Genetic Diversity and Relationships in *Malus* sp. Germplasm Collections as Determined by Randomly Amplified Polymorphic DNA

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ABSTRACT. Four subsets of apple (*Malus* Mill.) germplasm representing modern and old cultivars from the repository and apple genetics population of the Horticulture and Food Research Institute of New Zealand Limited were used in this study. A total of 155 genotypes randomly chosen from the four subsets were analyzed for random amplified polymorphic DNA (RAPD) variation. Nine decamer primers generated a total of 43 fragments, 42 of which were polymorphic across the 155 genotypes. Pairwise distances were calculated between germplasm subsets using the distance metric algorithm in S-PLUS, and used to examine intra- and inter-subset variance components by analysis of molecular variation (AMOVAR). A phenogram based on unweighted pair group method with arithmetic average (UPGMA) cluster analysis was constructed from the pairwise distances and a scatter plot was generated from principal coordinate analysis. The AMOVAR showed that most of the variation in the germplasm (94.6%) was found within subsets, suggesting that there is significant variation among the germplasm. The grouping of genotypes based on the phenogram and scatter plot generally did not reflect the pedigree or provenance of the genotypes. It is possible that more RAPD markers are needed for determining genetic relationships in apple germplasm. Nevertheless, the variation observed in the study suggests that the current practice of sublining populations in the first generation to control inbreeding may not be necessary in subsequent generations. If these results are confirmed by fully informative molecular markers, germplasm managers should reassess the structure of their genetics populations. There may be a need to combine sublines in order to capture the maximum genetic diversity available and to streamline breeding efforts.

The consequences of loss of genetic diversity have been experienced sporadically throughout history, often with significant human and economic costs, which has been a common problem with many of the world's important food crops (Way et al., 1990). The genus *Malus* (apples), is characterized by large diversity, but thus far this has not been translated in the cultivated (*Malus sylvestris* (L) Mill. var. *domestica* (Borkh.) Mansf.) varieties (Way et al., 1990). Modern commercial apple orchards are dominated by only a few cultivars and many breeding programs utilize only a few well known cultivars in crosses for commercial apple production (Noiton and Shelbourne, 1992; Way et al., 1990). As a result, levels of inbreeding and coancestry in modern apples have been projected to increase (Noiton and Alsapach, 1996).

Awareness of genetic diversity and management of crop genetic resources have been important components of plant improvement programs. The foundation of crop-based agriculture rests largely on the availability and knowledge of extant plant

genetic resources in germplasm collections and successive stages of development in breeding programs. More specifically, knowledge of genetic diversity and relationships among sets of germplasm and the potential merit of the genetic diversity is critical to plant improvement (Lee, 1995).

In 1990, the Horticulture and Food Research Institute of New Zealand Limited (HortResearch), established a population improvement program to broaden the genetic base and, at the same time study the genetics of horticulturally important traits in apples. An apple genetics population was developed from imported seed and pollen from apple cultivars and varieties from clonal repositories in major apple producing regions in Europe, North America, Australia, Asia and South Africa (Noiton and Shelbourne, 1992). Seeds of accessions of *Malus sieversii* (Lebed.) were also obtained from Kazakhstan—the presumed center of origin of the cultivated apples (Hokanson et al., 1997). These seedlings have been planted in research orchards in a recurrent selection breeding strategy and maintained at multiple sites to allow for evaluation of general and regionally important horticultural traits and biotic and abiotic resistances. We as germplasm managers now need to assess the level of diversity within this collection to help make informed decisions on management procedures as well as on the choice of breeding strategies to use in combining horticulturally important traits in our breeding program.

Historically, assessments of genetic composition of germplasm collections have been carried out at several levels including inferences based on reproductive biology, ecogeographic data, morphology, pigmentation, ontogeny, social history, pedigree records, breeding behavior, in situ and ex situ evaluation of traits, chromosome structure and behavior, protein markers, and molecular markers such as restricted fragment length polymor-

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Table 1. Composition of provenances and the number of cultivars/selections assigned to each provenance based on species or geographic area of origin of the cultivar or maternal parent of the selection.

Provenance	Countries included	Subset			
		1	2	3	4
<i>M. sylvestris</i> var. <i>domestica</i>					
North America	Canada, USA	11	5	3	9
UK	England	7	1	8	5
W. Europe	Belgium, France, Denmark, Italy, Netherlands, Norway, Sweden, Switzerland	12	13	8	4
E. Europe	Czechoslovakia, Estonia, Lithuania, Poland, Romania, Former Soviet Union, Russia, Ukraine, Yugoslavia	5	2	3	8
S. Hemisphere/Japan	New Zealand, Australia, Japan	14	0	0	1
<i>M. sieversii</i>					
Five accessions	Dzhungarsky, Alatau, Karmenka Gorge Kazakhstan	0	0	0	5
<i>M. kirghisorum</i>					
Three accessions	Ferganskiy Kherbet, Kyrgystan	0	0	0	3
<i>M. sieversii</i> / <i>M. kirghisorum</i>	Dzhungarsky Alatau, Kazakhstan	0	0	0	1
Species	<i>Malus</i> sp. (other than <i>M. sieversii</i>) and crabapples irrespective of country of origin	0	6	2	6
Unknown	Provenance unknown or too uncertain	1	3	6	3
		50	30	30	45

phisms (RLFPs). The emergence of new polymerase chain reaction (PCR) based molecular markers, such as randomly amplified polymorphic DNA (RAPD) markers, simple sequence repeats (SSRs), and amplified fragment length polymorphisms (AFLPs), has created opportunities to characterize germplasm collections at the molecular level, with markers that are presumably selectively neutral and not affected in their expression by environmental effects. The increased levels of variation detectable with these molecular markers have allowed germplasm managers, plant breeders, and geneticists to pursue with new vigor important questions relating to crops and their genetic resources (Bretting and Widrechner, 1995).

RAPD analyses have various scientific, operational, and budgetary advantages for genome mapping (Welsh and McClelland, 1990, 1991; Williams et al., 1990) and are used with a high degree of success as tools for harnessing genes from germplasm resources in our own apple genome mapping program (Gardiner et al., 1996a, 1999). These same advantages also appear valid for characterization of genetic variation for effective germplasm conservation and use, hence research in population genetics has shown the most growth with respect to the use of RAPD technology (Hedrick, 1992). RAPD markers have been used to create DNA fingerprints for the study of individual identity and taxonomic relationships in both eucaryotic and procaryotic organisms (Caetano-Anolles et al., 1991; Hu and Quiros, 1991; Kresovich et al., 1992); in apples, to differentiate cultivars (Koller et al., 1993), for paternity analysis (Harada et al., 1993), for determining genetic relationships in *Malus sylvestris* var. *domestica* cultivars and wild species (Dunemann et al., 1994), along with RFLPs and isozymes for tracing the parentage of 'Braeburn' (Gardiner et al., 1996b), and for phylogenetic studies (Zhou and Li, 2000).

The following research was conducted to examine the distribution of genetic diversity in the HortResearch apple germplasm collection using RAPD markers. The objectives of the investigation were to 1) assess the level of diversity between and within different subsets of apple germplasm, 2) determine the similarity of the selections/cultivars within the collections and 3) determine if the gene pool of apples had been effectively enlarged with the germplasm obtained from other countries and continents.

Materials and Methods

PLANT MATERIAL. The HortResearch apple germplasm was divided into four subsets. Subset 1 consisted of old cultivars and breeding lines that are maintained in the repository and the National Cultivar Center (NCC), and subsets 2, 3 and 4 consisted of sublines in the apple genetics population. The apple genetics population comprised open-pollinated (op) families as well as families derived from controlled crosses from primitive cultivars, cider apples, and wild species (Noiton et al., 1999), including wild accessions of *Malus sieversii* (Lebed.) and *M. kirghisorum* (Al.) Ponom. from Kazakhstan. The families were divided into four sublines and planted at the third leaf stage in randomized incomplete blocks of 20 single tree plots at three different sites over a period of 4 years as follows: 1991: 224 families (10,232 trees); 1992: 149 families (12,800 trees); 72 families (4,862 trees); and 1994: 86 families (3,665 trees). Sublining was used to control inbreeding and maximize, as far as possible, genetic variability and heterozygosity within the population (Noiton and Shelbourne, 1992). We crossed within sublines per generation since inbreeding is unlikely to be a serious problem for many generations. Nevertheless, we had planned to introduce new unrelated material after a few generations to circumvent inbreeding. Ideally, the families should have been grouped into sublines based on region of origin so that individuals from the same region will be confined to one subline. For example, families from countries in Western Europe should be in a different subline than families from countries in North America or Eastern Europe. But this never really happened in our own case, due in part to the fact that seeds were not all received at the same time. Hence, the division into sublines was based on year of planting to the effect that a subline includes families from any region where seeds were obtained. However, assessing the diversity within the collection still has merit as the information will assist us in developing the best selection, breeding, and management strategy for the entire germplasm collection. We assigned subline 91 to subset 2, subline 92 to subset 3, and sublines 93 and 94 to subset 4. At the time of this study, only families selected as parents for the second cycle of selections in the subline 91 were available. In total, 155

Table 2. Names of families or cultivars from which selections were taken for this study, their field code, country of origin, and the year the cultivar was introduced according to the National Apple Register of the United Kingdom (Smith, 1971), U.S. Department of Agriculture Germplasm Resources Information Network (GRIN), and other sources.

Name	Cultivar/family code	Origin	Year
		Families ^z	
Alexander (op)	91.004	Ukraine	1600
Autumn Arctic (op)	91.011	USA	1952
Belle de Lunteren (op)	91.013	Netherlands	
Ben Davis (op)	91.015	USA	1800
Bodil Neergård (op)	91.020	Denmark	1850
Borsdorfer (op)	91.022	Germany	1450
Calville d'Aout (op)	91.026	France	1832
Cravert Rouge (op)	91.037	France	1870
Druvåpple (op)	91.049	Sweden	
Grimes Golden (op)	91.071	USA	
Gul Rickard (op)	91.079	Germany	
Herfst Zoetzuur (op)	91.083	Unknown	
Holovouske Malinove (op)	91.085	Unknown	
John Downie (op)	91.092	UK	1875
Linne's apple from Stenb (op)	91.098	Unknown	
<i>M. baccata</i> 3 (op).	91.105	Siberia	1784
<i>M. brevipes</i> (op)	91.107	Japan	1883
<i>M. floribunda</i> (op)	91.109	Japan	1862
<i>M. sylvestris</i> (op)	91.111	Europe	
<i>M. ×atrosanguinea</i> (op)	91.112	China	1889
Robusta 5 (op)	91.113	China	1927
Reinette De Cusy (op)	91.139	France	1863
Roman Stem (op)	91.145	USA	1817
Rubin (op)	91.150	France	
Szampanska (op)	91.164	Russia	1852
White Angel (op)	91.175	USA	1947
Zomerzuur (op)	91.186	Netherlands	
Nonnetit Bastard x Bruyère	91.212	Denmark	1700
Torstein x De Casse	91.220	Norway	1973
Transparente de Croncels x St Germain	91.223	France/Spain	1869/1600
Anurka (op)	92.007	Italy	
Aromat de Vara (op)	92.009	Romania	
Beachamwell (op)	92.014	UK	1780
Bespodolenoë (op)	92.019	Unknown	
Cluster Golden Pippin (op)	92.026	UK	1750
Double Rose (Gros Api) (op)	92.029	France	1628
Doux Normandie (op)	92.030	UK	1783
Dutch Codlin (op)	92.033	Netherlands	
Friandise (op)	92.040	Netherlands	1760
Grahamovo (op)	92.044	England	
Graue Herbstrenette (op)	92.046	England	
Harvey (op)	92.051	UK	1629
Hibernal (op)	92.054	USA	1880
Håkonseple (op)	92.058	Germany	
Kitaika Solotaja Rannaja (op)	92.064	Unknown	
Leihmes (op)	92.067	Unknown	
<i>M. ×hartwigii</i> (op)	92.075	USA	1906
<i>M. ×robusta</i> 'Persicifolia' (op)	92.079	China	1910
Marie-Joseph d'Othee (op)	92.081	Belgium	1947
Nezname Stave (op)	92.088	Unknown	
Northwest Greening (op)	92.090	USA	1872
Pozdni Kraje (op)	92.105	Unknown	
Reinette Franche (op)	92.110	France	1510
Rondeurveit (op)	92.113	Unknown	

Table 2. Continued.

Name	Cultivar/family code	Origin	Year
Safranapfel (op)	92.117	Germany	1544–1889
Selena (op)	92.121	USA	1864
Shafran Letnij (op)	92.123	Russia	
Sussex Mother (op)	92.133	UK	1800
Titovka (op)	92.136	Ukraine	1870
Tower of Glamis (op)	92.137	UK	1800
Bashkirian Beauty (op)	93.003	USSR	1973
Binet Blanc Dore (op)	93.006	France	
Blahova Ruzena (op)	93.007	Czechoslovakia	
Dochdiany (op)	93.018	Poland	1959
Gladstone (op)	93.026	UK	1780–1883
Golden Russet (op)	93.028	USA	1800
Golden sweet (op)	93.029	USA	
Joe Trio (op)	93.034	USA	1936
Keo (op)	93.035	USA	1940
Kola (op)	93.037	USA	
Korichnoe Polosatoje (op)	93.038	Russia	1831–1915
Liestnaya Antonoffku 1836-A (op)	93.041	Unknown	
Longfield (op)	93.043	Russia	1870
Lord Derby (op)	93.044	UK	1862
<i>M. floribunda</i> 821 (op)	93.045	Japan	1908
<i>M. honanensis</i> (op)	93.046	China	1913
<i>M. prunifolia</i> 19651 (op)	93.047	China	1907
<i>M. prunifolia</i> 'Pendula' (op)	93.048	Species	
Mammoth crab (op)	93.049	UK	1939
Mildew Immune Seedling (op)	93.051	USA	
Mleevskaya Crasavitsa (op)	93.052	Ukraine	
Reinette Douce (op)	93.059	France	1831
Rosemary Russet (op)	93.060	Unknown	
Suisselpas Rozabols (op)	93.062	Unknown	
Toellisaare (op)	93.063	Former Soviet Union	
Trebu Seklandzis 7 (op)	93.064	Unknown	
Uralian Winter Apple (op)	93.065	Russia	1973
Vérité (op)	93.066	France	
Worcester Pearmain x Braeburn	93.070	UK/New Zealand	1873/1956
Yates (op)	93.071	USA	
<i>M. sieversii/kirghisorum</i> GMAL 3539/3551 ^y (op)	94.016	Kazakhstan	
<i>M. sieversii</i> GMAL 3543 (op)	94.020	Kazakhstan	
<i>M. sieversii</i> GMAL 3549 (op)	94.026	Kazakhstan	
<i>M. sieversii</i> GMAL 3554 (op)	94.031	Kazakhstan	
<i>M. sieversii</i> GMAL 3560 (op)	94.034	Kazakhstan	
<i>M. sieversii</i> GMAL 3566 (op)	94.038	Kazakhstan	
<i>M. kirghisorum</i> GMAL 3577 (op)	94.046	Kyrgystan	
<i>M. kirghisorum</i> GMAL 3581 (op)	94.050	Kyrgystan	
<i>M. kirghisorum</i> GMAL 3583 (op)	94.052	Kyrgystan	
Braeburn (op)	94.060	New Zealand	1956
Buchard (op)	94.061	Unknown	
Commercio (op)	94.064	USA	1865
Fogliona (op)	94.067	Italy	
Joybells (op)	94.077	UK	1908
Mother x Joybells	94.080	USA	1914

Table 2. continued next page.

genotypes were sampled randomly from the 4 subsets as follows: 50, 30, 30, and 45 genotypes respectively (Tables 1 and 2).

DNA ISOLATION. DNA was isolated using the method of Gardiner et al. (1996a, 1996b). The DNA was quantitated by visual comparison of DNA dilutions electrophoresed on 0.9% Ultrapure (Life Technologies, Gaithersburg, Md.) agarose gels in Trisacetate ethylenediaminetetraacetic acid (TAE) with 50 ng of lambda

phage DNA (Life Technologies) as standard. Electrophoresis runs were for 1.5 h at 2.5 V·cm⁻¹, and DNA staining was with 0.35 µg·mL⁻¹ ethidium bromide. Under these conditions, apple DNA runs as a clear band at the same position on the gel as the standard.

DNA AMPLIFICATION. Four hundred decamer oligonucleotide primers (Operon Technologies, Alameda, Calif.) including the 39 already developed for 'Braeburn' by Gardiner et al. (1996b), were

Table 2. Continued.

Name	Cultivar/family code	Origin	Year
		Cultivars	
Cox's Orange Pippin	NCC 1*	UK	1850
Pacific Beauty	NCC 2	New Zealand	1995
Pacific Queen	NCC 3	New Zealand	1995
Pacific Rose	NCC 4	New Zealand	1995
Pink Lady	NCC 5	Australia	
Southern Snap	NCC 6	New Zealand	1995
McIntosh	R13T229	Canada	1796
Delicious	R18T357-367	USA	1880
Red Chief	R1T10	USA	1972
Hi Early Red	R1T22	USA	1955
Fuji1-1	R1T34	Japan	1962
Antonovka	R1T46	Russia	<1862
Braeburn	R1T54	New Zealand	1956
Granny Smith	R1T56	Australia	1868
Royal Gala	R22T558-614	New Zealand	1965
Kentish Fillbasket	R2T13	UK	1820
Golden Delicious	R2T29	USA	1890
Ballarat Seedling	R2T47	Australia	1900
Close	R2T47	USA	1925
Democrat	R2T49	Australia	1900
Reinette du Canada	R2T56	France	1771
Kapai Red Jonathan	R30T966	USA	1826
Nonpareil	R3T15	France	1500
Fraasov Letni	R3T27	Czechoslovakia	
Nonnetit Bastard	R3T3	Denmark	1700
Alkmene	R3T39	Germany	1965
Shafran Kitajka	R3T51	Russia	1907
Alma Pippin	R4T20	South Africa	
Adam's Pearmain	R4T32	UK	1826
Rae Ime	R4T44	Estonia	
Flueritard Rouge	R4T56	France	1950
Rymers	R4T7	UK	1818
Cortland	R5T13	USA	1951
Rheinischer Krummstiel	R5T25	Germany	1821
Geheimrat Breuhahn	R5T37	Germany	1895
Maggi Grieve	R5T49	UK	1928
Idared	R5T59-60	USA	
Mr Fitch	R6T1	New Zealand	
Red Melba	R6T13	Canada	
Hladikovo Preuradne	R6T25	Czechoslovakia	
Guldborg	R6T37	Denmark	1870
Tydemann's Early Worcester	R6T49	UK	1929
Jamba	R7T14	Germany	1954
Mettias	R7T2	Unknown	
Lady Williams	R7T26	Australia	1935
Peasgood Nonsuch	R7T44	UK	1858
Pfirsichroter Sommerapfel	R7T56	Germany	1800
Reintte Marbree d'Auvergne	R8T33	France	
Redwell	R8T45	USA	1911
Glockenapfel Eckter	R8T9	Germany	1948

*Op = open pollinated.

[†]GMAL = Geneva *Malus*; A GMAL number references a local number assigned by the USDA, ARS Plant Genetic Resources Unit, Cornell University Geneva, N.Y., to an accession that has no plant introduction number.

*NCC = National Cultivar Center, and RT = row/tree number for cultivars in the repository.

prescreened using five cultivars ('Cox's Orange Pippin', 'Southern Snap', 'Alkmene', 'Fogliona' and 'Braeburn'), to select primers that amplified reliably. Out of these, nine that produced

polymorphic, reproducible, and scorable bands were chosen for further amplification of DNA from all genotypes (Table 3).

The RAPD-PCR runs generally were performed once; if there

Table 3. Summary of RAPD bands scored.

Primer	Sequence	Bands produced/ primer (no.)	Polymorphic bands produced/ primer (no.)
OPC06	GAACGGACTC	6	6
OPC10	TGTCTGGGTG	4	4
OPC13	AAGCCTCGTC	5	5
OPD03	GTCGCCGTCA	5	5
OPD20	ACCCGGTCAC	4	3
OPR16	CTCTGCGCGT	4	4
OPK14	CCCCTACAC	5	5
OPU08	GGCGAAGGTT	6	6
OPQ09	GGCTAACCGA	4	4

was a reaction failure for a particular primer in one or more lanes, the run was repeated. The reaction mixtures (16.5 μ L) contained 10 mM tris-HCL (pH 8.8), 50 mM KCl, 2.0 mM $MgCl_2$, 0.01% gelatin, 0.8% formamide, 0.1 mM each dNTP, 0.2 mM each primer and 1.5 ng of apple genomic DNA, 1.2 units of *Taq* DNA polymerase (Stratagene, La Jolla, Calif.) and were overlaid with 8 μ L paraffin. Reactions were set up in 96 well plates using a Biomek 2000 Laboratory Automation Workstation (Beckmann, Fullerton, Calif.) that also loaded reaction products on electrophoresis gels. Amplification was conducted in a thermal cycler (PCR Express; Hybaid, Ashford, United Kingdom) programmed for 4.5 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 37 °C, and 2 min at 72 °C, followed by a final extension cycle of 10 min at 72 °C. These reaction conditions produce consistently reproducible results for a range of apple DNA concentrations in our laboratory. Amplification products were electrophoresed on gels consisting of a mixture of 0.45% Separide (Life Technologies), 40.22% UltraPure agarose (Life Technologies) and 0.22% Nusieve GTG agarose (FMC, Rockland, Maine) in TAE at 4 V·cm⁻¹ for $\approx$ 5 h. Gels were stained with ethidium bromide at 0.35 μ g·mL⁻¹, visualized under ultra violet light and photographed in color (64T Kodak Ektachrome film; Eastman Kodak, Rochester, N.Y.). RAPD product sizes were estimated using a 100-base pair (bp) DNA standard ladder (Life Technologies). Reproducibility between gels and consistency of scoring was checked by effectively overlapping the gels—five samples, including ‘Cox’s Orange Pippin’, ‘Southern Snap’, ‘Alkmene’, ‘Fogliona’ and ‘Braeburn’, were repeated on each 42 well gel.

DATA ANALYSIS. Individual RAPD bands were scored as present or absent from enlarged color photographs. Faint bands of doubtful reproducibility were ignored, and those that were very faint compared to others in the same position were scored as absent (0) while the bright ones were scored as present (1).

All analyses were performed in S-Plus (Statistical Sciences, Inc., 1995) using the methods of Becker et al. (1988) and Chambers and Hastie (1992). Genetic distance was calculated using the ‘binary’ metric option. The distance (D) between any 2 genotypes is given by: $D = (\text{number of bands in common})/(\text{total number of bands})$. In this way, no assumptions were made on the number of loci or alleles. The distances were used to construct a phenogram using the unweighted pair group average (UPGMA) hierarchical clustering method.

Principal coordinate analysis was done with the presence and absence data matrix to obtain a graphical representation of the genotypes in dimension and space. It was done such that the Euclidean distance between genotypes was about the same as the

Table 4. Frequency of polymorphic RAPD bands in four subsets of New Zealand apple germplasm. P is the proportion of polymorphic bands for each primer. *Pt* is the overall proportion of polymorphic bands in each subset.

Primer band	P			
	Subset 1	Subset 2	Subset 3	Subset 4
OPC06_1	42	23	27	36
OPC06_2	46	27	53	29
OPC06_3	62	57	33	67
OPC06_4	36	47	57	63
OPC06_5	60	40	20	40
OPC06_6	60	23	47	63
<i>Pt</i>	100	100	100	100
OPC10_1	10	27	27	24
OPC10_2	88	90	90	89
OPC10_3	46	50	43	42
OPC10_4	38	37	30	47
<i>Pt</i>	100	100	100	100
OPC13_1	16	10	0	13
OPC13_2	50	67	47	42
OPC13_3	24	23	20	11
OPC13_4	8	0	0	0
OPC13_5	52	3	40	44
<i>Pt</i>	100	100	100	100
OPD03_1	54	63	30	40
OPD03_2	24	57	23	29
OPD03_3	12	0	7	18
OPD03_4	100	93	97	91
OPD03_5	24	23	37	22
<i>Pt</i>	100	60	100	100
OPD20_1	16	13	20	9
OPD20_2	12	37	17	9
OPD20_3	100	100	100	100
OPD20_4	44	27	30	44
<i>Pt</i>	75	75	75	75
OPK14_1	14	7	13	16
OPK14_2	2	0	3	7
OPK14_3	30	10	33	64
OPK14_4	32	7	17	43
OPK14_5	44	30	30	90
<i>Pt</i>	100	90	100	100
OPQ09_1	52	50	77	58
OPQ09_2	90	100	80	89
OPQ09_3	78	70	83	69
OPQ09_4	20	17	27	20
<i>Pt</i>	100	100	100	100
OPR16_1	26	33	47	24
OPR16_2	32	63	70	51
OPR16_3	4	3	30	13
OPR16_4	0	13	7	20
<i>Pt</i>	100	100	100	100
OPU08_1	70	50	77	53
OPU08_2	42	73	37	71
OPU08_3	62	30	70	73
OPU08_4	44	27	20	42
OPU08_5	32	33	20	38
OPU08_6	16	7	17	16
<i>Pt</i>	100	100	100	100

Fig 1. Representative RAPD profiles of 36 apple cultivars/selections generated using the 10-base primer OPC06. A 100-bp ladder was loaded in lanes 1, 21, and 39. Numbers (1–6) on the right show RAPD fragments scored for this primer that were used along with fragments from other primers to examine variation within and between germplasm subsets and to determine relationships of the 155 genotypes used in the study. Lanes 2–20, and 22–38, contain amplified DNA from the following genotypes in serial order ‘Pacific Beauty’, ‘Southern Snap’, ‘Pacific Queen’, ‘Pacific Rose’, Pink Lady, Royal Gala, Delicious, Kapi Red Jonathan, *M. sieversii* GMAL 3560 (op), *M. sieversii* GMAL 3554 (op), *M. sieversii/kirghisorum* GMAL 3539/3551 (op), ‘Worcester Pearmain’ x ‘Braeburn’, ‘Foglionia’ (op), *M. sieversii* GMAL 3543 (op), ‘Commercio’ (op), *M. kirghisorum* GMAL 3581 (op), *M. kirghisorum* 3583 (op), *M. brevipes* (op), Bechamwell (op), ‘Korichnoe Polosatoje’ (op), ‘Buchard’ (op), ‘Mother’ x ‘Joybells’, *M. sieversii* GMAL 3566 (op), *M. kirghisorum* GMAL 3549 (op), ‘Ballarat Seedling’, ‘Golden Delicious’, ‘Reinette du Canada’, ‘Kentish Fill basket’, ‘Granny Smith’, ‘Braeburn’, ‘Antonovka’, ‘Fuji-1’, ‘Hi Early Red’ and ‘Red Chief’.

raw pairwise distances between genotypes.

A V-fold cross validation with $V = 10$, was used to choose the number of variables to use for analysis of variance (ANOVA). This involved choosing a random sample of V points to be left out of the model for the fitting process (calculating distance matrix and multidimensional scaling). For various values of the number of principal coordinates, k, the cross validation stress defined as the mean squared error or relative errors in pairwise distances, $\text{mean}[(d.\text{obs} - d.\text{pred})/d.\text{pred}]^2$, was calculated. Where, d.obs, denotes the observed distance and d.pred, the predicted distance, for a given value of k. The process was repeated 15 times giving 15 stress values. The cross validation stress values continued to decrease almost until $k = 30$. Values for $k > 24$ could not be distinguished statistically.

Analysis of molecular variance (AMOVA) was carried out with 24 principal coordinates as well as with all 43, using the AMOVA procedure in S-PLUS to obtain gene diversity indices for all individuals within subsets and among subsets. The variance and the sums of squares of these analyses were combined to give a pooled analysis. Variance components were calculated from the mean squares using the method of moments estimates.

The F statistic (F_{st}) which is a measure of reduction in the number of heterozygotes, was computed using the equation $F_{st} = \sigma_p^2 / (\sigma_p^2 + \sigma_G^2)$ (Excoffier et al., 1992).

Results

From the nine random primers used (Table 3), 43 amplification products (bands) were stably generated (Table 4), 42 of which were polymorphic, thus reflecting the genetic diversity within the germplasm. Primer OPD20 produced a fragment (OPD20_3) of size between 1 and 1.1 kb, which was common to genotypes in all

subsets. One band from primer OPC13, OPC13_4, was unique to subset 1 and most (89%) of the other bands were present in all subsets but in varying frequencies. One band each was fixed in subsets 1 and 2, the band OPD03_4 in subset 1 and OPQ09_2, in subset 2. The level of intrasubset polymorphism varied from one RAPD primer to the other and from one subset to the other.

The polymorphisms observed with 36 representative genotypes using primer OPC06 is illustrated in Fig. 1. Though most of the bands were found in the majority of genotypes, no single fragment was common to all 36 genotypes. ‘Pacific Beauty’ and ‘Southern Snap’ had identical profiles (lanes 2 and 3) while ‘Pacific Queen’ (lane 4) lacked the fragment, OPC06_4, which was common to the rest of ‘Gala’ x ‘Splendour’ cultivars. ‘Pacific Rose’ shared a fragment of size 1.5 to 2.0 kb with ‘Gala’ (one of the parents), which was lacking in the other GS cultivars. The putative two sports of ‘Delicious’, ‘Red Chief’ and ‘Hi early Red’ (lanes 37 and 38) differed by one band (OPC06_1) and both lacked fragment OPC06_2 observed in ‘Delicious’ (lane 8).

The genetic distance between genotypes was generally high (data not presented), with most distance measures falling between 0.52 and 0.73. The highest was 0.952 and the lowest 0.148. It was not uncommon for individuals from different subsets to have a high genetic similarity. For example, ‘Ballarat Seedling’ (subset 1) and *M. xatrosanguinea* (op) in subset 2 had a similarity of 78.9% compared to a similarity of 4.8% between ‘Anurka’ (op). and *M. xrobusta* ‘Persicifolia’ Rehder (op). both from subset 3.

The phenogram (Fig. 2) from UPGMA cluster analysis showed several groupings which did not agree with the provenance or ancestry of the genotypes. However, it is difficult to establish the precise pedigree of the majority of apple cultivars, and even particularly the open-pollinated families used in subsets 2 to 4.

Fig. 2. Phenogram from UPGMA cluster analysis of 155 apple genotypes based on their phenotypes for 43 RAPD bands.

scattered all over the plot. However, in the lower left half of the plot, only a few genotypes were found and these were mostly from subsets 1 and 3. A plot of the first six principal coordinates, which explained 64% of the variation in pairs (plot not presented) did not improve the groupings.

The AMOVA results obtained with 24 and 43 RAPD markers were very close, so the results presented herein are for the 43 RAPD markers (Table 5). Although the F test for difference shows weak evidence for a variation among subsets (a *P* value of 0.0381), examination of the between subset variance component in Table 5 shows that this difference is not major. Most of the total genetic diversity (94.6%) was found within subsets. This pooled within subset variation was distributed almost equally among the subsets irrespective of the composition and differing number of individuals in the subsets.

Discussion

The GS cultivars however, had 3 ('Pacific Beauty', 'Pacific Queen' and 'Pacific Rose') out of the four used for the study grouped together with 'Royal Gala'. 'Southern Snap' was placed in another group starting with 'Belle de Lunteren' through to 'Northwest Greening'. This placement of one of the sibs is not inconsistent with the relationship of 'Cox's Orange Pippin' to 'Gala' ['Gala' = 'Kidd's Orange' ('Delicious' x 'Cox's Orange Pippin') x 'Golden Delicious']. 'Braeburn' is recognized as having a Cox-type flavor and cultivars known or suspected to have 'Cox' in their parentage occurred in the grouping starting from 'Pacific Beauty' to 'Treu Sekladzis 7' ('Pacific Beauty', 'Delicious', 'Pacific Queen', 'Pacific Rose', 'Royal Gala' and 'Cox' itself). Duplicate 'Braeburn' samples were not separable. The group starting with *M. floribunda* 821 through to 'Kola' contains many selections and families frequently used as parents in our resistance breeding program. The majority of genotypes in this group are crabapples.

Out of a total of 43 bands representing 43 principal coordinates, 16 principal coordinates explained 90% of the variation between subsets while 33 explained 99% of the variation. A plot of the first two principal coordinates (Fig. 3) which accounted for 38% of the variation did not separate out the subsets into distinct groups based on provenance or country of origin. The grouping of the individuals was similar to that obtained with the phenogram. Genotypes, irrespective of the subset they belonged to, were

Results indicate there are high levels of genetic variation within the HortResearch apple germplasm. Partitioning this variation into its within- and between-subset components indicated that 94.6% was maintained within subsets. This higher within subset diversity agrees with the finding of Lamboy et al. (1996) that *M. sieversii* accessions maintained 85% of their diversity within regions compared to 15% between regions. This study was not designed to specifically investigate population structure, and hence may not be directly comparable to such studies. Nevertheless, the results agree with the general observation that woody perennial outbreeding species maintain most of their variation within populations (Hamrick, 1994). Hamrick (1994) outlined a number of life history traits that affect partitioning of genetic diversity in tree species. These can be summarized in four general observations. First, temperate species on average, display higher levels of variation than tropical trees. Second, species with a widespread geographical distribution tend to display greater diversity than those of a more limited range. Also, taxa that are predominantly outcrossing maintain higher levels of genetic diversity than those that are primarily autogamous. Finally, selfing species tend to show a high degree of population substructuring, with diversity maintained among rather than within populations. Apparently, these four observations are true for apples and could well explain the high levels of variation obtained in this study.

The between subset component accounted for only $\approx 5\%$ (*P* =

Table 5. Analysis of molecular variance (AMOVA) based on 43 RAPD bands for 155 selections/cultivars of apples grouped into four subsets. The estimated within germplasm subset variation (%) is 0.244 for Subset 1, 0.245 for Subset 2, 0.242 for Subset 3, and 0.242 for Subset 4.

Source of variation	dF	SS	MS	Variation (%)	F ^z	1-Pr(F)	Vcomp ^y	F _{st} ^x
Between subset	3	2.1	0.699	0.054	2.88	0.0381	0.012	0.066
Within subset	151	36.7	0.243	0.946	NA ^w	NA	0.243	NA

^zVariance ratio.

^yVariance component.

^xF statistic.

^wNA = not applicable.

0.038) of the genetic variation between genotypes. This apparent lack of differentiation between the subsets could be attributed to high levels of mating and genetic recombination. Further evidence for lack of differentiation between subsets comes from the fact that all the subsets have most of the bands in common (though in different frequencies) and only one subset (subset 1, old cultivars and breeding lines) has a private allele (band, OPC13_4). One other possible explanation for the similarities between the subsets may be because of comigration of nonhomologous RAPD bands (Dunemann et al., 1994; Rieseberg, 1996). Though, Kazan et al. (1993) suggested that, within a genus, it is likely that fragments shared by any two species will be allelic. So, this theory needs further investigation. The similarities may also be caused by paralogous evolution or the wide range of variation across the species range. In any case, the open-pollinated nature of most of the material used would tend to enhance the similarities.

It was interesting to find that there was the same amount of genetic variation within subsets irrespective of the size and composition of each subset. We expected subset 1 to have much less variation than other subsets due to it containing only *M. ×domestica* genotypes including the five cultivars ('Cox's Orange Pippin', 'McIntosh', 'Delicious', 'Golden Delicious' and 'Jonathan') reported by Noiton and Alspach (1996) as contributing to the pedigree of modern apple cultivars. Also, all the genotypes were cultivars that have been clonally propagated. Other subsets had varying numbers of *M. sylvestris* var. *domestica* and other *Malus* species (largely open pollinated). Apparently, the high levels of coancestry reported in modern apples (Noiton and Alspach, 1996; Way et al., 1990) are not obvious in this subset. Dunemann et al. (1994) observed a high degree of variation between *M. ×sylvestris* var. *domestica* accessions with RAPD markers and suggested that a broad genetic base still exists in cultivated apples. Hokanson et al. (1998) using 8 microsatellite (SSR) markers detected high levels of variation with a mean of 12.1 alleles per locus with 66 accessions of *M. sylvestris* var. *domestica* from the U.S. Department of Agriculture germplasm collection. This germplasm included the 5 cultivars that have been used as recurrent parents in modern commercial apple breeding (Noiton and Alspach, 1996), as well as some old cultivars. We have reviewed our fruit evaluation database and found that the *M. sieversii* genotypes (op) used in this study (in subset 4) are all edible and have fruit about the same size as the cultivated varieties. Hence, as far as fruit size is concerned, subset 4 may not be much different from subset 1. This observation is in line with that of Juniper et al. (1999) where the fruit of *M. sieversii* show an extensive diversity from almost inedible crabs to fruit not very dissimilar to some modern cultivars. These results suggest there is still an appreciable amount of diversity within *M. sylvestris* var. *domestica*, which perhaps is retained in the old cultivars. It should be noted here that many apple cultivars are open pollinated seedlings and are of unknown or, at least, uncertain origin. Therefore, even among some widely grown cultivars, the genetic relationships are not yet fully known. It can be assumed that *M. sylvestris* var. *domestica* is a complex hybrid with several, as yet not fully identified, species involved in the process of domestication (Korban and Skirvin, 1984; Way et al., 1990). Hence, the genetic base of cultivated apples is probably still very broad.

The phenogram from UPGMA cluster analysis using the 155 genotypes produced several groupings most of which could not be explained by the pedigree or provenance of the genotypes. The estimated similarity between the genotypes was from 0.048 [between 'Anurka' (op) and *M. ×robusta* 'Persicifolia' (op)] to

0.85 (between 'Cox Orange Pippin' and 'Pacific Beauty') (data not presented). This range of similarities is very wide, therefore, we believe that these similarities are the result of the high polymorphism revealed by the RAPD markers. Other causes such as the approach taken to analyze the data, which involved scoring each band as a unique character and estimating the distances (or dissimilarities) using the shared band method, may not be ruled out. Although, the shared band method may not be a problem with identical clones since the two 'Braeburns', the cultivar in subset 1 and the seedling derived from this cultivar, in subset 4, had a genetic similarity of 100%. Nevertheless, the use of shared band analysis to investigate phenetic relationships in 18 potato (*Solanum tuberosum* L.) cultivars by Provan et al. (1996), produced groupings among the cultivars which did not agree with their coancestry, though the authors found sufficient polymorphism with the 16 SSR primers used which clearly distinguished between the cultivars. Dunemann et al. (1994) found that RAPD markers were able to separate the accessions of *M. sylvestris* var. *domestica* used in their study based on their pedigree, but there were slight variations in the placement of some taxa between the dendrograms generated with the simple matching coefficient and Jaccard's coefficient of similarity. They attributed this to <50 RAPD bands used in one of the analyses. However, the AMOVA results in this study were very close for 24 RAPD markers and the entire dataset of 43 RAPD bands suggesting that ≈24 RAPD bands are enough for partitioning the variation within the germplasm but more than 43 RAPD markers may be needed for determining relationships among the genotypes.

In conclusion, RAPD markers are a very useful tool for assessing genetic diversity in apples. It was relatively simple to generate a large number of polymorphisms to determine genetic relationships. The use of automation to run routine reactions enhances the reliability of the technique. However, due to the outbreeding nature of apples and the dominant nature of inheritance of RAPD markers, it may be useful to explore other fully informative molecular markers such as RFLPs or SSRs for a finer resolution of the relationships between the apple genotypes. Also, it would be useful to explore other methods for estimating similarities or distances than the shared band method.

We have demonstrated that old varieties are potential sources of diversity in apples, in addition to the wild species and current cultivars. The practice of grouping families into sublines is very popular in forestry. It is aimed at controlling inbreeding by restricting crosses within sublines in each generation. The level of variation obtained in this study suggests that, inbreeding is probably not a problem in apples and sublining the germplasm may not be necessary. If these results are confirmed by fully informative markers we may be able to merge the sublines into one population in the next generation to reduce costs and simultaneously maximize our selection and breeding efficiency.

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