MOLECULAR CHARACTERISATION OF ARC POME FRUIT COLLECTIONS IN SOUTH AFRICA

by

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Declaration

By submitting this thesis electronically, I Khethani Give Mhelembe, 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.

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Abstract

Apple (*Malus pumila* Mill.) and pear (*Pyrus communis* L.), commonly known as pome fruits, are important deciduous fruit crops in South Africa. The challenges of climate change, disease incidence, distant markets and fluctuating consumer preferences necessitate new cultivars. The Agricultural Research Council (ARC) Infruitec-Nietvoorbij conducts a breeding programme aimed at developing new cultivars that are well adapted, resistant to pests and diseases and good storage potential. A recent review of the pome fruit gene banks, the breeders' raw material, revealed misidentification and poor characterisation limitating the efficiency of its utilisation. To address these problems, the current study used microsatellite markers to investigate the trueness to type of accessions in the ARC gene banks. In addition, accessions of apple identified as true to type, were genotyped for the *ACS1* gene involved with ethylene production and fruit ripening.

Two sets of 12 microsatellite markers recommended by a European working group on *Pyrus*/*Malus*, one for apple and one for pear, were utilised to fingerprint 540 apple and 197 pear accessions. Eleven and eight of 12 markers, were used respectively to successfully discriminate across the apple and pear accessions, with the exception of clones and sports of particular cultivars. Where possible, fingerprints were compared with those of their reported parents. The use of recommended markers facilitated the comparison of ARC pear accessions with those of the collection in Brogdale (UK). Trueness to type of accessions were established and misidentified accessions were also detected. A similar comparison will be conducted for apple when the Brogdale apple accessions fingerprints become available. Several accessions were found to be false, 78 apple and 22 pear, and removal from the collection was recommended.

For *ACS1* genotyping of 292 apple accessions, customised fluorescently labelled ACS1-Pr were used rather than the published ACS1-5 primers. Of the 292 apple accessions, 29 were homozygous for the *b* allele associated with low ethylene and good storage potential. Novel size variation in one allele of the *ACS1* gene, was detected in some *Malus* species and ornamental hybrids. Successful amplification in a multiplex reaction was achieved and proves to be a cost effective method for simultaneous molecular fingerprinting and *ACS1* genotyping.

True to type material will facilitate confident use of genetic resources in the breeding programmes, and the *ACS1* genotypes will identify candidate parents for developing good storage performing cultivars for distant markets.

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List of symbols and abbreviations

%	Percentage
$\chi 2$	Chi-square
°C	Degrees Celsius
µl	Microlitre
µM	Micromolar
3’	Three Prime
5’	Five Prime
A	Adenine
AFLP	Amplified Fragment Length Polymorphism
ARC	Agricultural Research Council
BC	Before Christ
bp	Base pair
C	Cytosine
CAPS	Cleaved Amplified Polymorphic Sequences
CTAB	Cetyltrimethylammonium Bromide
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	Deoxyribonucleic Acid
DUS	Distinctness, Uniformity and Stability
EDTA	Ethylenediamine Tetra-acetate
g	Gram
g	Guanine
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

ng	Nanogram
NGS	Next Generation Sequencing
P	Probability
PCR	Polymerase Chain Reaction
pH	Concentration of Hydrogen Ions in a Solution
PIC	Polymorphism Information Content
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
SNP	Single Nucleotide Polymorphism
T	Thymine
TE	Tris-Ethylenediamine Tetra-acetate
UPGMA	Unweighted Pair Group Method with Arithmetic Average
OPOV	International Convention for the Protection of New Varieties of Plants
V	Volts
v/v	Volume to Volume

Chapter 1

INTRODUCTION

1.1. Background

The pome fruits, apples (*Malus*) and pears (*Pyrus*), are the second and third most important export fruit crops, respectively, in South Africa after grape (*Vitis*). More than 33 000 ha are planted under pome fruit, with more 40% of the produce for each individual crop being exported internationally (HORTGRO, 2013; PPECB, 2013). However, several shipments are rejected annually due to the presence of diseased fruits, excess chemical residues and over ripening (PPECB, 2013). The pome industry provides more than 43 000 jobs, with more than 100 000 dependents (HORTGRO, 2013).

The Agricultural Research Council (ARC) Infruitec-Nietvoorbij is charged with breeding new cultivars for the pome fruit industry to overcome among other challenges, the ones highlighted above. Breeding of new cultivars requires diverse and well maintained genetic resources (Bester *et al*., 2013). Therefore, a review of the status of ARC genetic resources was conducted by breeders and industry representatives. Misidentification of accessions and poor characterisation were identified as limiting factor to the success of the breeding programmes (Tobutt and Bester, 2011). Considerable effort has been invested to resolve misidentified accessions morphologically (Tobutt, personal communication). However, this technique is often unreliable as environmental conditions influence the phenotype observed, the process is time consuming in large gene banks and requires well trained horticulturists.

Funding for molecular studies secured from the Technology and Human Resources for Industry Programme (THRIP) has allowed the pome fruit genetic resources to be fingerprinted with molecular markers. A set of internationally recommended microsatellite markers, previously used at Brogdale, United Kingdom (UK), were selected to ensure comparability of accessions between gene banks. This fingerprinting is a step towards proper characterisation of the gene bank accessions to enable their efficient use in the breeding programmes

A know function gene, *ACS* involved in the biochemical pathway of ethylene production in apple (Sunako *et al*., 1999) was also included in the study. The published primer pairs were however redesigned to facilitate multiplexing with microsatellite markers. An experiment was also conducted to investigate whether the apple primer pairs would amplify in a set of pear accessions. Understanding the ripening potential of accessions is important to guide choice of parents for breeding delayed ripening cultivars needed for the export market.

Misidentification and poor characterisation of the ARC pome fruit accessions limits the efficiency of the breeding programme.

1.2. Aim and objectives

The primary aim of the current study was to fingerprint both the apple and pear accessions with a set of recommended markers by the European Cooperative Programme for Plant Genetic Resources (ECPGR). A further aim was to characterise the apple gene bank for the known function gene *ACS1*.

Thus objective 1, which forms part of chapter 3, was to fingerprint and compare ARC pear accessions with data generated for the international pear accessions at Brogdale. This was conducted to resolve potential misidentifications, trueness to type and parentage.

Objective 2, which forms part of chapter 4, was similar to the objective, to fingerprint and compare ARC apple accessions with the international Brogdale data to resolve misidentifications, trueness to type and parentage.

Objective 3, which forms part of chapter 5, was to characterise the true to type apple accessions for the *ACS1* gene.

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Chapter 2

LITERATURE REVIEW

2.1. Pome fruits

2.1.1. Botany of pome fruit

Apples (*Malus*) and pears (*Pyrus*) belong to the Rosaceae sub-family traditionally known as Maloideae together with other tree fruits such as *Eriobotrya* (loquat), *Mespilus* (medlar) and *Cydonia* (quince) (Mabberley, 1987; Janick, 2005; Hummer and Janick, 2009). Members of this sub-family are characterised by fruits consisting of two to five carpels enclosed in a fleshy covering known as pome (Janick *et al*., 1996). Other traditional sub-families of the Rosaceae are Prunoideae (*e.g*. peach) and Rosoideae (*e.g*. strawberry) (Hummer and Janick, 2009). However, recent plant systematic literature based on phylogenetic approaches argues that the Maloideae should be redefined as the subtribe Pyrinae, within the Pyreae tribe of the sub-family Spiraeoideae as they contain the same chromosome number with other members of Pyrinae (Potter *et al*., 2007; Judd *et al*., 2008).

Mabberley *et al*. (2001) justified the use of *Malus pumila* Mill. as the correct binomial name for the domesticated apple as opposed to *Malus* × *domestica* Borkh. previously asserted by Korban and Skirvin (1984). This was based on a genetic study which revealed no evidence of hybridisation of orchard apple with other *Malus* species, the original basis of the name *Malus* × *domestica*, thus rendering that name invalid. The wild apple from which the domesticated apple is derived is generally known as *M. sieversii* Ledeb. and approximately 27 other species of *Malus* are known (Forsline *et al*., 2003). Cultivated pears consist of European pears, scientifically known as *Pyrus communis* L., Chinese pears, known as *P. pyrifolia* (Burm.) Nakai, and Japanese pears, *P. ussuriensis* Maxim. (Itai, 2007). Twenty other species of *Pyrus* are known (Bell, 1990). Hybridisation within the genera, and some ornamental *Malus* and *Pyrus* species result from hybridisation between species (Janick and Moore, 1975; Moore and Ballington, 1990).

Diploid members of Maloideae have 17 chromosome pairs ($2n=2x=34$), perhaps the result of ancient gene duplication (Hancock *et al*., 2008), whereas other subfamilies of the Rosaceae have 7, 8 or 9 chromosome pairs (Wünsch and Hormaza, 2007). Hybridisation between two primitive forms of Rosaceae, Prunoideae and Spiraeoideae was suspected as the origin for this sub-family (Harris *et al*., 2002; Hancock and Lobos, 2008; Yamamoto and Chevreau, 2009); however, the study of Potter *et al*. (2007) questioned this hybrid origin as the sub-family appeared monophyletic.

Pome fruit seedlings usually have a long juvenile phase, which, if not grafted to rootstocks, can be up to six years in apple and longer in pear (Tartarini and Sansavini, 2002; Wilkie *et al*., 2008). Most apples and pears are self-incompatible (Brown and Maloney, 2003; Hancock *et al*., 2008) and are generally unable to set fruits if self-pollinated and need compatible pollinators (Bassil and Lewers, 2009); and some cultivars are cross-incompatible.

Apples and pears are the most commonly grown fruit crops in the Maloideae and are economically important both as fresh and processed fruits (Hummer and Janick, 2009). In addition, *Malus* and *Pyrus* have a diverse range of ornamental small-fruited species commonly known as crabs in apple, and some of these are important components of gardens in temperate regions due to their attractive blossom and profuse fruits (Janick *et al*., 1996; Hillier and Coombes, 2003).

2.1.2. Origin and distribution of pome fruits

Apples are believed to have been under cultivation since 1000 BC (Harris *et al*, 2002). Central Asia, especially Kazakhstan and Kyrgyzstan, is thought to be the centre of origin for *M. sieversii* (Janick *et al*., 1996). Historical studies indicate that animals and humans have played a role in the dispersal of the seeds from Asia, initially along the Silk Route to Europe and later to other parts of the world.

Pyrus is believed to have originated in the mountainous area of western and south-western China, and evolved as it spread along the mountains to the east and west (Bell, 1990; Bassil and Postman, 2010). Pears are believed to have been under cultivation since 900 to 800 BC and are now found in most parts of the temperate world (Lombard and Westwood, 1987; Chagné *et al*., 2014).

2.1.3. Horticulture of pome fruits

More than 10 000 cultivars of apple are known but there are very few that are widely grown on a commercial scale and the majority of these popular cultivars are chance seedlings or sports and mutations rather than outcomes of breeding programmes (Moore and Bellington, 1991; Janick *et al*., 1996). Likewise more than 900 cultivars of European pear are known (Wünsch

and Hormaza, 2007) but few cultivars are commercially grown (Bell, 1990; Itai, 2007) and most new cultivars are sports or mutations of existing popular cultivars.

The popularity of a limited number of pear cultivars such as 'Bartlett', 'Conference', and 'Packham's Triumph' and apple cultivars 'Delicious', 'Golden Delicious' and 'Granny Smith' has led to the replacement of traditional locally well-adapted cultivars and reduced genetic diversity (Urrestarazu *et al*., 2012). This reduction in genetic diversity has been further accelerated by the commercial release of mutants of popular cultivars (Brooks and Olmo, 1991, 1994). Most breeding programmes have used parents from this narrow genetic base (Kumar *et al*., 2010). Noiton and Alspach (1996) noted that the limited use of traditional cultivars in breeding programmes may be due in part to a lack of agronomic information on these cultivars.

Apple and pear cultivars are clonally (vegetatively) propagated. Scion cultivars are generally budded or grafted on to rootstocks propagated by cuttings or layering (Bell, 1990). Most cultivars are now grown on rootstocks which have been selected primarily for their adaptability to different environments and orchard management systems (Bell, 1990; Janick *et al*., 1996). Rootstocks 'M7', 'M793' and 'MM109' are commonly used in the South African apple industry while 'BP3' is widely used for pear. Recently there has been interest to commercialise 'M9' as a rootstock in South Africa; 'M9' rootstocks have dwarfing and precocity characteristics making them suitable for intensive planting or as interstocks to ensure early cropping in the orchard (Kotze and Steyn, personal communication).

Pome fruit crops enter a dormant state in winter; requiring exposure to cold conditions for a certain period known as the 'chilling requirement' to break the dormancy (Jonkers, 1979; Hauagge and Cummins, 1991; Mohamed, 2008). These crops can be grown in all temperate and some subtropical countries of the world with sufficient winter-chill. Production in tropical countries tends to be limited by warm winters; however, production is still possible with the use of dormancy breaking sprays (Hummer and Janick, 2009).

In many countries, efforts are being put into the breeding and selection of novel cultivars capable of coping with future challenges of fruit production such as environmental changes, emergence of diseases and pests or altered consumer demands (Lespinasse, 2007). Rootstocks adapted to different conditions of pome fruit production are also bred in some countries (Janick *et al*., 1996; Kumar *et al*., 2010).

2.1.4. History of pome fruits in South Africa

Pome fruits were introduced to what is now known as South Africa by the Dutch settlers. The Dutch explorers of the 16th century colonised the Western Cape around Table Mountain, where Jan van Riebeeck and company started a refreshment station for the ships *en route* to the spice lands to supply fresh food and fruits to prevent scurvy in sailors (Nel and Griesel, 2012). In the spring of 1652, the first apple tree was planted in Cape Town and then distributed across the Cape region. The first pear tree was planted around the late 1600s in the Company Garden in Cape Town (Roosi, 2005).

The industry expanded in the early 1900s with the advances in refrigeration for export. H.E.V. Pickstone, a principal instigator who set up a nursery in the Franschhoek Valley, imported and documented many apple and pear cultivars from foreign countries (Nel and Griessel, 2012). The pome fruit industry grew extensively over the years. By the later century, it was dominated by popular international cultivars such as 'Delicious', 'Golden Delicious', 'Granny Smith', and later 'Braeburn' and 'Gala', and their sports and 'Packham's Triumph', 'Forelle', 'Bartlett' and 'Conference' in the case of pears. Principal commercial producing areas were, and still are, Ceres, the Elgin Valley and the Langkloof in the Western Cape on account of the temperate climate of this region. Some parts of the Free State, Mpumalanga and the Northern Cape also produce pome fruits on a small scale (DAFF, 2011; Nel and Griesel, 2012).

The Agricultural Research Council (ARC) Infruitec-Nietvoorbij Institute, originally known as the Western Province Fruit Research Station (WPFRS) and then the Fruit and Fruit Technology Research Institute (FFTRI), was formed in 1940 to service the fruit growing industry of the Western Cape (Lötter, 2012). A breeding programme, which continues to this day, was started as described later. Several local cultivars have materialised from this initiative such as 'African Carmine' and 'Elegant' for apple and 'Cheeky', 'Flamingo' and 'Rosemarie' for pear.

There are 134 apple and 62 pear cultivars registered in the national list of fruit varieties in South Africa (DAFF, 2012). However, few cultivars are widely grown commercially with cultivars such as 'Golden Delicious', 'Granny Smith', 'Starking', 'Royal Gala', 'Fuji' and 'Pink Lady' grown in 82% of the apple production area in South Africa (HORTGRO, 2012). 'Packham's Triumph', 'Abate Fetel', 'Forelle' and 'Williams Bon Chretien' cultivars are grown in 87% of the pear production area in South Africa (HORTGRO, 2012).

2.1.5. Economic importance of pome fruits in South Africa

Fruit crops are a major role player in the economy of the Western Cape and South Africa and generate more than six billion South African Rand in export earnings with apples and pears respectively as the second and third most important deciduous fruit crops after grapes (HORTGRO, 2012). The apple and pear industries combined employ over 42 000 people with dependants numbering more than 160 000. Approximately 33 000 ha of land are planted under these crops especially in the Western Cape (HORTGRO, 2012). Some 65% of the total production is consumed locally either as fresh produce or for processing including cider production, juice production and drying with 35% exported for fresh consumption (PPECB, 2013).

South Africa's pome fruit industry exports more than 1100 metric tons per year. It is currently the 17th largest producer of apple worldwide and 4th in the southern hemisphere after Brazil, Chile and Argentina (FAO, 2013). For European pear, it occupies the 9th position globally and 2nd position in the southern hemisphere after Argentina (FAO, 2013). South Africa's fresh apple and pear exports are currently ranked 9th and 5th, in the world (World Apple Review, 2011; World Pear Review, 2011).

2.1.6. Health benefits of pome fruit

Pome fruits have traditionally been consumed fresh; however, they can also be dried, canned or processed into sauces, slices, and juice and can be used for pastries, cakes, tarts, and pies (Downing, 1989). The juice can be drunk fresh or fermented to make cider or wine (Janick *et al*., 1996). These fruits have important health properties. The notion "An apple a day, keeps the doctor away" is well supported by the fact that phytonutrients such as flavanols in apples help regulate blood sugar levels, help prevent heart disease through regulation of blood fat levels, are antioxidants that neutralise the effects of free radicals that damage body cells and are associated with decreased risk of asthma (Hollman and Arts, 2000; Kellerhals *et al*., 2004; Rahimi *et al*., 2005; Valko *et al*., 2007; WHF, 2013). Pears have similar desirable constituents (WHF, 2013).

2.2. Pome fruit breeding and genetics

2.2.1. Traditional fruit breeding

Juniper *et al*. (1996) described the selection of large sweet fruits by bears as the earliest form of apple improvement. Later, humans simply selected desirable fruits from different trees according to attributes such as size, colour, taste and appearance. This selection method can be regarded as the first step of breeding (Morgan *et al*, 1993; Ahmadi-Afzadi, 2012). The invention of grafting or budding of scions on rootstocks allowed the best selections to be propagated to establish an orchard rather than a random collection of seedlings (Morgan *et al*., 1993). As a consequence of the high heterozygosity in pome fruits, most of the desirable traits from one parent cannot be completely inherited as a whole by the offspring (Tartarini and Sansavini, 2003) and large numbers of seedlings are needed to obtain offspring with good combinations of desired traits (Kenis and Keulemans, 2005).

Around 1806, Thomas A. Knight introduced controlled breeding in England and this opened a new era of fruit improvement (Brown, 1992). Controlled breeding involves mating of parents with suitable complementary traits in order to combine desirable traits from both parents. Today, those principles still form the basis of fruit crop breeding, although the cultivars developed by Knight are no longer utilised. It was only when the knowledge of cultivar traits started to improve that some important cultivars emerged from controlled breeding (Janick *et al*., 1996).

There are many apple breeding programmes in the larger pome fruit producing countries of the world but fewer pear breeding programmes and fewer still concerned with rootstocks. The review on breeding objectives for apple scion and rootstock cultivars by Brown and Maloney (2003) indicates the commonalities in breeding programmes throughout the world and include aspects such as disease resistance and marketability as well as traits such as fruit appearance and also eating quality and outlines environment specific objectives for some countries.

Of particular importance in South Africa is developing cultivars adapted to low chilling conditions due to the mild winters as well as improved storage potential, which is key to successful export. In recent years, the demand for pome fruits in Europe during their winter season has increased creating the need to breed for early ripening cultivars in South Africa to meet the export opportunities. The recent demand for pears of blush type with a red skin colour for the export market has also made breeding for such cultivars a priority in South Africa

(Human, 2011). Improved rootstocks that crop early or have dwarfing properties, important in intensive planting systems, are also an important objective for breeders but are not currently the subject of breeding at ARC (Tobutt, personal communication).

2.2.2. Challenges to fruit breeding

The success of fruit breeding is hampered by several aspects, which are either natural or a result of human error. Pome fruit breeding programmes globally face similar limitations due to the long juvenile phase, large tree size and self-incompatibility (Brown and Maloney, 2003). The difficulty of distinguishing homozygotes from heterozygotes with respect to dominant single gene traits in parents and the challange of cross-incompatibility in sib-crossing are other limitations. In addition, incidences of incorrect clones due to mislabelling of germplasm accessions may occur, which lead to flawed genetic studies and inappropriate crosses for breeding (Garkava-Gustavsson *et al*., 2008).

2.2.3. Genetics of pome fruit breeding

With the introduction of controlled crossing, breeders started making crosses and raising seedlings with the aim to select better cultivars. Several breeders recorded inheritance of traits such as yield, growth habit and taste. The rediscovery of Gregor Mendel's work on inheritance of traits in pea plants around 1906 (Brown, 1992) brought a new perspective to interpreting phenotypic data. Subsequently, breeding programmes in parts of Europe and America started to analyse the segregation patterns observed in the seedlings genetically by comparison with Mendelian ratios and made crosses particularly for genetic studies. From the segregations observed, breeders could deduce the type of inheritance for certain traits. Various agronomic traits such as dwarfing, disease resistance, aphid resistance and other traits of interest have been studied in pome fruits over the years (Way *et al*., 1990; Brown, 1992; Bell *et al*., 1996). In apple, approximately, 25 major genes for agronomic traits have been identified (Brown, 1992) and approximately 15 in pear (Hancock and Lobos, 2008).

As described later, genetic studies in recent years have focused on the development of linkage maps and mapping of agronomic traits (Liebhard *et al*., 2002; Fernández-Fernández *et al*., 2008). This determination of gene positions relative to molecular markers in fruit crops is

paving the way for an advanced approach of fruit breeding known as Marker-Assisted Selection (MAS). This MAS method should enable breeders to use molecular markers to screen seedlings for the presence of desirable traits, thus reducing the need to wait for maturity to evaluate the seedlings morphologically (Kumar *et al*., 2010). However, there are few reports, Peace (2012), of MAS being routinely applied to apple and pear breeding programme.

2.2.4. Ethylene production

Cultivars vary greatly in ripening dates and this is associated with their rate of ethylene production. Some cultivars produce more ethylene sooner and hence ripen early, whereas others with slow ethylene production ripen later (Nakatsuka *et al*., 1998) and have long storage potential. Delayed fruit ripening is an important objective of breeding programmes as over-ripe consignments will be rejected (Oraguzie *et al*., 2004). Thus, knowledge of the ripening potential will facilitate selection of cultivars suitable for export and possibly result in an increased competitiveness in the market.

Apples and pears are regarded as climacteric fruit on the basis of a sudden increase in ethylene production during ripening (Biale and Young, 1981; Varanasi *et al*., 2011). These crops continue to show an increase in respiration rate even post-harvest (Harada *et al*., 2000; Oraguzie *et al*., 2004).

The biochemical pathway of ethylene production is controlled by two enzymes namely aminocyclopropane-1-carboxylic acid synthase (ACS) and the aminocyclopropane-1-carboxylic acid oxidase (ACO), as described below. The substrate S-adenosylmethionine is converted to 1-aminocyclopropane-1-carboxylic acid via the activity of ACS enzyme and then ACO converts 1-aminocyclopropane-1-carboxylic acid to ethylene (Fig. 2.1) (Yang and Hoffman, 1984).

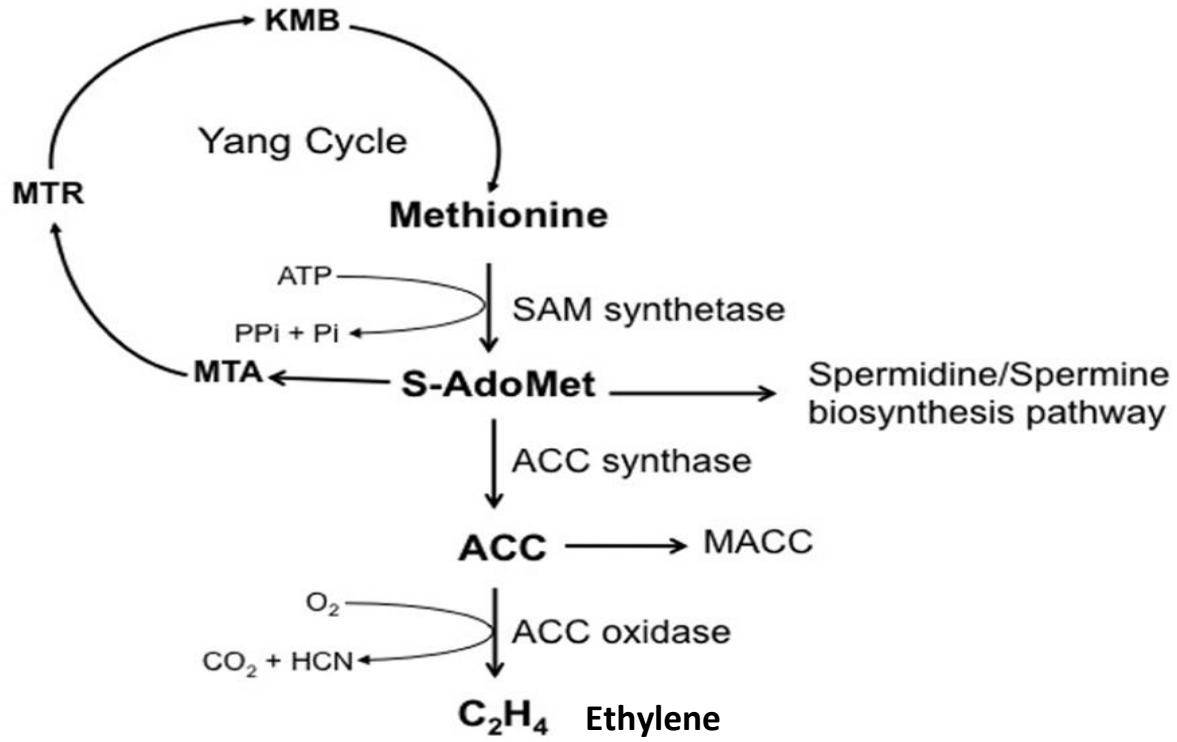

Fig. 2.1. The biochemical pathway of ethylene production to illustrate the roles of ACS and ACO in the pathway converting S-adenosylmethionine via 1-Aminocyclopropane carboxylate to ethylene (adapted from Yang and Hoffman, 1984).

The enzyme ACS is the rate limiting factor in the ethylene biosynthesis pathway, and in apple and pear fruit is encoded by the gene *ACS1* (Yang and Hoffman, 1984; Nakatsuka *et al*., 1998). The allelic variants of the *ACS1* gene *e.g*. *ACS1-1/1* or *ACS1-2/2* correlate with ripening time in apple (Sunako *et al*., 1999) but the relationship is not clear in pear, especially European pears (El-Sharkawy 2003, 2004; Oraguzie *et al*., 2010). The phenomena is explained in detail in section 2.7.

2.3. Breeding at the ARC Infruitec-Nietvoorbij

The Cultivar Development Division of the ARC houses South Africa's oldest pome fruit crop improvement programme, over 40 years old. Several objectives such as breeding for low-chill, prolonged storage potential and disease resistance are a major focus at ARC. However, yield, taste and consumer preferences are still considered. Genetic resource collections or gene banks, for apple, located in Drostersnes and Grabouw Experimental Farms (Elgin Valley), and for pear located in the Bien Donné Experimental Farm (Groot Drakenstein), serve as the breeders'

raw material. Over the years, fruit improvement at the ARC has been mostly conventional with minimal application of molecular markers. This limited application of markers in the breeding programme has had some successes over the years at the ARC resulting in important cultivars, 'Cheeky', 'Flamingo' and 'Rosemarie', grown in the industry. Most of the accessions in the national list of fruit crops managed by the Department of Agriculture, Forestry and Fisheries (DAFF) are imported cultivars or clones of existing cultivars with only a few ARC bred cultivars. The ARC has therefore recently recognised the need to integrate conventional breeding with molecular markers to improve the efficiency of the breeding programme and hence enhance cultivar development (Tobutt and Bester, 2011).

2.4. DNA Markers

A DNA marker can be defined as a distinctive section of DNA that shows allelic variation that is easy to detect in the laboratory (and that may be genetically linked to a trait of interest). DNA markers are widely used for diversity and functional studies and include non-functional and genic DNA markers. Non-functional markers, are traditional markers without direct functional significance, and include microsatellites (also known as simple sequence repeats (SSRs)), randomly amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs) and restriction fragment length polymorphisms (RFLPs). In contrast genic markers are derived from the sequences of known genes and can be used to distinguish functionally significant alleles for specific traits (Klug *et al.*, 2012). In pome fruits, one example is *ACS1* concerned with ethylene production.

Molecular markers are useful in various applications in fruit breeding including diversity studies, identification of different cultivars, developing linkage maps, mapping of single gene traits, identification of quantitative trait loci and MAS (Silfverberg-Dilworth *et al*., 2006; Bao *et al*., 2007; Celton *et al*., 2009).

2.4.1. Microsatellites (SSRs)

Microsatellites are short tandem nucleotide repeats (*e.g*. AT AT AT AT AT or TGC TGC TGC TGC) that show allelic length polymorphism and are abundant across the genome. The total length of repeated units is in the range of 10 to 60 bp and tends to vary (Gianfranceschi *et al*.,

1998; Hemmat *et al*., 2003; Guichoux *et al*., 2011). For each SSR marker, a diploid individual has two alleles that often differ in length; the variations in length correspond to the number of repeats present in each allele [*e.g.* $(AT)_3$ vs. $(AT)_5$]. Microsatellites result from mutations during DNA replication, and when located in non-coding regions, are not under any selective pressure during evolution or breeding (Gianfranceschi *et al*., 1998; Hemmat *et al*., 2003; Guichoux *et al*., 2011). Primers can be designed to flank the regions of the repeats so that the section can be amplified by polymerase chain reaction (PCR). The flanking regions of microsatellites tend to be conserved between the genomes of related species, thus enabling markers to be transferred between closely related species (Abbott et *al*., 1997; Gianfranceschi *et al*., 1998).

As microsatellites are inherited in a co-dominant manner enabling detection of both alleles in an organism, tend to be highly polymorphic with many alleles per locus and can be detected in small amounts of DNA using PCR from small amounts of leaf material (Hokanson *et al*., 1998; Gianfranceschi *et al*., 1998; Yamamoto *et al*., 2001; Liebhard *et al*., 2002). These characteristics have resulted in microsatellites' popularity over other molecular markers in molecular fruit breeding (Sehic *et al*., 2012; Pina *et al*., 2014).

Microsatellite have been widely used for genetic characterisation or fingerprinting (Potts *et al*., 2011; Dos Santos *et al*., 2011; Sehic *et al*., 2012; Pina *et al*., 2014) and in the development of genetic linkage maps in fruit improvement in recent years (Martínez-Gómez *et al*., 2003; Dirlewanger *et al*., 2006; Fernández-Fernández *et al*., 2008; Lu *et al*., 2010). The mapping of microsatellites has enabled sets of unlinked microsatellites to be selected for diversity and parentage analysis and fingerprinting studies in apple and closely related species such as pear (Liebhard *et al*., 2003; Brini *et al*., 2008; Fernández-Fernández *et al*., 2008; Baric *et al*., 2012). Apple microsatellite markers have been shown to be transferable to some extent to pear and other closely related species (Gianfranceschi *et al*., 1998; Gasi *et al*., 2010; Erfani *et al*., 2012).

2.4.2. Marker-Assisted Selection (MAS)

Marker-Assisted Selection is a method of selection based on molecular markers that are often genetically linked to a favourable agronomic trait, in the case of non-functional markers, or that can directly distinguish functional alleles, in the case of genic markers. Genic markers which represent the actual trait of interest are more precise than non-functional markers that

mostly allow a prediction of the presence of a trait of interest based on the genetic distance from the marker to the locus in question. In the latter case, a genetic map or at least the detection of linkage is required (Hamilton, 2009; Klug *et al*, 2012).

Use of MAS allows for the earlier selection of seedlings from breeding crosses, soon after germination instead of perhaps years later when the targeted traits may be expressed (Liebhard *et al*., 2003) and it may allow selection for traits that are difficult to measure directly such as resistance to exotic diseases (Currie, 2000; Ahmadi-Afzadi, 2012). As already noted, this method thus reduces the amount of land required to raise seedlings for selection and the associated costs of management (Moore and Ballington, 1990). Marker-Assisted Selection can also be useful for selecting breeding parents (Bus *et al*., 2000; Tartarini, 2003), which can reduce costs of genotyping seedlings. However, it should be noted that MAS is generally not practised in pome fruit breeding yet due to high cost associated with genotyping seedlings (Kumar et al., 2012). More routinely genic based markers for example for ACS (Castiglione *et al*., 1999; Itai *et al*., 1999), ACO (Costa *et al*., 2005) and S-incompatibility (Broothaerts, 1995; Janssens *et al*., 1995) are utilised within pome fruit breeding programmes.

2.5. Genetic resources in pome fruit

Commonly referred to as gene banks, genetic resources are the breeders' raw material for traits of interest necessary for fruit crop improvement and for associated genetic studies. Success in developing improved fruit tree cultivars is therefore dependent on access to genetic resources with sufficient variability to provide desirable gene combinations. Pome fruit gene banks typically consist of collections of wild species, popular cultivars, and sports of cultivars and usually include promising local or international breeders' selections. Genetic resources of fruit trees for breeding purposes are maintained in orchards as clonal gene banks (Sehic *et al*., 2012). This requires proper management as mislabelling and misidentification often occur during propagation and importing of accessions. The use of incorrect accessions leads to flawed genetic studies and compromise the efficiency of breeding programmes.

2.5.1. Importance of pome fruit genetic resources

As an outcome of the 1992 Biodiversity Convention meeting in Rio de Janeiro on plant genetic resources; governments recognised the need to conserve biological diversity, sustainable genetic resource utilisation as well as benefit sharing emanating from the use of these resources (Kellerhals, 2004). Preservation of genetic resources not only benefits breeding programmes but is also of significance for the heritage of the country (Fernández-Fernández, 2010; Nel and Griessel, 2012). In South Africa, the national government, through DAFF, partly funds the maintenance of fruit crop genetic resources at the ARC Infruitec-Nietvoorbij. Due to the importance of these resources as national assets, the maintenance of true to type collections is essential.

Knowledge of genetic diversity and relationships among accessions facilitates crop improvement as it allows more informed crosses to be initiated (Ganesh and Thangavelu, 1995; Erfani *et al*., 2012). To achieve efficient management and effective utilisation of the germplasm collections, accurate characterisation is important (Wünsch and Hormaza, 2007). The ability of a curator to achieve this goal is often hampered by rising costs, static budgets and large collection sizes (Hokanson *et al*., 1998). To increase the utility of the collection further, potentially useful accessions containing valuable genes within the collection needs to be well documented (Hokanson *et al*., 1998; Wünsch and Hormaza, 2007).

2.5.2. The ARC pome fruit collections

The breeding programme at the ARC Infruitec-Nietvoorbij maintains gene banks for both apples and pears in the Western Cape. The apple gene banks consist of approximately 540 accessions at Drostersnes and Elgin Experimental Farm located along the cooler Elgin Valley. The pear gene bank, consisting of approximately 197 accessions, is located at the Bien Donne experimental farm in the Franschhoek Valley. The collections comprise of wild species, commercial cultivars, mutants or sports of cultivars, international breeder's selections, local cultivars and ARC selections. Many of the accessions are mutations of popular cultivars. The ARC pome fruit collections are modest in comparison with those in many other countries in part due to South Africa's strict plant health policies, geographical isolation from other breeding programmes, political isolation during the apartheid era and the fact that pome fruits are not native to South Africa (Tobutt, personal communication).

A review of the available pome fruit genetic resources at ARC was recently conducted. As part of this exercise, the field collections were inspected to verify the reliability of the accessions. This was conducted with a view to increase confidence in the expected outcome of the breeding programme (Tobutt and Bester, 2011). Various misidentifications, such as mislabelling, were observed mostly by visual inspection. Furthermore, documentation could be improved. These issues hamper the breeding and genetics programme as they can lead to erroneous crosses and the wasting of time and money on raising seedlings that are not true to parentage (Evans *et al*., 2011).

2.6. Molecular fingerprinting of pome fruit genetic resources

Morphological characterisation (Westwood, 1981) was widely used for identifying pome fruit cultivars before the introduction of molecular markers and is still the only approved method for Plant Breeder's Rights determinations. There are however a limited number of suitable morphological characteristics and many of these may be greatly influenced by growth and environmental conditions (Zhang *et al*., 2011). Moreover, identification requires a lengthy and expensive evaluation especially during the growth period by skilled horticulturists to obtain morphological data (Ahmed *et al*., 2010). The identification of cultivars in a reliable and cost effective manner has therefore been an important application of molecular markers in recent years.

2.6.1. Identification methods for fingerprinting pome fruit

Molecular markers are more reliable in practice for cultivar identification (except for the discrimination of clones) and characterisation, because they are not influenced by variable environmental conditions or plant phenology and can differentiate between cultivars with similar phenotype (Tartarini, 2003). Protein based methods, *e.g*. isoenzymes, were some of the first molecular markers to be utilised for fruit tree characterisation but were also vulnerable to environmental influences (Weeden and Lamb, 1985) and were limited in number (Jang *et al*., 1991; Trujillo *et al*., 1995).

Various types of DNA markers can be used for tree fruit fingerprinting, including: RAPDs, inter-simple sequence repeats (ISSRs) and AFLPs. These markers were mostly used before the

emergence of microsatellites and single nucleotide polymorphisms (SNPs) (Wünsch and Hormaza, 2002). Although SNPs have gained popularity in recent years, many laboratories are not yet capable of handling SNP data and microsatellites are currently the markers most used in tree fruit fingerprinting.

2.6.2. Microsatellite markers for fingerprinting pome fruit

Various microsatellite markers have been developed in apple and pear have primarily been applied in fingerprinting studies (Guilford et *al.*, 1997; Hokanson *et al.*, 1998; Yamamoto *et al.*, 2001; Galli *et al*., 2005; Fernández-Fernández *et al*., 2006). Molecular fingerprinting of collections using microsatellites has had success in various other fruit crops as well including peach (Sosinki *et al*., 2000; Marchese *et al*., 2005), grape (Di Gaspero *et al*., 2000; Leão *et al*., 2009), papaya (De Oliveira *et al*., 2010) and many others. However opportunities to compare fingerprinting results between pome fruit collections have been hampered as, until recently, different laboratories used different sets of microsatellites.

This prompted the European Cooperative Programme for Genetic Resources (ECPGR) working group on pear and apple suggest a standardised sets of markers for the two crops (Tobutt and Evans, 2006; Evans *et al*., 2009; Fernández-Fernández, 2010). Standardised allele labelling systems, protocols, microsatellite markers and scoring were agreed upon to enhance comparison. To facilitate comparability, a set of 17 markers together with eight standard cultivars, were recommended per crop and high priority markers were specified for when all markers could not be used (Evans *et al*., 2009; Fernández-Fernández, 2010). The recommended sets have since been used successfully in several pome fruit studies, including diversity and fingerprinting projects for apple (Potts *et al*., 2011; Urrestarazu *et al*., 2012) and pear (Ahmed *et al*., 2010; Dos Santos *et al*., 2011; Sehic *et al*., 2012); making microsatellites the preferred markers for this work.

The first substantial use of the ECPGR recommended set of markers, protocol and standard cultivars, was conducted by East Malling Research (EMR), United Kingdom, with the fingerprinting of 2200 accessions of apple and 560 accessions of pear from the national collection at Brogdale (Fernández-Fernández, 2007, 2010): This work has been important in fingerprinting projects thereafter as a source of comparative data for apple (Potts *et al*., 2011; Urrestarazu *et al*., 2012) and pear (Ahmed *et al*., 2010; Dos Santos *et al*., 2011; Sehic *et al*.,

2012). Comparison of data between studies allow for trueness to type investigations due to the clonal nature of pome fruit.

2.7. Molecular characterisation of pome fruit genetic resources

Genic markers provide an opportunity to characterise available genetic resources for use both in breeding and as well as selection of cultivars for commercial orchard management and production practices.

The need for breeding new improved early and late ripening cultivars has long been recognised (Brown, 1960; Tancred 1995) particularly in countries that rely on shipping of products to distant markets (Gardener *et al*., 2007). The shelf-life of fruit and storability is highly influenced by the rate of ethylene production (Bassil and Lewers, 2009).

As previously explained, ethylene is synthesised from S-adenosyl-L-methionine (SAM) via the enzymes ACS and ACO. This is a two-step process: in the first step, ACS converts SAM to ACC and then ACO catalyses the oxidative fragmentation of ACC to form ethylene (Yang and Hoffman, 1984; Jiao *et al*., 1986). In apple, both enzymes proved to be candidates for MAS (Costa *et al*., 2005). The role of the *ACS* gene in ethylene production has been the most studied thus far (Itai *et al.*, 1999; Sunako *et al.*, 1999). However, there are other genes, such as *ACO*, involved in the ethylene production pathway.

The apple *ACS1* sequence contains three introns (I) located between four exonic regions (E) of the gene (Fig. 2.2) (Sunako *et al*., 1999). Length polymorphism in the promoter region (P) of the *ACS* gene is important for characterising variants of this gene. The variation in length of the promoter region occurs as a result of an insertion, 162 bp, and a concomitant 25 bp deletion totalling 138 bp. Primers have been designed to amplify the promoter region of the gene (Sunako *et al*., 1999). The presence or absence of the insertion (SINE) with the concomitant deletion (indel) determines the rate of ethylene production and subsequently, ripening. Cultivars homozygous for the shorter allele produce more ethylene than those homozygous for the longer allele. For genotypes that are heterozygous, a medium ripening pattern is observed (Sunako *et al*., 1999).

Castiglione *et al*. (1999) were the first to investigate the ripening phenomenon in apple with respect to ACS but observed no allelic forms of the *ACS* gene. Sunako *et al*. (1999) determined

the allelic forms of the *MdACS1* gene associated with the indel in the promoter region just described. Two allelic forms of *MdACS1* were found; late-season genotypes in the *MdACS1-2* class had the slowest rate of softening while early-season genotypes of the *MdACS1-1* class had the most rapid softening rate. Subsequently, three allelic combinations, *ACS1-1/1* (high ethylene production), *ACS1-1/2* (medium ethylene production) and *ACS1-2/2* (low ethylene production) have been correlated with early, mid or late ripening (Sunako *et al*., 1999; Harada *et al*., 2000; Oraguzie *et al*., 2004; Costa *et al*., 2005; Oraguzie *et al*., 2007; Zhu and Barritt, 2008; Zoufalá *et al*., 2009; Peace, 2014).

Fig. 2.2. Structure of ACS gene in apple (Sunako *et al*., 1999) showing promoter (P), four exons (E) and three introns (I) from the 5' to 3' end. An indel (In) occurs in the promoter region with arrows representing the primers amplifying the indel.

In Japanese and Chinese pears, Itai *et al*. (1999) developed an effective method for characterising genotypes with respect to ethylene production. Two cleaved amplified polymorphic sequence (CAPS) markers *PpACS1* and *PpACS2* of two ACS loci were associated with ethylene production; *PpACS1* is associated with high ethylene production and *PpACS2* with moderate ethylene production. The absence of these two markers indicated low ethylene producing genotypes (Itai *et al*., 2003).

El-Sharkawy *et al*. (2003; 2004) established a similar relationship in European pear where three genotypes of *PcACS1*, namely *aa*, *ab* and *bb*, successfully discriminated a few accessions into early, mid and late ripening respectively. However, no further studies have substantiated these findings. In a subsequent study on European pears, Oraguzie *et al*. (2010) were unable to genotype the European pears as in El-Sharkawy *et al*. (2003; 2004) suggesting further studies are required.

Table 2.1. A collation of the 267 apple cultivars and species. Information was sourced from several *ACS1* genotyping studies (Sunako *et al*., 1999; Harada *et al*., 2000; Oraguzie *et al*., 2004; Costa *et al*., 2005; Oraguzie *et al*., 2007; Zhu and Barritt, 2008; Zoufalá *et al*., 2009; Peace, 2014) that used the primers derived from Sunako *et al*. (1999). The initial study only characterised 48 accessions but paved the way for other *ACS1* characterisation studies.

Accession	Reference	Genotype	Accession	Reference	Genotype
Akane	c	*2/2*	Bramley's Seedling	a	*1/2*
Alice	d	*1/1*	Brighton	d	*1/1*
Alkmene	d	*2/2*	Calville Blanc d'Hiver	df	*1/2*
Aldenhamensis	b	*1/1*	Cambridge Pippin	d	*1/1*
Alma Pippin	d	*1/1*	Cameo	e	*1/2*
Amanishiki	a	*1/2*	Camoesa de Llobregat	d	*1/1*
Amassia	a	*1/1*	Catshead	f	*1/1*
Ambrosia	e	*2/2*	Chinook	ef	*2/2*
A. Summer Pearmain	a, b	*1/1*	Chisel Jersey	f	*1/1*
Ames 512	d	*1/1*	Civni	f	*2/2*
Angold	f	*1/2*	Close	d	*1/1*
Anna	f	*1/2*	Coop 15	e	*1/2*
Anbishas	a	*2/2*	Court Pendu Plat	f	*1/1*
Antonovka	a	*1/2*	Cox's Orange Pippin	a	*1/1*
Antonovka 172670-B	f	*1/1*	Creston	ef	*1/2*
Antonovka	f	*1/2*	Crimson Crisp	e, f	*2/2*
Api Rose	f	*1/1*	Cripp's Pink	e, f	*1/2*
Arlet	e	*1/2*	Cripp's Red	e f	*1/2*
Aurora Golden Gala	ef	*2/2*	Crofton	d	*1/2*
Autumn Gold	e	*1/2*	Dabinett	f	*1/1*
Ballarat Seedling	d	*1/2*	Danzinger Kantapfel	f	*1/1*
Bancroft	b	*1/1*	Delblush	e	*2/2*
Beacon	b	*1/2*	Delcorf	e, f	*1/1*
Beauty of Bath	d	*1/2*	Delcoros	f	*½*
Belgica	f	*1/2*	Delgollune	f	*1/2*
Bellefleur Krasny	f	*1/1*	Delicious	a, e, f	*1/2*
Bellefleur Kitaika	f	*1/1*	Delicious Red	d	*1/2*
Black Twig	d	*1/1*	Delicious Starking	b, c	*1/2*
Blanik	g	*1/2*	Delorgue	e	*1/2*
Blenheim Orange	f	*1/1*	Delorina	f	*1/2*
Bordes Cider	d	*1/1*	Democrat	g	*1/1*
Bozena Nemcova	d	*1/1*	Devonshire Quar.	d	*1/2*
Beninomai	c	*1/1*	Dima	g	*2/2*
Braeburn	d, e	*1/2*	Discovery	b, d, f	*2/2*

Accession	Reference	Genotype	Accession	Reference	Genotype
Dorset Golden	f	*1/2*	Guldborg	d	*1/1*
Dukat	g	*2/2*	Hacnine	c	*1/2*
Dumelow's Seedling	f	*1/2*	Hampshire	e	*1/1*
Early Strawberry	f	*1/1*	Haralson	f	*1/1*
Ecolette	f	*1/2*	Hatsuaki	a, c, e	*1/1*
Ed Gould Golden	f	*1/2*	Himekami	a, b, c	*2/2*
Edelborsdorfer	f	*1/1*	Himekomachi	b, e	*2/2*
Egremont Russet	f	*1/1*	Hokuto	e	*1/2*
Ein Shemer	df	*1/2*	Honeycrisp	e, f	*1/2*
Elise Ratke	d	*1/1*	Honeygold	g	*2/2*
Elliot	e	*1/2*	Hoozuri	c	*2/2*
Elstar	f	*2/2*	Huaguan	e, f	*2/2*
Emilia	f	*1/2*	Idared	f	*1/2*
Empire	e	*1/2*	Ikorovka Alaja	d	*1/1*
Enterprise	ef	*1/2*	Indo	a, b, c	*1/1*
Esopus Spitzenburg	f, g	*1/2*	Ingrid Marie	f	*1/2*
Fantasie	g	*1/2*	Iwakami	a, b	*2/2*
Fiesta	f, g	*1/2*	James Grieve	f	*1/1*
Florina	f	*1/2*	Jarka	g	*2/2*
Fortune	e	*1/1*	Jerseymac	b, d	*1/1*
Fraasove Letni	d	*1/2*	Jonagold	b, c	*1/2*
French Crab	df	*1/1*	Jonalord	g	*2/2*
Friandise	f	*1/2*	Jonathan	a, b, c	*1/2*
Freedom	c	*1/2*	Julia	g	*1/2*
Fu Jin	b	*1/1*	Julyred	a, b, f	*1/1*
Fuji	a, b, c, d, e, f	*2/2*	Kanki	c	*1/2*
Gala	c, e	*2/2*	Kaori	a, b	*2/2*
Gala Aurora Golden	e	*2/2*	Kempston	d	*1/1*
Gala Royal	d	*2/2*	Kidd's Orange Red	g	*1/2*
Gala Supreme	e	*2/2*	Kinsei	c	*1/2*
George Neale	d	*1/1*	Kitakami	a, c	*1/1*
Ginger Gold	e	*1/2*	Kitarou	c	*1/2*
Gloster	g	*2/2*	Klara	g	*1/1*
Gold Rush	e	*2/2*	Koningszuur	f	*1/1*
Golden Delicious	a, b, c, d, e, f	*1/2*	Korichnoe Polosatoje	f	*1/1*
Golden Melon	a, b	*1/2*	Koutarou	c	*1/2*
Goldrush	ef	*2/2*	Lady	f	*1/1*
Granny Smith	a, b, c, d, e, f	*1/1; 1/2**	Lady Williams	d	*1/2*
Gravenstein	f	*1/1*	Landsberger Reinette	f	*1/1*

Accession	Reference	Genotype	Accession	Reference	Genotype
Liberty	f	*1/2*	Norfolk Beefing	f	*1/1*
Lodi	d	*1/1*	Northern Spy	a, g	*1/1*
Lord Lambourne	f	*1/2*	Oaken Pin	f	*1/2*
Lord Suffolk	d	*1/2*	Ontario	f, g*	*2/2;1/2**
Lord Wosley	d	*1/2*	Orei	a	*1/2*
Lundbytorp	d	*1/2*	Orin	a, b, c, g	*1/2*
M. baccata	a	*1/1*	Oriole	d	*1/2*
M. florentina	a	*1/1*	Otava	g	*2/2*
M. floribunda	a	*1/1*	Pacific Beauty	d, e	*2/2*
M. hupehensis	a	*1/1*	Pacific Queen	d, e	*2/2*
M. prunifolia	a	*1/2*	Pacific Rose	d, e	*2/2*
M. pumila	a	*1/1*	Pink Lady	e	*1/2*
M. sargentii	a	*1/1*	Pinova	e	*2/2*
M. sieboldi	a	*1/1*	Prima	g	*1/2*
M. spectabilis	a	*1/1*	Priscilla	f	*1/2*
M. toringoides	a	*1/2*	Pristine	e	*1/2*
M. yunnanesis	a	*1/2*	Puritan	b	*1/1*
McIntosh	a, b, c, f	*1/1*	Rae Ime	d	*1/1*
Medaille d'Or	f	*1/1*	Ralls Janet	a, b, c, d	*2/2*
Megumi	a, b, c	*2/2*	Raritan	b	*1/2*
Melba	g	*1/1*	Red Delicious	d	*1/2*
Melrose	g	*2/2*	Red Dougherty	d	*1/1*
Merlijn	f	*1/2*	Redfree	g	*1/1*
Mikilife	d, e	*1/2*	Red Gold	a	*1/2*
Milwa	f	*2/2*	Red Malba	d	*1/1*
Min. von Hammerstein	f	*1/2*	Red Summer Rambo	f	*1/1*
Minneiska	f	*2/2*	Reinette de Thorn	d	*1/2*
Mollie's Delicious	f	*1/2*	Reinette du Canada	a	*1/1*
Monarch	d	*1/1*	R Marbree d'Auvergne	d	*1/1*
Monidal	e	*1/1*	Reinette Simirenko	d, f	*1/2*
Mr Fitch	d	*1/2*	Resista	g	*1/2*
Murray	f	*1/1*	Rokewood	d	*1/1*
Mutsu	a, b, c	*1/2*	Rome Beauty	a, d	*1/1*
Narihoko	a, b	*2/2*	Rosehask	d	*1/1*
Nevson	e	*2/2*	Rubimeg	g	*2/2*
Newtown Pippin	a, d, f	*1/2*	Rubin	g	*2/2*
Niagara	d	*1/2*	Rubinola	f, g	*2/2*
Nicogreen	f	*1/2*	Rubinstep	g	*2/2*
Nicoter	f	*2/2*	Rucla	f	*2/2*

Accession	Reference	Genotype	Accession	Reference	Genotype
Runkel	e	*1/2*	Suncrisp	e, f	*1/2*
Russian Seedling	f	*1/1*	Sundance	e, f	*2/2*
Sabina	e, f	*2/2*	Sundowner	e	*1/2*
Sansa	a, b, c, e, g	*2/2*	Sunrise	e, f	*1/2*
Santana	f	*1/2*	Tangier	b	*1/2*
Santarou	c	*1/1*	Toko	b	*1/2*
Scarlet Nonpareil	d	*1/2*	Tom Putt	f	*1/1*
Scarlet Pearmain	d	*1/2*	Topaz	f, g	*2/2*
Sekaichi	c	*1/2*	Tsugaru	a, b, c,	*1/2*
Selena	g	*1/1*	Tunda	f	*1/2*
Senshu	c, e	*2/2*	Viking	b	*1/1*
Shampion	g	*1/2*	Vista Bella	b	*1/1*
Shinsekai	c, e	*2/2*	Washington	d	*1/1*
Shizuka	e	*1/2*	White Winter Pearmain	a	*1/2*
Silken	e	*1/2*	Willie Sharp	d	*1/1*
Smoothee	c	*1/2*	Winston	d	*1/2*
Sonja	e	*2/2*	Winter Majetin	d, f	*1/1*
Spartan	G	*1/1*	Worcester Pearmain	f	*1/1*
Spatbluhender Taffet.	F	*1/1*	Yellow Newtown	a	*1/2*
Splendour	d, e	*2/2*	Yellow Transparent	f	*1/1*
Spokane Beauty	F	*1/1*	York Imperial	a	*1/2*
Springdale	D	*1/1*	Zari	f	*1/2*
State Fair	G	*2/2*	Zestar	e, f	*2/2*
Statesman	D	*1/1*	Zonga	f	*1/2*
Sturmer Pippin	a, d	*1/2*	Zvonkove	g	*1/2*
Summer Apple	F	*1/1*			

[a] Sunako *et al*. (1999), [b] Harada *et al*. (2000), [c] Oraguzie *et al*. (2004), [d] Oraguzie *et al*. (2007), [e] Zhu and Barritt (2008), [f]Peace (2014), [g]Zoufalá *et al*. (2009), *1/1* high ethylene producing, *1/2* medium ethylene, *2/2* low ethylene production, * inconsistency, Geno - genotype

Thus evidence exists that *ACS* genic markers can be used to identify ripening patterns in pome fruit cultivars in gene banks, providing useful knowledge of possible parents for breeding for delayed ripening. However, the genotypes of most accessions in the ARC's apple collection have not yet been genotyped for *ACS*.

2.8. Conclusion and purpose of study

The need to accurately identify germplasm clearly to enhance the usefulness of collections has been emphasised in the literature (Gulford *et al*., 1997; Hokanson *et al*., 1998). Microsatellites have been successful in fingerprinting of apple, pear and other fruit crops such as grapes (Leão *et al*., 2009) and peach (Marchese *et al*., 2005).

Markers for *ACS1* enable the genotyping of potential parents for breeding apple cultivars with low ethylene production associated with late ripening. Limited knowledge on the ripening pattern of European pears likely to be used as parents poses a challenge and an opportunity to the ARC breeding programme by providing an opportunity to develop markers useful for European pear genotyping in future.

The objectives of this study are: 1) to fingerprint the ARC apple collections using microsatellite markers to assist in resolving misidentifications, confirming trueness to type and verifying parentage, 2) to fingerprint the ARC's pear collections using microsatellite markers to simirlarly assist in resolving misidentifications, confirming trueness to type and verify parentage and 3) to characterise the *ACS1* genotypes of the apple accessions to understand ripening properties of potential parents using customised ACS primers.

2.9. References

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Chapter 3

MICROSATELLITE FINGERPRINTING OF THE ARC PEAR COLLECTION

3.1. Introduction

European pear (*Pyrus communis*) is the third most important deciduous fruit crop in South Africa after grape and apple. From the earliest plantings in the 1600s (Roosi, 2005), the area planted has expanded to 11 700 hectares in the more temperate regions of the country with over 360 000 tonnes annual production. Of this 46% is exported, mostly to Europe (HORTGRO, 2012; PPECB, 2013). Commercial production is dominated by a few introduced cultivars such as 'Abate Fetel', 'Beurre Bosch', 'Forelle', 'Packham's Triumph' and 'William's Bon Chretien' (Human, 2013), though the locally bred blushed cultivars 'Flamingo', 'Rosemarie' and, recently, 'Cheeky', have a significant area of production, ±791 hectares. There is an increasing need to breed high quality cultivars requiring low inputs that are well-adapted to the local growing conditions (Tobutt and Bester, 2011); not only to maintain competitiveness of the current production areas which are being threatened by climate change but also, potentially, to expand into non-traditional pear production areas.

The Agricultural Research Council (ARC) Infruitec-Nietvoorbij breeds new and diverse pear cultivars for the South African industry (Human, 2013). Success of the programme depends partly on the quality of the genetic resources available in the gene bank maintained at the Bien Donné Experimental farm in the Western Cape, South Africa. This gene bank, which is regarded as a national asset (Bester *et al*., 2013), was established over 40 years ago and consists of approximately 197 accessions (Human, personal communication). Most accessions are *P. communis,* and represent cultivars, or their clonal selections or sports, or seedling selections from the breeding programme. In addition, there are a few accessions of Japanese pear, *P. pyrifolia,* or other *Pyrus* species as well as some interspecific hybrids. Most accessions are planted as two adjacent trees, thought to be grafted on the rootstocks 'BP1' or 'BP3' (Human, personal communication). Most *Pyrus* accessions are diploid ($2n=2x=34$); however instances of triploids have been reported (Crane and Thomas, 1939; Crane and Lewis, 1942) and the cultivars 'Lucas' and 'Vicar of Winkfield' in the ARC gene bank are known triploids (Crane and Thomas, 1939; NCGR, 2013).

A recent review of the ARC pome fruit collections revealed inconsistencies that limit confident use of the material in the breeding programme (Tobutt and Bester, 2011). Misidentification of accessions was noted as a significant problem. Although morphological characterisation of the pear collection has been conducted to remedy the situation (Human, personal communication), some mislabelling are difficult to resolve visually especially if they concern lesser known

cultivars. Verification of trueness to type is therefore of high priority for effective utilisation of the genetic material. Molecular fingerprints of the accessions will provide baseline data in preparation for re-propagation of the gene bank and for verification purposes.

The advent of molecular markers and in particular the development of microsatellites, also known as Simple Sequence Repeats (SSR), provides a tool to fingerprint accessions in a rapid, reliable, and relatively cost effective manner without the need for expert morphological characterisation. Microsatellite markers are polymorphic, codominant and highly transferable between closely related species. Primers designed from apple (Gianfranceschi *et al*., 1998; Hokanson *et al*., 1998; Liebhard *et al*., 2002) have succesfully been used for genetic studies in pears but more recently microsatellite markers have been developed specifically in European pear (Bassil *et al*., 2004; Fernández-Fernández *et al*., 2006) and Japanese pear (Yamamoto *et al*., 2002). In the last decade, microsatellites have been used extensively for pear fingerprinting studies in several fruit research institutions across America, Asia and Europe (Volk *et al*., 2006; Miranda *et al*., 2010; Wolko *et al*., 2010; Yakovin *et al*., 2011; Tian *et al*., 2012).

However, the use of different sets of markers among laboratories prevents comparison of data. This prompted the European Cooperative Programme for Plant Genetic Resources (ECPGR)'s *Pyrus*/*Malus* working group to select a standard set of microsatellite markers and reference cultivars, and to harmonise the fingerprinting conditions, to enable comparison of microsatellite fingerprint data between laboratories (Tobutt and Evans, 2006; Evans *et al*., 2009). Seventeen markers that were publicly available and well-spaced across the genome were recommended; for each linkage group, one microsatellite marker that had a reasonable level of polymorphism, revealed a single locus and had no null alleles was selected (Tobutt and Evans, 2006). Subsequently, a subset of 12 microsatellite markers was identified as high priority for instances where not all markers could be used and three multiplexes, each comprised of four markers, were developed (Fernández-Fernández, 2010). The standard set of reference accessions to calibrate the scoring comprises five *P. communis* cultivars and three cultivars of other *Pyrus* species, maintained at the United Kingdom's National Fruit Collection at Brogdale.

Several studies have shown the utility for data sharing and comparison of the ECPGR's recommended markers and reference cultivars (Fernández-Fernández, 2010; Ahmed *et al*., 2010; Dos Santos *et al*., 2011; Sehic *et al*., 2012). Furthermore, the online availability of the fingerprints of 559 accessions from the Brogdale collection, genotyped with the subset of 12 microsatellites at East Malling Research (EMR) (http://www.emr.ac.uk/SPFeliFernández.htm)

has enabled other laboratories to compare their data when using the same microsatellites. However, no other study has successfully replicated the multiplex conditions used for the Brogdale collection by Fernández-Fernández (2010); thus some groups use the markers individually in simplex PCR reactions (Sehic *et al*., 2012).

The current study aims to fingerprint the ARC pear collection using the same subset of the recommended ECPGR microsatellite markers as Fernández-Fernández (2010), in order to resolve inconsistencies and provide reference data for future propagation. True to type material will improve the utility of the gene bank and allow breeders to conduct accurate crosses for breeding and genetic studies.

3.2. Materials and methods

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

3.2.1. Plant material

Samples of 197 accessions were collected from the pear gene bank; plot WG8 at Bien Donné, Groot Drakenstein, Western Cape, South Africa. This comprised of 119 accessions of *P. communis* cultivars, of which 80 were 'primary' cultivars and 42 were clones, duplicates or sports; 63 accessions of selections of *P. communis*, including duplicates; seven representatives of other *Pyrus* species or interspecific hybrids; and eight unknown accessions (Table 2.1). Approximately 53 of the 'primary' cultivars at ARC were also reported in the Brogdale collection. In total, 17 items were apparently duplicated in the gene bank, ten cultivars and seven selections, although it should be noted that the accessions usually did not have accession numbers, and therefore tree locations were used as identifiers. The rootsctocks 'BP1' and 'BP3' were included, material being sourced from the South African Plant improvement Organisation (SAPO). Another recently released cultivar, 'Celina', sourced from the Deciduous fruit Plant improvement Association (DPA) (commonly referred to as the Sagtevrugte Plantverbeterings Vereniging (SPV) in the pome fruit industry), was also included. In general, the first tree out of two of a kind was sampled; however, in instances where the first tree had died the second tree was sampled. Rather than obtaining reference materials from Brogdale, the ARC accessions of six of the eight recommended reference cultivars were used in this study; five of *P. communis* and one of *P. pyrifolia* (Table 2.2). The remaining two reference cultivars, *P.*

calleryana ‘Chantecler’ and *P. salicifolia* ‘Pendula’, were not present in the ARC pear gene bank and therefore not included in the current study.

Young expanding leaves were collected in spring (early September) and frozen at -80°C until required for DNA extraction. Leaf material was weighed to 0.3 g (±0.1 g) and placed in a labelled 2 ml Microcentrifuge tube and stored at -20°C until further use. Samples were prepared in duplicate to allow for repeat analysis.

Table 3.1. ARC pear accessions from Bien Donné Experimental Farm plot WG8, SAPO and SPV, fingerprinted in the current study indicating tree location, accession name and code. The accessions are grouped: ARC selections of *P. communis*, cultivars, clones, or sports of *P. communis*; *Pyrus* species and hybrids; and unknown accessions. Clones are grouped with primary cultivars. Items labelled B are present in both ARC and Brogdale collections, é represents a clone or a sport of a cultivar.

Tree No.	Accession name	Code	Tree No	Accession name	Code
ARC selections of *P. communis*			2_31	5-32-53	
1_12	3C_11_9		3_31	5-36-30	
1_24	3C_11_25		2_43	5-39-60	
1_40	3C_44_34		3_27	5-40-45	
2_16	3C_49_18		3_19	5-40-60	
3_43	3C_51_28		3_37	5-41-18	
6_7	3D_83_10		6_11	5-41-57	
6_16	5-03-29		3_41	8-6-34	
2_24	5-03-29		3_2	8-9-14	
1_28	5-16-89		1_10	8-20-58	
4_17	5-16-89		2_41	8-22-120	
5_1	5-16-122		3_29	8-23-81	
6_6	5-16-122		2_18	8-24-25	
3_33	5-17-169		5_3	8-24-51	
3_39	5-19-27		4_22	8-24-63	
3_11	5-24-21		2_29	8-25-25	
2_4	5-25-21		3_25	8-25-48	
2_28	5-31-79		2_22	8-25-57	
2_37	5-32-8		4_19	8-25-57	

Tree No.	Accession name	Code	Tree No.	Accession name	Code
1_38	8-25-72		3_46	Bartlett	B
1_16	8-26-91		5_4	Burger Bon C	B
3_23	8-28-59		2_35	Bon Chretien	B
2_20	8-30-145		3_40	Bon Chretien	B
4_18	8-30-145		3_20	Bon Chretien (Koo)	é
6_1	8-31-25		1_19	Bon Chretien A	é
1_44	8-31-37		1_21	Bon Chretien B	é
4_21	8-31-67		1_23	Bon Chretien C	é
1_30	8-31-158		1_25	Bon Chretien D	é
5_2	8-31-158		1_27	Bon Chretien E	é
1_34	8-33-53		1_29	Bon Chretien Sport	é
1_14	8-34-54		1_8	William's BC	B
1_22	8-34-91		3_28	William's BC	B
5_16	11B-2-25		1_26	El Dorado	é, B
5_13	11B-39-17		3_16	El Dorado	é, B
5_17	11B-7-17		5_5	El Dorado (VV)	é
5_18	11B-7-21		1_31	Bon Rouge	é
5_19	11B-7-26		2_12	Bon Rouge	é
5_20	11B-7-28		SAPO	BP 1	
6_15	11C-6-27		1_18	BP 2	
6_13	11C-9-11		1_6	BP 2	
5_21	11D-10-9		6_19	Cascade	B
5_14	15A-4-14		SPV	Celina	
5_15	15A-7-21		4_9	Ceres	
6_14	15B-5-2		4_11	Ann's Favourite	é
Cultivars of *P. communis*			1_33	Clapp's Favourite	B
6_22	Abate Fetel	B	3_17	Starkrimson	é, B
1_4	Abate Fetel	B	3_24	Starkrimson	é, B
1_1	Berg. de Esperance	B	3_14	Colonel Wilder	
4_3	Beth	B	6_24	Concorde	B
1_3	Beurre Bosch	B	3_42	Conference	B
3_21	Beurre Bosch	B	1_37	Conference	B
6_18	Golden Russet	é, B	1_35	Contesse de Paris	B
6_23	Boscova	é	4_2	Cristalli	
1_5	Beurre Clairgeau	B	1_7	Beurre d'Anjou	B
1_9	Beurre Giffard	B	6_3	Red d'Anjou	é
1_11	Beurre Hardy	B	1_20	December	
4_14	Beurre Hardy	B	1_39	December	
3_34	Beurre Hardy (Emla)	B	4_8	Delbard Precoce	
3_32	Beurre Hardy Sport	B	4_6	Delbard Premiere	
1_13	Beurre Six	B	6_29	Delete	
1_15	Beurre Superfin	B	6_28	Delmoip	
1_17	Beurre van Geerdt	B	6_26	Delmore	

Tree No.	Accession name	Code	Tree No.	Accession name	Code
6_17	Delwilmore		3_44	Onward	B
5_10.	Red Comice	B	6_9	Onward	B
1_41	Doyenne du Comice	B	2_34	Orange Bergamotte	B
3_38	Doy. du Comice (Emla)	B	3_22	Packham's Triumph	é
4_5	Dr Jules Guyot	B	3_18	Packham's Tr. (Brown)	é
1_43	Duch. d'Angouleme	B	4_15	Packham's Tr. (VV)	B
1_45	Duch. de Bordeaux	B	3_26	Passe Crassane	B
2_1	Emile d'Heyst	B	2_40	Passe Crassane	B
3_9	Emperor		2_36	Patrick Berry	B
1_2	Flamingo		2_38	Precoce de Trevoux	B
2_8	Flamingo		4_1	Reimer Red	B
4_20	Flamingo		2_42	Roosevelt	B
2_3	Fondante d'Automne	B	5_11	Rosemarie	
2_5	Forelle	B	6_4	Ruby Glo	
6_21	Forelle Malherbe	é	3_36	Saffran	
2_7	Ganzels Bergamotte	B	3_12	Saffran Winter	
3_15	General Leclerc	B	2_44	Stanley	
4_10	General Leclerc	B	2_46	Tongers	B
6_12	Glou Morceau	B	3_1	Twyford Monarch	
4_13	Harrow Delight	B	3_5	Vicar of Winkfield	B
2_13	Hertzogin Elza	B	3_10	Winter Nelis	
4_7	Highland	B	**Other *Pyrus* species and hybrids**		
6_27	Jana		SAPO	*P.* BP 3	
2_15	Jos. de Malines	B	2_26	*P. calleryana* Calleryana	
4_16	Kalbas Peer		5_7	*P. pyrifolia* Chojuro	B
6_20	Lily		2_9	*P.* hybrid Garber	
2_21	Louise Bonne A	B	5_9	*P. pyrifolia* Hosui	B
2_23	Louise Bonne B	B	2_17	*P.* hybrid Kieffer	B
2_25	Louise Bonne C	B	2_19	*P.* hybrid Le Conte	
2_27	Lucas	B	**Unknowns**		
2_30	Magnate		1_36	Unknown 1	
2_32	Marguerite Marillat		2_14	Unknown 2	
2_6	Morettini 64		3_3	Unknown 3	
4_4	Morettini 64		5_22	Unknown 5	
6_5	Mostert 51		5_23	Unknown 6	
5_12	Nassau Strydom		6_1	Unknown 7	
2_10	Old Home	B	6_2	Unknown 8	
3_4	Old Home	B	6_3	Unknown 9	

Berg = Bergamotte, Bon C = Bon Chretien, Doy = Doyenne, Duch = Duchesse, Jos = Josephine and Tr = Triumph

Table 3.2. Six South African accessions of reference pear cultivars recommended by ECPGR's *Pyrus/Malus* working group (Evans *et al.*, 2009) and their location in the ARC gene bank.

Tree No.	Species	Reference cultivar
1_4	*P. communis*	Abate Fetel
1_37	*P. communis*	Conference
1_41	*P. communis*	Doyenne du Comice
2_40	*P. communis*	Passe Crassane
3_28	*P. communis*	William's Bon Chretien
5_9	*P. pyrifolia*	Hosui

3.2.2. DNA extraction

Genomic DNA was extracted following a slightly modified method by De la Rosa *et al*. (2002). The microcentrifuge tubes containing frozen leaves were placed at room temperature to initiate thawing. Before complete thawing, a single 1 mm stainless steel ball-bearing was placed inside the tube. Extraction reagents of 0.8 ml prewarmed (65°C) CTAB buffer [2% (m/v) CTAB (Merck), 2% (m/v) PVP 40 (Merck), 1.4M NaCl (Merck), 20 mM EDTA at pH 8 (Merck), 100 mM Tris at pH 8 (Melford Laboratories)] and 0.08 ml β-mercaptoethanol (Merck) were added.

Samples were shaken by hand to mix the reagents and then ground thoroughly for 3 to 4 min using a Tissuelyser II ball mill (Qiagen). Samples were incubated for 2 hours at 65°C and the ball bearings removed using a stainless steel magnet. Thereafter 0.8 ml of chloroform-isoamyl alcohol (Merck) at a ratio 24:1 was added and the sample centrifuged (Labnet) for 15 min at 13 500 rpm using a centrifuge (Labnet). The top aqueous phase was recovered, 0.8 ml chloroform-isoamyl alcohol (24:1) was added again and the sample was centrifuged for 10 min at 13 500 rpm. The top aqueous phase was then recovered and precipitated with 0.5 ml cold isopropanol (Merck) overnight. After precipitation, samples were centrifuged at 4°C for 15 min at 13 500 rpm, the was solution discarded and the pellet washed in 0.5 ml of 70% (v/v) ethanol, dried for 30 to 45 min and resuspended in TE buffer until further use.

The quality and quantity of the DNA was determined with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) following the manufacturer's instructions. If a sample showed poor quality and quantity, the extraction was repeated. The DNA samples from the different extractions were diluted and adjusted to a final concentration of 100 ng/µl. Both resuspended and diluted DNA were stored at -20°C to avoid deterioration.

3.2.3. Primer selection and multiplex conditions

The subset of the 12 markers recommended by Fernández-Fernández (2010) from the Evans *et al*. (2009) set of 17 markers was used for the current study (Table 2.3). As mentioned, Fernández-Fernández (2010) combined the markers into three groups for multiplexing, based on product size range: Multiplex A (smaller sized products 82 to 164 bp), Multiplex B (medium sized products 84 to 195 bp) and Multiplex C (larger sized products 175 to 321 bp). The forward primers were fluorescently labelled (Applied Biosystems) with the dyes recommended by Fernández-Fernández (2010), and the reverse primers (Life Technologies) were unlabelled.

Table 3.3. The 12 microsatellite primer pairs, recommended by the ECPGR's *Pyrus*/*Malus* working group (Evans *et al*., 2009; Fernández-Fernández, 2010) used in the current study. The forward primers were labelled with the fluorescent dyes Fam, Ned, Vic or Pet.

Marker	Linkage group	Forward sequence	Reverse sequence	Label	Multiplex
[3]CH05c06	16	att gga act ctc cgt att gtg c	atc aac agt agt ggt agc cgg t	Fam	A
[4]EMPc11	11	gcg att aaa gat caa taa acc cat a	aag cag ctg gtt ggt gaa at	Ned	A
[4]EMPc117	7	gtt cta tct acc aag cca cgc t	cgt ttg tgt gtt tta cgt gtt g	Vic	A
[2]GD147	13	tcc cgc cat ttc tct gc	aaa ccg ctg ctg ctg aac	Pet	A
[3]CH03d12	6	gcc cag aag caa taa gta aac c	att gct cca tgc ata aag gg	Fam	B
[2]GD96	17	cgg cgg aaa gca atc acc t	gcc agc cct cta tgg ttc cag a	Ned	B
[3]CH01d09	12	gcc atc tga aca gaa tgt gc	ccc ttc att cac att tcc ag	Vic	B
[1]CH02b10	2	caa gga aat cat caa aga ttc aag	caa gtg gct tcg gat agt tg	Pet	B
[3]CH03g07	3	aat aag cat tca aag caa tcc g	ttt ttc caa atc gag ttt cgt t	Fam	C
[3]CH01d08	15	ctc cgc cgc tat aac act tc	tac tct gga ggg tat gtc aaa g	Ned	C
[3]CH01f07a	10	ccc tac aca gtt tct caa ccc	cgt ttt tgg agc gta gga ac	Vic	C
[3]CH04e03	5	ttg aag atg ttt ggc tgt gc	tgc atg tct gtc tcc tcc at	Pet	C

[1]Gianfranceschi *et al*. (1998), [2]Hokanson *et al*. (1998), [3]Liebhard *et al*. (2002), [4]Fernández-Fernández *et al*. (2006)

Initial optimisation revealed competition of fluorescent labels which necessitated the adjustment of the volumes per primer pair for the different multiplexes (Table 2.4). Primer dilutions for use in PCRs were made from a 100 µM primer stock solution adjusted to 10 µM working concentrations.

Table 3.4. Composition of primer mixes for Multiplexes A, B and C used in this study.

Marker	Forward primer (µl)	Reverse primer (µl)	Dilution (combined +DH_2O)
Mutiplex A			
CH05c06	1.0	1.0	
EMPc117	2.0	2.0	
EMPc11	1.0	1.0	
GD147	1.25	1.25	
Total	5.25	5.25	10.5+89.5 = 100 µl
Multipex B			
CH03d12	1.1	1.1	
CH01d09	1.0	1.0	
GD96	1.1	1.1	
CH02b10	1.4	1.4	
Total	4.6	4.6	9.2+90.8 = 100 µl
Multiplex C			
CH03g07	1.2	1.2	
CH01f07a	1.1	1.1	
CH01d08	1.1	1.1	
CH04e03	1.6	1.6	
Total	5.0	5.0	10.0+90.0 = 100 µl

3.2.4. Microsatellite genotyping

Amplification PCRs were performed in a final volume of 12.5 µl containing 1.5 µl of 100 ng template DNA, 6.25 µl of PCR mix (Qiagen), 1 µl of primer mix for Multiplex A, B or C (as diluted in Table 2.4) and 3.75 µl of RNase-free water. Amplification was carried out in GeneAmp (Applied Biosystems) or G-Storm (G-Storm Direct) thermal cyclers using the following conditions: an initial denaturation at 95°C for 15 min, followed by 29 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, with a final 15 min extension at 72°C.

Amplification products from a subset of accessions were first resolved electrophoretically (Hoefer Scientific Instruments PS 500X) on a 1% (m/v) agarose gel (Conda Laboratories) at 70V for 60 min in a 1X TBE buffer (Tris, Boric acid, EDTA) using a 1kb ladder (Thermo Scientific) to confirm amplification. Upon confirmation, the full set of PCR products was sized with capillary electrophoresis on a 3130 DNA capillary analyser (Applied Biosystems). Sizes of the amplified products were established in comparison with the internal size standard, GS500(-250)LIZ (Applied Biosystems). The software GENEMAPPER version 5.0 (Applied Biosystems) was used to visualise the peaks and aid allele scoring. Data were independently validated by a competent co-worker and collated in Microsoft Excel 2010.

3.2.5. Statistical analysis

Prior to genetic data analysis, the validity of the collated data generated by GENEMAPPER was tested using MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al*., 2004). The software examines the possibility of misscoring due to stuttering, allele dropout or presence of null alleles. 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 4.3 (Rousset, 2008).

Genetic diversity statistics were calculated, firstly for the entire set of accessions excluding false and triploid accessions (as identified by allele comparison of GENEMAPPER scores) and secondly for primary cultivars of *P. communis* excluding clones and ARC selections. No separate analyses were conducted on local ARC selections, Asiatic pears or interspecific hybrids as these individuals were limited in number. The number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He) and Shannon's information index (I) were calculated using GENALEX version 6.501 (Peakall and Smouse, 2012). The number of alleles per locus (Na) is a direct count of alleles amplified by a given marker for all the samples. 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. Expected heterozygosity (He) for each marker is calculated on the basis of the formula by Nei (1973), $He=1-\sum(pi)^2$, and *pi* is the probability that two alleles from the same locus are different when chosen at random from a given population. Shannon's information index, $I=\sum pi \ln pi$, similarly uses *pi* to provide an unbiased measure of allelic diversity per locus. Polymorphic information content (PIC) of markers, $PIC=1-\sum(pij)^2$, was calculated using CERVUS version 3.0.7 (Kalinowski *et al*., 2007) to determine how informative the markers were.

3.2.6. Classification of accessions

Fingerprint data of the Brogdale collection used for comparison were provided by Fernández-Fernández, EMR. Four classes have previously been proposed for grouping accessions fingerprinted in grape (De Andres *et al*., 2007) and have been adopted for classifying pear genotypes (Sehic *et al*., 2012). Classes proposed in the current study were motivated by these two studies: class 1, items present in the ARC collection but not the Brogdale collection; class 2, 'primary' cultivars present in both ARC and Brogdale collections and having consistent patterns; class 3, synonyms, sports and clones of 'primary' cultivars; class 4, items present in

ARC and Brogdale collection, but having inconsistent patterns; and class 5, unidentified accessions with no information.

To establish whether 'unknown' cultivars could be identified, cluster analysis was conducted. A pairwise genetic test was utilised to calculate the genetic distance matrix for codominant data using GENALEX on an individual-by-individual (*N*x*N*) basis. The matrix was subsequently converted to a MEGA input file and 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). No bootstrap analysis was conducted as this method was used only to identify 'Unknown' accessions and other possible mislabelling.

3.3. Results

The molecular fingerprints for the 197 accessions including 'BP1', 'BP3' and 'Celina' analysed in this study are attached as Appendix 3.1.

3.3.1. Marker performance

Four of the 12 markers used, CH01d08, CH01f07a, GD96, and GD147, amplified unsatisfactorily in the current study. The markers GD96 and GD147 (both Multiplex B) usually showed multiple peaks that were difficult to score, whereas markers CH01d08 and CH01f07a (both Multiplex C) showed no amplification at all. The other markers (CH01d09, CH02b10, CH03d12, CH03g07, CH04e03, CH05c06, EMPc11 and EMPc117) amplified well with one, two or three clear peaks per sample that were interpreted as alleles.

3.3.2. Statistical analysis

MICRO-CHECKER results revealed no evidence of scoring errors due to stuttering or presence of null alleles (Fig. 2.1) for any of the eight markers, and the data were considered valid for statistical analysis.

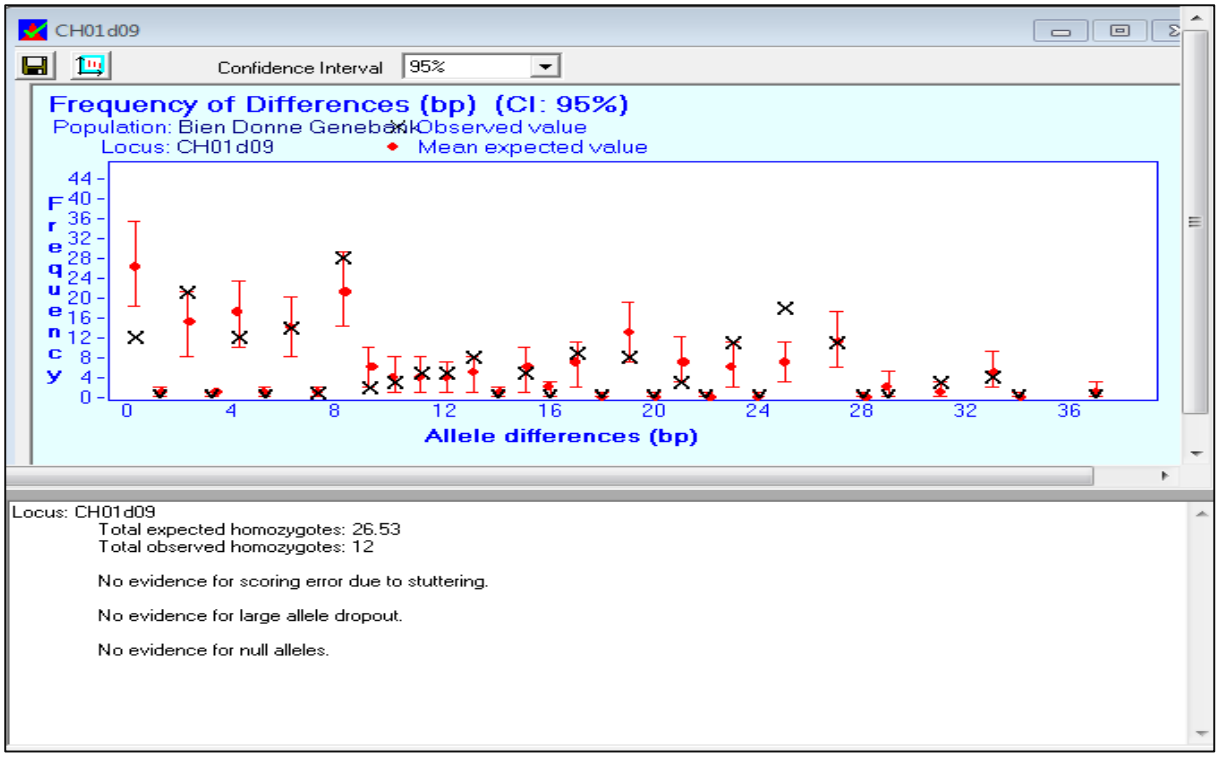

Fig. 3.1. MICRO-CHECKER output for marker CH01d09, showing no evidence of scoring error due to stuttering or null alleles. The other seven markers gave similar results.

Occasionally, some markers gave additional third peaks for some accessions and those accessions were excluded from the statistical analysis, which assume diploidy. All markers deviated significantly from HWE when both the entire set of accessions and only *P. communis* accessions were analysed (data not shown). For the entire set of accessions, excluding accessions found to be incorrect or triploids as described later, the number of alleles per locus ranged from 9, in the case of CH04e03, to 22, for CH03g07, (Table 3.5). In the second analysis, of only the 'primary' *P. communis* accessions, the number of alleles ranged from 5 per locus, for CH04e03, to 18, for CH03g07 (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 eight microsatellite markers for (a) complete set of 188 diploid accessions in the gene bank (above), and (b) 80 'primary' diploid *P. communis* cultivars (below).

		EMPc11	CH02b10	CH01d09	EMPc117	CH04e03	CH03g07	CH05c06	CH03d12
a	Na	14	19	18	17	9	22	13	13
	Ho	0.70	0.91	0.94	0.80	0.55	0.91	0.79	0.66
	He	0.64	0.84	0.85	0.74	0.56	0.81	0.72	0.70
	PIC	0.61	0.82	0.84	0.73	0.49	0.79	0.67	0.65
	I	1.45	2.11	2.22	1.85	1.08	2.081	1.59	1.47
b	Na	8	16	15	15	5	17	9	10
	Ho	0.69	0.95	0.98	0.81	0.53	0.94	0.71	0.73
	He	0.64	0.83	0.86	0.74	0.47	0.81	0.66	0.71
	PIC	0.59	0.81	0.83	0.72	0.41	0.78	0.60	0.65
	I	1.37	2.05	2.18	1.81	0.86	2.01	1.38	1.46

Overall, observed heterozygosity ranged from 0.55 for CH04e03 to 0.94 for CH01d09 with expected heterozygosity ranging from 0.56 for CH04e03 to 0.85 for CH01d09 for the entire collection. The mean observed heterozygosity was 0.78 and the mean expected heterozygosity, 0.73 (data not shown). When 'primary' *P. communis* cultivars were analysed separately, observed heterozygosity ranged from 0.53 for CH04e03 to 0.98 for CH01d09 and, likewise, expected heterozygosity from 0.47 to 0.86 with mean values of 0.79 and 0.72 for Ho and He, respectively. The PIC value ranged from 0.49 for CH04e03 to 0.84 for CH01d09 with a mean of 0.70 for the entire population, while a range from 0.41 for CH04e03 to 0.83 for CH01d09 was observed for 'primary' cultivars with a mean of 0.67. Shannon's information index was highest for marker CH01d09 and lowest for CH04e03 in both analyses with I values of 2.22 and 1.08 for the entire population, and 2.18 and 0.86 for the 'primary' cultivars, respectively.

3.3.3. Reference cultivar verification

The microsatellite patterns of the recommended ECPGR reference accessions from the Brogdale collection were compared with the South African accessions of the recommended cultivars to calibrate the scoring. For 'Hosui' and 'Passe Crassane', the microsatellite patterns of the ARC and Brogdale accessions did not match. The remaining reference cultivars provided a useful guide for calibration. Six out of eight markers presented adequate amplification, consistent shifts in allele size ranging from +1 to +3 bp were observed (Table 2.6). Data

adjustment for markers CH03g07 and EMPc11 was not straight forward and required accession by accession inspection. Microsatellite scores of accessions of cultivars also present in Brogdale, 'unknowns' and accessions in the ARC gene bank found to be mislabelled were adjusted and incorporated into an Excel sheet for comparison with the Brogdale data by genotype sorting.

Table 3.6. Comparison of the ECPGR genotypes of reference pear accessions from Brogdale with the genotypes of ARC accessions of the same cultivars for eight microsatellite markers.

Reference	CH01d09		CH02b10		CH03d12		CH03g07	
	Brogdale	ARC	Brogdale	ARC	Brogdale	ARC	Brogdale	ARC
Abate Fetel	149/151	151/153	126	129	106/110	108/112	240/243	243/246
Conference	155	157	122/126	125/129	106/123	108/125	224/254	227/257
D. du Comice	149/155	151/157	132/136	135/139	106/110	108/112	226/230	227/232
Passe Crassane*	151/155	140/151	130/132	135/144	123	112/125	224/240	227/243
Williams	147/155	149/157	120/126	123/129	106/123	108/125	224/240	227/243
Hosui*	138/153	130/151	122/132	125/135	97	98	248	252/258
Shift		+2		+3		+2		+1 to +3

* Accessions inconsistent with the Brogdale references

Table 3.6. Continued….

Reference	CH04e03		CH05c06		EMPc11		EMPc117	
	Brogdale	ARC	Brogdale	ARC	Brogdale	ARC	Brogdale	ARC
Abate Fetel	180/198	182/200	87/91	89/93	142/149	144/150	113/115	116/118
Conference	180/206	182/207	87/97	89/99	138/149	140/150	115/117	118/120
D. du Comice	180/198	182/200	87	89	149/153	150/155	113	116
Passe Crassane*	180	182	87/107	109/113	149	140/150	97/113	118
Williams	180/205	182/207	87/91	89/93	149	150	88/113	89/116
Hosui*	188	190	83/103	85/107	140/143	145	91/103	94/106
Shift		+2		+2		+1 to +2		+3

* Accessions inconsistent with the Brogdale references, D = Doyenne

3.3.4. Triploidy

The two known triploids, 'Lucas' and 'Vicar of Winkfield', showed a third allele for four of the eight markers scored (Table 3.7). However, four other accessions (not known to be triploid) also showed an additional third allele for at least half of the markers as well. These were 'Beurre Clairgeau', 'Duchesse de Bordeaux', 'Saffraan', and 'Winter Saffraan'. Two of these accessions, 'Beurre Clairgeau' and 'Duchesse de Bordeaux', are reportedly diploid (Crane and Thomas, 1939; NCGR, 2013) and the patterns observed cast doubt on the identity of these ARC accessions.

Table 3.7. Pear cultivars in the ARC gene bank showing a third allele for at least four of the eight microsatellites and which are presumed to be triploid.

Name	CH01d09	CH02b10	CH03d12	CH03g07	CH05c06	EMPc11	EMPc117
Beurre Clairgeau	142/153/159	123/129/133	120/125/161	227/237/243	93/97/111	144/150/158	98/116/120
Duchesse du Bordeaux	138/151/153	121/123/139	110/125	232/243	89/93/113	140/150/156	98/118
Lucas*	151/157/159	129/133/144	108/125	227/257	89/95	140/150/155	100/114/116
Saffraan	130/138/159	125/135	110/125	232/239/243	89/109	137/142/155	100/112/116
Vicar of Winkfield*	138/151/153	121/123/139	110/125	232/243	89/93/113	140/150/156	98/118
Winter Saffraan	130/138/153	123/135	110/125	227/243/257	89/93/107	144/150	110/116/120

Note. Scores for marker CH04e03 are omitted as none of the accessions fingerprinted showed a third allele for this marker, *Known triploids

In three cases, 'BP1', 'Kalbas Peer' and 'Winter Nelis', an occasional third allele was observed for one, two or three markers, and in these instances, genotyping was repeated. The third alleles still remained and the accessions were therefore subjected to pollen germination tests to confirm triploid status.

3.3.5. Trueness to type investigation

3.3.5.1. Class 1 accessions: items present in the ARC collection but not in the Brogdale collection

Ninety six *P. communis* accessions and three other *Pyrus* species or hybrids fingerprinted in this study were not present in the Brogdale collection and their microsatellite patterns could

therefore not be compared with reference fingerprints from East Malling (Table 3.8). Genotypes for these items have, presumably, not been reported previously.

The cultivars included three rootstocks and two scion cultivars bred at ARC, namely 'BP1', 'BP2', 'BP3', 'Flamingo' and 'Rosemarie', together with 63 ARC selections and four historically important local cultivars namely 'Ceres', 'Kalbas Peer', 'Saffraan', and 'Winter Saffraan'. Other accessions not present in the Brogdale collection but originating from foreign breeding programmes were 'Celina', 'Colonel Wilder', 'Cristalli', 'December', 'Delbard Precoce', 'Delbard Premiere', 'Delete', 'Delmoip', 'Delmore', 'Delwilmore', 'Duchesse d'Angouleme', 'Emperor', 'Jana', 'Lily', 'Magnate', 'Morettini 64', 'Mostert 51', 'Nassau Strydom', 'Orange Bergamotte', 'Patrick Barry', 'Ruby Glo', 'Stanley', and 'Twyford Monarch'.

In addition, two hybrids, 'Garber' and 'Le Conte', and the accession of *P. calleryana* could not be compared with accessions of the same name from Brogdale.

Table 3.8. Accessions present in the ARC gene bank but not in the Brogdale collection fingerprinted for the first time in the current study with eight microsatellite markers.

Name	CH01d09	CH02b10	CH03d12	CH03g07	CH04e03	CH05c06	EMPc11	EMPc117
***P. communis* selections and cultivars**								
3C-11-25	130/136	123/139	112/125	227/232	182/192	89/93	140/146	112/116
3C-11-9	124/134	123/135	108/110	215/243	182/201	93/99	150/155	106/116
3C-44-34	134/151	129/144	112/125	227/246	182	93/113	140	118/120
3C-49-18	130/157	129/135	108/125	227/232	182	89/93	150/155	116
3C-51-28	130/157	123/129	108/125	227/243	182/207	89	150	116
3D-83-10	130/134	123/133	108/125	232/243	182	89/93	150/155	116/120
5-03-29	124/149	129/135	125	215/227	207	93/109	137/150	89/116
5-16-122	149/151	123/131	110/125	215/243	207	93/109	137/150	116
5-16-89	151/157	123/135	108/110	227/248	182/207	93/109	150/155	89/106
5-17-169	151/157	123/131	108	215/227	207	89/99	137/150	116
5-19-27	140/157	129/135	108/125	227/243	182	89/113	140/150	89/91
5-24-21	149/151	123/144	108/112	227	182/207	93/113	150	116/118
5-25-21	149/151	123/144	112/125	243	182/207	93/113	140/150	116/118
5-31-79	157	123/135	108/125	232/243	182	89/93	150/155	89/118
5-32-53	130/149	129/135	108/125	232/243	182	89/93	150	89/116
5-32-8	130/157	123/135	108/125	243	182	89	150	89/116
5-36-30	157	129/135	108/125	232/243	182/207	89/93	150/155	89/116
5-39-60	124/157	123/135	108/110	215/227	182/207	93/99	150/155	106/116
5-40-45	149/151	129/135	108/110	227/248	182/201	93/109	137/150	89/106
5-40-60	149/151	131	110/125	215/227	207	89/109	150/155	116
5-41-18	124/149	129/131	108/110	215/227	182/207	89/99	137/150	106/116

Table 3.8. Continued…..

Name	CH01d09	CH02b10	CH03d12	CH03g07	CH04e03	CH05c06	EMPc11	EMPc117
5-41-57	136/149	121/123	125	227/260	192/207	93/121	146/150	112/116
8-20-58	140/149	123/144	112	243	182	89/109	150	89/120
8-22-120	157	123/129	108/125	243	182/207	89	150/155	116/118
8-23-81	124/157	129/135	108/110	215/243	201/207	93/109	137/150	89/116
8-24-25	124/149	131	108	215/243	182/207	93/99/109	150/155	116
8-24-51	124/149	129/131	108	215/243	182/207	93/99	150/155	116
8-24-63	124/149	129/131	125	215/243	200/207	93/109	150/155	89/116
8-25-25	151/157	123/135	125	243/248	182/201	93/109	150/155	89/116
8-25-48	149/161	121/133	125	227/232	182/207	93/121	144/150	102/112
8-25-57	149/151	123/131	108	227/248	182/201	89/99	137/150	106/116
8-25-72	124/149	123/131	108/110	227/248	207	93/99	138/150	116
8-26-91	151/157	129/135	125	227/243	182	93/113	150	89/118
8-28-59	149/157	123/129	108/125	232/243	182	89/93	150	116/118
8-30-145	124/149	129/131	108	215/227	207	93/109	137/150	106/116
8-31-23	124/157	129/135	125	215/226	182/207	89/99	150/155	116
8-31-67	149/151	129/131	110/125	227/248	207	93/99	150/155	116
8-31-158	124/149	123/131	108	215/243	182/207	93/99	150/155	106/116
8-33-53	124/157	129	124/125	227/248	207	93/99	150/155	106/116
8-34-54	130/149	129/135	108/125	232/243	182	89/93	150	89/116
8-34-91	149/161	121/133	125	227/232	182/207	93/121	144/150	102/112
8-6-34	136/149	121/123	108/125	243/260	182/207	89/121	150	112/116
8-9-14	130/149	129/135	125	232/243	182	89/93	150/155	89/118
11B-2-25	136/149	121/129	125	227	207	89/93	150	116
11B-39-17	157	123/133	108	227/243	182/207	93	150	116/120
11B-7-17	130	135/146	125/129	227/232	182	89/93	137/150	114/116
11B-7-21	130/157	135/146	110/125	232/245	182	89	146/155	114/116
11B-7-26	130	135/146	110/125	243/245	200	89	137/150	114/116
11B-7-28	149/157	135	110/125	227/232	182	89	137/155	114/116
11C-6-27	134/149	123/129	108/112	227	207	89/93	150	89/120
11C-9-11	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
11D-10-9	130/136	123	108/125	227/232	182/192	89/121	146/150	112/116
15A-4-14	134/149	123/129	108/112	227/243	182/207	89/93	150	89/116
15A-7-21	134/149	123	108/125	227/243	207	93	150	116/120
15B-5-2	140/149	129/139	108	227/228	182	89/93	150	116
BP 1	124/149	123/148	125	215/260	192/207	81/109/121	137/146	106/116
BP 2	132/149	121	132	260	182/207	93/101	144/150	116/120
BP 3	149/161	121/133	125	227/232	182/207	81/93	144/150	102/112
Celina	140/157	140/157	108/112	243/257	182	89/93	150	114/120
Ceres	149/151	123/135	108/110	227/243	182	89	140/150	116/118
Colonel Wilder	157/161	123/133	108/125	243/245	182	93	150/155	98/116
Cristalli	140/142	127/139	92/110	232/267	182	89/113	140/144	93/118
December	130/149	135/146	110	227/245	182	89	137/146	93/114

Table 3.8. Continued….

Name	CH01d09	CH02b10	CH03d12	CH03g07	CH04e03	CH05c06	EMPc11	EMPc117
Delbard Precoce	149/157	123	108/125	243/267	182	93/95	140/150	116/118
Delbard Premiere	128/149	123/135	108/131	243/269	182	89/101	144	110/116
Delete	149/151	123/139	112/125	227/228	182/207	89	150/155	116
Delmoip	153/157	133/139	108/125	227	182/200	89/109	144/150	116/118
Delmore	153/157	133/139	108/125	257/269	182/200	89/109	144/150	116/118
Delwilmore	149/153	129/135	108/125	232/243	182/200	93/109	150/155	116/118
Du. d'Angouleme	140/157	129/139	108/125	227/232	182/200	89/93	140/155	98/116
Emperor	124/149	131	124/125	215/245	201/207	93/109	150/155	89/116
Flamingo	124/149	123/131	108/110	215/227	182/207	89/99	150/155	116
Jana	130/151	129	125	232/243	182	89/109	150/155	94/118
Kalbas Peer	130/161	125/129	114/125/130	227/243/257	182/199	89/93	144	89/110/118
Lily	151/157	129/135	112/125	227	182/207	89/113	150	89/120
Magnate	138/153	137/152	125	246/248	182/207	89/93	140/150	116/120
Morettini 64	149	129/146	125	243/245	182	89/93	137/150	89/114
Mostert 51	138/149	123/139	108	239/260	182/192	89/93	146	112/120
Nassau Strydom	130/157	123/129	108	226/228	182/207	89	142/150	116/122
Orange Berg.	130/138	123/129	105	243/245	182/207	95/109	144/152	93/116
Patrick Berry	130/153	129/135	125	233/257	182	89/109	140	118/120
Rosemarie	130/149	135/146	110	227/245	182	89	137/146	93/114
Ruby Glo	124/149	123/131	108/110	215/243	201/207	89/99	150/155	106/116
Saffraan	130/138/159	125/135	110/125	232/239/243	198	89/109	137/142/155	110/112/116
Stanley	153/157	123/129	112/125	232/243	182/207	93/95	144/155	116/120
Twyford Monarch	130/153	129/133	108/125	243/257	182	89/93	144/155	94/116
Winter Saffraan	130/138/153	123/135	125	227/243/257	182/207	89/93/107	144/150	110/116/120
Other *Pyrus* species and hybrids								
P. hyb. Garber	128/136	121/135	125	243/260	182/188	89/121	148/158	112/120
P. hyb. Le Conte	136/138	135/137	108	239/258	182/188	89/111	147/150	112/120
P. *cal*. Calleryana	136/144	121/135	105	218/225	186/192	99	150/176	92/104

Berg = Bergamotte, Du = Duchesse, hyb = hybrid, *cal* = *calleryana*

3.3.5.2. Class 2 accessions: items present in both ARC and Brogdale collections and having consistent patterns

The fingerprints of 36 ARC accessions, 33 of *P*. *communis* and three other *Pyrus* species and hybrids, matched those of the Brogdale accessions for all microsatellite markers used in this study. Data for the items listed here are included in Appendix 3.1. Genotypes are not presented in the main text as they have already been published. Shifts (as previously noted) were considered during comparisons (data not shown).

The *P. communis* cultivars matching the Brogdale cultivars were 'Bartlett' (six out of eight markers used), 'Beurre Bosch' (syn 'Beurre Bosc' in Brogdale, but accession 3_21 was false), 'Beurre d'Anjou', 'Beurre Giffard', 'Beurre Hardy' (accession 3_32 was false), 'Beurre Six', 'Beurre Superfin', 'Beurre van Geerdt' (syn 'Beurre Jean Geert' at Brogdale), 'Beth', 'Cascade', 'Clapp's Favourite', 'Concorde', 'Conference' (3_42 'Conference' matched seven of eight markers used), 'Contesse de Paris' (syn. 'Comtess de Paris' from Brogdale), 'Doyenne du Comice', 'Duchesse d'Angouleme', 'Dr Jules Guyot' (syn. 'Doctor Jules Guyot' at Brogdale), 'Fondante d'Automne', 'Forelle', 'Ganzels Bergamotte' (syn. 'Gansels Bergamotte' in Brogdale), 'General Leclerc' (only accession 4_10 is true), 'Harrow Delight', 'Hertzogin Elza' (syn. 'Herzogin Elsa' in Brogdale), 'Highland', 'Louise Bonne' (syn. 'Louise Bonne of Jersey' from Brogdale), 'Lucas' (syn. 'Beurre Alexandre Lucas' in Brogdale), 'Maguerite Marillat', 'Packham's Triumph', 'Red Comice', 'Roosevelt', 'Starkrimson', 'Vicar of Winkfield' and 'William's Bon Chretien'.

The *P. pyrifolia* cultivar 'Chojuro' also had a microsatellite pattern identical to that of the accession in Brogdale and the hybrid 'Kieffer' matched 'Kieffer 4' from Brogdale.

3.3.5.3. Class 3 accessions: clones, synonyms and sports

Clones of seven cultivars of *P. communis* had microsatellite patterns identical to those of the 'primary' cultivar. Eleven clones of 'William's Bon Chretien', including 'Bon Rouge', 'Burger BC' and 'El Dorado', had identical microsatellite patterns (Table 3.9) which were consistent with 'Williams Bon Chretien' from Brogdale for the eight markers scored and 'Bartlett' matched for six out of eight markers used. Identical microsatellite patterns were observed for three 'Louise Bonne' clones, which matched 'Louise Bonne of Jersey' from Brogdale rather than 'Louise Bonne de Printemps' or 'Louise Bonne Sannier', and four clones of 'Clapp's Favourite', including 'Ann's Favourite' and 'Starkrimson', which matched 'Clapp's Favourite' of Brogdale. Three clones of 'Beurre Bosch', two clones of 'Beurre Hardy' (with the third, 'Beurre Hardy EMLA', matching seven out of eight markers) and three clones of 'Packham's Triumph' also gave consistent microsatellite patterns with the clones and their similar accessions at Brogdale. Cultivar 'Doyenne du Comice' had a microsatellite pattern consistent with 'Red Comice' from Brogdale and consistent with 'Doyenne du Comice EMLA' and its sport 'Red Comice' for six and seven of the eight markers, respectively.

However, two cultivars, 'Beurre d'Anjou' and 'Forelle', showed different microsatellite patterns from those of their sports 'Red d'Anjou' and 'Forelle Malherbe', respectively.

Table 3.9. Identical or near identical microsatellite patterns of 'William's Bon Chretien' and its clones and sports in the ARC *Pyrus* collection.

Name	CH01d09	CH02b10	CH03d12	CH03g07	CH04e03	CH05c06	EMPc11	EMPc117
Bartlett	134/157	123/129	108/125	227/243	182/207	89/93	150	116
Bon C. A	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Bon C. B	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Bon C. C	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Bon C. D	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Bon C. E	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Bon Chretien	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Bon C. (Koo)	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Bon C. Sport	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Bon Rouge	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Burger Bon C.	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
El Dorado	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
El D. (VV)	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116
Williams Bon C.	149/157	123/129	108/125	227/243	182/207	89/93	150	89/116

Bon C = Bon Chretien and D = Dorado

3.3.5.4. Class 4 accessions: items present in ARC and Brogdale collections but having inconsistent patterns.

Several accessions held at ARC had microsatellite patterns differing from those of the cultivars held at Brogdale under the same name (Table 3.10).

Inconsistent accessions of cultivars 'Beurre Bosch' (3_21), 'General Leclerc' (3_15) and 'Josephine de Malines' (2_15) matched the microsatellite pattern of rootstock 'BP3'.

For ten cultivars the scores of the ARC accessions did not match the Brogdale scores. These were: 'Passe Crassane' (the supposed male parent of ARC cultivar 'Cheeky' (Human, personal communication), 'Beurre Clairgeau', 'Beurre Hardy' (3_32), 'Duchesse de Bordeaux', 'Forelle Malherbe' (supposed mutant of Forelle), 'Glou Morceau', 'Old Home' (2_10 and 3_4), 'Onward' (3_44 and 6_9), 'Precoce de Trevoux', 'Red d'Anjou' and 'Winter Nelis'. This suggests that these ARC accessions are false if the Brogdale accessions are regarded as true to type.

The *P. pyrifolia* cultivar 'Hosui' also had a microsatellite pattern inconsistent with that of the Brogdale accession of 'Hosui'.

Table 3.10. Accessions present at ARC having microsatellite patterns different from those of the accessions of the same name at Brogdale, and therefore deemed to be false.

Location and name	CH01d09	CH02b10	CH03d12	CH03g07	CH04e03	CH05c06	EMPc11	EMPc117
***P. communis* cultivars**								
3_21 Beurre Bosch*	149/161	121/133	125	227/232	182/207	93/121	144/150	102/112
1_5 B. Clairgeau	142/153/155	123/129/133	120/125/161	227/236/243	182/200	93/97/111	144/150/158	98/116
3_32 B. Hardy Sport	130/140	135/139	108/112	228/232	182	89	140/150	116
6_3 Red d'Anjou	149/151	129/131	108/110	248/257	182	89/99	150/155	89/106
4_45 D. d Bordeaux	138/151/153	121/123/139	110/125	232/243	182	89/93/113	140/150/156	98/118
6_21 Forelle Mal.	130/149	123/131	108/125	243	182	89	150/155	116/120
3_15 G. Leclerc*	149/161	121/133	125	227/232	182/209	93/121	144/150	102/112
6_12 Glou Morceau	140/151	135/144	112/125	227/243	182	109/113	140/150	118
2_15 J. de Malines*	149/161	121/133	125	227/232	182/207	93/121	144/150	102/112
2_10 Old Home	149/153	123/133	112/125	227	182	89	150	116/118
3_4 Old Home	153/157	123/129	108/112	243/257	182/207	89/93	150	116
3_44 Onward	151/157	135/139	108/112	227/232	182	89	140/155	116
6_9 Onward	124/149	129/135	110/125	227/248	182/207	93/99	137/150	116
3_26 Passe Crassane	140/151	135/144	112/125	227/243	182	109/113	140/150	118
2_38 P. de Trevoux	130/157	123/135	108/125	232/243	182	89/113	150	89/118
3_10 Winter Nelis	130/151	135	110/125	232/243	182/207	89/109	140/155	116/118/120
Other *Pyrus* species								
5_9 Hosui	155	125/135	98	252/258	190	85/107	145	94/106

*Identical with rootstock 'BP3', B = Beurre, D. d = Duchesse de, G = General, J = Josephine, Mal = Malherbe and P = Precoce

3.3.5.5. Class 5 accessions: unidentified accessions

Eight unknown accessions in the gene bank were also fingerprinted (Table 3.11). The microsatellite patterns of the 'Unknowns' were compared with both ARC and Brogdale accessions to identify mislabelled these 'Unknowns'. Resolved 'Unknowns' are indicated in section 3.3.8.

Table 3.11. Molecular fingerprints of eight unknown accessions in the ARC gene bank.

Name	CH01d09	CH02b10	CH03d12	CH03g07	CH04e03	CH05c06	EMPc11	EMPc117
1_36 Unknown 1	130/157	123/135	108/125	243	182	89	150	89/116
2_14 Unknown 2	149/157	129/135	108/125	227/243	182/207	89/93	150	116/118
3_3 Unknown 3	152/158	129/148	110	227/245	182	89	137/146	93/114
5_22 Unknown 5	149/157	123/135	108/125	232/243	182	89/93	150/155	89/116
5_23 Unknown 6	149/151	123/144	112/125	243	182/207	93/113	140/150	116/118
6_1 Unknown 7	124/149	123/131	108/110	215/227	182/207	89/99	150/155	116
6_2 Unknown 8	124/149	123/131	125	215/243	182/207	93/99	150/155	89/106
6_3 Unknown 9	149/151	129	108/110	215/227	182/207	95/113	140/150	116/120

3.3.6. Verifying parentage

For accessions of known parentage, the fingerprints were compared with those of the parents in instances where the parents had been fingerprinted as well. Cultivars 'Cascade' ('Bartlett' × 'Doyenne du Comice'), 'Concorde' ('Doyenne du Comice' × 'Conference'), 'Flamingo' ('Bon Rouge' × 'Forelle), 'Highland' (Doyenne du Comice' × 'Bartlett') and 'Rosemarie' ('Bon Rouge' × 'Forelle') were investigated. The parentage of all cultivars except 'Rosemarie' was verified. The fingerprints of the 'Rosemarie' accession were not consistent with those of the reported parents 'Bon Rouge' and 'Forelle' (Fig. 3.2) suggesting that the 'Rosemarie' tree in the ARC gene bank is not true to type.

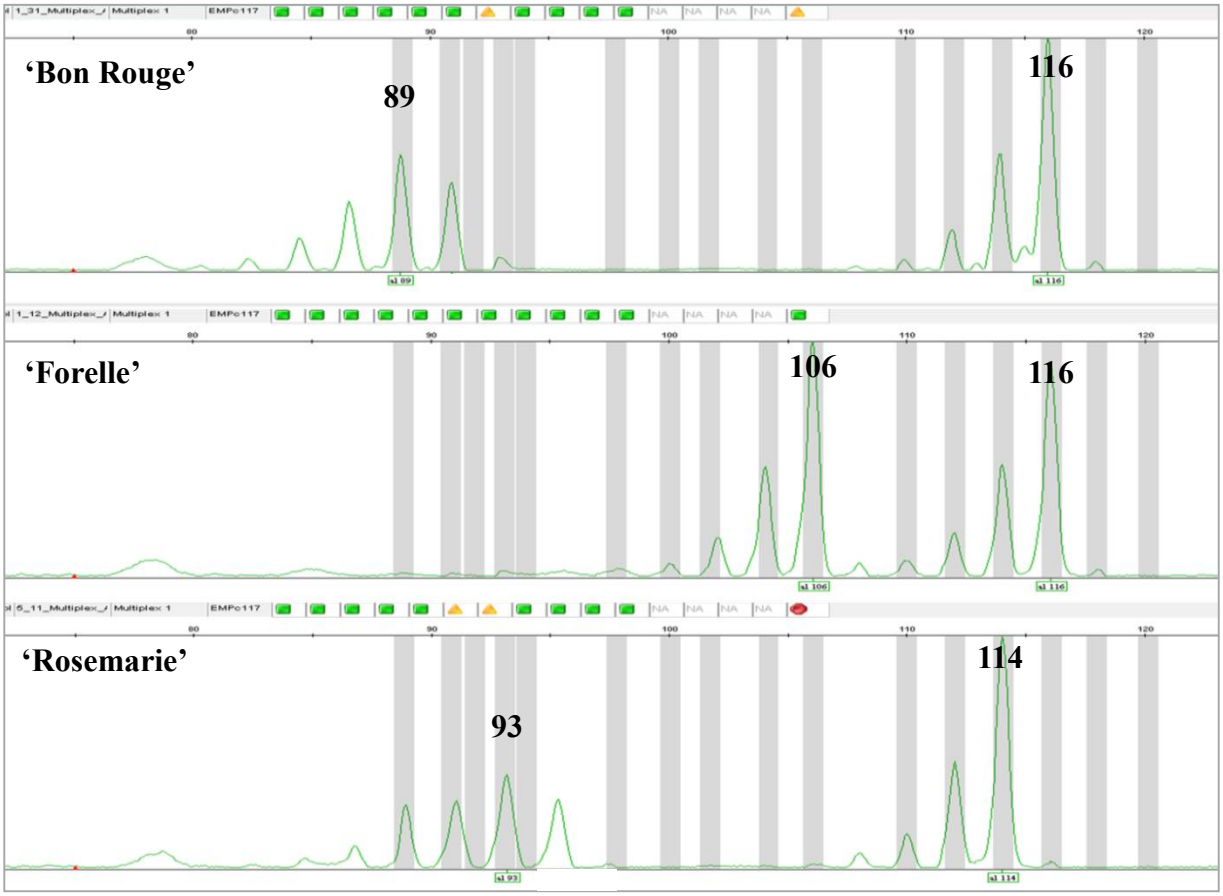

Fig. 3.2. GENEMAPPER output of microsatellite marker EMPc117 for ARC gene bank accessions, 'Bon Rouge', 'Forelle' and 'Rosemarie'; the 'Rosemarie' pattern is inconsistent with its reported parentage ('Bon Rouge' × 'Forelle'). Similar discrepancies were seen with the seven other markers.

A similar investigation was conducted for 42 ARC selections: 20 ‘Bon Rouge’ × ‘Forelle’ selections, eight ‘Bon Rouge’ × ‘Packham’s Triumph’ selections, six ‘Passe Crassane’ × ‘Bon Rouge’ selections, three ‘Bon Chretien × ‘Packham’s Triumph’, 15A-4-14 (‘Clapp’s Favourite’ × ‘Bon Chretien’), 11B-2-25 (‘Kieffer’ × ‘El Dorado’), 11B-7-26 (‘December’ × ‘Packham’s Triumph’), 3C-44-34 (‘Passe Crassane’ × ‘Packham’s Triumph’) and 3D-83-10 (‘Starkrimson’ × ‘Packham’s Triumph’). The parentage of 41 of the 42 selections was verified, the exception being 3C-44-34, for which the paternal parent, ‘Packham’s Triumph’, seemed unlikely as it did not have any alleles in common (data not shown).

3.3.7. Cluster analysis

Clustering of individuals using the UPGMA method (Fig. 3.3) proved very informative as it facilitated the detection of mislabelling in the gene bank as well as the identification of some ‘Unknown’ accessions. ‘Unknown 1’ and ‘Unknown 6’ were identical to ARC selections 5-32-8 and 5-25-21, respectively. Accessions of ‘Unknown 2’ and ‘Unknown 5’ were nearly identical to ‘William’s Bon Chretien’ and 79-05-31, respectively. Additionally, the false accession ‘Beurre Hardy Sport’ was identical to ‘Beurre Superfin’, ‘Glou Morceau’ was identical to ‘Passe Crassane’, ‘Onward’ (3_44) was identical to ‘Doyenne du Comice’ and the ARC selections 8-31-23, 5-41-57 and 11C-9-11, were identical to ‘Flamingo’, ‘Kieffer’ and ‘Williams’s Bon Chretien’, respectively. Furthermore, ARC selection 8-34-54 clustered with 5-32-53. The accessions ‘Beurre Bosch’, ‘General Leclerc’, ‘Josephine de Malines’ and the ARC selections 8-25-48 and 8-34-91 were all identical to rootstock ‘BP3’, indicating that for these samples the rootstock could have continued growing instead of the scion cultivar after propagation. Cultivars ‘Stanley’ and ‘Tongers’ that are known to be different unexpectedly clustered together.

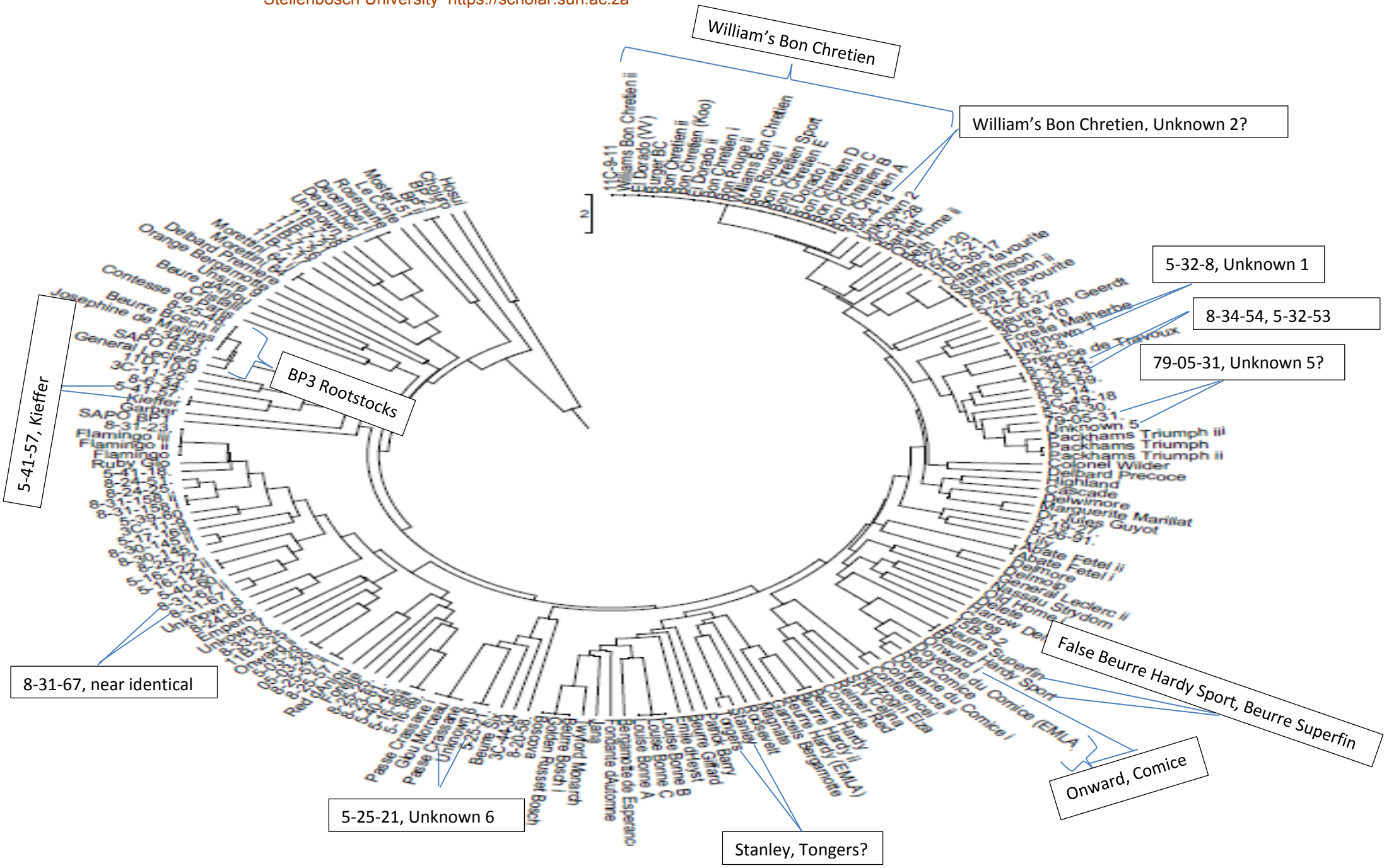

Fig. 3.3. UPGMA dendrogram of ARC pear accessions to identify unknown accessions as well as possible mislabelling. Suffixes i and ii indicate duplicates.

3.4. Discussion

In the present study microsatellite markers proved effective in investigating trueness to type of accessions in the ARC pear gene bank. The use of the recommended set of markers for fingerprinting was advantageous as it enabled the comparison of ARC fingerprints with those from the Brogdale collection for 53 accessions to verify identity. Of 53 accessions compared, 36 were confirmed as true to type while the remaining 17 appeared to be incorrectly identified.

3.4.1. Marker efficiency

Four of the set of 12 recommended markers used in this study (Table 3.12), CH01f07a, CH01d08, GD96 and GD147, gave poor amplification and were excluded from further analysis. Ahmed *et al*. (2010) also experienced poor amplification with marker CH01f07a, in addition to CH03d12 and EMPc117 and excluded these from the analyses of 56 *P*. *communis* and other *Pyrus* accessions. A more recent study by Sehic *et al*. (2012) used all 12 recommended markers on their study on 94 *P*. *communis* accessions, but due to problems encountered with multiplexing, the markers were amplified in single reactions. This, however, is laborious and very expensive especially when dealing with large sets of accessions. Despite using markers in simplex reactions, Sehic *et al*. (2012) still noted that marker GD96 gave poor amplification, similar to results observed in the current study, as did marker CH02b10.

Eleven of the 12 recommended markers were also used in simplex reactions by Dos Santos *et al*. (2011) for fingerprinting 221 *P*. *communis* accessions; marker CH01d09, while not problematic at ARC, gave poor amplification as did CH02b10, CH03d12, and EMPc117. The study of Fernández-Fernández (2010) on the fingerprinting of 559 *P*. *communis* and other *Pyrus* accessions is the only study that used the complete set of recommended markers successfully in multiplexes. Marker failure in different laboratories may be due to other multiplexing conditions, which indicates the need for further optimisation. Evans *et al*. (2014) when investigating 61 *P*. *communis* accessions also used the complete set of 12 recommended markers; however, full marker performance has not yet been reported as the study is in progress.

Table 3.12. Performance of the ECPGR recommended microsatellite markers in different pear genotyping studies. Markers are arranged alphabetically and then according to their genomic position.

Marker	LG	Fernández-Fernández 2010	Ahmed *et al*. 2010	Dos Santos *et al*. 2011	Urbanovich *et al*. 2011	Sehic *et al*. 2012	Current study
[3]CH01d08	15	Good	good	good	good	good	poor
[3]CH01d09	12	Good	good	poor	good	good	good
[3]CH01f07a	10	Good	poor	good		good	poor
[1]CH02b10	2	Good	good	poor		poor	good
[3]CH03d12	6	Good	poor	poor	good	good	good
[3]CH03g07	3	Good	good	good	good	good	good
[3]CH04e03	5	Good	good	good		good	good
[3]CH05c06	16	Good	good	good	good	good	good
[4]EMPc11	11	Good	good	good		good	good
[4]EMPc117	7	Good	poor	poor		good	good
[2]GD96	17	Good	good	good		poor	poor
[2]GD147	13	Good	good	good		good	poor

[1]Gianfranceschi *et al*. (1998), [2]Hokanson *et al*. (1998), [3]Liebhard *et al*. (2002), [4]Fernández-Fernández *et al*. (2006)

3.4.2. Allele scoring and comparison with Brogdale scores

Deviation in allele sizes, as observed between the current study and that of Evans *et al*. (2009), is not unexpected as different conditions, such as different automated sequencers, size standards and temperatures during sequencing are known to affect allele sizes. Such deviations necessitate the inclusion of reference cultivars for calibration between laboratories. Deviations of 0 to 3 bp were observed in the current study whereas Sehic *et al*. (2012) reported 0 to 6 bp deviations using the same fluorescently labelled markers. These deviations guide adjustment of microsatellite patterns to achieve comparable data between laboratories. However, complicated shifts were encountered with some markers, notably CH03g07 and EMPc11, in which shifts between the ARC alleles and the Brogdale alleles were not consistent. Similar complications were reported previously for CH03g07 and also for markers CH01d08, CH01d09, CH03d12 and CH04e03 (Sehic *et al*., 2012). These complications hamper the reliable conversion of data between laboratories.

The shifts described above aided the comparison of ARC and Brogdale scores, which was informative in establishing trueness to type of accessions of cultivars present in both collections. No ARC accessions that were ‘Unknown’ or were determined to be false matched any accession in the extensive Brogdale collection; suggesting these accessions may have been from a different source than UK, *e.g*. Corvallis collection. The accessions however, most probably represent mislabelled selections.

3.4.3. Diversity statistics

MICRO-CHECKER analysis conducted prior to analysis revealed no evidence of misscoring. This may be a consequence of careful selection of markers that had no null alleles as well as manual verification. The deviation from the HWE observed for all markers could perhaps be due to null alleles but is most probably associated with the inherent characteristics of the accessions in the gene bank. Although treated as a population in analysis, the gene bank represent different selected cultivars and accessions and is not a natural population.

When *P. communis* accessions were analysed separately, 5 to 18 alleles per marker were detected. This is in agreement with previous studies by Dos Santos *et al*. (2011) and Sehic *et al*. (2012) which found 7 to 20 and 6 to 15 alleles, respectively. When accessions of *P. communis* were analysed, markers CH01d09 and CH03g07 were the most polymorphic, amplifying 17 and 18 alleles, respectively while CH04e03 was the least polymorphic, amplifying only five alleles. Similar results for polymorphism were observed by Dos Santos *et al*. (2011) and Sehic *et al*. (2012). The higher numbers of alleles detected per marker in the current study when all accessions were included, 9 to 22, is attributed to the inclusion of other *Pyrus* species and interspecific hybrids.

Seven of the eight markers used in the current study proved very informative; CH04e03 was the least informative with PIC = 0.41 compared to 0.81 reported by Ahmed *et al*. (2010). Marker CH01d09 was the most informative marker with PIC = 0.83 compared to 0.93 reported by Ahmed *et al*. (2010). The observed and expected heterozygosities in the current study, 0.78 and 0.73 respectively, were consistent with those reported by Sehic *et al*. (2012) of 0.74 and 0.72 respectively. The minor difference between observed and expected heterozygosity is explained by the absence of null alleles in the markers used and the absence of genetically isolated groups within these gene bank collections (Wolko *et al*., 2010; Yakovin *et al*., 2011). The high values furthermore reflect the self-incompatible and highly heterozygous nature of pears (Brini *et al*., 2008; Sisko *et al*., 2009; Urbanovich *et al*., 2011; Sehic *et al*., 2012).

3.4.4. Triploidy

The occurrence of a third peak, indicating a third allele, across several markers is associated with triploidy. Cultivars ‘Lucas’ and ‘Vicar of Winkfield’ are known triploids (Crane and Thomas, 1939; Fernández-Fernández, 2010; NCGR, 2013) although the ARC gene bank list had not been annotated with this information. The ARC accessions of ‘Beurre Clairgeau’ and

'Duchesse de Bordeaux' also appeared to be triploid but were found to be false when compared with the Brogdale accessions; this suggests that these two accessions may be unknown triploid cultivars that were mislabelled. To try and identify these two accessions, their microsatellite patterns were compared with patterns of all triploid accessions in the ARC and Brogdale collections. The ARC accession of 'Duchesse de Bordeaux' was subsequently identified as 'Vicar of Winkfield' but no match was found for 'Beurre Clairgeau'. For cultivars 'Saffraan' and 'Winter Saffraan', both traditional South African pear, accessions for comparison were not available at Brogdale.

Accessions with occasional third peaks, consistent after retesting, for not more than three markers such as 'BP1', 'Kalbas Peer' and 'Winter Nelis', were thought to be diploid as occasional third peaks have been reported previously in some diploid accessions (Dos Santos *et al*., 2011; Sehic *et al*., 2012). 'BP1' and 'Kalbas Peer' were not available in the Brogdale collection for comparison. Pollen germination tests conducted for the suspected diploid 'Kalbas Peer' revealed pollen germination of 1 to 5% compared to 30 to 60% observed in the known diploid 'Doyenne du Comice', and 'Kalbas Peer' was therefore concluded to be triploid. Flowers of 'BP1' could not be acquired as it is a rootstock and is not present in the ARC gene bank. In future, as an alternative in cases where flowers are not obtainable, flow cytometry is also recommended (Tatum *et al*., 2005). The ARC accession of 'Winter Nelis' was not subjected to pollen germination tests as the accession had already been presumed false in the current study.

It must be noted that third alleles differing by 2bp to 4bp may arise due to chimeral mutations. Such alleles may be found for example in leaf tissue (which is formed from all three cell layers, L1, L2 and L3) but not in bark (which is formed from one cell layer, L1) (Whitham and Slobodchikoff, 1981).

Knowledge of triploidy should assist the breeder to avoid unproductive crosses as triploids rarely produce viable pollen or seeds (Crane and Thomas, 1939).

3.4.5. Consistency of clones

The inability of microsatellite markers to discriminate amongst clones and sports of cultivars makes them ideal markers to verify whether clones or sports are true to origin. A particular sport of 'William's Bon Chretien' cultivar, 'Bon Rouge' was easily compared with the 'primary' cultivar in the absence of the 'Bon Rouge' reference fingerprint from the Brogdale

collection. Accessions that differed markedly from the 'primary' cultivars were presumed false. For example 'Beurre Hardy Sport' and 'Forelle Malherbe' differed markedly from 'Beurre Hardy' and 'Forelle', respectively, across the eight microsatellite markers used. In discussion of the findings of the current study with the pear breeder, this study confirmed his suspicion that 'Forelle Malherbe' was false as the fruits have a russet phenotype instead of the supposed improved blush. Microsatellite markers also confirmed all clones of 'William's Bon Chretien' in the current study with the exception of 'Bartlett' that matched for only six of the eight markers used. Clones of 'Conference', 'Doyenne du Comice' and ARC selection 8-31-67 also differed slightly from the 'primary' cultivar. However, minor marker differences are common in clones and may result from misscoring but most probably result from mutations accumulating over the years. Utilising microsatellite markers, Donini *et al*. (2006) were able to confirm the common origin of over 64 'Cabernet Sauvignon' grape clones grown in different countries. Likewise, 29 clones of cherry were identified using microsatellite markers (Horvath *et al*., 2008).

If there is a need to discriminate amongst clones especially in Distinctiveness Uniformity and Stability (DUS) testing, it is possible that alternative markers such as Sequence-Specific Amplification Polymorphism (S-SAP) and Amplified Fragment Length Polymorphism (AFLP) could be employed (Venturi *et al*., 2006; Cretazzo *et al*., 2010). However, currently DUS testing utilises only morphological characteristics. Nevertheless, knowledge of these other marker methods may prove useful when the Plant Breeders Rights office start utilising molecular markers.

3.4.6. Parentage verification

Documentation of parentage for some cultivars in the ARC gene bank allowed trueness to parentage to be investigated, particularly for locally ARC bred cultivars that could not be compared with international cultivars. 'Flamingo', a seedling of 'Bon Rouge' × 'Forelle', appeared to be true to parentage whereas 'Rosemarie', reportedly of the same parentage, had a microsatellite pattern inconsistent with the alleged parents. As the alleged parents were found to be true to type, this implied that the 'Rosemarie' was false. Subsequently, comparison of the 'Rosemarie' microsatellite fingerprint with those of the other accessions revealed that the 'Rosemarie' in the ARC gene bank matched 'December' for all the markers used. True to parentage trees of 'Rosemarie' have subsequently been identified in a separate study growing

on the Grabouw Experimental Farm, Western Cape, South Africa (Ntladi, personal communication).

3.4.7. Cluster analysis

Clustering of accessions using UPGMA based on genetic distances was useful for determining the identity of two 'Unknown' accessions; 'Unknown 1' and 'Unknown 6' as ARC selections 5-32-8 and 5-25-21, respectively. Additionally, two other 'Unknown' accessions, 'Unknown 2' and 'Unknown 5' clustered closely with 'William's Bon Chretien' and 79-05-31, respectively, and fruit evaluation will be conducted in the next production season for comparison. As expected, clones and sports of cultivars clustered together if true to origin. Near identical clones clustered with their cultivar group but not precisely e.g. 'Bartlett' and 'William's Bon Chretien'; and likewise 'Conference' and ARC selection 8-31-67 will be compared. The fruit of cultivars 'Stanley' and 'Tongers', and ARC selections 8-34-5 and 5-32-53 will be compared during the next season. As observed in Miranda *et al*. (2010), Urbanovich *et al*. (2011) and Tian *et al*. (2012), different species tend to cluster together *e.g*. the Asian pears, 'Chojuro' and 'Hosui', formed a cluster distinct from the European pears. Of course, similar results were also obtained when 'manually' comparing microsatellite patterns across all markers to identify 'Unknown' cultivars by sorting in Excel; unknowns grouped with the same accessions as was found with the UPGMA method.

3.4.8. Implications of 'Passe Crassane' findings

The misidentifications of ARC 'Passe Crassane' detected in the current study raises questions with regard to the parentage of the ARC bred cultivar 'Cheeky', a successful new blushed cultivar, reportedly from the cross 'Passe Crassane' × 'Starkrimson' (Human, personal communication). Further comparisons showed no accessions in either the ARC or Brogdale collection matching ARC 'Passe Crassane'. Future attempts to repeat the cross for breeding or genetic studies would be unproductive, yielding flawed seedlings, if a different 'Passe Crassane' is used instead of the ARC 'Passe Crassane'. Morphological and molecular comparison of the ARC 'Passe Crassane' with true to name 'Passe Crassane' accessions from several trustworthy foreign sources is therefore advised.

3.4.9. DAFF DUS testing

Microsatellites have proven very useful in delineating inconsistencies in the ARC gene bank that were previously not detected morphologically. However, in accord with the rules of the International Union for the Protection of New Varieties of Plants (UPOV) (UPOV, 2000), microsatellite fingerprinting is currently not recognised for Distinctiveness, Uniformity and Stability (DUS) testing during cultivar registration for Plant Variety Rights (PVR), conducted in South Africa by the Department of Agriculture, Forestry and Fisheries (DAFF). Adopting this technique as a complementary test to morphological characterisation will facilitate distinctiveness testing of new pear cultivars. Adopting this technique has been advocated in grape DUS testing world-wide where morphological characterisation can also be confusing (Ibáñez *et al*., 2009).

3.4.10. Recommendations to the ARC breeding programme and gene bank management

Misidentified accessions, ‘Glou Morceau’, ‘Hosui’, ‘Onward’ and ‘Winter Nelis’, for which cultivars of the same name are registered on the national cultivar list have been sourced from reliable nurseries such as SAPO and the DPA for further comparisons and possible replacement in the gene bank. For ARC accessions that appear to be misnamed and are not available commercially in South Africa, further morphological characterisation and fingerprinting for comparison with other foreign accessions to investigate misidentifications or possible homonyms will be conducted.

Data generated in the current study will provide reference fingerprints useful for the planned re-propagation of the gene bank. These findings indicate which material is true to type. In due course, the new gene bank will be fingerprinted and the records compared with the fingerprints generated in the current study to verify correct labelling; this will be more efficient than waiting five years for morphological comparison of fruit once the trees start cropping.

Triploids, known to be infertile, were also detected and will be annotated as such in the gene bank list.

3.5. Concluding remarks

The microsatellite markers recommended by ECPGR used in the current study were mostly useful for investigating trueness to type for two thirds of the accessions in comparison with the Brogdale collection. Trueness to origin of clones and sports was established and parentages were confirmed. Sixteen *P. communis* cultivars, six ARC selections and the *P. pyrifolia* cultivar 'Hosui', were found to be not true to type and it is suggested that it is removed from the ARC collection. Triploids were successfully detected, including several not previously reported, and these accessions will be annotated accordingly in the gene bank database. Two 'Unknown' accessions were identified. Data recorded in the current study will be incorporated into the ARC gene bank database for each accession confirmed as true, which will be useful for reference when the collection is repropagated.

Adoption of this molecular technique is already proving useful for the deciduous fruit industry in South Africa, as, at the request of DPA, the trueness to type of 'BP1' and 'BP3' rootstocks in local nurseries was verified using the microsatellite markers utilised in the current study.

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Chapter 4

MICROSATELLITE FINGERPRINTING OF THE ARC APPLE COLLECTION

4.1. Introduction

The diploid, $2n=2x=34$, cultivated apple, *Malus pumila* Mill., is the second most important deciduous fruit crop grown in South Africa, after grape (PPECB, 2013). The annual production exceeds 800 000 tonnes and comes from approximately 22 000 hectares planted in the temperate regions of the country, especially in the Western Cape (HORTGRO, 2013). Approximately 40% of the produce is exported mostly to the northern hemisphere markets such as Europe (PPECB, 2013). As with pear, a few imported cultivars dominate the South African industry such as 'Braeburn', 'Fuji', 'Granny Smith', 'Golden Delicious', 'Pink Lady' and 'Starking' (HORTGRO, 2013).

The Agricultural Research Council (ARC) Infruitec-Nietvoorbij, South Africa, conducts an apple breeding programme to support the South African industry, and maintains genetic resource collections to address its breeding objectives (Bester *et al*., 2013). These objectives include adaptability to insufficient winter chill, disease resistance, storability, eating quality and yield (Tobutt and Bester, 2011). The ARC maintains two gene banks, located at the Drostersnes and Grabouw Experimental Farm in the Elgin Valley, Western Cape, comprising 540 accessions. Most accessions are cultivars or selections of *M. pumila* but accessions of other species such as *M. robusta* (Carrière) Rehder are also present along with *Malus* interspecific hybrids. Cultivars from which clones or sports are derived are regarded as 'primary' cultivars. There are 294 'primary' cultivars of *Malus*, with 229 'primary' *M. pumila* cultivars, and 65 cultivars of *M. pumila* selections and other *Malus* species and hybrids. Most of the accessions are unique but a few are duplicated. In a review of the ARC breeding programmes known as Fruit Route, misidentification of apple accessions was highlighted as a significant problem, presumably due in part to mislabelling at propagation or planting (Tobutt and Bester, 2011). Although morphological characterisation can resolve some of the obvious misidentifications, others are difficult to resolve phenotypically especially where new or lesser known cultivars are involved.

The development of microsatellite markers, also known as Simple Sequence Repeats (SSRs), has enabled the investigation of trueness to type of accessions in numerous fruit tree species. Although the initial development of microsatellite primers can be relatively time consuming and costly, once the primers have been developed the process of fingerprinting can be rapid and cost effective. Microsatellites are codominant, polymorphic markers that are transferable across related species. A large number of microsatellite markers have been developed from

apple over the last two decades primarily for use in genetic mapping (Liebhard *et al*., 2002; Silverberg-Dilworth *et al*., 2006) although these markers have also been utilised in genetic diversity and identification studies, early examples of which are those of Gianfranceschi *et al*. (1998) and Hokanson *et al*. (1998).

Although microsatellites have been used successfully in recent years for apple cultivar identification and diversity studies across several countries in Europe, Asia and America (Gianfranceschi *et al*., 1998; Hokanson *et al*., 1998; Oraguzie *et al*., 2005), a major limitation to comparing datasets across laboratories is the utilisation of different markers for fingerprinting by different laboratories. To alleviate this problem, a set of nine markers used to fingerprint the apple collection at the Institut National de la Recherche Agronomique (INRA), France (Laurens *et al*., 2004) was recommended as a standard set by Guarino *et al*. (2006). Subsequently, 12 markers including some of the original nine was used by East Malling Research group for fingerprinting 2200 apple accessions from the Brogdale collection in the United Kingdom (UK) (Fernández-Fernández, 2010). These 12 markers were proposed as an international standard set by the European Cooperative Programme for Plant Genetic Resources (ECPGR) *Pyrus/Malus* working group (Maggioni, 2011) and have recently been adopted by the ECPGR group (Lateur *et al*., 2013). In addition, eight reference accessions, maintained at INRA were selected for calibration (Fernández-Fernández, 2010); these comprise six cultivars of *M. pumila* and one selection each of *M. floribunda* Van Houtte and *M. robusta* 5.

Several studies have either used different subsets of markers derived from the initial recommended set of 12 markers (Pereira-Lorenzo *et al*., 2007; Garkava-Gustavsson *et al*., 2008; Van Treuren *et al*., 2010; Foroni *et al*., 2012; Patzak *et al*., 2012; Zhang *et al*., 2012; Garkava-Gustavsson *et al*., 2013) or the full set of 12 as used by Fernández-Fernández (2010) and obtained results that could be compared across laboratories (Xuan *et al*., 2010; Urrestarazu *et al*., 2012; Potts *et al*., 2011; Reim *et al*., 2013; Pina *et al*., 2014). Use of a standard set of fingerprinting markers across laboratories has also been proposed in other fruit crops in the ECPGR framework such as pear and cherry (Evans *et al*., 2007; Evans *et al*., 2009; Clarke and Tobutt, 2009).

Several classes have been proposed in grape fingerprinting for grouping accessions to organise fingerprint data into breeder friendly working categories in the gene bank (De Andres *et al*.,

2007). A recent study by Sehic *et al*. (2012) used a similar system for *Pyrus* and demonstrated the utility of a similar grouping of accessions in a different taxonomic group.

The current study aims to fingerprint the ARC's apple collection by employing the recommended ECPGR microsatellite markers, to resolve misidentifications and to provide reference data for future comparisons. The study was conducted to ensure the use of true to type material in the ARC's breeding programmes and genetic studies.

4.2. Materials and methods

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

4.2.1. Plant material

Samples for analyses of 540 apple accessions were collected from the gene banks at the Drostersnes and Grabouw Experimental Farms in the Elgin Valley, Western Cape, South Africa (Table 4.1). These comprise 489 accessions of *M. pumila* cultivars (257 'primary' cultivars, 211 clones and sports), 69 representatives of other *Malus* species or interspecific hybrids and three other selections. There are no accession numbers for most entries in the ARC apple gene banks and so tree locations are used as identifiers. Generally the first tree, of two of a kind in Drostersnes Experimental Farm and three of a kind in Grabouw Experimental Farm, were sampled. However, additional trees were sampled where discrepancies were observed.

Young expanding leaves were collected in spring (early September) and frozen at -80°C until required for DNA extraction. Leaf material was weighed to 0.3 g (±0.1 g) and placed in a labelled 2 ml microcentrifuge tube and stored at -20°C until further use. Samples were prepared in duplicate to allow for repeat analysis.

Table 4.1. Apple accessions fingerprinted from gene banks at Drostersnes and Grabouw Experimental Farms, plots DN7 and E1 respectively, indicating location of the tree. Accessions are arranged by primary cultivar group (according to gene bank information available in 2012), then alphabetically. Accessions present in the Brogdale collection have been coded B.

Location	Name	Code	Location	Name	Code
***M. pumila* cultivars and selections**			E1_11_1	Braeburn	B
DN7_16_4	20/1		DN7_15_9	Braeburn Hillwell	B
E1_1_17	20/1		E1_2_11	Braeburn Hillwell	B
DN7_17_4	28/1 = 2B-28-02		E1_10_15	Braeburn type	B
DN7_30_3	28/1		DN7_16_6	Braestar	B
E1_3_14	28/1		E1_11_21	Braestar	B
DN7_30_1	28/2		DN7_16_2	Braeburn	B
E1_3_13	28/2		E1_17_18	Calville De Saint Souve	B
DN7_15_8	28/2 = 2B-28-14		E1_7_19	Canvade	
E1_2_12	2B-12-25		E1_19_11	CC2/19	
DN7_17_9	4A-75-28 Rooi Granny		E1_17_16	Champion	
DN7_8_5	8A-1-Ouer		E1_18_14	Chantecler	B
DN7_15_5	Adina		E1_17_10	Charden	B
E1_3_12	Adina (syn Frankad)		E1_17_2	Haidegger Golden X	B
E1_3_10	African Carmine		E1_17_3	Haidegger Golden Y	B
E1_15_18	Akane	B	DN7_3_11	Climax	B
E1_9_19	Alfmission		E1_14_2	Climax	B
E1_7_3	Alkmene	B	E1_8_18	Climax	B
E1_14_11	Alsop's Beauty		E1_17_5	Coast	
DN7_18_3	Anna		E1_16_16	Commerce	
DN7_30_2	Anna		E1_16_15	Co-op 19	
E1_9_10	Anna		E1_7_12	Co-op 20	
DN7_24_9	Antonovka Seedling No6		E1_7_17	Coromandel Red	
DN7_16_1	Aport		E1_15_16	Red Cox	B
E1_19_12	Aport		DN7_5_3	Cox's Orange Pippin	B
E1_12_4	Arapkizi		E1_16_21	Crab A	
E1_17_11	Atties Favourite		E1_9_4	Crab C	
DN7_31_2	Austin		E1_7_14	Criterion	B
DN7_4_3	Austin		E1_16_18	Dakota	
E1_13_10	Baujade		E1_13_15	Dayton (=Co-op 21)	
E1_18_6	Beauty of Black Loop		DN7_24_1	Dayton Seedling No6	
E1_14_3	Belle de Boskoop	B	E1_9_14	Delblush	
E1_5_6	Rode Boskoop	B	DN7_3_2	2X Red Delicious X	B
E1_4_5	Schone Van Boskoop	B	DN7_3_3	2X Red Delicious Y	B
E1_5_17	Schone Van Boskoop	B	E1_16_6	Big Chief	B
E1_14_8	Belrene		E1_14_13	Classic	B
DN7_23_1	Ben Shogun		DN7_6_8	Dietrich	B
E1_13_11	Beni Osho		E1_6_15	Dietrich Starking	B
DN7_4_7	Beverly Hills		E1_6_18	Full Red	B
DN7_27_1	Bittenfelder		E1_12_8	Hardy Spur	B
E1_14_6	Blairmont		E1_15_9	Hi Early Delicious	B
E1_9_15	Blenheim Orange		E1_16_13	Lalla Delicious	B
DN7_31_3	Boiken	B	E1_6_20	Oregon Red Spur	B
E1_13_21	Boiken	B	E1_17_1	Oregon Red Spur 2	B

Tree No.	Name	Code	Tree No.	Name	Code
E1_14_4	Prime Red Delicious	B	E1_9_9	Fuji Akufi	B
E1_1_7R	Red Delicious	B	E1_8_22	Fuji Berthon	B
E1_18_5	Red Delicious Tasmania	B	E1_12_1	Fuji Irradiated	B
E1_7_18	Ryan Red	B	E1_16_1	Fuji Tac 114	B
E1_15_3	Ryan Red	B	E1_9_2	Gala	B
E1_15_4	Ryan Spur	B	E1_11_11	Gala Imperial Gala	B
E1_10_10	Shotwell Delicious	B	E1_14_7	Gala Royal Gala	B
E1_2_1	Stark Spur Supreme	B	E1_9_13	Gala To Red?	
DN7_4_9	Starking	B	DN7_5_11	Royal Gala	B
E1_8_3	Starking (RSA)	B	E1_13_7	Rubinstar	B
E1_17_17	Starking (USA)	B	DN7_6_7	Scarlet Gala	B
E1_3_3	Starking Red (Groend)	B	E1_4_11	Gavin	B
E1_4_15	Starkrimson	B	DN7_16_9	Ginger Gold	
E1_15_12	Super Chief Red Del	B	E1_2_14	Ginger Gold	
E1_8_23	Top Red	B	E1_9_18	Gloire de Hollande	
E1_1_1	Ultra Red	B	E1_7_20	Gloster	B
E1_8_10	Wellspur Delicious	B	E1_3_16	Belgold	B
E1_13_14	Early Red	B	E1_6_1	Compactagold	B
E1_13_18	Early Red No. 2	B	E1_12_16	Elbee	B
E1_13_20	Groth Red	B	DN7_2_1	Golden Delicious	B
E1_17_21	Morspur	B	DN7_7_3	Golden Delicious	B
E1_17_6	Starking Colorless	B	E1_16_12	Golden Delicious	B
E1_10_14	Starking Early (Moodie)	B	E1_14_12	Golden Deli (Hawaii)	B
E1_16_9	Starking Red (Moodie)	B	E1_6_10	Golden Delicious X	B
E1_8_7	Starking Stripeless	B	E1_6_11	Golden Delicious Y	B
DN7_18_4	Delkistar	B	E1_8_11	Golden Delicious Claz	B
E1_18_8	Democrat	B	E1_7_16	Golden Delicious Early	B
E1_12_13	Diva Gold		E1_11_20	Golden Delicious Fran	B
DN7_4_4	Drakenstein		E1_18_9	Golden Delicious Reinde F2	B
E1_14_17	Dukat	B	E1_11_17	Golden Delicious U.S.	B
DN7_4_2	E3 F2		E1_9_21	Golden Sheen (Belgold)	B
E1_9_12	Earligold		E1_11_5	Goldspur	B
DN7_22_2	Edgewood		E1_11_4	Goldspur Applewaite	B
E1_17_8	Edgewood		E1_11_14	Goldspur Aswell	B
E1_15_11	Eikhoff		E1_3_17	Lysgolden (=Goldenir)	B
E1_16_19	El Orange	B	E1_8_12	Panorama Golden X	B
E1_4_13	Elise	B	E1_8_13	Panorama Golden Y	B
E1_8_19	Elsie Grant		E1_15_6	Smoothee	B
E1_9_3	Elstar	B	E1_16_8	Spur Golden	B
DN7_4_5	Elstar Red	B	E1_11_10	Stark Spur Golden Del	B
E1_12_6	Elstar Red	B	E1_19_2	Heinderich Golden	B
E1_2_10	Elstar Red	B	E1_8_21	Yellow Delicious	B
E1_10_3	Empire	B	E1_11_13	Goldrush	
E1_9_17R	Fiesta		E1_9_20	Goldsmith (=Early Granny)	
E1_8_1	Flavorglo		E1_10_20	Goosen	
E1_7_13	Florentina		E1_9_8	Grand Richard	
E1_19_5	Forum		E1_11_15	Granearli	
E1_12_14	Fuji	B	DN7_19_1	Granny Smith	B
E1_10_12	Fuji A	B	E1_5_1	Granny Smith	B

Tree No.	Name	Code	Tree No.	Name	Code
E1_12_3	Granny Smith (Louterwater)	B	E1_16_7	Jonathan	B
E1_15_17	Granny Smith (RSA)	B	E1_4_7	Jonathan	B
E1_12_2	Granny Smith 14-7-70	B	E1_11_12	Jonnee	B
E1_16_14	Granny Smith Red		E1_4_4	Julia	
E1_15_10	Granny Smith Spur	B	E1_18_2	July Red	B
E1_12_10	Granny Smith USA	B	E1_16_2	Karmijn de Sonnaville	B
E1_11_7	Green Fielda	B	DN7_5_1	Kashawi	
E1_7_9	Red Gravenstein		E1_17_13	Kashawi	
E1_1_6	Greensleeves	B	E1_17_15	Kidd's Orange Red	B
E1_13_17	Harberts Reinette		E1_13_19	King of Tomkins County	B
E1_11_19	Himekami X		E1_1_4	Kirks X	
E1_11_23	Himekami Y		E1_1_5	Kirks Y	
DN7_33_1	HL 1004		E1_18_15	Klara	
DN7_32_1	HL 166C		E1_16_11	Kogetso	
E1_18_11	HL 237		E1_7_1	Koo	
E1_18_10	HL 318		DN7_6_4	Lady Williams	
E1_18_12	HL 938		E1_15_14	Lakeside	
DN7_24_8	Hofer Seedling		E1_17_12	Langkloof	
E1_1_13	Hokuto		E1_12_15	Laxton's Superb	B
DN7_4_6	Hoplan		E1_10_6	Lemon	
E1_17_20	Hoplan		DN7_19_2	Le Vant	
E1_14_10	Hops Late Red		E1_7_7	Le Vant	
E1_11_3	Howell		E1_10_19	Leyda	
DN7_22_3	Howell?		DN7_5_2	Liberty	
DN7_26_1	i5526 X 6407 INRA		E1_8_14	Liberty	
E1_7_15	Idared	B	E1_12_9	London Pippin	
DN7_5_10	Jersey Mac	B	E1_11_8	Longford	
E1_8_8	Jersey Mac	B	E1_13_6	Lord Lambourne	
DN7_33_2	Jester	B	E1_16_4	Lord Lambourne	
E1_15_1	Jester	B	DN7_20_10	M1	
DN7_20_7	Malling Jester X	B	E1_1_9	M1	
DN7_20_8	Malling Jester Y	B	DN7_1_8	M13	
E1_15_5	Jonafree (=Co-op 22)		E1_13_4	M13	
E1_18_7	Jonafree (=Co-op 22)		E1_1_11	M13	
E1_4_17	Jonafree (=Co-op 22)		DN7_1_10	M25	B
E1_9_1	Crown Gold	B	DN7_8_7	M26	B
DN7_17_5	Jonagold	B	DN7_20_11	M4	
DN7_3_1	Jonagold	B	E1_2_22	M4	
E1_13_12	Jonagold	B	DN7_21_11	M7	
E1_9_5	Jonagold	B	DN7_1_9	M7	
E1_8_15	Jonagold Costa X	B	DN7_5_4	M7 Elgin	
E1_8_16	Jonagold Costa Y	B	DN7_21_10	M793	
E1_10_11	Jonagold Jomured	B	DN7_2_10	M793	
E1_10_1	Jonagold Red	B	DN7_2_8	M793?	
E1_2_13	Jonagored	B	DN7_2_11	M9	B
E1_17_22	King Jonagold	B	DN7_21_9R	M9	B
E1_5_12	Russel Red		E1_13_3	M H 15-6	
E1_7_10	Schneica (=Jonica)	B	E1_7_2	Maayan	
E1_18_3	Blackjon	B	E1_14_16	Macobin	

Tree No.	Name	Code	Tree No.	Name	Code
E1_1_19	Maidens Blush		E1_6_22	P 18	
E1_17_4	Maigold		E1_17_14	Palmiet Red	
E1_11_2	Marajon		E1_16_10	Panorama Crab	
E1_12_5	Macspur	B	E1_1_15	Paragon	
E1_17_7	Macspur	B	DN7_8_8	Paulared	B
E1_6_13	Marshall McIntosh 6	B	E1_18_1	Paulared	B
E1_19_1	McIntosh	B	DN7_17_7	Pi-Au 9-24	
E1_9_7	McIntosh Early		DN7_21_7	Pi-Au 9-27	
DN7_3_10	Melba	B	E1_10_8	Pi-Au 9-27	
E1_3_11	Melba	B	DN7_19_3	Pilot	B
E1_15_15	Meldale		DN7_16_7	Pink Lady	
DN7_5_6	Melrose	B	E1_10_7	Pink Lady	
E1_3_15	Melrose	B	E1_3_2	Pink Lady	
E1_2_17	Meran	B	DN7_2_2	Pinova	B
DN7_17_3	Meran	B	DN7_31_1	Trajan (=Polka)	
DN7_1_6	Michal		E1_2_19	Pomme De Niege	
E1_11_6	Michinoku	B	E1_5_9	Porporate	
DN7_6_6	Milton		E1_11_16	Present of England	
E1_5_19	Missouri Pippin		DN7_6_9R	Prima	B
DN7_8_6	MM106	B	E1_17_9	Primgold	
DN7_21_12	MM109		E1_2_21	Prince Bismarck	
DN7_1_7	MM109		E1_10_4	Princesa	
DN7_20_6	MM111	B	DN7_6_10	Priscilla	
DN7_1_1	Mollie’s Delicious	B	DN7_6_2	Red Astrakhan	B
E1_4_10	Mollie's Delicious	B	E1_2_16	Red Astrakhan	B
E1_14_9	Monsa		E1_3_20	Red Astrakhan	B
E1_4_14	Morkel's Seedling		E1_2_5	Red Dutch	
E1_6_8	Mother	B	E1_16_5	Red Gem	
E1_10_17	Mutsu	B	E1_16_3	Redfree	
E1_15_13	Nebuta	B	E1_2_20	Redwine	
E1_9_6	New Gold		E1_14_18	Redwinter	
E1_5_5	New Year		DN7_7_8	Reglindis	
E1_2_4	Nickajack		E1_13_9	Reglindis	
DN7_24_2	No1 Dresden (Seedling 4)		E1_3_18	Reinette du Canada X	
DN7_24_3	No2 Dresden (Seedling 2)		E1_3_19	Reinette du Canada Y	
DN7_24_4	No3 Dresden (Seedling 1)		DN7_20_1	Remo	
DN7_4_8	Northern Spy	B	DN7_1_4	Resista X	
E1_12_12	Beaumont		DN7_1_5	Resista Y	
E1_16_17	Dunn's Seedling (Ohenimuri)		E1_8_5	Resista	
E1_8_9	Ohenimuri Early		DN7_7_9	Rewena	B
E113_13	Onderstam 5		E1_6_21	Rewena	B
DN7_18_5	Onderstem 5 X		E1_2_3	Rhode Island Greening	
DN7_18_6	Onderstem 5 Y		E1_2_7	Richared	
E1_13_16	Hunter Ontario	B	E1_17_19	Rokewood	B
E1_10_13	Jumbo Orin	B	E1_14_5	Clifton Rome	B
E1_3_21	Orleans Reinette		E1_6_12	Rome Beauty	B
E1_1_8	Ozark Gold	B	E1_1_12	Seeando Red Rome	B
E1_10_2	P 1		E1_4_1	Spur Red Rome	B
DN7_1_11	P 18		DN7_17_10	Russian Seedling	
E1_13_2	P 18		E1_13_8	Russian Seedling	

Tree No.	Name	Code	Tree No.	Name	Code
DN7_33_3	SA579-3		E1_11_22	Trajan (=Polka)	
E1_3_9	Sadie Frazer		DN7_15_10	Treco Red X	
DN7_5_9	Sansa		DN7_15_11	Treco Red Y	
E1_6_16	Sayaka		E1_7_8	Beni Tsugari	B
DN7_32_2	Scarlet		E1_1_2	Homei Tsugari X	B
E1_19_9	Selena		E1_1_3	Homei Tsugari Y	B
E1_5_15	Senator		DN7_20_5	Homei Tsugaru	B
E1_7_5	Senshu		E1_7_6	Natsuka	B
E1_2_6	Shampion	B	E1_14_1	Tuscan (=Bolero)	
DN7_22_1	Sharpe's Early		DN7_4_1	Twenty Ounce	B
DN7_3_9	Sharpe's Early		E1_11_18	Twenty Ounce	B
E1_4_9	Sharpe's Early		E1_4_16	Tydeman's Early	B
E1_8_2	Sharpe's Early		E1_1_18	Valmore	
E1_4_6	Sharpe's Late		E1_10_9	Veitchi Pumila	
E1_18_4	Shizuka		E1_6_9	Versveld	
DN7_2_4	Shlomit		E1_5_4	Viljoen's Red	
E1_11_9	Shlomit		DN7_6_1	Vista Bella	
E1_6_7	Shoreland Queen		E1_5_2	Vista Bella	
DN7_1_3	Sinclair		E1_6_6	Wainwright	
E1_5_7	Sir Isaac Newton	B	E1_14_19	Wemmershoek	
DN7_5_8	Sir Prise		E1_5_3	White Winter Banana	
E1_8_4	SPAB 919		E1_7_4	Widup	B
E1_5_14	Spartan	B	E1_3_4	William's Pride Co-op 23	
DN7_6_5	Splendour	B	E1_2_18	Winesap	
E1_4_8	Splendour	B	E1_5_13	Seeando Winesap	
E1_6_5	Jacored		E1_14_14	Spur Winter Banana	
DN7_7_4	Starkrimson		DN7_7_1	Winter Banana	
E1_9_16	Red Statesman		E1_14_15	Winter Banana	
E1_13_1	Black Stayman		E1_4_2	Wolf River	B
E1_3_5	Stayman Winesap		E1_19_8	X2765	
E1_6_2	Stark Scarlet Stayman		E1_19_3	X6163 P22 R19 A14	
DN7_5_7	Summerking Red		E1_19_10	X640 TNR42A45	
E1_4_3	Summerred		E1_19_4	X6688 K1 R87 A18	
E1_6_14	Summerred		E1_10_18	Yataka	
DN7_15_3	Sundowner X		DN7_2_3	Zabaoni	
DN7_15_4	Sundowner Y		E1_1_10	Zabaoni	
E1_10_5	Sundowner		E1_5_16	Zabaoni	
E1_2_2	Sunrise	B	E1_2_15	Zoba (=Lobo)	
E1_2_8	Suntan	B	E1_4_18	Zvonkove	
E1_3_8	Swartland		**Other *Malus* species and hybrids**		
E1_5_18	Swartland		DN7_15_7	*M. sieversii* Kaz-95-44	
E1_6_4	Sweet Cornelly		DN7_17_8	*M. sieversii* Kaz-95-57 X	
DN7_21_5	T 506		DN7_8_2	*M. sieversii* Kaz-95-57 Y	
E1_18_13	Takane		E1_13_5	*M. sieversii* Kaz-95-57 Z	
E1_12_7	Takane		DN7_16_5	*M. sieversii* Kaz-95-58 X	
E1_6_3	Tasman's Pride		DN7_8_1	*M. sieversii* Kaz-95-58 Y	
E1_10_16	Telamon (=Waltz)	B	DN7_18_2	*M. sieversii* Kaz-95-71 X	
E1_12_11	Telamon (=Waltz)	B	DN7_8_4	*M. sieversii* Kaz-95-71 Y	
E1_1_14	Tjeek		DN7_15_1	*M. sieversii* Kaz-95-71A	

Tree No.	Name	Code	Tree No.	Name	Code
DN7_18_1	*M. sieversii* Kaz-95-78 X		E1_19_7	*M.* Grandiflora Crab	
E1_8_20	*M. sieversii* Kaz-95-78 Y		E1_15_7	*M.* Jackson Crab	
DN7_17_2	*M. sieversii* Kaz-95-89 X		E1_15_8	*M.* L.P. Mornel Crab	
E1_8_6	*M. sieversii* Kaz-95-89 Y		E1_15_19	*M.* Maypole	B
DN7_15_6	*M. sieversii* Kaz-95-91 X		DN7_25_3	*M.* Maypole	B
DN7_8_3	*M. sieversii* Kaz-95-91 Y		DN7_20_2	*M. micromalus*	
E1_1_16	*M. sieversii* Kaz-95-91 Z		DN7_5_5	*M.* Mildew Resistant	
DN7_16_8	*M. sieversii* Kaz-95-122 X		DN7_20_4	*M.* Moeransi Profusion	
E1_8_17	*M. sieversii* Kaz-95-122 Y		E1_15_2	*M.* Pioneer Scarlet	
DN7_21_1	KSC 3		DN7_16_3	*M. platycarpa*	
DN7_21_2	KSC 11		E1_6_19	*M. platycarpa*	
E1_7_21	KSC 11		DN7_2_5	*M. purpurea*	
E1_7_11	KSC 13		E1_9_11	*M. purpurea*	
DN7_21_3	KSC 25		DN7_2_7	*M. spectabilis*	
DN7_21_6	Malus 44		DN7_6_3	*M. spectabilis*	
E1_3_1	Malus 44		DN7_17_1	*M. robusta*	
DN7_34_1	*M.* Aldenhamensis		DN7_4_10	*M. robusta*	
DN7_25_2	*M. baccata*		E1_5_10R	*M. robusta*	
E1_5_8	*M.* Butterball		DN7_7_6	*M. sieboldii*	
DN7_20_3	*M. coronaria*		DN7_20_9	*M.* Veitch's Scarlet	
E1_3_7	*M. floribunda*		DN7_7_10	*M. zumi*	
E1_5_11	*M. floribunda*		DN7_24_5	No4 Dresden (Seedling 3)	
E1_17_6R	*M. floribunda*		DN7_24_6	No5 Dresden (Seedling ?)	
DN7_25_1	*M. fusca*		DN7_24_7	No5 Dresden (Seedling ?)	
DN7_16_10	*M.* Golden Hornet		DN7_25_4	S202	
E1_3_6	*M.* Golden Hornet		DN7_15_2	Spy 227	
E1_4_12	*M.* Golden Hornet		E1_6_17	Spy 227	
DN7_2_9	*M.* Grandiflora Crab		DN7_21_4	T 585	

Accessions with discrepancies are indicated by suffixes X, Y and Z, D material in Drostersnes Experimental Farm gene bank plot DN7, E material in Grabouw Experimental Farm gene bank plot E1.

Rather than obtaining reference materials from INRA, France, as recommended by Laurens *et al*. (2004) and Evans *et al*. (2007), the ARC accessions of six of the eight recommended accessions were used in this study; four cultivars of *M. pumila,* one accession of *M. floribunda* Van Houtte and one accession of *M. robusta* (Table 4.2). The remaining two *M. pumila* reference cultivars, ‘Michelin’ and ‘Worcester Pearmain’, were not available.

Table 4.2. Six ARC accessions of reference cultivars recommended by ECPGR (Fernández-Fernández, 2010) and their location in the Drostersnes and Grabouw Experimental Farm gene banks. Accessions of the two remaining reference cultivars were not present in the ARC gene bank.

Tree location	Species	Name
E1_17_6	*M. floribunda*	*M. floribunda* 821
E1_9_17	*M. pumila*	Fiesta
DN7_ 21_9	*M. pumila*	M9
DN7_6_9	*M. pumila*	Prima
E1_1_7	*M. pumila*	(Red) Delicious
E1_5_10	*M. robusta*	*M. robusta* 5

4.2.2. DNA extraction

Genomic DNA was extracted following a slightly modified method by De la Rosa *et al*. (2002). The microcentrifuge tubes containing frosen leaves were placed on the bench to initiate thawing. Before complete thawing, a single 1 mm stainless steel ball-bearing was placed inside the tube. Extraction reagents of 0.8 ml prewarmed (65°C) CTAB buffer [2% (m/v) CTAB (Merck), 2% (m/v) PVP 40 (Merck), 1.4M NaCl (Merck), 20 mM EDTA at pH 8 (Merck), 100 mM Tris at pH 8 (Melford Laboratories)] and 0.08 ml β-mercaptoethanol (Merck) were added.

Samples were shaken by hand to mix the reagents and then ground thoroughly for 3 to 4 min using a Tissuelyser II ball mill (Qiagen). Samples were incubated for 2 hours at 65°C and the ball bearings were removed using a stainless steel magnet. Thereafter 0.8 ml of chloroform-isoamyl alcohol (Merck) at ratio 24:1 was added and the samples centrifuged (Labnet) for 15 min at 13 500 rpm. The top aqueous phase was recovered; 0.8 ml of chloroform-isoamyl alcohol (24:1) was added again and the sample centrifuged for 10 min at 13 500 rpm. The top aqueous phase was again recovered and precipitated with 0.5 ml of cold isopropanol overnight. After precipitation, samples were cold centrifuged at 4°C for 15 min at 13 500 rpm, the solution was discarded; and the pellet was washed in 0.5 ml of 70% (v/v) ethanol, dried for 30 to 45 min and resuspended in TE buffer until further use.

The quality and quantity of the DNA was determined with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) following the manufacturer's instructions. If a sample showed poor quality and quantity, the extraction was repeated. The DNA samples from the different extractions were diluted and adjusted to a final concentration of 100 ng/µl.

4.2.3. Primer selection and multiplex conditions

The full set of 12 microsatellite markers recommended by the ECPGR *Pyrus/Malus* working group (Fernández-Fernández, 2010) was used for the current study (Table 4.3). These markers were used in the multiplexes recommended by Fernández-Fernández (2010) with markers combined into three groups on the basis of the size range of their products: Multiplex A (smaller sized products: 89 to 151 bp), Multiplex B (medium sized products: 130 to 206 bp) and Multiplex C (larger sized products: 175 to 257 bp). Both the forward primers, fluorescently labelled, and the reverse primers, unlabelled, were supplied by Applied Biosystems.

Table 4.3. The 12 microsatellite markers recommended by the ECPGR's *Pyrus*/*Malus* working group (Fernández-Fernández, 2010) used for fingerprinting the ARC apple collection.

Marker	Linkage group	Forward sequence	Reverse sequence	Label	Multiplex
[2]CH01f02	12	acc aca tta gag cag ttg agg	ctg gtt tgt ttt cct cca gc	Fam	B
[2]CH01f03b	9	gag aag caa atg caa aac cc	ctc ccc ggc tcc tat tct ac	Vic	B
[2]CH01h01	17	gaa aga ctt gca gtg gga gc	gga gtg ggt ttg aga agg tt	Ned	A
[2]CH01h10	8	tgc aaa gat agg tag ata tat gcc a	agg agg gat tgt ttg tgc ac	Vic	A
[2]CH02c09	15	tta tgt acc aac ttt gct aac ctc	aga agc agc aga gga gga tg	Pet	C
[2]CH02c11	10	tga agg caa tca ctc tgt gc	ttc cga gaa tcc tct tcg ac	Ned	C
[2]CH02d08	11	tcc aaa atg gcg tac ctc tc	gca gac act cac tca cta tct ctc	Vic	C
[2]CH04c07	14	ggc ctt cca tgt ctc aga ag	cct cat gcc ctc cac taa ca	Fam	A
[2]CH04e05	7	agg cta aca gaa atg tgg ttt g	atg gct cct att gcc atc at	Fam	C
[1]GD12	3	ttg agg tgt ttc tcc cat tgg a	cta acg aag ccg cca ttt ctt t	Ned	B
[1]GD147	13	tcc cgc cat ttc tct gc	gtt taa acc gct gct gct gaa c	Pet	B
[3]Hi02c07	1	aga gct acg ggg atc caa at	gtt taa gca tcc cga ttg aaa gg	Pet	A

[1]Hokanson *et al*. (1998), [2]Liebhard *et al*. (2002), [3]Silverberg-Dilworth *et al*. (2006)

Initial optimisation revealed competition of fluorescent labels which necessitated the use of different volumes per primer for the different multiplexes (Table 4.4). Primer dilutions for use in PCRs were made from a 100 µM primer stock solution adjusted to a working concentration of 10 µM.

Table 4.4. Composition of microsatellite multiplex primer mixes A, B and C used for fingerprinting the ARC apple collection.

Marker	Forward (µl)	Reverse (µl)	Dilution (combined +DH_2O)
Mutiplex A			
CH01h01	1.15	1.15	
CH04c07	1.75	1.75	
CH01h10	1.2	1.2	
Hi02c07	1	1	
Total	5.1	5.1	10.2+89.8 = 100 µl
Multipex B			
CH01f02	1.2	1.2	
GD12	1.2	1.2	
CH01f03b	1	1	
GD147	1.4	1.4	
Total	4.8	4.8	9.6+90.4 = 100 µl
Multiplex C			
CH04e05	1.75	1.75	
CH02c11	1.25	1.25	
CH02d08	1.75	1.75	
CH02c09	1.65	1.65	
Total	6.4	6.4	12.8+87.2 = 100 µl

4.2.4. Microsatellite genotyping

PCRs were performed in a final volume of 12.5 µl containing 1.5 µl of 100 ng template DNA, 6.25 µl of PCR mix (Qiagen), 1 µl of multiplex primer mix A, B or C as diluted in Table 4.4 and 3.75 µl of RNase-free water. Amplification was carried out in GeneAmp (Applied Biosystems) and G-Storm (G-Storm Direct) thermal cyclers using the following conditions: an initial denaturation at 95°C for 15 min, followed by 29 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, and a final 30 min extension at 60°C.

PCR products from a subset of accessions were first resolved electrophoretically on a 1% (m/v) agarose gel (Conda Laboratories) at 70V (Hoefer Scientific Instruments PS 500X) for 60 min in a 1X TBE buffer (Tris, Boric acid, EDTA) using a 1kb ladder (Thermo Scientific) to verify amplification. Upon confirmation, the full set of PCR products was sized with capillary electrophoresis on a 3130 DNA capillary analyser (Applied Biosystems) at the Central Analytical Facility's DNA sequencing unit of Stellenbosch University. Sizes of the amplified products were established in comparison with the internal size standard, GS500(-250)LIZ (Applied Biosystems). The software GENEMAPPER version 5.0 (Applied Biosystems) was used to visualise the peaks and aid allele scoring. Data were independently verified by a competent co-worker. Single peaks were attributed to homozygotes, two peaks to

heterozygotes. More than two peaks at several markers were indicative of polyploidy. Verified data were collated in Microsoft Excel 2010.

4.2.5. Statistical analysis

Prior to analysis for genetic diversity, the validity of the collated data was verified using MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al*., 2004), which tests the possibility of misscoring due to stuttering, allele dropout or presence of null alleles. Additionally, deviation of markers from the Hardy-Weinberg Equilibrium (HWE) was tested using Markov chain exact tests (1000 dememorisation, 100 batches and 1000 iterations per batch), computed with GENEPOP version 4.3 (Rousset, 2008).

Genetic diversity statistics were calculated using GENALEX version 6.501 (Peakall and Smouse, 2012), firstly for the entire collection and secondly, for the 238 'primary' cultivars of *M. pumila* excluding duplicates and triploids. The number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He) and Shannon's information index (I) were calculated. The number of alleles per locus (Na) is a direct count of the alleles amplified by a given marker for all the samples. 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. Expected heterozygosity (He) for each marker is calculated based on the formula by Nei (1973), $He=1-\sum(pi)^2$, and is the probability that two alleles from the same locus are different when chosen at random from a given population. Shannon's information index, $I=\sum pi \ln pi$ is an unbiased measure of allelic diversity per locus. The polymorphism information content (PIC) of markers, $PIC=1-\sum(pij)^2$, was calculated using CERVUS version 3.0.7 (Kalinowski *et al*., 2007) to determine how informative the markers were.

4.2.6. Trueness to type investigation

Ideally, classes used Chapter 2 could have been used. However, fingerprinting data from the Brogdale collection could not be sourced and no direct comparison could be made.

To assist with classification of accessions, an alternative approach was employed comprising two strategies. First the accessions were sorted in Microsoft Excel according to 'primary' cultivar *e.g.* several variants of 'Golden Delicious' were grouped together. Secondly the genotypes were sorted in Microsoft Excel in numerical order, allele by allele, for each of the 'diploid' columns for all the amplified markers. This facilitated the detection of accessions with matching microsatellite patterns, and various false accessions were detected. This strategy can however be invalidated by minor variant scores in the first few columns.

This strategy enabled some investigation of trueness to type in the absence of comparative verified apple fingerprinting data. Five classes were used for arranging the fingerprints: class 1, items which could be compared with their clones or sports and had a matching pattern; class 2, items with two or more representatives having matching microsatellite patterns but which could not be validated by comparison with other clones or sports of the original cultivar; class 3, items that were compared with their clones or sports but had inconsistent patterns; class 4, items with single entries in the collection which could not be compared; and class 5, items with only two representatives having different microsatellite patterns.

Parentage analysis was also conducted for accessions with known parentage to confirm their identity particularly for accessions in class 3, 4 and 5. Accessions arising from the reported parentage were coded either as 'V', meaning verified, or 'F', meaning false or not arising from the reported parentage.

Additionally, a genetic distance matrix for codominant data was calculated using GENALEX pairwise genetic test on an individual-by-individual (*N*x*N*) basis. The genetic distance matrix was subsequently converted to a MEGA input file. A dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster method in MEGA version 6 (Tamura *et al*., 2013), using default settings. No bootstrap analysis was performed as this was conducted only to verify possible mislabelled accessions.

4.3. Results

A complete set of microsatellite fingerprints of 540 apple accessions in the ARC apple gene bank for 11 markers is presented in Appendix 4.1. The data were used to estimate genetic diversity statistics and for various comparisons to verify trueness to type of accessions.

4.3.1. Marker performance

Eleven of the 12 markers used in this study (CH01f02, CH01f03b, CH01h01, CH01h10, CH02c09, CH02c11, CH02d08, CH04c07, CH04e05, GD12 and Hi02c07) successfully amplified the DNA samples and gave easy to score patterns across the 540 accessions fingerprinted. However, marker GD147, in Multiplex B, often gave a non-allelic product at 142 bp which hindered scoring and was therefore excluded from subsequent analysis. For some accessions in this study more than two alleles were detected by all markers used and those accessions were left out from subsequent statistical analyses as the software assumes diploidy.

4.3.2. Statistical analysis

Preliminary MICRO-CHECKER analysis detected no evidence of misscoring due to the presence of null alleles, stuttering or allele dropout for nine of the 11 markers used. Markers CH04c07 and GD12, showed apparent excess homozygotes which suggest the possibility of null alleles (Fig. 4.1). However, there was no scoring error attributed to stuttering or allele dropout for the two markers and these were therefore retained for further analyses.

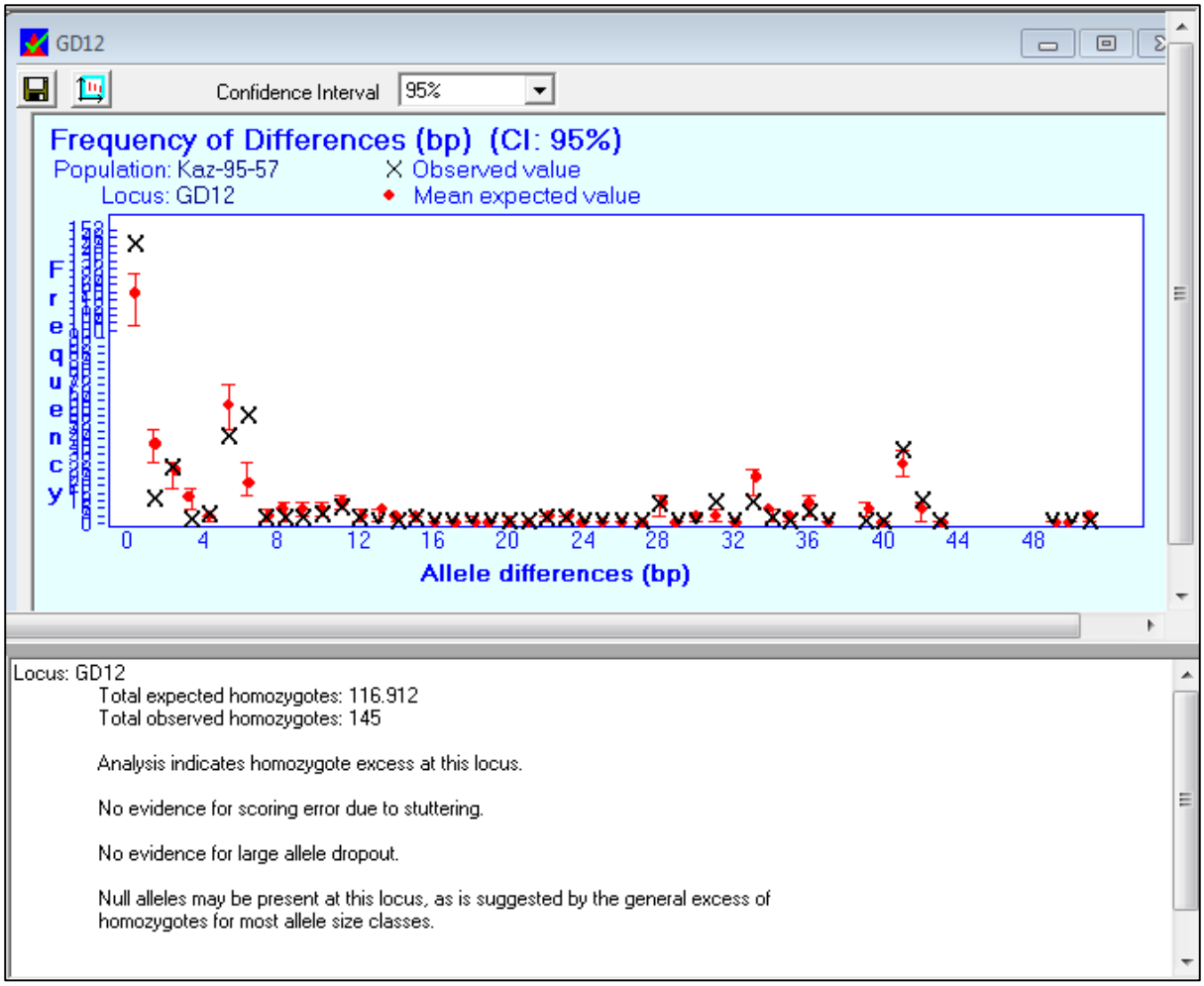

Fig. 4.1. MICRO-CHECKER results for marker GD12 indicating excess homozygotes and evidence of possible null alleles. Similar results were observed for marker CH04c07.

All markers except for Hi02c07, which had a P value of 0.05, deviated significantly from HWE when 'primary' accessions of *M. pumila* accessions were analysed (data not shown). The number of alleles per locus ranged from 14 in the case of CH01h10, to 25, for CH04e05, when the entire population, excluding possible triploids, was analysed (Table 4.5). A reduction was observed when representatives of other *Malus* species were excluded from the analysis, with the number of alleles per locus ranging from 12, for markers CH01h10 and CH02c09, to 22, for CH04e05.

Table 4.5. Number of alleles (Na), polymorphic information content (PIC), observed heterozygosity (Ho), expected heterozygosity (He) and Shannon's information index (I) detected using 11 microsatellite markers for 296 diploid *Malus* accessions (above) and 238 *M. pumila* cultivars (below).

	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
Na	20	16	16	14	16	16	19	17	25	18	21
PIC	0.87	0.77	0.83	0.67	0.81	0.88	0.84	0.82	0.79	0.66	0.72
Ho	0.86	0.85	0.89	0.71	0.79	0.92	0.88	0.73	0.79	0.60	0.73
He	0.87	0.80	0.85	0.70	0.83	0.89	0.85	0.83	0.80	0.68	0.74
I	2.31	1.86	2.09	1.61	1.99	2.34	2.23	2.15	2.10	1.73	1.79
	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
Na	20	14	15	12	12	13	15	17	22	15	20
PIC	0.85	0.77	0.83	0.63	0.82	0.88	0.83	0.84	0.73	0.70	0.73
Ho	0.87	0.85	0.89	0.68	0.80	0.92	0.87	0.83	0.77	0.68	0.73
He	0.87	0.78	0.83	0.67	0.81	0.88	0.84	0.85	0.78	0.73	0.73
I	2.24	1.76	1.97	1.45	1.86	2.26	2.13	2.19	1.95	1.78	1.72

An average Ho of 0.79, and He of 0.80, were realised when all *Malus* accessions were analysed. When only *M. pumila* accessions were analysed, means of 0.81 for Ho, and 0.80 for He, were obtained. Shannon's information index was highest for marker CH02c11 in the two analyses, with values of 2.34 and 2.26, respectively, and likewise lowest for marker CH01h10 with values of 1.61 and 1.53, respectively. Marker CH02c11 was the most polymorphic for both analyses (0.88, 0.88), while GD12 (0.66) and CH01h10 (0.63) were the least informative for the entire set and the *M. pumila* analyses.

4.3.3. Verifying reference cultivars

Although the data from the Brogdale accessions were not available, the EMR scores for the ECPGR recommended reference cultivars at INRA were verified against the ARC scores to establish calibration required for future comparison. Two markers, CH02c09 and CH04c07, gave identical scores, while four of the 11 markers used, CH01h01, CH02c11, CH02d08 and Hi02c07, gave simple shifts of +2, +3, +2 and +1 bp, respectively when compared with EMR reference scores (Table 4.6). Markers CH01f02, CH01f03b, CH01h10, CH04e05 and GD12, gave more complex shifts. The reference accession 'Fiesta' from ARC, was inconsistent when compared with the EMR accession of 'Fiesta', indicating that the ARC accession is probably false.

Table 4.6. Comparison of six apple reference cultivars recommended by ECPGR *Pyrus/Malus* working group, kept at INRA, France, with the South African accessions of the same cultivars with respect to microsatellite fingerprints.

Reference	CH01f02		CH01f03b		CH01h01		CH01h10	
	INRA	ARC	INRA	ARC	INRA	ARC	INRA	ARC
M. floribunda 821	175/179	175/179	149	150	103/137	104/139	102/110	104/111
Fiesta*	181/204	170/173	159/171	138/160	116/128	114/116	96	91/98
M9	169/171	170/172	159/171	160/171	112/118	114/120	96/113	98/115
Prima	179/206	179/206	137/159	138/160	112/116	114/118	89/96	91/98
(Red) Delicious	179/183	179/183	137/178	138/179	114	116	89/96	91/98
M. robusta 5	175/179	175/179	171	171	86/96	88/98	87/110	89/111
Shift		0 to +1		0 to +1		+2		+1 to +2

*ARC accession clearly different from INRA reference cultivar.
Underline indicates scores that deviate from the normal shift for a given marker between ARC and INRA accessions.

Table 4.6. Continued….

Reference	CH02c09		CH02c11		CH02d08		CH04c07	
	INRA	ARC	INRA	ARC	INRA	ARC	INRA	ARC
M. floribunda 821	231/251	231/251	221/225	224/228	214/218	216/220	108	108
Fiesta*	233/249	233/257	215/227	220	224/253	212/226	106/122	112
M9	245	245	213/233	213/235	212/253	214/255	106/114/129	106/114/129
Prima	233/243	233/243	227/231	230/234	253	255	104/106	106/108
(Red) Delicious	245/255	245/255	205/231	208/234	210/216	212/218	117/133	117/133
M. robusta 5	248	248	203/217	206/220	210/212	212/214	106/108	106/108
Shift		0		+3		+2		0

*ARC accession clearly different from INRA reference cultivar.
Underline indicates scores that deviate from the normal shift for a given marker between ARC and INRA accessions

Table 4.6. Continued….

Reference	CH04e05		GD12		Hi02c07	
	INRA	ARC	INRA	ARC	INRA	ARC
M. floribunda 821	187/196	188/198	148/172	150/173	114/135	115/136
Fiesta*	198/226	175/204	136/148	150/152	116/150	117
M9	196/220	198/221	148/159	150/161	116	117
Prima	173/208	175/209	182/190	183/191	110/118	111/119
(Red) Delicious	173/202	175/204	147/153	149/155	114/116	115/117
M. robusta 5	181	182	149/151	152	116/118	117/119
Shift		+1 to +2		+1 to +2		+1

*ARC accession clearly different from INRA reference cultivar.

4.3.4. Triploids

Nineteen accessions of *M. pumila* in the gene bank showed third peaks for four or more markers and were presumed triploid; these are ‘Adina’ (syn Frankad), ‘Alfmission’, ‘Baujade’, ‘Belle de Boskoop’, ‘Blenheim Orange’, ‘Charden’, ‘Forum’, ‘Harberts Reinette’, ‘Jonagold’, ‘King of Tomkins County’, ‘Lemon’, ‘Mutsu’, ‘Paragon’, ‘Red Gravenstein’, ‘Reinette du Canada’, ‘Rhode Island Greening’, ‘Stark Scarlet Stayman’, ‘Tekane’, and X6688 K1 R87 A18 (Table 4.7). The literature indicates all of these *M. pumila* accessions are known triploids except for ‘Adina’ (syn ‘Frankad’), ‘Alfmission’, ‘Forum’, ‘Harberts Reinette’, ‘Lemon’, ‘Tekane’, and X6688 K1 R87 A18 although this information had not always been incorporated into the gene bank database. Likewise, representatives of two other *Malus* species, *M. coronaria* (L.) Mill. and *M. platycarpa* Rehder, also had frequent third peaks.

Five accessions having third alleles for three markers or less as well as the triploids ‘Paragon’, ‘Red Gravenstein’ and ‘Stark Scarlet Stayman’, were tested for pollen germination with the known diploid, ‘Pink Lady’, included as a control. The accession ‘Karmijn de Sonnaville’, known to be triploid, but which did not show an additional third allele in the current study, was also included in the pollen germination tests.

Table 4.7. ARC apple accessions showing third alleles for at least four markers used and presumed to be triploid together with the cultivar 'Karmijn de Sonnaville', known to be triploid, but not showing additional alleles.

Name	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
M. pumila											
*Adina (syn Frankad)	170/204	171/179	114/116/118	91/109/115	233/243	218/234	226/255	95/106	175	149/191	109/115/117
Alfmission	170/183/206	138/171/179	118/114	98/103/109	233/251/256	208/234/238	212/255	106/120/133	175/209	150/155/161	111/115/117
Baujade	170/173/179	138/160/177	114/116/120	91/98	233/239/255	220/228/234	212/226/229	95/108/112	175/200	150/152/191	117/119
‡Belle de Boskoop	183/204	138/171/179	104/127/131	91/98	233/249/255	220/231/234	214/226/229	106/112	175/209	140/149/183	117/119
‡Blenheim Orange	179/183	160/179	112/120/129	91/98	243/255/257	218/228/234	224/229/255	95/106	175/202/221	150	107/119/151
‡Charden	172/179	138/171/179	116/118/131	91/98/109	243/253/257	208/220/234	224/226/229	95/106/112	175	150/155/191	111/117
Forum	170/179/206	160/171/179	112/116/131	91/98/109	239/243/245	235/238	212/224/255	97/108/133	175	150/155	111/117
Harberts Reinette	183/185	138/179	112/118/131	91/98	233/245/257	208/218/234	226/255	106/112/120	175/202	150	111/119/151
‡Jonagold	170/179/206	138/171	116/118/131	91/109	243/249/257	220/230/234	224/226/229	95/112	175	150/191	111/119
‡Karmijn de Sonnevil	204/206	160/171	118/131	91/98	233/257	218/230	229/255	106/112	175/200	150/155	117/151
‡King of Tomkins Co	179/182/206	138/171	116/118	98	233/255/257	230/234	212/226/229	106/112	175	150/152/155	115/117/151
Lemon	183/206	160/171/179	120/127/131	98/106	243/245/255	228/234	214/224/247	106/120	175/223	150/183	119
‡Mutsu	170/179/182	138/171/177	116/118	91/98/109	243/245/257	220/230/234	212/224/226	95/112/133	175	150/191	111/117
†*Paragon	179/183	138/171/179	116/120	98	233/243	216/234/238	212/218/255	106/120	175/204	149/155	115/117
‡*Red Gravenstein	182/183	138/171	114/116/131	91/98	255/257	216/218/224	226/255	97/106/108	198/209	150	117/151
‡Reinette Du Canada	179/182	138/160	112/116/131	91/98	233/245/255	208/216/230	212/229/255	106110/112	175/204	150/152	119
‡Rhode Island Green	173/182/204	160/162/179	114/118/131	91/109	255/257	230/235/238	229/255	97/110/112	202/204/224	150/183	107/117
†*Stark Scarlet Stay	179/183	138/171	116/120	98	233/243/255	231/234/238	212/218	106/120	175/202	149/150/155	111/115
Tekane	183	171/177/179	116/118	91/98/109	233/243/245	220/230/234	212	106/117/133	175/202	149/155	115/117
*X6688 K1 R87 A18	179/206	160/171	113/116	98/109	233/239/243	218/228/234	226/229/255	97/112	175/202	150/155	115/119
Other *Malus*											
M. coronaria	167/186	171/177	116/129/131	85/91	234/236/242	205/224/231	226	96/106/118	202	140/155/173	109/119
M. platycarpa	178/186/189	171/174	100/112/116	85/89/98	243/245/255	209/215/216	227	95/104/126	202	150/155/168	109/117/124

*Accessions showing third alleles for three or fewer markers used, ‡triploids already known, †triploids reported in literature.

4.3.5. Trueness to type investigation

4.3.5.1. Class 1. A number of accessions could be compared with their clones, sports or duplicates and showed microsatellite patterns consistent with those of their 'primary' cultivars. Twenty four of 39 clones or sports of 'Delicious' (Table 4.8) were consistent with each other and can be considered true. Other clones or sports that could be confirmed are those of 'Belle de Boskoop' (4 of 4), 'Braeburn' (5 of 7), 'Cox' (2), 'Elstar' (3), 'Fuji' (6 of 8), 'Gala' (5), 'Golden Delicious' (16 of 22) (Table 4.9), 'Granny Smith' (8), 'Jester' (4), 'Jonagold' (11), 'Jonathan' (3), 'McIntosh' (2), 'Ohenumuri' (2), 'Rome Beauty' (3), 'Summerred' (3), 'Tsugaru' (4), 'Winesap' (2) and 'Winter Banana' (3) with the number of confirmed clones or sports in brackets. These accessions were designated 'T' in Appendix 4.1.

4.3.5.2. Class 2. Items with two or more representatives having matching microsatellite patterns but which could not be validated were supposedly the same clone and in all likelihood be replicate accessions. For the following *M. pumila* cultivars, the names could not be confirmed as true: 'Aport' (2), 20/1 (2), 28/1 (3), 28/2 (3), 'Anna' (3), 'Climax' (2), 'Jonafree' (4), 'Ginger Gold' (2), 'Edgewood' (2), 'Le Vant' (2), 'Liberty' (2), 'Lord Lambourne' (2), 'M1' (2), 'M4' (2), 'M7' (3), 'M793' (2), 'M9' (2), 'M13' (3), 'Melrose' (2), 'Meran' (2), 'MM109' (2), 'Mollies Delicious' (2), 'Onderstem 5' (2), 'P18' (2), Pi-Au 9-27 (2), 'Pink Lady' (3), 'Trajan' (2), 'Red Astrakhan' (3), 'Reinette du Canada' (2), 'Rewena' (2), 'Russian Seedling' (2), 'Sharpe's Early' (2), 'Shlomit' (2), 'Sundowner' (2), 'Splendour' (2), 'Takane' (2), 'Telamon' (2), 'Vista Bella' (2) and 'Zabaoni' (3).

Representatives of other *Malus* species also having matching microsatellite patterns were *M.* 'Golden Hornet' (3), *M.* 'Grandiflora Crab' (2), Kaz-95-57 (3), Kaz-95-58 (2), Kaz-95-71 (2), Kaz-95-89 (2), Kaz-95-91 (3), KSC3 (2), KSC11 (2), *M.* 'Lemonei' (2), 'Malus 44' (2), *M. floribunda* (3), *M. platycarpa* (2), *M. purpurea* (2), *M. robusta* accession I14439 (2), *M. spectabilis* (Aiton) Borkh. (2), and *M.* Maypole (2).

4.3.5.3. Class 3. Accessions in this class when compared with their 'primary' accessions or duplicates were found to have inconsistent microsatellite patterns. One or several accessions of 'Braeburn' (2), 'Climax', 'Delicious' (15) (Table 4.10), 'Elstar', 'Fuji' (2), 'Gala' (3), 'Golden Delicious' (6) (Table 4.11), 'Jonagold' (3), 'Jonathan', 'M793', 'McIntosh' (2),

'Onderstam 5', 'P 18', 'Rome Beauty', 'Sundowner' and 'Tsugaru' had inconsistent microsatellite patterns. An accession of *Malus*, Kaz-95-71, in Appendix 4.1, had a microsatellite pattern inconsistent with the other two clones of Kaz-95-1. These accessions were designated 'F' in Appendix 4.1.

Table 4.8. Microsatellite patterns of 24 'Delicious' clones and sports showing identical or near identical patterns, indicating these accessions are indeed forms of 'Delicious'. 'Early Red' had an additional allele for marker CH04c07 that was not present in other accessions.

Name	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
M. pumila											
2X Red Delicious A	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Big Chief	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Classic	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Dietrich	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Early Red	179/183	138/179	116	91/98	245/255	208/234	212/218	108/117/133	175/204	149/155	115/117
Full Red	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Hardy Spur	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Jacored	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Oregon Red Spur 2	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Prime Red Delicious	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Red Delicious	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Redwine	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Richared	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Ryan Red	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Ryan Spur	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Shotwell Delicious	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Starking	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Starking Colourless	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Starking Early	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Starking Red	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Starking Red Groend	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Starking Stripeless	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Starking (USA)	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
Viljoen's Red	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117

Table 4.9. Microsatellite patterns of 16 'Golden Delicious' clones and sports in the ARC gene bank showing identical patterns, confirming their identity as variants of 'Golden Delicious'.

Name	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
M. pumila											
Compactagold	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Golden Delicious	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Golden Delicious A	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Golden Delicious B	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Golden Delicious Claz	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Golden Delicious Reinde	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Golden Delicious U.S.	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Goldspur	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Goldspur Applewaite	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Heinderich Golden	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Panarama Golden A	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Panarama Golden B	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Smoothee	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Spur Golden	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Stark Spur Gold Del	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Yellow Delicious	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117

Table 4.10. Microsatellite patterns of 15 supposed clones or sports of 'Delicious' showing patterns different from the correct 'Delicious' pattern and therefore indicating that these individuals are not true to type.

Name	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
M. pumila											
†Red Delicious	179/183	138/179	116	91/98	245/255	208/234	212/218	117/133	175/204	149/155	115/117
2X Red Delicious B	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
Dietrich Starking	183	160/179	112/129	91/98	255/257	218/234	229/257	95/106	175/202	150	107/151
Hi Delicious Early	179	138	116/118	91/109	243/255	220/234	218/226	95/117	175/204	150/155	117
Groth Red	183	160/179	112/129	91/98	255/257	218/234	229/257	95/106	175/202	150	107/151
Lalla Delicious	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Oregon Red Spur	183/191	138/171	116/120	91/98	243/255	234/238	212/255	120/133	175/217	150/155	115/119
Red Delicious Tasmania	183/191	138/171	116/120	91/98	243/255	234/238	212/255	120/133	175/217	150/155	115/119
Ryan Red	189/206	171/177	112	98/103	243/257	218/234	218/224	108	175	150	115/117
Starkrimson	179	138	116	91/109	255/257	208/220	218/224	112/117	175	150/155	111/117
Starkimson	170/179	171/179	118/131	91	233/257	234	226/255	112/133	175	150/191	117
Starking (RSA)	170/183	171/179	116	91/98	255/257	220/234	212/224	112/133	175/204	149/191	111/115
Super Chief Red Del	179	138	116	91/109	255/257	208/220	218/224	112/117	175	150/155	111/117
Top Red	170/183	171/179	116	91/98	255/257	220/234	212/224	112/133	175/204	149/191	111/115
Ultra Red	182/220	138/171	116/131	91/98	233/255	228/231	226/255	95	175/221	150	115/117
Wellspur Delicious	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117

†Correct 'Delicious' pattern.

Table 4.11. Microsatellite patterns of eight supposed clones or sports of 'Golden Delicious' showing patterns different from the correct 'Golden Delicious' pattern and therefore indicating that these individuals are not true to type.

Name	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
M. pumila											
†Golden Delicious	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Belgolden	170/183	138/179	116	98/109	243/245	220/234	212/224	95/133	175	150/155	111/115
Elbee	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Golden Delicious Early	170/183	138/171	116/131	91/109	233/243	234/238	212/224	95/112	175	150	111/117
Golden Delicious France	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Golden Delicious Hawaii	170/179	138/179	116/118	91	255/257	208/234	218/224	95/133	175/204	149/150	115/117
Golden Sheen	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Goldspur Aswell	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Lysgolden =Goldenir	170/183	138/179	116	98/109	243/245	220/234	212/224	95/133	175	150/155	111/115

†Correct 'Golden Delicious' pattern.

4.3.5.4. Class 4. Many items with single entries in the collection could not be compared with other identical accessions (Table 4.12).

Table 4.12. Accessions with a single entry in the ARC apple collection, which could not be compared with any other accessions.

Class 4 accessions arranged alphabetically		
M. pumila	Earligold	Longford
2B-12-25	Eikhoff	M25
4A-75-28 (Rooi Granny)	Elise	M26
8A-1-Ouer	Elsie Grant	MH 15-16
African Carmine	Empire	Maayan
Akane	Russel Red	Macobin
Alfmission	Florentina	Maiden's Blush
Alkmene	Forum	Maigold
Alsop's Beauty	Gavin	McIntosh Early
Antonovka Seedling No6	Gloire de Hollande	Meldale
Arapkizi	Gloster	Michal
Atties Favourite	Goldrush	Michinoku
Baujade	Goldsmith	Milton
Belrene	Goosen	Missouri Pippin
Beni Osho	Granearli	MM111
Beverly Hills	Granny Smith Red	Monsa
Bittenfelder	Red Gravenstein	Morkel's Seedling
Blairmont	Greensleeves	Mother
Blenheim Orange	Harberts Reinette	Mutsu
Calville de Saint Souve	HL 1004	Nebuta
Canvade	HL 166C	New Gold
CC2/9	HL 237	New Year
Champion	HL 318	Nickajack
Chantecler	HL938	No1 Dresden (Seedling 4)
Charden	Hofer Seedling	No2 Dresden (Seedling 2)
Coast	Hokuto	No3 Dresden (Seedling 1)
Commerce	Hops Late Red	Northern Spy
Co-op 9	I5526 X 6407 INRA	Hunter Ontario
Co-op 20	Idared	Jumbo Orin
Coromandel Red	Julia	Orleans Reinette
Crab A	July Red	Ozark Gold
Crab C	Karmijn de Sonnaville	P 1
Criterion	King of Tomkins County	Palmiet Red
Dakota	Klara	Panorama Crab
Dayton (Co-op 21)	Kogetso	Paragon
Dayton Seedling No6	Koo	Pilot
Delblush	Lady Williams	Pinova
Delkistar	Lakeside	Pomme de Niege
Democrat	Langkloof	Porporate
Diva Gold	Laxton's Superb	Present of England
Drakenstein	Lemon	Prima
Dukat	Leyda	Primgold
E3 F2	London Pippin	Prince Bismarck

Table 4.12. Continued…

Class 4 accessions arranged alphabetically		
Princesa	Sweet Cornelly	Zoba
Priscilla	T 506	Zvonkove
Red Dutch	Tasman's Pride	**Other *Malus* species**
Redfree	Tjeek	Kaz-95-44
Red Gem	Tuscan	*M.* Aldenhamensis
Redwine	Valmore	*M. baccata*
Redwinter	Veitchi Pumila	*M.* Butterball
Remo	Versveld	*M. coronaria*
Rhode Island Greening	Viljoen's Red	*M. fusca*
Rokewood	Wainwright	*M.* Jackson's Crab
SAPB 919	Wemmershoek	*M.* L.P. Mornel Crab
Shampion	White Winter Pearmain	*M. micromalus*
Spartan	Widup	*M.* Mildew Resistant
Statesman (Red)	William's Pride	*M.* Moeransi Profusion
Stayman (Black)	Wolf River	*M.* Pioneer Scarlet
Stayman Winesap	X2765	*M.* Veitch's Scarlet
Stark Scarlet Stayman	X6163 P22 R19 A14	No4 Dresden (Seedling 3)
Sunrise	X640 TNR42A45	S202
Suntan	X6688 K1 R87 A18	T 585

4.3.5.5. Class 5. Several items had two representatives with different microsatellite patterns. The *M. pumila* accessions were 'Austin', 'Boiken', 'Heidegger Golden A', 'Heidegger Golden B', 'Himekami X', 'Himekami Y', 'Howell', 'Jesrsey Mac', 'Kirks X', 'Kirks Y', 'Melba', 'Paula Red', 'Reglindis', 'Resista', 'Sharpe's Early', 'Swartland' and 'Twenty Ounce'. A few *Malus* accessions, Kaz-95-122, Kaz-95-78, 'Spy 227' and 'No5 Dresden' (Seedling ?) also displayed different microsatellite patterns between two representatives.

4.3.6. Parentage analysis

Fifteen accessions of known parentage were investigated for trueness to parentage, 'African Carmine', 'Dukat', 'Gala', 'Himekami', 'Karmijn De Sonnaville', 'Kidd's Orange', 'Kogetso', 'Melrose', 'MM106', 'MM111', 'Pink Lady', 'Shampion', 'Sundowner', 'Trajan' and 'Tuscan' (ASHS, 1997; Morgan and Richards 2002) (Table 4.13). For four of the 15 accessions, 'Dukat', 'MM106', 'MM111' and 'Tuscan', the microsatellite patterns were not consistent with those of their supposed parents and were therefore deemed false (designated 'F'). For the other accessions trueness to type were confirmed, and these were considered as verified (designated 'V').

Table 4.13. Parentage analysis of 15 apple accessions of known parentage (ASHS, 1997; Morgan and Richards 2002). Colour codes represent alleles matching between an accession and its supposed parents. Eleven of the 15 accessions were true to reported parentage and were coded 'V'; those not true to parentage were coded 'F'.

Name	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
True to parentage											
Golden Delicious♀	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Dietrich Starking♂	179/183	138/179	116	91/98	245/255	208/234	212/218	118/133	175/204	149/155	117
African Carmine (V)	170/179	138	116/118	91	243/255	208/234	212/226	112/118	175/204	155/191	117
Red Delicious♀	179/183	138/179	116	91/98	245/255	208/234	212/218	118/133	175/204	149/155	117
Cox's Orange Pippin♂	204/206	160	118/131	98	233/257	218	255	106/112	175/200	150/155	117/151
Kidd's Orange (V)	179/204	160/179	116/131	91/98	233/255	218/234	212/255	106/133	175/204	149/150	115/117
Cox's Orange Pippin♀	204/206	160	118/131	98	233/257	218	255	106/112	175/200	150/155	117/151
Jonathan♂	206	171	114/131	91/98	249/257	230/235	229/255	110/112	175/202	150	117/151
Karmijn de Sonnaville (V)	204/206	160/171	118/131	91/98	233/257	218/230	229/255	106/112	175/200	150/155	117/151
Jonathan♀	206	171	114/131	91/98	249/257	230/235	229/255	110/112	175/202	150	117/151
Fuji A♂	183	171/179	116	91/98	233/234	230/234	212	106/117	175/202	149/155	115/117
Himekami A (V)	183/206	171	116/131	91	233/257	230/235	212/255	112/117	175/202	150/155	117/151
Jonathan♀	206	171	114/131	91/98	249/257	230/235	229/255	110/112	175/202	150	117/151
Golden Delicious♂	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Kogetso (V)	179/206	138/171	116/131	91	243/249	230/234	226/229	95/112	175/202	150/191	111/151
Red Delicious♀	179/183	138/179	116	91/98	245/255	208/234	212/218	118/133	175/204	149/155	117
Jonathan♂	206	171	114/131	91/98	249/257	230/235	229/255	110/112	175/202	150	117/151
Melrose (V)	183/206	171/179	114/116	91/98	255/257	230/234	212/229	110/133	175	149/150	117/151
Lady Williams♀	183/206	171/179	112/120	98/118	233/243	208/228	212	106	175/213	140/150	117/119
Golden Delicious♂	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Pink Lady (V)	179/206	138/171	112/118	91/98	233/257	208/234	212/224	95/106	175	150/191	117
Lady Williams♀	183/206	171/179	112/120	98/118	233/243	208/228	212	106	175/213	140/150	117/119
Golden Delicious♂	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Sundowner (V)	170/206	138/171	112/118	91/98	243/257	228/234	212/224	106/112	175	150	111/117

Table 4.13. Continued…..

Name	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
Kidd's Orange♀	179/204	160/179	116/131	91/98	233/255	218/234	212/255	106/133	175/204	149/150	115/117
Golden Delicious♂	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Gala (V)	170/204	171/179	118/131	91/109	233/243	218/234	226/255	95/133	175	149/191	115/117
Lord Lambourne♀	206	138/160	112/120	98	233/245	228/238	251/255	108	175/202	150	151
Golden Delicious♂	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Shampion (V)	170/206	160/171	118/120	91/98	233/243	220/238	226/255	108/112	175/202	150/191	111/151
Golden Delicious♀	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
M McIntosh♂	173/204	160/171	114/116	91/98	233/257	228/230	212/229	106	202/209	150/183	111/119
Trajan (=Polka) (V)	179/204	160/171	116/118	98/109	243/257	220/230	226/229	95/106	175/209	150/191	111/117
Not true to parentage											
Greensleeves♀	170//206	160/171	118	91/98	233/243	220/238	226/229	95/108	175/200	150/191	117/120
M McIntosh♂	173/204	160/171	114/116	91/98	233/257	228/230	212/229	106	202/209	150/183	111/119
Tuscan (=Bolero) (F)	179/204	160/171	114/131	98/104	233/255	208/220	212/255	106	209/221	150/152	117
Cox's Orange Pippin♀	204/206	160	118/131	98	233/257	218	255	106/112	175/200	150/155	117/151
Golden Delicious♂	170/179	138/171	116/118	91/109	243/257	220/234	224/226	95/112	175	150/191	111/117
Dukat (F)	189/206	160/179	118/120	98/103	233/257	218/228	218/255	106	175/200	150/155	115/151
Northern Spy♀	183	171/179	104/131	91/98	233/245	208	212/249	106/110	175/209	150	117
M793♂	173/182	138/171	131	98	245/255	208/231	212/229	110/114/129	175	150	117
MM111 (F)	182/187	171/179	104/118	91/104	233/245	208	212	106/110	175/221	150	115/117
Northern Spy♀	183	171/179	104/131	91/98	233/245	208	212/249	106/110	175/209	150	117
M1♂	173/204	138/160	129/131	98	255	231/238	212/229	114/120/129	175	149/150	111/117
MM106 (F)	170/179	160/162	112/114	98/104	245	216/231	214/226	106	175/198	161	117/119

4.3.7 Resolving misidentifications

In addition to the data classification described above, further sorting of genotypes in Microsoft Excel and the UPGMA clustering method was employed because different microsatellite patterns were observed for some duplicate clones. The two approaches were very useful for identifying false accessions and unknown clones or sports of several 'primary' cultivars. Similar clustering was observed when using either method. A group of 37 different accessions (Table 4.14) having identical or near identical microsatellite patterns with 'Onderstam 5' was observed when sorting with Excel and clustering with UPGMA (Fig. 4.2). Twenty eight matched for all markers and nine of the 37 accessions matched for 11 markers used except for Hi02c07. Accessions 'Kirks A', 'M793' and *M. fusca* (Raf.) C.K.Schneid differed from the group only for markers CH04c07, CH01h01 and CH02c09, respectively. Unexpectedly, accessions 'Treco Red' and 'Yataka' that were thought to be different grouped with 'Gala' and 'Fuji', respectively.

Table 4.14. Near identical microsatellite pattern for 37 different apple accessions. It is concluded that these 'accessions' may be 'Onderstam 5' rootstock.

Name	CH01f02	CH01f03b	CH01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
M. pumila											
2X Red Delicious	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
8A-1-Ouer	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
Austin	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
Climax	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
E3 F2	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
Eikhoff	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Elbee	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Empire	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Golden Delicious France	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Golden Sheen	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Goldspur Aswell	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Howell?	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Idared	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Jonnee	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Julia	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Kashawi	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Kirks A	183/187	171/179	118/131	98/104	233	208/216	212/249	106/114	209/221	150	115/117
Lalla Delicious	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
M793	183/187	171/179	118/133	98/104	233	208/216	212/249	106	209/221	150	115/117
Macobin	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Melba	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
Onderstam 5	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Onderstam 5 A	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
P18	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Paulared	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
Present of England	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Sharpe's Early	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117

Table 4.14. Continued……..

Name	CH01f02	CH01f03b	C/H01h01	CH01h10	CH02c09	CH02c11	CH02d08	CH04c07	CH04e05	GD12	Hi02c07
Sinclair	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
SPAB 919	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Statesman (Red)	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Stayman (Black)	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Sundowner A	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	117
Suntan	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Twenty Ounce	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Tydeman's Early	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Wellspur Delicious	183/187	171/179	118/131	98/104	233	208/216	212/249	106	209/221	150	115/117
Other *Malus* species											
Malus fusca	183/187	171/179	118/131	98/104	222/233	208/216	212/249	106	209/221	150	115/117

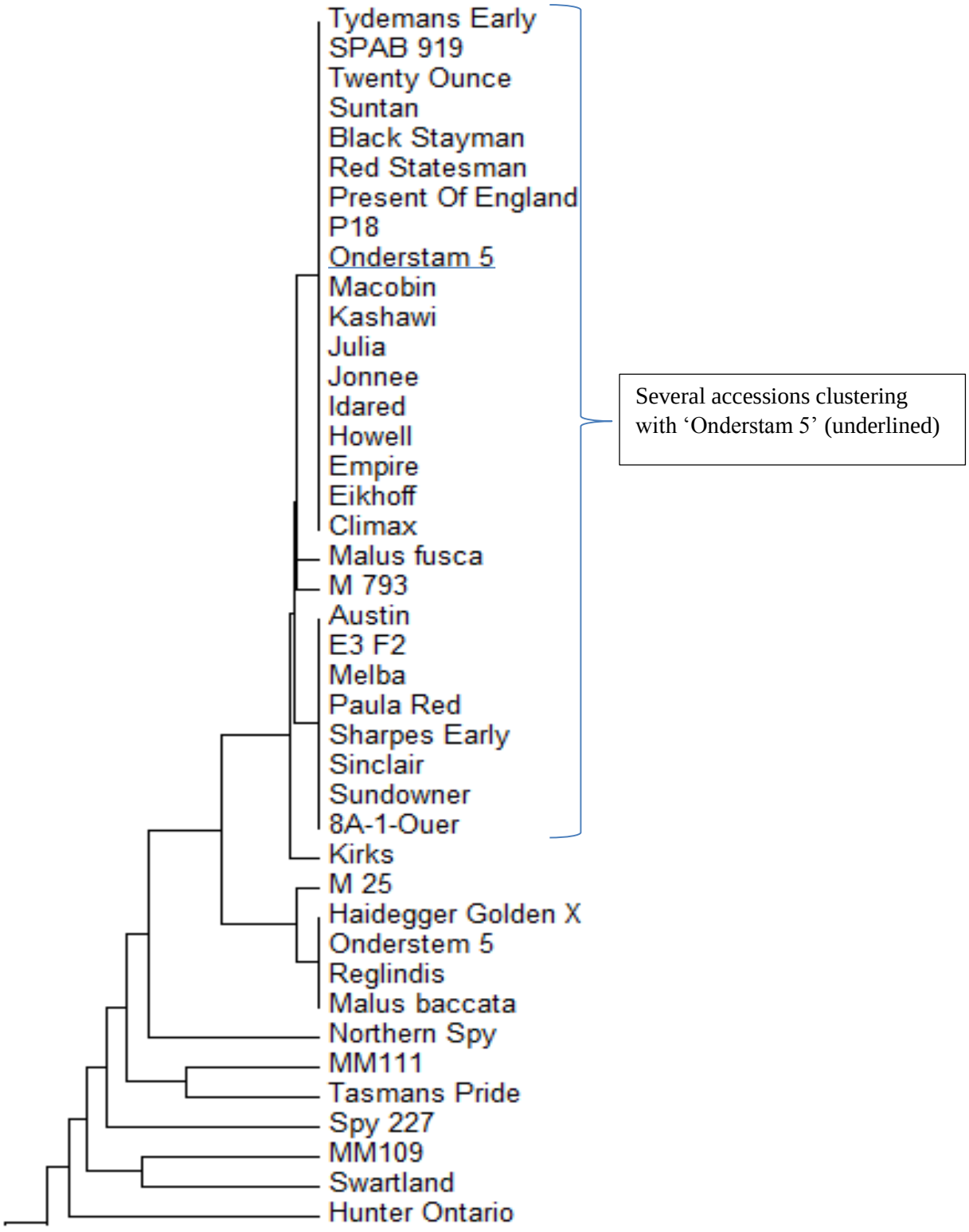

Fig. 4.2. A dendrogram of ARC accessions constructed using UPGMA, having identical or near identical microsatellite patterns with 'Onderstam 5' suggesting the rootstock may have grown in place of the scion cultivar. This is an excerpt from the larger dendrogram (Appendix 4.3).

4.4. Discussion

The microsatellite markers, and methods employed to compare genotypes such as sorting entries with Microsoft Excel and UPGMA, proved very useful in this study.

4.4.1. Marker performance

One marker, GD147, out of the 12 markers used, gave poor amplification with a confusing non-allelic product, 142 bp, which hindered scoring and was therefore excluded from further analysis. However, there are no reports of marker GD147 failing in previous studies. The unsatisfactory performance of this specific marker in the current study may perhaps be attributed to manufacturing errors. Two other markers, GD12 and CH04c07, showed the possibility of misscoring due to null alleles as observed from MICRO-CHECKER results but were still included in diversity statistical analysis with CERVUS and GENALEX along with the other nine markers. Urrestarazu *et al*. (2012) also detected evidence of misscoring due to null alleles for two markers, CH01h01 and Hi02c07, and Pina *et al*. (2014) likewise for marker CH01f02. These three markers showed no evidence of misscoring due to null alleles in the current study.

The deviation from the HWE observed for ten of the 11 markers, all from different linkage groups could perhaps be due to null alleles but was more likely associated with the inherent characteristics of the accessions in the gene bank. The gene bank represents a collection of accessions rather than a natural population, and as such HWE deviations are not unexpected.

Marker CH02c11 was the most polymorphic, whereas GD12 and CH01h10 were the least polymorphic for the analyses in this study. Potts *et al*. (2011) also found CH01h10 to be the least polymorphic; however, Potts *et al*. (2011) included representatives of several other *Malus* species. Noting levels of polymorphism are important for example in cases where all markers cannot be used, the least polymorphic markers could be omitted.

4.4.2. Scoring

Deviations in base pairs for expected allele sizes are common in microsatellite analysis when markers are used across different laboratories, due to use of different sequencing equipment and chemicals (Sutton *et al*., 2011; Sehic *et al*., 2013). The extent of these deviations also tends to differ amongst loci. Deviations detected in this study differ from those reported by Xuan *et*

al. (2010) and Reim *et al*. (2013) using the accessions of the recommended cultivars. The variation amongst laboratories emphasises the importance of including reference cultivars and laboratory specific calibration.

4.4.3. Diversity statistics

Diversity statistics were calculated for all 11 markers including those with evidence of null alleles. The number of alleles per marker ranged from 12 to 22 when only *M. pumila* accessions were analysed. Xuan *et al*. (2010) used the same set of markers for a collection of 95 accessions of *M. pumila* and detected between 11 alleles per marker, for CH01f03b, and 19 alleles per marker, for CH01f02 and CH02d08. A similar range was observed by Urrestarazu *et al*. (2012) for a collection of 495 accessions, where between 12 alleles per marker, for CH01h02 and GD12, and 30 alleles per marker, for CH04f10, were observed; the additional alleles in that study are most probably associated with the larger population size. A greater range of allele sizes, from 14 to 25 per marker was observed in the analysis of the complete set of *Malus* accessions as some of the other *Malus* had additional alleles that were absent from the set of *M. pumila* accessions.

The observed heterozygosity in the current study ranged from 0.68 to 0.92 per marker, which is in agreement with the observed heterozygosity of 0.71 to 0.92 per marker reported by Xuan *et al*. (2010) with the same set of markers. Average observed and expected heterozygosities of 0.82 and 0.81 were realised in the current study. Similar values, 0.78 and 0.82, were reported by Pina *et al*. (2014) when analysing 183 accessions comprising mostly *M. pumila* cultivars. Pereira-Lorenzo *et al*. (2007), Urrestarazu *et al*. (2012) and Garkava-Gustavsson *et al*. (2013) also reported average expected heterozygosities of 0.80, 0.82 and 0.77, respectively, when gene bank collections were analysed. The expected heterozygosity in the current study and those of aforementioned studies are higher than the He reported in the studies of Guarino *et al*. (2006), Garkava-Gustavsson *et al*. (2008), Gharghani *et al*. (2009) and Gasi *et al*. (2010) that were dominated by indigenous or ancient cultivars from particular regions. The higher heterozygosity observed in studies dominated by *M. pumila* may be due to the use of informative markers but most likely results from cross-hybridisation and selection in breeding programmes, which introduces heterozygosity into the collection (Lamboy and Alpha, 1998).

4.4.4. Trueness to type

The existence of multiple clones or sports of popular 'primary' cultivars enabled confirmation of the trueness to type for some clones as well as the detection of false clones. Microsatellites are not appropriate markers for differentiating between clones or sports of a particular 'primary' cultivar as the clones or sports should be identical except for occasional somatic mutations. This limitation was useful in this study as it enabled the consistency among clones or sports derived from popular cultivars to be examined. The minor variation observed in some clones or sports may have arisen from mutation as a result of slippage during mitosis (Guichoux *et al*., 2011). Accessions that could not be confirmed as true or false but that are present in the Brogdale collection and were genotyped with the same markers by Fernández-Fernández (2010) will be compared when the EMR data become available. Such accessions are coded (B) in Appendix 4.1. A number of misidentifications were observed in the current study but such findings are common even in well characterised gene banks curated to international standards *e.g*. Brogdale, UK, Centre for Genetic Resources (CGN), Netherlands, and Washington State University, USA (Sehic *et al*., 2013).

The trueness to type investigation has already been useful as a guide for genetic studies in progress at ARC. Subsequently, a research project had to be revised as some of the parents that were included were proved to be false by the current study (Mbulawa, personal communication).

4.4.5. Ploidy

Five of the 19 potential triploids of *M. pumila*, 'Belle de Boskoop', 'Blenheim Orange', 'Charden', 'Jonagold' and 'Mutsu', detected in the current study had been noted as triploids on the gene bank list. Additionally, 'Baujade', 'King of Tomkins County', 'Red Gravenstein', 'Reinette du Canada' and 'Rhode Island Greening', detected as triploids in this study were known to be triploids but had not been annotated as such. Dermen (1965) reported that 'Paragon' and 'Stark Scarlet Stayman' are triploids. The accessions of two *Malus* species, *M. coronaria* and *M. platycarpa,* appeared to be triploid and tetraploid, respectively.

Accessions that had third alleles in three or less markers, 'Adina' (syn Frankad), 'Paragon', 'Red Gravenstein', 'Stark Scarlet Stayman' and X6688 K1 R87 A18, were tested for pollen germination as poor germination is indicative of triploidy. 'Paragon', 'Red Gravenstein', 'Stark Scarlet Stayman' and X6688 K1 R87 A18 showed pollen germination between 1 and 5%

whereas the control, 'Pink Lady', showed 80 to 90% germination. These results confirm their triploid nature. However, 'Adina' (syn 'Frankad') showed germination between 60 and 70%, which indicates that it is diploid and that the additional third peaks, might be a combination of mutations and chimerism. If occasional microsatellite mutations occur via slippage in somatic tissue then different cell layers would have slightly different diploid genotypes. Leaf samples, to which all three layers contribute, could therefore show three alleles for those loci (Guichoux *et al*., 2011).

Accession 'Karmijn de Sonnaville' showed low germination, from 1 to 10%, indicative of triploidy as reported, even though it did not show any additional peaks. Absence of third peaks in triploids such as 'Karmijn de Sonnaville' is perhaps due to both parents having an allele in common. The triploid would therefore have three alleles *e.g*. 89/105/105, but only two peaks would be detected in the microsatellite analysis.

Six accessions, 'Alfmission', 'Forum', 'Harberts Reinette', 'Lemon', 'Tekane' and X6688 K1 R87 A18, were detected as potential triploids for the first time in this study.

As triploids produce aneuploid pollen and ovules of low fertility (Dermen, 1965), they are of limited or no use as parents in a breeding programme. Annotating the gene bank list with this information will accordingly increase the efficiency of the crossing programme.

4.4.6. DNA profile sorting

Sorting of accessions according to microsatellite profiles using Microsoft Excel proved very useful for detecting false accessions that clustered with other cultivar groups which they were not supposed to be identical. These are denoted 'N' in Appendix 4.2 for not true to type. However, sorting using microsatellite profiles in this manner has limitations. For example, for two entries with different names, to have matching profiles may be confirmation of identity but does not establish which name is correct, especially if their parentage is unknown. In the most noteworthy example of microsatellite sorting, 37 accessions grouped with an unknown rootstock, 'Onderstam 5'. It may be that this rootstock had grown in place of the scion cultivar during previous propagation. Accessions 'Treco Red' and 'Yataka' clustered with 'Gala' and 'Fuji', respectively, and it was confirmed from literature (ASHS, 1997) that these are clones or sports of 'Gala' and 'Fuji.

Clustering of all *Malus* accessions using UPGMA method confirmed the results observed with Excel sorting. Hence, either of the two methods can be useful for DNA profile sorting and identifying false accessions.

4.4.7. Parentage

Testing of parentage confirm the identity of various accessions, particularly for those accessions that had a single entry, such as 'African Carmine', 'Dukat', 'Karmijn de Sonnaville', 'Kidd's Orange', 'Kogetso', 'MM106', 'MM111', 'Shampion' and 'Tuscan'. Accession 'Himekami' with two suspect entries 'Himekami A' and 'Himekami B' was also tested. Additionally, accessions 'Gala' (and five other variants of it), 'Sundowner' and 'Trajan' (syn 'Polka') had patterns consistent with the reported parents. Accessions that matched the reported parentage were regarded as true to type and denoted 'V'. Accessions 'Dukat', 'Himekami B', 'MM106', 'MM111' and 'Tuscan' had a pattern inconsistent with the reported parentage, suggesting they are false and were denoted 'F'.

4.4.8. DUS testing

The awarding of Plant Breeder's Rights (PBRs) for new apple varieties in South Africa relies on Distinctiveness, Uniformity and Stability (DUS) tests conducted by the Department of Agriculture, Forestry and Fisheries (DAFF). These tests require true to type control cultivars for morphological comparisons. Interestingly, the apple gene bank, E1, at the Grabouw Experimental Farm, in which various trees are misidentified, is currently used as a source of 'true to type' reference cultivars for DUS testing (Tobutt, personal communication). Trueness to type of the gene bank material is therefore not only important to the ARC breeders, but also to the DAFF office.

4.4.9. Recommendations to ARC gene bank management

A list of the false accessions detected in this study will be passed to the breeder. The trees will be cut down and, where possible, replaced with true to type accessions from reliable sources, *e.g.* the South African Plant Improvement Organisation (SAPO) Trust, and confirmed with molecular markers in comparison with reference fingerprints. The microsatellite profiles will be incorporated into the gene bank database for each accession confirmed as true and will be used as reference data for future comparison. Additionally, newly identified triploids are being annotated as such in the gene bank list to avoid their future utilisation in crossing.

4.5. Concluding remarks

In the current study, the set of microsatellite markers recommended by ECPGR proved informative for true to type investigation of the ARC apple collections. Sorting of accessions with Microsoft Excel and UPGMA clustering proved convenient for organising data to match fingerprints and to detect false accessions. Of 540 apple accessions, 69 *M. pumila*, two *M. pumila* selections and seven other *Malus* representatives were found not to be true to type. Many of the errors might never have been detected morphologically, to the detriment of the apple breeding programme and underpinning genetic studies. East Malling Research has confirmed it will supply the Brogdale data set (Fernández-Fernández, personal communication) and then further comparisons can be made for the remaining 213 accessions. Discarding the misnamed accessions will allow new additions to the gene bank without increasing the cost of managing the accessions. Six new triploids were detected for the first time in the current study. The accession records will be updated with this characterisation data which will bring the South African collections in line with good international practice. Baseline data were generated during the current study which will be invaluable when the collection is repropagated.

4.6. References

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Chapter 5

MOLECULAR CHARACTERISATION OF ARC APPLE ACCESSIONS WITH RESPECT TO THE *ACS1* GENE

5.1. Introduction

The domestic apple (*Malus pumila* Mill.) belongs to the sub-tribe Pyrinae of the rosaceous sub-family Spiraeoideae, together with pear (*Pyrus communis* L.) and other pome fruits. It is an important deciduous fruit crop in South Africa, second to grape in terms of producing hectares; approximately 22 000 hectares are grown, producing approximately 800 000 tonnes of which 40% is exported internationally, mostly to the northern hemisphere markets such as Europe (HORTGRO, 2012; PPECB, 2013). The Agricultural Research Council's Institute Infruitec-Nietvoorbij is breeding new cultivars for South African growers and an important objective is the good storage potential necessary for supplying distant markets (Tobutt and Bester, 2011). Other southern hemisphere countries face a similar challenge of breeding for cultivars with prolonged storability (Gardiner *et al*., 2007).

Apples are climacteric in nature; their ripening is associated with increased ethylene production and cellular respiration which continues even after harvest (Gorny and Kader, 1997; Alexander and Grierson, 2002) and their storability is greatly influenced by their rate of ethylene production (Bassil and Lewers, 2009). The enzyme ACS is an important component of the biochemical pathway of ethylene synthesis (Yang and Hoffman, 1984), and is responsible for the conversion of S-adenosyl-L-methionine (SAM) to 1-Aminocyclopropane-1-carboxylic acid (ACC). This is generally accepted as the rate limiting factor in the ethylene production pathway and manipulating this can influence the amount of ethylene produced which in turn affects the rate of ripening (Lau *et al*., 1986).

Fruit export with ships takes several weeks to reach international markets. During this time the fruit continues to produce ethylene, and if not stored correctly this will result in accelerated ripening and the arrival of over mature, soft, fruit. Several pre- and post-harvest methods are utilised to inhibit ethylene production during storage to prolong the shelf life of apple fruits. Chemicals known to inhibit ethylene production pre-harvest, Naphthaleneacetic (NAA) and Aminoethoxyvinylglycine (AVG), and post-harvest, 1-methylcyclopropane (1-MCP), contribute to prolonging fruit storage ability when used correctly (Fan *et al*., 1999; Schupp and Greene, 2004; Yuan and Carbaugh, 2007). However, these present additional costs to the producer and incorrect application may lead to rejection of shipments at foreign markets that tend to be cautious of chemical residues (Johnson *et al*., 2002; Defilippi *et al*., 2005). The breeding of low ethylene producing cultivars can counter this problem by slowing down the ripening process and avoid post-harvest fruit losses making it an important objective in the

ARC breeding programme. Likewise, other breeding programmes such as the Washington State University are already breeding for low ethylene producing cultivars (Peace, 2014).

Allelic variation at the *ACS1* locus with respect to the insertion of a SINE (short interspersed element) in the promoter region, 162 bp in length, as well as a concomitant deletion of 25 bp, correlates to some extent with apple fruit storage potential. Accessions that are homozygous for the indel tend to show delayed ethylene production (Sunako *et al*., 1999) and enhanced storability. Primers have been designed to distinguish the two alleles, denoted as *1* and *2*, to genotype seedlings as early as one week from germination (Sunako *et al*., 1999; Kumar *et al*., 2012). Amplification products, reportedly of 489 bp and 655 bp, respectively, can be distinguished when separated with agarose gel electrophoresis. However, the 166 bp difference between the two alleles is inconsistent with the events described in Sunako *et al*. (1999), which explain a difference of only 138 bp, indicating the possible mis-scoring of the actual allele sizes with agarose gel electrophoresis. Using the primers designed by Sunako *et al.* (1999), the genotypes of 262 accessions of domestic apple and 18 accessions of other *Malus* species and hybrids have been reported in literature (Table 1.1) (Sunako *et al*., 1999; Harada *et al*., 2000; Oraguzie *et al*., 2004; Oraguzie *et al*., 2007; Zhu and Barritt, 2008; Zoufalá *et al*., 2009; Peace, 2014).

The ARC Infruitec-Nietvoorbij maintains the South African apple collection of domestic apple and related species in the Elgin Valley, Western Cape; these are primarily used as a gene bank for the breeding programme but are also regarded as a national asset (Bester *et al*., 2013). The collection contains 540 accessions which are comprised mostly of *M. pumila* accessions with some accessions of *M. sieversii* Ledeb. and representatives of other *Malus* species and hybrids. The developments in the molecular genetics of apple over the last twenty years provide an opportunity to enhance the collection, not only by fingerprinting accessions with microsatellite markers for verifying trueness to type (Chapter 3) but also genotyping accessions for various agronomically significant functional genes that have been sequenced and for which primers are available such as *ACS1* (Sunako *et al*., 1999). In the ARC collection, 225 items, 171 *M. pumila* cultivars, 20 *M. pumila* selections and 34 representatives of other *Malus*, have not been genotyped for *ACS1* previously.

The use of fluorescently labelled primers for distinguishing *ACS1* alleles via an automated sequencer has not been reported. Although fluorescent sizing is costly, it can reveal variation in product size not distinguishable with agarose gel electrophoresis and provide scope for

multiplexing with other markers. In the case of the *S* locus in *Prunus*, (which is multi-allelic) fluorescent primers have proved very informative (Vaughan *et al*., 2006; Sonneveld *et al*., 2006). For automated sequencing systems using the GS500(-250)LIZ ladder, the amplification products need to be smaller than 500 bp for accurate sizing, whereas the *ACS1-2* allelic product amplified with the Sunako *et al*. (1999) primers is 655 bp and is out of the appropriate size range. To be distinguished via fluorescent sizing it would be convenient if the amplified products were smaller than the products described in the Sunako *et al*. (1999) study.

In European pear, El-Sharkawy *et al*. (2003; 2004) described the *ACS1* phenomenon to explain differences in ripening. Two variants of *Pc-ACS1*, *a* and *b*, with a difference in size of 39 bp were proposed as an explanation for the differences in ripening but this could not be validated by Oraguzie *et al*. (2010). There have been no other studies to substantiate these findings.

The purpose of the current study was primarily to determine the genotypes of the ARC Infruitec-Nietvoorbij apple collection with respect to *ACS1* using a PCR approach; information which will enhance the characterisation of the accessions and provide information useful to the breeder designing crosses to improve storability. Primers designed for fluorescent sizing were used to detect possible minor variations in allele size and investigate possibilities for multiplexing. The utility of these markers were also tested in a set of pear accessions.

5.2. Materials and methods

5.2.1. Plant material

A total of 292 of the 540 ARC apple accessions growing in the collections at Drostersnes and Grabouw Experimental Farms, in the Elgin Valley that had previously been fingerprinted (Chapter 3), were selected for *ACS1* genotyping (Table 5.1). Two hundred and thirty five accessions of *M. pumila* cultivars, excluding clones and duplicates, 20 *M. pumila* selections and 37 *Malus* species and hybrids were included for analyses. Accessions shown to be false (Chapter 3) were omitted. Other studies had genotyped 67 accessions that were nominally the same as the items in the gene bank, 64 *M. pumila* cultivars and three other *Malus* species and hybrids (Sunako *et al*., 1999; Harada *et al*., 2000; Oraguzie *et al*., 2004; Oraguzie *et al*., 2007; Zhu and Barritt, 2008; Zoufalá *et al*., 2009 or Peace, 2014) and these scores (Table 1.1) were used for comparison. Although some of the accessions, 17 *M. pumila* cultivars, one selection

and two *Malus* species, were determined to be triploid (and therefore infertile) in the previous chapter, they were included in the current study for scientific interest.

Table 5.1. The 292 apple accessions in the ARC gene bank, previously fingerprinted using microsatellite markers and not identified as false, genotyped for *ACS1*. Just one item per cultivar group is included. Duplicate accessions were included in cases where two accessions of a kind gave different microsatellite fingerprints. Items for which genotypes had already been reported in the literature are coded L. Triploids are coded T. Duplicate accessions with different microsatellite fingerprints that could not be confirmed are labelled with the suffixes X, Y or Z.

Location	Name	Code	Location	Name	Code
***M. pumila* selections**			E1_12_12	Beaumont	
DN7_16_4	20/1		E1_14_3	Belle de Boskoop	T
DN7_30_1	28/1		E1_14_8	Belrene	
E1_3_13	28/2		E1_13_11	Beni Osho	
E1_2_12	2B-12-25		DN7_4_7	Beverly Hills	
DN7_17_9	4A-75-28		DN7_27_1	Bittenfelder	
19_11	CC2/19		E1_14_6	Blairmont	
DN7_32_1	HL 166C		E1_9_15	Blenheim Orange	T, L
E1_18_11	HL 237		DN7_31_3	Boiken X	
E1_18_10	HL 318		E1_13_21	Boiken Y	
E1_18_12	HL 938		E1_11_1	Braeburn	L
DN7_33_1	HL 1004		E1_17_18	Calville De Saint Souve	
DN7_26_1	i5526 X 6407 INRA		E1_7_19	Canvade	
E1_13_3	M H 15-6		E1_17_16	Champion	
DN7_17_7	Pi-Au 9-24		E1_18_14	Chantecler	
DN7_21_7	Pi-Au 9-27		E1_17_10	Charden	T
DN7_33_3	SA579-3		E1_14_2	Climax	
E1_19_8	X2765		E1_17_5	Coast	
E1_19_3	X6163 P22 R19 A14		E1_16_16	Commerce	
E1_9_10	X640 TNR42A45		E1_7_17	Coromandel Red	
E1_19_4	X6688 K1 R87 A18	T	DN7_5_3	Cox’s Orange Pippin	L
***M. pumila* cultivars**			E1_16_15	Co-op 19	
DN7_15_5	Adina		E1_7_12	Co-op 20	
E1_3_12	Adina (syn Frankad)		E1_16_21	Crab A	
E1_3_10	African Carmine		E1_9_4	Crab C	
E1_15_18	Akane	L	E1_7_14	Criterion	
E1_9_19	Alfmission	T	E1_16_18	Dakota	
E1_7_3	Alkmene	L	E1_13_15	Dayton (=Co-op 21)	
E1_14_11	Alsop's Beauty		DN7_24_1	Dayton Seedling No6	
DN7_18_3	Anna	L	E1_9_14	Delblush	L
DN7_24_9	Antonovka Seedling No6	L	E1_14_4	Red Delicious	L
DN7_16_1	Aport		DN7_18_4	Delkistar	
E1_12_4	Arapkizi		E1_18_8	Democrat	L
17_11	Atties Favourite		E1_12_13	Diva Gold	
DN7_31_2	Austin		E1_4_4	Drakenstein	
E1_13_10	Baujade	T	E1_16_17	Dunn’s Seedling	

Location	Name	Code	Location	Name	Code
E1_9_12	Earligold		E1_10_6	Lemon	T
DN7_22_2	Edgewood		DN7_19_2	Le Vant	
E1_15_11	Eikhoff		E1_10_19	Leyda	
E1_4_13	Elise		DN7_5_2	Liberty	L
E1_8_19	Elsie Grant		E1_12_9	London Pippin	
DN7_4_5	Elstar Red	L	E1_11_8	Longford	
E1_7_13	Florentina		E1_13_6	Lord Lambourne	L
E1_19_5	Forum	T	DN7_20_10	M1	
E1_9_9	Fuji (Akufi)	L	DN7_20_11	M4	
E1_9_2	Gala	L	DN7_21_11	M7	
E1_4_11	Gavin		DN7_2_11	M9	
DN7_16_9	Ginger Gold	L	DN7_1_8	M13	
E1_9_18	Gloire de Hollande		DN7_1_10	M25	
E1_7_20	Gloster	L	DN7_8_7	M26	
E1_16_12	Golden Delicious	L	DN7_2_10	M793	
E1_11_13	Goldrush	L	E1_7_2	Maayan	
E1_9_20	Goldsmith		E1_1_19	Maidens Blush	
E1_10_20	Goosen		E1_17_4	Maigold	
E1_9_8	Grand Richard		E1_19_1	McIntosh	L
E1_11_15	Granearli		E1_9_7	McIntosh Early	
DN7_19_1	Granny Smith	L	E1_3_11	Melba	L
E1_7_9	Gravenstein (Red)	T, L	E1_15_15	Meldale	
E1_1_6	Greensleeves		DN7_5_6	Melrose	L
E1_13_17	Harberts Reinette	T	E1_2_17	Meran	
E1_11_19	Himekami A	L	DN7_1_6	Michal	
DN7_24_8	Hofer Seedling		E1_11_6	Michinoku	
E1_1_13	Hokuto	L	DN7_6_6	Milton	
DN7_4_6	Hoplan X		E1_5_19	Missouri Pippin	
E1_17_20	Hoplan Y		DN7_21_12	MM109	
E1_14_10	Hops Late Red		DN7_1_1	Mollie’s Delicious	L
E1_11_3	Howell		E1_14_9	Monsa	
DN7_5_10	Jersey Mac	L	E1_4_14	Morkel's Seedling	
DN7_33_2	Jester		E1_6_8	Mother	
E1_15_5	Jonafree (=Co-op 22)		E1_10_17	Mutsu	T, L
DN7_17_5	Jonagold	T	E1_	Nebuta	
E1_16_7	Jonathan	L	E1_9_6	New Gold	
E1_18_2	July Red	L	E1_5_5	New Year	
E1_16_2	Karmijn de Sonnaville	T	E1_2_4	Nickajack	
DN7_5_1	Kashawi		DN7_24_2	No1 Dresden (Seedling 4)	
E1_17_15	Kidd's Orange Red	L	DN7_24_3	No2 Dresden (Seedling 2)	
E1_13_19	King of Tomkins County	T	DN7_24_4	No3 Dresden (Seedling 1)	
E1_1_5	Kirks B		DN7_4_8	Northern Spy	L
E1_18_15	Klara	L	E1_8_9	Ohenimuri Early	
E1_16_11	Kogetso		E1_13_13	Onderstam 5	
E1_7_1	Koo		E1_13_16	Ontario (Hunter)	L
DN7_6_4	Lady Williams	L	E1_10_13	Orin (Jumbo)	L
E1_15_14	Lakeside		E1_3_21	Orleans Reinette	
E1_17_12	Langkloof		E1_1_8	Ozark Gold	L
E1_12_15	Laxton's Superb		E1_10_2	P 1	

Location	Name	Code	Location	Name	Code
DN7_1_11	P 18		DN7_5_8	Sir Prize	
E1_16_10	Panorama Crab		E1_5_14	Spartan	L
E1_1_15	Paragon	T	DN7_6_5	Splendour	L
E1_18_1	Paulared		E1_9_16	Statesman (Red)	L
DN7_19_3	Pilot		E1_6_2	Stark Scarlet Stayman	T
DN7_16_7	Pink Lady	L	DN7_5_7	Summerking Red	
DN7_2_2	Pinova	L	DN7_4_3	Summerred	
E1_15_2	Pioneer Scarlet		DN7_10_5	Sundowner	L
E1_2_19	Pomme De Niege		E1_2_2	Sunrise	L
E1_5_9	Porporate		E1_3_8	Swartland X	
DN7_6_9	Prima	L	E1_5_18	Swartland Y	
E1_17_9	Primgold		E1_6_4	Sweet Cornelly	
E1_2_21	Prince Bismarck		DN7_21_5	T 506	
E1_10_4	Princesa		E1_12_7	Takane	T
DN7_6_10	Priscilla	L	DN7_21_5	T 506	
DN7_6_2	Red Astrakhan		E1_12_7	Takane	T
E1_2_5	Red Dutch		E1_6_3	Tasman's Pride	
E1_16_5	Red Gem		E1_10_16	Telamon (=Waltz)	
E1_16_3	Redfree	L	E1_1_14	Tjeek	
E1_2_20	Redwine		E1_11_22	Trajan (=Polka)	
E1_14_18	Redwinter		E1_1_2	Tsugari Homei	L
DN7_7_8	Reglindis X		E1_14_1	Tuscan (=Bolero)	
E1_13_9	Reglindis Y		E1_4_1	Twenty Ounce	
E1_3_18	Reinette du Canada	T, L	E1_1_18	Valmore	
DN7_20_1	Remo		E1_10_9	Veitchii Pumila	
DN7_1_4	Resista X	L	E1_6_9	Versveld	
DN7_1_5	Resista Y	L	E1_5_2	Vista Bella	L
E1_8_5	Resista Z	L	E1_6_6	Wainwright	
DN7_7_9	Rewena		E1_14_19	Wemmershoek	
E1_2_3	Rhode Island Greening	T	E1_5_3	White Winter Pearmain	L
E1_17_19	Rokewood	L	E1_7_4	Widup	
E1_6_12	Rome Beauty	L	E1_3_4	William's Pride Co-op 23	
DN7_17_10	Russian Seedling	L	E1_2_18	Winesap	
E1_3_9	Sadie Frazer		DN7_7_1	Winter Banana	
DN7_5_9	Sansa	L	E1_4_2	Wolf River	
E1_6_16	Sayaka		DN7_2_3	Zabaoni	
DN7_32_2	Scarlet		E1_2_15	Zoba (=Lobo)	
E1_19_9	Selena	L	E1_4_18	Zvonkove	L
E1_5_15	Senator		**Other *Malus* species and hybrids**		
E1_7_5	Senshu	L	DN7_15_7	*M. sieversii* Kaz-95-44	
E1_2_6	Shampion	L	DN7_17_8	*M. sieversii* Kaz-95-57	
DN7_22_1	Sharpe's Early X		DN7_16_5	*M. sieversii* Kaz-95-58	
E1_8_2	Sharpe's Early Y		DN7_18_2	*M. sieversii* Kaz-95-71	
E1_4_6	Sharpe's Late		DN7_15_1	*M. sieversii* Kaz-95-71A	
E1_18_4	Shizuka	L	DN7_18_1	*M. sieversii* Kaz-95-78	
DN7_2_4	Shlomit		DN7_17_2	*M. sieversii* Kaz-95-89	
E1_6_7	Shoreland Queen		DN7_15_6	*M. sieversii* Kaz-95-91	
DN7_1_3	Sinclair		E1_8_17	*M. sieversii* Kaz-95-122	
E1_5_7	Sir Isaac Newton		E1_7_11	KSC 3	

Location	Name	Code	Location	Name	Code
DN7_21_2	KSC 11		DN7_16_3	*M. platycarpa*	T
DN7_21_3	KSC 25		E1_9_11	*M. purpurea*	
DN7_21_6	Malus 44		DN7_7_10	*M. zumi*	
DN7_34_1	*M.* Aldenhamensis	L	E1_15_19	*M.* Maypole	
E1_5_8	*M.* Butterball		DN7_5_5	*M.* Mildew Resistant	
DN7_20_3	*M. coronaria*	T	DN7_20_4	*M.* Moeransi Profusion	
E1_17_6	*M. floribunda*	L	DN7_20_9	*M.* Veitch's Scarlet	
DN7_16_10	*M.* Golden Hornet		E1_6_17	*M.* Spy 227	
DN7_2_6	*M.* Lemonei		DN7_24_5	No4 Dresden (Seedling 3)	
DN7_20_2	*M. micromalus*		DN7_24_6	No5 Dresden (Seedling ?) X	
DN7_17_1	*M. robusta*		DN7_24_7	No5 Dresden (Seedling ?) Y	
E1_5_10	*M. robusta* 5		DN7_25_4	S202	
DN7_2_7	*M. spectabilis*		DN7_21_4	T 585	

In general, the first tree of three of a kind for the Drostersnes orchard or of two of a kind for the Grabouw orchard was sampled. Tree locations were used as identifiers in the absence of accession numbers. Young expanding leaves were collected in spring (early September) and frozen at -80°C until required for DNA extraction. Leaf material was weighed to approximately 0.3 g (±0.1 g) and placed in a labelled 2 ml Microcentrifuge tube and stored at -20°C until further use. Samples were prepared in duplicate to allow for repeat analysis.

5.2.2. DNA extraction

Genomic DNA was extracted following a slightly modified method by De la Rosa *et al*. (2002). The microcentrifuge tubes containing frozen leaves were placed at room temperature to initiate thawing. Before complete thawing, a single 1 mm stainless steel ball-bearing was placed inside the tube. Extraction reagents comprising 0.8 ml prewarmed (65°C) CTAB buffer [2% (m/v) CTAB (Merck), 2% (m/v) PVP 40 (Merck), 1.4M NaCl (Merck), 20 mM EDTA at pH 8 (Merck), 100 mM Tris at pH 8 (Melford Laboratories)] and 0.08 ml β-mercaptoethanol (Merck) were added.

Samples were shaken by hand to mix the reagents and then ground thoroughly for 3 to 4 min using a Tissuelyser II ball mill (Qiagen). Samples were incubated for 2 hours at 65°C and the ball bearings removed using a stainless steel magnet. Thereafter 0.8 ml of chloroform-isoamyl alcohol (Merck) at a ratio 24:1 was added and the sample centrifuged (Labnet) for 15 min at 13 500 rpm. The top aqueous phase was recovered, 0.8 ml chloroform-isoamyl alcohol (24:1) was added again and the sample was centrifuged for 10 min at 13 500 rpm. The top aqueous

phase was then recovered and precipitated with 0.5 ml cold isopropanol (Merck) overnight. After precipitation, samples were centrifuged at 4°C for 15 min at 13 500 rpm, the solution was discarded and the pellet washed in 0.5 ml of 70% (v/v) ethanol, dried for 30 to 45 min and resuspended in TE buffer until further use.

The quality and quantity of the DNA was determined with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) following the manufacturer's instructions. If a sample showed poor quality and quantity, the extraction was repeated. The DNA samples from the extractions were diluted and adjusted to a final concentration of 100 ng/µl. Both resuspended and diluted DNA were stored at -20°C to minimise deterioration.

5.2.3. Primer design

Primers were designed to generate products shorter than 500 bp, ACS1-Pr′F 5′agc ata tgg acc agg gtg ggt c3′ and ACS1-Pr′R 5′ggc gtt cac cat tac ctg gca taa3′, to allow for possibility of multiplexing with microsatellite markers. These are based on the only NCBI sequence of the *ACS1* promoter region in apple, AB010102.1, and flanked the indel (Fig. 5.1). The forward primer was fluorescently labelled with PET (Applied Biosystems). The product sizes expected from the sequences were 198 bp (allele *a*) and 335 bp (allele *b*).

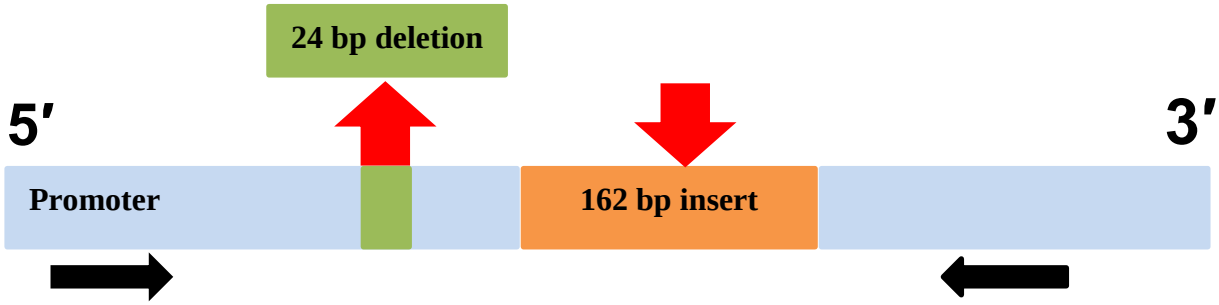

Fig. 5.1. Structure of the promoter region of the *ACS1* gene in apple (Sunako *et al*., 1999) showing the 162 bp SINE insertion and the concomitant 24 bp deletion that distinguish alleles *a* and *b*. Horizontal arrows show the primers amplifying across the indels which results in a difference of approximately 138 bp between the two alleles.

5.2.4. *ACS1* genotyping

PCRs were performed in a final volume of 12.5 µl containing 1.5 µl of 100 ng template DNA, 6.25 µl of Qiagen PCR mix (Qiagen), 1 µl of the *ACS1* primer mix and 3.75 µl of RNase-free water. Amplification was carried out in GeneAmp (Applied Biosystems) and G-Storm (G-Storm Direct) thermal cyclers using the following conditions: an initial denaturation at 95°C for 15 min, followed by 29 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, and a final 30 min extension at 60°C.

PCR products from a subset of accessions were first resolved electrophoretically on a 1% (m/v) agarose gel (Conda Laboratories) at 70V (Hoefer Scientific Instruments PS 500X) for 60 min in a 1X TBE buffer (Tris, Boric acid, EDTA) using a 1kb ladder (Thermo Scientific) to verify amplification. Upon confirmation, the full set of PCR products was sized with capillary electrophoresis on a 3130 DNA capillary analyser (Applied Biosystems). Sizes of the amplified products were established in comparison with the internal size standard, GS500(-250)LIZ (Applied Biosystems). The software GENEMAPPER version 5.0 (Applied Biosystems) was used to visualise the peaks and aid allele scoring. Data were independently verified for validity and collated in Microsoft Excel.

Accessions that exhibited single peaks on the GENEMAPPER traces were assumed to be homozygous; those with only the 202 bp product were designated *aa*, or *aaa* in case of triploids and those with only the 339 bp peak were designated *bb*, or *bbb* in triploids. Heterozygous accessions with both the 202 bp and 339 bp peaks, were designated *ab*, or in the case of triploids *ab*-. Occasional variants of the *a* allele were observed and designated with a subscript *e.g.* $a_{204.}$ Alleles *a* and *b* in the current study correspond to alleles *1* and *2* of Sunako *et al*. (1999) for high and low ethylene production, respectively.

5.2.5. Hardy-Weinberg Equilibrium (HWE) statistics

The deviation from HWE was calculated manually for the three genotypic classes, *aa*, *ab* and *bb* and confirmed by an online HWE calculator (http://had2know.com/academics/hardy-weinberg-calculator-2-alleles.html). Analyses were conducted for the 235 *M. pumila* cultivars and the 20 selections; *Malus* species and hybrids were not included.

5.2.6. Investigation of discrepancies with reported sizes

An experiment using a fluorescent version of the ACS1-5 primers reported by Sunako *et al.* (1999), was conducted to test the reported product sizes, 489 bp (allele *1*) and 655 bp (allele *2*). The primer was labelled with VIC (Applied Biosystems). Five cultivars of *M. pumila,* 'Delicious', 'Golden Delicious', 'Granny Smith', 'Jersey Mac' and 'Jonagold' were tested using conditions as described for ACS1-Pr primers. A GS1200LIZ standard was used for accurate scoring as the product size of allele *2* was outside the GS500(-250)LIZ range. The analyses were repeated in triplicate to verify the observed sizes.

5.2.7. Pear *ACS1* investigation

Nine pear accessions were analysed using both the ACS1-Pr (current study) and ACS1-5 (Sunako *et al.*, 1999) primers to investigate whether an indel in the promoter similar to that associated with ripening in *Malus* could be detected in *Pyrus*. The *P. communis* cultivars 'Beurre Bosch', 'Beurre Superfin', 'Beurre d'Anjou', 'Beurre Six', 'BP2', and 'Flamingo', the *Pyrus* hybrids 'Garber' and 'Kieffer' and an accession of *P. calleryana* were used. Detection of alleles *a* and *b* (or *1* and *2*) would indicate the presence of a 138 bp indel similar to that which is associated with different patterns of ethylene production in *Malus*.

5.2.8. ACS1-Pr multiplexing with apple microsatellite markers

Simultaneous amplification of microsatellite markers and ACS1-Pr was done to investigate the suitability of ACS1-Pr for multiplexing. The ACS1-Pr primers, labelled with PET, were used in a multiplex with three markers from apple multiplex C, giving large product sizes of 175 to 257 bp (Chapter 3); CH04e05 (FAM), CH02c11 (NED) and CH02d08 (VIC). The fourth marker, CH02c09 (PET), was removed to minimise dye competition during automated electrophoresis. Five *M. pumila* cultivars, 'Braeburn', 'Delicious', 'Golden Delicious', 'Granny Smith' and 'Jonagold' and two accessions of *Malus* species, *M. floribunda* and *M. robusta* 5, were analysed using the conditions described in Chapter 3.

5.3. Results

5.3.1. Performance of fluorescent primers, *ACS1* genotyping and novel variants in apple

The redesigned primers, ACS1-Pr, successfully amplified all *Malus* samples tested giving either one or two products per item. In *M. pumila*, allele sizes of 202 bp and 339 bp were observed for *a* and *b*. Novel variants for the *a* allele, namely 204, 205 and 206 bp, were however also detected in some other *Malus* species.

Of the 292 *Malus* accessions analysed in this study, 148 were homozygous for the *a* allele, 115 were heterozygous *ab* and 29 were homozygous for the *b* allele.

For the 235 *M. pumila* cultivars, 110 accessions were homozygous for allele *a*, 101 were heterozygous for *ab* and 24 homozygous for allele *b*. Of the 20 *M. pumila* selections, eight were *aa*, eight *ab* and four had *bb* genotypes. Only four of the 37 *Malus* species and hybrids were heterozygous for the *ab* alleles, while the remaining accessions were homozygous for allele *a*; no *bb* genotypes were observed. Interestingly, there were several variants detected for allele *a* in most *Malus* species and hybrids (Fig. 5.2), with sizes of 204, 205 and 206 bp, which had not been detected in previous studies using agarose gel electrophoresis.

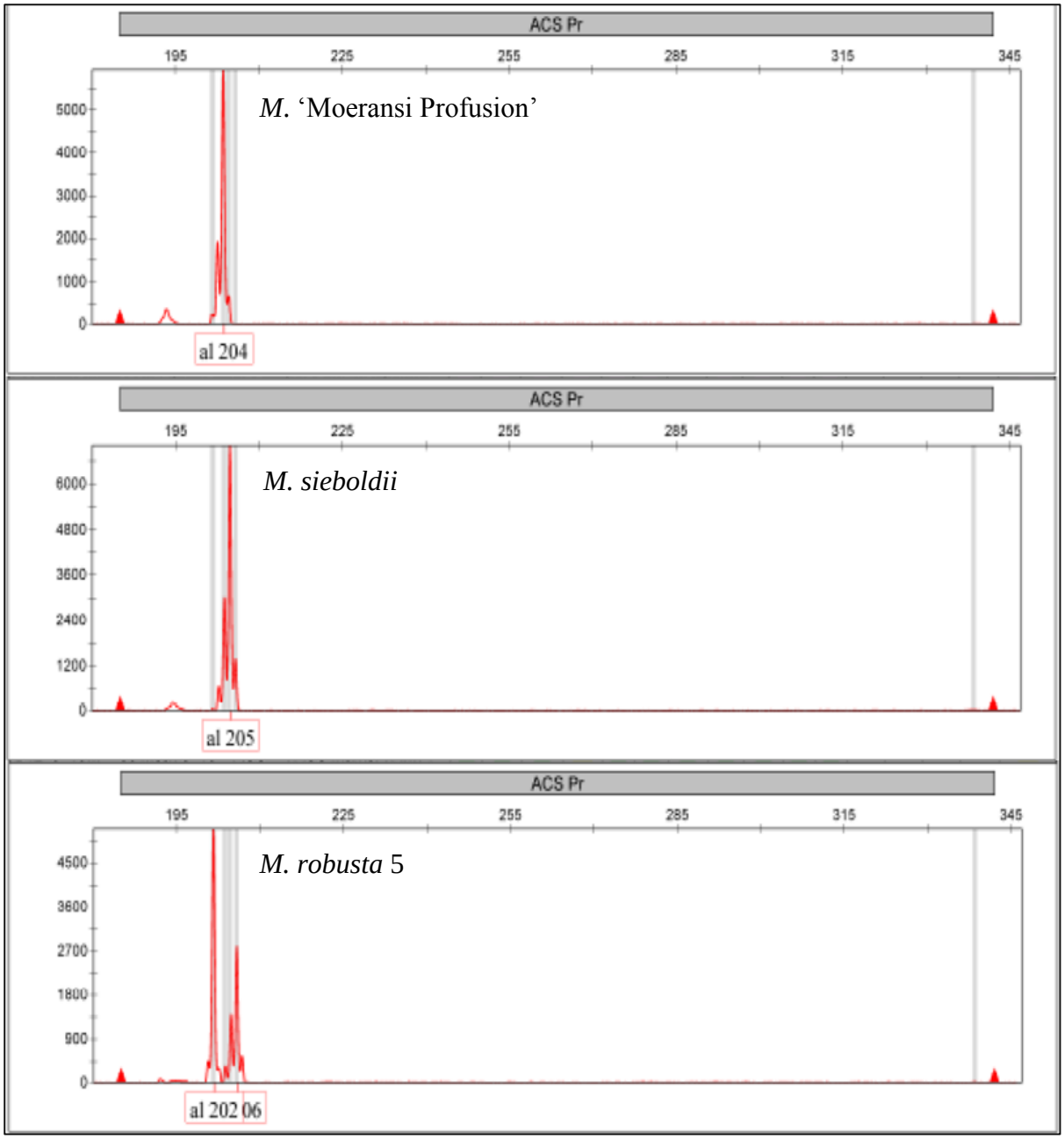

Fig. 5.2. Products of *Malus* accessions *M.* 'Moeransi Profusion', *M. sieboldii* and *M. robusta* 5, amplified with ACS1-Pr primers and visualised with GENEMAPPER, showing variants of allele *a*, of 204, 205, and 206 bp, in addition to the 202 bp allele, that were observed in various *Malus* accessions.

The *ACS1* genotypes of 64 *M. pumila* cultivars in the ARC gene bank were compared with published *ACS1* genotypes; for ten of the 64 accessions, the *ACS1* genotypes were inconsistent, while the remaining 54 matched (Table 5.2). No inconsistencies were found when comparing the genotypes of the three *Malus* accessions with the reported genotypes; however, for one of four triploids, 'Blenheim Orange', the *ACS1* genotype was inconsistent.

Table 5.2. *ACS1* genotypes of 64 *M. pumila* cultivars and three *Malus* species or hybrids in the ARC collection genotyped with fluorescently labelled primers and compared with the genotypes reported in the literature. The published genotypes *1/1*, *1/2* and *2/2* are equivalent to *aa*, *ab* and *bb*.

Location	Accession name	Alleles	ARC	Reported	Reference‡	Notes
***M pumila* cultivars**						
E1_15_18	Akane	339	*bb*	*2/2*	O'04	
E1_7_3	Alkmene	339	*bb*	*2/2*	O'07	
E1_9_10	Anna	202/339	*ab*	*1/2*	P'14	
DN7_24_9	Antonovka Seedling	202	*aa*	*1/1, 1/2*	S'99, P'14	
E1_9_15	Blenheim Orange†	202/339	*ab-*	*1/1*	P'14	*
E1_11_1	Braeburn	202/339	*ab*	*½*	O'07, ZB'08	
DN7_5_3	Cox's Orange Pippin	202/339	*ab*	*1/1*	S'99	*
E1_9_14	Delblush	202	*aa*	*2/2*	ZB'08	*
E1_18_8	Democrat	202	*aa*	*1/1*	Z'09	
DN7_4_5	Elstar (Red)	339	*bb*	*2/2*	P'14	
E1_9_9	Fuji Akufi	339	*bb*	*2/2*	All	
E1_9_2	Gala	339	*bb*	*2/2*	O'04, ZB'08	
DN7_16_9	Ginger Gold	202/339	*ab*	*1/2*	ZB'08	
E1_7_20	Gloster	339	*bb*	*2/2*	Z'09	
E1_16_12	Golden Delicious	202/339	*ab*	*1/2*	All	
E1_11_13	Goldrush	202/339	*ab*	*1/2*	ZB'08, P'14	
DN7_19.1	Granny Smith	202/339	*ab*	*1/1, 1/2*	O'04/Others	
E1_7_9	Gravenstein (Red) †	202	*aaa*	*1/1*	P'14	
E1_11_19	Himekami	339	*bb*	*2/2*	S'99, ZB'08	
E1_1_13	Hokuto	202/339	*ab*	*1/2*	ZB'08	
E1_1_2	Homei Tsugaru	202/339	*ab*	*1/2*	S'99, H'00, O'04	
E1_13_16	Hunter Ontario	202/339	*ab*	*1/2, 2/2*	Z'09/P'14	
DN7_5_10	Jersey Mac	202	*aa*	*1/1*	H'00, O'07	
DN7_17_5	Jonagold†	202/339	*ab-*	*1/2*	H'00, O'04	
E1_16_7	Jonathan	202/339	*ab*	*1/2*	S'99, H'00, O'04	
E1_18_2	July Red	202	*aa*	*1/2*	S'99, H'00, P'14	*
E1_10_13	Jumbo Orin	202	*aa*	*1/2*	S'99, H'00, O'04, Z'09	*
E1_17_15	Kidd's Orange Red	202/339	*ab*	*1/2*	Z'09	
E1_18_15	Klara	202	*aa*	*1/1*	Z'09	
DN7_6_4	Lady Williams	202/339	*ab*	*1/2*	O'07	
DN7_5_2	Liberty	202	*aa*	*1/2*	P'14	*
E1_13_6	Lord Lambourne	202/339	*ab*	*1/2*	P'14	
E1_19_1	McIntosh	202	*aa*	*1/1*	S'99, H'00, O'04', P'14	
E1_3_11	Melba	202	*aa*	*1/1*	Z'09	
DN7_5_6	Melrose	339	*bb*	*2/2*	Z'09	
DN7_1_1	Mollie's Delicious	202/339	*ab*	*1/2*	P'14	
E1_10_17	Mutsu†	202/339	*ab-*	*1/2*	S'99, H'00, O'04	
DN7_4_8	Northern Spy	202	*aa*	*1/1*	S'99, Z'09	
DN7_16_7	Pink Lady	202/339	*ab*	*1/2*	ZB'08	
DN7_2_2	Pinova	339	*bb*	*2/2*	ZB'08	

**ACS1* genotype inconsistent with the published genotype, † triploid, ‡S'99 Sunako *et al*. (1999), H'00 Harada *et al*. (2000), O'04 Oraguzie *et al*. (2004), O'07 Oraguzie *et al*. (2007), ZB'08 Zhu and Barritt (2008), Z'09 Zoufalá *et al*. (2009), P'14 Peace (2014).

Location	Accession name	Alleles	ARC	Reported	Reference‡	Notes
DN7_6_9	Prima	202/339	*ab*	*1/2*	Z'09	
DN7_6_10	Priscilla	202/339	*ab*	*1/2*	P'14	
E1_14_4	Red Delicious	202/339	*ab*	*1/2*	O'07	
E1_9_16	Red Statesman	202	*aa*	*1/1*	O'07	
E1_16_3	Redfree	202	*aa*	*1/1*	Z'09	
E1_3_18	Reinette du Canada†	202	*aaa*	*1/1*	S'99	
DN7_1_4	Resista A	202	*aa*	*1/2*	Z'09	*
DN7_1_5	Resista B	202	*aa*	*1/2*	Z'09	*
E1_8_5	Resista	202/339	*ab*	*1/2*	Z'09	
E1_17_19	Rokewood	202	*aa*	*1/1*	O'07	
E1_6_12	Rome Beauty	202/339	*ab*	*1/1*	S'99, O'07	*
DN7_17_10	Russian Seedling	202	*aa*	*1/1*	P'14	
DN7_5_9	Sansa	202	*aa*	*2/2*	All	*
E1_19_9	Selena	202	*aa*	*1/1*	Z'09	
E1_7_5	Senshu	339	*bb*	*2/2*	0'04, ZB'08	
E1_2_6	Shampion	202/339	*ab*	*1/2*	Z'09	
E1_18_4	Shizuka	202/339	*ab*	*1/2*	ZB'08	
E1_5_14	Spartan	202	*aa*	*1/1*	Z'09	
DN7_6_5	Splendour	339	*bb*	*2/2*	O'07, ZB'08	
E1_10_5	Sundowner	202/339	*ab*	*1/2*	ZB'08	
E1_2_2	Sunrise	202/339	*ab*	*1/2*	ZB'08, P'14	
E1_5_2	Vista Bella	202	*aa*	*1/1*	H'00	
E1_5_3	White Winter Pearmain	202/339	*ab*	*1/2*	S'99	
E1_4_18	Zvonkove	202/339	*ab*	*1/2*	Z'09	
Malus						
DN7_34_1	*M.* Aldenhamensis	202/204	aa_{204}	*1/1*	H'00	
DN7_17_6	*M. floribunda*	202/204	aa_{204}	*1/1*	S'99	
DN7_2_7	*M. spectabilis*	202	*aa*	*1/1*	S'99	

**ACS1* genotype inconsistent with the published genotype, † triploid, ‡S'99 Sunako *et al*. (1999), H'00 Harada *et al*. (2000), O'04 Oraguzie *et al*. (2004), O'07 Oraguzie *et al*. (2007), ZB'08 Zhu and Barritt (2008), Z'09 Zoufalá *et al*. (2009), P'14 Peace (2014).

Genotypes for 225 accessions observed during the current study (Table 5.3) have not been genotyped previously; of these 171 were *M. pumila* cultivars, 20 were selections of *M. pumila* and 34 were *Malus* species and hybrids. In the case of heterozygous, *ab*, triploids, it was not possible to distinguish whether the two peaks represented *aab* or *abb*.

Table 5.3. *ACS1* genotypes of 225 apple accessions in the ARC collection genotyped with fluorescently labelled ACS1-Pr primers and reported for the first time in the current study.

Location	Accession Name	*ACS1*	Location	Accession Name	*ACS1*
***M pumila* selections**			E1_18_14	Chantecler	*aa*
DN7_16_4	20/1	*ab*	E1_17_10	Charden†	*ab-*
DN7_30_1	28/1	*aa*	E1_14_2	Climax	*aa*
E1_3_13	28/2	*aa*	E1_17_5	Coast	*ab*
E1_2_12	2B-12-25	*ab*	E1_16_16	Commerce	*ab*
DN7_17_9	4A-75-28	*ab*	E1_16_15	Co-op 19	*bb*
E1_19_11	CC2/19	*aa*	E1_7_12	Co-op 20	*ab*
DN7_33_1	HL 1004	*aa*	E1_7_17	Coromandel Red	*ab*
DN7_32_1	HL 166C	*ab*	E1_16_21	Crab A	*aa*
E1_18_11	HL 237	*ab*	E1_9_4	Crab C	*aa*
E1_18_10	HL 318	*bb*	E1_7_14	Criterion	*ab*
E1_18_12	HL 938	*ab*	E1_16_18	Dakota	*ab*
DN7_26_1	i5526 X 6407 INRA	*bb*	E1_13_15	Dayton (=Co-op 21)	*aa*
E1_13_3	M H 15-6	*aa*	DN7_24_1	Dayton Seedling No6	*aa*
DN7_17_7	Pi-Au 9-24	*aa*	DN7_18_4	Delkistar	*ab*
DN7_21_7	Pi-Au 9-27	*aa*	E1_12_13	Diva Gold	*ab*
DN7_33_3	SA579-3	*ab*	DN7_4_4	Drakenstein	*aa*
E1_19_8	X2765	*aa*	E1_16_17	Dunn's Seedling	*aa*
E1_19_3	X6163 P22 R19 A14	*ab*	E1_9_12	Earligold	*aa*
E1_19_10	X640 TNR42A45	*bb*	DN7_22_2	Edgewood	*ab*
E1_19_4	X6688 K1 R87 A18	*bb*	E1_15_11	Eikhoff	*aa*
***M. pumila* cultivars**			E1_4_13	Elise	*ab*
DN7_15_5	Adina	*bb*	E1_8_19	Elsie Grant	*ab*
E1_3_12	Adina (syn Frankad)	*bb*	E1_7_13	Florentina	*aa*
E1_3_10	African Carmine	*ab*	E1_19_5	Forum†	*ab-*
E1_9_19	Alfmission	*ab*	E1_4_11	Gavin	*bb*
E1_14_11	Alsop's Beauty	*aa*	E1_9_18	Gloire de Hollande	*aa*
DN7_16_1	Aport	*aa*	E1_9_20_	Goldsmith	*ab*
E1_12_4	Arapkizi	*aa*	E1_10_20_	Goosen	*bb*
E1_17_11	Atties Favourite	*ab*	E1_9_8	Grand Richard	*aa*
DN7_31_2	Austin	*aa*	E1_11_15	Granearli	*ab*
E1_13_10	Baujade†	*ab-*	E1_1_6	Greensleeves	*ab*
E1_12_12	Beaumont	*aa*	E1_13_17	Harberts Reinette	*aa*
E1_14_3	Belle de Boskoop†	*aaa*	DN7_24_8	Hofer Seedling	*bb*
E1_14_8	Belrene	*aa*	DN7_4_6	Hoplan X	*aa*
E1_13_11	Beni Osho	*aa*	E1_17_20	Hoplan Y	*ab*
DN7_4_7	Beverly Hills	*aa*	E1_14_10	Hops Late Red	*ab*
DN7_27_1	Bittenfelder	*bb*	E1_11_3	Howell	*bb*
E1_14_6	Blairmont	*ab*	DN7_33_2	Jester	*ab*
DN7_31_3	Boiken X	*aa*	E1_15_5	Jonafree (=Co-op 22)	*ab*
E1_13_21	Boiken Y	*ab*	E1_16_2	Karmijn de Sonnaville†	*ab-*
E1_17_18	Calville de Saint Souve	*ab*	DN7_5_1	Kashawi	*aa*
E1_7_19	Canvade	*aa*	E1_13_19	King of Tomkins County	*ab*
E1_17_16	Champion	*aa*	E1_1_5	Kirks B	*aa*

Location	Accession Name	*ACS1*	Location	Accession Name	*ACS1*
E1_16_11	Kogetso	*ab*	E1_5_9	Porporate	*ab*
E1_7_1	Koo	*aa*	E1_17_9	Primgold	*ab*
E1_15_14	Lakeside	*ab*	E1_2_21	Prince Bismarck	*aa*
E1_17_12	Langkloof	*aa*	E1_10_4	Princesa	*bb*
E1_12_15	Laxton's Superb	*aa*	DN7_6_2	Red Astrakhan	*ab*
DN7_19_2	Le Vant	*ab*	E1_2_5	Red Dutch	*aa*
E1_10_6	Lemon†	*ab-*	E1_16_5	Red Gem	*aa*
E1_10_19	Leyda	*aa*	E1_2_20	Redwine	*ab*
E1_12_9	London Pippin	*bb*	E1_14_18	Redwinter	*bb*
E1_11_8	Longford	*aa*	DN7_7_8	Reglindis X	*ab*
DN7_20_10	M1	*aa*	E1_13_9	Reglindis Y	*aa*
DN7_20_11	M4	*aa*	DN7_20_1	Remo	*ab*
DN7_21_11	M7	*aa*	DN7_7_9	Rewena	*ab*
DN7_2_11	M9	*aa*	E1_2_3	Rhode Island Greening†	*ab-*
DN7_1_8	M13	*aa*	E1_3_9	Sadie Frazer	*aa*
DN7_1_10	M25	*aa*	E1_6_16	Sayaka	*ab*
DN7_8_7	M26	*aa*	DN7_32_2	Scarlet	*ab*
DN7_2_10	M793	*aa*	E1_5_15	Senator	*ab*
E1_7_2	Maayan	*aa*	E1_8_2	Sharpe's Early X	*aa*
E1_1_19	Maidens Blush	*ab*	DN7_22_1	Sharpe's Early Y	*aa*
E1_17_4	Maigold	*ab*	E1_4_5	Sharpe's Late	*aa*
E1_9_7	McIntosh Early	*aa*	DN7_2_4	Shlomit	*aa*
E1_15_15	Meldale	*ab*	E1_6_7	Shoreland Queen	*ab*
E1_2_17	Meran	*bb*	DN7_1_3	Sinclair	*aa*
DN7_1_6	Michal	*ab*	E1_5_7	Sir Isaac Newton	*aa*
E1_11_6	Michinoku	*aa*	DN7_5_8	Sir Prize	*ab*
DN7_6_6	Milton	*ab*	E1_6_2	Stark Scarlet Stayman†	*aaa*
E1_5_19	Missouri Pippin	*aa*	DN7_5_7	Summerking Red	*ab*
DN7_21_12	MM109	*aa*	DN7_4_3	Summerred	*ab*
E1_14_9	Monsa	*ab*	E1_3_8	Swartland X	*aa*
E1_4_14	Morkel's seedling	*ab*	E1_5_18	Swartland Y	*aa*
E1_6_8	Mother	*ab*	E1_6_4	Sweet Cornelly	*ab*
E1_15_13	Nebuta	*ab*	DN7_21_5	T 506	*aa*
E1_9_6	New Gold	*ab*	E1_12_7	Takane	*ab*
E1_5_5	New Year	*aa*	E1_6_3	Tasman's Pride	*aa*
E1_2_4	Nickajack	*aa*	E1_10_16	Telamon (=Waltz)	*ab*
DN7_24_2	No1 Dresden Seedling 4	*aa*	E1_1_14	Tjeek	*aa*
DN7_24_3	No2 Dresden Seedling 2	*aa*	E1_11_22	Trajan (=Polka)	*ab*
DN7_24_4	No3 Dresden Seedling 1	*aa*	E1_14_1	Tuscan (=Bolero)	*aa*
E1_8_9	Ohenimuri Early	*aa*	E1_4_1	Twenty Ounce	*ab*
E1_13_13	Onderstam 5	*aa*	E1_1_18	Valmore	*ab*
E1_3_21	Orleans Reinette	*bb*	E1_10_9	Veitchi Pumila	*aa*
E1_1_8	Ozark gold	*ab*	E1_6_9	Versveld	*ab*
E1_10_2	P 1	*aa*	E1_6_6	Wainwright	*aa*
DN7_1_11	P 18	*aa*	E1_14_19	Wemmershoek	*aa*
E1_16_10_	Panorama Crab	*ab*	E1_7_4	Widup	*aa*
E1_1_15	Paragon†	*aaa*	E1_3_4	William's Pride	*ab*
E1_18_1	Paulared	*aa*	E1_2_18	Winesap	*aa*
DN7_19_3	Pilot	*bb*	DN7_7_1	Winter Banana	*ab*
E1_2_19	Pomme de Niege	*ab*	E1_4_2	Wolf River	*ab*

Location	Accession Name	*ACS1*	Location	Accession Name	*ACS1*
DN7_2_3	Zabaoni	*aa*	DN7_2_6	*M.* Lemonei	*aa*
E1_2_15	Zoba (=Lobo)	*aa*	DN7_20_2	*M. micromalus*	*aa*
Other *Malus* species and hybrids			DN7_16_3	*M. platycarpa*†	*aaa*
DN7_15_7	Kaz-95-44	*aa*	E1_9_11	*M. purpurea*	aa_{204}
DN7_17_8	Kaz-95-57	*aa*	DN7_17_1	*M. robusta*	$a_{205}a_{205}$
DN7_16_5	Kaz-95-58	*aa*	E1_5_10	*M. robusta* 5	aa_{206}
DN7_18_2	Kaz-95-71	*aa*	DN7_7_10	*M. zumi*	aa_{204}
DN7_15_1	Kaz-95-71A	*aa*	E1_15_19	*M.* Maypole	*aa*
DN7_18_1	Kaz-95-78	*aa*	DN7_5_5	*M.* Mildew Resistant	aa_{204}
DN7_17_2	Kaz-95-89	*aa*	DN7_20_4	*M.* Moeransi Profusion	$a_{204}a_{204}$
DN7_15_6	Kaz-95-91	*ab*	E1_15_2	*M.* Pioneer Scarlet	aa_{205}
E1_8_17	Kaz-95-122	*ab*	E1_6_17	*M.* Spy 227	aa_{204}
E1_7_11	KSC 3	aa_{205}	DN7_20_9	*M.* Veitch's Scarlet	$a_{204}a_{205}$
DN7_21_2	KSC 11	aa_{204}	DN7_24_5	No4 Dresden Seedling 3	$a_{202}a_{205}$
DN7_21_3	KSC 25	*aa*	DN7_24_6	No5 Dresden Seedling ? X	$a_{205}b$
DN7_21_6	Malus 44	*ab*	DN7_24_7	No5 Dresden Seedling ? Y	aa_{205}
E1_5_8	*M.* Butterball	aa_{204}	DN7_25_4	S202	aa_{205}
DN7_20_3	*M. coronaria*†	aa_{204}-	DN7_21_4	T 585	aa_{205}
DN7_16_10	*M.* Golden Hornet	$a_{204}b$			

†triploid

5.3.2. Hardy-Weinberg Equilibrium statistics

For the *M. pumila* cultivars, the number of cultivars expected in the three classes, *aa*, *ab* and *bb*, expected under HWE, namely 109.6 *aa*, 101.8 *ab* and 23.6 *bb*, accorded almost exactly with the numbers observed, 110 *aa*, 101 *ab* and 24 *bb*, with a X^2 value = 0.91 with 1 degree of freedom. Of the 20 selections analysed, the 8 *aa*, 8 *ab* and 4 *bb* genotypes observed were in close accordance with 7.2 *aa*, 9.6 *ab* and 3.2 *bb* genotypes expected under HWE, with a X^2 value = 0.46.

5.3.3. Investigation of discrepancies with reported sizes

When the fluorescently labelled ACS1-5 primers (Sunako *et al.*, 1999) were tested on five cultivars, allele scores of 514 bp and 652 bp were consistently observed for alleles *a*, (or *1*), and *b*, (or *2*) respectively (Fig. 5.3). These are slightly different from the published allele sizes of 489 bp (allele *1*) and 655 bp (allele *2*), substantially so in the case of allele *1*. Note that the difference in size between the two alleles detected with the fluorescently labelled primers in the current study was 138 bp whereas the difference between the published sizes of the two alleles (489 and 655 bp) was 166 bp. The difference observed with the fluorescently labelled

ACS1-5 primers is in close agreement with the difference observed in the current study with the ACS-Pr primers, namely 137 bp.

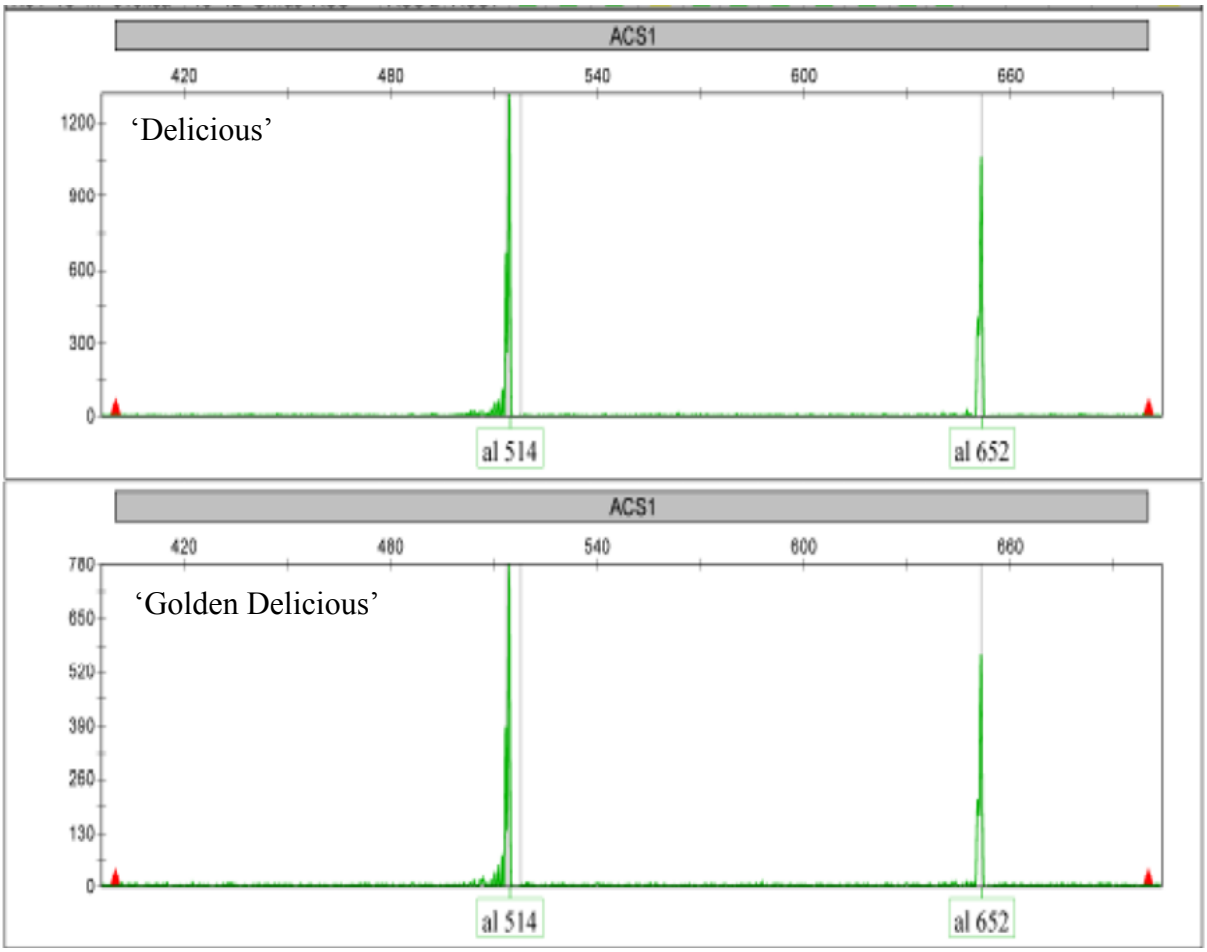

Fig. 5.3. Products of 'Delicious' and 'Golden Delicious' cultivars amplified with the fluorescently labelled version of the ACS1-5 primers and visualised with GENEMAPPER. Allele sizes of 514 bp, *a*, and 652 bp, *b*, were observed. Similar allele sizes were observed for 'Granny Smith' (*ab*), 'Jersey Mac' (*aa*) and 'Jonagold' (*ab*).

5.3.4. Pear *ACS1* investigation

In pear, the primers ACS1-Pr amplified a product of 199 bp only in *P. calleryana* and the hybrids 'Garber' and 'Kieffer'. No amplification was observed in the six *P. communis* accessions. Likewise, the fluorescently labelled primers of Sunako *et al.* (1999) ACS1-5 amplified a product of 488 bp only in *P. calleryana*, 'Garber' and 'Kieffer' with no amplification observed for the six *P. communis* samples tested (Fig. 5.4).

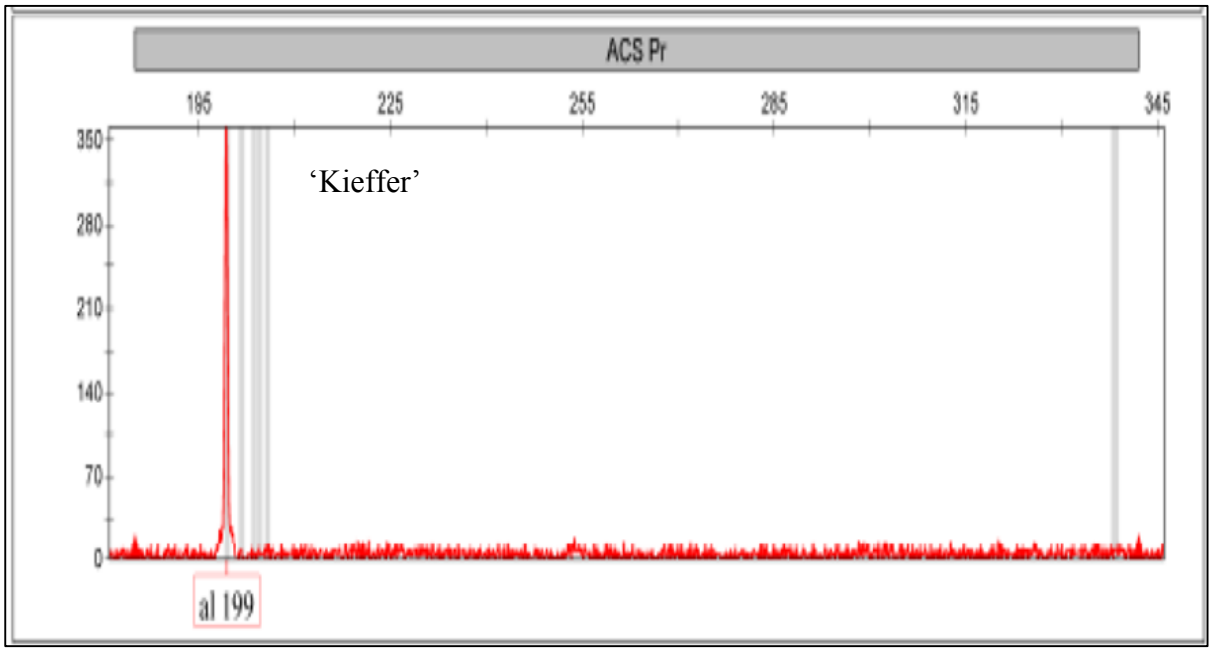

Fig. 5.4. Product of pear cultivar 'Kieffer' amplified with primers ACS1-Pr and visualised using GENEMAPPER. No amplification was observed for *P. communis* cultivars, whereas 'Kieffer' and other *Pyrus* species and hybrids showed a product of 199 bp. Similar results were observed using ACS1-5 primers but with a product of 488 bp.

5.3.5. Multiplexing ACS1-Pr with microsatellite markers

The ACS1-Pr primers and the three apple microsatellites, multiplexed together, amplified across all eight accessions tested and gave scores identical with those reported earlier (Table 5.4).

Table 5.4. Microsatellite and *ACS1* GENEMAPPER scores for seven accessions amplified in a multiplex reaction to establish the compatibility of ACS1-Pr primers in multiplex.

Name	CH02c11	CH02d08	CH04e05	ACS1-Pr
***M. pumila* cultivars**				
Braeburn	208/234	218/255	202/204	202/339
Delicious	208/234	212/218	175/204	202/339
Golden Delicious	220/234	224/226	175	202/339
Granny Smith	228/231	212/251	175	202/339
Jonagold	220/230/234	224/226/229	175	202/339
Other *Malus* species				
M. floribunda	224/228	216/220	188/198	202/204
M. robusta 5	206/220	212/214	182	202/206

5.4. Discussion

5.4.1. Fluorescently labelled markers and novel alleles

This study is thought to be the first to use fluorescently labelled primers to determine the *ACS1* genotypes in apple. The redesigned fluorescently labelled primers, ACS1-Pr, proved convenient and informative. Product sizes of 202 bp and 339 bp with a difference of 137 bp were detected when using ACS1-Pr. The difference was consistent with the indel phenomenon reported by Sunako *et al*. (1999), but not with the difference between the allele sizes reported in that study and subsequent studies, which were sized by agarose gel electrophoresis and were reported to differ by 166 bp. This is discussed in the next section. Additionally, in various *Malus* accessions minor variation in the length of the amplification product of the *a* allele was detected, which had not previously been reported as it would not have been detectable using agarose gel electrophoresis. However, it is not known if this variation has a functional significance. No variants were detected for allele *b*.

5.4.2. Discrepancies in reported sizes

The difference in size for alleles *a* and *b* detected in the current study using the fluorescently labelled ACS1-5 primers, 514 bp and 652 bp, differing by 138 bp. This differed markedly from allele sizes 489 bp and 655 bp differing by 166 bp reported by Sunako *et al*. (1999) using unlabelled ACS1-5 primers and several subsequent studies using these primers (Harada *et al*., 2000; Oraguzie *et al*., 2004; Oraguzie *et al*., 2007; Zhu and Barritt, 2008; Zoufalá *et al*., 2009). The insertion and deletion phenomena explained by Sunako *et al*. (1999), account for a difference of 138 bp between the *a* and *b* alleles. Fluorescent labelling can introduce a minor shift in product length (Sutton *et al*., 2011), so the 137 bp difference observed when using a different fluorescent label, PET, for the ACS1-Pr primers rather than the expected 138 bp is not surprising. The apparent mis-scoring of alleles in all previous studies may be attributed to misinterpretation of the indel phenomenon, in particular neglecting to account for 24 bp deletion associated with the 162 bp insertion, as well as the use of agarose gel electrophoresis, which is not the most accurate method for sizing amplification products.

5.4.3. Comparison of ARC genotypes with published *ACS1* genotypes

There are several possible explanations why ten of the 64 *M. pumila* accessions had *ACS1* genotypes inconsistent with the published genotypes. It is possible that seven of the cultivars are false as no reference genotypes are available for confirming trueness to type during fingerprinting (Chapter 3), namely 'Delblush', 'July Red', 'Jumbo Orin', 'Liberty', 'Resista X', 'Resista Y' and 'Sansa'. However, three accessions, 'Blenheim Orange', 'Rome Beauty' and 'Cox's Orange Pippin', did appear to be true to type based on microsatellite fingerprinting (Chapter 3). It should be noted that some of the reports in the literature may be incorrect. In the case of 'Granny Smith', the genotype which was reported as *aa* by Oraguzie *et al*. (2004) but *ab* by other studies (Sunako *et al*., 1999; Harada *et al*., 2000; Peace, 2014). For all ten of these accessions, it is recommended that the microsatellite fingerprints and the *ACS1* genotypes are verified on material sourced from reliable international gene banks.

No inconsistencies were observed for the three *Malus* accessions when compared with reported genotypes for similar named accessions in other studies. However, in two cases the variant allele a_{204} was detected in addition to a_{202}.

5.4.4. ARC *ACS1* genotypes

A total of 235 *M. pumila* cultivars, 20 selections of *M. pumila* and 36 *Malus* species and hybrids were genotyped but only 24 *bb* cultivars were observed. The collation of *ACS1* genotypes from the literature (Table 1.1) also indicates a scarcity of *bb* genotypes associated with low ethylene production in international apple collections.

The *ACS1* genotypes of 171 'primary' cultivars of *M. pumila* and 20 selections of *M. pumila* in the ARC gene bank genotyped in the current study had not been previously reported. There were only 18 *bb* genotypes, homozygous for low ethylene production, observed in the 191 cultivars and selections of *M. pumila*. Nineteen of the 171 *M. pumila* accessions were triploid and genotypes of five of these accessions, 'Blenheim Orange', 'Jonagold', 'Mutsu', 'Red Gravenstein' and 'Reinette du Canada' had been reported previously. It was not possible to distinguish *aab* from *abb* genotypes in triploids by differential peak sizes.

For 34 *Malus* species and hybrids analysed, genotypes have not been previously reported. A more detailed survey of other *Malus* species and hybrids may reveal how length variation with respect to the *a* allele occurred in relation to speciation, and to establish if it exists in only sections of *Malus*.

The knowledge of *ACS1* genotypes associated with ripening will benefit not only the ARC breeding programme but, once published, will guide choice of parents for breeding low ethylene producing cultivars in other apple improvement programmes.

5.4.5 Hardy-Weinberg Equilibrium statistics

The results observed for both *M. pumila* cultivars and selections of *M. pumila* indicate no evidence of deviation from HWE, and thus no differential selection for a particular genotypic class.

5.4.6. Genetic resources and breeding

Prolonged storage ability, which is necessary for fruit shipped to international markets, is an important objective in the ARC breeding programme. The low proportion of homozygous *bb* genotypes in the gene bank, 24 of the 291 accessions, indicates the need for additional lower ethylene producing accessions for use as parents in breeding. Additional cultivars identified as homozygous for the *bb* genotype (Table 1.1) can be incorporated into the ARC gene bank if available in South Africa. Two of these, 'Huaguan' and 'Minneiska', are registered in the South African national variety list. Knowledge of *ACS1* genotypes of the gene bank material will inform the design of crosses. Combining cultivars with *bb* genotypes will ensure the seedlings do not segregate for the trait. The Washington State University apple breeding programme is already intercrossing homozygous *bb* parents (Peace, 2012). In cases where heterozygous *ab* parents are crossed with each other or with *bb* homozygotes, MAS can be used to select seedlings with the homozygous *bb* genotype.

5.4.7. Marker-Assisted Selection (MAS)

Detection of the desirable low ethylene production trait at an early age using *ACS1* primers can be used instead of phenotypic characterisation, which can only be conducted once the tree starts bearing fruits after several years. Even so, phenotypic characterisation of ripening is complex as expression may differ due to environmental conditions (Kellerhals *et al*., 2000; Zhu and Barritt, 2008). Adoption of *ACS1* markers for Marker-Assisted Selection will enable selection of homozygous *bb* seedlings from appropriate parents as early as one week from germination (Costa *et al*, 2005). Although, *ACS1* is an important factor influencing fruit firmness before harvest (Atkinson, 1998), other genes such as *1-Aminocylopropane-1-oxidase*, *ACO1* (ethylene production), *ACS3* (*ACS1* accelerator), *Polygalacturonase*, *PG* (post-harvest softening) and *Expansin*, *Exp7* (fruit softening) (Costa *et al*., 2005; Costa *et al*., 2008; Wang *et al*., 2009;

Varanasi *et al*., 2011; Nybom *et al*., 2012) are also important in the ripening pathway and affect the overall storability of apple fruits. Markers linked to these genes are available or are being developed. Washington State University is already using *ACS1* and *ACO* in Marker-Assisted Selection (Peace, 2012).

5.4.8. Relevance to export market

Excessive application of ethylene inhibitors during storage and pre-storage may leave excess residues on fruits which could exceed the maximum acceptable level and subsequently result in rejection of shipments (Johnson *et al*., 2002). Release of new cultivars homozygous for the *bb* genotype, should have good storage and firmness associated with natural low ethylene production. This will ensure successful exports with minimum application of ethylene inhibitors and less dependence on post-harvest environments (Gorny and Kader, 1996; Zhu and Barritt, 2008). The current study will facilitate the use of appropriate parents, homozygous for the *b* allele or heterozygous, *ab*, to increase the chance of obtaining good quality late keeping cultivars for the export market.

5.4.9. Pear *ACS1* investigation

The ACS1-Pr and ACS1-5 primers amplified a single product in the *Pyrus* species, *P. calleryana,* and the hybrids 'Garber' and 'Kieffer'. The failure of these primers to amplify in the European pears could be attributed to the absence of appropriate primer binding sites in the *P. communis* promoter region or, perhaps, to a large insertions preventing amplification.

The 199 bp allele observed in the two *Pyrus* hybrids and *P. calleryana* may essentially be the same as the *a* allele in *Malus*, with the small difference in product length a consequence of minor differences in the promoter sequence length. The absence of the *b* allele in three indivduals giving successful amplification could indicate that the insertion of the SINE known in *Malus* occurred after the evolutionary divergence from *Pyrus*. However, a wider range of accessions, and species, needs to be analysed to test this proposition.

With the recent publication of the *P. communis* genome (Chagné *et al*., 2014), it will be interesting to examine the sequence in the region of the ACS1 promoter to understand why there is a failure of the *Malus* ACS1-Pr and ACS1-5 primers and to guide more appropriate primer design.

5.4.9. Multiplexing ACS1-Pr with apple microsatellites

The use of markers for agronomic traits in multiplex reactions with microsatellites, or with other known function genes, is cost effective compared to genotyping large collections with single markers. Fingerprinting and characterisation of the ARC Phase 2 selections will benefit from the findings of the current study as microsatellite fingerprinting and *ACS1* genotyping can be achieved in one reaction thus saving costs.

5.5. Concluding remarks

The fluorescently labelled ACS1-Pr primers were useful for assigning *ACS1* genotypes to the accessions in the ARC apple gene bank collection, 225 of which were genotyped for the first time. Novel variants were detected for allele *a* in *Malus* species accessions. Discrepancies in the reported allele sizes were highlighted and correct sizes were proposed. The multiplexing potential of the ACS1-Pr primers was demonstrated. The *ACS1* phenomena in pear, however, still require further studies. More effort is needed to acquire good quality homozygous *bb* genotypes for use in the ARC breeding programme. Breeding cultivars homozygous for the *b* allele will assist in addressing the storage challenge faced by South African fruit exporters. Additionally, second economy farmers who can not affort sophisticated post-harvest environment will be able to store the fruits longer. The functional and evolutionary significance of the variation observed in *Malus* species and hybrids in the current study is not yet understood. Characterising germplasm collections for other 'known function' genes such as *ACO* and *S*-incompatibility in addition to *ACS1* will provide further information needed by breeders to plan crosses and align their breeding programmes with industry needs.

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Chapter 6

GENERAL DISCUSSION AND FUTURE CONSIDERATIONS

6.1. Introduction

Apples and pears are important export crops in South Africa contributing greatly to the economy of the country. Global warming, diseases, and ever changing consumer preferences locally and in export markets necessitate constant development of new cultivars to meet these challenges; better storage potential resulting in extended shelf life is particularly important. Reliable genetic resources are the breeders' raw material for conducting informed crosses to develop new cultivars and to undertake the underpinning genetic studies. Five years ago, the Agricultural Research Council's (ARC) pome fruit gene banks at ARC Infruitec-Nietvoorbij, comprising 540 apple and 197 pear accessions, were identified as inadequate, unverified and poorly characterised as well as limited in range. Funding for molecular studies secured from the Technology and Human Resources for Industry Programme (THRIP) has allowed the pome fruit genetic resources to be fingerprinted with microsatellite markers to confirm trueness to type. In the case of apple, accessions that were true to type were additionally characterised for an agronomic trait, *ACS1*, concerned with ethylene production and ripening. The current study is the first application of molecular markers to deciduous tree fruit gene banks in South Africa.

6.2. Microsatellite fingerprinting of pome fruit collections

A key point in the fingerprinting of the apple and pear collection was the use of standard microsatellite markers and reference cultivars recommended by the ECPGR *Pyrus*/*Malus* working group. In addition verification was facilitated by the existence of fingerprints for the United Kingdom (UK) collections at Brogdale to which comparison could be made. The pear fingerprints from Brogdale were available for the current study and comparison to the apple fingerprints will be made as soon as these are provided.

The use of markers in recommended multiplex reactions, four per multiplex reaction, proved cost effective considering the combined sample size of 737 accessions in the current study. Approximately, 75% saving in sequencing cost was realised for fingerprinting both the apple and pear collections. Calculations are based on the current economical and technological conditions. It should be noted that one apple microsatellite and four of the pear microsatellite markers consistently failed or gave unsatisfactory amplification in the current study, although it is not clear that multiplexing was the cause of the failure, and similar failures for certain microsatellites have been reported in other studies using the recommended multiplexes.

However, for most cases even four polymorphic markers are enough to discriminate between accessions although more markers may be needed for discrimination of siblings (Hokanson *et al*., 1998).

Although microsatellites are polymorphic, highly transferable and cost effective markers once developed, they are not appropriate for discriminating among clones of cultivars (Guichoux *et al*., 2011). This characteristic enabled confirmation of trueness to origin of clones in the current study *e.g*. 24 clones or sports of the apple cultivar 'Delicious' and 11 clones or sports of the pear cultivar 'William's Bon Chretien'. In cases where clonal discrimination is necessary, other marker types, such as S-SAP, AFLP, and SNPs, have been suggested as an alternative as demonstrated in grape (Venturi *et al*., 2006) and pear (Dondini *et al*., 2007).

The advent of Next Generation Sequencing (NGS) technologies enables characterisation and detection of Single Nucleotide Polymorphisms (SNPs), which can be used for fingerprinting as an alternative to microsatellite markers. These markers might supersede microsatellite fingerprinting in the near future due to the speed of use, high accuracy of allele calling and the ability to discriminate among clones (Vignal *et al*., 2002; Korir *et al*., 2012). However, despite the advances and generation of large number of SNPs in apple (Chagné *et al*., 2012) and pear (Montanari *et al*., 2013), a simple method for applying this technique to cultivar identification has not yet been developed (Korir *et al*., 2012). In addition, the data generated by SNP markers requires some level of bioinformatics understanding and is thus not user-friendly to fruit breeders and gene bank curators, who often have limited molecular genetics and bioinformatics backgrounds.

6.3. *ACS1* genotyping of apple collection

The newly designed fluorescently labelled ACS1-Pr primers were informative in apple for detecting the differences in *ACS1* genotypes caused by indels in the promoter region of the gene that correlate with ethylene production: *aa*, high ethylene, *ab*, medium ethylene and *bb*, low ethylene. The current study represents the first use of fluorescently labelled *ACS1* primers for characterising apple genotypes with an automated sequencer. It proved to be a sensitive method. Minor variations in the *a* allele, which were not previously reported, were detected and, in addition, discrepancies in the reported relative sizes of the principal alleles were identified and clarified. Fluorescent genotype proved to be time- and cost efficient as

genotyping by agarose gel electrophoresis can be laborious when dealing with large sample sizes and does not allow precise determination of product sizes.

Moreover, the taxonomic distribution of the *a* allele variants and the *b* allele paves way for further study in relation to the speciation of *Malus*. The reasons for success of the primers in a small set of non *P. communis* accessions but their failure in *P. communis* would also be interesting to study. A comparative study of the *ACS1* gene in different pear species with the published pear and apple genomes might guide design of pear specific primers.

6.4. Gene bank curation

The microsatellite fingerprinting will facilitate proper management of the ARC's apple and pear gene bank collections. Names can be confirmed and corrected. Fingerprints recorded in the current study will be incorporated into the gene bank databases for both fruit crops providing useful characterisation data in accord with good gene bank management practice. However, the possibility of developing a web-based database is being considered. When the collections are repropagated, they will be verified by comparison with reference fingerprints rather than phenotypically after several years.

All the accessions identified as false in the current study will be discarded; 78 of the 540 apple accessions (69 *M. pumila*, two *M. pumila* selections and seven other *Malus* representatives), and 22 of the 197 pear accessions (16 *P. communis*, five ARC selections of *P. communis* and one *P. pyrifolia*). Removal of false accessions will create space for the acquisition of more useful true to type accessions (Tobutt, personal communication).

Microsatellite markers are currently being employed for fingerprinting the ARC stone fruit gene banks (Kwalimba and Nyawo, personal communication). Similar studies of other perennial fruit crop gene banks such as those of cherry, fig and olive maintained by the Horticulture Division, ARC Infruitec-Nietvoorbij are desirable, although currently ARC does not conduct breeding programmes for these crops. Recently, the ARC Institute for Tropical and Subtropical Crops (ITSC) has started fingerprinting the citrus rootstock collection in collaboration with ARC Infruitec-Nietvoorbij (Bijzet *et al*., 2014). Other fruit crop collections, such as guava, litchi, mango and avocado, still need to be fingerprinted.

Similarly, the *ACS1* genotypes recorded in the current study will prove useful for guiding breeder choice of parents in the gene bank. Incorporation of characterisation data is already a common practice in international gene banks. The ARC collections will benefit from further characterisation of other useful agronomic traits, such as *ACO* also involved in the ethylene production pathway with allelic variation known to correlate with ethylene production. Cultivars that are homozygous for *ACS1* (*bb*) and *ACO* (*bb*) produce low levels of ethylene and display prolonged storage potential (Zhu and Barritt, 2008). Another gene, *S*-incompatibility, for which primers are also available to discriminate alleles, determines cross-compatibility in pome fruits. Accessions with the same genotype are cross-incompatible, accessions with one allele in common are semi-compatible and accessions with no allele in common are cross-compatible (Kobel *et al*., 1939; Ishimuzi *et al*., 1998).

6.5. Application to pome fruit breeding

Henceforth, crosses in the apple and pear breeding programme at Infruitec-Nietvoorbij can be made with greater confidence since the parents are known to be true to type; moreover, it will be simple and fast to detect false accessions once the microsatellite data is incorporated into the gene bank database. Futhermore, seedlings can now be verified for trueness to parentage at an early age.

Knowledge of *ACS1* genotypes will be very useful. Homozygous *bb* cultivars can be intercrossed to ensure that all seedlings arising from the cross are low ethylene producers with no segregation for the trait, dispensing the need for Marker-Assisted Selection (MAS) and allowing the breeder to focus on other traits of importance *e.g*. taste. In addition, seedlings from heterozygous parents, *ab*, can be screened at an early age using MAS, and the undesirable seedlings with potentially high ethylene production, *aa* and *ab*, can be discarded (Zhu and Barritt, 2008).

Genotyping for the *ACS1* trait will also be useful for predicting the storage capability of the advanced selections in Phase 2 at the ARC, which are being evaluated for release to the industry.

6.6. Application to the pome fruit industry

The data set generated will serve as reference data for identification of apples or pears in future. It would be desirable to fingerprint all the cultivars on the South African national variety list and a proposal to the Department of Agriculture, Forestry and Fisheries for this work is currently being considered by ARC.

In the last year, microsatellite genotyping has proved very useful in the rootstock trade in South Africa. As a direct application of microsatellite fingerprinting resulting from this project, more than 110 supposed 'M9' apple rootstocks plus several control rootstocks were analysed using the recommended microsatellite markers, at the request of HORTGRO Science on behalf of the industry. Approximately 25% of the supposed 'M9' rootstocks proved not to be 'M9'. Such mistakes are not easily detectable in the orchards and even for the nursery there are limited morphological descriptors. In pear, analyses were conducted to test the trueness to type of rootstocks, 'BP1' and 'BP3', at the request of the Deciduous fruit Plant improvement Association (DPA), also known as the Sagtevrugte Plantverbeterings Vereniging (SPV). During the finalisation of this thesis, a further request was received from the South African Plant improvement Organisation (SAPO) to test the trueness to type of two apple rootstocks, 'M9' and 'M793', for some growers.

Although a high quality assurance system is employed in propagation of plant material commercially, the investigation for HORTGRO Science above proved that mislabelling does occur. Thus HORTGRO Science and deciduous fruit nurseries now acknowledge the importance of microsatellite fingerprinting as a technique to supplement the morphological characterisation being employed for rootstock cultivar identification (Kotze and Steyn, personal communication). The stone fruit rootstock industry will also benefit from similar studies that are currently in progress at ARC Infruitec-Nietvoorbij (Kwalimba and Nyawo, personal communication). The ARC ITSC institute, which propagates plant material for the tropical and subtropical industry may need to consider adopting microsatellite markers as a supplement to morphological characterisation.

Development of naturally low ethylene producing cultivars, homozygous *bb*, holds promise for the pome fruit export market as it will reduce the costs involved with post-harvest preservation of pome fruits during export. Cost of pre-harvest chemicals used for delaying ethylene production can be greatly reduced thus saving on the operational costs incurred by the pome fruit farmers.

Microsatellite fingerprinting can be useful for identifying unknown apples and pears in private gardens and heritage gardens. In addition, commercial farmers can now investigate whether the plant supplied is what it is supposed to be.

6.7. Limitations of the study

Four microsatellite markers in pear and one apple marker, failed to amplify satisfactorily in the current study. Although the failed markers did not hamper discrimination of accessions in the current study, the missing data could be useful in future. Thus further optimisation is still recommended for the markers that failed in order to obtain a full data set to facilitate comparison with other studies that used the same 12 markers. The shifts observed between the ARC and the Brogdale pear fingerprints was in some cases complex, complicating data set comparisons. The accumulated apple data still needs to be compared with the Brogdale fingerprints to confirm trueness to type as those data were not available at time of preparing this thesis.

6.8. Future considerations

The current study is a model for future work and the DNA extracted is available for further characterisation studies. Other agronomic genes such as *S*-incompatibility concerned with cross-compatibility in apples and pear, and *ACO*, need to be characterised together with other recently sequenced genes in apple such as Malic acid (*Ma*) (Powell *et al*., 2014), concerned with fruit acidity. Characterisation of gene banks for known function genes is useful in preparation for MAS of seedlings.

Genetic resources are the breeders' raw material and use of true to type accessions contributes to the efficiency of breeding programmes. The current study has demonstrated the utility of microsatellite fingerprinting for resolving misidentifications, not only in the ARC gene bank but in the industry as well. The *ACS1* genotypes detected presents candidate parents for breeding low-ethylene producing cultivars that store well and will save costs to producers and the export industry. Genotyping for *ACS1* benefited from trueness to type studies as false accessions were omitted. The fingerprinting and *ACS1* characterisation used automated sequencing and it was demonstrated that these markers can be combined into the same

multiplex reaction and data can be recorded similarly in gene bank databases. The acquisition of both fingerprinting and genotyping data will enable better annotation of the ARC gene banks and provide an example of good gene bank management practice for other perennial gene banks. The current study represents a first crucial step in incorporating molecular genetics into the ARC pome fruit breeding programme.

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Appendices

Appendix 3.1. Genotypes for eight microsatellite markers of pear accessions in the ARC gene bank at Bien Donne Experimental Farm (WG8) with tree location and name. Accessions supposed to be clones derived from the same cultivar are grouped together, In note column, F indicates false accessions. In class column, 1, 2, 3, 4, and 5 represent categorisation of pear fingerprint data.

Tree	Name	Note	CH01d09			CH02b10			CH03d12			CH03g07			CH04e03		CH05c06			EMPc11			EMPc117			Class
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	1	2	3	1	2	3	1	2	3	
ARC selections of *P. communis*																										
1_12	3C-11-9		124	134		123	135		108	110		215	243		182	201	93	99		150	155		106	116		1
1_24	3C-11-25		130	136		123	139		112	125		227	232		182	192	89	93		140	146		112	116		1
1_40.	3C-44-34	F	134	151		129	144		112	125		227	246		182		93	113		140			118	120		1
2_16	3C-49-18		130	157		129	135		108	125		227	232		182		89	93		150	155		116			1
3_43	3C-51-28		130	157		123	129		108	125		227	243		182	207	89			150			116			1
6_7	3D-83-10		130	134		123	133		108	125		232	243		182		89	93		150	155		116	120		1
5_16	11B-2-25		136	149		121	129		125			227			207		89	93		150			116			1
5_13	11B-3-17		157			123	133		108			227	243		182	207	93			150			116	120		1
5_17	11B-7-17		130			135	146		125	129		227	232		182		89	93		137	150		114	116		1
5_18	11B-7-21		130	157		135	146		110	125		232	245		182		89			146	155		114	116		1
5_19	11B-7-26		130			135	146		110	125		243	245		200		89			137	150		114	116		1
5_20.	11B-7-28		149	157		135			110	125		227	232		182		89			137	155		114	116		1
6_15	11C-6-27		134	149		123	129		108	112		227			207		89	93		150			89	120		1
6_13	11C-9-11		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		1
5_21	11D-10-9		130	136		123			108	125		227	232		182	192	89	121		146	150		112	116		1
5_14	15A-4-14		134	149		123	129		108	112		227	243		182	207	89	93		150			89	116		1
5_15	15A-7-21		134	149		123			108	125		227	243		207		93			150			116	120		1
6_14	15B-5-2		140	149		129	139		108			227	228		182		89	93		150			116			1
6_16	5-03-29		124	149		129	135		125			215	227		207		93	109		137	150		89	116		1
2_24	5-03-29		124	149		129	135		125			215	227		207		93	109		137	150		89	116		1
5_1	5-16-122		149	151		123	131		110	125		215	243		207		93	109		137	150		116			1
6_6	5-16-122		149	151		123	131		110	125		215	243		207		93	109		137	150		116			1
1_28	5-16-89		151	157		123	135		108	110		227	248		182	207	93	109		150	155		89	106		1
4_17	5-16-89		151	157		123	135		108	110		227	248		182	207	93	109		150	155		89	106		1
3_33	5-17-169		151	157		123	131		108			215	227		207		89	99		137	150		116			1
3_39	5-19-27		140	157		129	135		108	125		227	243		182		89	113		140	150		89	91		1
3_11	5-24-21		149	151		123	144		108	112		227			182	207	93	113		150			116	118		1
2_4	5-25-21		149	151		123	144		112	125		243			182	207	93	113		140	150		116	118		1
2_28	5-31-79		157			123	135		108	125		232	243		182		89	93		150	155		89	118		1
2_37	5-32-8		130	157		123	135		108	125		243			182		89			150			89	116		1
2_31	5-32-53		130	149		129	135		108	125		232	243		182		89	93		150			89	116		1
3_31	5-36-30		157			129	135		108	125		232	243		182	207	89	93		150	155		89	116		1
2_43	5-39-60		124	157		123	135		108	110		215	227		182	207	93	99		150	155		106	116		1
3_27	5-40-45		149	151		129	135		108	110		227	248		182	201	93	109		137	150		89	106		1
3_19	5-40-60		149	151		131			110	125		215	227		207		89	109		150	155		116			1

Tree	Name	CH01d09			CH02b10			CH03d12			CH03g07			CH04e03		CH05c06			EMPc11			EMPc117			Class
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	1	2	3	1	2	3	1	2	3	
3_37	5-41-18	124	149		129	131		108	110		215	227		182	207	89	99		137	150		106	116		1
6_11	5-41-57	136	149		121	123		125			227	260		192	207	93	121		146	150		112	116		1
1_10.	8-20-58	140	149		123	144		112			243			182		89	109		150			89	120		1
2_41	8-22-120	135	149		123	129		108	125		243	260		182	207	89	121		136	150		116	118		1
3_29	8-23-81	124	157		129	135		108	110		215	243		201	207	93	109		137	150		89	116		1
2_18	8-24-25	124	149		131			108			215	243		182	207	93	99	109	150	155		116			1
5_3	8-24-51	124	149		129	131		108			215	243		182	207	93	99		150	155		116			1
4_22	8-24-63	124	149		129	131		125			215	243		200	207	93	109		150	155		89	116		1
2_29	8-25-25	151	157		123	135		125			243	248		182	201	93	109		150	155		89	116		1
3_25	8-25-48	149	161		121	133		125			227	232		182	207	93	121		144	150		102	112		1
2_22	8-25-57	149	151		123	131		108			227	248		182	201	89	99		137	150		106	116		1
4_19	8-25-57	149	151		123	131		108			227	248		182	201	89	99		137	150		106	116		1
1_38	8-25-72	124	149		123	131		108	110		227	248		207		93	99		138	150		116			1
1_16	8-26-91	151	157		129	135		125			227	243		182		93	113		150			89	118		1
3_23	8-28-59	149	157		123	129		108	125		232	243		182		89	93		150			116	118		1
2_20.	8-30-145	124	149		129	131		108			215	227		207		93	109		137	150		106	116		1
4_18	8-30-145	124	149		129	131		108			215	227		207		93	109		137	150		106	116		1
1_30.	8-31-158	124	149		123	131		108			215	243		182	207	93	99		150	155		106	116		1
5_2	8-31-158	124	149		123	131		108			215	243		182	207	93	99		150	155		106	116		1
6_1	8-31-23	124	157		129	135		125			215	226		182	207	89	99		150	155		116			1
1_44	8-31-67	149	151		129	131		110	125		227	248		207		93	99		150	155		116			1
4_21	8-31-67	149	151		129	131		110	125		227	248		207		93	99		150	155		116			1
1_34	8-33-53	124	157		129			124	125		227	248		207		93	99		150	155		106	116		1
1_14	8-34-54	130	149		129	135		108	125		232	243		182		89	93		150			89	116		1
1_22	8-34-91	149	161		121	133		125			227	232		182	207	93	121		144	150		102	112		1
3_41	8-6-34	136	149		121	123		108	125		243	260		182	207	89	121		150			112	116		1
3_2	8-9-14	130	149		129	135		125			232	243		182		89	93		150	155		89	118		1
Cultivars or derivatives of *P. communis*, European pear																									
1_4 R	Abate Fetel i	151	153		129			108	112		243	246		182	200	89	93		144	150		116	118		2
6_22	Abate Fetel ii	151	153		129			108	112		243	246		182	200	89	93		144	150		116	118		2
1_1	Bergamotte de Esperance	153	157		133	135		125			227	243		182		89	109		150			100	116		2
4_3	Beth	140	157		123	139		108	112		232	243		182		89	93		150			116			2
1_3	Beurre Bosch	130	153		129	133		125			243	257		182		89			144	150		94	116		2
3_21	Beurre Bosch	149	161		121	133		125			227	232		182	207	93	121		144	150		102	112		4
6_18	Golden Russet	130	153		129	133		125			243	257		182		89			144	150		94	116		3
6_23	Boscova	130	153		129	133		125			243	257		182		89			144	150		94	116		3
1_5	Beurre Clairgeau	142	153	155	123	129	133	120	125	161	227	236	243	182	200	93	97	111	144	150	158	98	116		4, 3*x*
1_9	Beurre Giffard	138	153		129	133		108	120		227	243		182		89			146	150		114	120		2
1_11	Beurre Hardy	130	161		131	135		112	125		243	257		182		89	93		150	155		116	120		2
4_14	Beurre Hardy	130	161		131	135		112	125		243	257		182		89	93		150	155		116	120		2

Tree	Name		CH01d09			CH02b10			CH03d12			CH03g07			CH04e03		CH05c06			EMPc11			EMPc117			Class
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	1	2	3	1	2	3	1	2	3	
3_34	Beurre Hardy (Emla)		130	161		131	135		112	125		243	257		200		89	93		150	155		116	120		3
3_32	Beurre Hardy Sport	F	130	140		135	139		108	112		228	232		182		89			140	150		116			4
1_13	Beurre Six		134	151		135	144		110	125		243	257		182	207	93	109		150			116	118		2
1_15	Beurre Superfin		130	140		135	139		108	112		228	232		182		89			140	150		116			2
1_17	Beurre van Geerdt		130	138		123	129		108	125		243			182		89	93		150	155		116	120		2
3_46	Bartlett		134	157		123	129		108	125		227	243		182	207	89	93		150			116			2
5_4	Burger BC		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		2
2_35	Bon Chretien i		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		2
3_40.	Bon Chretien ii		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		2
3_20.	Bon Chretien (Koo)		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
1_19	Bon Chretien A		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
1_21	Bon Chretien B		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
1_23	Bon Chretien C		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
1_25	Bon Chretien D		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
1_27	Bon Chretien E		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
1_29	Bon Chretien Sport		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
1_8	William's Bon Chretien		130	149		123	129		108	125		227	243		182	207	89	93		150			89	116		2
3_28 R	William's Bon Chretien		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		2
1_26	El Dorado		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
3_16	El Dorado		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
5_5	El Dorado (VV)		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		3
1_31	Bon Rouge		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		2
2_12	Bon Rouge		149	157		123	129		108	125		227	243		182	207	89	93		150			89	116		2
SAPO	BP 1		124	149		123	148		125			215	260		192	207	81	109	121	137	146		106	116		1
1_06	BP 2		132	149		121			132			260			182	207	93	101		144	150		116	120		1
1_18	BP 2		132	149		121			132			260			182	207	93	101		144	150		116	120		1
6_19	Cascade		149	157		108	123		108			232	243		200	207	89	93		150	155		116			2
SPV	Celina		140	157		140	157		108	112		243	257		182		89	93		150			114	120		1
4_9	Ceres		149	151		123	135		108	110		227	243		182		89			140	150		116	118		1
4_11	Ann's Favourite		134	157		123	133		108	112		227	243		182	207	93			150			116	120		1
1_33	Clapp's Favourite		134	157		123	133		108	112		227	243		182	207	93			150			116	120		2
3_17	Starkrimson		134	157		123	133		108	112		227	243		182	207	93			150			116	120		2
3_24	Starkrimson		134	157		123	133		108	112		227	243		182	207	93			150			116	120		2
3_14	Colonel Wilder		157	161		123	133		108	125		243	245		182		93			150	155		98	116		1
6_24	Concorde		151	157		125	135		108	112		229	257		182		89			140	150		116	120		2
1_37 R	Conference		157			125	129		108	125		227	257		182	207	89	99		140	150		118	120		2
3_42	Conference		157			125	129		108	125		227	257		182	207	89	99		140	150		118	118		2
1_35	Contesse de Paris		130	151		123	139		128			232	257		182		89	113		140	150		118	120		2
4_2	Cristalli		140	142		127	139		92	110		232	267		182		89	113		140	144		93	118		1
1_7	Beurre d'Anjou		151	153		133	139		110	125		248	257		182		95	113		140	150		116	120		2
6_3	Red d'Anjou	F	149	151		129	131		108	110		248	257		182		89	99		150	155		89	106		4

Tree	Name		CH01d09			CH02b10			CH03d12			CH03g07			CH04e03		CH05c06			EMPc11			EMPc117			Class
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	1	2	3	1	2	3	1	2	3	
1_20.	December		130	149		135	146		110			227	245		182		89			137	146		93	114		1
1_39	December		130	149		135	146		110			227	245		182		89			137	146		93	114		1
4_8	Delbard Precoce		149	157		123			108	125		243	267		182		93	95		140	150		116	118		1
4_6	Delbard Premiere		128	149		123	135		108	131		243	269		182		89	101		144			110	116		1
6_29	Delete		149	151		123	139		112	125		227	228		182	207	89			150	155		116			1
6_28	Delmoip		153	157		133	139		108	125		227			182	200	89	109		144	150		116	118		1
6_26	Delmore		153	157		133	139		108	125		257	269		182	200	89	109		144	150		116	118		1
6_17	Delwilmore		149	153		129	135		108	125		232	243		182	200	93	109		150	155		116	118		1
5_10.	Red Comice		151	157		135	139		108	112		225	225		182	200	89			150	155		116			2
1_41 R	Doyenne du Comice		151	157		135	139		108	112		228	232		182	200	89			150	155		116			2
3_38	Doyenne du Comice (Emla)		151	157		135	139		108	108		227	232		182	200	89			150	155		116			2
4_5	Dr Jules Guyot		149	153		123	129		108			232	243		200	207	93	99		140	150		116			2
1_43	Duchesse d'Angouleme		140	157		129	139		108	125		227	232		182	200	89	93		140	155		98	116		2
1_45	Duchesse de Bordeaux		138	151	153	121	123	139	110	125		232	243		182		89	93	113	140	150	156	98	118		4, 3*x*
2_1	Emile d'Heyst		138	151		123	133		125			227	245		182		89			144	155		100	116		2
3_9	Emperor		124	149		131			124	125		215	245		201	207	93	109		150	155		89	116		1
1_2	Flamingo		124	149		123	131		108	110		215	227		182	207	89	99		150	155		116			1
2_8	Flamingo		124	149		123	131		108	110		215	227		182	207	89	99		150	155		116			1
4_20.	Flamingo		124	149		123	131		108	110		215	227		182	207	89	99		150	155		116			1
2_3	Fondante d'Automne		151	153		123	129		112	125		243	257		182		89			150			114	118		2
2_5	Forelle		124	151		131	135		110	124		215	248		201	207	99	109		137	155		106	116		2
6_21	Forelle Malherbe	F	130	149		123	131		108	125		243			182		89			150	155		116	120		4
2_7	Ganzels Bergamotte		153	161		121	125		125			243	257		182		89	93		150	155		116	120		2
3_15	General Leclerc	F	149	161		121	133		125			227	232		182	209	93	121		144	150		102	112		4
4_10.	General Leclerc	F	140	151		123	129		108	125		228	232		182	207	89			140	144		116	118		2
6_12	Glou Morceau	F	140	151		135	144		112	125		227	243		182		109	113		140	150		118			4
4_13	Harrow Delight		142	149		123	125		110	125		227	236		182		89	93		140	150		112	116		2
2_13	Hertzogin Elza		140	157		133	139		108	112		245	257		182		89	93		140	150		100	120		2
4_7	Highland		149	155		123	139		108	125		227	232		182	200	89	93		150			116			2
6_27	Jana		130	151		129			125			232	243		182		89	109		150	155		94	118		1
2_15	Josephine de Malines	F	149	161		121	133		125			227	232		182	207	93	121		144	150		102	112		4
4_16	Kalbas Peer		130	161		125	129		114	125	130	227	243	257	182	199	89	93		144			89	110	118	1
6_20.	Lily		151	157		129	135		112	125		227			182	207	89	113		150			89	120		1
2_21	Louise Bonne A		140	153		129	133		125			227	239		182		89	105		140	150		116	120		2
2_23	Louise Bonne B		140	153		129	133		125			227	239		182		89	105		140	150		116	120		2
2_25	Louise Bonne C		140	153		129	133		125			227	239		182		89	105		140	150		116	120		2
2_27	Lucas		151	157		129	133	144	108	125		227	257		182		89	95		140	150	155	100	114	116	2, 3*x*
2_30.	Magnate		138	153		137	152		125			246	248		182	207	89	93		140	150		116	120		1
2_32	Marguerite Marillat		149	153		123	129		108	125		232	243		182	207	93	99		140	150		116			2
2_6	Morettini 64		149			129	146		125			243	245		182		89	93		137	150		89	114		1
4_4	Morettini 64		149			129	146		125			243	245		182		89	93		137	150		89	114		1

Tree	Name		CH01d09			CH02b10			CH03d12			CH03g07			CH04e03		CH05c06			EMPc11			EMPc117			Class
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	1	2	3	1	2	3	1	2	3	
6_5	Mostert 51		138	149		123	139		108			239	260		182	192	89	93		146			112	120		1
5_12	Nassau Strydom		130	157		123	129		108			226	228		182	207	89			142	150		116	122		1
2_10.	Old Home	F	149	153		123	133		112	125		227			182		89			150			116	118		4
3_4	Old Home	F	153	157		123	129		108	112		243	257		182	207	89	93		150			116			4
3_44	Onward	F	151	157		135	139		108	112		227	232		182		89			140	155		116			2
6_9	Onward	F	124	149		129	135		110	125		227	248		182	207	93	99		137	150		116			2
2_34	Orange Bergamotte		130	138		123	129		105			243	245		182	207	95	109		144	152		93	116		1
3_22	Packham's Triumph		130	157		123	135		108	125		232	243		182		89	93		150	155		116	118		2
3_18	Packham's T. (Brown)		130	157		123	135		108	125		232	243		182		89	93		150	155		116	118		3
4_15	Packham's T. (VV)		130	157		123	135		108	125		232	243		182	200	89	93		150	155		116	118		3
2_40 R	Passe Crassane	F	140	151		135	144		112	125		227	243		182		109	113		140	150		118			2
3_26	Passe Crassane	F	140	151		135	144		112	125		227	243		182		109	113		140	150		118			2
2_36	Patrick Barry		130	153		129	135		125			233	257		182		89	109		140			118	120		1
2_38	Precoce de Trevoux		130	157		123	135		108	125		232	243		182		89	113		150			89	118		2
4_1	Reimer Red		151	157		123	139		108	112		228	257		182		89			150			89	120		2
2_42	Roosevelt		134	157		129	133		112	125		243	245		182	207	93			140	150		120			2
5_11	Rosemarie		130	149		135	146		110			227	245		182		89			137	146		93	114		1, 4
6_4	Ruby Glo		124	149		123	131		108	110		215	243		201	207	89	99		150	155		106	116		1
3_36	Saffraan		130	138	159	125	135		110	125		232	239	243	198		89	109		137	142	155	110	112	116	1, 3*x*
3_12	Winter Saffraan		130	138	153	123	135		125			227	243	257	182	207	89	93	107	144	150		110	116	120	1, 3*x*
2_44	Stanley		153	157		123	129		112	125		232	243		182	207	93	95		144	155		116	120		1
2_46	Tongers		153	157		123	129		125			232	243		182	207	93	95		144	155		116	120		2
3_1	Twyford Monarch		130	153		129	133		108	125		243	257		182		89	93		144	155		94	116		1
3_5	Vicar of Winkfield		138	151	153	121	123	139	110	125		232	243		182		89	93	113	140	150	156	98	118		2, 3*x*
3_10.	Winter Nelis	F	130	151		135			110	125		232	243		182	207	89	109		140	155		116	118	120	1
Interspecific hybrids																										
SAPO	BP 3		149	161		121	133		125			227	232		182	207	93	121		144	150		102	112		1
2_9	Garber		128	136		121	135		125			243	260		182	188	89	121		148	158		112	120		1
2_17	Kieffer		136	149		121	123		125			227	260		192	207	93	121		146	150		112	116		2
2_19	Le Conte		136	138		135	137		108			239	258		182	188	89	111		147	150		112	120		1
***P. pyrifolia* or Asiatic pears**																										
5_7	Chojuro		140			119			98			250	258		190		85	107		145	152		106			2
5_9 R	Hosui	F	155			125	135		98			252	258		190		85	107		145			94	106		4
P. *calleryana* (PC)																										
2_26	Calleryana		136	144		121	135		105			218	225		186	192	99			150	176		92	104		4
To be determined																										
1_36	Unknown 1		130	157		123	135		108	125		243			182		89			150			89	116		5
2_14	Unknown 2		149	157		129	135		108	125		227	243		182	207	89	93		150			116	118		5
3_3	Unknown 3		152	158		129	148		110			227	245		182		89			137	146		93	114		5
5_22	Unknown 5		149	157		123	135		108	125		232	243		182		89	93		150	155		89	116		5
5_23	Unknown 6		149	151		123	144		112	125		243			182	207	93	113		140	150		116	118		5

Tree	Name	CH01d09			CH02b10			CH03d12			CH03g07			CH04e03		CH05c06			EMPc11			EMPc117			Class
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	1	2	3	1	2	3	1	2	3	
6_1	Unknown 7	124	149		123	131		108	110		215	227		182	207	89	99		150	155		116			5
6_2	Unknown 8	124	149		123	131		125			215	243		182	207	93	99		150	155		89	106		5
6_3	Unknown 9	149	151		129			108	110		215	227		182	207	95	113		140	150		116	120		5

Brackets represent clones or sports of a ‘primary’ (Main cultivar), 3x- Triploid

Appendix 4.1. Genotypes for eleven microsatellite markers of apple accessions in the ARC gene bank at Drostersnes (DN7) and Grabouw (E1) Experimental Farms with tree location and name. Accessions supposed to be clones derived from the same cultivar are grouped together. Note column indicates if an accession is believed not to be true to type. F, false by parentage, N, false based on clonal comparison. Triploids presented in Table 4.7 are excluded.

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
***M. pumila* cultivars and selections**																								
E1_19_6	?		179	206	138	171	114	118	98		233	245	230	234	212	255	104	120	175	202	150	183	111	117
DN7_16_4	20/1		170	204	160	171	114	120	98	109	245	257	220	238	226	229	97	106	175	202	150	155	151	
E1_1_17	20/1		170	204	160	171	114	120	98	109	245	257	220	238	226	229	97	106	175	202	150	155	151	
DN7_17_4	28/1 = 2B-28-02		170	204	160	171	104	114	91	98	239	245	228		226	229	97	108	202		155	183	115	
DN7_30_3	28/1		170	204	160	171	104	114	91	98	239	245	228		226	229	97	108	202		155	183	115	
E1_3_14	28/1		170	204	160	171	104	114	91	98	239	245	228		226	229	97	108	202		155	183	115	
DN7_30_1	28/2.		172	183	160	171	116	120	98		239	251	216	238	226	253	108	133	175	213	155	183	115	
E1_3_13	28/2.		172	183	160	171	116	120	98		239	251	216	238	226	253	108	133	175	213	155	183	115	
DN7_15_8	28/2 = 2B-28-14		172	183	160	171	116	120	98		239	251	216	238	226	253	108	133	175	213	155	183	115	
E1_2_12	2B-12-25		170	206	138	171	112	116	98	109	243		208	220	212	224	95	106	175		150		111	117
DN7_17_9	4A-75-28 Rooi Granny		170	206	138	171	114	116	98	109	243	249	220	235	224	255	95	112	175		150		117	151
DN7_8_5	8A-1-Ouer	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
DN7_15_5	Adina	N	170	204	171	179	118	131	91	109	233	243	218	234	226	255	95	133	175		149	191	117	
E1_3_10.	African Carmine	V	170	179	138		116	118	91		243	255	208	234	212	226	112	118	175	204	155	191	117	
E1_15_18	Akane		206		138	171	114	131	91	98	245	257	224	230	229	251	108	112	175		150		151	
E1_7_3	Alkmene		189	206	160	179	120	131	98		255		218	224	229	255	106	112	200	221	150		117	151
E1_14_11	Alsop's Beauty		183	206	171		118	131	91	98	233	255	231	234	226	255	106	110	202	221	150		115	
DN7_18_3	Anna		170	183	160		104	120	91	98	245	251	228	238	226		97	108	202	213	155	156	115	151
DN7_30_2	Anna		170	183	160		104	120	91	98	245	251	228	238	226		97	133	202	213	155	156	115	151
E1_9_10.	Anna		170	183	160		104	120	91	98	245	251	228	238	226		97	133	202	213	155	156	115	151
DN7_24_9	Antonovka Seedling No6		170	193	138		114	118	98	115	245	249	216	234	247	255	95	108	209	230	140	150	119	151
DN7_16_1	Aport		170	189	171	179	112	114	98	103	239		224	228	218		108	110	198	223	150		115	
E1_19_12	Aport		170	189	171	179	112	114	98	103	239		224	228	218		108	110	198	223	150		115	
E1_12_4	Arapkizi		173	179	138		116		98	115	233	255	218	220	212		97	110	175	198	152	155	107	117
E1_17_11	Atties Favourite		179		138		116	118	91	109	243	255	220	234	218	226	95	118	175	204	150	155	117	
DN7_31_2	Austin		182	206	157	171	131		91	98	249	255	208	228	255		95	112	175	182	150		115	122
DN7_4_3	Austin		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
E1_18_6	Beauty of Black Loop	N	179	183	138	179	116		91	98	245	255	208	234	212	218	118	133	175	204	149	155	115	117
E1_14_8	Belrene		183		160	179	112	129	91	98	255	257	218	234	229	257	95	106	175	202	150		107	151

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_13_11	Beni Osho		179	187	179		131		98	106	233	245	208	218	214	229	120	133	175		149	155	117	124
DN7_4_7	Beverly Hills		182	204	145	160	114	116	91	98	233	243	230	238	212		104		202	204	152	183	118	151
DN7_27_1	Bittenfelder		183	204	145	160	104	114	98		245	255	220	228	212	226	97	106	175	213	150	155	111	115
E1_14_6	Blairmont		182	204	171		116	118	98		233	255	218	230	212	255	108	118	175		152	156	115	119
E1_13_21	Boiken X		179	220	138	179	120		98		243	257	234	238	214	229	106	133	217	223	155	183	117	119
DN7_31_3	Boiken Y		179		138		112	116	98		233	255	231	234	212	255	106	133	175	182	149	155	117	
E1_11_1	Braeburn	N	179	206	171		116	120	91	98	233	243	220	228	212	226	106	112	175		140	150	111	119
DN7_15_9	Braeburn Hillwell		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
E1_2_11	Braeburn Hillwell		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
E1_10_15	Braeburn Type	N	182	183	138	171	114	116	91	98	255	257	216	218	226	255	97	106	198	209	150		117	151
DN7_16_6	Braestar		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
E1_11_21	Braestar		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
DN7_16_2	Braeburn	N	170	204	171	179	118	131	91	109	233	243	218	234	226	255	95	133	175		149	191	117	
E1_17_18	Calville de Saint Souve		182		160	177	114	120	98		243	255	208	230	214	255	112	133	202		150	155	117	151
E1_7_19	Canvade		182	204	145	160	114		91	98	233	245	224	238	216	229	106		175	204	152	183	117	151
E1_19_11	CC2/19		183		138	160	120	131	98	118	243	245	208	228	212	251	112		175		140	155	117	
E1_17_16	Champion		170	183	179		120	131	98		233	245	216	234	212	255	114	120	202	221	150	155	107	115
E1_18_14	Chantecler		170	182	138	179	118	131	91	98	243	253	208	220	224	255	95	106	175		150		117	
E1_17_2	Haidegger Golden X		183	187	171	179	110	131	98		233		208	216	218	249	106	110	209	221	150		115	117
E1_17_3	Haidegger Golden Y		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
E1_14_2	Climax		170	182	145	171	114	131	91	100	243	245	208	218	212	255	106		175		150	156	115	117
DN7_3_11	Climax		170	182	145	171	114	131	91	100	243	245	208	218	212	255	106		175		150	156	115	117
E1_8_18	Climax	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_17_5	Coast		179	183	138	179	131		91		233	245	230	238	212		106	112	175		155		109	117
E1_16_16	Commerce		170	182	179	183	116	131	98	109	233		228		212	255	97	106	182	202	150	152	115	119
E1_16_15	Co-op 19		179	182	138	157	112	116	98		245	257	234	235	212	255	104	117	175	202	149	155	117	151
E1_7_12	Co-op 20		206		160	171	114	118	98		243	255	234	235	229	255	104		175	202	150	191	117	151
E1_7_17	Coromandel Red		179	183	138	171	116	120	91	98	233	243	208	220	212	226	95	106	175		140	191	117	
E1_15_16	Red Cox		204	206	160		118	131	98		233	257	218		255		106	112	175	200	150	155	117	151
DN7_5_3	Cox's Orange Pippin		204	206	160		118	131	98		233	257	218		255		106	112	175	200	150	155	117	151
E1_16_21	Crab A		173	187	171		120	129	98		233	257	231	234	212	255	106	120	202	217	150		115	117
E1_9_4	Crab C		179	193	160	162	120	127	98		233	257	208	234	212	255	106		204	219	152		115	117
E1_7_14	Criterion		179	183	138		116	118	91	98	243	245	208	234	218	226	95	117	175	204	150	155	117	
E1_16_18	Dakota		172	179	145	179	112	116	98		239	245	216	234	212	255	110	117	175	223	149	152	117	

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_13_15	Dayton (=Co-op 21)		179	206	145	160	116	118	91		243		230		255		106	108	175	209	183	191	119	
DN7_24_1	Dayton Seedling No6		179		138	179	118	139	89	98	245	255	208	234	214	224	104	108	175	202	150	155	117	119
E1_9_14	Delblush		170	183	171	179	116	118	91	109	243	255	234		218	226	95	133	175		155	191	115	117
DN7_3_2	2X Red Delicious X		179	183	138	179	116		91	98	245	255	208	234	212	218	118	133	175	204	149	155	117	
DN7_3_3	2X Red Delicious Y	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
E1_16_6	Big Chief		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_14_13	Classic		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
DN7_6_8	Dietrich		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	117	
E1_6_15	Dietrich Starking	N	183		160	179	112	129	91	98	255	257	218	234	229	257	95	106	175	202	150		107	151
E1_6_18	Full Red		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_12_8	Hardy Spur		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_15_9	Hi Early Delicious	N	179		138		116	118	91	109	243	255	220	234	218	226	95	117	175	204	150	155	117	
E1_16_13	Lalla Delicious	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_6_20.	Oregon Red Spur	N	183	191	138	171	116	120	91	98	243	255	234	238	212	255	120	133	175	217	150	155	115	119
E1_17_1	Oregon Red Spur 2		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_14_4	Prime Red Delicious		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_1_7 R	Red Delicious		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_18_5	Red Delicious Tasmania	N	183	191	138	171	116	120	91	98	243	255	234	238	212	255	120	133	175	217	150	155	115	119
E1_2_7	Richared		179	183	138	179	116		91	98	245	255	208	234	212	218	118	133	175	204	149	155	115	117
E1_7_18	Ryan Red		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_15_3	Ryan Red	N	189	206	171	177	112		98	103	243	257	218	234	218	224	108		175		150		115	117
E1_15_4	Ryan Spur		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_10_10.	Shotwell Delicious		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_2_1	Stark Spur Supreme	N	170	206	171	179	114	131	98	103	239	257	228	230	218	255	110	112	200	202	150		115	117
DN7_4_9	Starking		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	117	
E1_8_3	Starking (RSA)	N	170	183	171	179	116		91	98	255	257	220	234	212	224	112	133	175	204	149	191	111	115
E1_17_17	Starking (USA)		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_3_3	Starking Red (Groend)		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
DN7_7_4	Starkrimson	N	170	179	171	179	118	131	91		233	257	234		226	255	112	133	175		150	191	117	
E1_4_15	Starkrimson	N	179		138		116		91	109	255	257	208	220	218	224	112	117	175		150	155	111	117
E1_15_12	Super Chief Red Del	N	179		138		116		91	109	255	257	208	220	218	224	112	117	175		150	155	111	117
E1_8_23	Top Red	N	170	183	171	179	116		91	98	255	257	220	234	212	224	112	133	175	204	149	191	111	115
E1_1_1	Ultra Red	N	182	220	138	171	116	131	91	98	233	255	228	231	226	255	95		175	221	150		115	117
E1_8_10.	Wellspur Delicious	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_6_5	Jacored		179	183	138	179	116		91	98	245	255	208	234	212	218	118	133	175	204	149	155	115	117
E1_13_14	Early Red		179	183	138	179	116		91	98	245	255	208	234	212	218	108	117	175	204	149	155	115	117
E1_13_18	Early Red No. 2		173	182	145	160	114	116	98	103	233	245	224	230	212	216	104	110	204	209	150	152	119	151
E1_13_20.	Groth Red	N	183		160	179	112	129	91	98	255	257	218	234	229	257	95	106	175	202	150		107	151
E1_17_21	Morspur		173	204	160	171	114	116	91	98	233	257	228	230	212	229	106		202	209	150	183	111	119
E1_17_6	Starking Colorless		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_10_14	Starking Early (Moodie)		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_16_9	Starking Red (Moodie)		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_8_7	Starking Stripeless		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
DN7_18_4	Delkistar		179	206	138	171	116	118	91	98	245	257	230	234	212	229	110	133	175	204	149	150	111	117
E1_18_8	Democrat		170	183	179		114	131	98	103	239	257	218	228	218	255	108	112	198	200	150		115	119
E1_12_13	Diva Gold		170	182	138		116	120	91		243	245	208	234	226	229	97	112	175		150	191	117	
DN7_4_4	Drakenstein		179	182	138	160	112	116	91	98	233	245	231	234	218	255	106	133	175	204	150	155	117	
E1_14_17	Dukat		189	206	160	179	118	120	98	103	233	257	218	228	218	255	106		175	200	150	155	115	151
DN7_4_2	E3 F2	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
E1_9_12	Earligold		170	183	171		112	116	91		249	257	218	220	216	226	95	97	175	209	150	183	111	151
DN7_22_2	Edgewood		182	206	160	171	114	118	98		233	257	230	234	229	255	110		175	202	150		117	
E1_17_8	Edgewood		182	206	160	171	114	118	98		233	257	230	234	229	255	110		175	202	150		117	
E1_15_11	Eikhoff		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_16_19	El Orange + Ellison's?	N	204	206	160		118	131	98		233	257	218		255		106	112	175	200	150	155	117	151
E1_4_13	Elise		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
E1_8_19	Elsie Grant		183	206	160	171	120	131	98	118	233	243	220	230	212	255	106		175		140	150	107	117
E1_9_3	Elstar	N	170	179	138	171	116	118	91	109	243	249	220	230	224	226	95	112	175		150	191	111	117
DN7_4_5	Elstar Red		170		138		112	118	109		243	257	224	234	224	255	95	112	175	198	150		117	151
E1_12_6	Elstar Red		170		138		112	118	109		243	257	224	234	224	255	95	112	175	198	150		117	151
E1_2_10.	Elstar Red		170		138		112	118	109		243	257	224	234	224	255	95	112	175	198	150		117	151
E1_10_3	Empire	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_5_12	Russel Red		170	183	179		112	131	98	103	239	257	218	228	218	255	108	112	198	200	150		115	119
E1_9_17 R	Fiesta	N	170	173	138	160	114	116	91	98	233	257	220		212	226	112		175	204	150	152	117	
E1_7_13	Florentina		183		160	179	112	129	91	98	255	257	218	234	229	257	95	106	175	202	150		107	151
E1_19_5	Forum		170	179	160	171	112	116	91	98	239	243	235	238	212	224	97	108	175		150	155	111	117
DN7_23_1	Ben Shogun		183		171	179	116		91	98	233	245	230	234	212		106	118	175	202	149	155	115	117
E1_12_14	Fuji	N	206		160	171	112	120	98		243	255	228	234	253	255	106		198	202	150	152	117	119
E1_10_12	Fuji A		183		171	179	116		91	98	233	245	230	234	212		106	117	175	202	149	155	115	117

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_9_9	Fuji Akufi		183		171	179	116		91	98	233	245	230	234	212		106	117	175	202	149	155	115	117
E1_8_22	Fuji Berthon		183		171	179	116		91	98	233	245	230	234	212		106	117	175	202	149	155	115	117
E1_12_1	Fuji Irradiated	N	179		138	171	116	118	91		255	257	220	234	212	226	95	133	175	204	155	191	117	
E1_16_1	Fuji Tac 114		183		171	179	116		91	98	233	245	230	234	212		106	117	175	202	149	155	115	117
E1_10_18	Yataka		183		171	179	114	116	91	98	233	245	230	234	212		106	117	175	202	149	155	115	117
E1_9_2	Gala	V	170	204	171	179	118	131	91	109	233	243	218	234	226	255	95	133	175		149	191	115	117
E1_11_11	Gala Imperial Gala		170	204	171	179	118	131	91	109	233	243	218	234	226	255	95	133	175		149	191	115	117
E1_14_7	Gala Royal Gala	N	170	173	138	160	114	116	91	98	233	257	220		212	226	112		175	204	150	152	117	
E1_9_13	Gala To Red?	N	170	173	138	160	114	116	91	98	233	257	220		212	226	112		175	204	150	152	117	
DN7_5_11	Royal Gala		170	204	171	179	118	131	91	109	233	243	218	234	226	255	95	133	175		149	191	117	
DN7_6_7	Scarlet Gala		170	204	171	179	118	131	91	109	233	243	218	234	226	255	95	113	175		149	191	115	117
DN7_15_10	Treco Red X		170	204	171	179	118	131	91	109	233	243	218	234	226	255	95	133	175		149	191	117	
DN7_15_11	Treco Red Y	N	175	182	150	171	104	131	98		227	233	208	224	215	249	106		188	209	150		115	117
E1_4_11	Gavin		187	206	160	171	112	120	91	104	245	249	224	235	255		104	108	200	202	150	155	115	119
DN7_16_9	Ginger Gold		170	187	171		112	118	91		233	243	216	234	226	255	95	128	175		150		117	151
E1_2_14	Ginger Gold		170	187	171		112	118	91		233	243	216	234	226	255	95	128	175		150		117	151
E1_9_18	Gloire de Hollande		183		138		104	127	98		249	255	231	234	226	247	106	120	175	202	163	183	107	117
E1_7_20.	Gloster		179	191	138	179	116	118	98		243	255	208	216	214	218	120	133	175	204	149	155	117	119
E1_3_16	Belgold		170	183	138	179	116		98	109	243	245	220	234	212	224	95	133	175		150	155	111	115
E1_6_1	Compactagold		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_12_16	Elbee	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
DN7_2_1	Golden Delicious		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
DN7_7_3	Golden Delicious		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_16_12	Golden Delicious		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_14_12	Golden Delicious (Hawaii early)	N	170	179	138	179	116	118	91		255	257	208	234	218	224	95	133	175	204	149	150	115	117
E1_6_10	Golden Delicious X		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_6_11	Golden Delicious Y		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	
E1_8_11	Golden Delicious Claz		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_11_20	Golden Delicious Fran	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_18_9	Golden Delicious Reinde F2		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_11_17	Golden Delicious U.S.		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_9_21	Golden Sheen (=Belgolden)	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_11_5	Goldspur		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_11_4	Goldspur Applewaite		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_11_14	Goldspur Aswell	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_3_17	Lysgolden (=Goldenir)		170	183	138	179	116		98	109	243	245	220	234	212	224	95	133	175		150	155	111	115
E1_8_12	Panorama Golden A		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_8_13	Panorama Golden B		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_15_6	Smoothee		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_16_8	Spur Golden		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_11_10	Stark Spur Golden Del		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_7_16	Golden Delicious Early		170	183	138	171	116	131	91	109	233	243	234	238	212	224	95	112	175		150		111	117
E1_19_2	Heinderich Golden		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_8_21	Yellow Delicious		170	179	138	171	116	118	91	109	243	257	220	234	224	226	95	112	175		150	191	111	117
E1_11_13	Goldrush		179	206	138	179	118	131	98	109	233	243	228	234	224	251	95	106	175		150	155	117	119
E1_9_20	Goldsmith (=Early Granny)		170	183	138	179	116		98	109	243	245	220	234	212	224	95	133	175		150	155	111	115
E1_10_20	Goosen		170	183	160	171	116	131	91	109	233	251	228	238	212		97	112	175	202	150		115	117
E1_9_8	Grand Richard	F	182		171	179	104	131	91	98	233	245	208		212	249	106	110	175	209	150		117	
E1_11_15	Granearli		170	183	138	179	116		98	109	243	245	220	234	212	224	95	133	175		150	155	111	115
DN7_19_1	Granny Smith		182	206	160	179	112	131	98		233	243	228	231	212	251	106	112	175		150	155	115	117
E1_5_1	Granny Smith		182	206	160	179	112	131	98		233	243	228	231	212	251	106	112	175		150	155	115	117
E1_12_3	Granny Smith (Louterwater)		182	206	160	179	112	131	98		233	243	228	231	212	251	106	112	175		150	155	115	117
E1_15_17	Granny Smith (RSA)		182	206	160	179	112	131	98		233	243	228	231	212	251	106	112	175		150	155	115	117
E1_12_2	Granny Smith 14-7-70		182	206	160	179	112	131	98		233	243	228	231	212	251	106	112	175		150	155	115	117
E1_15_10	Granny Smith Spur		182	206	160	179	112	131	98		233	243	228	231	212	251	106	112	175		150	155	115	117
E1_12_10	Granny Smith USA		182	206	160	179	112	131	98		233	243	228	231	212	251	106	112	175		150	155	115	117
E1_11_7	Green Fielda		182	206	160	179	112	131	98		233	243	228	231	212	251	106	112	175		150	155	115	119
E1_16_14	Granny Smith Red		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
E1_7_9	Red Gravenstein		182	183	138	171	114	116	91	98	255	257	216	218	226	255	97	106	198	209	149	150	117	151
E1_1_6	Greensleeves		170	206	160	171	118		91	98	233	243	220	238	226	229	95	108	175	200	150	191	117	120
E1_11_19	Himekami X	V	183	206	171		116	131	91		233	257	230	235	212	255	112	117	175	202	150	155	117	151
E1_11_23	Himekami Y	F	183	187	171	179	110	131	98		233		208	216	218	249	106	110	209	221	150		115	117
DN7_33_1	HL 1004		170	191	171	179	118		98	109	255	257	220	238	214	226	95	120	175		150		111	117
DN7_32_1	HL 166C		183		171	177	116	118	91	98	233	243	220	230	212		106	117	175	202	149	155	115	117
E1_18_11	HL 237		183	191	138	171	116	120	91	98	243	255	234	238	212	255	120	133	175	217	150	155	115	119
E1_18_10	HL 318		182	206	160	171	118	120	98		243	249	234	235	251		112		175		150		117	151
E1_18_12	HL 938		170	183	138		116		91	109	245	257	220	234	218	226	95	133	175		155	191	117	
DN7_24_8	Hofer Seedling		170	187	138	179	120	131	103	115	239	243	216	228	212	218	110	112	200	209	150	152	117	

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_1_13	Hokuto		183		171	177	116	118	91	98	233	243	220	230	212		106	117	175	202	149	155	115	117
E1_17_20	Hoplan		170	206	160	171	120	131	91	98	233	243	216	218	212	255	112	117	175		150		115	117
DN7_4_6	Hoplan		183	189	160	179	114	131	98	103	239	257	208	228	218	255	106	110	202	223	150		115	119
E1_14_10	Hops Late Red		183		138	179	112	116	91	98	233	255	231	234	218	251	106	117	175	204	150	155	119	
E1_11_3	Howell		179	183	171	179	116		91	109	243	245	220	234	212	224	112	133	175		149	150	117	
DN7_22_3	Howell?	F	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
DN7_26_1	i5526 X 6407 INRA		170	206	138	171	114	118	91		243	249	234		212	224	108	133	202	217	150		113	117
E1_7_15	Idared		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
DN7_5_10	Jersey Mac		170	206	138	179	114	116	91	103	239	243	230	238	255		108	110	175	209	150	191	117	119
E1_8_8	Jersey Mac	N	170	179	145	171	114	116	91	103	239	243	230	238	255		108	110	175	209	152	183	117	119
DN7_33_2	Jester		170	206	138	171	112	118	104	109	233	243	224	234	226	251	95	108	175	202	150		115	117
E1_15_1	Jester		170	206	138	171	112	118	104	109	233	243	224	234	226	251	95	108	175	202	150		115	117
DN7_20_7	Malling Jester		170	206	138	171	112	118	104	109	233	243	224	234	226	251	95	108	175	202	150		115	117
DN7_20_8	Malling Jester		170	206	138	171	112	118	104	109	233	243	224	234	226	251	95	108	175	202	150		115	117
DN7_7_2	Co-op 22		206		171	179	112	120	91	98	243	249	208	234	212	253	106		175	198	150		117	
E1_15_5	Jonafree (=Co-op 22)		206		171	179	112	120	91	98	243	249	208	234	212	253	106		175	198	150		117	
E1_18_7	Jonafree (=Co-op 22)		206		171	179	112	120	91	98	243	249	208	234	212	253	106		175	198	150		117	
E1_4_17	Jonafree (=Co-op 22)		206		171	179	112	120	91	98	243	249	208	234	212	253	106		175	198	150		117	
E1_18_3	Blackjon		206		171		114	131	91	98	249	257	230	235	229	255	110	112	175	202	150		117	151
E1_16_7	Jonathan		206		171		114	131	91	98	249	257	230	235	229	255	110	112	175	202	150		117	151
E1_4_7	Jonathan		206		171		114	131	91	98	249	257	230	235	229	255	110	112	175	202	150		117	151
E1_11_12	Jonnee	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_4_4	Julia		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_18_2	July Red		170	182	145	177	116	120	91	98	243		230	238	216	255	104	110	175	223	152	183	124	
E1_16_2	Karmijn de Sonnaville	V	204	206	160	171	118	131	91	98	233	257	218	230	229	255	106	112	175	200	150	155	117	151
DN7_5_1	Kashawi		172	183	157	171	114	116	98	103	253	257	208	216	212	218	106	120	200	217	150	181	111	147
E1_17_13	Kashawi		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_17_15	Kidd's Orange Red	V	179	204	160	179	116	131	91	98	233	255	218	234	212	255	106	133	175	204	149	150	115	117
E1_1_4	Kirks X	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106	114	209	221	150		115	117
E1_1_5	Kirks Y		182	220	138	171	116	131	91	98	233	255	228	231	226	255	95		175	221	150		115	117
E1_18_15	Klara		179	187	179		131		98	106	233	245	208	218	214	229	120	133	175		149	155	117	124
E1_16_11	Kogetso	V	179	206	138	171	116	131	91		243	249	230	234	226	229	95	112	175	202	150	191	111	151
E1_7_1	Koo		179	204	160	179	116	118	98		255	257	220	230	226	255	110	120	175	209	150		115	128

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
DN7_6_4	Lady Williams		183	206	171	179	112	120	98	118	233	243	208	228	212		106		175	213	140	150	117	119
E1_15_14	Lakeside		179	183	179	183	116	131	98	104	233	257	208		212	255	106	112	175		150	152	117	
E1_17_12	Langkloof		179	183	138	171	118	131	98		233	245	208	234	226	253	106	110	175	202	150		117	128
E1_12_15	Laxton's Superb		179	206	160	179	131		98		233	257	218	224	218	255	106	112	198	200	150	155	107	149
DN7_19_2	Le Vant		170	182	160	171	118	131	98	109	233	243	220	231	226	251	112		175		150		117	119
E1_7_7	Le Vant		170	182	160	171	118	131	98	109	233	243	220	231	226	251	112		175		150		117	119
E1_10_19	Leyda		179	183	160	179	120	131	98		255	257	208	234	229	255	106		175	209	150	152	107	117
DN7_5_2	Liberty		179	204	171	179	114	120	91	111	233	239	228	238	212	255	106	108	202		150	152	118	151
E1_8_14	Liberty		179	204	171	179	114	120	91	111	233	239	228	238	212	255	106	108	202		150	152	118	151
E1_12_9	London Pippin		179	206	171	177	118	120	98	109	233	243	228	234	212		110	133	175	209	152	163	107	151
E1_11_8	Longford		179	183	160	179	120	131	98		255	257	208	234	229	255	106		175	209	150	152	107	117
E1_13_6	Lord Lambourne		206		138	160	112	120	98		233	245	228	238	251	255	108		175	202	150		151	
E1_16_4	Lord Lambourne		206		138	160	112	120	98		233	245	228	238	251	255	108		175	202	150		151	
DN7_20_10	M1		173	204	138	160	129	131	98		255		231	238	212	229	114	120	175		149	150	111	117
E1_1_9	M1		173	204	138	160	129	131	98		255		231	238	212	229	114	120	175		149	150	111	117
DN7_1_8	M13		172	206	138	171	112	131	91	98	245	251	220	231	226		106	120	202	209	163		107	115
E1_13_4	M13		172	206	138	171	112	131	91	98	245	251	220	231	226		106	120	202	209	163		107	115
E1_1_11	M13		172	206	138	171	112	131	91	98	245	251	220	231	226		106	120	202	209	163		107	115
DN7_20_11	M4		170	204	138	171	116		98	115	245	249	218	235	212	255	97	106	198		183		117	
E1_2_22	M4		170	204	138	171	116		98	115	245	249	218	235	212	255	97	106	198		183		117	
DN7_21_11	M7		170		162	177	118	120	98	104	243	245	231	235	251	253	106		198	209	149	150	107	111
DN7_1_9	M7		170		162	177	118	120	98	104	243	245	231	235	251	253	106		198	209	149	150	107	111
DN7_5_4	M7 Elgin		170		162	177	118	120	98	104	243	245	231	235	249	251	106		198	209	149	150	107	111
DN7_1_10	M25		183	187	171	179	110	131	98		233		208	216	218	249	106	110	209	221	150		117	
DN7_8_7	M26		170	179	160	162	112	114	98	104	245		216	231	214	226	106		175	198	161		117	119
DN7_2_10	M793		173	182	138	171	131		98		245	255	208	231	212	229	110	114	175		150		117	
DN7_21_10	M793	N	183	187	171	179	118	133	98	104	233		208	216	212	249	106		209	221	150		115	117
DN7_2_8	M793?		173	182	138	171	131		98		245	255	208	231	212	229	110	114	175		150		117	
DN7_2_11	M9		170	172	160	171	114	120	98	115	245		213	235	214	255	106	114	198	221	150	161	117	
DN7_21_9 R	M9		170	172	160	171	114	120	98	115	245		213	235	214	255	106	114	198	221	150	161	117	
E1_13_3	M H 15-6		172	183	157	171	114	116	98	103	253	257	208	216	212	218	106	120	200	217	150	181	111	147
E1_7_2	Maayan		173	179	138	179	114	116	98		243	245	208	230	212	218	112	117	175	209	149	189	117	122
E1_14_16	Macobin		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_1_19	Maidens Blush		179		138	171	116		91	98	245	257	234		213	226	95	133	175	204	149	150	115	117

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_17_4	Maigold		179	206	138	171	116	118	91	115	243	245	220	230	224	229	106	112	175		150		111	117
E1_19_1	M McIntosh		173	204	160	171	114	116	91	98	233	257	228	230	212	229	106		202	209	150	183	111	119
E1_17_7	Macspur	N	170	204	171		114	116	91		233		228	230	212		106		202		150	152	111	151
E1_12_5	Macspur		173	204	160	171	114	116	91	98	233	257	228	230	212	229	106		202	209	150	183	111	119
E1_6_13	Marshall Mcintosh 6	N	170	183	171	179	116		91	98	255	257	220	234	212	224	112	133	175	204	149	191	111	115
E1_9_7	McIntosh Early		170	183	179		114	131	98	103	239	257	218	228	218	255	108	112	198	200	150		115	119
E1_3_11	Melba		182	206	157	171	118	131	91	98	233	249	228	234	218	255	104	110	202	209	150		117	119
DN7_3_10	Melba		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
E1_15_15	Meldale		183		160	179	112	116	91	98	233	255	208	228	212	253	106	117	175	204	150	155	115	117
DN7_5_6	Melrose		183	206	171	179	114	116	91	98	255	257	230	234	212	229	110	133	175		149	150	117	151
E1_3_15	Melrose		183	206	171	179	114	116	91	98	255	257	230	234	212	229	110	133	175		149	150	117	151
E1_2_17	Meran		170	206	138	171	114	118	91		243	249	220	235	224	229	95	112	175	202	150	191	117	
DN7_17_3	Meran		170	206	138	171	114	118	91		243	249	220	235	224	229	95	112	175	202	150	191	117	
DN7_1_6	Michal		173	179	157	179	114	116	98		243	245	208	238	218		120	133	204	223	155	189	115	122
E1_11_6	Michinoku		179	204	138	171	118	131	91	98	243	249	230	234	212	255	95	112	175	202	150		111	117
DN7_6_6	Milton		170	183	171	179	116	118	91	98	243	245	220	234	212	226	95	117	175		150	155	117	
E1_5_19	Missouri Pippin		170	183	171	183	118	131	98		233	255	224	231	229		117	135	198	221	140		115	117
DN7_8_6	MM106	F	170	179	160	162	112	114	98	104	245		216	231	214	226	106		175	198	161		117	119
DN7_21_12	MM109		182	187	171		104	110	98		233	255	208	216	212	249	106	110	175	221	150		115	117
DN7_1_7	MM109		182	187	171		104	110	98		233	255	208	216	212	249	106	110	175	221	150		117	
DN7_20_6	MM111	F	182	187	171	179	104	118	91	104	233	245	208		212		106	110	175	221	150		115	117
DN7_1_1	Mollies Delicious		170	182	171	177	116	120	98	109	243	257	234	238	226	255	108	133	175	202	150	183	111	117
E1_4_10	Mollies Delicious		170	182	171	177	116	120	98	109	243	257	234	238	226	255	108	133	175	202	150	183	111	117
E1_14_9	Monsa		179		138		116	118	91	109	243	255	220	234	218	226	95	117	175	204	150	155	111	117
E1_4_14	Morkel's Seedling		179	183	138	171	116	120	91	118	245		208	230	212	255	112	133	202	204	140	155	107	115
E1_6_8	Mother		183	206	162	179	112	131	98	109	233		234	238	212	226	112	114	175		150	155	111	117
E1_15_13	Nebuta		170	206	138	179	114	131	91	98	243		234		212	255	95	112	175	202	150	155	117	
E1_9_6	New Gold		170	206	138	160	116	118	91	98	233	243	218	234	226	247	108	112	175		150	191	117	119
E1_5_5	New Year		182	220	157	171	131		91	98	233	255	208	238	212	255	112	120	202	221	150		115	151
E1_2_4	Nickajack		170	182	138	183	116	118	91		233	255	220	231	212	229	106	112	175	202	150		117	
DN7_24_2	No1 Dresden (Seedling 4)		201	218	160	171	131		98		239	255	235	238	253	255	106	110	175	190	150		115	
DN7_24_3	No2 Dresden (Seedling 2)		182		160		112	114	91		233	255	230	234	224	255	95	112	175	202	150	183	107	151
DN7_24_4	No3 Dresden (Seedling 1)		170	193	138	177	114	118	103	115	249	257	224	234	247	251	95	108	209	230	161	183	119	153
DN7_4_8	Northern Spy		182		171	179	104	131	91	98	233	245	208		212	249	106	110	175	209	150		117	

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_12_12	Beaumont		179	183	160	183	114	131	91	104	233	257	220	230	212	224	95	112	175	202	150	155	107	115
E1_16_17	Dunn's Seedling (syn Ohenimuri)		183	206	179	183	112	131	98	104	233	257	208	220	212	255	106	112	175	202	150	152	115	119
E1_8_9	Ohenimuri Early		183	206	179	183	112	131	98	104	233	257	208	220	212	255	106	112	175	202	150	152	115	119
E1_13_13	Onderstam 5		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
DN7_18_6	Onderstem 5 B		183	187	171	179	110	131	98		233		208	216	218	249	106	110	209	221	150		115	117
DN7_18_5	Onderstem 5 A		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_13_16	Hunter Ontario		182		157	179	131		91	98	233	245	208	230	226	249	106		198	209	150		117	
E1_10_13	Jumbo Orin		179	183	138	179	116	118	91	98	243	257	230	234	224	229	95	106	175		150	161	117	119
E1_3_21	Orleans Reinette		189	206	171	177	112		98	103	243	257	218	234	218	224	108		175		150		115	117
E1_1_8	Ozark Gold		170	179	171	179	118	131	98	109	255	257	234	235	224	253	112	133	175		149	191	117	
E1_10_2	P 1		204		171	179	114	116	98	115	233	245	216	235	255		97	112	198	221	140	183	117	
DN7_1_11	P 18		170	204	138		114	116	115		249		218	224	247	255	95	97	198	226	161	183	117	153
E1_6_22	P 18		170	204	138		114	116	115		249		218	224	247	255	95	97	198	226	161	183	117	153
E1_13_2	P 18		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_17_14	Palmiet Red		179	183	138	171	110	116	98		233	255	234	238	212	218	120		175	204	149	155	115	
E1_16_10	Panorama Crab		179	191	179		116	118	91	98	243	255	208	238	212	214	106	133	204	217	149	155	115	119
E1_18_1	Paula Red		172	204	171		114	116	98		233	239	216	228	229	253	106	108	175	202	156	183	115	119
DN7_8_8	Paula Red		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
DN7_17_7	Pi-Au 9-24		172	182	150	160	120	139	111	115	245	251	216	228	212	255	106	126	221	224	150	157	115	117
DN7_21_7	Pi-Au 9-27		172	182	157	160	108	120	98	105	245		228	235	212	214	114	126	188	198	157	161	115	117
E1_10_8	Pi-Au 9-27		172	182	157	160	108	120	98	105	245		228	235	212	214	114	126	188	198	157	161	115	117
DN7_19_3	Pilot		204	206	160	171	131		91	103	257		228	230	255		112		175	200	150		117	151
DN7_16_7	Pink Lady	V	179	206	138	171	112	118	91	98	233	257	208	234	212	224	95	106	175		150	191	117	
E1_10_7	Pink Lady	V	179	206	138	171	112	118	91	98	233	257	208	234	212	224	95	106	175		150	191	117	
E1_3_2	Pink Lady	V	179	206	138	171	112	118	91	98	233	257	208	234	212	224	95	106	175		150	191	117	
DN7_2_2	Pinova		170	204	138	177	118	131	103	109	243	257	228	234	218	224	108	112	175	200	150	191	111	117
E1_15_2	Pioneer Scarlet		162	218	150	171	108		96	104	249	255	231	241	212	220	108	110	223	242	149	158	115	136
DN7_31_1	Polka		179	204	160	171	116	118	98	109	243	257	220	230	226	229	95	106	175	209	150	191	111	117
E1_2_19	Pomme de Niege		179		138		116	118	91	109	243	255	220	234	218	226	95	117	175	204	150	155	117	
E1_5_9	Porporate		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
E1_11_16	Present of England		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
DN7_6_9 R	Prima		179	206	138	160	114	118	91	98	233	243	230	234	255		106	108	175	209	183	191	111	119
E1_17_9	Primgold		170	172	138	145	114	118	91	109	239	257	234		224	229	106	112	175	223	156	191	111	115
E1_2_21	Prince Bismarck		179	183	138	171	118	131	98		233	245	208	234	226	255	106	110	175	202	150		117	128

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_10_4	Princesa		170	179	160	171	104	131	91	98	233	245	228	235	226	255	106	133	202		150	155	115	151
DN7_6_10	Priscilla		173	183	171	179	116	118	91	111	233	245	228	234	212	218	104	133	202	204	149	191	111	117
DN7_6_2	Red Astrakhan		187	206	145	164	118	120	98	109	243	257	218	238	212	226	106	133	175		150	152	117	119
E1_2_16	Red Astrakhan		187	206	145	164	118	120	98	109	243	257	218	238	212	226	106	133	175		150	152	117	119
E1_3_20	Red Astrakhan		187	206	145	164	118	120	98	109	243	257	218	238	212	226	106	133	175		150	152	117	119
E1_2_5	Red Dutch		183	204	138	171	116	120	91	104	233	245	220	224	229	255	106	110	175	209	155		117	
E1_16_5	Red Gem		204	206	171	179	104	114	98		245	257	208	235	229	255	110	112	175		150		117	
E1_16_3	Redfree		179	206	171		112	116	98		233	239	220	228	229	255	108		175	202	152	183	117	
E1_2_20	Redwine		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
E1_14_18	Redwinter		182	206	171	177	118	131	98		245		220	230	212	255	106	120	202		149	150	117	119
DN7_7_8	Reglindis		179	206	177	179	120	131	91	98	239	255	228	234	229	255	95	108	175	200	150	191	117	119
E1_13_9	Reglindis		183	187	171	179	110	131	98		233		208	216	218	249	106	110	209	221	150		115	117
DN7_20_1	Remo		204	206	160	179	114	120	91	98	233	257	238		255		106	110	200	202	150		119	151
E1_8_5	Resista		170	206	160	171	120	131	91	98	233	243	216	218	212	255	112	118	175		150		115	117
DN7_1_4	Resista X		179	182	138	183	131		98		245	255	208	231	212	229	110	114	175		150		117	
DN7_1_5	Resista Y		173	182	138	171	116	131	98	109	233	257	220	228	226	255	95	106	175	221	150		111	115
DN7_7_9	Rewena		206		177	179	112	131	91	98	255	257	224	238	218	255	108	112	202	221	150		117	119
E1_6_21	Rewena		206		177	179	112	131	91	98	255	257	224	238	218	255	108	112	202	221	150		117	119
E1_2_3	Rhode Island Greening		173	182	160	162	114	118	91	109	255	257	230	235	229	255	97	110	202	204	150	183	107	117
E1_17_19	Rokewood		206		160	179	118	131	98		233	257	218	220	255		106	112	175	202	150		107	119
E1_14_5	Clifton Rome	F	179	183	138	171	112	120	98	118	243	245	208	230	212	255	106	112	175	202	140	161	107	117
E1_6_12	Rome Beauty		206		160	171	112	120	98		243	255	228	234	255		106		198	202	150	152	117	119
E1_1_12	Seeando Red Rome		206		160	171	112	120	98		243	255	228	234	255		106		198	202	150	152	117	119
E1_4_1	Spur Red Rome		206		160	171	112	120	98		243	255	228	234	255		106		198	202	150	152	117	119
DN7_17_10	Russian Seedling		172		156	167	108		103		255		208	216	212	218	97	106	198	215	152	156	115	119
E1_13_8	Russian Seedling		172		156	167	108		103		255		208	216	212	218	97	106	198	215	152	156	113	117
DN7_33_3	SA579-3		173	183	160	171	104	114	91	98	233	245	228	230	226	229	97	104	202	209	150	155	111	115
E1_3_9	Sadie Frazer		179	220	171		114	116	91		233	247	208	234	253	255	106	110	175	221	150		115	128
DN7_5_9	Sansa		170	179	145	171	131		91	109	233	257	218	224	251	253	95	108	175		152	183	115	151
E1_6_16	Sayaka		179	206	138	171	114	116	91	98	243	249	234	235	218	255	95	110	175	202	150	191	117	151
DN7_32_2	Scarlet		170	182	130	138	118	131	98	109	249	257	234	235	226	229	95	112	175		150	191	111	119
E1_19_9	Selena		170	179	138	171	114	120	98		233		230	234	212	255	106		175	223	150	183	119	124
E1_5_15	Senator		170	179	160	179	116	118	98		233		208	220	212	255	106		175	221	150	155	107	117
E1_7_5	Senshu		183		138	179	116		98		233	243	230		212	226	106	133	175		150	155	111	117

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_2_6	Shampion	V	170	206	160	171	118	120	91	98	233	243	220	238	226	255	108	112	175	202	150	191	111	151
DN7_22_1	Sharpe's Early		179	204	162	171	120	131	98		233		220	238	212	255	106	112	175		150	183	117	
E1_4_9	Sharpe's Early		182	206	157	171	118	131	91	98	233	249	228	234	218	255	104	110	202	209	150		117	119
E1_8_2	Sharpe's Early		182	206	157	171	118	131	91	98	233	249	228	234	218	255	104	110	202	209	150		117	119
DN7_3_9	Sharpe's Early		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
E1_4_6	Sharpe's Late		179	204	162	171	120	131	98		233		220	238	212	255	106	112	175		150	183	117	
E1_18_4	Shizuka		170	179	171	179	118	131	98	109	255	257	234	235	224	253	112	133	175		149	191	117	
DN7_2_4	Shlomit		172	183	138	157	116	120	98		243	255	216	234	214	218	112	133	204	223	155	189	117	
E1_11_9	Shlomit		172	183	138	157	116	120	98		243	255	216	234	214	218	112	133	204	223	155	189	117	
E1_6_7	Shoreland Queen		179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
DN7_1_3	Sinclair		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
E1_5_7	Sir Isaac Newton		182	187	171	177	120	131	98	109	233	257	228	235	255		106		175		150	191	115	117
DN7_5_8	Sir Prize		187	206	138	171	112	118	98		233	243	220	234	212		106	112	175	198	149	150	117	119
E1_8_4	SPAB 919	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_5_14	Spartan		173	179	171	179	114	116	91	98	233	245	208	230	213		106	133	204	209	150	155	115	119
DN7_6_5	Splendour		179	183	138	171	116	118	91	98	243	245	234		218	226	112	133	175	204	149	191	117	
E1_4_8	Splendour		179	183	138	171	116	118	91	98	243	245	234		218	226	112	133	175	204	149	191	117	
E1_9_16	Red Statesman		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_13_1	Black Stayman		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_3_5	Stayman Winesap	N	179	206	160	179	116	131	98		245	257	208	234	218	255	110	133	202	204	149	155	117	
DN7_5_7	Summerking Red		172	204	145	171	114	118	98	109	257		216	220	212	229	106		175	223	150		111	151
E1_4_3	Summerred		172	204	145	171	114	118	98	109	257		216	220	212	229	106		175	223	150		111	151
E1_6_14	Summerred		172	204	145	171	114	118	98	109	257		216	220	212	229	106		175	223	150		111	151
DN7_15_4	Sundowner		170	206	138	171	112	118	91	98	243	257	228	234	212	224	106	112	175		150		111	117
E1_10_5	Sundowner		170	206	138	171	112	118	91	98	243	257	228	234	212	224	106	112	175		150		111	117
DN7_15_3	Sundowner	F	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		117	
E1_2_2	Sunrise		170		138	171	114	118	91	98	243	255	216	220	212	226	97	106	175		150	152	117	118
E1_2_8	Suntan	N	183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_3_8	Swartland		182	206	160	171	104	112	98	103	233	239	208	224	212	255	106	110	175		150		115	117
E1_5_18	Swartland		185	206	160	171	113	131	91	98	243	255	218	230	212	255	95	106	175		140	155	111	151
E1_6_4	Sweet Cornelly		170	173	138	160	114	118	98	109	233	243	220	224	212	224	110	112	175	204	152	191	117	151
DN7_21_5	T 506		183		138	160	112	131	98		233	245	228	230	212	255	112		175		140	150	117	119
E1_6_3	Tasman's Pride		182	187	171		104	118	91	104	233		208	216	212	218	106	110	198	213	150		115	117

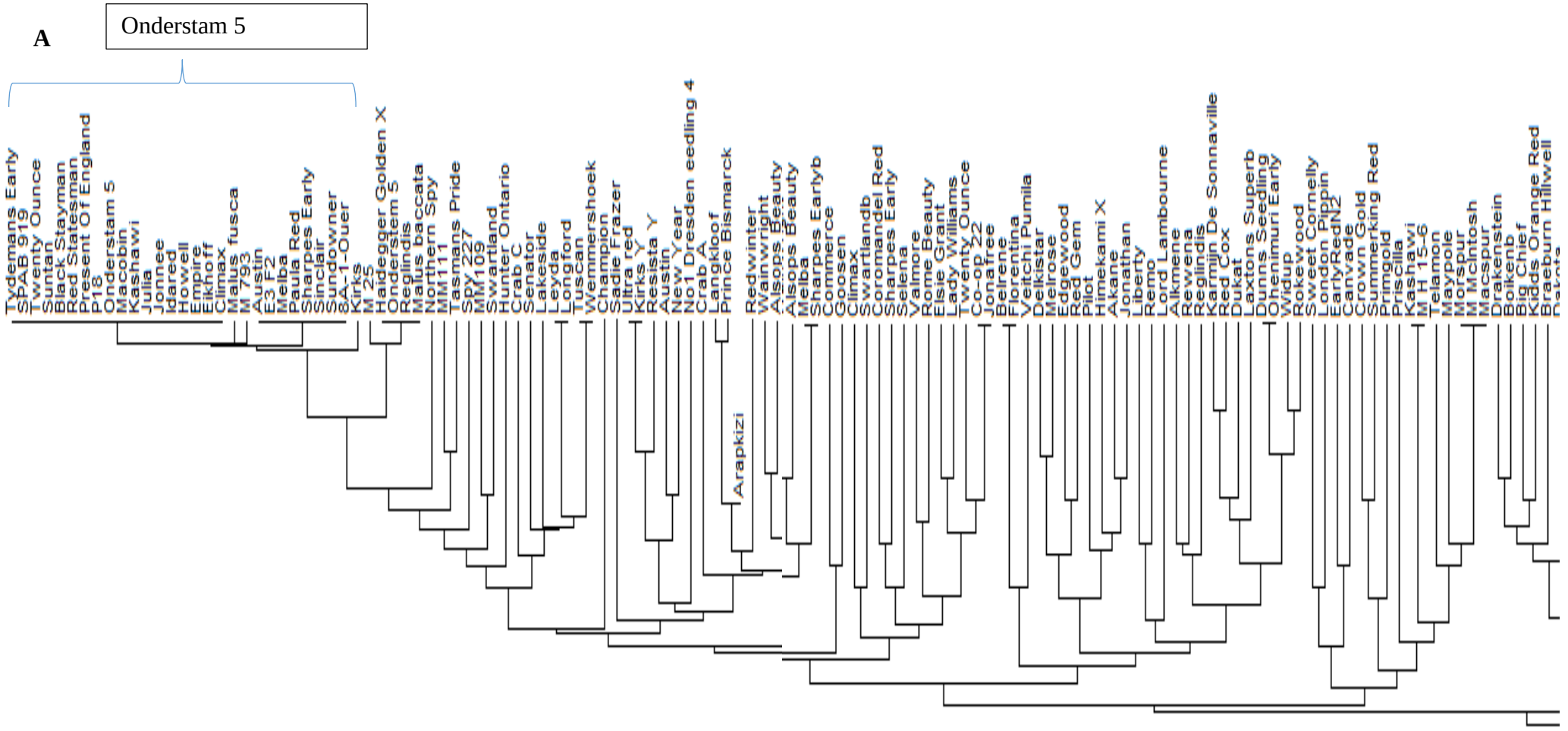
Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_10_16	Telamon (syn Waltz)		170	204	171		114	116	91	109	233	243	230	234	212	224	106	112	175	202	150	191	111	
E1_12_11	Telamon (syn Waltz)		170	204	171		114	116	91	109	233	243	230	234	212	224	106	112	175	202	150	191	111	
E1_1_14	Tjeek		183	220	160	171	116	131	91		245	257	208	224	255		106	120	175	209	150	155	115	117
E1_11_22	Trajan (=Polka)	V	179	204	160	171	116	118	98	109	243	257	220	230	226	229	95	106	175	209	150	191	111	117
E1_7_8	Beni Tsugaru	F	170	173	138	160	114	116	91	98	233	257	220		212	226	112		175	204	150	152	117	
DN7_20_5	Homei Tsugaru		179	206	138	171	114	118	91		243	249	230	234	226	255	95	112	175	202	150		111	117
E1_1_2	Homei Tsugaru A		179	206	138	171	114	118	91		243	249	230	234	226	255	95	112	175	202	150		111	117
E1_1_3	Homei Tsugaru B		179	206	138	171	114	118	91		243	249	230	234	226	255	95	112	175	202	150		111	117
E1_7_6	Natsuka		179	206	138	171	114	118	91		243	249	230	234	226	255	95	112	175	202	150		111	117
E1_14_1	Tuscan (=Bolero)		179	204	160	171	114	131	98	104	233	255	208	220	212	255	106		209	221	150	152	117	
DN7_4_1	Twenty Ounce		204	206	171	179	104	120	98		243	249	216	228	212	255	106		175	202	150	152	117	
E1_11_18	Twenty Ounce		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_4_16	Tydeman's Early		183	187	171	179	118	131	98	104	233		208	216	212	249	106		209	221	150		115	117
E1_1_18	Valmore		183	206	160	171	112		98		233	243	228	238	218	255	106	117	175	202	152	155	115	119
E1_10_9	Veitchi Pumila		182	218	150	179	116	129	98	111	256	257	216	218	212	229	106	110	202	209	150		151	
E1_6_9	Versveld		183	204	138	171	131		91	98	233	245	230		212	226	106	120	175		149	155	109	115
E1_5_4	Viljoen's Red		179	183	138	179	116		91	98	245	255	208	234	212	218	117	133	175	204	149	155	115	117
DN7_6_1	Vista Bella		182	206	145	160	114	116	91	98	233	243	228	238	255		97	104	175	209	152	183	117	
E1_5_2	Vista Bella		182	206	145	160	114	116	91	98	233	243	228	238	255		97	104	175	209	152	183	117	
E1_6_6	Wainwright		182	204	160	171	112	118	91	98	233		231	234	226	255	106	110	202	221	150	152	115	117
E1_14_19	Wemmershoek		179	204	160	171	114	131	98	104	233	255	208	220	212	255	106		209	221	150	152	117	
E1_5_3	White Winter Pearmain		179	183	171	179	116	131	91	98	233	245	208	238	212		112	133	175		150	155	111	117
E1_7_4	Widup		183	206	179	183	118	131	98	104	233	257	218	220	255		106	112	175	202	150	152	107	119
E1_3_4	William's Pride (=Co-op 23)		170	179	145	171	114	120	91	103	233	257	234	235	229	255	104	108	175	209	152	183	111	119
E1_2_18	Winesap		179	183	138	171	110	116	98		233	255	234	238	212	218	120		175	204	149	155	115	
E1_5_13	Seeando Winesap		179	183	138	171	110	116	98		233	256	234	238	212	218	117	120	175	204	149	155	115	
E1_14_14	Spur Winter Banana		179	182	160		114	118	91	98	233	257	220	230	224		95	112	175	202	155	183	107	117
DN7_7_1	Winter Banana		179	182	160		114	118	91	98	233	257	220	230	224		95	112	175	202	155	183	107	117
E1_14_15	Winter Banana		179	182	160		114	118	91	98	233	257	220	230	224		95	112	175	202	155	183	107	117
E1_4_2	Wolf River		170	206	138	179	112	114	98	103	239	255	224	230	212	218	110		175	198	150	191	115	119
E1_19_8	X2765		170	198	162	177	118	120	98	104	243	245	231	235	251	253	106		198	209	149	150	107	111
E1_19_3	X6163 P22 R19 A14		170	204	171	179	118	131	91	98	243	245	231	234	212	224	95	106	175		149	183	117	119
E1_19_10	X640 TNR42A45		170	206	138	171	114	118	91		243	249	234		212	224	133		202	217	150		113	117
DN7_2_3	Zabaoni		170	173	138	177	114	116	103		245	249	216	218	212	218	97	106	202	226	183	189	117	122

Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
E1_1_10	Zabaoni		170	173	138	177	114	116	103		245	249	216	218	212	218	97	106	202	226	183	189	117	122
E1_5_16	Zabaoni		170	173	138	177	114	116	103		245	249	216	218	212	218	97	106	202	226	183	189	117	122
E1_2_15	Zoba (=Lobo)		170	173	171		112	116	98		233	239	228	230	229	255	104	108	202	223	150		111	115
E1_4_18	Zvonkove		191	220	171	179	118	120	98		243	255	216	238	214	255	106	120	175	217	150	155	111	119
Other *Malus* species and hybrids																								
E1_7_11	KSC 3		172	175	138	150	114	122	89	98	255		206	216	216	247	110		175	184	150	152	134	151
DN7_21_1	KSC 3		172	175	138	150	114	122	89	98	255		206	216	216	247	110		175	184	150	152	134	151
DN7_21_2	KSC 11		172	187	138	148	114	118	89	103	245	249	206	216	216	247	108	110	175	184	152		119	124
E1_7_21	KSC 11		172	187	138	148	114	118	89	103	245	249	206	216	216	247	108	110	175	184	153		119	124
DN7_21_3	KSC 25		170	187	138		114		98	118	245	255	216	224	229	247	128	135	175	226	150	161	119	151
DN7_21_6	Malus 44		183	206	160	171	104	120	91	111	243	251	228	234	224	226	108	133	175	202	155	191	115	
E1_3_1	Malus 44		183	206	160	171	104	120	91	111	243	251	228	234	224	226	108	133	175	202	155	191	115	
DN7_34_1	*Malus* Aldenhamensis		206	216	171	181	104	116	96	104	231	245	234	241	212	224	110	130	188	240	158	159	115	
DN7_25_2	*Malus baccata*		183	187	171	179	110	131	98		233		208	216	218	249	106	110	209	221	150		115	117
E1_5_8	*Malus* Butterball		175	206	150	171	104	112	98	104	227	239	228		212	215	110		182	198	152	159	115	130
E1_3_7	*Malus floribunda*		175	179	150		104	139	104	111	231	251	224	228	216	220	108		188	198	150	173	115	136
E1_5_11	*Malus floribunda*		175	179	150		104	139	104	111	231	251	224	228	216	220	108		188	198	150	173	115	136
E1_17_6 R	*Malus floribunda*		175	179	150		104	139	104	111	231	251	224	228	216	220	108		188	198	150	173	115	136
DN7_25_1	*Malus fusca*	N	183	187	171	179	118	131	98	104	222	233	208	216	212	249	106		209	221	150		115	117
DN7_16_10	*Malus* Golden Hornet		172	177	150	164	104	116	91	104	231	255	235		214	224	106		194	198	149	161	117	138
E1_3_6	*Malus* Golden Hornet		172	177	150	164	104	116	91	104	231	255	235		214	224	106		194	198	149	161	117	138
E1_4_12	*Malus* Golden Hornet		172	177	150	164	104	116	91	104	231	255	235		214	224	106		194	198	149	161	117	138
DN7_2_9	*Malus* Grandiflora Crab	N	175	179	150		104	139	104	111	231	251	224	228	216	220	108		188	198	150	173	115	136
E1_19_7	*Malus* Grandiflora Crab	N	175	179	150		104	139	104	111	231	251	224	228	216	220	108		188	198	150	173	115	136
E1_15_7	*Malus* Jackson Crab	N	175	206	150	171	114	116	96	104	249	251	228	241	212	216	104	126	188	242	150		115	
E1_15_8	*Malus* L.P. Mornel Crab	N	175	206	150	171	116	139	96	104	249	251	228	241	212	216	104	126	188	242	150		115	
DN7_2_6	*Malus* Lemonei		175	206	150	171	116	139	96	104	249	251	228	241	212	216	104	126	188	242	150		115	
E1_3_22	*Malus* Lemonei Crab		175	206	150	171	116	138	96	104	249	251	228	241	212	216	104	126	188	242	150		115	
DN7_25_3	*Malus* Maypole		162	173	157	160	108	114	91	111	245	257	216	230	212	229	106	108	198	202	150	152	111	
E1_15_19	*Malus* Maypole		162	173	157	160	108	114	91	111	245	257	216	230	212	229	106	108	198	202	150	152	111	
DN7_20_2	*Malus micromalus*		179	182	150	157	108	139	105	111	245	251	228		212	220	108	126	188	224	150	157	113	115
DN7_5_5	*Malus* Mildew Resistant		175	179	138	150	108	116	98	115	251	255	208		216	218	118		194	204	155	165	117	126
DN7_20_4	*Malus* Moeransi Profusion		162	175	150	157	104	108	104	109	231	251	224	241	215		110		194	198	149	157	115	122
DN7_2_5	*Malus purpurea*		162	179	150	171	104	116	104	105	231	245	224	241	214	216	104	108	198	242	150		115	136
E1_9_11	*Malus purpurea*		162	179	150	171	104	116	104	105	231	245	224	241	214	216	104	108	198	240	150		115	136

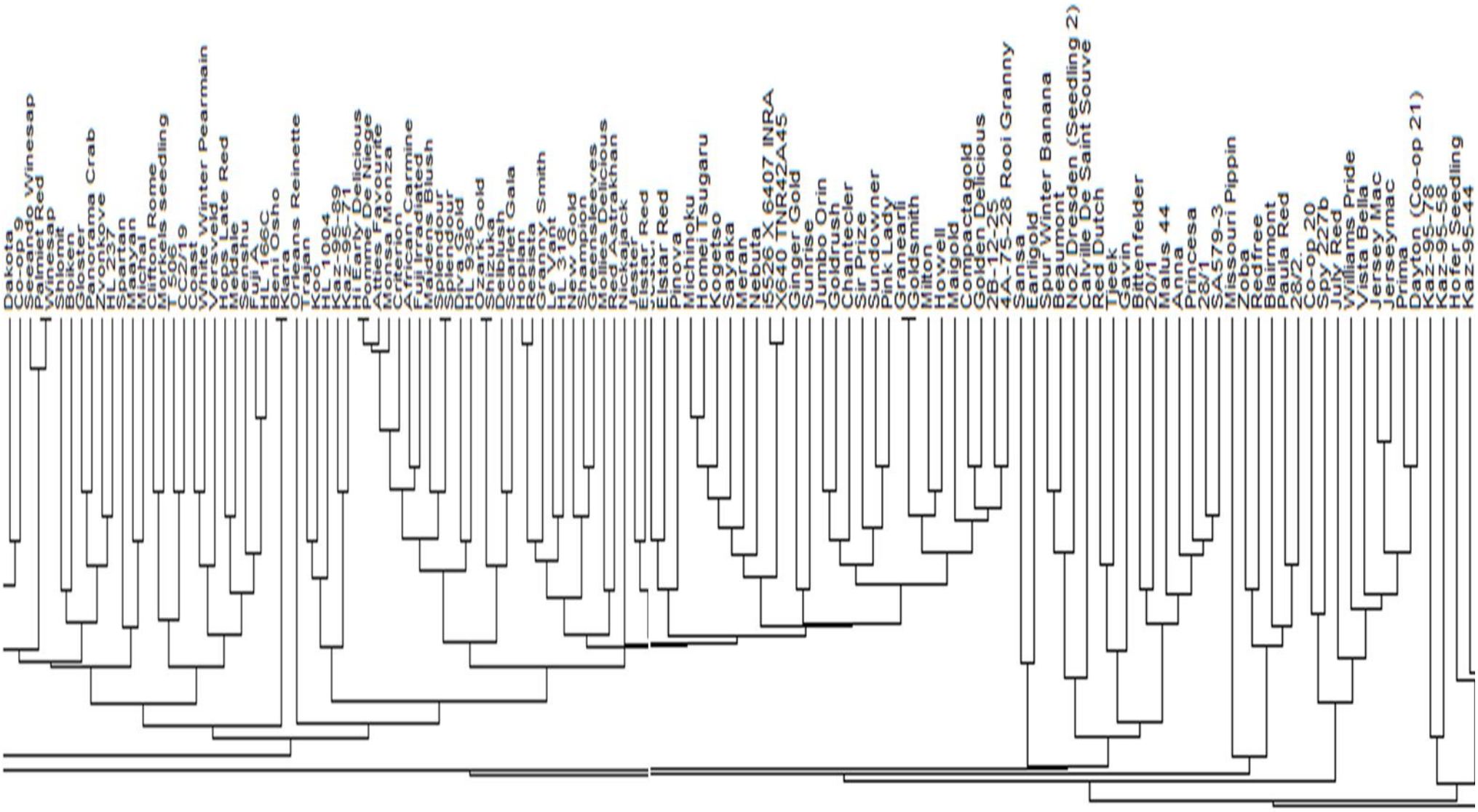
Tree	Name	Note	CH01f02		CH01f03b		CH01h01		CH01h10		CH02c09		CH02c11		CH02d08		CH04c07		CH04e05		GD12		Hi02c07	
			1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
DN7 4_10 R	*Malus robusta*		175	179	171		104		89	111	248		206	220	212	214	106	108	182		152		117	119
DN7_17_1	*Malus robusta*	I14439	175		150	171	110	116	109	111	251		206	231	214		97	108	182	204	152	161	117	119
E1_5_10	*Malus robusta*	I14439	175		150	171	110	116	109	111	251		206	231	214		97	108	182	204	152	161	117	119
DN7_7_6	*Malus sieboldii*		179		150	185	118	129	106	113	257		222	232	222	232	108		195	202	163		122	
DN7_15_7	*Malus sieversii* Kaz-95-44		187		145	179	114		98		239	249	224	228	218	226	110		198	209	150	156	111	115
DN7_17_8	*Malus sieversii* Kaz-95-57		162	170	179	185	108	114	98	103	239	245	218	228	212	218	108		198	223	150	161	113	115
DN7_8_2	*Malus sieversii* Kaz-95-57		162	170	179	185	108	114	98	103	239	245	218	228	212	218	108		198	223	150	161	113	115
E1_13_5	*Malus sieversii* Kaz-95-57		162	170	179	185	108	114	98	103	239	245	218	228	212	218	108		198	223	150	161	113	115
DN7_16_5	*Malus sieversii* Kaz-95-58		162	183	153	183	108	118	98	103	243		208	228	208	247	110	117	175	198	156	161	111	117
DN7_8_1	*Malus sieversii* Kaz-95-58		162	183	153	183	108	118	98	103	243		208	228	208	247	110	117	175	198	156	161	111	117
DN7_18_2	*Malus sieversii* Kaz-95-71		179		145	171	118		98		249	257	224	238	229	247	97	108	175	198	150		107	117
DN7_8_4	*Malus sieversii* Kaz-95-71		179		145	171	118		98		249	257	224	238	229	247	97	108	175	198	150		107	117
DN7_15_1	*Malus sieversii* Kaz-95-71A		170		157	171	108	114	96	103	239	251	224	228	212	218	110		198	223	150	161	113	115
DN7_18_1	*Malus sieversii* Kaz-95-78		162	172	157		108	112	98	118	249		218	228	212	214	108	110	198	207	150	161	117	126
E18_20	*Malus sieversii* Kaz-95-78		170	179	157	160	106	116	98	105	251		208	231	212	214	104	108	204		159	161	113	117
DN7_17_2	*Malus sieversii* Kaz-95-89		179	191	145	171	114	118	98	103	249	255	224	238	212	229	97	108	175		150		117	151
E1_8_6	*Malus sieversii* Kaz-95-89		179	191	145	171	114	118	98	103	249	255	224	238	212	229	97	108	175		150		117	151
DN7_15_6	*Malus sieversii* Kaz-95-91		162	172	179		108	112	98	105	239		224	239	212	218	97	108	198	205	150	183	115	
DN7_8_3	*Malus sieversii* Kaz-95-91		162	172	179		108	112	98	105	239		224	239	212	218	97	108	198	205	150	183	115	
E1_1_16	*Malus sieversii* Kaz-95-91		162	172	179		108	112	98	105	239		224	241	212	218	97	108	198	205	150	183	115	
E1_8_17	*Malus sieversii* Kaz-95-122	N	170	179	138	171	116	118	91	109	243	249	220	230	224	226	95	112	175		150	191	111	117
DN7_16_8	*Malus sieversii* Kaz-95-122		170	179	157	160	106	116	98	105	251		208	231	212	214	104	108	204		159	161	113	117
DN7_2_7	*Malus spectabilis*		162	216	150	181	108	122	100	109	232	251	218	239	212	224	108	130	188	194	155	159	115	126
DN7_6_3	*Malus spectabilis*		162	216	150	181	108	122	100	109	232	251	218	239	212	224	108	130	188	194	155	159	115	126
DN7_20_9	*Malus* Veitch's Scarlet		175		150	157	106	118	106	111	227	257	224		215	224	110	126	194	202	163		115	
DN77_10	*Malus zumi*	N	175	179	150		104	139	104	111	231	251	224	228	216	220	108		188	198	150	173	115	136
DN7_24_5	No4 Dresden (Seedling 3)		191	206	150	179	106	129	98	106	233	257	218	238	208	226	108		190	202	141	155	111	117
DN7_24_6	No5 Dresden (Seedling ?)		179	204	171	179	114		89	106	257		206	230	212	255	97	108	182	202	150	152	117	
DN7_24_7	No5 Dresden (Seedling ?)		191	206	150	179	106	129	98	106	233	257	218	238	208	229	112	120	188	190	141	152	109	117
DN7_25_4	S202		175	206	171		131		91	111	245	248	206	235	214		106	114	182	209	150	152	115	119
E1_6_17	Spy 227		175	182	150	171	104	131	98		227	233	208	224	215	249	106		188	209	150		115	117
DN7_15_2	Spy 227	N	179	206	138	171	116	118	98	109	243		234		224	255	95	104	175	209	191		111	119
DN7_21_4	T 585		175	204	171		116		98	111	245	248	218	220	212		106		182	198	152	183	117	

{ Brackets represent clones or sports of a 'primary' (Main cultivar),

Appendix 4.2. UPGMA dendrogram of the ARC apple collection based on genotypes at 11 microsatellite loci split into three, A, B and C, for better viewing and interpretation.



B



C