510	*Sc*
Peeka *	355	347	2 800	510	*Sc*
Peeka (RC)	337, 355	347	2 800	510	*Sc*
Pu Sha Shin	381, 398	389	490, 1 337		*S14S18*
Real de Timola	355	347	2 800	510	*Sc*
Rouge de Fournes	355, 413	347	2 000, 2 800	510	*S1Sc*
Royal	355	347	2 000, 2 800	510	*(S1)Sc*
Royal (RC)	337, 355	n.a	2 000, 2 800	510	*(S1)Sc*
Royal Blenheim	355,338	347	2 800	510	*Sc*
Royal FR	355	347	2 000, 2 800	510	*(S1)Sc*
Sancastresse	355, 402	347	2 800	510	*Sc*
Satungsky	355	347	2 000, 2 800	510	*(S1)Sc*
Satunska (RS)	381	207, 370	510, 2 200		*(S(c)S(d))*
Shimizugou (RS)	381	207, 370	510, 2 200		*(S(c)S(d))*
Soldonné	332,355	347	900, 2 800	510	*S2Sc*
Soldonne (RC)	332, 355	n.a	900, 2 800	510	*S2Sc*
Soledane	n.a	347	900, 1 350, 2 800	510	*(S2S13)Sc*
Suaprieight	355, 402	347	2 200, 2 800	510	*ScS(d)*
Suaprieight (RC)	355, 402	n.a	2 200, 2 800	510	*ScS(d)*
Suaprinine (RC)	402	393	2 000, 2 200		*(S1)S(d)*
Suapriseven	193, 355	n.a	2 800	510	*Sc*
Suapriseven (RC)	355, 402	347	2 200, 2 800,	510	*ScS(d)*
Suapritwo	355	347	2 000, 2 800	510	*(S1)Sc*
Super Gold *	355,402	347, 393	2 200, 2 800	510	*(S(d))Sc*
Supergold (RC)	355, 402	n.a	2 800	510	*Sc*
T.B. Therma	355	347	2 000, 2800	510	*(S1)Sc*
Travatt	332, 355	347	900, 2 800	510	*S2Sc*
Trigems	402	393	2 000, 2 200		*(S1)S(d)*
Vesna (RS)	381	207, 370	510, 2 200		*(S(c)S(d))*
Zinostojky	337, 355	347, 327	1 200, 2 800	510	*(S5)Sc*

Note: * ARC developed cultivars and selections. † indicates no first intron amplification observed. () indicates preliminary genotypes. (RC) indicates accessions from reference collection. (RS) indicated rootstocks.

Based on the results presented in table 4.4, a summary of the observed first and second intron product sizes and subsequently deduced *S*-alleles is presented in table 4.5.

Table 4.5. Correlation of first and second intron amplification product sizes observed in the current study in order to deduce apricot *S*-alleles; PCR products were amplified using Sutherland *et al*., 2004 (Su.) (EM-PC2consFD + EM-PC3consRD), Vilanova *et al*., 2005 and Romero *et al.*, 2004 (V. & R.) (SRc-F + SRc-R) and Sonneveld *et al*., 2005 (S.) primers (PaconsI-F + PaconsI-R2).

***S*-alleles**	**1st intron (bp) (SRc-F+SRc-R) (V. & R.)**	**1st intron (bp) (PaconsI-F +PaconsI-R2) (S.)**	**2nd intron (bp) (EM-PC2consFD +EM-PC3consRD) (Su.)**	**Notes**
Sc/8	355	347	2 800	
S1	413	404	2 000	
S2	332	n.a	900	
S4	324	n.a	310	Halasz *et al*. (2007) reported 320 bp fragment to be *S3*
S5	238/337	229/327	1 200	
S6	n.a	415	1 300	
S7	402	393	820/2200	Two second intron products could share the same size first intron product. See discussion.
S9	203	194	500	Zhang *et al*. (2008) reported *S9* as 659 bp
S10	270	n.a	260	
S11	305	295	1 700	
S13	379	370	1 350	Zhang *et al*. (2008) reported as 401 bp
S14	371/ 381	362	490	Two previously unreported first intron products
S18	398	389	1 337	
S19	337	n.a	1 980	Halasz *et al*. (2010) reported as 500 bp
S(a)	332/413	322	690	
S(b)	376	367	1 500	
S(c)			510	
S(d)		393	2 200	
S(e)		212	2 700	

Note: n.a indicates no amplification.

4.3.2.1 First intron amplification in apricot

Both SRC and Pacons primer sets were useful in identifying of *S*-genotypes in apricot (Table 4.4). However the product sizes between the two primer sets differed from one another by eight or nine base pairs, most likely due to the positioning of the primers along the sequence.

The SRc-F & SRc-R primer pairs

In apricot, amplification products of the first intron amplified with the SRc primers ranged between 203 bp and 413 bp (Table 4.5). The *Sc/8* allele that corresponds with a product size of 355 bp was present in 69 accessions. Eleven product sizes corresponding to 11 S-alleles were amplified in this study, namely: *S1*; *S2*; *S4*; *S5*; *S9*, *S10*; *S11*; *S13*; *S18*; *S19* and *Sc/8.* Two product sizes not corresponding with any of the published *S*-alleles were recorded in Table 4.5. When amplifying with the SRc primers, the *Sc/8* (355) allele was the most common allele, followed by the *S2* allele (332 bp). The least common *S*-alleles were *S10*, *S13*, *S18* and *S19*, each amplified in 1 accession. In the case of each of the other alleles, no more than 5 accessions showed amplification for each *S*-allele.

The PaconsI-F + PaconsI-R2 primer pairs

In order to deduce first intron products amplified using the PaconsI-F + R2 primers, product sizes were compared with both the first intron products that were amplified with the SRc primers and with the second intron products. Amplification products of the first intron ranged from 194 bp to 415 bp (Table 4.5; Figure 4.2). The *Sc/8* allele that corresponds with a product size of 347 bp was present in 63 accessions. Ten product sizes corresponding to ten *S*-alleles were amplified in this study, namely: *S1*; *S5; S6*; *S7*, *S9*; *S11*; *S13*; *S14*; *S18* and *Sc/8.* Four product sizes not corresponding with any of the published *S*-alleles were recorded in Table 4.5. When amplifying with the Pacons primers, the *Sc/8* (347) allele was the most common allele, followed by an unpublished product size *S(d)* allele (332 bp). The least common *S*-alleles were *S1*, *S13*, *S14* and *S18*, each amplified in 1 accession.

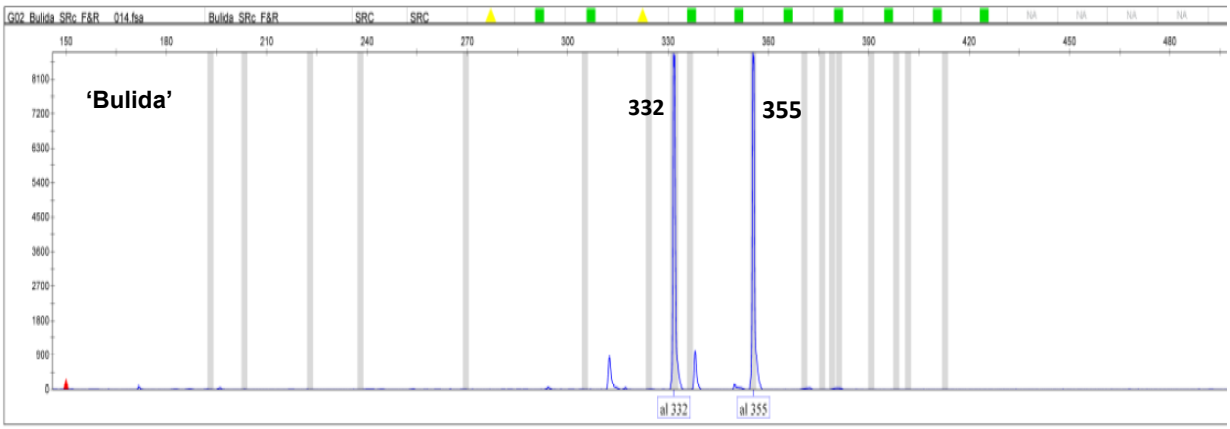


Fig 4.2. GENEMAPPER output of first intron products amplified using the SRC primers, for the ARC reference collection cultivar 'Bulida' (*S2Sc*). Similar output was observed in other accessions.

4.3.2.2 Second intron amplification in apricot

Primers flanking the second intron yielded PCR products ranging between 260 bp to 2 800 bp (Table 4.5) (See appendix A). In 88 accessions, two bands were observed and in 30 accessions a single band was observed (Figure 4.3). The *Sc/8* allele that corresponds with a product size of 2 800 bp was present in 71 accessions, indicating that it was the most frequent, followed by the *S1* allele present in 22 accessions. Fourteen *S*-alleles were amplified namely: *S1*; *S2*; *S4*; *S5*; *S6*; *S7*; *S10*; *S11*; *S13*; *S14*; *S18*; *S19*; *S20*/*S9*.

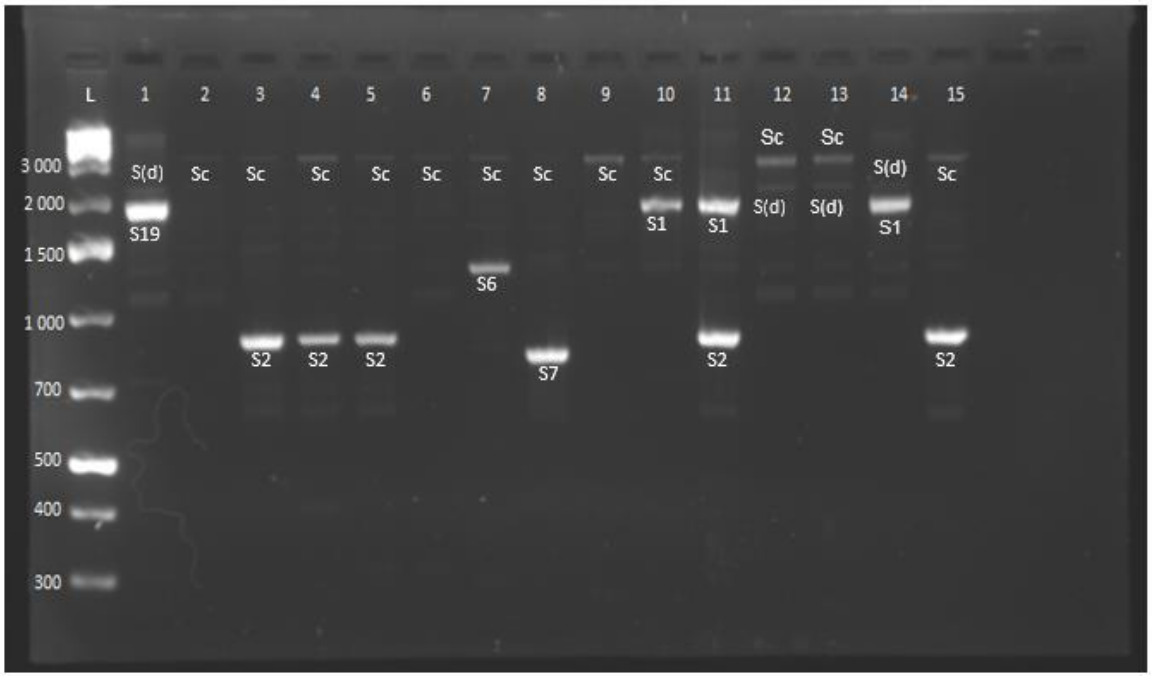


Fig 4.3. Second intron products from apricot reference collection loaded as (from the left): ladder (L), 1-Grandir (*S19 S(d)*), 2- Supergold (*Sc*), 3- Soldonne (*S2Sc*), 4- Bulida (*S2Sc*), 5- Ladisun (*S2Sc*), 6- Paletyn (*Sc*), 7- Cape bebeco (*S6Sc*), 8- Charisma (*S7Sc*), 9- Peeka (*Sc*), 10- Royal (*S1Sc*), 11- Alpha (*S1S2*), 12- Suapriseven (*S(d)Sc*), 13- Suaprieight (*S(d)Sc*), 14- Suaprinine (*S1S(d)*), 15- Atricot (*S2Sc*).

4.3.2.3 Inconsistencies with amplification of first and second intron products

Overall, out of the 30 previously published alleles, 14 alleles were amplified in the current study using first intron primers. However, there were some inconsistencies regarding first intron amplification. Although the *S6* allele was amplified using the Pacons primers, there was no amplification with the SRc primers. Similarly, the SRc primers amplified *S2, S4, S10* and *S19* alleles; however there was no amplification of these alleles using the Pacons primers.

The *S1*, *S6*, *S7* and *S14* alleles showed some inconsistent amplification. In 19 accessions, second intron amplification resulted in a PCR product of approximately 2 000 bp, previously

reported to be the *S1* allele. First intron amplification of the *S1* allele showed inconsistent amplification, with peak sizes of 413 bp and 404 bp amplified using the SRc and Pacons primer pairs, respectively. Only two accessions showed both alleles when amplified using both primer pairs. However, in three accessions only the 413 bp product was amplified using the SRc primers and failed to amplify using the Pacons primers. The second intron amplification of the *S6* allele yielded a product size of approximately 1 300 bp in 17 accessions. No amplification corresponding to the *S6* allele was observed with the SRc primer set. However a 415 bp product was observed using the PaconsI-F + PaconsI-R2 primer set, presumably corresponding with the *S6* allele. Consistent amplification for *S*6 first and second introns was observed in eight accessions, and nine accessions only showed the second intron amplification product of 1 300 bp. Two accessions only amplified the first intron fragment of 415 bp and not the second intron product. The *S7* allele, previously reported to have a second intron product size of 820 bp, was present in seven accessions with a first intron product size of 402 bp when amplified with the SRc primers. Interestingly, the 402 bp product corresponded with an unpublished product size of the second intron, estimated to be 2 000 bp.

Combining the two datasets for the first intron and single dataset for the second intron amplifications, full *S*-genotypes of 62 accessions were deduced and in 47 accessions only one of the alleles concurring with first or second intron data could be reported. Incomplete genotypes were scored in which only one allele amplified with first and second intron products and the other allele did not amplify in either the first or the second intron. *S*-alleles deduced from product size in only one intron are in brackets (Table 4.4). In 12 accessions, the *S*-genotypes could not be identified. In three of the accessions, no first intron regions were amplified and possible genotypes were only deduced from the second intron alleles. In three accessions, there was no correspondence between the first and second intron alleles. Six of the accessions were rootstocks and hence did not correspond with any of the *P. armeniaca* alleles used as references in this study.

4.3.2.4 Identification of SFBc/8 alleles in apricot

A product of approximately 355 bp/2 800 bp was amplified in 71 apricot accessions with the *S-RNase* primers, indicating the *Sc* or *S8* allele (Halasz *et al*., 2007). Using the apricot fluorescently labelled *SFB* primers (AprSFBSc/8-F + AprSFBSc/8-R) gave a product of approximately 500 bp in 69 accessions, which was deduced to be Sc and a product of 150 bp in two accessions (1006B and 1002B), which was deduced to be *S8*.

4.4 Discussion

In this study, identification of *S*-genotypes of 116 apricot accessions was attempted using consensus primers amplifying the first and second intron region of the *S-RNase* gene. The degenerate primers (EM-PC2consFD + EM-PC3consRD) (Sutherland *et al*., 2004a) were useful in amplifying the second intron region. Furthermore, the fluorescent primers used for precise product length determination of first intron products, reported by Sonneveld *et al.* (2006), was useful in discriminating between first intron products which are characterised by short size variability.

In apricot, using the described approach, 14 PCR products were amplified, corresponding to 14 previously published *S*-alleles: *Sc/8*, *S1, S2*, *S4*, *S5*, *S6*, *S7*, *S9*, *S10*, *S11*, *S13, S14, S18* and *S19*. Amplification products corresponding with alleles: *S12*, *S15*, *S16*, *S17*, *S19* up to *S30* were not observed in the tested accessions. Five amplification products, namely *S(a)*, *S(b), S(c), S(d)* and *S(e)*, that did not correspond with published *S*-alleles, were also identified.

4.4.1 Primers amplifying the S-RNase

4.4.1.1 First intron amplification

First intron amplification in apricot was done using two sets of primers SRc-F + SRc-R and PaconsI-F + PaconsI-R, in which the forward primers were both fluorescently labelled. Both these primer pairs were useful in identifying apricot *S*-genotypes, especially in the case of accessions where only one allele was amplified with either one of the primers. For example, '1031B' showed amplification of only one of the alleles with each primer set; therefore by combining these data it was possible to confirm presence of both alleles in relation to second intron amplification products.

Additionally, scoring of alleles amplified using the Pacons primers proved to be slightly challenging due to the presence of more peaks than expected. This may possibly have been caused by an issue on the capillary analysis or scoring error due to background noise. Nevertheless, identification of alleles was based on the careful observation of correlation between second intron alleles relative to first intron products. Failure to amplify particular alleles may be due to differences in primer sequences which are not matching the nucleotide sequences of the alleles. Complementarity between primers and target sites is often crucial for PCR amplification, as mismatches can severely reduce priming efficiency (Stadhouders *et al*., 2010). Therefore, to facilitate improved S-genotyping in apricot, it would be useful to design consensus primers that can be used to amplify the first intron products of a wide range of *Prunus* species.

4.4.1.2 Second intron amplification

In apricot, 14 *S*-alleles (Table 4.5) were identified. Other studies have revealed inconsistencies with amplification of some alleles. This problem was overcome by using additional primer sets. Furthermore allele-specific primers could be used in order to give conclusive results (Tao *et al*., 2000). Using allele-specific primers to confirm certain alleles has proven to be a useful method in sweet cherry (Sonneveld *et al*., 2001), Japanese apricot (Tao *et al*., 2000) and Japanese plum (Sapir *et al*., 2004). In the current study, for the identification of self-compatible apricot accessions, a *SFBSc/S8* allele-specific primer was used which distinguished the *Sc* allele from *S8,* which codes for self-compatibility. These primers have been used to identify self-compatibility alleles in various studies (Halasz *et al.,* 2010; Halasz *et al*., 2013; Kodad *et al*., 2013; Yilmaz *et al*., 2016).

Mousari *et al*. (2011) reported on the *S*-genotyping of almond cultivars, in which they initially used the EM-PC2consFD + EM-PC3consRD to amplify the second intron alleles. When inconsistencies were observed, *e.g.* amplification of only one band or unclear correspondence between the band sizes observed for the second intron and those previously reported, another primer set was used (*i.e.* PaconsI-F + EM-PCcons5RD) to resolve inconsistencies and allow precise identification of alleles.

The problems encountered in this study with the amplification of second intron products using the EM-PC2consFD + EM-PC5consRD may be associated with the low complementarity of the sequence of the primers set with apricot DNA template (Stadhouders *et al*., 2010).

4.4.2. Self-compatibility in apricot

In this study, 71 of the 116 apricot accessions had a *Sc/ S8* allele (characterised with a 2800 bp band for the second intron and a 355 bp/347 bp first intron product). Further investigation, using the *SFB*c/8 primer to clarify whether the accessions had the *Sc* or *S8* allele, concluded that 69 accessions had the *Sc* allele (500 bp) and two selections (1002B and 1006B) had the 150 bp variant of the *SFB* corresponding with *S8* rather than the SC variant (Table 4.7). Some accessions were homozygous for the *Sc* allele and some were heterozygous *i.e.* showing a *Sc* allele and a self-incompatibility allele (eg *S1*). The presence of at least one *Sc* allele, either homozygous or heterozygous, confers self-compatibility (Vilanova *et al*., 2005). These results suggest that most of the accessions are self-compatible and confirm that South African apricots belonging to the European group are primarily self-compatible (Mehlenbacher *et al*., 1991).

Despite the limitations of self-compatible cultivars in terms of decreased diversity in the breeding programme, they ensure good production as they crop well without the need for a compatible pollinator cultivar. Self-compatible cultivars are also useful for breeding SC cultivars that crops reliably. It should be noted that most of the South African cultivars having the *Sc* allele were reported to have very good productivity/yield (Horstmann, personal communication).

4.4.3. Self-compatible mutation

In addition to the SFB Sc/S8 explored in this study, a number of other mutations have been reported. Regarding the pollen-part mutation (PPM), self-compatible mutants within the non-functional *SFB* gene have been identified in sweet cherry (Ushijima *et al*., 2004); sour cherry (Hauck *et al*., 2006); Japanese apricot (Ushijima *et al*., 2004) and peach (Tao *et al*., 2007). Other spontaneous and induced self-compatible mutants have been identified in a number of *Prunus* species. For example, Zuriaga *et al*. (2013) reported on a *Mu*-like element insertion upstream of the *S6m-RNase* in sour cherry (Yamane *et al*., 2003) and a similar mutation in the Japanese plum *Se-RNase* (Watari *et al*., 2007) (discussed below) reduce the *S-RNase* expression level, leading to insufficient accumulation of *S-RNase* protein structure in the style and breakdown of the rejection mechanism. Marchese *et al*. (2007) also reported on a pollen part mutation of the *S*(5) allele, designated *S*(5)', which conferes self-compatibility. The natural mutation of the second intron of *S*(5) was reported to result from the presence of a microsatellite smaller than that associated with *S*(5).

In Rosaceae, non-*S* mutations on modifier genes have been reported. In apricot, Vilanova *et al*. (2006) and Zuriaga *et al*. (2013) confirmed that the apricot cultivar 'Canino' (*S2Sc Mm*) possesses two different mutations conferring SC. One was the insertion in the *SFBc* gene that produces a *SFBc* truncated protein, mentioned previously, and the other was a mutation in a modifier gene (M) not linked to the *S*-locus. These two mutations therefore cause the loss of pollen-*S* function independently. Zuriaga *et al*. (2013) suggested that the non-*S* M-locus is also involved in pollen-*S* function breakdown in the apricot cultivar 'Katy' (*S1S2*).

In a recent study by Munoz-Sanz *et al*. (2017), it was confirmed that self-(in)compatibility in apricot relies mainly on the *S*- but also on the *M*-locus. The findings from Munoz-Sanz *et al*., study showed that the *Mo*- haplotype associated with SC is shared by 'Canino", 'Katy' and many other cultivars. However, its prevalence is lower than that for Sc in terms of frequency and geographic distribution. The study also reported on other mutations conferring SC, deserving further investigation.

4.4.4 Self-incompatibility

Although apricot cultivars grouped under the European eco-geographical group, including the South African cultivars, are generally self-compatible, a number of self-incompatibility genotypes are known in this group. In the current study, 14 known and five potentially new self-incompatibility alleles were detected (Table 4.5), deduced from the first and second intron sizes. The most frequent self-incompatible allele was *S2* (900 bp) in 30 accessions, followed by an unidentified *S*-allele (*Sd*) in 19 accessions with a product size of 2 200 bp. Five new allele sizes including *Sd*, not reported previously, were also observed. These allele sizes were provisionally assigned in alphabetical order, namely: *Sa* (690 bp), *Sb* (1 500 bp), *Sc* (510 bp), and *Se* (2 700 bp). It would be useful to characterise these new *S*-alleles by cloning and sequencing them and to align them with other known alleles, in order to confirm their authenticity (Romero *et al*., 2004).

Variation with scoring of alleles in comparison with previously published alleles, based on each researcher's perspective of their observed results, was observed (Table 4.2). For certain *S*-alleles, different researchers have published different product sizes. For example, Halasz *et al*. (2013) reported the second intron product of the *S7 S-RNase* allele to be 820 bp whereas Kodad *et al*. (2013) reported it as 750 bp. Even though both these groups identified *S*-alleles of apricot cultivars from the same region, they reported some similar *S*-alleles to differ in size.

The presence of some self-incompatible alleles in the current study was mainly observed in cultivars brought into the breeding programme from other countries (data not shown), to introduce certain desirable traits and increase genetic diversity within the breeding programme. The integration of these non-South African cultivars into the breeding programme has led to the release of newly bred cultivars that are self-incompatible, a problem for South African growers who are not familiar with planting pollinator cultivars (Horstmann, personal communication).

The study found 22 cultivars and 23 selections to display self-incompatibility alleles. Most accessions had different *S*-genotypes (for example: 'Grandir' *S19S*(d), 'Pu sha shin' *S11S18*, 'Jushiro Los' *S1S14*, 'Heiwa' *S(d)S1*), indicating that these accessions would most likely be cross-compatible and would therefore be suitable as cross-pollinators in orchards. In this study no cultivars were observed to share the same self-incompatibility genotypes. If these were present, they would be expected to be cross-incompatible and unsuitable for cross-pollination. Knowledge of the SI genotypes is important for growers and breeders.

4.5 Conclusion

The current study reported on the genotyping of the ARC accessions and cultivars from the apricot collection. Consensus primers were used to amplify the first and second intron regions of the *S-RNase* gene, while allele-specific primers were used to amplify the *Sc/8 SFB* region. In the current study we were able to identify 14 previously published SI alleles. Additionally, five product sizes not previously published were observed. Many accessions in the ARC apricot collections displayed the *Sc* allele and by using the *SFBSc/8* primers; it was possible to discriminate between the *Sc* and *S8* alleles. The *SFB* primers proved to be a useful marker for identifying SC cultivars.

The findings obtained in the current study provide useful information for farmers and breeders. Knowledge of the *S*-genotypes of commercially important SA cultivars offers some level of certainty for the commercial farmers to expect good yield if they plant cultivars with different SI genotypes or self-compatible cultivars.

4.6 References

Alburquerque N., Egea J., Perez-Tornero O. and Burgos L. (2002): Genotyping apricot cultivars for self-(in) compatibility by means of RNases associated with *S*-alleles. Plant Breeding, 121: 343-347.

Beppu K., Yamane H., Yaegaki H., Yamagachi M., Kataoka L. and Tao R. (2003): Determination of *S*-haplotypes of Japanese plum (*Prunus salicina* Lindl.) cultivars by PCR and cross pollination tests. Journal of Horticultural Science and Biotechnology, 78: 315-318.

Beppu K., Yamane H., Yaegaki H., Yamagachi M., Kataoka L. and Tao R. (2002): Diversity of *S-RNase* genes and *S*-haplotypes in Japanese plum (*Prunus salicina* Lindl.). Journal of Horticultural Science and Biotechnology, 77: 658-664.

Bošković R. and Tobutt KR. (1996): Correlation of stylar ribonuclease zymograms with incompatibility alleles in sweet cherry. Euphytica, 90: 245-250.

De Nettancourt D. (2001): The basic features of self-incompatibility. In: De Nettancourt D. (ed) Incompatibility and incongruity in wild and cultivated plants. Springer, Berlin, pp 1-24.

Donoso J.M., Aros D., Meneses C. and Infante R. (2009): Identification of *S*-alleles associated with self-incompatibility in apricot (*Prunus armeniaca* L.) using molecular markers. Journal of Food, Agriculture and Environment, 7: 270-273.

Doyle J. J. and Doyle J. L. (1990): Isolation of plant DNA from fresh tissue. Focus, 12, 13-15.

East E.M. and Mangelsdorf A.J. (1925): A new interpretation of the hereditary behaviour of self-sterile plants. Proceedings of the National Academy of Science of the United States of America, 11: 166-171.

Entani T., Iwano M., Shiba H., Che F.S., Isoga A. and Takayama S. (2003): Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: identification of a pollen expressed F-box gene with allelic diversity. Gene Cells, 8: 203-213.

Entani T., Iwano M., Shiba H., Che F.S., Isoga A. and Takayama S. (2003): Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: identification of a pollen expressed F-box gene with allelic diversity. Gene Cells, 8: 203-213.

Gharesheikhbayat R. (2010): Self-incompatibility in apricot (*Prunus armeniaca*); New achievement and molecular aspects of *S*-locus allele segregation (published doctoral dissertation). Alma Mater Studiorum – University of Bologna, pp 3-16.

Halasz J., Hegedus A., Szabo Z., Nyeki J. and Pedryc A. (2007): DNA-based S-genotyping of Japanese plum and pluot cultivars to clarify incompatibility relationships. Hortscience, 42: 46–50.

Halasz J., Hegedus A., Szikriszt B., Ercisli S., Orhan E. and Unlu H.M. (2013): The *S*-genotyping of wild-growing apricots reveals only self-incompatible accessions in the Erzincan region of Turkey. Turkish Journal of Biology, 37: 733-740.

Halasz J., Pedryc A., Ercisli S., Yilmaz K.U. and Hegedus A. (2010): *S*-genotyping supports the genetic relationships between Turkish and Hungarian apricot germplasm. Journal of American Society of Horticultural Science, 135: 410-417.

Halasz J., Pedryc A., Ercisli S., Yilmaz K.U. and Hegedus A. (2010): *S*-genotyping supports the genetic relationships between Turkish and Hungarian apricot germplasm. Journal of American Society of Horticultural Science, 135: 410-417.

Halasz J., Szikriszi B., Ercisli S., Yilmaz K.U., Dogan A., Szabo Z., Nyeki J., Pedryc A. and Hegedus A. (2012): *S*-Locus genotyping on stone fruits. Acta Horticulturae, 967: 113-119.

Hauck N.R., Yamane H., Tao R. and Iezzoni A.F. (2006): Accumulation of non-functional *S*-haplotypes results in the breakdown of gametophytic self-incompatibility in tetraploid *Prunus*. Genetics, 172: 1191-1198.

Ikeda K., Igic B., Ushijima K., Yamane H., Hauck N.R., Nakano R., Sassa H., Iezzoni A.F., Kohn J.R. and Tao R. (2004): Primary structural features of the *S* haplotype-specific F-box protein, *SFB*, in *Prunus*. Sexual Plant Reproduction, 16: 235-243.

Kodad O., Hegedus A., Company R.C. and Halasz J. (2013): Self-(in)compatibility genotypes of Moroccan apricot indicate differences and similarities in the crop history of European and North African apricot germplasm. Plant Biology, 13: 1-11.

Marchese A., Boskovic R. I., Carus T., Raimondo A., Cutuli M. and Tobutt K. R. (2007): A new self-compatibility haplotype in the sweet cherry 'Kronio', *S5*', attributable to a pollen-part mutation in the *SFB* gene. Journal of Experimental Botany, 58: 4347-4356.

Mehlenbacher S.A., Cociu V. and Hough L.F. (1991): Apricot (*Prunus*). In: Genetic Resources of Temperate Fruit and Nut Crops. Moore J.N. and Ballington J.R. (eds). ISHS Acta Horticulturae, 290: 65-107.

Mousari A., Fatahi R., Zamani Z., Imani A., Dicenta E. and Ortega E. (2011): Identification of self-incompatibility genotypes in Iranian almond cultivars. Acta Horticulturae, 912: 303-311.

Munoz-Snaz J. V., Zuriaga E., Lopez I., Badenes M.L. and Romero C. (2017): Self-(in)compatibility in apricot germplasm is controlled by two major loci, S and M. BMC Plant Biology, 17: 1-16.

Nunes M.D.S., Santos R.A.M., Ferreira S.M., Vieira J., and Vieira C.P. (2006). Variability patterns and positively selected sites at the gametophytic self-incompatibility pollen *SFB* gene in a wild self-incompatible *Prunus spinosa* (Rosaceae) population. New Phytologist, 172: 577–587.

Ortega E., Sutherland B.G., Dicenta F., Bošković R. and Tobutt K.R. (2005): Determination of incompatibility genotypes in almond using first and second intron consensus primers: detection of new *S* alleles and correction of reported *S* genotypes. Plant Breeding, 124: 188-196.

Ortega E., Sutherland B.G., Dicenta F., Bošković R. and Tobutt K.R. (2005): Determination of incompatibility genotypes in almond using first and second intron consensus primers: detection of new *S* alleles and correction of reported *S* genotypes. Plant Breeding, 124: 188-196.

Roalson E.H. and McCubbin A.G. (2003): *S-RNase* and sexual compatibility: structure, functions, and evolutionary perspectives. Molecular Phylogenetics and Evolution, 29: 490-506.

Romero C., Vilanova S., Burgos L., Martinez-Calvo J. Vicente M., Llacer G. and Badenes M.L. (2004): Analysis of the *S*-locus structure in *Prunus armeniaca* L.: Identification of *S*-haplotypes *S-RNase* and F-box genes. Plant Molecular Biology, 56: 145-157.

Sapir G., Stern R.A., Shafir S. and Goldway M. (2008): *S-RNase* based *S*-genotyping of Japanese plum (*Prunus salicina*) and its implication on the assortment of cultivar-couples in the orchard. Scientia Horticulturae, 118: 8-13.

Sassa H., Hirano H., Nshio T. and Koba T. (1997): Style-specific self-compatibility mutation caused by deletion of the S-RNase gene in Japanese pear (*Pyrus serotina*). The Plant Journal, 12: 223-227.

Sonneveld T., Robbins T.P. and Tobutt K.R. (2006): Improved discrimination of self-incompatibility *S-RNase* alleles in cherry and high throughput genotyping by automated sizing of first intron polymerase chain reaction products. Plant Breeding, 125: 305-307.

Sonneveld T., Robbins T.P. and Tobutt K.R. (2006): Improved discrimination of self-incompatibility *S-RNase* alleles in cherry and high throughput genotyping by automated sizing of first intron polymerase chain reaction products. Plant Breeding, 125: 305-307.

Sonneveld T., Tobutt K.R. and Robbins T.P. (2003): Allele-specific PCR detection of sweet cherry self-incompatibility (*S*) alleles *S1* to *S16* using consensus and allele-specific primers. Theoretical and Applied Genetics, 107: 1059-1070.

Stadhouders R., Pas S.D., Ander J., Voermans J., Mes T.H. and Schutten M. (2010): The effect of primer template mismatches on the detection and quantification of nucleic acids using the 5' nuclease assay. Journal of Molecular Diagnostics, 12: 109-117.

Tao R., Habu T., Yamane H., Sugiura A. and Iwamoto K. (2000): Molecular markers of self-compatibility in Japanese apricot (*Prunus mume*). HortScience, 35: 1121-1123.

Tao R., Habu T., Yamane H., Sugiura A. and Iwamoto K. (2000): Molecular markers of self-compatibility in Japanese apricot (*Prunus mume*). HortScience, 35: 1121-1123.

Tao R., Yamane H., Sassa H., Mori H., Gradziel T.M., Dandekar A.M. and Sugiura A (1997): Identification of stylar RNases associated with gametophytic self-incompatibility in almond (*Prunus dulcis*). Plant and Cell Physiology, 38: 304-311.

Tao R., Yamane H., Sugiura A., Maruyama H., Sassa H. and Mori H. (1999): Molecular typing of *S*-alleles through identification, characterisation and cDNA cloning for *S-RNases* in sweet cherry. Journal of the American Society for Horticultural Science, 124: 224-233.

Ushijima K., Sassa H., Dandekar A.M., Gradziel T.M and Tao R. (2003): Structural and transcriptional analysis of the self incompatibility locus of almond. Identification of a pollen expressed F-box gene with haplotype-specific polymorphism. Plant Cell, 15: 771-781.

Ushijima K., Yamane H., Watari A., Kakehi E. and Ikeda K. (2004): The *S*-haplotype-specific F-box gene, *SFB*, is defective in self-compatible haplotypes of *P. avium* and *P. mume*. Plant Journal, 39: 573-586.

Vilanova S., Romero C., Liacer G. and Badenes M.L. (2005): Identification of Self-(in)compatibility alleles in apricot by PCR and sequence analysis. Journal of the American Society for Horticultural Science, 130: 893-898.

Watari A., Hanada T., Yamane H., Esumi T. and Tao R. (2007): A low transcriptional level of *Se-RNase* in the *Se*-haplotype confers self-compatibility in Japanese plum. Journal of American Society of Horticultural Science, 132: 396-406.

Wu J., Gu C., Zhang S.L., Zhang S.J., Wu H.Q., and Heng W. (2009): Identification of S-haplotype-specific *S-RNase* and *SFB* alleles in native Chinese apricot (*Prunus armeniaca* L.). Journal of Horticultural Science and Biotechnology, 84: 645-652.

Yaegaki H., Shimada T., Moriguchi T., Hayama H., Haji T., and Yamaguchi M. (2001): Molecular characterisation of *S*-RNase genes and *S*-genotypes in the Japanese apricot (*Prunus mume* Sieb. et Zucc.). Sexual Plant Reproduction, 13: 251-257.

Yamane H., Ikeda K., Hauck N.R., Iezzoni A.F. and Tao R. (2003): Self incompatibility (S) locus region of the mutated *S6*-haplotype of sour cherry (*Prunus cerasus*) contains a functional pollen *S* allele and non-functional pistil S-allele. Journal of Experimental Botany, 54: 2431-2437.

Yamane, H., Tao R., Sugiura A., Hauck N.R., and Iezzoni A.F. (2001): Identification and characterisation of *S-RNases* in tetraploid sour cherry (*Prunus cerasus*). Journal of American Society Horticultural Science, 126: 661-667.

Yamane, H., Tao R., Sugiura A., Hauck N.R., and Iezzoni A.F. (2001): Identification and characterisation of *S-RNases* in tetraploid sour cherry (*Prunus cerasus*). Journal of American Society Horticultural Science, 126: 661-667.

Yilmaz K.U., Basbug B., Gurcan K., Pinar H., Halasz J., Ercisli S., Uzun A. and Cocen E. (2016): S-genotype profiles of Turkish apricot germplasm. Notulae Botanicae Horti Agrobotanic Cluj-Napoca, 44: 67-71.

Zhang L., Chen X., Chen X.L., Zhang C., Liu X., Ci Z.J., Zhang H., Wu C. and Liu C. (2008): Identification of self-incompatibility (*S*-) genotypes of Chinese apricot cultivars. Euphytica, 160: 241-248.

Zuriaga E., Munoz-Sanz J.V., Molina L., Gisbert A.D., Badenes M.L. and Romero C. (2013): An *S*-locus independent pollen factor confers self-compatibility in 'Katy' apricot. PLoS ONE 8: 1-14.

Chapter 5:

Molecular characterisation of the self-incompatibility locus in plum accessions within the ARC's germplasm collection

Parts of this chapter, especially the introduction as well as materials and methods are duplicated equivalent to chapter 4 but have been included for completeness.

5.1 Introduction

Among the stone fruit belonging to the genus *Prunus* in the family Rosaceae, is Japanese plum (*P. salicina*). In South Africa, this crop is grown commercially, especially for the export market. Some cultivars are self-compatible and can be grown conveniently in single cultivar orchards whereas others are self-incompatible and need to be planted with appropriate pollinator cultivars to set fruit. Knowledge of self-(in)compatibility relationships of cultivars is therefore important to growers when planning an orchard and selecting cross-pollinators. This information is also useful to plant breeders designing crosses.

As in other species of the genus *Prunus*, in plum self-incompatibility (SI) is gametophytic (De Nettancourt, 2001) and is regulated by a single multi-allelic *S*-locus. This comprises at least two genes, one expressed in the style and one in the pollen. Incompatibility occurs when the *S*-allele of a pollen grain (haploid) coincides with either of the *S*-alleles of the style (diploid) (East and Mangelsdorf, 1925). In such a case the pollen tube growth is terminated in the style

making fertilisation impossible. Two cultivars may be cross-incompatible, semi-compatible or fully compatible depending on whether they share two, one or zero alleles. Self-compatibility (SC) can result from mutations in the stylar or pollen reproductive organs so that there is some loss of recognition or other function, though there is also evidence of some non-*S* mutations that can confer self-compatibility.

Prunus species have been reported to have at least two genes at the *S*-locus, one encoding a stylar glycoprotein with ribonuclease activity (*S-RNase*) (Bošković and Tobutt, 1996) and the other a cognate *S*-haplotype-specific-Fbox gene (*SFB*/*SLF*) (Entani *et al*., 2003; Ushijima *et al*., 2003; Romero *et al*., 2004). The *S-RNase* gene is expressed in the style and comprises a signal peptide (SP), five highly conserved regions (C1 to C5), a hyper-variable region (HVR) located between C2 and C3, and two introns (Sassa *et al*., 1997; Ushijima *et al*., 2003). Characterisation of *S-RNase* genotypes is often based on these two introns, which exhibit allele-specific length polymorphisms. The first intron, unique to *Prunus*, lies between the sequence responsible for the secretion signal peptide and the C1 region and the second intron is located between the C2 and C3 region in the HVR (Fig. 4.1) (Yamane *et al*., 2001). The second gene encodes the pollen *S*-haplotype-specific F-box gene (*SFB*/*SLF*) (Entani *et al*., 2003; Romero *et al*., 2004). The *SFB*/*SLF* gene is expressed in the pollen and contains one intron at the 5' end, an F-box motif and two hyper-variable regions, HVa and HVb at the C-terminus (Ikeda *et al*., 2004; Nunes *et al*., 2006) (Fig. 4.1). Romero *et al*. (2004) sequenced the *S*-locus region in three different apricot *S*-haplotypes and showed that the *S-RNase* and *SFB* genes were in very close proximity (with a physical distance of 2.9kb) allowing them to be inherited as a unit (Roalson and McCubbin, 2003). Several *S-RNase* and *SFB* protein gene pairs have been determined from *Prunus*: *P. salicina* (Beppu *et al*., 2003), *P. dulcis* (Tao *et al*., 1997), *P. cerasus* (Yamane *et al*., 2001), *P. mume* (Entani *et al*., 2003*)* and *P. armeniaca* (Romero *et al*., 2004).

PCR based *S*-genotyping in *Prunus* species was initially based on amplification of the *SRNase* second intron; the primer set Pru-C2, Pru-C4R and Pru-C5, was designed from conserved regions (Tao *et al*., 1999) and has been used for the *S*-genotyping of sour cherry (Yamane et al., 2003); Japanese apricot (Tao *et al*., 2000; Yaegaki *et al*., 2001) and Japanese plum (Beppu *et al*., 2002). However these primers, designed from a small set of allele sequences from just one or two species, failed to amplify all alleles (Sutherland *et al*., 2004a). Sutherland *et al*. (2004a) hence designed degenerate consensus primers, flanking the second intron, from conserved regions of 27 *S*-RNase gene sequences from five *Prunus* species, which proved to be more broadly applicable.

Regarding the first intron, amplification products were small and therefore difficult to discriminate on agarose gel electrophoresis. However in almond, Ortega *et al*. (2005) reported a more precise approach to determine and confirm S genotypes, through PCR amplification of the first intron alleles using a fluorescently labelled forward primer (PaconsI-F), designed from cherry, used in combination with a reverse primer (EM-PC1consRD) designed from 22 published sequences of *Prunus S-RNases*. Product sizes of the first intron region were generally under 500 bp. Sonneveld *et al*. (2006) similarly distinguished the first intron products of sweet cherry *S*-alleles using the same fluorescently labelled primer (PaconsI-F) in combination with a different reverse primer (PaconsI-R2).

In recent years, much progress has been made in identification via PCR of *S*-genotypes in Japanese plum, often using *SRNase* primers developed in other *Prunus* species. In all, 36 different *S*-alleles have been identified (Guerra *et al*., 2012). Initially, Beppu *et al*. (2002, 2003) and Guerra *et al*. (2009) reported on 19 *S-RNase* alleles that were detected and labelled alphabetically. Five of these were also labelled using numbers: *Sa* (*S1*); *Sc* (*S4*); *Se* (*S5*); *Sf* (*S6*) and *Sk* (*S3*) by Sapir *et al*. (2004). Zhang *et al*. (2008), also described 17 *S-RNase* alleles using numbers (*S7*, *S8*, *S10*, *S11* and *S15* to *S27*). In plum, there is a self-compatibility *S-RNase* allele designated as *Se* (Beppu *et al*., 2005). According to Hegedus and Halasz (2007), evidence from expression analysis carried out by RT-PCR showed that the Se allele was transcribed in the style. Therefore, it appeared that neither a deletion of the *S-RNase* gene, nor prevention of transcription of the *S-RNase* gene was the cause of self-compatibility in the Se-haplotype. In addition, Guerra *et al*. (2015) reported that self-compatibility was also associated with other S-haplotypes: *Sb* (Guerra *et al*., 2009; Beppu *et al*., 2010), *Sg* (Beppu *et al*., 2012) and *Ss* (Beppu *et al*., 2012). However, numerous Japanese plum cultivars displaying the *Se* or *Sb* alleles have been described as self-incompatible. This phenomenon of self-compatibility in Japanese plum is not well understood.

Examples of *S*-genotyping of plum cultivars include; PCR-based S-genotyping of 43 Japanese plum cultivars grown in Israel, using the Sutherland *et al*. (2004a) primers to amplify the second intron, as reported by Sapir *et al*. (2008). *S*-genotyping in Japanese plum has also been reported by Halasz *et al*. (2007), Sapir *et al*. (2008) Guerra *et al*. (2009) and Guerra and Rodrigo (2015).

Little is known about the self-(in)compatibility genotypes of plums grown in South Africa. The Agricultural Research Council (ARC) Institute at Infruitec-Nietvoorbij, located in the Western Cape, conducts apricot and plum breeding programmes, which supplies the industries. It holds a plum gene bank of accessions for use in breeding and the National reference collection, which contains most of the cultivars grown commercially in South Africa. In addititon, some

plum and apricot hybrids are present. There is a need to identify the self-(in)compatibility genotypes of the plum cultivars that are used in the breeding programme (in order to inform the breeders when planning crosses) and/or are released to growers (to inform growers when planning orhards to identify suiable compatible cultivars).

In this study, the objective was to use appropriate fluorescently labelled primers for the first intron (Romero *et al*., 2004; Vilanova *et al*., 2005; Sonneveld *et al*., 2006), and for the second intron, non-labelled primers (Sutherland *et al*., 2004a) to determine the S-genotypes of accessions of the ARC Infruitec-Nietvoorbij plum collections.

5.2 Materials and methods

5.2.1 Plant material

The plant material was grown at the ARC's Bien Donne Experimental Farm located in Groot Drakenstein, Western Cape, South Africa. It consists of 39 plum cultivars and hybrids from the gene banks (SV8A and SV8B), 57 plums from the stone fruit reference collection (BD10), 8 plum rootstocks (ZN7) and 6 plums from the plum crossing collection (ZN5) (Table 3.2; Chapter 3). The 70 plum (*P. salicina*) trees had generally been supplied by the South African Plant improvement Organisation (SAPO). In this section, only diploid species were evaluated and polyploids were excluded because they resulted in complex patterns and multiple bands, which made correlation of amplification products difficult; hence resulting in mis-identification of *S*-alleles. The same plant material was used in both the microsatellite analyses and the currect chapters. The selections/cultivars and their locations and species are recorded in Table 3.1. The accessions were planted in sets of three trees, and if they were morphologically uniform, just one was sampled, usually the first. Occasionally there were two accessions of the same cultivar; these were distinguished as 1 and 2 if they were in different orchards, and A and B when in the same orchard. These trees are considered to be the same: they were planted separately if more than 3 trees were supplied by SAPO. In addition, two of the plum cultivars were grown in two or more plots in the gene banks.

5.2.2 DNA extraction

Genomic DNA was extracted using the same protocol as in apricot. Briefly, young leaves of the accessions were collected in spring and stored in a -80^{o} freezer, until used. DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990), with slight modifications. Three leaf discs were put in a 2 ml Eppendorf tube with

3 ball bearings of 3 mm size, 400 µl of CTAB extraction buffer (heated to 65°C) and 4 µl β-Mercapto-ethanol (Sigma Aldrich) was added to each tube within a fume hood. Samples were initially mixed by inversion. The leaves were homogenised in a Tissuelyser II ball mill (Qiagen) at 30Hz for 4 min; leaves that were not completely homogenised were run for an additional 2 min. This step was followed by incubation of the tubes in a 60°C water-bath for 1 hour, with inversion every 10 to 20 min. After the removal of the ball bearings with a magnet, 400 µl of chloroform-isoamyl alcohol (24:1 ratio) was added, mixed by inverting and centrifuged at 13 500 rpm for 15 min using a centrifuge (Labnet). The top aqueous phase was aliquoted into a new 2 ml Eppendorf tube, 400 µl of chloroform-isoamyl alcohol (24:1 ratio) was again added and the samples were then centrifuged at 13 500 rpm for 10 min. The top aqueous phase was transferred a final time into a new Eppendorf tube and precipitated with 320 µl cold isopropanol overnight in a refrigerator. After overnight incubation, the samples were centrifuged for 15 min at 13 500 rpm and the supernatant was discarded; the pellet was then washed with 70% cold ethanol. The ethanol was removed and the pellet was dried for 45 min, re-suspended in TE buffer and stored in the refrigerator until required.

The quality and quantity of DNA was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) according to the manufacturer's instructions. If samples showed poor quality and quantity, extractions were repeated. Good quality DNA (with A260/A280 ratio between 1.8 and 2.0) was then diluted to a concentration of approximately 20-30 ng/µl and the samples were stored at -20°C until further use to minimise degradation of the DNA.

5.2.3 Primers amplifying the S-RNase gene

Pairs of consensus primers flanking the first or second intron of the *S-RNase* gene were used in this study (Table 5.1; Fig. 4.1). To amplify the first intron, the consensus forward primer PaConsI-F (Sonneveld *et al*., 2003) designed from the region coding for the signal peptide region of cherry cultivars and labelled with a HEX fluorescent dye, was used in combination with reverse primer PaConsI-R2 (Sonneveld *et al*., 2006) designed from the C1 region of the DNA sequences of cherry. To amplify the second intron, the consensus primers, designed from the second (C2), third (C3) and fifth (C5) conserved regions of 27 *S-RNase* allele sequences of five *Prunus* species (Sutherland *et al*., 2004a) were used in the following two combinations: EM-PC2consFD + EM-PC3consRD; and EM-PC2consFD + EM-PC5consRD. However, the primer combination, amplifying the second intron, designed from the conserved regions C2 + C5 (EM-PC2cons-FD + EM-PC5consRD) displayed poorer amplification in most

accessions, showing non-specific amplification and in some case no amplification, therefore the results for this primer pair are not reported.

Table 5.1. Sequences of primers, used to amplify first or second intron alleles of the *Prunus S-RNase* gene.

Primer	Target region	Sequence	Ta°	References
PaConsI-F	1st intron	5'-(c/a)ct tgt tct tg(c/g) ttt (t/c)gc ttt ctt c-3'	54	Son. *et al*., 2003
PaConsI-R2	1st intron	5'-gcc att gtt gca caa att ga-3'	54	Son. *et al*., 2006
EM-PC2consFD	2nd intron	5'-tca cma tyc atg gcc tat gg-3'	58	Su. *et al*., 2004a
EM-PC3consRD	2nd intron	5'-aws trc crf gyt tgt tcc att c-3'	58	Su. *et al*., 2004a
EM-PC5consRD	2nd intron	5'-caa aat acc act tca tgt aac ar-3'	58	Su. *et al*., 2004a

Ta° – annealing temperature, Son- Sonneveld *et al*., 2003, 2006. Su. Sutherland *et al*., 2004a

5.2.4 S-RNase genotyping

5.2.4.1 PCR amplification of first intron

PCR amplification of plum DNA was conducted following a procedure described by Sonneveld *et al*. (2003). Genomic DNA of approximately 20-30 ng/µl (2 µl) was used in a 25 µl reaction, which contained 5 µl of 1X PCR buffer (Promega), a final concentration of 2.5 mM $MgCl_2$ (2.5 µl), 0.2 mM dNTPs (0.5 µl), 0.2 µM of each of the two primers (0.5 µl), 0.625 U of *Taq* DNA polymerase (0.125 µl) (Promega) and 13.875 µl of dH_2O. PCR amplification was carried out in the following conditions: 2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 54°C and 1 min at 72°C, and a 5 min final extension at 72°C. The PCR was set up in ice, then transferred to a GENEAMP (Applied Biosystems) or a G-storm (G-Storm Direct) thermal cycler.

The first subset of PCR products was resolved by 1.5% (m/v) agarose gel electrophoresis (Conda laboratory) at 80 V for 90 min. To verify amplification, the products were electrophoresed with 2 µl of 6X loading dye (Fermentas) against a 1 kb ladder (Fermentas) in a 1% (m/v) TBE buffer (Tris-Boric Acid-EDTA). The forward primer was fluorescently labelled with a HEX dye, which allowed more precise identification of allele sizes when analysed on capillary electrophoresis. Upon verification, the full set of the first intron amplification products, all smaller than 500 bp, were separated by capillary electrophoresis on a 3130 DNA capillary analyser (Applied Biosystems) at the Central Analytical Facility's DNA sequencing unit at

Stellenbosch University. Sizes of peaks were estimated in comparison with the internal standard GS500(-250)LIZ (Applied Biosystems) and data were captured in Excel. GENEMAPPER software version 5.0 (Applied Biosystems) was used to visualise the peaks and score the alleles. Data were checked and allele scores were compared and verified with previously reported allele sizes to compile provisional genotypes (Table 5.2).

5.2.4.2 PCR amplification of second intron

To amplify the second intron, a protocol described by Sutherland *et al*. (2004a) was followed. In a 20 µl volume the following were added: 20-30 ng/µl DNA (2 µl), 4 µl of 1X PCR buffer, 2 mM $MgCl_2$ (2 µl), 0.2 mM dNTPs (0.4 µl), 2 µl of Q solution, 0.5 U of *Taq* DNA polymerase (0.14 µl), 0.3 µM of each of the two primers (0.6 µl) and 8.25 µl of dH_2O. PCR conditions were set up as follows: initial denaturation for 2 min at 94°C and 35 cycles of 94°C for 10 s, 58°C for 2 min and 68°C for 10 s, with an increment of 10 s per cycle on the extension step after the 10^{th} cycle. The PCR was set up in ice and centrifuged before running the samples on a GENEAMP thermal cycler.

Amplified products were resolved using gel electrophoresis on a 1.5% TBE buffer agarose gel at 70 V for 2 h and stained using ethidium bromide. Usually this separation technique is useful for the separation of products larger than 500 bp that cannot be analysed with the standard set-up on a 3130 DNA capillary analyser. Provisional identification of bands was done by comparison to previously published *S*-alleles product sizes using a 1 kb plus ladder (Fermentas) (Table 5.2).

5.2.4.3 Reference *S*-alleles and deducing of genotypes

A number of studies have used primers amplifying the first or second introns to determine the *S*-genotypes of plum cultivars. The previously published sizes of known *S*-alleles were used as references to facilitate the correlation of data, *i.e.* bands and peaks (Table 5.2). In plum, first intron amplification products have been previously amplififed using PaconsI-F + EM-PC1cons-RD (Halasz *et.al*. 2007); therefore, the reported product sizes were used for reference. Guerra *et al*. (2012) also reported on Japanese plum first intron amplification products using PaconsI-F + PaconsI-R2 primers that were assigned *Sa* to *Sf*, *Sh*, *Sk* and *So* to *Ss*. Second intron amplification in plum has been conducted more extensively using the primers: PruC2 + PCE-R (Beppu *et al*., 2002; 2003) and EM-PC2cons-FD + EM-PC3consRD (Halasz *et al*., 2007). In the current study, correlation of first and second intron amplification products, amplified using the Pacons (first intron) and EM-PC (second intron) primers, was on

the basis of correspondence between product sizes of published *S*-alleles and product sizes amplified in the current study.

Table 5.2. Previously reported first (fluorescently detected) and second intron (detected using agarose gel electrophoresis) product sizes of Japanese plum *S*-alleles amplified using a range of primers flanking the two introns, used as reference for correlation of amplified PCR products.

	1st intron (bp)			2nd intron (bp) (EM-PC2cons-FD+EM-PC3consRD) (detected using gel electrophoresis)		2nd intron (bp) (PruC2-PCER)		2nd intron (bp) (PruC2+PCER)
S-allele	**Halasz *et al*., 2007 (PaconsI-F + EM-PC1consRD)**	**Guerra *et al*., 2012 ABI310/ABI3130*x1 (PaconsI-F+ PaconsI-R2)**	**Current study (PaconsI-F+ PaconsI-R2)**	**Halasz *et al*., 2007**	**Current study**	**Beppu *et al*., 2002; 2003**		**Guerra *et al*., 2009**
Sa	-	388/389	-	-	-	470	-	-
Sb	367	367/369	369	1550	1550	1540	-	-
Sc	343	343/343	343	1200	1200	1170	-	-
Sd	-	423/424	-	-	-	1290	-	-
Se	372	372/373	373	1450	1450	1420	-	-
Sf	295	328	-	1250	-	1120	-	-
Sg	-	-	-	-	-	1230	-	-
Sh	388	388/390	390	550	550	520	-	-
Si	-	-	-	-	-	410	-	-
Sj	-	-	-	-	-	-	1660	-
Sk	382	381/384	384	350	350	-	360	-
Sl	-	-	-	-	-	-	870	-
Sm	-	-	-	-	-	-	320	-
Sn	-	-	-	-	-	-	640	-
So	-	421/422	-	-	-	-	-	1340
Sp	-	370	-	-	-	-	-	600
Sq	-	204/206	-	-	-	-	-	1270
Sr	-	206/208	-	-	-	-	-	760
Ss	-	-	-	-	-	-	-	580

* ABI310/ABI3130x1 product sizes detected using two different capillary analysers. (-): no reported amplification

5.3 Results

For first intron amplification, in general two peaks were observed, however some batches were slightly problematic. In some batches, there were some difficulties in terms of distinguishing between real peaks and background pull-up or noise. Some accessions showed three peaks, as discussed later. In general, second intron amplififcation resulted in one or two primary bands in the case of Japanese plum accessions. In some cases, shadow bands were observed but only the two main bands were taken into account. In some accessions, three primary bands were observed. *S*-alleles were scored initially on the basis of correlation with previously published second intron *S*-alleles and the first intron *S*-alleles were then deduced from these. However for data presentation, first intron data is presented before second intron data.

5.3.1 S-genotype verification

The *S*-genotypes of the 13 plum cultivars for which genotypes have previously been reported (Table 5.3), were compared to calibrate scoring of alleles. The observed genotypes were not completely consistent with reported *S*-genotypes; four of the cultivars showed one of the reported alleles, two amplified completely different alleles and seven cultivars had the expected / reported genotypes.

Table 5.3. Comparison of a set of ARC apricot and plum cultivars with previously reported *S*-genotypes.

Plum cultivar	Reported genotype	Reference	Observed genotype
Black Amber	*SbSc*	Halasz *et al*., 2007	*SdSh*
Casselman	*SeSh*	Guerra *et al*., 2009	*ScSh*
Flavor King	*SbSe*	Halasz *et al*., 2007	*SbSe*
Fortune	*SbSc*	Guerra *et al*., 2009	*SbSg*
Harry Pickstone	*SbSk*	Beppu *et al*., 2003	*SbSk*
Hiromi Red	*SbSh*	Guerra *et al*., 2009	*SbSk*
Laetitia	*SeSh*	Guerra *et al*., 2009	*SeSh*
Laroda	*SbSc*	Beppu *et al*., 2002	*SbSd*
Pioneer	*SeSb*	Guerra *et al*., 2009	*SeSb*
Santa Rosa	*ScSe*	Beppu *et al*., 2002	*ScSe*
Simka	*SeSk*	Beppu *et al*., 2003	*SbSj*
Songold	*SkSh*	Guerra *et al*., 2009	*SkSh*
Souvenir	*SeSh*	Guerra *et al*., 2009	*SeSh*

5.3.2 S-genotyping of plum accessions

First intron amplification was attempted using two sets of consensus fluorescent primer pairs, namely SRc-F + SRc-R (Romero *et al*., 2004; Vilanova *et al*., 2005) and PaconsI-F + PaconsI-R2 (Sonneveld *et al*., 2005). The SRc-F + SRc-R primer pair (designed for *S*-genotyping in apricot) yielded poor amplification products, revealing complex patterns which made scoring of alleles difficult. These were for genotyping in plum. However, the Pacons primer set was useful in identifying and confirming *S*-genotypes of 59 cultivars and 12 selections of diploid plum accessions (*P. salicina*) and 12 *P. armeniaca* x *P. salicina* interspecific hybrids. In the case of four accessions ('Betty Anne', 'Mac Verma', 'Purple King' and 'By 69 1637') three primary bands were observed.

Amplified products of the second intron were assigned to SI alleles previously reported as *Sa* to *Sn* and *Se* for the self-compatible allele according to previous reports (Table 5.2). However, self-compatibility was reported to be also associated with other alleles (Beppu *et al*., 2012), *e.g*. *Sg* and *Sb*. *S*-alleles identified in this study are reported in Table 5.6, with PCR products amplified in the current study but that have not been previously reported, designated with roman numerals.

The self-(in)compatibility relationship of a set of plum cultivars were previously investigated by De Klerk and Smith (2013) and classified as SI or SC. These cultivars were used to compare the data from the current study and to confirm the validity of the data. The comparison however showed few similarities between the two datasets (Table 5.4).

Table 5.4. Comparison of plum cultivars according to result reported by De Klerk and Smith (2013) and results observed in the current study, indicating self-compatible cultivars associated with the presence of the *Se*, *Sb* and *Sg* allele in various cases. Sc indicates self compatible cultivars and SI indicates self incompatible cultivars.

***P. salicina* cultivars/selections**	**SI/SC reported by De Kerk and Smith (2013)**	**Current study**
African Delight	SI	*Se* allele ∴ SC
Casselman	SC	No *Se* allele ∴ SI
Flavor King	SI	*Se* allele ∴ SC
Flavor Rich	SI	*Se* allele ∴ SI
Fortune (1)	SI	No *Se* allele ∴ SI
Golden Kiss	SI	*Se* allele ∴ SC
Harry Pickstone*	SC	No *Se* allele ∴ SI
Lady Red	SI	No *Se* allele ∴ SI
Laetitia (1)*	SI	*Se* allele ∴ SC

Pioneer (1)*	SC	*Se* allele ∴ SC
Purple Majesty	SI	*Se* allele ∴ SC
Ruby Red	SI	No *Se* allele ∴ SI
Ruby Star	SC	Inconclusive result
Santa Rosa	SC	*Se* allele ∴ SC
Sapphire (1)	SI	*Se* allele ∴ SC
Simka	SC	Scored 374
Songold	SI	No *Se* allele ∴ SI
Southern Belle	SI	Inconclusive result
Souvenir (1)	SI	Inconclusive result
Sun Kiss	SI	Inconclusive result
Sundew	SI	Inconclusive result
Sunset	SI	*Se* allele ∴ SC

Table 5.5. Preliminary interpretation of 7 plum accessions and 13 plum hybrids PCR products, deduced from first and second intron PCR products amplified using Pacons and EM-PC primer pair, respectively.

Selections\Cultivars	**Tree location**		**1st intron amplification products (bp)**	**2nd intron amplification products (bp)**	**Deduced S-genotypes**	**Accessions showing Se allele**
P. salicina	**Orchard and R/T**		**PaconsI-F + PaconsI-R2**	**EM-PC2cons-FD + EM-PC3cons-RD**		
ARC PR-1 * (RC)	BD10	28/40	369, 374	1 450, 1 550	*SeSb*	Se
ARC PR-2 * (RC)	BD10	29/46	374, 390	550, 1 450 1 660	*ShSe*	Se
ARC PR-3 * (RC)	BD10	30/07	374, 390	550, 1 450	*ShSe*	Se
ARC PR-4 * (RC)	BD10	28/01	369, 374	1 450, 1 550	*SeSb*	Se
African Delight *	ZN5A	04/52	374, 390	550, 1 450	*ShSe*	Se
Autumn Sun * (RC)	BD10	30/31	374, 384	350, 1 450	*SkSe*	Se
Betty Anne (RC)	BD10	30/04	343, 374, 390	550, 1 200, 1 450	*ShScSe*	Se
Black Amber	SV8B	02/01	369, 383, 390	550, 1 200	*Sh*	
Black Egg (RC)	BD10	28/43	350, 369, 390	410, 1 550	*Sb*	
Casselman (RC)	BD10	29/43	343, 390	550, 1 200	*ShSc*	
Celebration (1) * (RC)	BD10	30/01	369, 390	550, 1 550	*ShSb*	
Celebration (2) *	SV8B	02/16	369, 390	550, 1 550	*ShSb*	
Crocodile Dundee (RC)	BD10	29/01	384, 390	350, 550	*SkSh*	
Explorer	SV8B	02/04	343, 369	550, 1 200	*(Sh)Sc*	
Extreme (RC)	BD10	28/07	369, 374	1 450, 1 550	*SeSb*	Se
Flavor Rich (RC)	BD10	31/34	369, 374	1 450, 1 550	*SeSb*	Se
Fortune (1) (RC)	BD10	29/04	343, 369	1 200, 1 550	*ScSb*	
Fortune (2)	ZN5A	04/07	343, 369	1 200, 1 550	*ScSb*	

Gaviota (RC)	BD10	28/25	369, 373	n.a	-	
Golden King (RC)	BD10	30/37	355, 369, 374, 390	550, 1 450	-	Se
Golden Kiss * (RC)	BD10	28/31	374, 390	550, 1 450	*ShSe*	Se
Green Red	BD10	30/43	329, 353, 374	1 250, 1 450	*SgSe*	Se
Harry Pickstone * (RC)	BD10	29/07	369, 384	350, 1 550	*SkSb*	Se
Hiromi Red (RC)	BD10	28/16	369, 390	550, 1 550	*ShSb*	
Lady Red * (RC)	BD10	29/22	351, 369, 390	550, 1 450, 1 550	*ShSb*	
Lady West * (RC)	BD10	29/19	340, 350, 369	1250, 1 450, 1 550	*ScSb*	
Laetitia (1) * (RC)	BD10	29/28	374, 390	550, 1 450	*ShSe*	Se
Laetitia (2) *	ZN5A	03/26	374, 390	550, 1 450	*ShSe*	Se
Lamoon (RC)	BD10	29/37	343, 390	550, 1 200	*ShSc*	
Late Lamoon (RC)	BD10	30/10	343, 390	550, 1 200	*ShSc*	
Laroda	SV8B	01/46	351, 354, 369, 374	1 290, 1550	*Sb*	
Mac Verma	SV8B	02/26	374, 390	1 290, 1 450, 3 000	-	Se
Methley (RC)	BD10	28/10	353, 369	1 230, 1 550	*SgSb*	
Mirell (RC)	BD10	30/46	343, 390	550, 1 200	*ShSc*	
Pioneer (1) * (RC)	BD10	28/04	369, 374	1 450, 1 550	*SeSb*	Se
Pioneer (2) *	ZN5A	04/02	369, 374	1 450, 1 550	*SeSb*	Se
Purple King	SV8B	02/29	374, 390	550, 1 290, 3 000	-	
Purple Majesty (RC)	BD10	28/22	355, 374, 390	550, 1 450	*ShSe*	Se
Red Gold * (RC)	BD10	29/31	384, 390	350, 550	*SkSh*	
Reubennel * (RC)	BD10	29/16	350, 369	1 250, 1 550	*SbSg*	
Ruby Prince * (RC)	BD10	31/07	355, 374, 384	350, 1 450	*SkSe*	Se
Ruby Red * (RC)	BD10	29/10	347, 369	1 200	*Sc*	
Ruby Star * (RC)	BD10	29/40	374, 384	350, 1 450	*SkSe*	Se
Ruby Sun * (RC)	BD10	31/01	343, 369	1 200, 1 550	*ScSb*	
Santa Rosa (RC)	BD10	28/19	343, 374	1 200,1 450	*ScSe*	Se
Sapphire (1) * (RC)	BD10	28/13	374	1 450	*Se*	Se
Sapphire (2) *	ZN5A	03/01	374	1 450	*Se*	Se
Satin Gold * (RC)	BD10	31/10	369, 374	1 450, 1 550	*SeSb*	Se
Sensation * (RC)	BD10	28/46	369, 374	1 450,1 550	*SeSb*	Se
Simka (RC)	BD10	31/13	374, 384	350, 1 450	*SkSe*	Se
Songold * (RC)	BD10	29/34	384, 390	350, 550	*SkSh*	
Southern Belle (RC)	BD10	30/16	343, 374	1 200, 1 450	*ScSe*	Se
Souvenir * (1) (RC)	BD10	28/28	374, 390	550, 1 450	*ShSe*	Se
Souvenir (2)	ZN5A	04/07	374, 390	550, 1 450	*ShSe*	Se
Sun Kiss * (RC)	BD10	28/34	343, 390	550, 1 200	*ShSc*	
Sundew * (RC)	BD10	28/37	369, 390	550, 1 550	*ShSb*	
Sunset (RC)	BD10	31/16	374, 384	350, 1 450	*SkSe*	Se
Suplum 6 (RC)	BD10	30/13	343, 390	550, 1 200	*ShSc*	
Suplum 11 (RC)	BD10	29/25	353,369,374,390	550, 1 450	*ShSe*	Se
Suplum 25 (RC)	BD10	29/13	225, 238, 369	1 550	*Sb*	
Suplum 28 (RC)	BD10	31/19	369, 374	1 450, 1 550	*SeSb*	Se
Sweet Aroma (RC)	BD10	31/22	343, 390	550, 1 200	*ShSc*	

Winner (RC)	BD10	31/25	343, 374	1 200, 1 450	*ScSe*	Se
17-10-212 *	SV8B	02/23	369, 390	550, 1 550	*SkSd*	
17-32-118 *	SV8B	01/37	369, 390	350, 1 550	*(Sk)Sb*	
4A-8-13 *	SV8B	02/13	238, 369	350, 1 550	*(Sk)Sb*	
4C-8-20 *	SV8B	02/10	328, 343	350, 1 290	*(SkSd)*	
Hybrids						
S5A-25-5 † *	SV8B	01/16	369, 390	2 000, 1 550	*(Sii)Sb*	
S5A-25-20 † *	SV8B	01/07	369, 374	1 550, 2 800	*Sb(Sc(ap))*	
S5A-26-11 † *	SV8B	01/22	374, 390	550, 1 450, 1 750	*Sh*	
S5A-26-13 † *	SV8B	01/04	369, 374	1 550, 2 800	*Sb(Sc(ap))*	
S5A-26-28 † *	SV8B	01/10	350, 369	1 550, 2 800	*Sb(Sc(ap))*	
S5A-26-30 † *	SV8B	01/19	374	500, 2 800	*(S(iv)Sc(ap))*	
S5A-26-35 † *	SV8B	01/01	369, 374	1 550, 2 800	*Sb(Sc(ap))*	
S5A-33-25 † *	SV8B	01/28	347, 369	1 550	*Sb*	
S5A-34-25 † *	SV8B	01/13	369, 374	500, 2 800	*(S(iv)Sc(ap))*	
S5A-34-28† *	SV8B	01/31	374, 390	550, 2 800	*Sh(Sc(ap))*	
S5A-34-37† *	SV8B	01/25	347, 369	550, 1 550	*(Sh)Sb*	
S5A-35-34 † *	SV8B	01/34	347, 369	1 550, 2 800	*Sb(Sc(ap))*	
By 69-1637P † *	SV8B	03/04	374, 390	550, 1 660, 2 000	-	
Flavor Fall (RC)	BD10	31/28	369, 374	1 450, 1 550	*SeSb*	Se
Flavor King (RC)	BD10	31/31	369, 374	1 450, 1 550	*SeSb*	Se
Green Red (RC)	BD10	30/43	207, 369, 422	1 100, 1 550	-	

* ARC developed cultivars / selections; † (*P. salicina x P. armeniaca*). Accessions designated 1 & 2 were planted on different plots, A & B were designated to duplicated accessions planted on the same plot. (RC) indicates accessions that are the Reference Collection. Tree location labelled as BD10, SV8B and ZN5A indicate trees located in the reference collection, gene bank and the rootstock collection, respectively. – represents cultivars in which genotypes were unresolved.

Table 5.6. Correlation of first and second intron products of plum *S*-alleles, compared with PCR products observed in current study, amplified with Sutherland primers (EM-PC2consFD + EM-PC3consRD) and Sonneveld primers (PaconsI-F + PaconsI-R2).

***S*-alleles**	**1st intron (bp)**	**2nd intron (bp)**
Sb	369	1 550
Sh	390	550
Se	374	1 450
Sc	343	1 200
Sg	353/350	1 230/1 250
Sk	384	350
Sr	No peak in first intron amp	780
Si	No peak in first intron amp	410
Sd	No peak in first intron amp	1 290
Sn	No peak in first intron amp	640
S(c)	No peak in first intron amp	2800

New PCR products identified in current study		
S(i)		3 000
S(ii)		2 000
S(iii)		1 750
S(iv)		500
S(v)		1 100
Sc(ap) (observed in hybrids)	347	2 800

In the present study, 81 plum accessions were genotyped, of which 42 accessions lacked the self-compatibility allele *Se*.

5.3.2.1 First intron amplification in plum

In plum, first intron amplification resulted in product sizes ranging between 329 bp and 422 bp (Table 5.6). In the accessions analysed, three accessions had one peak, 67 accessions had two peaks and in the case of 13 plum accessions ('Betty Anne', 'Black Egg', 'Flavor Fall', 'Golden King', 'Green Red', 'Lady Red', 'Lady West', 'Purple Majesty', 'Ruby Prince', 'Ruby Red', 'Ruby Sun', 'Sulpum 11', 'Suplum 25'), more than two peaks were observed (Table 5.5). Observed amplification products were compared with the sizes of the first intron products of *S*-alleles (Table 5.6). The *Sb* allele, corresponding with a 369 bp products was observed in 41 accessions, making it the most frequent allele. The least freaquent allele was the *Sg* allele, observed in four accessions. Six PCR products, associated with the *Sb*, *Sh*, *Se*, *Sc*, *Sg* and *Sk* alleles, were amplified in the current study. There were six amplification producs that did not correspond with any of the reported *S*-allele (Table 5.6).

There is limited information on first intron alleles in plum; identification of first intron alleles was based on observation of patterns of correlation between first and second intron amplification products. *S*-alleles in brackets (Table 5.5) were scored using only one dataset (*i.e.* either first or second intron amplification products).

5.3.2.2 Second intron amplification in plum

Second intron primers amplified PCR products ranging in size between 350 bp and 3 000 bp (See Appendix B). In eight accessions ('By69-1637', 'S5A-26-11', 'ARC PR-2', 'Betty Anne', 'Lady Red', 'Lady West', 'Purple King' and 'Reubunnel') more than two bands were observed and two accessions had one band (Figure 5.1). *Sb* was the most frequently amplified allele, observed in 39 accessions with a product size of 1550 bp. The least frequent allele was the

Sn allele, associated with a 640 bp product, observed in two accessions. Eleven S-alleles (*Sb*, *Sh*, *Se*, *Sc*, *Sg*, *Sk*, *Sr*, *Si*, *Sd*, *Sn* and *Sc*) were observed in the current study. There were six amplification products that did no match any of the published *S*-allele sizes (Table 5.6).

In the case of some of the hyrids of *P. armeniaca* with *P. salicina*, a band of approximately 2 800 bp was observed, which is presumed to indicate the presence of the self-compatibility (*Sc*) allele inherited from apricot. PCR products (five) which did not correspond with any of the previously published *S*-allele sizes are listed in Table 5.6.

Fig. 5.1 Second intron products, amplified using the EM-PC primers. DNAs of plum accessions from the reference collection arranged as: 16-Sensation (*SeSb*), 17- Crocodile Dundee (*SkSh*), 18- Fortune (*ScSb*), 19- Harry Pickstone (*SkSb*), 20- Ruby Red (*Sc*), 21- Suplum 25 (*Sb*), 22- Reubennel (*SgSb*), 23- Lady West (*ScSb*), 24- LadyRed (*ShSb*), 25- Suplum 11 (*SeSh*), 26- Laetitia (*ShSe*), 27- Red Gold (*SkSh*), 28- Songold (*SkSh*), 29-Lamoon (*ShSc*), 30- Ruby Star (*SkSe*).

5.4 Discussion

In this study, identification of *S*-genotypes of 87 plum accessions was attempted using consensus primers amplifying the first and second intron region of the *S-RNase* gene. The

degenerate primers (EM-PC2consFD + EM-PC3consRD) (Sutherland *et al*., 2004a) were useful in amplifying the second intron region. Furthermore, the fluorescent primers PaconsI-F + PaconsI-R2 used for precise product length determination of first intron products, reported by Ortega *et al*. (2005), was useful in discriminating between first intron products which are characterised by short size variability. Using these described approaches, amplification of nine *S*-alleles was observed, *i.e. Sb*, *Sh*, *Se*, *Sc*, *Sg*, *Sk*, *Sr*, *Si* and *Sd*.

5.4.1 Primers amplifying the S-RNase gene

5.4.1.1 First intron amplification

First intron amplification in plum was initially attempted using two sets of primers SRc-F + SRc-R and PaconsI-F + PaconsI-R, in which the forward primers were both fluorescently labelled. However, the SRc primers yielded poor amplification products, revealing complex patterns which made scoring of alleles difficult. Even with the PaconsI-F + PaconsI-R primers, scoring of alleles proved to be slightly challenging on some ocassions due to the presence of supplementary peaks, possibly caused by an issue on the capillary analysis. Nevertheless, identification of alleles was usually confirmed by correlation with first intron products. Failure to amplify particular alleles may be due to differences in primer sequences which are not matching the nucleotide sequences of the alleles. Complementarity between primers and target sites is often crucial for PCR amplification, as mismatches can severely reduce priming efficiency (Stadhouders *et al*., 2010). Interpretation of plum first intron data proved to be challenging due to the limited information available. The presence of more than the expected number of peaks made scoring of alleles difficult, however combining the available second intron data and the previously published first intron products, it was possible to designate preliminary genotypes from the observed products sizes.

5.4.1.2 Second intron amplification

For second intron amplification, primers EM-PC2consFD + EM-PC3consRD resulted in good amplification but EM-PC2consFD + EM-PC5consRD gave poor amplification products. Other studies have revealed inconsistencies with amplification of some alleles. This problem was overcome by using additional primer sets. Furthermore allele-specific primers can be used in order to give conclusive results. Using allele-specific primers to distinguish between certain alleles has proven to be a useful method in sweet cherry (Sonneveld *et al*., 2001) and Japanese apricot (Tao *et al*., 2000) as well as Japanese plum (Sapir *et al*., 2004). In plum, Sapir *et al*. (2004), designed allele-specific primers for the five Japanese plum alleles labelled

with numeric codes (*S1, S3, S4, S5* and *S6*). These primers were not only useful in revealing correspondence between the two nomenclatures, but in addition, the primer specific to *S5* (which is equal to the *Se* allele) can be used as a marker for determining self-compatible cultivars. However this is not applicable in all cultivars, as Sapir (2008) later identified cultivars that showed the *Se* allele but were not self-compatible.

Mousavi *et al*. (2011) reported on the *S*-genotyping of almond cultivars, in which they initially used the EM-PC2consFD + EM-PC3consRD to amplify the second intron alleles. When inconsistencies were observed, *e.g.* amplification of only one band or unclear correspondence between the band sizes observed for the second intron and those previously reported, another primer set was used (*i.e.* PaconsI-F + EM-PCcons5RD) to resolve inconsistencies and improve identification of alleles.

5.4.2 Self-compatibility in Japanese plum

The *Se* allele that is often associated with self-compatibility was observed in 39 accessions, with a product size of 347 bp and 1 450 bp for the first and second intron, respectively. The association of the *Se* allele with self-compatibility in Japanese plum was initially established by Beppu *et al*. (2002), based on the presence of the *Se* allele in several self-compatible cultivars. Guerra *et al*. (2009) also reported *Se* in the SC cultivars 'Laetitia' (*SeSh*), 'Casselman' (*ScSe*), 'Santa Rosa' (*ScSe*) and 'Simka' (*SeSk*).

In an attempt to further understand the occurence of the self-compatibility phenotype in plum, a study by Beppu *et al*. (2005) suggested that neither a deletion of the *S-RNase* gene nor prevention of transcription of the *S-RNase* gene was the cause of self-incompatibility in the *Se* haplotype. However later work by Watari *et al*. (2007) showed that the *Se-RNase* transcript levels in the pistil are much lower than those of the SI *S*-haplotypes of Japanese plum and that little, if any, Se-RNase protein was accumulated. The findings by the Watari *et al*. (2007) study may suggest that the low accumulation of Se-RNase may be the possible cause of SC observed in the *Se* haplotype.

Interestingly, Guerra *et al*. (2009) noted other cultivars namely, 'Nubiana' (*SbSh*) and 'Zanzi Sun' (*SbSc*), were self-compatible, but did not display the *Se* allele. These results suggested that additional sources of self-compatibility, other than the *Se* allele, may be operating in Japanese plum cultivars. In a later study, Guerra *et al*. (2015) reported that self-compatibility was also associated with several other *S*-haplotypes: *Sb* (Guerra *et al*., 2009; Beppu *et al*., 2010), *Sg* (Beppu *et al*., 2012) and *Ss* (Beppu *et al*., 2012). Moreover, numerous Japanese plum cultivars displaying the *Se* or *Sb* alleles have been described as self-incompatible.The

mechanism of action behind self-compatibility in plum is not well understood and further investigation to understand this system in more depth is essential. One way would be to investigate the cosegregation of the *Se*-allele in a controlled cross and another would be to investigate gene expression *i.e.* using transcriptomic and quantitative approaches.

5.4.3 Self-incompatibility in Japanese plum

There were minor inconsistencies in the literature regarding some of the intron products for several S-alleles. For example, the second intron product of the *Sb* allele was reported by Beppu *et al*. (2002) as 1 540 bp whereas Halasz and Hegedus (2007) reported it to be 1 550 bp, which is a minor discrepancy if tested on agarose gel elctrophoresis. Additionally, the second intron product size of the *Sc* allele was reported by Beppu *et al*. (2002) to be 1 170 bp whereas Halasz and Hegedus (2007) reported it as 1 200 bp. This inconsistency may be the result of different primers and/or different primer binding sites used for amplification between the different publications. Hegedus and Halasz (2007) reportedly used the EM-PC2cons + EM-PC3cons primers and Beppu *et al*. (2002) used the PruC2 + PCER primers. In such cases, it is important that information regarding *S*-alleles must be compared between different research groups in order to maintain consistency of results. This can be accomplished through publishing genotypes of reference cultivars and exchanging DNA between laboratories.

In this study self-(in)compatibility genotypes of 12 of the 81 plum accessions analysed had previously been published. Only the *S*-genotypes of the cultivars ‘Harry Pickstone’ (*SbSk*) and ‘Songold’ (*SkSh*) corresponded with the findings of the current study. The genotypes of the other seven cultivars ‘Black Amber’, ‘Casselman’, ‘Fortune’, ‘Laroda’, ‘Pioneer’, ‘Sapphire’ and ‘Simka’ were different from S-genotypes reported previously. For the cultivars ‘Laroda’ (*SbSc*), ‘Pioneer’ (*SeSb*), ‘Casselman’ (*ScSe*) and ‘Simka’ (*SeSj*), only one of the alleles was consistent with the reported genotype.

Two cultivars, namely ‘Lady West’ and ‘Betty Anne’, showed three bands and peaks in the first and second intron amplification products, suggesting that they were possibly triploid, trisomic or have three rather than two copies of the locus. Other plum accessions also had multiple amplification products in either the first or second intron; hence the genotypes of the accessions were inconclusive.

Consensus and allele-specific primers, designed to amplify the first and second introns of the *S-RNase* region of *Prunus* species, have already proved to be an efficient and reliable method

for identification of S-alleles in cherry (Sonneveld *et al*., 2003), almond (Ortega *et al*., 2005), apricot (Halasz *et al*., 2005) and Japanese plums (Beppu *et al*., 2012).

5.5 Conclusion

The genotyping of the ARC accessions and cultivars from the apricot and plum collection with consensus primers amplifying the first or second intron regions of the *S-RNase* gene were reasonably useful. In the current study we were able to identify 14 previously published SI alleles in apricot and nine published SI alleles in plum. Additionally, five PCR products not previously published were observed in plum. Even though self-compatibility in plum is associated with the *Se* allele, the findings in the current study showed evidence that the *Se* haplotype may not be the only source of SC because some cultivars known to be SC, lacked the *Se* allele.

The findings obtained in the current study provide useful information for farmers and breeders. Knowledge of the *S*-genotypes of commercially important SA cultivars offers some level of certainty for the commercial farmers to expect good yield if they plant cultivars with different SI genotypes or self-compatible cultivars.

5.6 References

Beppu K., Komatsu N., Yamane H., Yaegaki H., Yamaguchi M., Tao R., Kataoka I. (2005): *Se*-haplotype confers self-compatibility in Japanese plum (*Prunus salicina* Lindl.). Journal of Horticultural Science and Biotechnology, 80: 760-764.

Beppu K., Kumai M., Yamane H., Tao R. and Kataok I. (2012): Molecular and genetic analysis of the *S*-haplotype of the self-compatible Japanese plum (*Prunus salicina* Lindl.) 'Methley'. Journal of Horticultural Science and Biotechnology, 87: 493-498.

Beppu K., Yamane H., Yaegaki H., Yamagachi M., Kataoka L. and Tao R. (2002): Diversity of *S-RNase* genes and *S*-haplotypes in Japanese plum (*Prunus salicina* Lindl.). Journal of Horticultural Science and Biotechnology, 77: 658-664.

Beppu K., Yamane H., Yaegaki H., Yamagachi M., Kataoka L. and Tao R. (2003): Determination of *S*-haplotypes of Japanese plum (*Prunus salicina* Lindl.) cultivars by PCR and cross pollination tests. Journal of Horticultural Science and Biotechnology, 78: 315-318.

Bošković R. and Tobutt KR. (1996): Correlation of stylar ribonuclease zymograms with incompatibility alleles in sweet cherry. Euphytica, 90: 245-250.

De Klerk J. and Smith C. (2013): Pollination requirements of Japanese plum (*Prunus salicina*) cultivars. Technology, Oct-Nov: 57-62.

De Nettancourt D. (2001): The basic features of self-incompatibility. In: De Nettancourt D. (ed) Incompatibility and incongruity in wild and cultivated plants. Springer, Berlin, pp 1-24.

Doyle J. J. and Doyle J. L. (1990): Isolation of plant DNA from fresh tissue. Focus, 12, 13-15.

East E.M. and Mangelsdorf A.J. (1925): A new interpretation of the hereditary behaviour of self-sterile plants. Proceedings of the National Academy of Science of the United States of America, 11: 166-171.

Entani T., Iwano M., Shiba H., Che F.S., Isoga A. and Takayama S. (2003): Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: identification of a pollen expressed F-box gene with allelic diversity. Gene Cells, 8: 203-213.

Guerra M.E., Lopez-Corrales M. and Wünsch A. (2012): Improved S-genotyping and new incompatibility groups in Japanese plum. Euphytica, 186: 445-452.

Guerra M.E., Rodrigo J., López-Corrales M., Wünsch, A. (2009): *S-RNase* genotyping and incompatibility group assignment by PCR and pollination experiments in Japanese plum. Plant Breeding, 128: 304-311.

Guerra ME and Rodrigo J. (2015): Japanese plum pollination: A review. Scientia Horticulturae, 197: 674-684.

Halasz J., Hegedus A., Szabo Z., Nyeki J. and Pedryc A. (2007): DNA-based S-genotyping of Japanese plum and pluot cultivars to clarify incompatibility relationships. Hortscience, 42: 46–50.

Halasz J., Kurilla A., and Hegedus A. (2014): Preliminary characterisation of the self-incompatibility genotypes of European plum (*Prunus domestica* L.) cultivars. International Journal of Horticultural Science, 20: 23-26.

Halasz J., Pedryc A., Ercisli S., Yilmaz K.U. and Hegedus A. (2010): *S*-genotyping supports the genetic relationships between Turkish and Hungarian apricot germplasm. Journal of American Society of Horticultural Science, 135: 410-417.

Halasz J., Szikriszi B., Ercisli S., Yilmaz K.U., Dogan A., Szabo Z., Nyeki J., Pedryc A. and Hegedus A. (2012): *S*-Locus genotyping on stone fruits. Acta Horticulturae, 967: 113-119.

Hegedus A. and Halasz J. (2007): Recent findings on the tree fruit self-incompatibility studies. International Journal of Horticultural Science, 13: 7-15.

Ikeda K., Igic B., Ushijima K., Yamane H., Hauck N.R., Nakano R., Sassa H., Iezzoni A.F., Kohn J.R. and Tao R. (2004): Primary structural features of the *S* haplotype-specific F-box protein, *SFB*, in *Prunus*. Sexual Plant Reproduction, 16: 235-243.

Nunes M.D.S., Santos R.A.M., Ferreira S.M., Vieira J., and Vieira C.P. (2006). Variability patterns and positively selected sites at the gametophytic self-incompatibility pollen *SFB* gene in a wild self-incompatible *Prunus spinosa* (Rosaceae) population. New Phytologist, 172: 577–587.

Ortega E., Sutherland B.G., Dicenta F., Bošković R. and Tobutt K.R. (2005): Determination of incompatibility genotypes in almond using first and second intron consensus primers: detection of new *S* alleles and correction of reported *S* genotypes. Plant Breeding, 124: 188-196.

Roalson E.H. and McCubbin A.G. (2003): *S-RNase* and sexual compatibility: structure, functions, and evolutionary perspectives. Molecular Phylogenetics and Evolution, 29: 490-506.

Romero C., Vilanova S., Burgos L., Martinez-Calvo J. Vicente M., Llacer G. and Badenes M.L. (2004): Analysis of the *S*-locus structure in *Prunus armeniaca* L.: Identification of *S*-haplotypes *S-RNase* and F-box genes. Plant Molecular Biology, 56: 145-157.

Sapir G., Stern R.A., Eisikowitch D. and Goldway M. (2004): Cloning of four new Japanese plum *S*-alleles and determination of the compatibility between cultivars by PCR analysis. Journal of Horticultural Science and Biotechnology, 79: 223-227.

Sapir G., Stern R.A., Shafir S. and Goldway M. (2008): *S-RNase* based *S*-genotyping of Japanese plum (*Prunus salicina*) and its implication on the assortment of cultivar-couples in the orchard. Scientia Horticulturae, 118: 8-13.

Sassa H., Hirano H., Nshio T. and Koba T. (1997): Style-specific self-compatibility mutation caused by deletion of the S-RNase gene in Japanese pear (*Pyrus serotina*). The Plant Journal, 12: 223-227.

Sonneveld T., Robbins T.P. and Tobutt K.R. (2006): Improved discrimination of self-incompatibility *S-RNase* alleles in cherry and high throughput genotyping by automated sizing of first intron polymerase chain reaction products. Plant Breeding, 125: 305-307.

Sonneveld T., Tobutt K.R. and Robbins T.P. (2003): Allele-specific PCR detection of sweet cherry self-incompatibility (*S*) alleles *S1* to *S16* using consensus and allele-specific primers. Theoretical and Applied Genetics, 107: 1059-1070.

Stadhouders R., Pas S.D., Ander J., Voermans J., Mes T.H. and Schutten M. (2010): The effect of primer template mismatches on the detection and quantification of nucleic acids using the 5' nuclease assay. Journal of Molecular Diagnostics, 12: 109-117.

Sutherland B. G., Robbins T.P. and Tobutt K.R. (2004): Primers amplifying a range of *Prunus S*-alleles. Plant Breeding 123: 582-584.

Tao R., Habu T., Yamane H., Sugiura A. and Iwamoto K. (2000): Molecular markers of self-compatibility in Japanese apricot (*Prunus mume*). HortScience, 35: 1121-1123.

Tao R., Yamane H., Sassa H., Mori H., Gradziel T.M., Dandekar A.M. and Sugiura A (1997): Identification of stylar RNases associated with gametophytic self-incompatibility in almond (*Prunus dulcis*). Plant and Cell Physiology, 38: 304-311.

Tao R., Yamane H., Sugiura A., Maruyama H., Sassa H. and Mori H. (1999): Molecular typing of *S*-alleles through identification, characterisation and cDNA cloning for *S-RNases* in sweet cherry. Journal of the American Society for Horticultural Science, 124: 224-233.

Ushijima K., Sassa H., Dandekar A.M., Gradziel T.M and Tao R. (2003): Structural and transcriptional analysis of the self incompatibility locus of almond. Identification of a pollen expressed F-box gene with haplotype-specific polymorphism. Plant Cell, 15: 771-781.

Vilanova S., Romero C., Liacer G. and Badenes M.L. (2005): Identification of Self-(in)compatibility alleles in apricot by PCR and sequence analysis. Journal of the American Society for Horticultural Science, 130: 893-898.

Watari A., Hanada T., Yamane H., Esumi T. and Tao R. (2007): A low transcriptional level of *Se-RNase* in the *Se*-haplotype confers self-compatibility in Japanese plum. Journal of American Society of Horticultural Science, 132: 396-406.

Yaegaki H., Shimada T., Moriguchi T., Hayama H., Haji T., and Yamaguchi M. (2001): Molecular characterisation of *S*-RNase genes and *S*-genotypes in the Japanese apricot (*Prunus mume* Sieb. et Zucc.). Sexual Plant Reproduction, 13: 251-257.

Yamane H., Ikeda K., Hauck N.R., Iezzoni A.F. and Tao R. (2003): Self incompatibility (S) locus region of the mutated *S6*-haplotype of sour cherry (*Prunus cerasus*) contains a

functional pollen *S* allele and non-functional pistil S-allele. Journal of Experimental Botany, 54: 2431-2437.

Yamane H., Ikeda K., Ushijima K., Sassa H. and Tao R. (2003): A pollen-expressed gene for a novel protein with an F-box motif that is very tightly linked to a gene for *S-RNase* in two species of cherry, *Prunus cerasus and Prunus avium*. Plant Cell Physiology, 44: 764-769.

Yamane, H., Tao R., Sugiura A., Hauck N.R., and Iezzoni A.F. (2001): Identification and characterisation of *S-RNases* in tetraploid sour cherry (*Prunus cerasus*). Journal of American Society Horticultural Science, 126: 661-667.

Zhang L., Chen X., Chen X.L., Zhang C., Liu X., Ci Z.J., Zhang H., Wu C. and Liu C. (2008): Identification of self-incompatibility (*S*-) genotypes of Chinese apricot cultivars. Euphytica, 160: 241-248.

Chapter 6:
General discussion and future considerations

6.1 Introduction

Apricot and Japanese plum form part of the economically important stone fruit industry in South Africa, of which a large portion is designated for the export market. The Agricultural Research Council (ARC) Infruitec-Nietvoorbij conducts an apricot and plum breeding programme that develops cultivars with good eating quality, disease resistance and that are

well adapted to South African environmental conditions. The breeder was aware that the ARC apricot and plum gene bank (120 apricot and 81 plum accessions), contains accessions that may be mis-identified and is poorly characterised. Funding, made available by the Technology and Human Resource for Industry Programme (THRIP), allowed for the molecular fingerprinting and molecular characterisation of the gene bank. This is helping ro verify trueness to type of the accessions and to determine the self (in)-compatibility genotypes. In addition, the molecular characterisation of the self-(in)compatibility locus (*S*-locus) has allowed identification of self-compatible and self-incompatible genotypes of the apricot and plum collections.

6.2 Fingerprinting of apricot and plum collections using microsatellite markers

In the absence of a generally agreed list of microsatellite primers that can be used for the fingerprinting of *Prunus* species, a set of fluorescently labelled peach-derived microsatellite primers with a reported high transferability between related species was used. The grouping of such primers into multiplexes with sufficient efficiency and minimum background “pull-up” when sized automatically posed a challenge and required careful optimisation. Although the use of markers in multiplex reactions proved to be cost effective for the fingerprinting of apricots and plums, some of the markers failed to produce scorable results without a clear indication that multiplexing was the cause of the failure.

Although microsatellites are polymorphic, highly transferable and cost effective once developed, new technologies exploring Single Nucleotide Polymorphisms (SNPs) may supersede them as the genome sequences of apricot and plum is now available. Strategies that could alternatively be used in the fingerprinting of *Prunus* species include SNP genotyping assays and genotyping by Sequencing (Bianchi *et al*., 2015). This technique offers a significant decrease in the time and cost of genotyping in apricot and plum (Salazar *et al*., 2015).

6.3 Characterisation of the self-(in)compatibility (SI) locus

The *S*-genotyping of the ARC apricot and plum gene bank collection was based on the use of previously published primers flanking one or other of the two *SRNase* introns of the *S* locus and reported product sizes of various alleles. In apricot, up to 30 *S*-alleles corresponding with the second intron of the *S-RNase* gene have been reported and were used as reference alleles for the current study. Comparison with the previous *S*-allele data proved to be very useful in the characterisation of product sizes identified in the current study and emphasises the

importance of published findings and sharing of information amongst research laboratories. The use of previously published and unlabelled primers was essential for the identification of the larger second intron products in apricots and plums. Fluorescently labelled primers were useful in characterising smaller product sizes of the first intron. The current study represents the first use of the fluorescent PaconsI-F + PaconsI-R2 primers were useful on a wider range of apricot and plum accessions and proved to be time and cost effective whilst allowing precise identification of smaller products with little variation.

The current study observed novel product sizes that have not been previously reported and paves the way for further studies for further characterisation of the amplicons and possibly adding new alleles to the existing list of published *S*-alleles in apricot and plum. Confussion of genotypes with similarities on self-(in) compatibility status by De klerk and Smith (2013) provides a stepping-stone for further investigation into the origin of the self-compatible phenotype. Analysing the sequences of the SC cultivars that lack the *Se* allele may identify possible mutations present in the *SRNase* and even the *SFB* genes.

6.4 Use of microsatellites for gene bank management

Microsatellite fingerprints were used to confirm and correct the names and parentages of some of the accessions in the gene banks and reference collections. The findings of this study will facilitate the improvement of the gene bank management by providing useful baseline data prior to repropagation. The possible development of an internet-based database is worth considering as it may provide a means for rapid verification by comparing genotypes but it would be important to include some reference to calibrate allele sizes.

Microsatellite markers have been used recently to fingerprint the ARC apple and pear gene banks (Mhelembe, 2015) as well as peaches and nectarines (Kwalimba, personal communication). The ARC's Institute for Tropical and Subtropical Crops (ITSC) has recently started fingerprinting the citrus rootstock collection (Bijzet *et al*., 2014). Similar studies on other fruit collections at ARC such as cherry, almond and olive is desirable, even though the ARC does not currently run breeding programmes for these crops.

The characterisation of the self-(in)compatibility locus will be useful for producers when selecting cross-pollinators and breeders when selecting parents in both apricot and plum. Similarly, knowledge of the apple, pear and almond *S*-genotypes is necessary to ensure effective cropping of commercial cultivars and effective breeding. Other agronomic genes such as the ACS1 may provide useful information to the apricot and plum breeding programme. The ACS1 gene codes for the enzyme 1-aminocyclopropane-1-carboxylate

(ACC) synthase, which is an important component in ethylene production (Yang and Hoffman, 1984). Allelic variation effect ethylene production and Mhlelembe (2015) has investigated this gene in the ARC apple collection. If similar variants of the ACS1 gene exist in apricot, they could be useful for the breeding of low ethylene producing cultivars with delayed.

6.5 Application to the apricot and plum breeding and fruit production industry

For the accessions confirmed as true to type by microsatellite fingerprinting data, crosses in the apricot and plum breeding programme can be made with confidence and crosses false accessions can be excluded. Additionally the parentages of cultivars can be verififed.

S-genotyping will facilitate identification of pollinators for the self-incompatible cultivars and also help identify self-compatible cultivars that do not require pollinators. This information will be useful, particularly to the production industry when planting orchards and choosing pollinators. It will also be useful to the breeder planning crosses by identifying cultivars that are cross compatible. Additionally, the current findings can be incorporated with the lists of self-incompatibility groups (Table 2.6) (Guerra and Rodrigo, 2015; Halasz *et al*., 2010). Knowledge of the SI-genotypes of SI cultivars that will be introduced into the breeding programme can also be useful to maintain genetic diversity within the gene bank.

SC cultivars can be used to breed for self-compatibility. And it should be noted that, in general, if an SI cultivar is pollinated with an SC cultivar will ultimately share an Sc allele, then al the seedlings should be SC. These cultivars can also be good candidates for cultivars that have high productivity without the need of a pollinator.

6.6 Limitations of the current study

Not all the microsatellites initially selected were used successfully in the study, three markers in apricot and five markers in plum were removed from further analysis. Despite the failure of some markers to result in scorable amplification products, it was possible to distinguish the accessions. However the missing data could be useful in future. Therefore, further optimisation for the markers that failed is still necessary in order to obtain a full data set to facilitate comparison with other studies that used the same markers and have a larger set of data. For improved discrimination of cultivars, additional markers could be used. The absence of a generally recommended set of microsatellite primers that can be used for apricot and plum fingerprinting creates difficulties with selection of primers for multiplexing and prolongs multiplex optimisation for efficient and successful fingerprinting.

The limited available genome information regarding first intron SI alleles in both apricot and plum caused difficulties when correlating first intron product size and second intron products. We were, however, able to correspond first intron products with second intron alleles and propose provisional first intron alleles. It would therefore be useful for researches in the field to publish first intron alleles in order to facilitate accurate and reliable SI-genotyping of apricot and plum cultivars.

6.7 Future considerations

The current study demonstrates the use of microsatellites as a rapid and cost effective tool for cultivar identification and parentage verification. Developing a generally recommended multiplex set of primers for fingerprinting apricot and plum accessions would be useful for the stone fruit industry. It could simplify the reproductivity of data that would be comparable amongst laboratories. This study also displays the effective use of molecular methods for characterisation of a germplasm collection. The molecular approach can be used for characterisation of other horticulturally important genes for which functional markers are available, such as endo-PG and ACS, which are involved in the fruit ripening process.

The data from the fingerprinting and *S*-genotyping chapters were recorded and will be used to annotate of the ARC gene banks to enhance appropriate gene bank management practice.

6.8 References

Bianchi V.J., Rubio M., Trainotti L., Verde I., Bonghi C and Martinez-Gomez P. (2015): Prunus transcription factors: breeding perspectives. Frontiers in Plant Science, 6: 443.

Bijzet Z., Safodien S. and Booyse M. (2014): Simple Sequence Repeat (SSR) markers to determine the level genetic diversity of citrus rootstocks in a mega environment. Abstract of: 10th Southern African Plant Breeding Symposium, March 10-12, Thaba 'Nchu, South Africa, p 23.

De Klerk J. and Smith C. (2013): Pollination requiremtns of Japanese plum (*Prunus salicina*) cultivars. Tegnologie, 57-62.

Guerra ME and Rodrigo J. (2015): Japanese plum pollination: A review. Scientia Horticulturae, 197: 674-684.

Guerra M.E., Rodrigo J., López-Corrales M., Wünsch, A. (2009): *S-RNase* genotyping and incompatibility group assignment by PCR and pollination experiments in Japanese plum. Plant Breeding, 128: 304-311.

Halasz J., Pedryc A., Ercisli S., Yilmaz K. U. and Hegedus A. (2010): *S*-genotyping supports the genetic relationship between Turkish and Hungarian apricot germplasm. Journal of American Society of Horticultural Science, 135: 410-417.

Mhelembe K.G. (2015): Molecular Characterisation of ARC pome fruit collections in South Africa. Unpublished MSc thesis Stellenbosch University, pp: 1-171.

Salazar J. A., Rubio M., Ruiz D., Tartarini S., Martínez-Gómez P. and Dondini L. (2015): SNP development for genetic diversity analysis in apricot. Tree Genetics andGenomes, 11: 15.

Yang S.F. and Hoffman N.E. (1984): Ethylene biosynthesis and its regulation in higher plants. Annual Review of Plant Physiology, 35:155-189.

APPENDICES

Appendix A: Gel electrophoresis images of second intron PCR products amplified using EM-PC2consFD + EM-PC3consRD primer pair. Amplified products were correlated to previously reported S-alleles by comparing sizes of amplified bands in measured against a 1kb plus ladder.

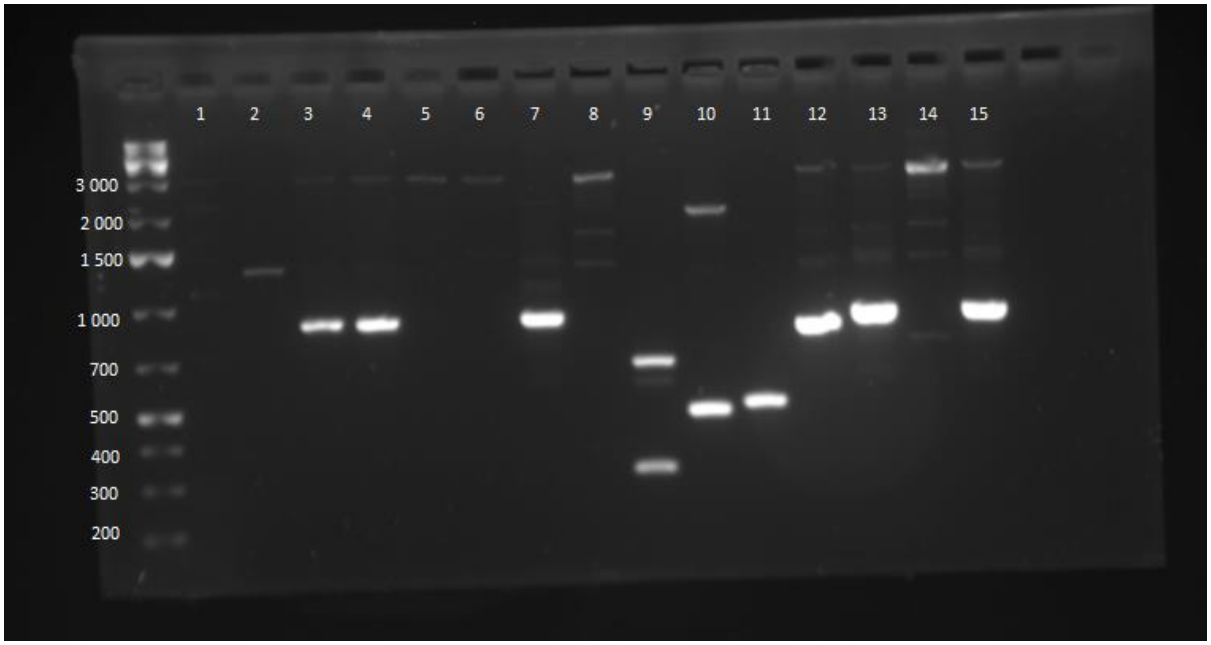


Fig A.1: Apricot gene bank accessions 1 to 15

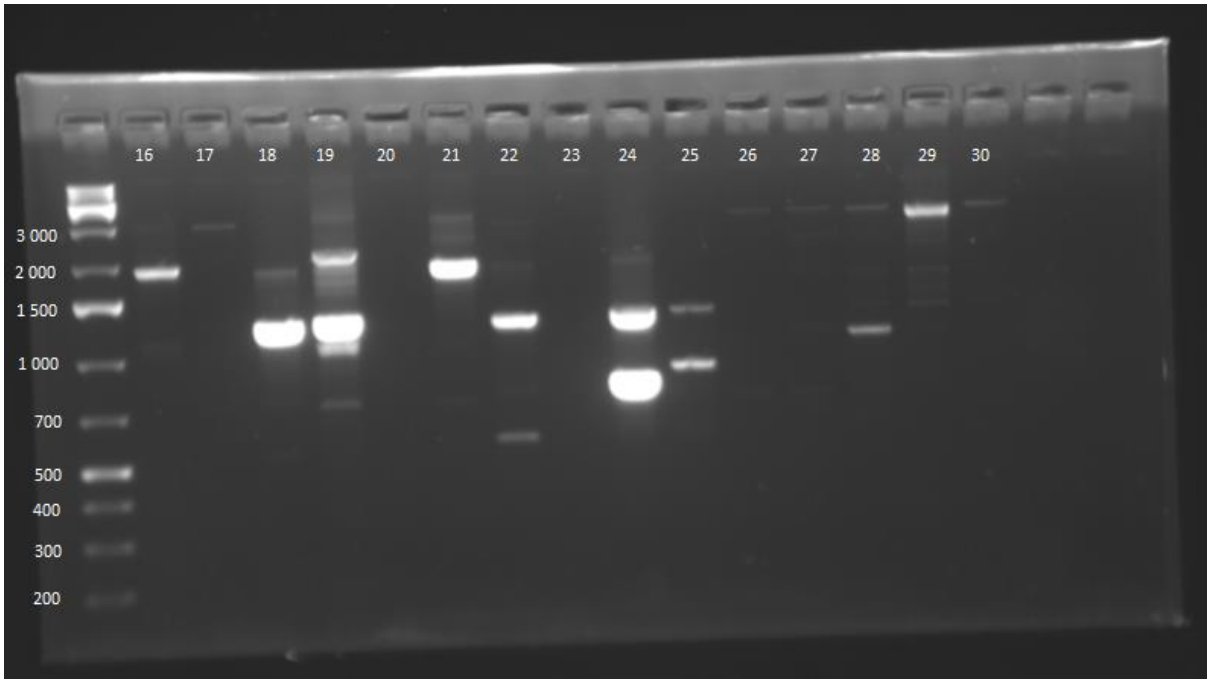


Fig A.2: Apricot gene bank accessions 16 to 30

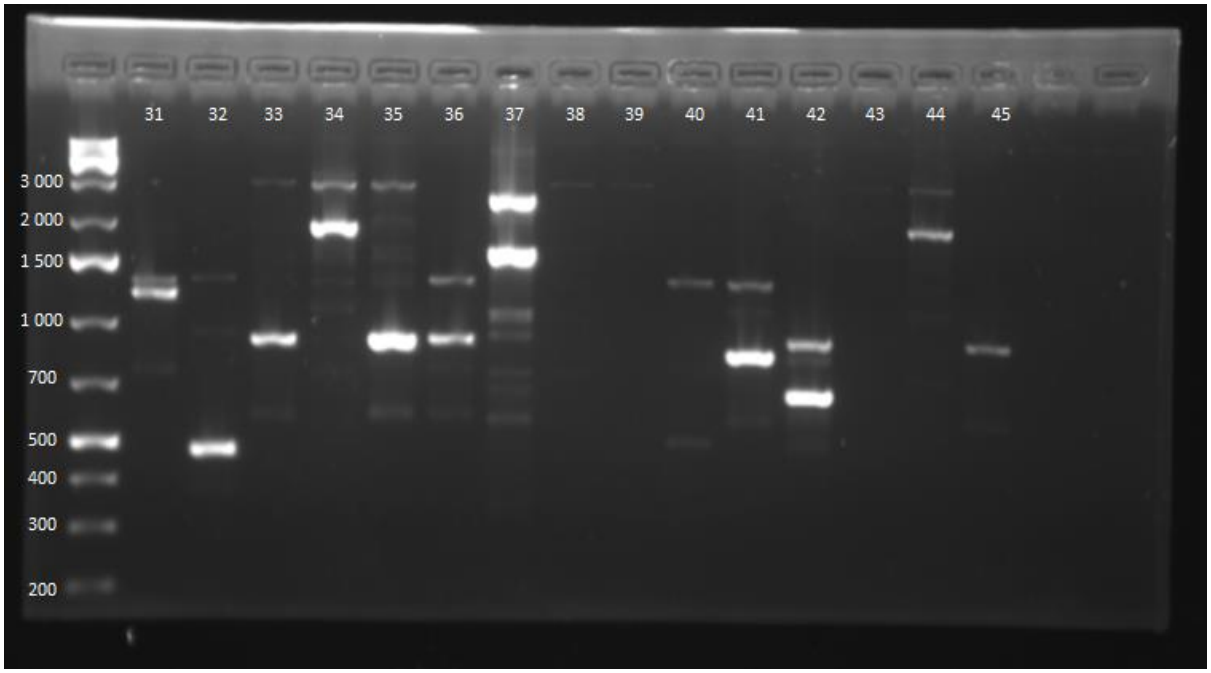


Fig A.3: Apricot gene bank accessions 36 to 45

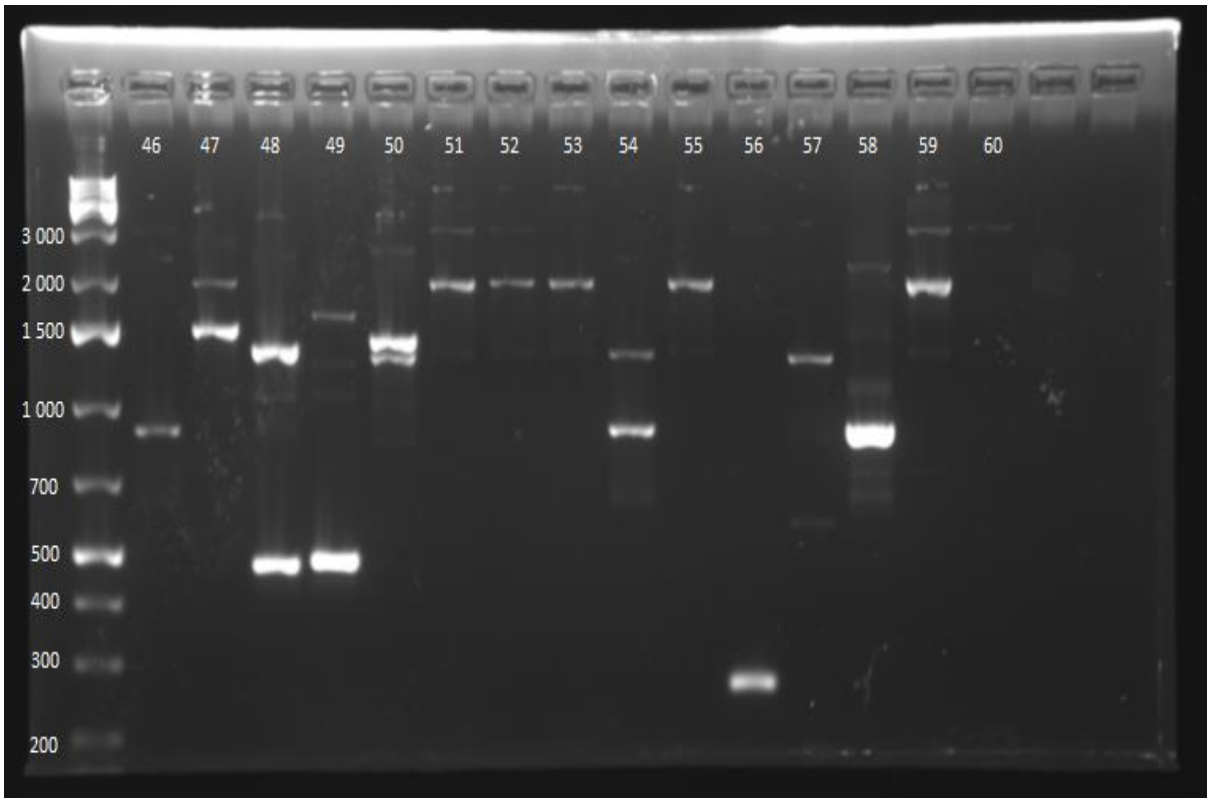


Fig A.4: Apricot gene bank accessions 46 to 60

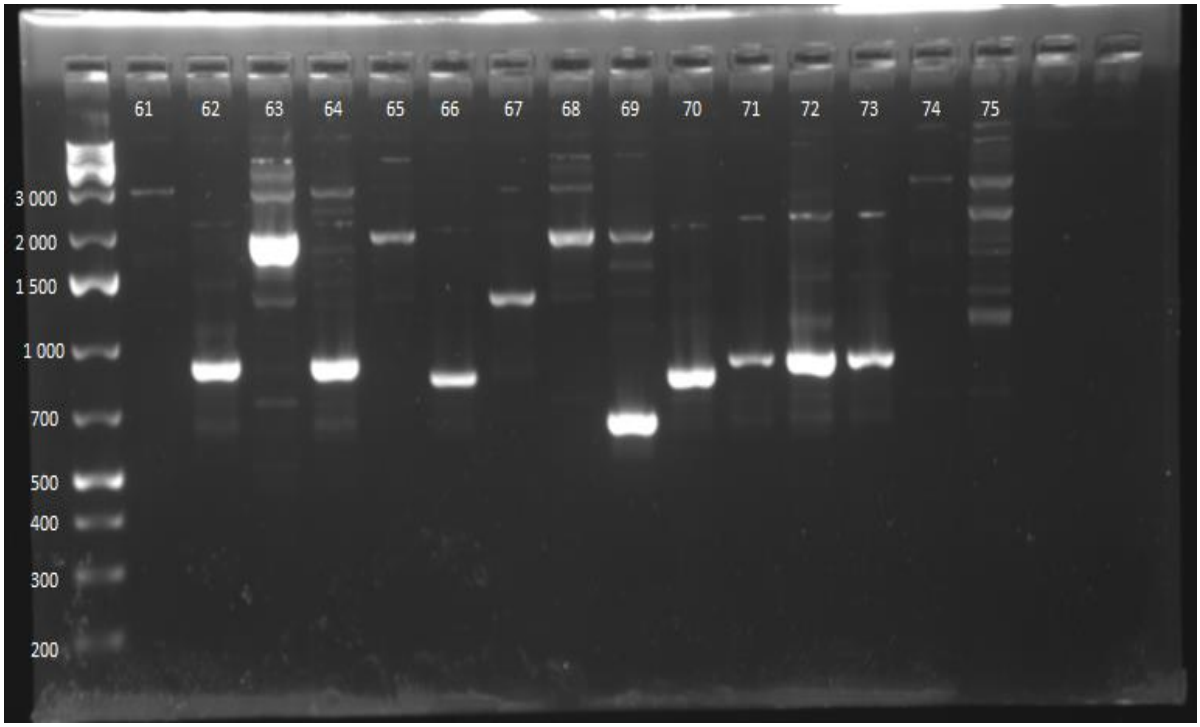


Fig A.5: Apriot gene bank accessions 61 to 75

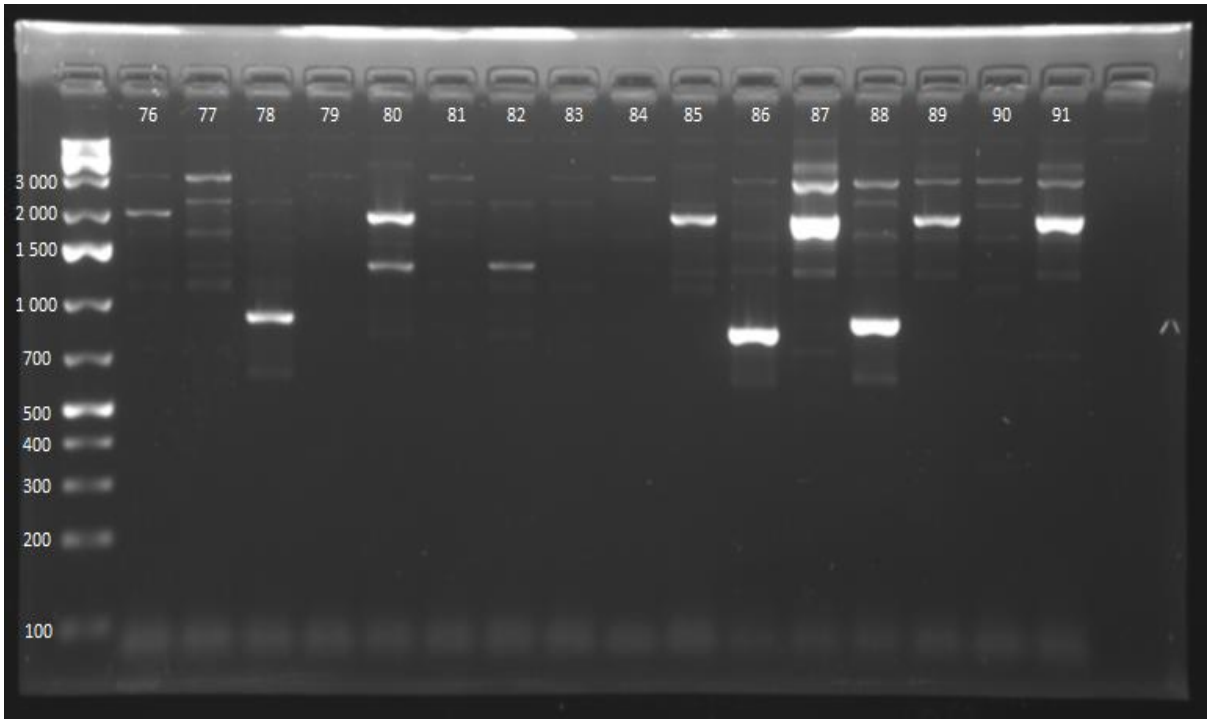


Fig A.6: Apricot gene bank accessions 75 to 91

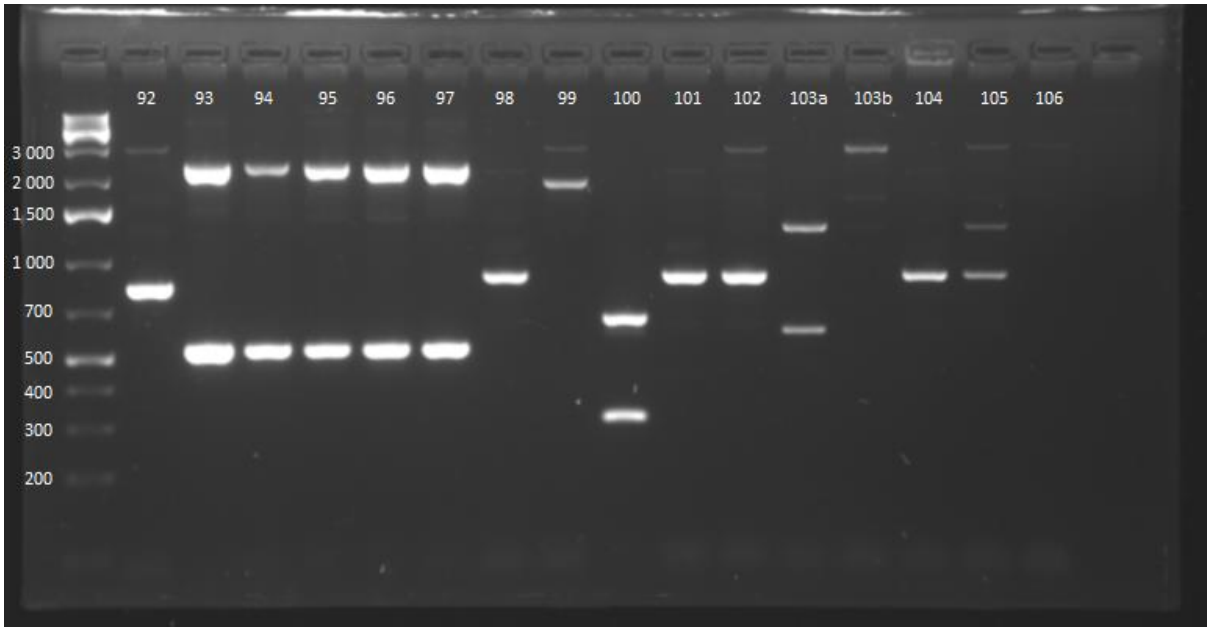


Fig A.7: Apricot gene bank accessions 92 to 106

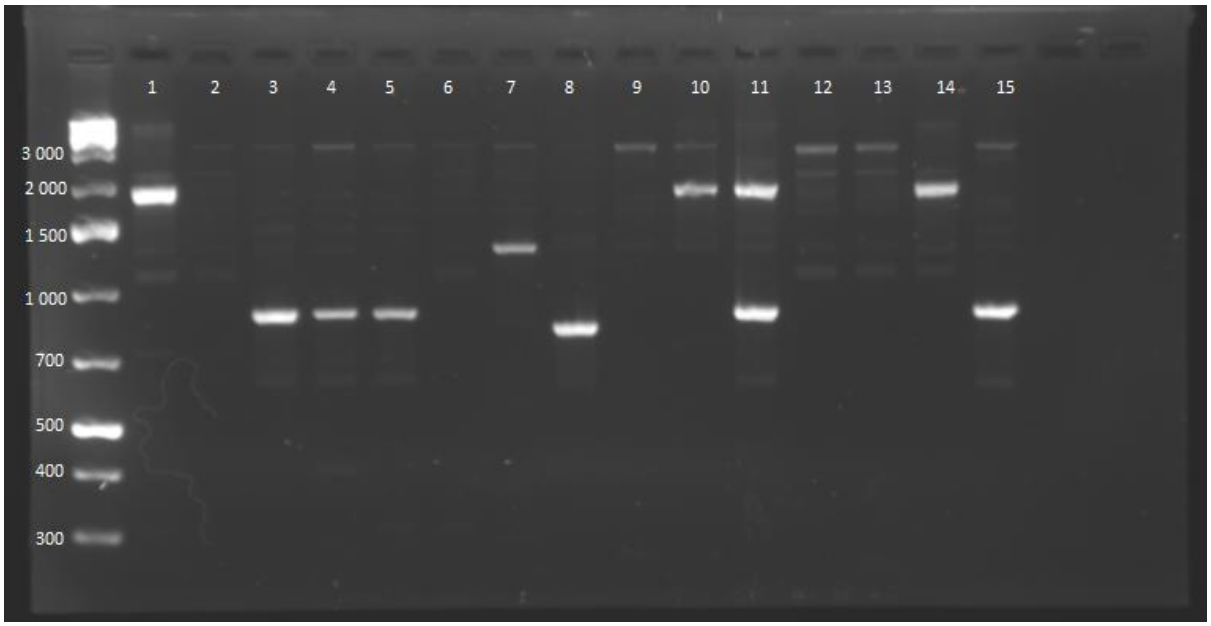


Fig A. 8: Apricot reference collection cultivars 1 to 15

Table A: Listed below are apricot accessions on gel images according to arrangement on apricot gene bank and refence collection.

#	Gene bank accessions	5	1007A	10	Junshiro Los
1	Supergold*	6	15-ED-103	11	1002B
2	Cape Bebeco	7	Icapl	12	1004B
3	Soldonné*	8	1006B	13	1002A
4	Ladisun*	9	Ken Psar	14	1031B

15	Nja-19
16	Alpa
17	Barracca
18	1007B
19	Ng5
20	1037A
21	"Rootstock"
22	Mume Bungo
23	1012A
24	1031B
25	1018B
26	1038A
27	Sancastresse
28	Zinostojky
29	1005A
30	1028Ba
31	1008A
32	Armeniaca anshu
33	Travatt
34	Satungsky
35	1032B
36	Moorpark nuwe
37	Olimp
38	1003A
39	1030A
40	1023B
41	1013B
42	Goldstrike
43	Early Baidy
44	Kathy
45	1020A
46	Moorpark
47	Heiwa
48	Pu Sha Shin
49	1017B
50	Frater
51	Royal FR
52	1027B
53	1031A
54	1020B
55	1028Bb
56	NG3
57	Hybrid Bungo
58	Castleton
59	Malan Royal*
60	Real de Timola
61	Royal Blenheim
62	AP93-9
63	AP94-10
64	77-LA-579
65	AP94-34
66	Malice apricot
67	4A/3/36
68	Suapritwo
69	118-LE-133
70	12D-39- 5
71	8A-10-14
72	12D-31- 5
73	12D-10-15
74	Fantasme avikour
75	3B- 9-25
76	Rouge de Fournes
77	Suaprieight
78	S5A- 2- 4
79	Autumn Glo
80	4A/3/39
81	Suapriseven
82	AP95-56
83	Palsteyn*
84	Peeka*
85	Trigems
86	Charisma*
87	T.B. Therma (Tardif Bordaneil)
88	Comedie
89	Royal
90	Guillia (Giulia)
91	20A-8-1
92	20C-24-20
93	Ivresse
94	Vesna
95	Shimizugou
96	Satunska
97	Glada
98	Dof 1-A-586
99	Gessekai
100	Clone 0.11
101	Giada
102	Comedie
103	LE 3241 (a&b)
104	Goldrich
105	Bergarouge
106	Soledane

#	Reference collection cultivars
1	Grandir
2	Supergold
3	Soldenne
4	Bulida
5	Ladisun
6	Palsteyn
7	Cape bebeco
8	Charisma
9	Peeka
10	Royal
11	Alpa
12	Suapriseven
13	Suaprieight
14	Suaprinine
15	Atricot

Appendix B: Gel electrophoresis images of second intron PCR products amplified using EM-PC2consFD + EM-PC3consRD primer pair. Amplified products of plum gene bank and reference collection accessins were correlated to previously reported *S*-alleles by comparing sizes of amplified bands in measured against a 1kb plus ladder.

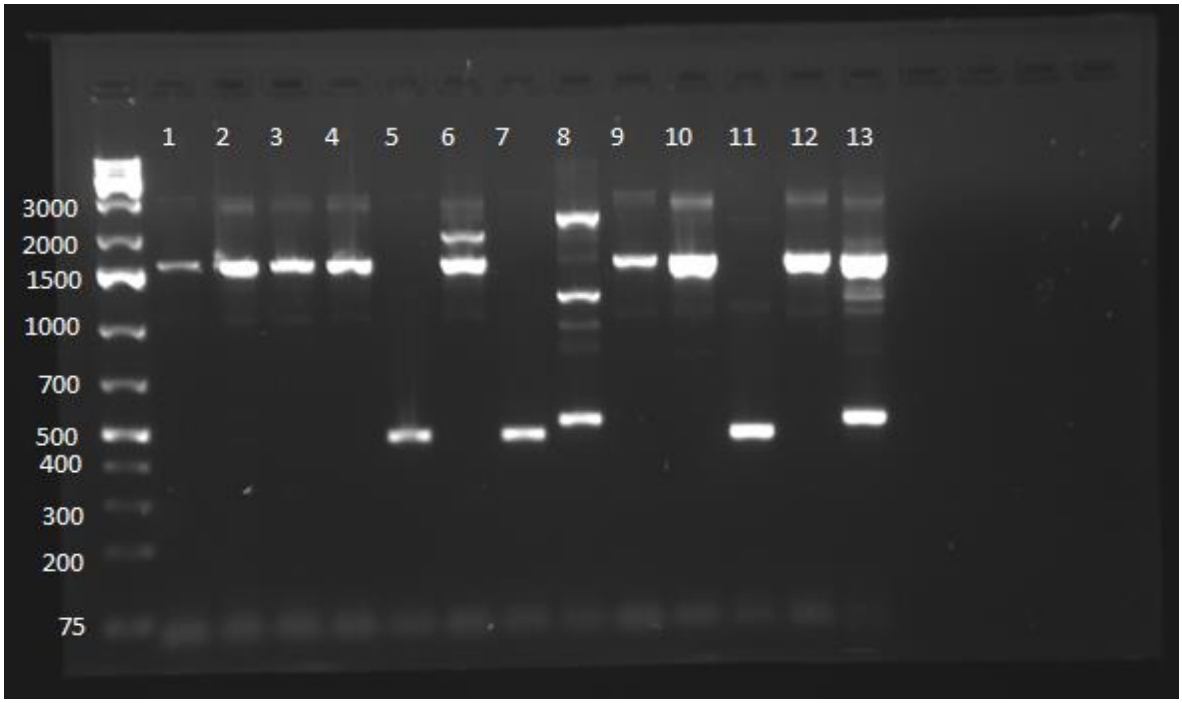


Fig B.1: Plum gene bank accessions 1 to 13

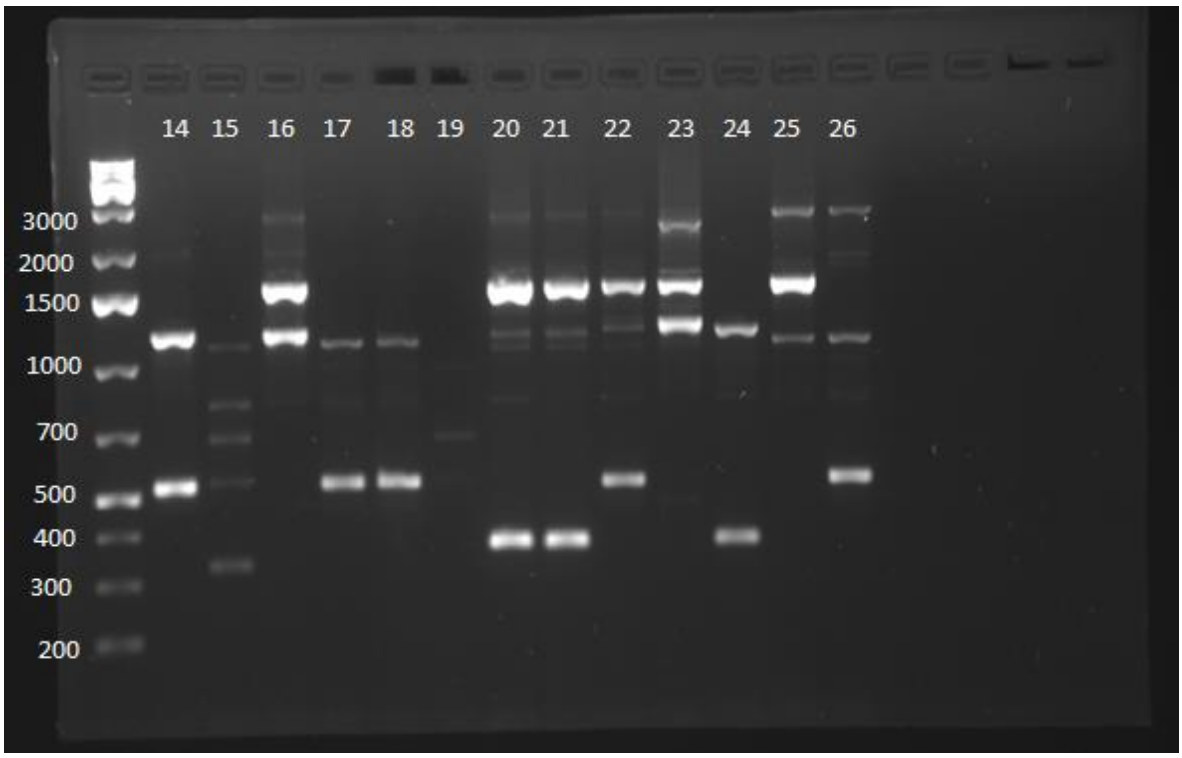


Fig B.2: Plum gene bank accessions 14 to 26

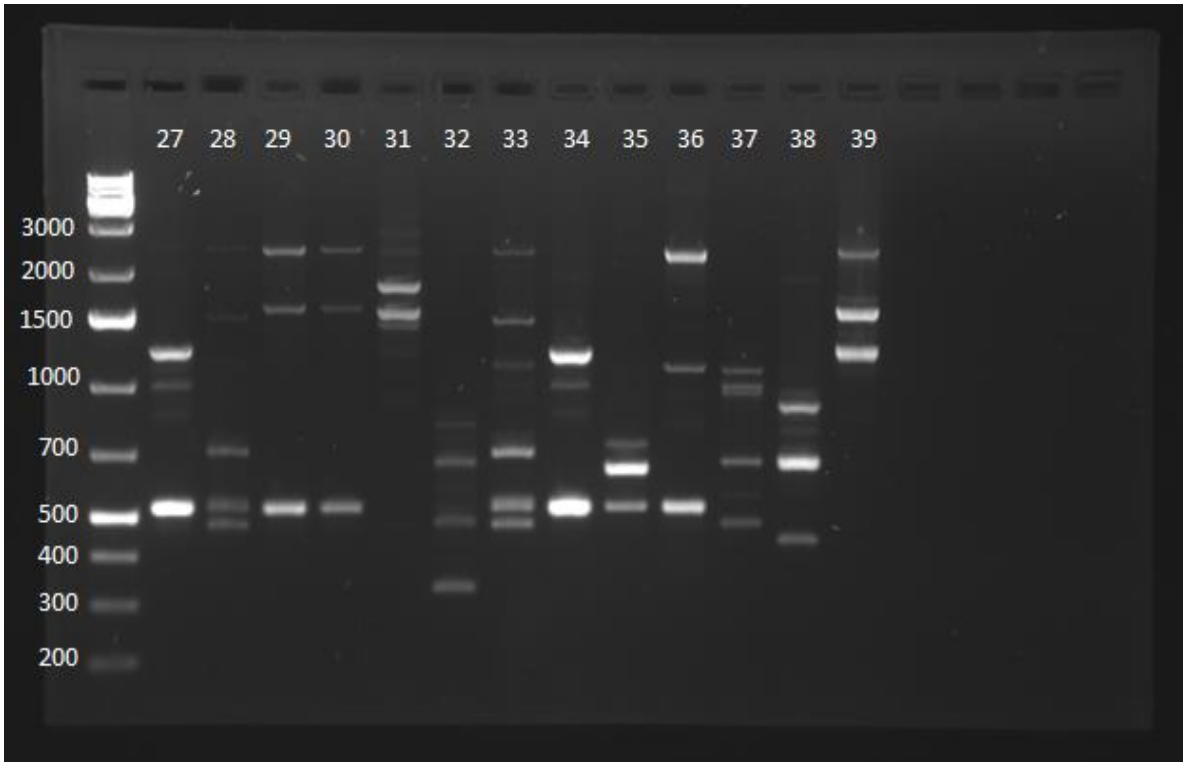


Fig B.3: Plum gene bank accessions 27 to 39

Table B: Plum accessions listed according to arrangement in gene bank plots and above gel images.

#	Species	Gene bank accessions
1	hybrid	S5A-26-35
2	hybrid	S5A-26-13
3	hybrid	S5A-25-20
4	hybrid	S5A-26-28
5	hybrid	S5A-34-25
6	hybrid	S5A-25- 5
7	hybrid	S5A-26-30
8	hybrid	S5A-26-11
9	hybrid	S5A-34-37
10	hybrid	S5A-33-25
11	hybrid	S5A-34-28
12	hybrid	S5A-35-34
13	*P. salicina*	17-32-118
14	*P. cerasifera*	Adara
15	*P. domestica*	Oheida
16	*P. salicina*	Loroda
17	*P. salicina*	Black Amber
18	*P. salicina*	Explorer
19	*P. domestica*	Jubileum
20	*P. salicina*	4C- 8-20
21	*P. salicina*	4A- 8-13
22	*P. salicina*	Celebration
23	*P. salicicna*	Ferely
24	*P. salicina*	17-10-212
25	*P. salicina*	Mac Verma
26	*P. salicina*	Purple King
27	*P.cerasifera*	Ademir (1)
28	*P. domestica*	P. marianna (1)
29	*P. insititia*	Adesoto (1)
30	*P. insititia*	Pixy (1)
31	Hybrid(P. besseyi)	Ferlenain (P2038)
32	*P. insititia*	Pixy (2)
33	*P. mariana*	P. marianna (2)
34	*P.cerasifera*	Ademir (2)

35	plumcot hybrid	By 69-1637P
36	*P. salicina*	Sel 6-64
37	*P. insititia*	Adesoto (2)
38	*P. domestica*	Eruni
39	french hybrid	Fereley Jaspi

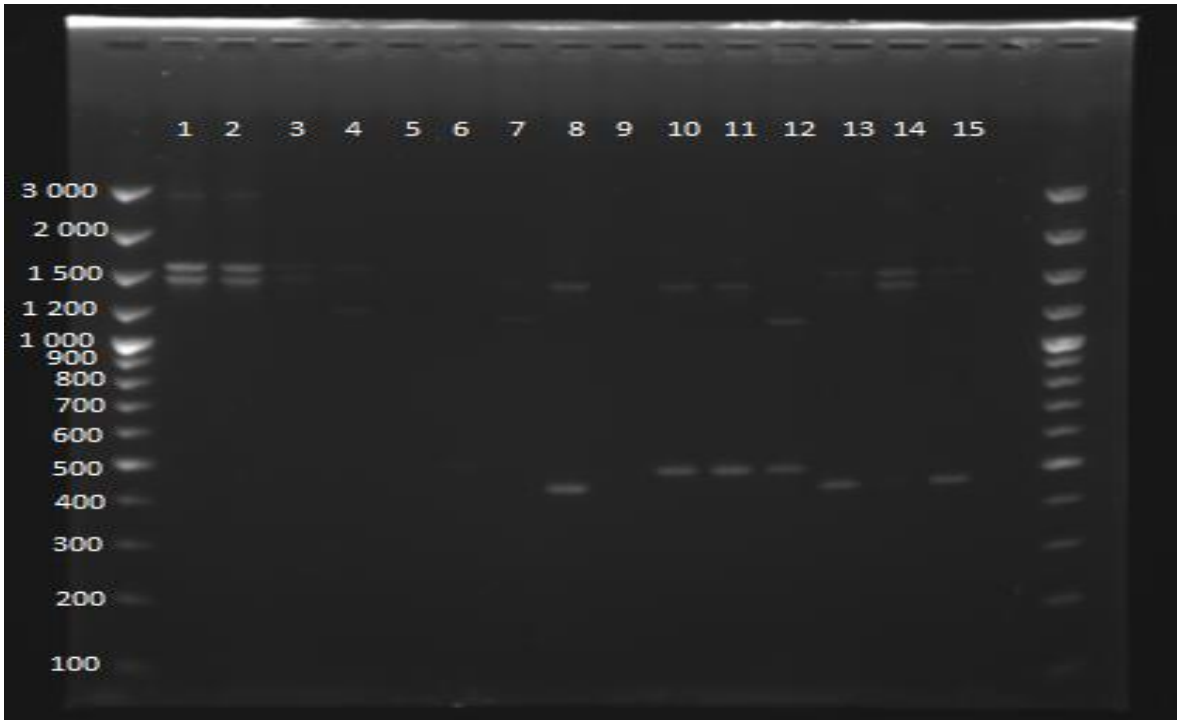


Fig B.4: Plum reference collection accessions 1 to 15

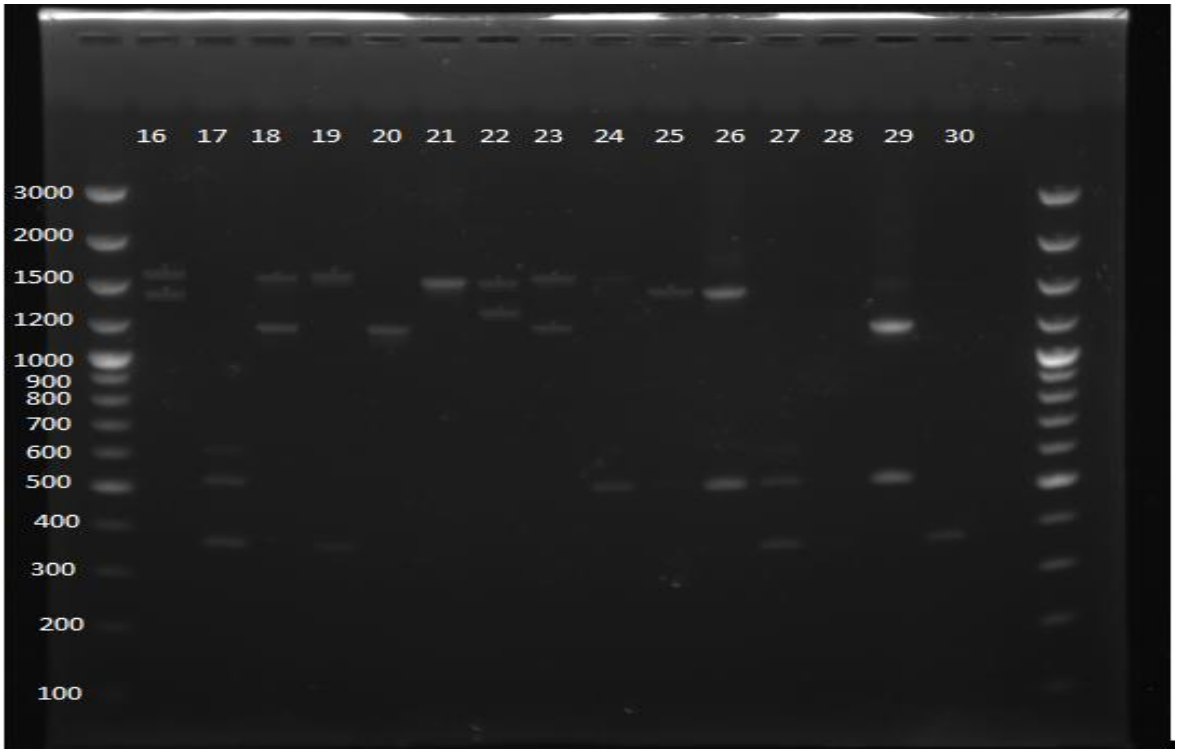


Fig B.5: Plum reference collection accessions 16 to 30

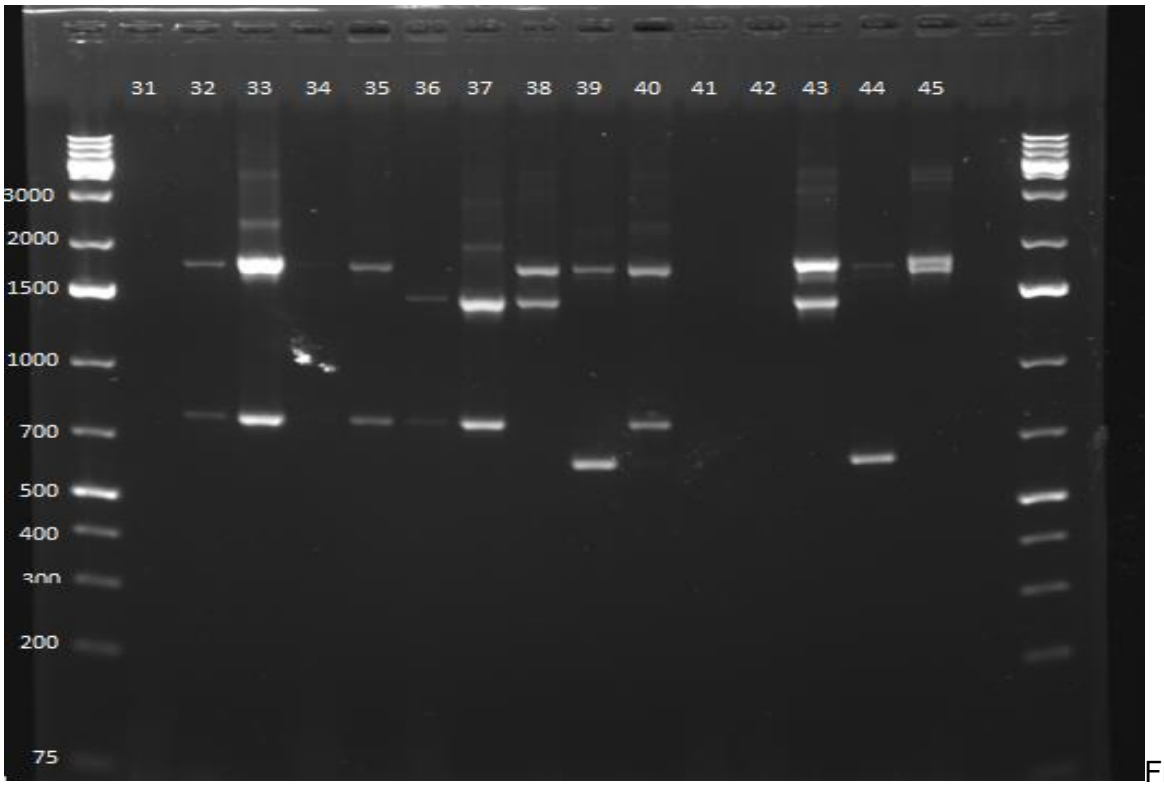


Fig B.6: Plum referece collection accessions 31 to 45

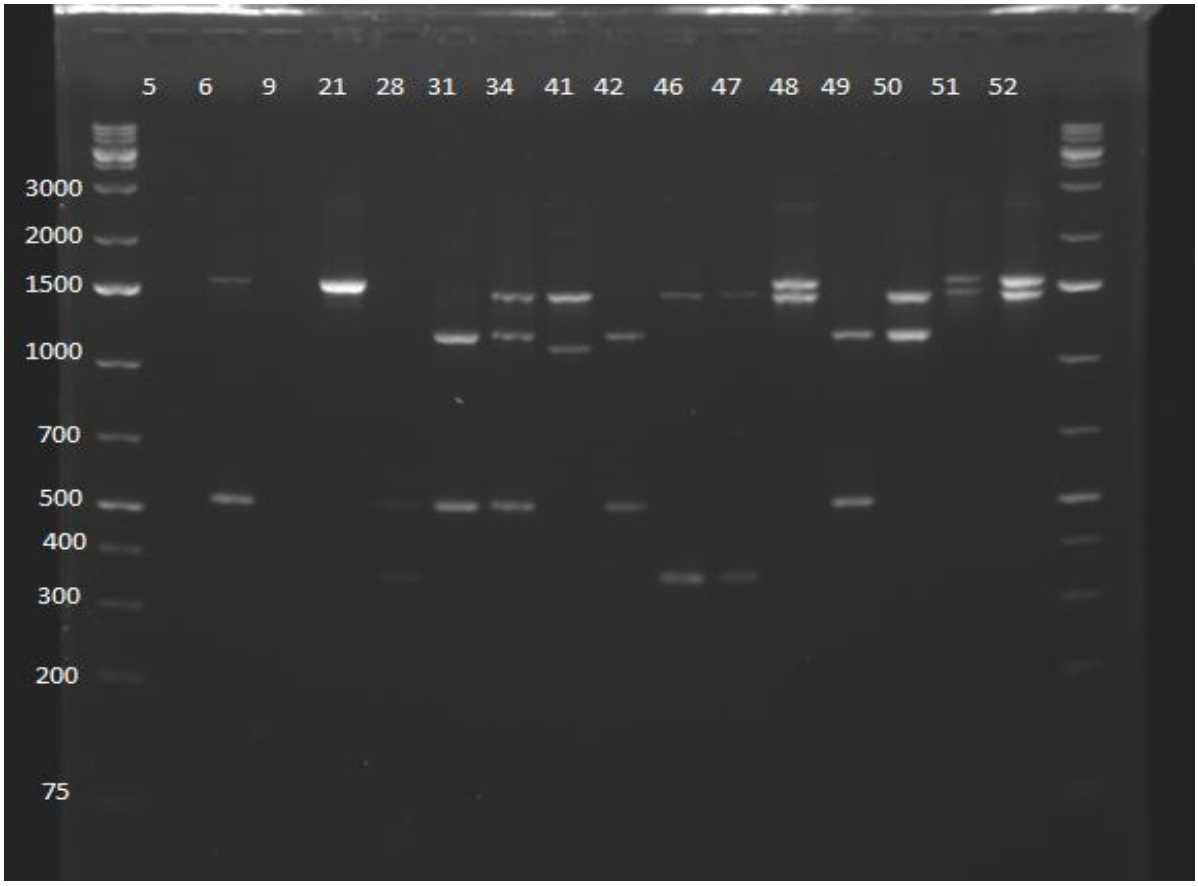


Fig B.7: Plum reference collection accessions 46 to 52, including samples 5 to 42 repeated due to previous failed amplification

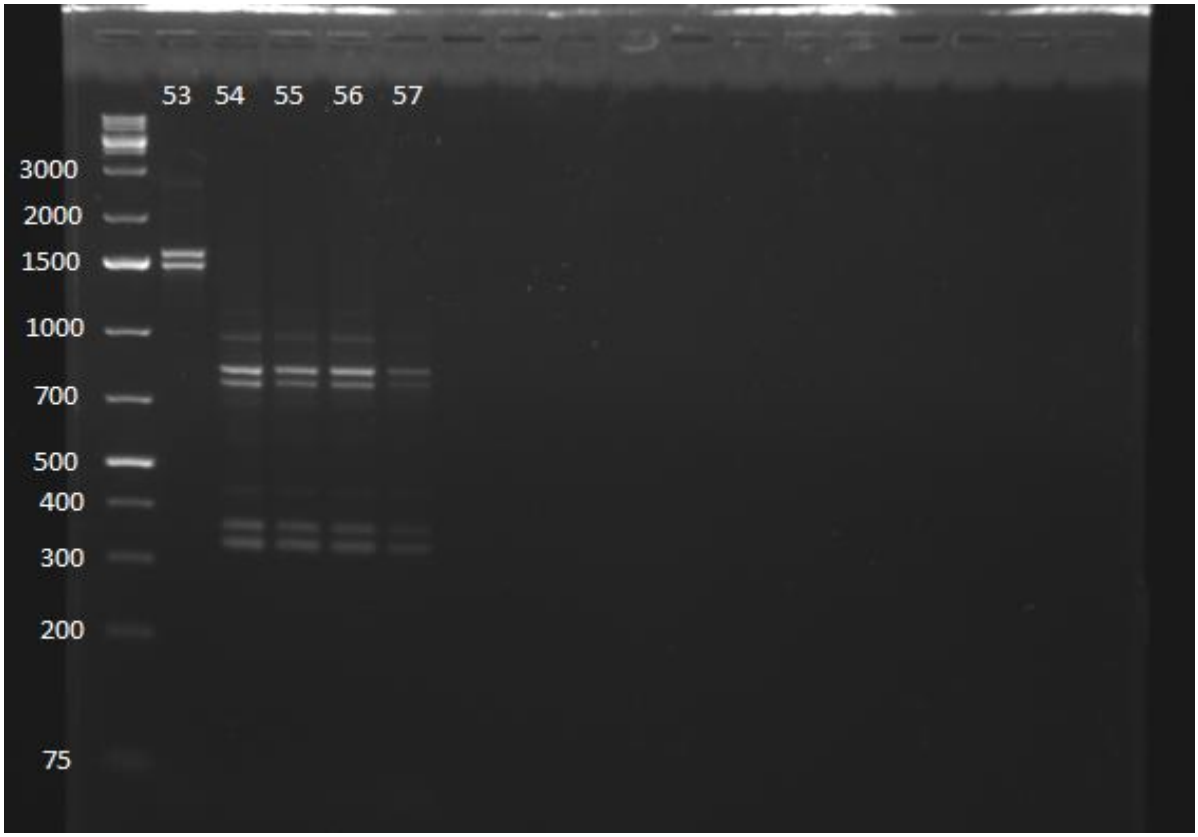


Fig B.8: Plum reference collesction accessions 53 to 57

Table C: Plum reference collection accessions, according to arrangement in on gel images.

#	Reference collection cultivars
1	ARC PR-4
2	Pioneer
3	Extreme
4	Methley
5	Sapphire
6	Hiromi Red
7	Santa Rosa
8	Purple Majesty
9	Gaviota
10	Sovenir
11	Golden Kiss
12	Sun Kiss
13	Sundew
14	ARC PR-1
15	Black Egg
16	Sensation
17	Crocodile Dendee
18	Fortune
19	Harry Pickstone
20	Rruby red
21	Suplum 25
22	Reubennel
23	Lady West
24	Lady Red
25	Suplum 11
26	Laetitia
27	Red Gold
28	Son Gold
29	Lamoon
30	Ruby Star
31	Casselman
32	ARC PR-2
33	Celebration
34	Betty Anne
35	ARC PR-3
36	Late Lamoon
37	Suplum 6
38	SouthernBelle
39	Autumn Sun
40	Golden king
41	Green Red
42	Mirell
43	Ruby Sun
44	Ruby Prince
45	Satin Gold
46	Simka
47	Sunset
48	Suplum 28
49	Sweet Aroma
50	Winner
51	Flavor Fall
52	Flavor King
53	Flavor rich

54	Erfdeel
55	Prune D' agen
56	French Prune
57	V D Merwe

Appendix C: Alignment of apricot amino acid sequences of *S1*, *S2*, *S4*, *S8*, *S10*, *S13*, *S21*, *S25*, *S26*, *S30* and *Sc*, indicating the three conserved regions (C1,C2 and C3) as well as the hyper-variable region (HVR) reported in the rosaceous S-RNases. Gaps are marked by dashes and the C1, C2, HVR and C3 regions are highlighted. The alignment was prepared

using the CLUSTAL method from uploaded sequences of the second intron *S*-alleles; therefor only the position of the second intron could be identified. The alignment was compared to alignment from almond sequence (Ortega *et al*., 2006).