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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: GC0140
2. Project title: Fingerprinting the National Apple & Pear Collections
3. Contractor organisation(s):
   East Malling Research
   New Road
   East Malling
   Kent
   ME19 6BJ
4. Total Defra project costs (agreed fixed price): £ 319,372.00
5. Project: start date ..... 01 April 2007
   end date ..... 31 March 2010

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Aims and Objectives

Defra holds the National Fruit Collections (NFC) sited in Brogdale (Kent) and curated scientifically by the University of Reading. These collections constitute valuable genetic resources for the genetic improvement of the UK's principal fruit crops They contain approximately 2200 apple and 560 pear accessions, including dessert, culinary, ornamental and cider/perry types, as well as collections of other fruit crops. In recent years, the use of DNA markers for characterisation of germplasm collections has become increasing common. The markers most commonly used, known as Simple Sequence Repeats (SSRs), are areas of the genome of repetitive sequence (e.g. AT AT AT AT AT AT) and variable length (eg. 'AT AT AT' vs 'AT AT AT AT'). These variations in length can be detected in the laboratory and compiled to create a genetic 'fingerprint' of an individual tree. Such fingerprints are invaluable aids to the management of collections, eg when checking for trueness to type after propagation or for detecting likely duplicates. In addition, the determination of incompatibility (S) genotype by molecular methods is proving useful for fingerprinting Rosaceous tree fruits as well as providing agronomically useful data. Continuing the work started in Defra funded project GC0139, this proposal aim to complete the characterisation of the pear collection and similarly characterise the apple accessions. The main objectives were to:

- Use microsatellites to fingerprint the apple accessions in the National Fruit Collection and provide curators with a valuable data set which distinguishes clearly all or most of the varieties tested thus enabling checking of identities and detection of synonyms.
- Complete the data set of microsatellite fingerprints of the pear accessions in the National Fruit Collection and provide curators with a valuable data set which distinguishes clearly all or most of the varieties tested thus enabling checking of identities and detection of synonyms.

The work was broken down into the following technical aims:

1. to extract DNA samples from the accessions of the apple collection and the remaining half (275) of the pear collection
2. to optimise PCR conditions for ~12 informative microsatellite primers in apple, developing multiplexes if appropriate
3. to determine the microsatellite fingerprints of the accessions of the apple collection and the remaining half of the pear collection
4. to verify ploidy levels of all accessions which appear from microsatellite analysis not to be

diploid

5. to collate the data into Excel spreadsheets and provide to the scientific curator, e.g. to allow the search for duplicates, to submit the data to the freely-accessible ECPGR *Malus* and *Pyrus* databases and to produce papers on ploidy and fingerprinting.

Main findings

A total of 559 pear and 2,162 apple accessions were analysed with twelve SSRs chosen from the marker sets recommended by the ECPGR for each genus. A set of eight control genotypes for each crop were also included in the analysis to allow for the internal harmonization of data and to aid comparison of results with other studies. Analysis of the SSR data identified a total of 443 and 1,613 unique accessions of pear and apple respectively with the remaining individuals having at least one other accession with identical SSR profile. A total of 43 and 193 groups of suspected duplicates were identified in pear and apple respectively and they are presented in Tables 2 & 3. Some of these groups are made out of the known clones of popular cultivars - e.g. 15 'Williams', 7 'Conference' and 5 'Comice' clones in the case of pear and 20 'Jonagold', 21 'Golden Delicious' and 20 'Cox's Orange Pippin' clones in the case of apple were identified - whilst other groups could indicate previously unknown and/or unwanted replication, mislabelling etc. All data generated from this project has been tabulated and sent to Curator of the National Fruit Collection where morphological data will be used to determine if the accessions within the groups are in fact identical. These fingerprints will prove to be an extremely valuable reference set for testing the trueness-to-type of the recently re-propagated pear collection and the soon to be re-propagated apple collection. The re-propagated accessions fingerprinted using the same methodology used in this study and the two data sets compared – thus avoiding in most cases the need for laborious and time-consuming morphological comparison.

Additionally, 2,095 accessions were analysed using the consensus primers to amplify alleles of the *S*-locus and twenty-two putative new alleles were identified. At least one allele was amplified for each accession and 1,696 genotypes were fully characterised. Unfortunately, 399 accessions remained not fully resolved with one or more of their incompatibility alleles still undetected. Further research will be needed to confirm the new alleles identified and to improve the methodology in order to fully characterise the S genotype of all accessions.

Apples and pears are generally diploid (their cells contain two copies of each chromosome) however certain cultivars are polyploid i.e. they present three or four copies of each chromosome. Cytometric analysis was undertaken to confirm ploidy levels where SSR analysis indicated that an accession could be polyploid. A total of 48 pear and 304 apple accession have been confirmed as polyploids (Tables 6 & 7).

Future work and prospects

EMR has been recently commissioned to fingerprint two other UK fruit collections and result from these analyses will be compared to the data for the NFC. This would allow rationalising the germplasm kept in these other collections and present new candidates for accession into the NFC. As other international groups adopt the harmonised fingerprinting protocols for apple and pear it will be possible to compare data between collections which could lead to the identification of accession errors and even to the rationalisation of germplasm collections across Europe. Furthermore, different species of *Pyrus* and *Malus* evolved in a wide range of environmental conditions are known to have contributed to the current range of cultivated pears and apples respectively. Extending the range of material analysed with SSRs to include related species may be able to shed some light on the origins of domestic pears and apples the speciation within these genera. It could also be interesting to compare the genetic diversity found in cultivar collections with wild germplasm sampling in the species centres of origin. This would allow the incorporation of valuable novel material into germplasm collections thus increasing their value as genetic resources.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or

Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:

- the scientific objectives as set out in the contract;
- the extent to which the objectives set out in the contract have been met;
- details of methods used and the results obtained, including statistical analysis (if appropriate);
- a discussion of the results and their reliability;
- the main implications of the findings;
- possible future work; and
- any action resulting from the research (e.g. IP, Knowledge Transfer).

Introduction

Defra holds the National Fruit Collections (NFC), which are located at Brogdale, Kent, and curated scientifically by University of Reading. These collections are not only of heritage interest but constitute valuable genetic resources for projects concerned with the genetic improvement of the UK's principal fruit crops with respect to the requirements of, e.g., sustainable production and climate change. They contain approximately 2200 accessions of apple (*Malus*) and approximately 560 accessions of pear (*Pyrus*) - in both cases dessert, culinary, ornamental and cider/perry types - as well as collections of cherry, currant and gooseberry, grape, hazelnut and plum. In recent years, the use of DNA markers for characterisation of germplasm collections has become increasing common (Hokanson *et al.* 1998, Yamamoto *et al.* 2002, Guarino *et al.* 2006). Currently, the markers most commonly used are known as microsatellites. Microsatellites or Simple Sequence Repeats (SSRs) are non-coding sections of DNA consisting of two or three coding units repeated a variable number of times (e.g. AT AT AT AT AT AT or TGC TGC TGC TGC). For each SSR marker, an individual has two alleles, often of different length. The variations in length correspond to the number of repeats present in each allele (eg. 'AT AT AT' vs 'AT AT AT AT AT') and they arise from errors during DNA replication known as mutations. Mutations in non-coding DNA, such as SSR, have no noticeable effect in the organism and therefore they are not subject to selection pressure during evolution or in breeding. Consequently, many variants of these genetic regions can coexist in a given population making them ideal markers to detect diversity and for fingerprinting purposes. Such fingerprints are invaluable aids to the management of collections, e.g. when checking for trueness-to-type after propagation or for detecting likely duplicates. In addition, the determination of incompatibility (S) genotype by molecular methods is proving useful for fingerprinting Rosaceous tree fruits as well as providing agronomically useful data.

This project aimed to complete the fingerprint of the pear collection initiated in Defra-funded project GC0139 and to fingerprint the entire apple collection. Many informative SSRs have been developed for apple and pear (Gianfranceschi *et al.* 1998; Liebhard *et al.* 2002; Hemmat *et al.* 2003; Silfverberg-Dilworth *et al.* 2006; Yamamoto *et al.* 2002; Fernández-Fernández *et al.* 2006) and various research groups have used different sets for fingerprinting. This project, like GC0139, has benefited from decisions to nominate standard microsatellite sets to fingerprint these crops reached by international experts at an ECPGR (European Collaborative Programme for Crop Genetic Resources) workshop organised by EMR in 2006. Moreover, a small complementary project carried out in collaboration between EMR and Imperial College (London) has developed consensus markers for the amplification of the S locus in apple allowing us to provide considerable data on the self-incompatibility genotypes of the accession in the NFC.

The fingerprinting data arising from this project will be a great aid to the efficient management of the collections. It will allow duplicates to be detected, which can lead to rationalisation, and it will provide the reference dataset against which new fingerprints can be checked after repropagation of the collections. The traditional method of doing this, morphological comparison, is time consuming and could take several years during which both the old and new collections would need to be maintained. In addition, because the sets of microsatellites used have been accepted as the standard European set for genotyping, the fingerprints can be compared with those of European collections to aid verification and, potentially, rationalisation at the European level.

Aims and Objectives

1. Use microsatellites to fingerprint the apple accessions in the National Fruit Collection and provide curators with a valuable data set which distinguishes clearly all or most of the varieties tested thus enabling checking of identities and detection of synonyms
2. Complete the data set of microsatellite fingerprints of the pear accessions in the National Fruit Collection and provide curators with a valuable data set which distinguishes clearly all or most of the varieties tested thus enabling checking of identities and detection of synonyms.

The work was broken down into the following technical aims:

6. to extract DNA samples from the accessions of the apple collection and the remaining half (275) of the pear collection
7. to optimise PCR conditions for ~12 informative microsatellite primers in apple, developing multiplexes if appropriate
8. to determine the microsatellite fingerprints of the accessions of the apple collection and the remaining half of the pear collection
9. to verify ploidy levels of all accessions which appear from microsatellite analysis not to be diploid
10. to collate the data into Excel spreadsheets and provide to the scientific curator, e.g. to allow the search for duplicates, to submit the data to the freely-accessible ECPGR *Malus* and *Pyrus* databases and to produce papers on ploidy and fingerprinting.

Technical aim 1- DNA extraction: to extract DNA samples from the accessions of the apple collection and the remaining half (275) of the pear collection

DNA was extracted from the remaining pear samples using 0.2g tissue following a modified CTAB protocol (De La Rosa *et al.* 2002). The extracts were quantified and partially qualified by electrophoresis through agarose. The quality of the DNA was further assessed by checking the amplification the samples with a fully optimised PCR. A total of 2,162 apple accessions, including 75 from the observation plot added to the analysis in 2009 at the request of the collection curator, were collected. Leafy shoots were taken from one of each pair of accessions of the apple collection at Brogdale by the sub-contractor or the curator and were labelled to indicate the row number and tree position within each row. A list of the genotypes collected was produced. Foil parcels and tubes were labelled according to the collection list provided, leaves of each sample were removed and frozen in liquid nitrogen for storage in a -80^{o}C freezer at EMR. Apple DNA was carried out following the technique described above.

Technical aim 2 - Choice of primers and optimisation of PCR conditions: to optimise PCR conditions for ~12 informative microsatellite primers in apple, developing multiplexes if appropriate

2.1. Optimisation for SSR fingerprinting of the pear collection:

Pear fingerprinting was carried out as in Defra-funded project GC0139 of which this work is a continuation.

2.2. Optimisation for SSR fingerprinting of the apple collection:

The choice of a set of microsatellites for fingerprinting apples was discussed at an ECPGR workshop (December 2006). Markers were chosen from each linkage group (where possible) that are robust, preferably single locus and that have been proved to be polymorphic in previous studies. From those recommended, we chose 12 namely, CH04c07, CH01h10, CH01h01, Hi02c07, CH01f02, CH01f03b, GD12, GD147, CH04e05, CH02d08, CH02c11 and CH02c09, that when labelled with four different fluorescent dyes, could be combined into three 'multiplexed' reactions. These 12 primers have now been chosen as the ECPGR core set. PCR conditions were optimised for the multiplexes to obtain robust and reliable amplification.

2.3. Optimisation of methodology for self(in)compatibility allele genotyping in apple

Consensus primers for the *S* genotyping of the apple collection have been developed using a reference set of cultivars which represent the *S* alleles 1 to 32. The reference set are cultivars in which the *S* genotype has been reported and confirmed in the literature.

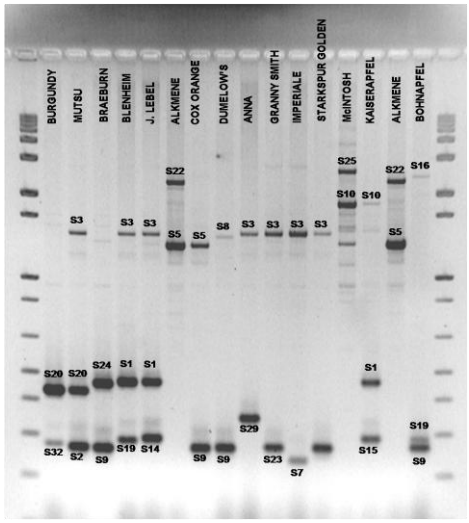

Figure 1. Apple S-RNases amplified with consensus primers, separated and sized in an agarose gel

Allele sizes for each of the published *S* alleles are presented in Table 1 alongside some of the cultivars they were originally described as provided by Imperial College (London) consultant.Two different methods were combined to allow the detection and accurate sizing of alleles across a wide range of sizes. Originally, PCRs were carried out using non-labelled primers and product was then separated by electrophoresis on agarose gels (Fig.1) allowing the identification of large alleles such as S_3 (~1,400 bp) or S_{22} (~2,200 bp). Where it was not possible to score all alleles in agarose gels, PCRs where repeated using fluorescently labelled primer pairs and products from these reactions was also run on a semi-automated ABI 3100 sequencer thus allowing the accurate discrimination of alleles under 500 bp such as S_{18} (279 bp) and S_{19} (282 bp). Where samples were unclear or failed on the first round of PCRs reactions were repeated using fluorescently labelled markers followed by electrophoresis in agarose gels as well as on the semi-automated ABI 3100 instrument.

Table 1. *S-alleles* previously described in apple, product size detected using Imperial College's protocol and cultivar(s) in which each allele can be found

Allele	Size (bp)	Cultivars	Allele	Size (bp)	Cultivars
S_1	450	Blenheim, Jacques Lebel	S_{19}	279	Blenheim Orange, Empire
S_2	259	Mutsu, Prima, Reinette de Champagne	S_{21}	287	Ribston Pippin, Melba
S_3	1400	Mutsu, Granny Smith, Citron d'Hiver	S_{22}	2200	Alkmene, Bismarck
S_4	250	Gravenstein, Reinette de Champagne	S_{23}	258	Granny Smith, Glockenapfel
S_5	1300	Cox's Orange Pippin, Alkmene	S_{24}	445	Braeburn, Red Rome
S_6	280	Citron d'Hiver, Oxford Hoard	S_{25}	2600	McIntosh, Laxtons Pearmain
S_7	231	Jonathan, Imperiale, Monroe	S_{26}	273	*Malus baskatong*
S_8	1350	Dumelow's Seedling, James Grieve	S_{29}	339	Anna
S_9	256	Cox's Orange Pippin, Jonathan	S_{31}	383	Perrine York, Bosbury Pippin
S_{10}	1800	McIntosh, Spartan, Prima	$S_{14} = S_{15}$	285	Gravenstein, Jacques Lebel
S_{16}	2400&2800	Bohnapfel	$S_{28} = S_{30}$	286	Red Delicious, Gloster 69
S_{18}	282	Starking Delicious	$S_{20} = S_{32}$	424	Gravenstein, Mutsu

Technical aim 3 – Genotyping of the apple and pear collections: to determine the microsatellite fingerprints of the accessions of the apple collection and the remaining half of the pear collection.

3.1. – SSR fingerprinting of the pear collection

The remaining pear DNA samples were amplified with the chosen primers in multiplex reactions, using a thermocycler machine and ensuring the presence of the selected control samples within each plate (as listed in GC0139). The amplification products were loaded on to an ABI semi-automated Genetic Analyzer for fragment electrophoresis and sizing. Then the data were compiled using GENESCAN and GENOTYPER software. The quality and reliability of the fingerprints was assured by using standardised methodologies with optimised primers and regular control samples. Hard copies of all traces from the software were printed facilitating clear comparisons between peak intensity and fragment size of the amplified products. A degree of 'judgement' was used to score different markers; unreliable peaks or those largely outside the range of the marker were ignored, where double peaks are generated in PCR (due to imperfect A-addition), a decision was made on whether to consistently score left-hand or right-hand peak regardless of their relative size and where strong stuttering was present and care was taken to discriminate between stutters and nearby alleles.
Each plate was scored independently and scores were confirmed by a second researcher. When necessary, corrections to scores were made on the printed files and transferred to the score files. Repeats were performed on any sample that failed or where the traces were unclear. Any variation in peak size between plates was normalised by comparing the control samples. After being checked twice by two independent researchers and data were considered validated and remained stored within EMR's EMQA system.

Allele sizes provided by GENOTYPER software are estimations by comparison with internal size standard and therefore are expressed as a range of values with decimals (121.78, 121.89, 121.91, 122.01 and 122.12 would all correspond to an allele of 122 bp). In order to compare fingerprints for different accession it was necessary to round those alleles. Rounding was done by transferring all peak sizes to a different file and plotting the spread of each allele in a graph to help visualise the size range for each allele. Once the range of sizes for each allele was determined, the EXCEL function 'Vertical look up'(VLOOKUP) was used to replace the original score by the rounded allele size. Data were compared using GenAlEx software. The analysis of all the pear data produced in this project together with that from project GC0139 showed a number of accessions with identical fingerprints (Table 2) including known clones and some suspected replications.

Table 2. Groups of pear accessions indistinguishable by microsatellite analysis

Comice[1]	**Williams**	05 21 Fertility
01[2] 19[3] Red Comice	06 07 Nye Russet Bartlett	05 23 Improved Fertility
02 19 Doyenne du Comice	07 08 Parburton	**Hosui**
03 19 Doyenne du Comice	08 08 Arnold	06 29 Shinsui Asian
05 29 Comice Bodson	09 08 Double Williams	08 09 Gros Blanquet
Conference	10 00 Instone 1	09 03 Bianchettone
06 17 Conference BronzeeB557	10 08 Max Red Bartlett	03 04 Early Seckel
07 18 Conference Primo	11 07 Redbald	04 04 Early Seckel
08 17 Conference Van Wetten	12 07 Biggar Russet Bartlett	06 37 Liegels Butterbirne
09 17 Conference	13 07 Knock out Russet Bartlett	17 41 Virgoloso
10 17 Conference Russet Wheldon	14 07 Moyer Russet Bartlett	12 41 Sos
34 03 Saels	15 07 Sandar Williams Creuse	18 37 Easter Beurre
04 25 Williams d'Hiver	16 07 Williams Bon Chretien	11 37 Rogue Red
06 31 Williams d'Hiver	17 07 Williams Bon Chretien	19 00 G Rosired
01 11 Laxtons Superb	18 07 Russet Bartlett	21 15 Onward
02 12 Mercer	19 07 Striped Williams	21 27 Belle de Soignies
14 19 Duchesse Bererd	06 13 Spadona d'Estate	16 03 Citron des Carmes
15 19 Duchesse d'Angouleme	13 00 E Krystali	17 03 Citron des Carmes Panache
17 37 Duchesse Panachee	19 35 Butirra	18 11 Saint Jean Panachee
19 15 Csatar	05 03 Grand Champion	22 21 Oldfield
20 15 Soldat Labourer	06 03 Gorham	P1 11 Oldfield
11 27 Crassane Panachee	13 16 Autumn Bergamot	11 31 Unknown S R Peart
22 35 Crassane	O3 39 Autumn Bergamot (accessed as Achan)	21 33 Beurre Rance
04 21 Ferdinand Gaillard	06 05 Maxine	06 27 Red Beurre Hardy
09 27 Constant Lesueur	07 05 Starking Delicious	07 27 Beurre Hardy
11 29 Southworth	03 25 Ritson	16 13 Howlett
12 31 Vermont Beauty	13 17 Danas Hovey	23 19 Madame Treyve
04 05 Magness	05 19 Dubbele Kreeftpeer	15 31 Italy 154
05 05 Magness	14 38 Double de Guerre	18 00 G Porporata
06 35 Furedi	01 01 Andre Desportes	14 35 Blickling
19 37 Egri	22 11 Alexandrina Bivort	23 31 Jean de Witte
34 08 Little Swans Egg	O4 35 Constance Mary	20 17 Belle de Bruxelles
O[3]1 39 Muirfowl Egg	O0 Miles	27 31 Belle de Bruxelles
08 29 Fondante d'Automne	19 03 Clapps Favourite	10 09 Hessle
12 17 Bergamotte Heimbourg	20 03 Starkrimson	O4 33 Denny's Farm
21 23 Seigneur Esperen	20 05 Large Clapps	O4 13 Goudnap
07 09 Green Pear of Yair A	07 01 Buzas Korte	O4 17 Grey Honey
O5 3 Port Allen 1	13 13 Voros Buza Korte	O5 17 Maggie Duncan
O5 5 Port Allen 2		
O5 7 Port Allen 3		

[1] Controls samples used to standardise allele sizes across different plates appear in bold in the table.
[2] First two digits indicate row number
[3] Second two digits indicate position of the tree within the row
[4] The letter 'O' indicate the samples came from the observation plot not the main collection

3.2. – SSR fingerprinting of the apple collection

DNA from 2,162 accessions from apple collection, including 96 sample from the cider collection and 75 from the observation plot was amplified with a set of 12 SSR markers amplified in three multiplex reactions, using a thermocycler (Fernández-Fernández et al. in preparation). Eight control samples ('Delicious', 'Fiesta', 'Malling 9' (rootstock), 'Michelin', 'Prima', 'Worcester Pearmain', '*Malus robusta* 5'

and '*Malus floribunda* 821' all ex-INRA at Angers) were included in each plate as per recommendation of the ECPGR 2006 workshop.

Methodology for scoring, checking, and rounding data was the same as the one used for pear as outlined above. The analysis of all SSR data produced showed a number of accessions with identical fingerprints including known clones and some suspected replications (Table 3).

Table 3. Groups of apple accessions indistinguishable by microsatellite analysis

02[1] 01[2] Nico 03 01 Lodi	03 11 Reverend W Wilks 04 13 Autumn Harvest	05 15 Grenadier 05 17 Guldborg
10 05 Melba 10 07 Hunter Melba 10 09 Red Melba	04 05 Wrixparent 11 09 White Transparent 15 19 Perrine Yellow Transparent	11 25 Beauty of Bath 11 31 Crimson Beauty of Bath 11 33 Tims Early
06 03 Karinable 10 03 Maidstone Favourite	10 25 Savstaholm 10 27 PJ Bergius	10 31 Tewkesbury Baron 10 33 Thomas Jeffrey
13 17 Laxtons Fortune 13 19 Fisher Fortune 13 21 Red Fortune	24 31 Lord Derby 24 33 Lord Derby spur type O[3]2 13 Harvest Lemon	29 03 Lord Lambourne 29 05 Lady Lambourne 29 07 Russet Lambourne
12 23 Discovery 14 05 Discovery 14 07 Discovery 19 23 Discovery	14 03 James Grieve 14 09 Erich Neumanns Roter 14 13 James Grieve 19 17 Redcoat Grieve	**Worcester Pearmain**[4] 14 15 Worcester Pearmain 16 21 Worcester Pearmain O 2 31 Hick's Fancy
12 03 Benoni 12 05 Red Benoni	13 01 Laxtons Epicure 13 03 Epicurean	13 25 George Cave 13 27 George Cave
39 19 Sunset 39 21 Sunset sport	31 21 Barchards Seedling 40 17 Welford Park Nonsuch	42 31 Ellisons Orange McCarroll 42 34 Red Ellison
36 27 Millicent Barnes 36 29 Millicent Barnes sport	35 03 Guelph 37 17 Peacemaker	35 09 Histon Favourite 38 15 Richardson Tomalin
29 11 Blackmack 29 13 McIntosh 29 15 Alexis 29 22 Black Mickey 29 25 Kimball McIntosh 29 28 Johhnson McIntosh 29 30 Rogers McIntosh 29 31 Starkspur McIntosh 49 14 Red Fameuse	52 01 Jonathan 52 03 Blackjon 52 05 Jonathan Matthews 52 07 Jonathan a 52 09 Jonathan b 52 15 Kapai Red Jonathan 52 19 Jonathan 15 Welday 52 21 Jonathan 19 Welday 37 01 Nouvelle Europe	40 35 Red Elstar 42 35 Elstar 47 27 Daliest 19 103 Reinstar 21 99 Elnica 24 91 Elshof 25 111 Bel-el 40 01 Wealthy 40 05 Double Red Wealthy
35 19 Jefferis 35 21 Jennifer Wastie	38 29 Sandew 38 31 San Peinte	40 07 Loop Wealthy 40 09 Stevenson Wealthy
15 37 Cox's Orange Pippin 15 41 Cox's Orange Pippin LA 79 15 43 Cox's Orange Pippin Otago 15 45 Cox's Orange Pippin Vison 15 50 Clarkes Royal 15 51 Queen Cox Maclean 15 55 Queen Cox 15 57 King Cox 15 59 Crimson Cox 16 37 Cox's Orange Pippin 16 39 Cherry Cox 16 43 Kortegaard Cox 16 45 Cox's Orange Pippin Potter 16 47 Rouge des Flandres 16 49 Frydeland Cox 16 53 Queen Cox 16 55 Cox's Orange Pippin LA 62D 16 59 Cox's Orange Pippin Wisley 17 57 Cox's Orange Pippin spur type 18 89 Cox La Vera 18 91 Red Cox (93-019) 20 111 Vegi Cox	07 29 Chips 15 23 Queenbys Glory 28 33 Keeds Cottage 30 11 Nanny 17 30 Margaret 18 21 Summer Apple 25 01 Peasgoods Nonsuch 25 03 Crimson Peasgood 15 01 Millers Seedling 15 03 Red Millers Seedling 25 19 Winter Codlin 26 09 Woodford **Fiesta** 29 33 Fiesta 06 26 Willy Sharp 31 27 Beacon 31 33 Biesterfelder-Renette 32 05 Calville Rouge du Mont d'Or 32 15 Charles Ross 32 17 Red Charles Ross 34 09 Geeveston Fanny 34 11 Red Geeveston Fanny	13 39 Testerspur Golden Delicious 13 41 Golden Auvilspur 13 43 Starkspur Golden Delicious 13 45 Goldenspur 13 47 Yellowspur 13 49 Golden Delicious B 13 55 Courtagold 14 37 Golden Delicious 14 39 Golden Delicious 14 41 Horst No 2 14 43 Goldensheen 14 47 Nugget 14 49 Double Golden Delicious 14 51 Golden Delicious Russet Form 14 55 Lys Gold 14 57 Ed Gould Golden 14 59 Smoothee 15 39 Penco 48 83 Skinlite 24 89 Elbee 26 85 Golden Delicious (Vinson)

continued

Table 3 (continued). Groups of apple accessions indistinguishable by microsatellite analysis

43 21 Ingrid Marie 43 23 Ingrid Marie 43 23 Red Ingrid Marie	43 29 King of the Pippins 43 31 King Russet 43 35 Rote Goldparmane	38 19 Rivers Nonsuch 45 29 Renown 42 87 Lambs Seedling
24 03 Christie Manson 44 23 Green Roland	39 23 Ten Commandments 44 29 Merchant Apple	25 05 Red Musk 45 33 Seabrooks Red
26 89 Jonagold 1905 O 4 27 Queen Anne	45 05 Norfolk Royal 45 07 Norfolk Royal Russet Sport	**Prima** 47 31 Prima
07 09 Evagil 47 11 Sharleston Pippin 47 33 Yellow Pitcher	28 11 Devon Crimson Queen 48 33 Sops in Wine C^{5}6 19 Sops in Wine (B)	53 13 Dugamel 55 15 Melrose 57 17 Marstar
O 2 17 Charleston O 4 05 Kane's Seedling	30 35 Rosa del Caldaro 49 29 Mela Carla	51 27 Kidds Orange Red 51 29 Captain Kidd
52 23 Crimson Superb 52 25 Laxtons Superb	54 10 Spartan 54 11 Spartan Scotland	48 25 Missouri 53 17 Ozark Gold
52 29 Maxton 52 31 Russet Superb	54 13 Spartan Sweden 54 16 Spartan No3	53 25 Red Statesman 53 28 Statesman Red Sport
52 33 Laxtons Superb NFT clone 52 35 Laxtons Superb	54 17 Hunter Spartan 54 19 Spartan 10C-6-43-l	44 17 McLivers Winesap 54 31 Dermen Winesap
56 09 Jincoa Zagarra 10 39 Eri Zagarra	37 11 Orleans 11 45 Quindell	34 03 Foulden Pearmain 20 55 Old Pearmain
10 37 Dukat 12 43 Dukat spur type 43 85 Shenandoah	24 01 Chelmsford Wonder 28 51 Lundbytorp 27 110 Mitchelsons Seedling	23 48 Marosszeki Piros Paris 31 105 Martini 31 107 Red Martini
48 19 New Fiji 19 59 Fuji	16 51 Kent 23 37 Kent	50 25 Ben Davis 26 47 Black Ben
18 95 Fuji INRA Nagafu O 2 39 Fuji Brak	06 15 Red Victoria 24 59 Sweet Caroline	20 39 Gustavs Dauerapfel 27 54 Gyogyi Piros
Delicious 30 61 Starks Late Delicious	27 61 Jonagold 28 57 Minister von Hammerstein	29 47 Pixie 29 49 Pixie red sport
43 55 Pagsup spur type 43 59 Oregon Spur 44 45 Richared Delicious 44 49 Starking 44 51 Starkrimson 44 57 Wellspur 24 103 Lancraig	35 81 Northern Spy 35 83 Double Red Northern Spy 35 85 Hunter Kinkead Spy (4n) 35 87 Kinkead Red Spy 35 89 Loop Spy (4n) 35 91 Crimson Spy 42 107 Geneva Ontario (4n)	36 73 Rome Beauty 36 75 Barkley Red Rome 36 77 Double Red Rome Beauty 36 79 Glengyle Red 36 81 Red Rome (Australia) 36 83 Ruby Rome Beauty 36 85 SeeandO Red Rome
24 95 Hared 26 113 Averdal	25 50 Granny Smith spur type 35 49 Granny Smith	21 53 Aromatic Russet 38 37 Parkers Pippin
38 49 Pomme d'Amour 38 51 Pomme de Fer	54 05 Rosioare Calugaresti 40 49 Sikulai Alma	50 35 Colonel Vaughan 41 43 Winter Marigold
30 05 Maltster 41 57 Leeders Perfection	42 65 Cavallotta 42 67 Champ Gaillard	33 35 Flower of the Town 43 61 Langes Perfection
44 05 Lemon Queen 28 71 Nottingham Pippin	42 01 Caroline 28 85 Patrick	29 101 Barnack Beauty 29 103 Barnack Beauty sport
56 23 Mrs Phillimore 29 79 Sweet Merlin	18 63 Newtown Pippin 29 89 Yellow Newtown Pippin	44 07 Limoncella 30 81 Cola
35 59 Hoe 31 113 Megumi	31 101 Lucullus 32 87 Prins Bernhard	31 79 Herceg Batthyanyi Alma 32 99 Reinette Franche
33 105 Api Rose 33 113 Blandurette	23 31 Broad Eyed Pippin 33 96 Betty Geeson	33 97 Winston 33 99 Winston sport
45 49 Gala 45 51 Tenroy	45 65 Green Purnell 45 67 Gros Api	55 23 Baxters Pearmain 45 63 Golden Reinette
20 93 Imperial Gala 23 89 Galaxy	58 03 Apez Zagarra 46 37 Anisa	48 39 Beurriere 48 61 Normandie
24 105 Prince Gala Regal Prince	49 38 Cravert	16 61 Belle de France
30 91 Daru Sovari	49 39 Cravert Rouge	50 62 Crawley Beauty
31 76 Harang Alma 35 109 Sovari Nobil	51 B Telamon 51 F Trajan	28 111 Sandow 28 113 Hunter Sandow (4n)
45 13 Orenco 34 109 Ivo	34 93 Feuillot 34 95 Fosters Seedling	18 25 Taunton Cross 35 73 Laxtons Pearmain
25 23 Desse de Buff 36 105 Franc Roseau	53 20 Perrine York 36 99 York-a-Red	49 44 Belle des Buits 37 102 Paradis

continued

Table 3 (continued). Groups of apple accessions indistinguishable by microsatellite analysis

22 15 Meri Cretesti 38 81 Cretesc	37 31 Prinz Albrecht von Preussen 38 97 Fairy	38 106 Maid of Kent 39 83 Bismarck
21 35 Grosse de Saint Clement 40 89 Grandmere	41 99 Bedfordshire Foundling 42 99 Missing Link	55 19 Winter Peach 43 111 Devonshire Buckland
44 95 Newton Wonder 44 97 Crimson Newton Wonder	32 53 Bouquepreuve 45 98 Bonnet de Comte	47 03 Gronsvelder Klumpke 46 99 Rheinischer Krummstiel
44 101 Red Newton Wonder 44 99 Marston Scarlet Wonder	46 77 Gazerau 46 79 Gelber Trierer Weinapfel	46 45 Franc Bon Pommier 47 75 Brabant Bellefleur
36 103 Bastien 47 88 Lagree	18 105 Royal Blush 18 107 Rodluvan	24 45 Reinette Grise de Saintonge 18 113 Rushock Pearmain
17 37 Alkmene 19 112 Red Alkmene 23 83 Ceeval	O 2 37 Fallbarrow Favourite O 3 15 Weaten Loaves (Hedge) O 3 17 Weaten Loaves (Leaning)	44 105 Present van Engeland O 3 7 Lady's Finger (Gorman) O 3 9 Lady's Finger (Wass Helmsley)
34 111 King Charles Pearmain 19 81 Polan 2	25 47 Falstaff 20 97 Red Fallstaff	30 07 Milton 21 105 Karina
42 83 Jeanne Hardy 21 83 Belle de Pontoise	21 109 Reinette de Caux 23 111 Mangasuper	20 99 Chantecler 25 113 Chantegrise
19 29 Rubin 21 89 Bohemia	48 05 Delcorf 23 87 Dalili	07 02 Coopers Seedling 26 109 Grimoldby Golden
25 65 Aroma 22 113 Amorosa	43 45 Cockle Pippin 25 105 Grey Pippin	56 15 Kingston Black C2 23 Kingston Black (B)
18 37 False Burr Knot Howard C3 27 Genet Moyle	C5 1 Pethyre C5 21 Broadleaf Norman	41 59 Hockings Green C5 17 John Broad
C3 5 False (Balls Bittersweet) C3 7 EB54	C1 23 Reine des Pommes C6 3 Langworthy	07 25 Venus Pippin O 1 19 Plum Vite
33 101 Wyken Pippin O 1 1 Whiting Pippin	26 07 Kings Acre Bountiful O 1 33 White Melrose (Anton's Hill)	26 03 White Melrose O 1 35 White Melrose (Priorwood)
39 85 Costard (Howlett) O 1 9 Costard (supposed)	36 17 Mabbotts Pearmain O 2 15 Stead's Reinette	17 105 Onibury Pippin O 2 23 Onibury Pippin
O 1 5 Sam's Crab O 2 25 Sam's Crab	O 2 29 Costard O 2 35 Sweet Cleeve	45 21 Rank Thorn O 3 11 Rankthorn
23 07 Transparente de Croncels O 3 19 Weaten Loaves (tree 2)	O 3 23 Wanstall Pippin O 3 25 Wanstall Pippin	43 17 Herrings Pippin O 3 29 Red Rolo
51 03 Court of Wick O 4 1 Sykehouse Russet (Far)	15 21 Polly O 4 29 Reynold's Peach	45 93 Annie Elizabeth O 5 23 Greasy Butcher
04 03 Tordai Alma 22 09 Kirkes Lord Nelson	17 59 Acklam Russet 29 57 Reinette de Macon	54 21 Stafner Rosen 42 41 Baldwin
22 07 Loddington 26 17 Stones Mosaic	24 29 Ladys Delight 36 35 New German	32 35 Csikos Orias Halasi 39 31 Vajki Alma
20 15 Hibernal 42 23 Luzhanka 42 25 Doux dArgent original sample	27 25 Old English Round 55 11 Crimson King 2 13 Crimson King	24 18 Granges Pearmain Barnes 40 41 Sandlin Duchess 39 87 Dicks Favourite
25 55 New Jonagold 17 109 Veekmans Jonaster	26 87 Jonagold O 5 19 Abbot's Early	38 43 Pepin de Bovelingen 38 64 Rambour Podolskii
18 101 Excel 18 93 Wilmuta	39 79 Belle de Boskoop (3n) 39 81 Red Belle de Boskoop	32 95 Reinette du Canada (3n) 32 97 Reinette Grise du Canada (3n)
18 99 Jonagored Supra 19 107 Jonagold (EMLA)	21 87 Bielaar 24 113 Botden	24 05 Contessa 40 75 Beauty of Kent
19 113 Red Jonaprince 20 102 Jonagold Boerekamp	41 105 Bramleys Seedling (3n) 41 107 Bramley (m Crimson)(3n)	AO 1 21 Profit 1 AO 1 23 Profit 2
20 103 Josegold 20 107 Prince Jonagold DH	20 07 Earl Cowper 47 101 Tower of Glamis	43 51 Cornish Pine AO 1 25 Red Ribbed Greening
20 89 Decosta 21 111 Jonagold clone AW2001	45 01 Mother 17 111 Queen Mary	27 07 Gravenstein 27 09 All Red Gravenstein
23 103 Jomured 23 113 Orei	21 31 Excelsior Seabrook 26 107 Rougemont	27 11 Morkrod 23 01 Tom Putt
24 107 Rubinstar 24 99 Jored	38 94 Smalls Admirable 5 24 Captain Broad (B)	23 05 Sidney Strake C3 1 Tom Putt (B)
26 91 Jonagored 26 93 Crowngold 26 95 King Jonagold 26 97 Jonica	36 37 Holstein 36 45 Holstein Mahler 36 47 Holstein Palloks 36 49 Holstein sport	25 13 Thomas Rivers AO 1 13 Mainds Costard AO 2 29 Costard

continued

Table 3 (continued). Groups of apple accessions indistinguishable by microsatellite analysis

54 30 Winesap	33 37 Blenheim Orange	55 07 New York E18
25 39 Wang Young	33 39 Blenheim Orange Wisley	55 09 New York E232
32 45 Blaxtayman	33 41 Red Blenheim	11 37 Libovicka Reneta
32 47 Dark Red Staymared	33 65 Aldenham Blenheim	11 47 Ruzena Blahova
32 49 Scarlet Staymared	**Malling 9**	50 05 Roter Stettiner
48 01 Mutsu Spur Type	29 20 Dermen McIntosh	26 61 Cigany Alma
49 05 Crispin	25 83 M9	

[1] First set of digits indicate row number
[2] Second set digits indicate position of the tree within the row
[3] The letter 'O' indicate the samples came from the observation plot not the main collection
[4] Controls samples used to standardise allele sizes across different plates appear in bold in the table
[5] The letter 'C' indicate the samples came from the cider plot not the main collection

Many of these groups correspond to known clones and were, therefore, expected. On the other hand, some accessions expected to be clonal have turned out not to be so. For example, 'Merrigold', tree 53 in row 13, (reportedly a mutant of Golden Delicious) has shown a different genotype to all other 'Golden Delicious' clones for every locus analysed. It does however share an allele at each locus with them suggesting it is likely to be a seedling of 'Golden Delicious' rather than a sport.

In addition to the full matches in Table 3, there are a small group of accessions that present identical amplification patterns with the exception of one or two alleles (Table 4). It is unlikely that non-clonal genotypes would show differences in only one or two loci, however the accuracy of these genotypes was confirmed repeating the relevant amplifications. Moreover, the discrepant SSR alleles are always consecutive i.e. +/- 2 bp. Three explanations are possible. If the accessions are clonal, allele differences could be due to somatic mutations or they could be PCR artefacts due to *Taq* polymerase 'copying errors' during amplification (extreme cases of the process responsible 'stuttering' in SSR genotyping). On the other hand, these accessions could indeed be non-clonal. To determine the most likely explanation for this phenomenon, careful phenotypic comparison between these accessions will be necessary as well as further SSR analysis; testing markers closely linked to those showing discrepancies as well as some totally unlinked.

Table 4. Accessions presenting *quasi* identical genotypes and discrepancies in their scores

Accessions	**Discrepancies**			
	Locus	**Alleles**	**Locus**	**Allele**
17 19 Lady Sudeley	**Hi02c07**	114/150		
17 21 Red Sudeley		114/152		
49 27 Mauss Reinette	**CH01h01**	115/121		
28 49 Love Beauty		115/123		
09 01 Akero	**Hi02c07**	110/148		
43 65 Idaho Delicious		110/150		
29 107 Boiken	**CH01h01**	96/98		
46 88 Hoskreiger		96/96[1]		
10 15 Pfirsichroter Sommerapfel	**CH01f03b**	158/176		
48 107 Rozovoe iz Tartu		158/178		
43 29 King of the Pippins	**CH02d08**	228/254		
43 31 King Russet		228/254		
43 35 Rote Goldparmane		228/254		
25 109 Baunen		228/256		
34 09 Geeveston Fanny	**Ch01h01**	111/135	**GD147**	135/150
34 11 Red Geeveston Fanny		111/135		135/150
49 07 Democrat		113/137		137/150
All other Golden Delicious clones	**CH02d08**	222/224	**CH01c11**	217/231
14 39 Golden Delicious		222/224		217/231
13 37 Golden Morspur		224/224[1]		219/231
46 45 Franc Bon Pommier	**CH01f02**	178/205	**GD147**	137/150
47 75 Brabant Bellefleur		178/205		137/150
48 45 De Flandre		178/207		137/150
47 67 Reaux		178/205		139/150

[1] Where only one allele was detected it is assumed that this allele was homozygous (i.e. that both alleles at that locus are identical)

3.3. – Self(in)compatibility (S-locus) allele genotyping of the apple collection

A total of 2,095 accessions (including eight control samples used for the SSR fingerprinting but excluding the genotypes in the observation plot) were analysed using the consensus primers to amplify alleles of the *S*-locus.

All alleles previously described (Table 1) were amplified in one or more genotypes. Additionally, 22 products of unique length were amplified and considered as putative new alleles (Table 5). At least one PCR product was amplified for each accession and 1,696 genotypes were fully characterised (i.e. the number of S alleles amplified corresponded to the level of ploidy). Unfortunately, 399 accessions remained not fully resolved with one or more of their incompatibility alleles still undetected. A range of possible explanations could account for our inability to fully characterise these accessions; due to limitations in the technique itself or to biological causes. Consensus primers used in this protocol are, by definition, not fully specific and this could hamper the amplification of certain templates or, perhaps, they could be subject to problems of preferential amplification where a better-matched template is amplified to the detriment of those less compatible. In fact, some of the alleles (e.g. S_{16}) are comparatively weak and could have been missed which could account for their low-frequency. Similarly, primer design was based on a limited number of known sequences for this locus; one or more 'new' alleles alleles with more divergent sequences present in the set of apparently hemi/homozygous genotype could be impossible to amplify using these primers. It is also possible that a small proportion of the problematic cultivars may indeed turn out to be homozygous, in particular if they were not fully functional, or even hemizygous.

Table 5. Putative new apple *S*-alleles identified during the analysis of the self(in)compatibility locus in the NFC accessions

Putative allele	Size (bp)[1]	Cultivar(s) in which allele were detected
S_{33}	500	Chataignier
S_{34}	640	Shinfield Seedling, Oxford Yeoman, Lady Isabel
S_{35}	650	Red Transparent, Verallot, Prince George
S_{36}	840	George Neal, Sharleston Pippin, Yellow Pitcher
S_{37}	850	Glebe Gold, Macoun, Rose Rouge
S_{38}	980	Calville Duquesne,Signe Tillisch, Cox's Pomona
S_{39}	1000	Adersleber Calville
S_{40}	1200	Keeds Cottage, Lady Lambourne, Rose de Benauge
S_{41}	1250	Directeur Lesage, Sweet Cornelly, Hockings Green
S_{42}	2000	Welcome, Patrick
S_{43}	168	Red Victoria, Tydemans Early Worcester, Maidstone Favourite
S_{44}	228	Shin Indo
S_{45}	262	Scarlet Pearmain, Melmoth, Schoner aus External
S_{46}	269	Lavina, Verdona, Stable Jersey
S_{47}	274	Cola Gelata, Trotuse, Nobil de Geoagiu
S_{48}	276	Akero, Ringstad, Limoncella
S_{49}	281	McLivers Winesap, Greasy Pippin, Chieftain
S_{50}	290	Crimson King, Lille, Old Rock Pippin
S_{51}	1500	Royal George, Dymock Red
S_{52}	292	Shoreditch White
S_{53}	294	Richardson Ashworth
S_{54}	300	Grosse Mignonnette d'Herbassy, Carmignole Musquee

[1] Allele sizes over 500bp were estimated from electrophoresis in agarose gel and would need to be confirmed through sequencing of the relevant fragments

Technical aim 4 – To verify ploidy levels: to verify ploidy levels of all accessions which appear from microsatellite analysis not to be diploid

A list of accessions which appeared not to be diploid following analysis of the microsatellite data was produced for confirmation through cytometric analyses. Fresh leaf samples of these accessions were collected and sent to Plant Cytometry Services in the Netherlands for ploidy determination. Tables 6 and 7 summarise the confirmed ploidy levels for 48 pear and 304 apple accessions respectively. Additionally, three other apple accessions (48_01 Mutsu Spur Type, 29_85 Wheelers Russet and 26_91 Jonagored) are very likely to be polyploids as they present three alleles for several SSR as well as for the S-locus, cytometric analysis did not confirmed this hypothesis but this could be due to a mistake during sampling. The proportion of confirmed polyploid accession is considerably higher than expected in the apple collection (~14%) even when compared with the 8.5% of polyploids identified in the pear collection.

Table 6. Non-diploid pear accessions confirmed by cytometric analysis; all triploid (3x) unless stated

01 07 Merton Pride	12 15 Alliance Franco Russe	18 33 Lucas Bronzee
04 26 Catillac	13 13 Voros Buza Korte	19 27 Palkonyai Cukor
05 27 Pitmaston Duchess	13 19 Doyenne Boussoch	20 40 Nar Armud **(4x)**
07 01 Buzas Korte	13 41 Spinacarpi	21 33 Beurre Rance
07 41 Saint Remy	14 13 Windsor	23 17 Doctor Lucius
08 00 C Graparon	15 42 Uvedales St Germain	24 35 Sucree de Montlucon
08 01 Abas Beki	16 17 De Sirole	26 39 Black Worcester
08 09 Gros Blanquet	16 25 Marechal de Cour	30 01 Conseiller a la Cour 1996-026
08 35 Pitmaston Duchess	16 31 JI 3884	32 05 Ingeborg
08 39 Vicar of Winkfield	17 01 Beurre d'Amanlis	P1 13 Parsonage
09 03 Bianchettone	17 13 JI 3807	P2 05 Gelbmostler
10 31 Triomphe de Jodoigne	17 31 JI 4244 **(4x)**	P2 09 Hellens Early
11 22 Beurre Diel	17 33 Beurre Alexandre Lucas	P2 11 Sweet Huffcap
11 31 Unknown S R Peart	17 39 Madernassa	P2 13 Wassenbirne
11 41 Sini Armud	18 01 Beurre d'Amanlis Panache	P2 17 Barland
12 09 Jargonelle	18 31 JI 3897	Roumi

Table 7. Non-diploid apple accessions confirmed by cytometric analysis; all triploid (3x) unless stated

04 03 Tordai Alma	23 19 Yorkshire Aromatic	29 63 Ribston Pippin	38 83 Doctor Ramburg
06 19 Severn Bank	23 21 Colloget Pippin	29 77 Suntan	38 94 Smalls Admirable
07 13 Hodges Seedling	23 29 Bloody Butcher	30 19 Ohio Nonpareil	39 101 Peter Lock
07 15 Jacques Lebel	23 41 Lappio	30 57 Spigold	39 105 Poor Mans Profit
08 29 Close	24 05 Contessa	30 79 Ciodo	39 112 Warners King
11 41 Trezeke Meyers	24 09 Dewdneys Seedling	31 63 Arkansas	39 31 Vajki Alma
12 01 Bellaqueeny	24 107 Rubinstar	31 81 Hohenzollern	39 75 Welschisner
16 07 Stibbert	24 113 Botden	31 91 King Byerd	39 87 Dicks Favourite
17 108 Plympton King	24 29 Ladys Delight	32 109 Rode Wagenaar	39 89 Essching
17 11 Morgan Sweet	24 35 Measdays Favourite	32 113 Roter Eiserapfel	39 95 Lord Clyde
17 47 Leathercoat Russet	24 39 Reinette Coulon	32 35 Csikos Orias Halasi	39 99 Oxford Yeoman
17 59 Acklam Russet	24 99 Jored	32 45 Blaxtayman	40 107 Mere de Menage
18 39 Daniel Fele Renet	25 07 Rossie Pippin	32 49 Scarlet Staymared	40 109 Minshull Crab
19 37 Nobil de Geoagiu	25 33 Isaac Newtons Tree	32 59 Burgess Seedling	40 41 Sandlin Duchess
19 45 Breitling	25 39 Wang Young	32 81 Pomme d'Enfer	40 75 Beauty of Kent
19 49 Charden	25 43 Winter Pearmain	32 93 Reinette Dubuisson	40 79 Bukhovitsa
19 53 Domnesc	25 45 Dubbele Zoete Aagt	32 95 Reinette du Canada	40 82 Byfleet Seedling
19 55 Double Rose	25 53 Tillington Court	33 21 Endsleigh Beauty	40 83 Byford Wonder
20 01 Bietigheimer	25 55 Jonagold	33 27 Fall Pippin	40 93 Hambledon Deux Ans
20 07 Earl Cowper	26 101 Bossom	33 37 Blenheim Orange	40 97 Kentish Fillbasket
20 13 Hannan Seedling	26 107 Rougemont	33 41 Red Blenheim	41 101 Belle de Tours
20 15 Hibernal	26 11 Genet Moyle	33 79 Galloway Pippin	41 103 Bovarde
20 17 Meads Broading	26 15 Jupiter	34 21 Glass Apple	41 109 Catshead
20 19 Mobbs Royal	26 17 Stones Mosaic	34 36 Green Custard	41 113 Reinette de Bailleul
20 21 False Morning Pippin	26 25 Glasbury Night	34 53 Edwards	41 27 Philadelphia
20 25 Notarisappel	26 61 Cigany Alma	34 57 Fraise de Buhler	41 73 Payette
20 61 Reinette Descardre	27 07 Gravenstein	34 61 Fremy	41 75 Pladei
21 05 Baron Wood	27 09 All Red Gravenstein	35 15 Huntingdon Codlin	41 87 Striped Beefing
21 19 Cockpit	27 11 Morkrod	35 35 Lady Hopetown	41 91 Tylers Kernel
21 21 Improved Cockpit	27 13 Orbai Alma	35 79 Marroi Rouge	41 95 Yorkshire Greening
21 25 Cure	27 25 Old English Round	35 93 Orleans Reinette	42 05 Carswells Orange
21 27 Doctor Hogg	27 37 Dredges Fame	36 15 Lorna Doone	42 103 Norfolk Beefing
21 31 Excelsior Seabrook	27 51 Graue Herbstrenette	36 35 New German	42 109 Ontario
21 87 Bielaar	27 71 Harberts Reinette	36 37 Holstein	42 113 Ponsford
22 07 Loddington	27 85 Kaiser Wilhelm	36 45 Holstein Mahler	42 23 Luzhanka
22 09 Kirkes Lord Nelson	27 87 Kings Acre Pippin	36 47 Holstein Palloks	42 41 Baldwin
22 37 Braddick Nonpareil	27 91 Lady Henniker	36 49 Holstein sport	42 43 Baldwin Double Red
22 49 Friedrich der Grosse	27 93 La Gaillarde	36 61 Kolacara	42 47 Belledge Pippin
23 01 Tom Putt	28 05 Coul Blush	37 35 Puffin	42 49 Bohnapfel
23 05 Sidney Strake	28 109 Roxbury Russet	37 37 Lemoen	42 61 Carrara Brusca
23 113 Orei	28 95 Pinner Seedling	38 109 Ponyik Alma	42 63 Carters Pearmain
23 15 Withington Fillbasket	29 100 Ashmeads Kernel	38 113 Verdona	42 73 Hamblings Seedling
23 17 False Woodford	29 57 Reinette de Macon	38 75 Alnarps Favourite	42 75 Hanwell Souring

continued

Table 7. (continued). Non-diploid apple accessions confirmed by cytometric analysis; all triploid (3×) unless stated

42 85 Jubile dArgovie	46 85 Horneburger Pfannkuchen	23 103 Jomured (4n)
42 95 Maggie Sinclair	50 55 Marie Doudou	24 18 Granges Pearmain Barnes
43 05 Galantine	52 11 Montmedy	24 44 Reinette Grise de Portugal
43 101 Brettacher Samling	53 29 Reinette Courthay	25 107 Minier's Dumpling
43 105 Coeur de Boeuf	54 01 Rosa du Perche	26 93 Crowngold (m of Jonagold)
43 27 Jersey Beauty	54 07 Sir Prize	26 95 King Jonagold (m of Jonagold)
43 39 Citron dHiver	54 21 Stafner Rosen	26 97 Jonica (m of Jonagold)
43 41 Claygate Pearmain	54 30 Winesap	31 13 Washington Strawberry
43 51 Cornish Pine	55 11 Crimson King	31 37 Szabadkai Szercsika
43 73 Rambour Papeleu	57 13 Vicar of Beighton	32 47 Dark Red Staymared
43 77 Reinette a la Reine	1 15 Bulmers Norman	32 97 Reinette Grise du Canada
43 81 Reinette de IHopital	1 19 Court Royal	33 39 Blenheim Orange Wisley
43 83 Scotch Bridget	1 3 Belle Fille de la Manche	33 65 Aldenham Blenheim
43 89 Dubbele Belle Fleur	1 7 Muscadet de Dieppe	33 73 Roter Munsterlander Borsdorfer
44 13 Luxemburger Renette	1 9 Omont	36 03 Ladys Finger of Offaly
44 63 DEylau	2 13 Crimson King	36 58 King of Tompkins County
44 80 Galloway Pippin	2 27 Morgan Sweet	37 41 False Long Bider
45 103 Calville des Femmes	3 15 Collington Big Bitters	38 04 False Rambour d'Ete
45 11 Oranje de Sonnaville	3 1 Tom Putt (B)	38 11 Reinette van Ekenstein
45 37 Entz Rosmarin	3 29 Gros Doux Blanc	38 43 Pepin de Bovelingen
45 47 Fukunishiki	3 9 Black Vallis	38 64 Rambour Podolskii
45 83 Teint Frais	4 35 Hereford Broadleaf	38 73 Alfa 68 (4n)
45 87 Verdese	5 13 Vilberie	38 91 False (received as Reinette Tendre)
45 89 Westons Seedling	5 24 Captain Broad (B)	39 41 Reinette dAnjou
46 05 Summer Blenheim	5 35 Four Square	39 45 Reinette de Bretagne
46 109 Belle de Longue	6 16 Skyrme s Kernel	39 47 Reinette de Brucbrucks
46 53 Gros Croquet	6 21 Strawberry Norman	39 61 Rose de Bouchetiere
46 55 Gros Locard	6 29 unknown (acc. as Hollow Core)	39 67 Roundway Magnum Bonum
46 81 Gooseberry	15 07 Norfolk Summer Broadend	39 79 Belle de Boskoop
47 101 Tower of Glamis	17 109 Veekmans Jonaster	39 81 Red Belle de Boskoop
47 106 Catherine	18 101 Excel	40 101 Lady of the Wemyss
47 59 Hommel Orne	18 93 Wilmuta	40 111 Nemes Szercsika Alma
47 65 Marie-Madeleine	18 99 Jonagored Supra	41 105 Bramleys Seedling
48 11 Misen Jaromerska	19 107 Jonagold (EMLA)	41 107 Bramley (m Crimson)
48 63 Reinette de France	19 113 Red Jonaprince	41 79 Rhode Island Greening
48 77 Belle de Boskoop	20 102 Jonagold Boerekamp	41 81 Rhode Island Greening (4n)
49 05 Crispin	20 103 Josegold	43 99 Belle-Fleur Large Mouche
49 17 Fekete Tanyeralma	20 107 Prince Jonagold	45 77 Serveau (4n)
49 33 Polly Prosser	20 51 Improved Ashmeads Kernel	46 111 Belle-Fleur de France
50 05 Roter Stettiner	20 59 Pommerscher Krummstiel	47 104 Mather 2 (4n)
50 13 Honey Pippin	20 89 Decosta	50 21 Beauty of Hants Myers
50 37 Pomme de Glace	20 95 Jorayca	50 47 Pomme de Choux a Nez Creux
50 51 Warrens Seedling	21 103 Joseph Musch	55 07 New York E18 (4n)
50 53 Bassard	21 111 Jonagold	55 09 New York E232 (4n)

Technical aim 5 - Collating genotypes: to collate the data into Excel spreadsheets and provide to the scientific curator, e.g. to allow the search for duplicates, to submit the data to the freely-accessible ECPGR *Malus* and *Pyrus* databases and to produce papers on ploidy and fingerprinting

EXCEL spreadsheets were prepared giving the genotypes at each analysed microsatellite locus for the pear and apple collection and they were sent to the scientific curator of the collection at University of Reading (also responsible for the ECPGR *Malus* database) in June 2008 and March 2010 respectively. A similar file detailing all available S allele genotypes for the apple collection was also prepared and sent in March 2010. Data was also sent to Marc Lateur curator of the ECPGR *Pyrus* database.

Publications concentrating in the methodology used for these studies and containing partial data sets have been prepared and will be submitted to relevant journals in the near future. It is expected that

the full data sets will be made available through the ECPGR data bases soon. In the meantime they are available from EMR on request.

Discussion of results and potential future work

Data arising from this work will prove a useful tool for the more efficient management of the NFC germplasm. The maintenance of a large number of replicated accessions unnecessarily increases the cost of managing and curating the collection. It would be appropriate to evaluate the interest of different clones prior to re-propagation. This process is due to take place in the next couple of years for the apple collection and it would be an excellent opportunity for rationalisation. In some cases, most if not all the replicates detected are known mutants of the same cultivar however there are some unexpected results. For example, 29 20 Dermen McIntosh should not be a clone of M9 suggesting the grafted cultivar probably died and the rootstock is growing in its place, 26 87 Jonagold was expected to be clonal to the rest of Jonagold mutants but does not appear so, etc.

Although every reasonable precaution was taken to ensure the accuracy of the work, it is worth pointing out the possible sources of error in this fingerprinting exercise. It is possible that some samples were not collected from the correct tree either in the first place or later on when re-sampling was done for cytometric analysis. Great care was taken in accurate labelling, lists written and double checked etc. Errors can also occur in the laboratory either during sampling for storage, during DNA extraction or handling either at the PCR stage or during loading for electrophoresis. Again best laboratory practice was followed to minimise problems but human error remains a possibility. Therefore the duplicates here indicated should be compared to literature records to determine if the relevant accessions where known or suspected to be clonal. Phenotypic observation should also be undertaken and, if in doubt, DNA analysis should be repeated in a case by case basis.

The genotyping of the self(in)compatibility locus in such a large number of accessions has provided a much better understanding of the S-allele variability. The detection of 22 new putative alleles and the suggestion of even more alleles we have not been able to detect with the current protocol open up interesting avenues for future research. It would be useful to clone and sequence these putative new alleles fragment in order to confirm and fully characterise them. These new sequences could then inform the design of more inclusive consensus primers that might allow the amplification, perhaps using less strict PCR conditions, of some of the alleles currently not being detected. Test crossing pollen of accessions where only one allele was detected with other accession containing that same allele (e.g. transferring S_3S_x pollen onto an S_1S_3 style) would clarify they may indeed be homozygous; if S_3 is the only allele in the pollen it will not germinate in a style with that allele therefore following such a test cross a homozygous accession would not be able to pollinate (no fruit would be set) whereas if seeds are produced from the cross we can postulate a new allele and the resulting seedlings would carry it. Similarly, the presence of these not-detected new alleles could be confirmed through various intercrosses, e.g. if a cross between an S_3S_x and an S_4S_x genotypes was to produce 25% seedlings with no detectable S-alleles and 50% with only either S_3 or S_4 then the two S_x alleles are different. On the other hand, if the resulting progeny segregates 50:50 S_3S_4 to S_4S_x then both undetected alleles are the same. The most productive approach would be to separate the stylar proteins in this accessions and stain for RNase activity - if the RNase phenotypes show a single band this would indicate the other allele is 'null' (inactive) and therefore the accessions are hemizygous where as if all the problematic cultivars share a particular band that would be presumably the S_x band. However it is also possible that at least a proportion of the accessions showing only one S allele in this study could carry some already defined alleles that were out-competed during PCR or that some of the ‘weaker’ they were missed in this analysis due to the sensitivity of agarose detection system.

As other international groups adopt the harmonised fingerprinting protocols for apple and pear it will be possible to carry out comparison between collections. This could lead to the identification of errors in cultivar accession and even more importantly, to the rationalisation of germplasm collections across Europe. To this end we continue to promote the use of ECPGR-agreed fingerprinting sets by assisting overseas groups starting germplasm fingerprinting on *Prunus, Malus* and *Pyrus.* Furthermore, EMR has been commissioned to fingerprint two other UK fruit collections – the apples held by the National Trust in Cornwall and the perry pears maintained by the Shambles Museum in Gloucester – result from these analysis will be compared to the data for the NFC. This will assist management and could lead to the rationalisation of the germplasm kept by those organisations and present new candidates for accession into the NFC.

Different species of *Pyrus* and *Malus* are known to have contributed to the current range of cultivated pears and apples respectively. These species evolved in a wide range of environmental conditions and consequently have variety of different physiological characteristics, e.g. drought tolerance, pest and disease resistances, cold hardiness. Extending the range of material analysed with microsatellites to include related species may be able to shed some light on the origins of domestic pears and apples the speciation within these genera. It could also be interesting to compare the genetic diversity found in cultivar collections with wild germplasm sampling in the species centres of origin. This would allow the incorporation of valuable novel material into germplasm collections.

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References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Published:

EVANS K.M. (2009) Introduction to molecular markers and DNA fingerprinting. *DNA day EMRA Members Day Report*

FERNÁNDEZ- FERNÁNDEZ F. (2009) An introduction to mapping for marker-assisted selection in fruit crops *DNA day EMRA Members Day Report*

EVANS KM, FERNÁNDEZ- FERNÁNDEZ F, GOVAN C. (2009) Harmonising fingerprinting protocols to allow comparisons between germplasm collections - *Pyrus. Acta Hort.* (ISHS) 814: 103-106

FERNÁNDEZ- FERNÁNDEZ F (2010). Fingerprinting Fruit. National Orchard Forum Newsletter 15

FERNÁNDEZ- FERNÁNDEZ F, GOVAN C, VAN DE WEG E, EVANS KM (in preparation for *Molecular Breeding*). The use of a standardised set of multiplexed microsatellites (SSRs) for genotyping *Malus* cultivars and species

TOBUTT KR, CLARKE JB, KM EVANS, GOVAN CL, FERNÁNDEZ-FERNÁNDEZ F. Harmonising fingerprinting protocols to allow comparison between germplasm collections: general principles illustrated using the case of pear and cherry (in preparation for *Plant Breeding*)

Technology transfer:

- K. Tobutt and K. Evans hosted a visit from Prof. Cameron Peace (Washington State University) to discuss possible future collaborations between the USA and UK apple molecular biology teams (April 2007)
- F. Fernández-Fernández visited Dr Susan Brown (Cornell University - USA) and discussed fingerprinting technology (July 2007)
- F. Fernández-Fernández discussed the use of automated software to score AFLP markers and the suitability of these markers for fingerprinting with Yiz Lem Wan (Cornell University - USA) (Jul 2007)
- K. Evans outlined the project to the National Fruit Collections Advisory Committee (Jul 2007)
- K. Tobutt, K. Evans, F. Fernández-Fernández, J. Clarke and C. Govan hosted a visit from Dr Gennaro Fazio (the apple rootstock breeder and geneticist from the USDA ARS at Cornell University, Geneva, USA) and discussed fingerprinting set and its uses for breeding (Sep 2007)
- K. Evans discussed the aims of the project with the ECPGR *Malus/Pyrus* working group at their meeting in Zaragoza (Sep 2007)
- K. Tobutt and K. Evans manned a stand about fingerprinting the NFC at the public EMR 'Apple Day' weekend (Sep 2007)
- F. Fernández-Fernández attended the 'Cornwall Fruit Focus' event organised by the Eden Project (Boldeva - Cornwall) in December 2007. Apple cultivation in Cornwall, opportunities for organic fruit production and the use of molecular markers for fingerprinting local germplasm were discussed
- F. Fernández-Fernández met with Sean MacAntsaoir from Agri-Food and Biosciences Institute (Northern Ireland) to discuss harmonization of fingerprinting set in apple and pear (Apr 2008)
- F. Fernández-Fernández and K. Evans attended the AAB Plant Genetic Resources Meeting at Wellesbourne and presented a poster on apple, pear and cherry fingerprinting sets (May 2008)
- K. Evans sent pear collection SSR data set to NFC curator (Jun 2008)
- K. Evans presented an interim report to National Fruit Collection Advisory Committee on the progress of the fingerprinting (Jul 2008)
- F. Fernández-Fernández provided general information regarding the use of SSRs for fingerprinting in apple Dr Andrew Ormerod from the Eden Project who has an interest in the characterisation of Cornish genotypes (Jul 08)
- K. Evans and F. Fernández-Fernández meet with Dr Joan Morgan and Mrs Alison Lean regarding fingerprinting results for pears to discuss duplicates and possible hypothesis to test in analysis (Oct 2008)
- F. Fernández-Fernández sent information regarding SSR multiplexes for apple and cherry to Dr Andrea Patocchi (Frei Forschungsanstalt Agroscope Changins-Wädenswil ACW) (Nov 2008)
- F. Fernández-Fernández provided general information regarding fingerprinting project to Mr Edward Milner currently writing a book on UK tree genetic resources (Nov 2008)
- F. Fernández-Fernández provided information regarding the use of SSRs for fingerprinting in apple to Mr Chris Groves from the National Trust (Dec 2008)
- F. Fernández-Fernández updated the National Fruit Collections Advisory Committee on the progress of the project (Jan 2010)
- F. Fernández-Fernández sent apple collection SSR and *S-allele* data set to NFC curator (Mar 2010)

International co-operations:

- Tobutt, K. Evans, F. Fernández-Fernández, J. Clarke and C. Govan organised and hosted a two-day international meeting on 'Molecular genetics of rosaceous plants' with colleagues from the Rosaceous Genomics project (HH3724SSF) at Aylesford Friars where DNA fingerprinting with common SSR sets was discussed (Dec 2007)
- F. Fernández-Fernández sent details of SSR multiplexes for apple, pear and cherry to Prof. Santiago Pereira Lorenzo (Universidad de Santiago de Compostela – Spain) (Jan 2009)
- F. Fernández-Fernández sent optimised protocols for apple fingerprinting SSR multiplexes to Ms Ana Ramos (Universidad de Santiago de Compostela – Spain) (Jan 2009)
- F. Fernández-Fernández sent optimised protocols for pear fingerprinting SSR multiplexes to Dr Patricia Ritschel from Embrapa, Brazil. (Feb 2009)

Talks:

- K. Evans and F. Fernández-Fernández presented talks entitled 'Introduction to molecular markers & DNA fingerprinting' and 'An introduction to mapping for marker-assisted selection in fruit crops' at the East Malling Research Association (EMRA) DNA day in November 2007
- K. Evans gave a presentation on the uses of genetic fingerprinting in fruit crops to the directors of the Oxford Farming Conference Directors during a visit to EMR (Jul 2008)
- K. Evans presented an interim project report to National Fruit Collections Advisory Committee at Brogdale (Jul 2008)
- F. Fernández-Fernández gave a presentation entitled: "Fruity Fingerprints? – use of molecular fingerprinting in fruit crops" to the RHS Northern Fruit Group in Harrogate (Feb 2009)