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P. G. Lanham · R. M. Brennan · C. Hackett · R. J. McNicol

RAPD fingerprinting of blackcurrant (*Ribes nigrum* L.) cultivars

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Abstract *Ribes nigrum* germplasm was screened for random amplified polymorphic DNA (RAPD) markers. Fifty-four markers were identified which generated individual fingerprints for each of 21 cultivars. Genetic variation within *R. nigrum* germplasm, as detected by RAPDs, demonstrated that the genetic basis for improvement of blackcurrant is narrower than would be expected by the analysis of parentage.

Key words *Ribes nigrum* · RAPD · Genetic variation

Introduction

The genus *Ribes* consists of approximately 150 species of spiny and non-spiny shrubs, distributed mainly in northern temperate regions of Europe and North America. Most of the world's commercial production of *Ribes* fruits is in northern Europe, and the major crop is the blackcurrant, *R. nigrum* L., which has become domesticated in northern Europe within the last 400 years only.

The most important cultivar in the UK in the late 19th and early 20th centuries was 'Baldwin' which dominated UK production in this period and still occupies a significant proportion of the UK hectarage. Cultivars 'Baldwin' and 'Boskoop Giant' were used to develop a further group of cultivars, released in the early 20th century, until Tydeman (1938) investigated the inheritance of commercially desirable characters in crosses between western European cultivars and concluded that further improvements required the involvement of species other than *R. nigrum*. His ideas have been applied through the increasing use of Nordic and Russian germplasm in modern blackcurrant breeding.

One of the main areas where improvements have been made by the incorporation of Nordic germplasm, including other *Ribes* spp., is in low temperature hardiness, particularly during the flowering period. The Finnish cultivar 'Brödtorp' and Swedish cultivars 'Öjebyn and 'Sunderbyn II' were local selections from the wild which have been highly successful in their native countries and also in the former USSR (Pavlova and Vorodina 1978). As parental lines in western breeding programmes, together with other Swedish types such as 'Janslunda', they have played a major part in the development of late-flowering cultivars with improved low temperature hardiness which now dominate UK production. The most commercially successful of these new cultivars are the 'Ben' series bred at the Scottish Crop Research Institute, from 'Ben Lomond' to the most recent, 'Ben Connan'.

In the USSR the indigenous *R. nigrum* var *sibiricum* Wolf. and *R. dikuscha* Fisch. have been used as donors of hardiness and disease resistance in crosses with western European cultivars (Mosolova 1963; Melekhina 1964; Lihonas and Pavlova 1969), and the first important cultivar to be produced in this way was 'Primorskij Čempion' (*R. nigrum* x *R. dikuscha*). Selection among wild ecotypes of *R. nigrum* var *sibiricum* extended the northern limits of blackcurrant production in the USSR to inside the Arctic Circle (Vitkovskij 1964a). Today the USSR has a very large range of cultivars, the most popular of which, in the European areas, are 'Bieloruskaja Slodkaja' and 'Minaj Smyrev' (Volunez 1988). Other widely grown cultivars include the Finnish 'Brödtorp', 'Golubka' (a *R. dikuscha* derivative), 'Narjadnaja', 'Pilot Aleksandr Mamkin' (a *R. pauciflorum* hybrid), 'Stakhanovka Altaya' and 'Vystavochnaja' (Pavlova and Vorodina 1978).

Random amplified polymorphic DNA (RAPD) markers, utilising polymerase chain reaction (PCR) amplification from single primers of arbitrary nucleotide sequence, were developed by Williams et al. (1990) and Welsh and

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P. G. Lanham (✉) · R. M. Brennan · R. J. McNicol
Soft Fruit Genetics Department,
Scottish Crop Research Institute, Invergowrie,
Dundee DD2 5DA, UK

C. Hackett
Scottish Agricultural Statistics Service,
Scottish Crop Research Institute, Invergowrie,
Dundee DD2 5DA, UK

McClelland (1990) as molecular markers for use in genetic analysis. RAPDs have since been shown to be useful in genetic fingerprinting (Quiros et al. 1991; Wilde et al. 1992; Collins and Symons 1993; Eskew et al. 1993; Harada et al. 1993; Yang and Quiros 1993; Graham et al. 1994), phylogenetic analysis (Demeke et al. 1992; Halward et al. 1992; Kresovitch et al. 1992; Kazan et al. 1993; Yu and Pauls 1993), parentage analysis (Welsh et al. 1991; Roy et al. 1992; Scott et al. 1992) and genetic mapping (Martin et al. 1991; Reiter et al. 1992; Barua et al. 1993; Paran and Michelmore 1993; Williams et al. 1993). In this communication we report the fingerprinting of cultivars of *Ribes nigrum* using RAPDs, and we compare the relatedness of these cultivars to each other based on RAPD data to that based on pedigree analysis.

Materials and methods

The *R. nigrum* cultivars screened for RAPD markers are listed in Table 1 and were obtained from the germplasm collection maintained at the Scottish Crop Research Institute as an integral part of the ongoing *Ribes* breeding programme. They represent a broad cross-section of the available genetic base utilised in germplasm improvement of *R. nigrum*, including European, Scandinavian and Russian subgroups, cultivars developed directly from wild accessions (e.g. 'Öjebyn') and seedlings derived from interspecific hybridisations (e.g. 'B1834').

DNA isolation

DNA was isolated from *R. nigrum* by a method based on that of Gawel and Jarret (1991). One gram of fresh leaf material was ground in liquid nitrogen in the presence of acid-washed sand and polyvinylpyrrolidone (insoluble). Five milliliters of extraction buffer [1% (w/v) hexadecyltrimethylammonium bromide (CTAB); 100 m*M* TRIS-HCl, pH 8.0; 1.4 *M* NaCl; 20 m*M* ethylenediaminetetra-acetic acid; 1% (w/v) dithiothreitol] was added and the mixture incubated at 65°C for 30 min. One chloroform extraction was performed followed by precipitation of nucleic acid from the aqueous phase by the addition of an equal volume of isopropanol, incubation at room temperature for 15 min and centrifugation at 3,646 *g* for 20 min. The dried pellet was redissolved in 0.5 ml TE buffer (10 m*M* Tris-HCl pH 8.0; 1 mM ethylenediaminetetra-acetic acid), treated with RNAse and the DNA precipitated by the addition of five volumes of ice-cold 100% ethanol. Precipitated DNA was hooked out and redissolved in 200 µl of TE.

Polymerase chain reaction

The 10-mer random primers used in this study were supplied by Operon Technologies and are listed in Table 2. PCR reactions contained (50 µl final volume) approximately 50 ng target DNA; 200 m*M* primer; dATP, dCTP, dGTP, dTTP each at 100 µm; 1×*Taq* polymerase buffer and 0.5 units of *Taq* polymerase. Each reaction was overlaid with mineral oil and subjected to 45 repeats of the following cycle: 92°C for 1 min; 35°C for 3 min; 72°C for 2 min. After the final cycle, samples were incubated for a further 5 min at 72°C to ensure completed extension along the entire length of the target molecules.

PCR products were electrophoresed through 1.5% agarose gels run in 0.5×TBE (44.5 m*M* Tris; 44.5 m*M* boric acid; 1 m*M* ethylenediaminetetra-acetic acid), stained with ethidium bromide and visualised by illumination with UV light (302 nm). Each reaction was performed a minimum of three times, and only reproducible bands were scored. Polymorphism was scored on a presence or absence basis. The index proposed by Nei and Li (1979) was used to calculate the similarities, S_{ij}, between the cultivars i and j:

$$S_{ij} = \frac{2N_{ij}}{(N_i + N_j)}$$

where N_{ij} = the number of bands in common between cultivars i and j and N_i and N_j are the number of bands for cultivars i and j, respectively. A second measure of similarity was derived from the pedigree data, using the proportion of genetic material from common founding clones. For example, the pedigrees for 'Ben Sarek', 'Ben More' and 'Amos Black' are: 'Ben Sarek' ['Goliath' (50%) ×'Öjebyn' (50%)]; 'Ben More' [Unknown (50%)×{'Goliath'

Table 1 *R. nigrum* germplasm which was screened for RAPD markers

Cultivar	Country of origin
243/7	United Kingdom
Amos Black	United Kingdom
B1834	United Kingdom
B1835	United Kingdom
Baldwin	United Kingdom
Ben Alder	United Kingdom
Ben Connan	United Kingdom
Ben Lomond	United Kingdom
Ben Loyal	United Kingdom
Ben More	United Kingdom
Ben Nevis	United Kingdom
Ben Sarek	United Kingdom
Ben Tirran	United Kingdom
C1/9/10	United Kingdom
Ceres	Poland
Kosmiczeskaja	Commonwealth of Independent States
Narjadnaja	Commonwealth of Independent States
Öjebyn	Sweden
Polar	Sweden
Storklas	Sweden
Sunderbyn	Sweden

Table 2 Random primers used to screen *R. nigrum* germplasm for RAPDs

Primer	Sequence
OPA-04	AATCGGGCTG
OPA-06	GGTCCCTGAC
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPA-20	GTTGCGATCC
OPB-01	GTTTCGCTCC
OPB-02	TGATCCCTGG
OPB-03	CATCCCCCTG
OPB-04	GGACTGGAGT
OPB-05	TGCGCCCTTC
OPB-06	TGCTCTGCCC
OPB-07	GGTGACGCAG
OPB-08	GTCCACACGG
OPB-09	TGGGGGACTC
OPB-10	CTGCTGGGAC
OPB-11	GTAGACCCGT
OPB-12	CCTTGACGCA
OPB-14	TCCGCTCTGG

(25%)×'Öjebyn' (25%)]} and 'Amos Black' ['Goliath' (50%)× 'Baldwin' (50%)]. We would calculate the similarities between 'Ben Sarek' and 'Amos Black' as 50%, between 'Ben Sarek' and 'Ben More' as 50% and between 'Ben More' and 'Amos Black' as 25%. The similarities with 'Öjebyn', an accession from the Swedish wild population of *R. nigrum*, are 50% for 'Ben Sarek', 25% for 'Ben More' and 0% for 'Amos Black'. This method was preferred over Malecot's (1948) coefficient of co-ancestry due to the incomplete blackcurrant pedigrees. The rankings of these similarities are more important than their absolute values.

Both sets of similarities were used to cluster the cultivars using average linkage cluster analysis. All statistical calculations were carried out using Genstat 5.2.

Results

Out of a total of 210 amplification products scored, 54 (26%) were polymorphic, i.e. RAPDs (Table 3). Four primers, OPA-04, OPA-14, OPB-04 and OPB-06, detected no polymorphism although they did successfully amplify a range of monomorphic bands (data now shown). Some primers detected 1 polymorphism only while OPA-06 detected 6. The average number of RAPDs detected per polymorphic primer was 2.8 (Table 3). Figure 1 gives examples of RAPD markers detected in *R. nigrum* germplasm.

Table 3 Summary of the detection of RAPD markers in *R. nigrum* germplasm

Total number of primers	23
Number of polymorphic primers	19
Total number of bands amplified from polymorphic primers	210
Size range of amplification products	0.5–3.5 kb
Average number of bands per polymorphic primer	11
Total number of polymorphic bands (RAPDs) identified	54
Average number of RAPDs per polymorphic primer	2.8
Percentage of total bands which were polymorphic	26

Figure 2 gives a graphical representation of the scores of all 54 RAPD markers in the form of a bandmap (Powell et al. 1991). The order of the cultivars on the left hand side of this figure is the result of cluster analysis (see Fig. 3) of similarity indices calculated from the RAPD scores; those cultivars with the greatest similarity tend therefore to occur near each other on the bandmap. The bandmap demonstrates that all cultivars had distinct profiles and were successfully fingerprinted using RAPDs. The bandmap also orders the markers by their frequency of occurrence whereby the commonest RAPDs are to the left and the rarest are to the right. Four RAPD markers (numbers 18, 36, 45, 52), which were present in 1 cultivar, were identified, 2 of them in 'Ceres'. The lowest number of positive scores for any 1 cultivar was 20 (B1834), and the highest was 32 ('Polar', column B in Fig. 1), indicating that the RAPD markers were evenly distributed throughout the germplasm tested.

Cluster analysis of RAPD scores grouped the *R. nigrum* cultivars into four main clusters with '243/7' and 'Ceres' not being placed in any particular group (Fig. 3a). Also, the 'Narjadnaja'/'Kosmiczeskaja' group is strongly separated from all other cultivars, which reflects the different genetic background of these cultivars. In both of these cases, the cultivars concerned are of completely different geographical origin and incorporate wildtype germplasm from within these areas. The seedling '243/7' is an SCRI breeding line of complex parentage incorporating elements not common to other genotypes within the study. Cluster analysis based on pedigrees is presented in Fig. 3b. While there are similarities between Fig. 3a and 3b, there are also some very obvious differences. 'Ceres', '243/7' and 'Narjadnaja', are separated from other cultivars by both types of analysis while 'Kosmiczeskaja' is linked to 'Amos' Black by pedigree but not by RAPDs. The groupings based on pedigree are different from those based on RAPDs. Some cultivar pairs (e.g. 'Ben Connan'/'Ben Loyal') are identical by pedigree (100%) but distinguished

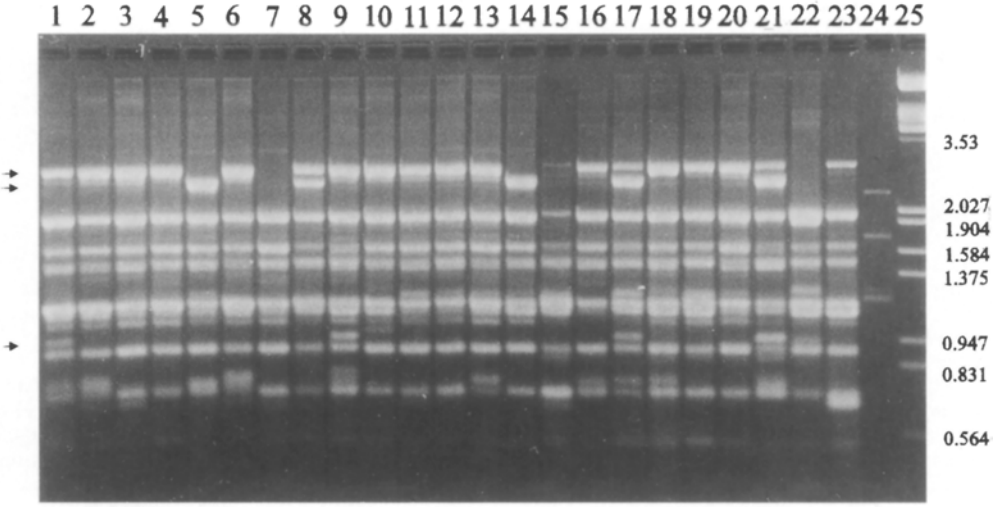

Fig. 1 PCR products amplified from *R. nigrum* DNA using primer OPA-06, GGTCCCTGAC. Examples of RAPDs are indicated by *arrows*. Molecular sizes are in kilobase-pairs. *Lane* 1 'Narjadnaja', *2* 'Pilot Mamkin', *3* 'Ben Lomond', *4* 'Baldwin', *5* 'Ben More', *6* 'Brödtorp', *7* 'Sunderbyn', *8* '243/7', *9* 'Polar', *10* 'Öjebyn', *11* 'Ben Alder', *12* 'Ben Tirran', *13* 'C1/9/10', *14* 'Amos Black', *15* 'B1835', *16* 'Ben Connan', *17* 'Kosmiczeskaja', *18* 'Ben Loyal', *19* 'B1834', *20* 'Ben Nevis', *21* 'Ben Sarek', *22* 'Ceres', *23* 'Storklas', *24* No target DNA control, *25* molecular size standards

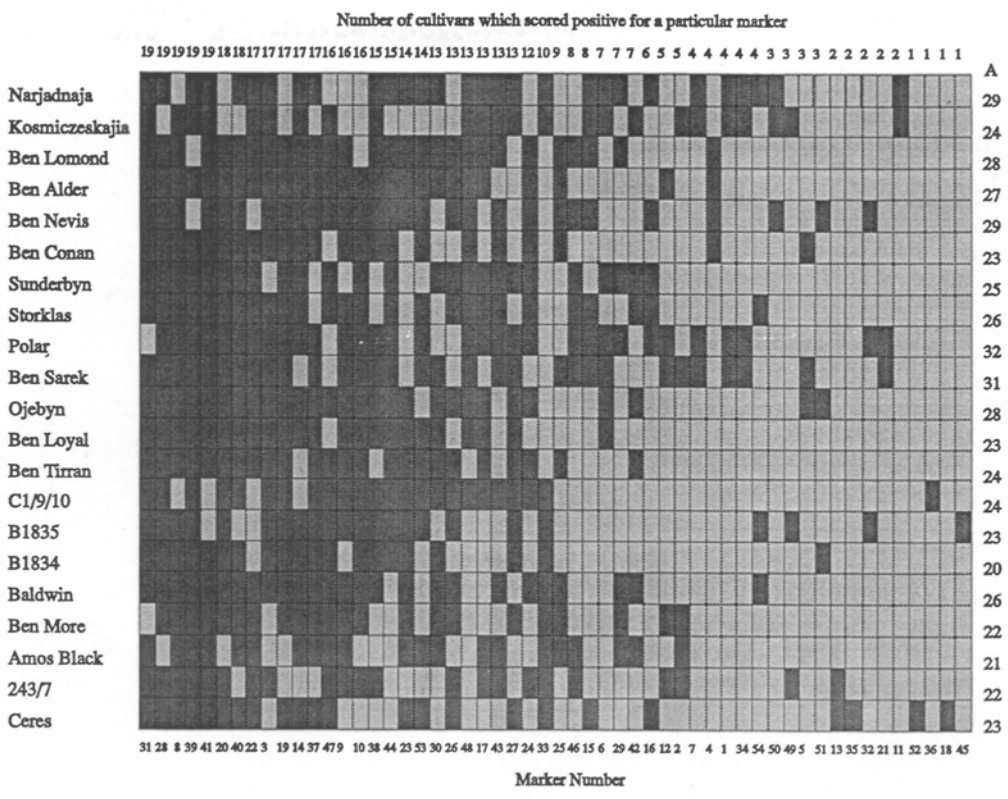

Fig. 2 Bandmap of RAPD markers detected in *R. nigrum* germplasm. A *dark-grey box* represents the presence of a marker; a *light-grey box,* the absence of a marker. The row of *numbers* along the *bottom* of the bandmap identifies each RAPD marker by number. The row of *numbers* along the *top* gives the number of genotypes for which that RAPD scored positive, e.g. RAPD number 31 occurred in 19 genotypes. *Column A* on the *right-hand side* of the bandmap indicates the number of markers present in each genotype, e.g. 'Narjadnaja' scored positive for 29 RAPDs

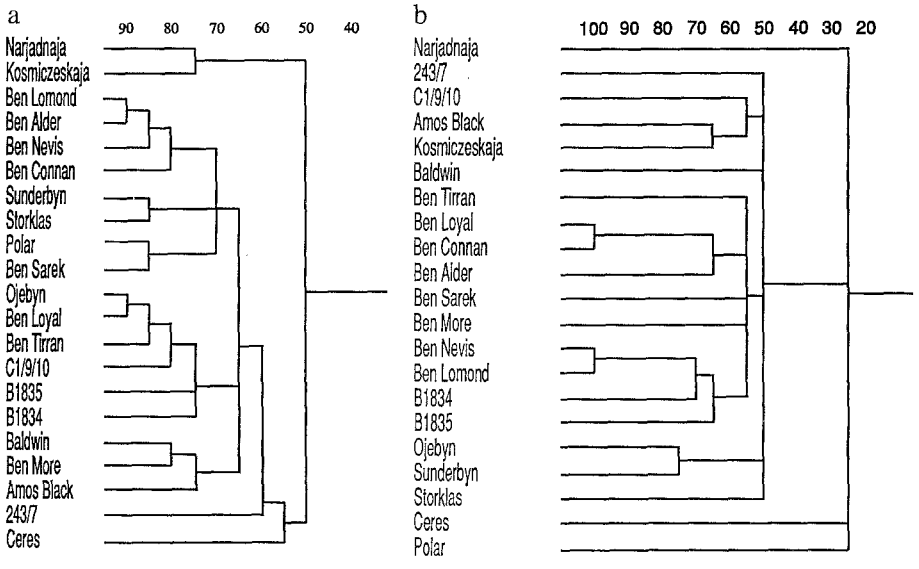

Fig. 3a Dendrogram of *R. nigrum* genotypes based on RAPD data. **b** Dendrogram of *R. nigrum* genotypes based on parentage

by RAPDs (65%). This latter observation demonstrates the power of RAPD markers in the detection of subtle genetic differences between closely related cultivars.

A similarity matrix of the *R. nigrum* cultivars based on RAPD scores is presented in Table 4a. Similarities ranged from 24% ('243/7':'Narjadnaja' and 'Ben More': 'Kosmiczeskaja') to 77% ('Ben Alder':'Ben Lomond'). In the majority of closely similar pairings, some relationship in terms of parentage exists, e.g. 'Ben Lomond' is a parent of 'Ben Alder', 'Ben Connan' and 'Ben Tirran', while 'Ben Nevis' and 'Ben Lomond' are sister seedlings (Fig. 4a). A similarity matrix based on parentage is presented in Table 4b. Similarities ranged from 100% ('Ben Lomond':'Ben Nevis', 'Ben Connan':'Ben Loyal') to 0% (many examples). As in the case of cluster analysis (Figs 3a, 2b), certain anomalies were apparent in the similarities detected using RAPDs and those that would be expected from the known parentages, e.g. 'Narjadnaja' and 'Ben Sarek' have 0% similarity when pedigrees are compared, whereas they have 50% similarity based on RAPD scores. This discrepancy reflects the ability of RAPD markers to detect not only subtle differences between cultivars but

Table 4 Similarity matrix of *R. nigrum* genotypes based on RAPD data (a) and pedigree analysis (b)

a) Based on RAPD data

Narjadnaja	1	100																			
Kosmiczeskaja	2	56	100																		
Ben Lomond	3	46	30	100																	
Ben Alder	4	40	28	77	100																
Ben Nevis	5	38	26	73	65	100															
Ben Connan	6	37	27	65	72	63	100														
Sunderbyn	7	42	36	56	53	46	45	100													
Storklas	8	41	35	64	61	62	58	70	100												
Polar	9	56	40	54	51	52	53	54	57	100											
Ben Sarek	10	50	41	51	53	46	59	44	46	70	100										
Ojebyn	11	43	37	60	72	54	59	66	59	50	51	100									
Ben Loyal	12	53	34	59	72	49	64	55	53	57	59	76	100								
Ben Tirran	13	36	26	63	76	51	57	58	61	44	49	73	68	100							
C1/9/10	14	43	26	58	65	51	52	48	52	44	45	63	62	66	100						
B1835	15	33	27	42	52	44	44	33	44	34	35	50	53	52	57	100					
B1834	16	40	29	45	57	48	48	45	39	37	42	66	65	57	57	59	100				
Baldwin	17	41	32	64	66	53	58	55	58	41	43	64	53	67	56	53	59	100			
Ben More	18	31	24	47	58	38	41	47	37	38	43	52	50	59	44	45	56	66	100		
Amos Black	19	39	36	53	45	35	38	44	42	33	37	40	42	50	36	29	37	57	54	100	
243/7	20	24	39	43	48	38	41	42	50	38	39	43	36	44	44	41	35	50	42	39	100
Ceres	21	37	27	42	43	41	35	41	44	34	32	42	44	47	38	48	48	44	50	38	32	100
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21

b) Based on pedigree analysis

Narjadnaja	1	100																			
Kosmiczeskaja	2	25	100																		
Ben Lomond	3	0	13	100																	
Ben Alder	4	0	19	59	100																
Ben Nevis	5	0	13	100	59	100															
Ben Connan	6	0	31	59	75	59	100														
Sunderbyn	7	0	0	19	20	19	28	100													
Storklas	8	0	0	44	33	44	41	50	100												
Polar	9	13	0	19	19	19	19	25	25	100											
Ben Sarek	10	0	25	19	35	19	60	38	38	19	100										
Ojebyn	11	0	0	19	23	19	35	75	38	19	50	100									
Ben Loyal	12	0	31	59	75	59	100	28	41	19	59	35	100								
Ben Tirran	13	0	19	56	56	56	56	10	23	10	16	10	56	100							
C1/9/10	14	13	52	25	25	25	25	0	0	13	6	0	25	44	100						
B1835	15	2	16	63	41	63	45	23	48	20	23	23	45	38	17	100					
B1834	16	2	9	72	54	72	54	14	39	16	14	14	54	53	23	69	100				
Baldwin	17	0	44	13	6	13	6	0	0	0	0	0	6	13	54	16	9	100			
Ben More	18	0	25	19	59	19	50	19	19	19	50	25	50	16	6	19	14	0	100		
Amos Black	19	0	69	13	19	13	31	0	0	0	50	0	31	19	56	16	9	50	25	100	
243/7	20	25	25	33	31	33	31	0	0	13	13	0	31	52	54	10	22	8	13	21	100
Ceres	21	13	25	13	63	13	6	9	0	0	0	0	6	13	13	13	9	13	0	13	8	100
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21

also unexpected similarities. The RAPD results are more realistic in that any two *R. nigrum* cultivars which have nothing in common in their pedigrees (and would thus score 0% similarity) would be very unlikely to have nothing in common at the molecular genetic level.

Discussion

This is the first report of the use of PCR-based protocols to assess genetic variability and to fingerprint genotypes from the *Ribes* genus, and from the data presented it is clear that the use of RAPDs provides a powerful means of fingerprinting individual genotypes of *R. nigrum* in a reproducible manner.

The pedigree cluster analysis (Fig. 4b) shows that the genetic base utilised in the breeding programmes for this species is fairly restricted in scope. This is emphasised by the observation that 74% of the products amplified from random primers were monomorphic for blackcurrant cultivars (Table 3). The introgression of genes from hitherto unexploited sources should be investigated. The need for greater uniformity in hybrid populations can also act against genetic diversity in breeding programmes (Smith 1969).

Two pairs of sister seedlings, 'Ben Nevis'/'Ben Lomond' and Ben Connan'/'Ben Loyal', showed high degrees of similarity in the RAPD analysis, at 73% and 64%

respectively. The lower values for the latter pair may be due to the inclusion of monomorphic markers, and resulted in 'Ben Connan' and 'Ben Loyal' being placed in different groups in the cluster analysis presented in Fig. 3. The reported methods are able to distinguish between sibs while reflecting their close relationship, and this technique may have applications for the horticultural industry and for germplasm collections throughout the world in differentiating between sister seedlings/cultivars.

Parentages for some of the genotypes are not as descriptive as would be ideal for two reasons: firstly, cultivars such as 'Baldwin' are over 150 years old and are of unknown derivation, and several of the parental types such as 'Victoria' are merely thought to be selections from 'Baldwin'. The exact parentage is therefore impossible to define beyond this. Also, some of the older types are described as open-pollinated seedlings from a particular genotype, so that the male lineage is effectively lost. Secondly, some Scandinavian cultivars such as 'Öjebyn' and 'Sunderbyn II' are direct accessions from wild populations of *R. nigrum*. Whilst in the latter instance they are obviously closely related (the RAPD analysis suggests a 66% similarity), it is impossible to evaluate the real variation within the wild population without sampling a large number of individuals. Centres of genetic diversity for *R. nigrum* exist in northern Scandinavia and parts of the former Soviet Union and China, and from this study it is important from a breeding viewpoint that further wild types are investigated for potential use. In terms of germplasm collections, it is important that random sampling of wild populations is provided to avoid an imbalance with respect to recessive genes in heterozygous plants.

Ribes, in common with most woody perennial fruit genera, is highly heterozygous in terms of genetic background (Zagaja 1983). The level of intraclonal variability was not assessed in this study, but the results presented show a high degree of reproducibility.

Differentiation between distinct groups (Fig. 3a) shows good separation into related genotypes, notably:
1) the Russian group ('Narjadnaja' and 'Kosmiczieskaja'),
2) a 'Ben Lomond'-related group ('Ben Lomond', 'Ben Alder', 'Ben Nevis', 'Ben Connan'),
3) a Scandinavian group ('Sunderbyn II', 'StorKlas' and 'Polar')
4) a *R. nigrum* × *R. grossularia* group ('B1834' and 'B1835').

The two breeding lines, 'B1834' and 'B1835', are both fifth backcrosses from *R. nigrum* × *R. grossularia* containing the *Ce* resistance gene against the blackcurrant gall mite *Cecidophyopsis ribis*. The parentages show a 69% similarity, although the *R. nigrum* parent at the fifth backcross differs from 'Ben Lomond' in 'B1834' to 'Malling' Jet in 'B1835'. This accounts for the 31% dissimilarity, since 'Malling Jet' contains *R. bracteosum* in its parentage, which is not found in 'Ben Lomond'. With RAPDs, the similarity is 59%, showing good agreement with the pedigree data.

The Polish cultivar 'Ceres' proved to be an isolated case, reflecting a complex parentage incorporating *R. dikuscha* and the Russian cultivar 'Barchatnaja', itself a derivative of *R. nigrum* var *sibiricum*. A closer relationship with 'Narjadnaja' might have been expected. Also, one would have expected 'C1/9/10' and one of its parents, '243/7', to show a closer link using RAPDs. However, overall the correlation between the pedigree analysis and RAPD analysis is reasonable.

This communication reports the successful fingerprinting of blackcurrant cultivars using RAPDs and demonstrates the usefulness of these markers in estimating the extent of genetic variation which exists in *R. nigrum* germplasm. The use of RAPD analyses in *Ribes* is continuing in order to derive specific linkages between RAPD markers and genes controlling agronomically important characters, notably pest resistance genes such as *Ce*. The incorporation of marker-assisted selection for characters hitherto difficult and expensive to assess represents a major advance in the genetic improvement of woody perennial crop species.

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