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Genetic diversity in a collection of apple (*Malus* × *domestica* Borkh.) cultivars as revealed by RAPD markers

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Summary: A collection of 151 apple cultivars was investigated with 7 RAPD primers generating 71 informative bands, to evaluate genetic variability and relatedness. All cultivars presumably derived through genetic recombination were distinguished whereas identical DNA profiles indicated that some cultivars had arisen as sports. A cluster analysis and a PCO did not reveal any distinct geographic patterns, but there was a weak tendency for Swedish and foreign cultivars to differentiate. Many cultivars however clustered together with either one of their parents or with siblings. Overall genetic diversity among the 151 cultivars was estimated with Nei's diversity index (H), 0.269, and with Shannon's index (H'), 0.594. The cultivars were also analysed in six groups, according to time of origination and country of origin, with an average H = 0.262 and H' = 0.546. No major differences in genetic diversity were observed over time or space, although the group with recent, foreign cultivars had the lowest diversity (H = 0.235, H' = 0.493). Comparison between the entire material and a subset with 94 mandate cultivars chosen for preservation in Sweden, showed similar genetic diversity: H_{ENTIRE} = 0.268, H'_{ENTIRE} = 0.593 and $H_{MANDATE}$ = 0.263, $H'_{MANDATE}$ = 0.575. No major differences in band frequencies were observed between these two sets, but 5 RAPD bands were missing in the set with mandate cultivars..

Key words: apple, genetic diversity, germplasm, *Malus domestica*, RAPD

Introduction

Apple, *Malus* x *domestica* Borkh., is one of the economically most important fruit crops in temperate zones. It is also a very diverse fruit crop, with numerous different cultivars all over the world, and the number is increasing rapidly (*Sansavini* et al., 2004). Many apple cultivars have arisen as open-pollinated seedlings, often of unknown origin. The exploitation of naturally occurring mutations (bud-sports) in adapted cultivars has also been important. In the last century, an increasing number of cultivars have, however, been created by crosses performed by plant breeders (*Janick* et al., 1996).

Apple cultivars are maintained by vegetative propagation and are monoclonal, which means that all individuals, belonging to the same cultivar, are genetically identical. By contrast, genetic diversity among cultivars, obtained by sexual recombination, is expected to be rather high because of self-incompatibility which enforces outbreeding and results in heterozygosity (*Kitahara* et al., 2005). Many recently developed apple cultivars have been designed to incorporate genetically determined resistance towards apple scab and some other fungal diseases and insect pests (*Crosby* et al., 1992; *Holb*, 2000). Genes for disease resistance have been obtained from wild relatives to our cultivated apple, thus contributing to the genetic diversity in apple.

From its centre of origin in Central Asia, the apple was introduced into Europe by Romans and for the last 2000 years, the domesticated apple has diversified and flourished worldwide (*Harris* et al., 2002). Apples were brought to Sweden from Central and Southern Europe, and the first apple trees were planted around the 12th century, mainly in monastery orchards. Apple growing with the aim to produce fruits for commercial sale started in the 16th century. In addition to foreign cultivars brought in from e.g. Germany and England, new local cultivars originating as chance seedlings were also grown when found to possess desirable characters like large and tasty fruits. Such seedlings were selected, propagated by grafting and distributed to other growers.

Modern plant breeding, based on controlled crosses, has been undertaken in Sweden first at Alnarp (1920–1960) and then at Balsgård (from around 1950), both nowadays part of the Swedish University for Agricultural Sciences. Both foreign and indigenous cultivars have been used in these plant breeding programs. At Balsgård, there is presently about 1000 different apple cultivars in a germplasm collection, which includes old and new Swedish varieties as well as foreign varieties which are adapted to the Swedish climate and/or contain genes of special interest for plant breeding.

At present, publicly funded conservation of clonally propagated plant genetic resources in Sweden is managed by a governmentally appointed unit, the 'National Program for Diversity of Cultivated Plants', which has defined a set of mandate cultivars. Mandate cultivars are indigenous varieties which have been named, bred, propagated and marketed in Sweden. Some foreign cultivars with a long history of being grown in Sweden are also included (*Hjalmarsson & Wallace*, 2004). In apple, 220 mandate cultivars have been appointed. These cultivars are presently conserved mainly in smaller

clone archives all over the country, usually at outdoor museums or other public places. About 100 of these cultivars are also present in the Balsgård collection.

Extensive collections of clonally propagated crops are difficult and expensive to maintain. Accurate and permanent genetic identification of individual genotypes is therefore of outmost importance. All unnecessary duplicates, synonyms and mis-labelled genotypes can then be identified and removed. Proper characterisation also ensures that genotypes are true-to-type, and enables users to refer character screenings to unambiguously identifiable genotypes. Furthermore, estimates of genetic relatedness among genotypes may be useful for character screening: instead of screening all available accessions, only those genotypes which appear to be the most promising according to relatedness information, can be targeted.

Historically, so called pomological (morphological) characters have been used for identification of apple cultivars (*Nilsson*, 1986), but most of these characters are heavily influenced by the environment. During the past few decades, molecular markers have therefore became increasingly popular in the characterization of apple collections, e.g., isozymes (*Weeden & Lamb*, 1985), RFLP (*Nybom & Shaal*, 1990), RAPD (*Koller* et al., 1993), AFLP (*Xu & Korban*, 2000), ISSR (*Goulao* & Oliveira, 2001) and SSR (*Gianfranceschi* et al., 1998, *Liebhard* et al., 2002). The most user-friendly of these methods in terms of need for technical equipment, skills and funding is RAPD, which has been used for identification of apple cultivars (*Koller* et al., 1993; *Mulcahy* et al., 1993) and rootstocks (*Autio* et al., 1998), to study genetic diversity in the genus *Malus* (*Dunemann* et al., 1994; *Zhou & Li*, 2000), and for paternity analysis (*Harada* et al., 1993). RAPD has also been used in the early stages of genomic mapping projects (*Conner* et al., 1997). Specific RAPD bands have been used as markers of horticulturally important traits and have sometimes been converted into co-dominant SCAR markers (*Cheng* et al., 1996; *Yang* et al., 1997; *Kim* et al., 2003). Possible problems with reproducibility within the same laboratory can be avoided if the same protocol is applied and followed carefully (*Mulcahy* et al., 1993) and only strong, clear and consistently amplified bands are scored (*Koller* et al., 1993).

In this study, RAPD-markers were applied to some of the cultivars in the Balsgård apple collection to: i) discriminate among cultivars; ii) detect duplicates and mis-labellings; iii) identify genetic relationships among cultivars; iv) evaluate the genetic diversity and possible effects over time (ancient, old and more recent cultivars) and space (Swedish and foreign cultivars).

Materials and methods

Plant material

In total 151 apple cultivars were analysed (*Table 1*). Of these, 94 are mandate cultivars with 68 originating in Sweden. These 151 cultivars were divided into groups based on their historical age: ancient (originated before 1800), old

Table 1 Apple cultivars analysed and their origination, marked with '?' if only putative. Cultivars were divided into groups (I–VI), which are defined in Table 3. Cultivars regarded as 'mandate cultivars' in Sweden are marked with 'M' after the group number

	Cultivar	Origin	Group	Descendence
1	'Alexander'	Russia	II, M	
2	'Alfa 68'	Sweden	V, M	'Boskoop' x 'Filippa'
3	'Algott' (B:0654)	Sweden	V	'Astrakan, Gyllenkrok's' x 'Worcester Pearmain'
4	'Alice'	Sweden	V, M	Seedling of 'Ingrid Marie'¾
5	'Annero'	Sweden	III, M	
6	'Antonovka Kamenichka'	Ukraina	IV	
7	'Antonovka Pamtorutka'	Russia	IV, M	
8	'Aroma'	Sweden	V, M	'Ingrid Marie' x 'Filippa'
9	'Arvidsäpple'	Sweden	III, M	
10	'Aspa'	Sweden	III, M	
11	'Astrakan, Gyllenkrok's'	Sweden	I, M	
12	'Astrakan, White'	Russia	II, M	
13	'Astrakan, Red'	Sweden	I, M	
14	'Astrakan, Stor Klar'	Sweden	III, M	Progeny of 'Astrakan, White' ?
15	'Birgit Bonnier'	Sweden	V, M	'Cortland' x 'Lord Lambourne'
16	'Blenheim Orange'	England	II	
17	'Boiken'	Germany	II	
18	'Borgherre'	Netherlands? Germany?	II, M	
19	'Borsdorfer'	Germany	II, M	
20	'Boskoop'	Netherlands	IV	
21	'Brunnsäpple, Halland'	Sweden	I, M	
22	'Cellini'	England	IV, M	Seedling of 'Langton's Nonesuch'
23	'Charlamovsky'	Russia	II, M	
24	'Classic Red Delicious'	USA	IV	
25	'Close'	USA	VI	
26	'Cortland'	USA	VI	'Ben Davis' x 'McIntosh'
27	'Cox's Orange Pippin'	England	IV, M	Seedling of 'Ribston'?
28	'Cox's Pomona'	England	IV, M	Seedling of 'Ribston'?
29	'Discovery'	England	VI	'Worcester Pearmain' x 'Beauty of Bath'
30	'Domö Favorit'	Sweden	III, M	
31	'Drakenberg'	Sweden	III, M	
32	'Dronning Louise'	Denmark	IV	
33	'Edsele'	Sweden	V, M	
34	'Elise' (syn. 'Roblos')	Netherlands	VI	'Septer' x 'Cox's Orange Pippin'
35	'Elstar'	Netherlands	VI	'Golden Delicious' x 'Ingrid Marie'
36	'Eva-Lotta'	Sweden	V, M	'Cortland' x 'James Grieve'
37	'Fagerö'	Sweden	III, M	
38	'Farmors Juläpple'	Sweden	V, M	
39	'Fiholms Ribston'	Sweden	V	
40	'Filippa'	Denmark	IV, M	
41	'Flädie'	Sweden	III, M	Seedling of 'Gravensteiner' ?
42	'Fredrik'	Sweden	V	'Aroma' x selection from USA
43	'Frida'	Sweden	V	'Aroma' x selection from USA
44	'Frösåker'	Sweden	III, M	
45	'Fullerö'	Sweden	III, M	
46	'Förlovningsäpple'	Sweden	III, M	
47	'Gelber Richard'	Germany	II	
48	'Golden Delicious'	USA	IV	

	Cultivar	Origin	Group	Descendence
49	'Goldparmain'	England	IV, M	
50	'Granatäpple, Kungsbacka'	Sweden	I, M	
51	'Gravensteiner'	Italy?		
		Denmark?	II, M	
52	'Gravensteiner, Red'	Italy?		
		Denmark?	II, M	
53	'Gravensteiner of Fusa'	Norway	IV	
54	'Grågylling'	Sweden	I, M	
55	'Grågylling from Skokloster'	Sweden	I	
56	'Guldborg'	Denmark	IV	
57	'Göteborgs Flickäpple'	Sweden	III, M	
58	'Hanaskog'	Sweden	III, M	Seedling of 'Oranie' ?
59	'Hannaäpple'	Sweden	V	
60	'Hedenlunda'	Sweden	III, M	
61	'Himmelstalund'	Sweden	III, M	
62	'Holsteiner Cox'	Germany	VI	Seedling of 'Cox's Orange Pippin'
63	'Holländaräpple'	Sweden	III, M	
64	'Hugoäpple'	Sweden	V	
65	'Hausmütterchen'	Germany	II, M	
66	'Höstkalvill, Gul'	Germany	IV, M	
67	'Ingrid Marie'	Denmark	VI	Possibly 'Cox's Orange Pippin' x unknown
68	'Ivö'	USA/Sweden	VI, M	Synonym: 'Monroe seedling', came to Sweden as budwood
69	'James Grieve'	Scotland	IV	Seedling of 'Pott's Seedling'
70	'Jonathan'	USA	IV	Seedling of 'Esop Spizenburg'
71	'Josefiner'	Sweden	III, M	
72	'John-Georg'	Sweden	V	'Golden Delicious' x 'James Grieve'
73	'Julyred'	USA	VI	
74	K:1016	Sweden	V	'Aroma' x selection from USA
75	K:1016, Red	Sweden	V	Possible sport of K:1016
76	K:1160	Sweden	V	'Katja' x 'Priscilla'
77	K:1343	Sweden	V	Seedling of Coop14 (USA)
78	'Kalmar Glasäpple'	Sweden	I, M	
79	'Katja'	Sweden	V, M	'James Grieve' x 'Worcester Pearmain'
80	'Kavlås'	Sweden	III, M	
81	'Kesäter'	Germany	II, M	
82	'Kramforsäpple'	Sweden	V	
83	'Kim'	Sweden	V, M	'Cortland' x 'Ingrid Marie'
84	'Kingston Black'	England	II	
85	'Kinnekulle Kantäpple'	Sweden	V, M	
86	'Landskronaäpple'	Sweden	V, M	
87	'Langton's Nonesuch'	England	IV	
88	'Larsmässcäpple'	Sweden	I	
89	'Laxton's Superb'	England	IV	'Cox's Orange Pippin' x 'Wyken Pipping'
90	'Linda'	Canada	VI	Seedling of 'Langford Beauty'
91	'Linnaeus' Apple'	Sweden	I, M	
92	'Lobo'	Canada	IV	Seedling of 'McIntosh'
93	'Maglemer'	Denmark	II, M	
94	'Mank's Codlin'	England	IV	
95	'McIntosh, Rogers'	Canada	II	Seedling of 'Fames'
96	'Melon'	Germany	II, M	
97	'Melon, Red'	Germany	IV, M	
98	'Melonkalvill'	Sweden	III, M	
99	'Menigasker'	Sweden	III, M	
100	'Mio'	Sweden	V, M	'Worcester Pearmain' x 'Oranie'
101	'Mutsu'	Japan	VI	'Golden Delicious' x 'Indo'
102	'Mälsåker'	Sweden	III, M	
103	'Nanna'	Norway	VI	'Katja' x 'Buckley Giant'
104	'Norrstack'	Sweden	III, M	
105	'Norrviken'	Sweden	III, M	
106	'Oranie'	Sweden	I,M	
107	'Oretorp'	Sweden	V, M	
108	'Pigeon'	Denmark	IV	
109	'Prima'	USA	VI	PRI 14-510 x NJ 123249
110	'Prinsessäpple'	Netherlands	II, M	
111	'Queen Cox'	England	VI	Sport of 'Cox's Orange Pippin'
112	'Reinette de Blenheim'	England	II	
113	'Rescue'	Canada	VI	Seedling of 'Blushed Calville'
114	'Ribston'	England	II	
115	'Ringstad'	Sweden	III, M	
116	'Risäter'	Sweden	III, M	
117	'Rosen Crab'	Russia	IV	
118	'Rödluvan'	Sweden	V, M	'Lobo' x 'Barhatnoe'
119	'Sandbergs Röda'	Sweden	V, M	
120	'Signe Tillisch'	Denmark	IV, M	
121	'Silva'	Sweden	V, M	'Melba' x 'Stenbock'
122	'Siv'	Norway	VI	'Katja' x 'Buckley Giant'
123	'Snövit'	Sweden	V, M	'Stenbock' x 'Pfirsichroter Sommerapfel'
124	'Sparreholm'	Sweden	III, M	
125	'Spässerud'	Sweden	I, M	
126	'Stenkyrke'	Sweden	I, M	
127	'Stäringe Karin'	Sweden	III, M	
128	'Svanetorp'	Germany	IV, M	
129	'Suislepper'	Estonia	IV, M	
130	'Summerred'	Canada	VI	Seedling of 'Summerland'
131	'Sylvia'	Sweden	V, M	'Astrakan, Gyllenkrok's' x 'Worcester Pearmain'
132	'Särsö'	Sweden	V, M	
133	'Sävstaholm'	Sweden	III, M	
134	'Sörmlandsäpple'	Sweden	I	
135	'Titovka'	Russia	IV	
136	'Transparente Blanche'	Russia?	II, M	
137	'Trogsta'	Sweden	III, M	
138	'Vallda'	Sweden	III, M	
139	'Veseäpple'	Sweden	I, M	
140	'Villands Glasäpple'	Sweden	III, M	
141	'Vista Bella'	USA	VI	NJ 77359 x 'Julyred'
142	'Vitgylling'	Netherlands	II, M	
143	'Vittsjö'	Sweden	III, M	
144	'Vrams Järnäpple'	Sweden	I, M	
145	'Värmlands Sötäpple'	Sweden	III, M	
146	'Wealthy, Red'	USA	IV	
147	'Worcester Pearmain'	England	IV	Seedling of 'Devonshire Quarrenden'
148	'Åkerö'	Sweden	I, M	
149	'Åkerö, Gripsholm'	Sweden	V, M	Sport of 'Åkerö'
150	'Ökna Lökäpple'	Sweden	I, M	
151	'Ökna Vita Vintergylling'	Sweden	III, M	

(1800–1900) and new (after 1900) and their geographic origination (Swedish or foreign) according to pomological literature (*Dahl*, 1929; *Nilsson*, 1986; *Svensson & Kastman*, 2005). In total, six groups were thus defined: ancient Swedish (17), ancient foreign (21), old Swedish (34), old foreign (29), new Swedish (31) and new foreign (19) (*Table 2*).

Table 2 Number of investigated apple cultivars (within parentheses the original number before duplicates and mislabelled samples had been deleted) used to calculate within-group genetic diversity, measured by Nei's diversity index (H) and Shannon's index (H') (including standard error) in historically and geographically different groups

Group	No. of cultivars	Nei's diversity index, H	Shannon's index, H'
I Ancient Swedish	16 (17)	0.260 (0.022)	0.539 (0.042)
II Ancient foreign	19 (21)	0.276 (0.023)	0.568 (0.044)
III Old Swedish	33 (34)	0.248 (0.023)	0.523 (0.045)
IV Old foreign	28 (29)	0.281 (0.022)	0.585 (0.042)
V New Swedish	29 (31)	0.270 (0.021)	0.567 (0.038)
VI New foreign	18 (19)	0.235 (0.023)	0.493 (0.044)
		x = 0.262	x = 0.546

RAPD analysis

Young leaves were collected in April-May and stored at -80 °C until use. Leaves were ground to a powder with liquid nitrogen in pre-cooled mortars. Approximately 100 mg of the powder was used for isolation of total plant DNA using the Qiagen Dneasy™ Plant Mini Kit and following the Qiagen protocol.

PCR reactions were performed in volumes of 25 µL, containing 20 ng of DNA, 1 x reaction buffer IV (Advanced Biotechnologies), 2.5 mM $MgCL_2$ (Advanced Biotechnologies), 0.5 µM primer (Operon Technologies), 0.2 µM PCR Nucleotide Mix (Roche Diagnostics Corp.) and 1.0 unit *Taq* DNA Polymerase (Advanced Biotechnologies). The steps for PCR amplification were: one cycle of 5 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C and finally by one cycle of 7 min at 72 °C. DNA fragments were separated by electrophoresis in 1.8% agarose gel with a Tris-EDTA-Acetic acid buffer (TEA). The gels were stained with ethidium bromide and the amplification products were visualised under UV light and documented with Polaroid photography for further analyses. Molecular Weight Marker VI (Roche Diagnostics Corp.) was used to determine the size of the DNA fragments.

A total of 186 decamer primers (Operon Technologies) were checked for polymorphism, reproducibility and clarity of the obtained patterns on a subset of 4 cultivars. Seven primers were subsequently chosen for further analysis of all the 151 cultivars (*Table 3*). To check the reproducibility between runs, DNA of the same three plants was included in every run. In addition, amplification of two different samples was carried out twice in each PCR run.

Table 3 Selected primers used for RAPD analysis

Primer	Sequence (5′ to 3′)	Number of scored bands	Number of polymorphic bands	Polymorphic bands (%)	Size range	RAPD primer index
OPA-08	GTGACGTAGG	15	15	100.0	180–1300	3.69
OPA-19	CAAACGTCGG	12	12	100.0	240–1200	2.66
OPF-11	TTGGTACCCC	7	4	57.1	240–1500	1.75
OPF-19	CCTCTAGACC	13	13	100.0	453–2170	4.20
OPK-16	GAGCGTCGAA	14	12	85.7	270–1230	2.90
OPM-09	GTCTTGCGGA	8	8	100.0	430–1176	1.97
OPY-17	GACGTGGTGA	9	7	77.8	250-850	1.79

Statistical analyses

Amplification products were scored manually. Each RAPD band was treated as an independent locus with two alleles, presence (1) or absence (0) of a band. Seventyone polymorphic RAPD bands were entered into a binary matrix.

The informativeness of each RAPD primer was evaluated using the Polymorphic Index Content (PIC) (*Ghislain* et al., 1999) calculated as in *Garkava-Gustavsson* et al. (2005).

To assess levels of molecular relatedness, Jaccard's coefficient of similarity was calculated for all pairwise comparisons between cultivars. A distance matrix was then used to perform a cluster analysis based on average linkage between groups (unweighted pair group method algorithm, UPGMA) (SPSS Data Analysis Package 11.0 for Macintosh). A large dendrogram, representing the relatedness among all 151 analysed cultivars, was produced. Another Jaccard similarity matrix was obtained for 143 individual genotypes (duplicates were not included) and used to perform a Principal coordinate analysis (PCO) (NTSYS–pc statistical package, *Rolf*, 1998). A two-dimensional plot was produced.

To evaluate the amount of genetic diversity within groups of cultivars, in the entire plant set and in the set of mandate cultivars, two diversity indices were used: Nei's gene diversity index, H (*Nei*, 1987) and Shannon's diversity index, H' (*Bussell*, 1999). The Nei's gene diversity was calculated as in *Marita* et al. (2000) and Shannon's diversity index as in *Garkava-Gustavsson* et al. (2005). Shannon's index was also used for partitioning of diversity in its within- and betweengroup components. The index was calculated for each locus $G'_{GROUP/ENTIRE(i)} = (H'_{ENTIRE(i)} - H'_{GROUP(i)})/H'_{ENTIRE(i)}$, where $H'_{GROUP(i)}$ is the average Shannon's index per locus, calculated by averaging $H'_{GROUP(i)}$ over all groups. Mean value of $G'_{GROUP/ENTIRE}$ (basically the same as G'-statistics) was then calculated by averaging $G'_{GROUP/ENTIRE(i)}$ over all markers. In addition, frequencies of individual RAPD bands were calculated in both the entire plant material (151 cvs) and in the subset of Swedish (but not necessarily indigenous) mandate cultivars (94 cvs) in order to reveal any overall changes, as in *Garkava-Gustavsson* et al. (2005).

Results

RAPD polymorphism

Out of 186 oligonucleotide primers initially screened with four apple cultivars, 9 primers showed high levels of polymorphism and good

reproducibility. Two of them, OPF-13 and OPG-06, were difficult to score unambiguously because of differences in band intensity and were therefore excluded. Thus, seven primers which detected distinct, clearly resolved and consistently reproducible amplification products were selected for further analyses. These seven primers generated a total of 77 reliable fragments. The band size ranged from 180 bp to 2170 bp. Number of polymorphic bands ranged from 4 to 15, while the proportion of polymorphic bands varied from 57.1% to 100% (*Table 3*). In total, 71 bands were polymorphic in the entire set of cultivars. Five of these were unique, i.e. present in one cultivar but not in any other. In addition, nine bands were rare, here defined as present in less than 5% of all cultivars.

Based on pairwise comparisons with Jaccard's coefficient of similarity, we checked band-by-band DNA-profiles for all cultivars for which the Jaccard value was equal to 1. As expected, the sports 'Melon, red', 'Gravensteiner, red', 'K:1016, red' and 'Åkerö from Gripsholm' had profiles identical with those obtained for their progenitors ('Melon', 'Gravensteiner', K:1016 and 'Åkerö'). We also compared RAPD banding patterns for two trees labelled with the synonymous names 'Blenheim Orange' and 'Reinette de Blenheim' respectively, and these were also identical as expected. The cultivar 'Fagerö' was identical to 'Grågylling' as suspected since these have been described as very similar, with 'Fagerö' possibly being a red sport of the latter (*Nilsson*, 1986). It was somewhat more surprising to find that 'Spässerud', an old Swedish cultivar from the province of Värmland, was identical to the slightly younger cultivar 'Särsö' originating from the province of Östergötland but described as being rather similar to 'Spässerud' (*Nilsson*, 1986). In this case we performed some additional analyses, in which we compared RAPD profiles of 'Spässerud' and 'Särsö' from our collections with profiles of 'Spässerud' from three different locations in Sweden (Mårbacka, Årnäs and Gränna), and with 'Särsö' from Finland. All these samples showed identical RAPD profiles. The cultivars 'Grågylling from Skokloster' and 'Alexander' also had identical DNA profiles, but these cultivars are quite distinct according to pomological literature. Observation of the two trees in the Balsgård collection indicated that both represent true 'Alexander', thus suggesting that the tree previously regarded as'Grågylling from Skokloster' was mislabelled. Based on our results, the now documented sports or duplicates 'Gravensteiner, red', 'Melon, red', 'K:1016, red', 'Åkerö from Gripsholm', 'Reinette de Blenheim', 'Fagerö', 'Särsö' and 'Grågylling from Skokloster' were deleted from the statistical analyses of genetic diversity.

Another case, where we expected to find identical profiles, was the comparison of 'Cox's Orange Pippin' with its sport 'Queen Cox'. One of the bands amplified by primer OPM-09 was, however, found only in 'Cox's Orange Pippin'. Whether this band was amplified from a region that truly differs between the ancestral cultivar and its derivative, or whether the band difference is artefactual is not yet known. We decided, however, to retain both of these cultivars in the further analyses.

Cluster analysis and Principal coordinate analysis (PCO)

A UPGMA dendrogram, illustrating the molecular relatedness in the entire set of 151 cultivars (*Figure 1*) was constructed. No major clusters were observed in the dendrogram, and there was little grouping that could be associated with geographic origination or historical age: Swedish and foreign, old and new cultivars were completely intermingled.

Many cultivars grouped in accordance to their known descendance: either together with one of their parents or together with the other cultivars with common ancestors. 'Cox's Orange Pippin' of course clusters closely with its sport 'Queen Cox' from which it differed by only one DNA band, and also with another offspring, namely 'Holsteiner Cox'. By contrast, its alleged mother 'Ribston', its alleged sibling 'Cox's Pomona' and three other offspring, namely 'Elise', 'Ingrid Marie' and 'Laxton's Superb' occur further apart in the dendrogram.

The analysed material contained three cases of sibling cultivars. First, K:1016 and 'Fredrik' belong to the same cluster as their mother 'Aroma', while a third sibling, 'Frida', occurs somewhat further apart, as also 'Filippa' which is the mother of 'Aroma'. Second, B:0654 and 'Sylvia', which derive from a cross between 'Astrakan, Gyllenkrok's' and 'Worcester Pearmain', cluster together with one another but not with either of the parents. Interestingly, 'Worcester Pearmain' does not cluster closely with any of its other offspring ('Discovery' and 'Katja') either. Finally, the siblings 'Siv' and 'Nanna' occur quite far apart from one another, and from their mother 'Katja', which instead clusters with 'Mio' with which it shares one parent, namely the above-mentioned 'Worcester Pearmain'.

Other cases of clustering between parents and offspring involve 'Boskoop' and its offspring 'Alfa 68', 'Cortland' and its offspring 'Birgit Bonnier' and 'Eva-Lotta', 'Golden Delicious' and its offspring 'Elstar', 'John-Georg' and 'Mutsu', and 'Ingrid Marie' and its offspring 'Alice' and 'Kim'. By contrast, cases where a parent does not cluster closely with its offspring include 'Cortland' and its offspring 'Kim', 'Filippa' and its offspring 'Alfa', 'Ingrid Marie' and its offspring 'Aroma' and 'Elstar', 'James Grieve' and its offspring 'Eva-Lotta', 'John-Georg' and 'Katja', 'Julyred' and its offspring 'Vista Bella', 'Katja' and its offspring K:1160, 'Lobo' and its offspring 'Rödluvan', and 'McIntosh' and its offspring 'Cortland' and 'Lobo'. Interestingly, of all 12 cultivars having both parents included in the analyses, all but the three Balsgård varieties B:0654, 'Katja' and 'Sylvia' (all of them offspring of 'Worcester Pearmain') clustered with one of the parents.

Most of the older, indigenous mandate cultivars have an unknown origin. According to the dendrograms, a closer relationship might be suspected between the following pairs: 'Annero' and 'Sparreholm', 'Astrakan, Stor Klar' and 'Arvidsäpple', 'Aspa' and 'Kalmar Glasäpple', 'Farmors Juläpple' and 'Astrakan, red', 'Åkerö' and 'Vitgylling', 'Hedenlunda' and 'Frösåker', 'Sävstaholm' and 'Kramforsäpple'.

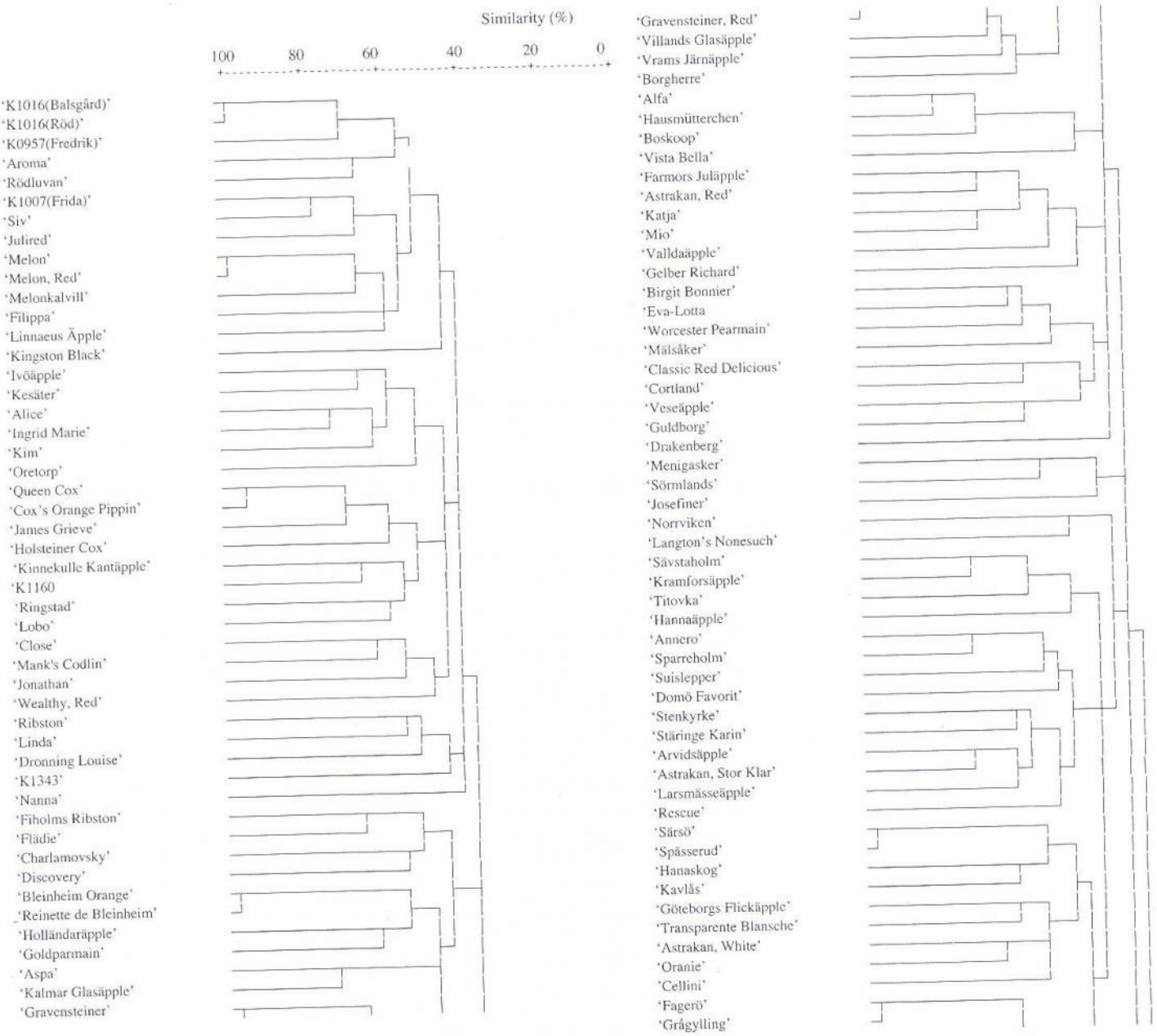


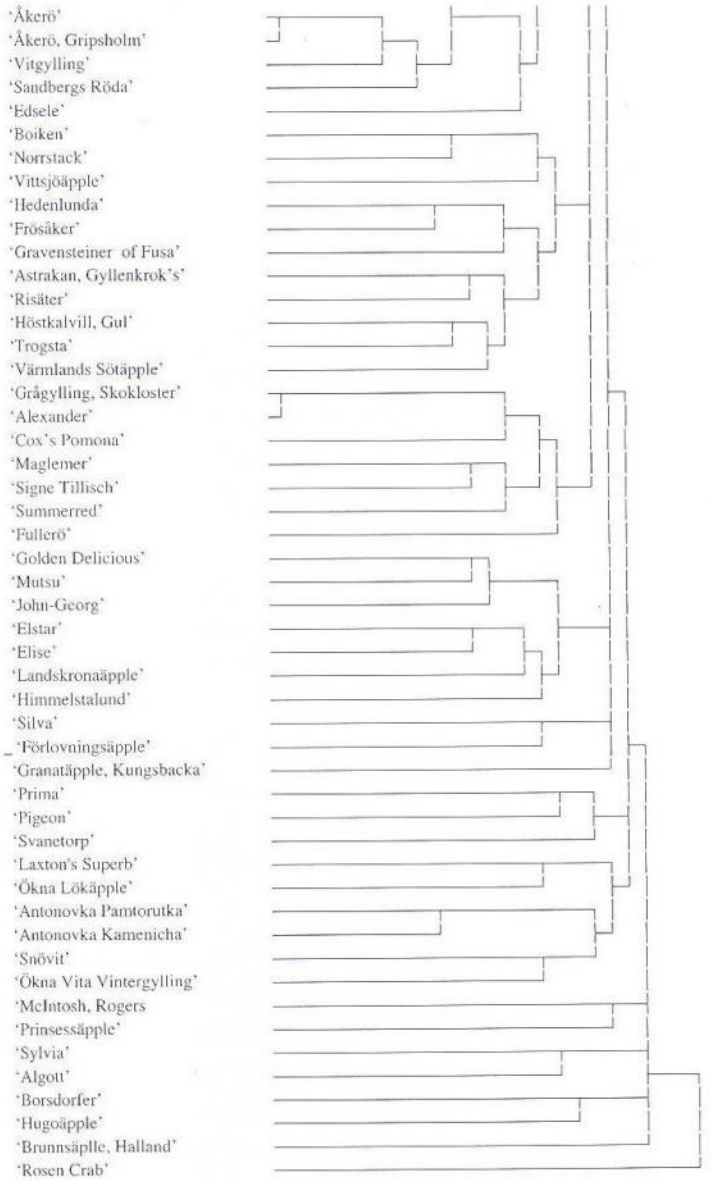
Figure 1. A dendrogram, illustrating molecular relationships among 151 analysed apple cultivars

The PCO analysis, applied to 143 individual genotypes, explained only 11% of the diversity on the first two principal components and confirmed the general pattern of intermingling among cultivars (*Figure* 2). There was, however, a clear tendency for the foreign cultivars (especially the most recent ones) to group in the leftmost half of the plot whereas the Swedish cultivars were more evenly distributed.

Genetic diversity

Genetic diversity value in the entire set of 151 cultivars measured with Nei's diversity index, H_{ENTIRE}, was 0.269. Corresponding value for Shannon's index was $H'_{ENTIRE} = 0.594$. The diversity estimators yielded only slightly lower values when calculated for the subset of 94 mandate cultivars: $H_{MANDATE} = 0.263$ and $H'_{MANDATE} = 0.575$. These diversity estimators were also calculated for each of the 6 groups of cultivars, yielding the mean values of $H = 0.262$, and $H' = 0.548$ (Table 3). The highest level of genetic diversity was observed for group 4, old foreign cultivars ($H = 0.281$; $H' = 0.585$) and the lowest for group 6, new foreign cultivars ($H = 0.235$; $H' = 0.493$). Only 14.6% of the total diversity resided between groups, which indicates a high diversity within groups compared to a rather low degree of differentiation between groups.

Another way to compare diversity in the entire set of cultivars with the subset of mandate cultivars is to analyse RAPD-band frequencies. Plotting these band frequencies, from the most common band to the least common band in both data sets, shows that there were very few discrepancies (*Figure 3*). Bands, that were common in the entire set, remained common also in the subset of mandate cultivars, and the rare bands did in most cases neither increase nor



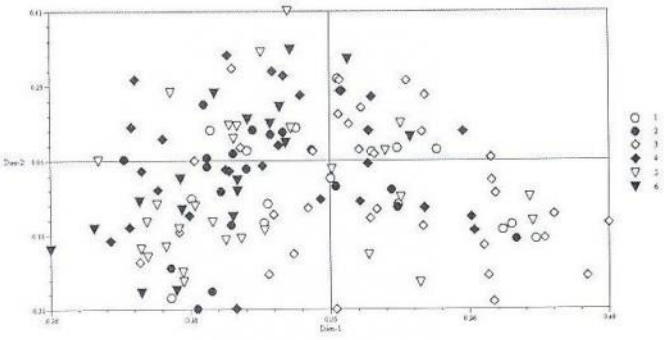


Figure 2. A two-dimensional plot of PCO analysis of 143 individual apple genotypes belonging to different historical and geographical groups (all duplicates are removed).

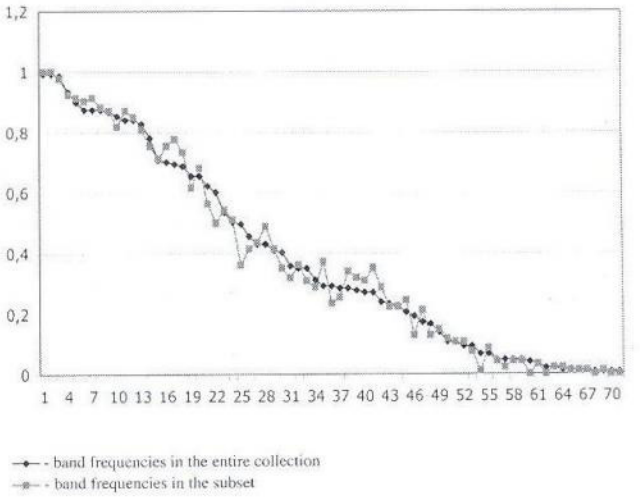


Figure 3 Comparison of RAPD band frequencies in the entire set of apple cultivars (151 cvs) and in the subset of Swedish mandate cultivars (94 cvs).

decrease in frequency. Five RAPD bands were, however, absent in the subset of mandate cultivars, showing that some gene regions are lacking in this subset.

Discussion

The purpose of our study was to obtain a quick preliminary evaluation of genetic diversity in the Balsgård germplasm collection and to find possible duplicates, synonyms and mislabellings, and therefore we chose to use RAPD. To ensure good reproducibility, great care was taken in primer selection: a large number of primers were screened and only those providing consistently amplified bands were chosen. In a different study, the same set of apple cultivars have been analysed with SSR markers (*Garkava-Gustavsson* et al., unpublished). Compared to RAPD, SSR-based analysis lends itself better to the setting up of shared marker score sheets but the high mutation rates can make these markers less useful for relatedness studies (*Weising* et al., 2005).

Identification and relatedness among cultivars

Unambiguous identification of research material becomes especially important when costly screenings are made of e.g. content of phenolic compounds and allergenic proteins (*Nybom* et al., in press) and disease resistance (*Mattisson & Nybom*, 2005). For plant breeders and other users, it is similarly important to have access to correctly identified plant material, which does, in fact, contain the genes and traits expected from previous investigations and analyses. RAPD markers have previously proven to be useful for identification of putative duplicates and misclassifications in the collection of e.g. yam cultivars (*Dansi* et al., 2000).

As expected, we could easily distinguish all the cultivars except for most of the somatic mutations (sports) like 'Gravensteiner, red, 'Melon, red', 'K:1016, red, 'Åkerö from Gripsholm' and 'Fagerö'. These sports were omitted from further diversity analyses. We also obtained identical RAPD

profiles for the Swedish cultivars 'Spässerud' (first described in 1903) and 'Särsö' (first described in 1917). Apparently, these cultivars are therefore either completely identical, or – perhaps more likely – one of them has been derived as a sport of the other since fruit flesh colour is reported to be somewhat different (*Nilsson*, 1986). We found one obvious case of mislabelling; the tree labelled 'Grågylling from Skokloster' was instead 'Alexander'. A similar case was revealed in a small pilot study (*Garkava-Gustavsson & Nybom*, 2003), in which 'Astrakan, white' appeared to be identical to 'Astrakan, stor klar'. The RAPD-profile of our 'Astrakan, white' was compared to a profile derived from analysis of a tree in the collection at Pikkiö in Finland, and appeared to be different. We concluded that our 'Astrakan, white' was mislabelled, and in this study it was therefore substituted with 'Astrakan, white' from Finland. Interestingly we did, however, find a one-band difference between our samples of 'Cox's Orange Pippin' and its alleged sport 'Queen Cox'. Since sexual recombination usually results in much larger band profile differences, it appears that our material of 'Queen Cox' does, indeed, represent a sport. Whether the band difference is artefactual or actually reflects a genetic difference in a mutated region must be further investigated.

RAPD markers have usually failed to differentiate among sports also in previous studies of apple identification (*Harada* et al., 1993; *Goulao* et al., 2001). Nevertheless, RAPD markers were efficient in differentiating between the original cultivar and its new radiomutants in chrysantemum (*Lema-Ruminska* et al., 2004). Experimentally applied mutagenics are, however, likely to cause considerably more changes in the genome than in the case of spontaneous sport mutations.

By contrast, RAPD as well as many other DNA-based markers have proven very useful in determining whether different cultivars have arisen by recombination or as sports. Using SSR, the nature of genetic identity was determined for seven pairs of apple cultivars in the core subset collection at USDA-ARS (*Hokansson* et al., 1998). The authors concluded that five pairs contained sport mutations and/or their progenitors, one accession was mislabelled and another one had probably a synonymous name or was a sport mutation.

The clustering of cultivars in our dendrogram, and in our two-dimensional PCO plot, showed very weak associations with geographic origin or the historical age of the investigated cultivars. Similarly, an SSR-based dendrogram, produced for the subset of 68 indigenous Swedish mandate cultivars, failed to produce well defined clusters (*Garkava-Gustavsson* et al., unpublished). A corresponding lack of association between geographical origin and clustering in a dendrogram has also been reported for e.g., mulberry (*Vijayan* et al., 2005), pear (*Monte-Corvo* et al., 2000) and olive (*Grati-Kamoun* et al., 2006). The fact that the Swedish apple cultivars were scattered all over the dendrogram, as well as the PCO plot, indicates that they represent a more or less random set of molecular phenotypes and derive from a broad gene pool, obtained through hybridisation between highly heterozygous apple genotypes during several centuries of apple cultivation. Some of the most popular cultivars have a slow turnover and are more than a hundred years old. The pattern of diversity observed in our study is typical of longlived crops, for which germplasm has been shared extensively around the world.

By contrast, *Pereira-Lorenzo* et al. (2007), using SSR markers, revealed regional differentiation among local Spanish cultivars based on PCA (Principal Component Analysis) and cluster analysis. In Spain, apple growing and cultivar development has, however, a much longer history than in Sweden. Still the differentiation values (Fst) were low, suggesting the origination of local cultivars from a common gene pool, high gene flow between regions and minimal genetic isolation among populations.

We found a strong tendency for cultivars to cluster with one of their parental cultivars. Many other studies have similarly reported that apple genotypes cluster on UPGMA dendrograms in accordance with pedigree information (*Goulao* et al., 2001; *Gardner & Hokanson*, 2005). These results suggest that closely clustering cultivars can be regarded as genetically more similar than average cultivars. Consequently, information on clustering cultivars can become useful in character screenings; only those genotypes which appear to be most promising can be targeted instead of screening all available accessions.

Genetic diversity

Diversity values obtained with Shannon's index in our study were about twice as high as the values obtained with Nei's diversity index. The mean value of Shannon's index for 6 groups of cultivars was 0.546, and slightly higher, 0.593, when calculated across all cultivars. These values are similar to the mean value for wildgrowing populations of the outcrossing flowering quince *Chaenomeles speciosa* (0.552) (*Bartish* et al., 2000), somewhat higher than the mean value for collection sites of cultivated clones of the outcrossing Ethiopian crop plant *Ensete ventricosum* (0.498) (*Birmeta* et al., 2002) and somewhat lower than the mean value for wildgrowing populations of *E. ventricosum* (0.630) (*Birmeta* et al., 2004).

Mean value for Nei's genetic diversity was 0.262 in our study of six groups of apple cultivars and 0.268 when calculated for the entire set. These values are very similar to those obtained by *Marita* et al. (2000) for randomly sampled clones of cacao (0.305) and *Capsicum* (0.269), and approximately twice as high as for a collection of sour orange accessions (0.122) (*Siragusa* et al., 2006). All in all, apple appears to hold average values of genetic diversity when compared to other outcrossing crop species, and variously defined subsets hold almost the same amount of diversity as a larger set with cultivars originating at different points of time from a large array of different countries.

During the last twenty years, the number of apple breeding programs and released cultivars has increased remarkably. However, of the 1000 new cultivars released in these two decades, a majority are sports (mutant clones) (*Sansavini* et al., 2004) and therefore do not contribute to a

widening of the gene pool. Despite the recent activity in apple breeding, it has instead been suggested that the actually utilized gene pool is becoming dangerously narrow (*Noiton & Alspach*, 1996).

In our study, a small tendency towards a narrowing of the gene pool can be seen in the lower diversity for recent foreign cultivars compared to the other investigated groups of cultivars. The 18 cultivars belonging to the group 'recent foreign', represent 8 countries (USA, Canada, England, Netherlands, Norway, Denmark and Japan). Both parents are known for 8 of these cultivars while one parent is known for 6 cultivars, three have completely unknown pedigrees and one ('Queen Cox') is a sport. Pedigree analysis shows that 'Cox's Orange Pippin', 'Golden Delicious', 'McIntosh' and 'Worcester Pearmain' are the most commonly occurring cultivars in the pedigrees for the recent foreign group.

Interestingly, a corresponding decrease of genetic diversity could not be seen in the group with recent Swedish cultivars, which is probably due to the rather broad range of cultivars used in breeding programs. Ancient indigenous cultivars like 'Astrakan, Gyllenkrok's' have been used along with old foreign cultivars and novel selections.

Choice of mandate cultivars

Many studies have been devoted to the development and comparison of strategies and methods for assembling gene-banks and core collections in different plant species (*Dwivedi* et al., 2005; *Garkava-Gustavsson* et al., 2005; *van Raams-donk & Wijnker*, 2000). At Balsgård, an active germplasm collection with approximately 1000 cultivars is being used in plant breeding and research. In addition, a set of cultivars, mostly of Swedish origination, have been granted 'mandate cultivar' status in Sweden and are now conserved mainly at outdoor museums and other public places, although many of them are also present in the Balsgård collection. The mandate cultivar status was accorded on historical merits and no priority has been given to the preservation of genetic diversity or to the availability of particular genes of interest for research and breeding. We therefore compared a set of cultivars from the active collection (151 cvs) with a subset containing only mandate cultivars (94 cvs). Both diversity values (Nei's genetic diversity and Shannon's index) were only marginally lower in the collection with mandate cultivars, suggesting that it has the same amount of variation as a randomly sampled collection would have. By contrast, collections created to preserve maximum genetic variation should have more diversity than randomly assembled collections. Thus, the mean genetic diversity in a collection of *Theobroma cacao*, created by the Maximum genetic diversity program was 0.377 compared to 0.305 in the case of random sampling (*Marita* et al., 2000). For *Capsicum* corresponding values were 0.361 and 0.269 (*Marita* et al., 2000) and for *Vaccinium vitis-idaea* 0.356 and 0.303 (*Garkava-Gustavsson* et al., 2005).

We also compared band frequencies in our two sets of cultivars, and there were no major changes although 5 bands were missing in the set of mandate cultivars. However, bands that were common in the entire collection remained common in the subset, and rare bands remained rare. By contrast, when a subset of lingonberry (*Vaccinium vitis-idaea*) genotypes had instead been choosen by a Maximum diversity algorithm procedure, all bands were preserved and moreover, the rare bands increased in frequency while common bands decreased (*Garkava-Gustavsson* et al., 2005).

Usually, mandate cultivars, aimed at preservation of mainly indigenous genetic resources, contain genotypes chosen for their cultural and historical values. This has clearly been the case for the mandate apple cultivars in Sweden. Although one could expect some overall similarities due e.g., to their adaptation to a cold climate, these cultivars appear to genetically constitute a random sampling and contain the same amount of variation as the larger set of Swedish and foreign cultivars analysed in our study.

A rather different approach for preservation of genetic resources has been taken in several other countries. Although conservation of historic cultivars is one of the purposes for gene banks in e.g., the National Plant Germplasm System (NPGS, www.ars-grin.gov/npgs) in USA, one of the main goals is to provide plant material for basic plant genetic research and breeding (*Postman* et al., 2006). Consequently, this gene bank as well as others, e.g., the National Fruit Collections at Brogdale in England (www.brogdale.org) and The Vavilov Institute in Russia (www.vir.nw.ru), contain carefully chosen cultivars from all around the world, including genotypes with especially important genes governing e.g., resistance against various diseases. The core collections developed at e.g., PGRU, which is a part of the NPGS, can therefore be regarded as sources of genes, rather than sources of genotypes and clones (*Volk* et al., 2005). Obviously in countries like Sweden, preservation of mandate cultivars must be complemented with preservation of germplasm that has been selected to increase genetic variation and to provide important genes in order to better fulfill the needs of researchers and plant breeders.

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