Theor Appl Genet (1998) 96:917–921

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Characterization of the genetic resources of redcurrant (*Ribes rubrum*: subg. *Ribesia*) using anchored microsatellite markers

Received: 18 October 1997 / Accepted: 1 December 1997

Abstract Redcurrant, *Ribes rubrum* L., germplasm was screened for molecular polymorphism using anchored microsatellite primers in polymerase chain reactions (PCRs). Eighty markers were detected using only three primers and 16 redcurrant genotypes were fingerprinted using these markers. The genetic relatedness of these genotypes, as determined by the anchored microsatellite markers, and the implications for redcurrant breeding programmes are examined.

Key words *Ribes* · Markers · Fingerprint · Redcurrant · Germplasm

Introduction

Commercial cultivars of redcurrant are derived from four Ribes species of the sub-genus *Ribesia* namely; *R. rubrum* L., *R. petraeum* Wulf. *R. sativum* Syme (*R. vulgare* Jancz), and *R. multiflorum* Kit. (Keep 1975; Brennan 1996) either singly or in combination. Whitecurrants are similarly composed, being a variant colour form of redcurrant. Most redcurrant species are found in Europe, although North American representatives exist. For the breeding of new cultivars, the main sources are the Netherlands, Scandinavia and the UK, with occasional cultivars from the USA such as 'Red Lake'. The main objectives are the improvement of cropping potential using *Ribes multiflorum* and resistance to leafspot. The fresh fruit of red- and whitecurrant are generally used for culinary purposes, although a small amount of processing for jellies or juice is also done.

The fingerprinting of plant species and cultivated varieties using molecular markers has been widely reported (Collins and Symons 1993; Harada et al. 1993; Graham et al. 1994). Genotypes of *Ribes nigrum* L. (blackcurrant) have previously been characterised using random amplified polymorphic DNA markers (RAPDs, Lanham et al. 1995). Microsatellite sequences (simple sequence repeats, SSRs) are abundant throughout eukaryotic genomes (Tautz and Renz 1984) and have proved valuable as genetic markers (Tautz 1989; Morgante and Olivieri 1993; Morchen et al. 1996). Anchored microsatellites were developed to circumvent the labour-intensive procedures required to develop SSRs as molecular markers (Wu et al. 1994; Zietkiewicz et al. 1994). Detection of these markers utilises polymerase chain reaction (PCR) primers consisting of a simple repeat sequence anchored at either the 5′ or 3′ end by a short arbitrary, often redundant, sequence. They have been found to detect polymorphism at multiple genomic loci in a single gel lane and to segregate as dominant Mendelian traits (Zietkiewicz et al. 1994). They have been successfully used in fingerprinting oilseed rape (*Brassica napus* L. ssp. *oleifera*) and were shown to be a more informative type of marker than RAPDs (Charters et al. 1996).

In the present communication, genetic variation among redcurrant genotypes and the fingerprinting of individual varieties using anchored microsatellites is reported. The genotypes chosen reflect a broad geographical and genetic base and include the progenitor species, *Ribes petraeum*, *R. sativum* and *R. multiflorum*. The genetic relatedness of different cultivars to each other and to the wild species is also discussed.

Materials and methods

Redcurrant germplasm was obtained from the Scottish Crop Research Institute *Ribes* germplasm collection, and included 13 named

Communicated by P. M. A. Tigerstedt

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cultivars of *R. rubrum* and one accession each of *R. petraeum*, *R. multiflorum* and *R. sativum* (Table 1).

DNA was extracted by a modified protein-precipitation method (Milligan 1992). Young leaves were harvested and 1-gram amounts were ground in liquid N_2 in a mortar and pestle, then 6 ml of extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM ethylene-diamine-tetra-acetic acid, pH 8.0; 500 mM NaCl; 2% sodium dodecyl sulphate,; 1% polyvinylpyrollidone-360; and 10 mM dithiothreitol) were added, the mixture transferred to a 15-ml centrifuge tube and incubated at 65°C for 20 min. Two microliters of 5 M potassium acetate were then added followed by vigorous mixing and incubation on ice for 5 min. Centrifugation at 4500 g was carried out for 20 min. DNA was pelleted from the supernatant by the addition of 4 ml propanol-2-ol, overnight incubation at −20°C, and centrifugation at 4,500 g for 15 min. Pellets were re-dissolved in 220 µl sterile distilled water and treated with RNAse (10 µg) for 20 min at 65°C. The following components were added: potassium acetate, pH 5.5, final concentration 50 mM; Na_2EDTA, final concentration 10 mM; and Caylase (Cayla, Toulouse, France), final concentration 0.5 mg/ml. Then overnight incubation was carried out at 37°C. One chloroform extraction was performed, and the DNA further purified by the addition of 75 µl of 3 M sodium acetate, pH 7.6, 500 µl of ice-cold propan-2-ol and overnight precipitation at −20°C.

An initial screening for useful primers was completed using DNA from 'Rolan', 'Rotet', 'Rovada' and 'White Pearl'. All primers were supplied by UBC (University of British Colombia) and were from set #9. Polymerase chain reaction (PCR) conditions were as follows: 50 ng of target DNA; 300 µM of primer; 200 nM each of dATP, dCTP, dGTP, dTTP; 1 × *Taq* polymerase buffer (HT-Biotechnologies) and 0.4 u of Super*Taq* (HT-Biotechnologies). Final reaction volumes were 25 µl. All reactions were overlaid with mineral oil and subjected to 30 rounds of the following amplification cycle: 94°C for 1 min, 55°C for 2 min, 72°C for 1 min. A final incubation at 72°C for 5 min was also included. PCR products were separated on 1.5% agarose gels buffered with 0.5 × TBE (44.5 mM Tris; 44.5 mM boric acid; 1 mM ethylene-diamine-tetra-acetic acid), stained with Ethidium bromide and visualised by illumination with UV light (302 nm). A total of 62 primers were tested and those giving the greatest number of amplification products were chosen for further use.

Fingerprinting was carried out using the same PCR conditions as above with the exception that the final reaction volumes were 10 µl and the primers were end-labelled with ^{33}P gamma-ATP (111 TBq/mmol, Amersham) by incubation with T4 kinase (Boehringer Mannheim) in a 1 × kinase buffer (supplied by the manufacturer) at 37°C for 1 h. PCR products were separated on 6% polyacrylamide denaturing gels and detected using standard autoradiography.

Scoring was on the basis of the presence or absence of bands. Results were analysed by the 'Bandmap' program (Powell et al. 1991; Lanham et al. 1995) using the index proposed by Nei and Li (1979) to calculate similarities, S_{ij}, between cultivars i and j:

$$S_{ij} = 2N_{ij}/(N_i + N_j),$$

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. The similarities were used to cluster the genotypes using average linkage cluster analysis. All statistical calculations were carried out using Genstat 5.3.2.

Table 1 *Ribes* germplasm which was fingerprinted using anchored microsatellites

Species		Origin
R. rubrum	*Redcurrant*	
	'Fay's Prolific'	New York, USA
	'Laxton's Perfection'	The Netherlands
	'Red Lake'	Minnesota, USA
	'Redstart'	UK
	'Rolan'	The Netherlands
	'Rotet'	The Netherlands
	'Rovada'	The Netherlands
	'Versailles'	France
	'Jonkheer van Tets'	The Netherlands
	'Augustus'	The Netherlands
	Whitecurrant	
	'Werdavia'	Germany
	'White Pearl'	The Netherlands
	'Zitavia'	Germany
R. petraeum	R7-4-2	Western/Central Europe
R. multiflorum	R7-6-3	Western Europe
R. sativum	R7-3-3	Eastern Europe

Results

The results of the initial screening produced 13 primers all of which efficiently amplified redcurrant DNA. Three were chosen for further study (Table 2). A total of 80 polymorphisms were detected in the redcurrant germplasm; examples of polymorphic bands are given in Fig. 1. The sizes of bands ranged from 100 bp to 2.0 kb. The primers varied in the number of polymorphisms identified, with 891 detecting the greatest number, 32 (Table 3). However, 816 amplified fewer loci but detected proportionately more polymorphism, 51% compared to 47% and 38% for 888 and 891 respectively. All the redcurrant genotypes were successfully fingerprinted (Fig. 2, Table 4). Each of the three primers alone detected enough polymorphism to generate individual profiles for all genotypes (data not shown).

The similarity matrix generated by the 80 polymorphisms analysed ranged from 13% ('White Pearl'/*R. multiflorum*) to 90% ('Red Lake'/'Laxton's Perfection'; 'Jonkheer van Tets'/'Laxton's Perfection'). As might be expected, *R. multiflorum*, which has been introduced comparatively recently into redcurrant breeding programmes, had the most unique profile, its highest similarities being 51% ('Rovada') and 50% ('Augustus'). The range of similarities among the cultivars varied from 61% ('Rovada'/'Redstart'; 'White Pearl'/'Rotet') to 90% ('Red Lake'/'Laxton's Perfection'; 'Jonkheer van Tets'/'Laxton's Perfection').

Cluster analysis of the data divided the genotypes into four groups (Fig. 2). The first contains 'Fay's Prolific', 'Versailles', 'Laxton's Perfection', 'Jonkheer van Tets', 'Red Lake', and 'Redstart'. These genotypes join together closely at the 81% level yet come from diverse

Table 2 Primers which were used in polymerase chain reactions. B = C or G or T; D = A or G or T; H = A or C or T; V = A or C or G

Primer	Sequence
816	5′ CAC ACA CAC ACA CAC AT 3′
888	5′ BDB CAC ACA CAC ACA CA 3′
891	5′ HVH TGT GTG TGT GTG TG 3′

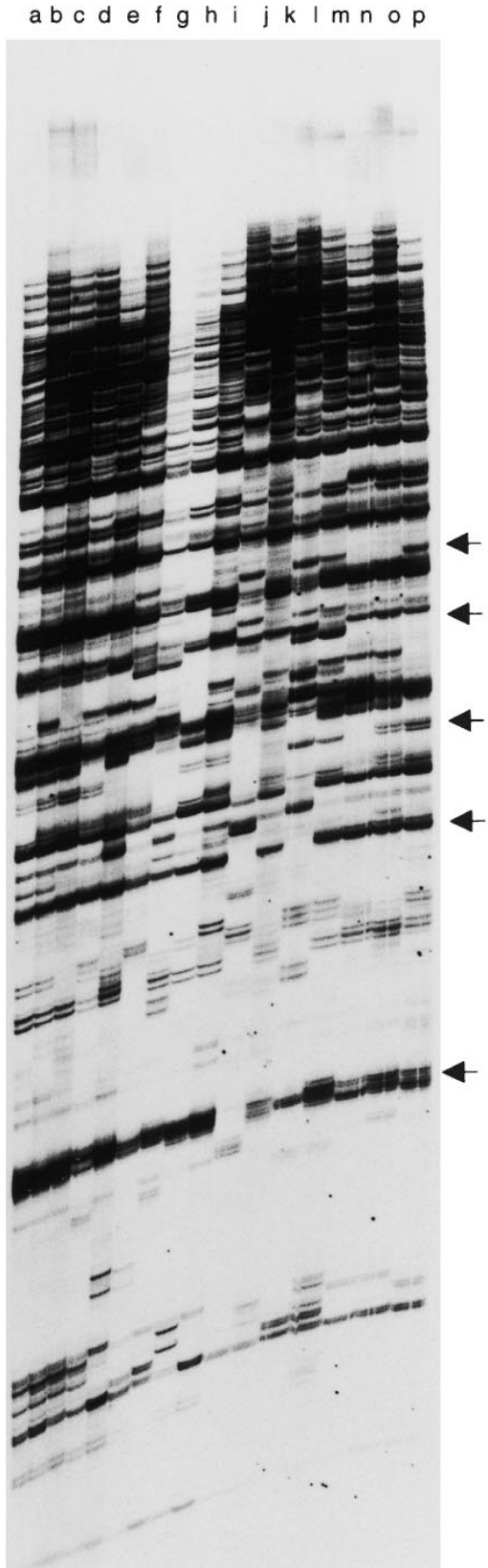

Fig. 1 Polymerase chain reaction products from redcurrant DNA amplified with primer 891. Some of the polymorphisms detected by this primer are indicated by the *arrows* on the right. The genotypes are run on the lanes as follows: *a* 'Fay's Prolific'; *b* 'Laxton's Perfection'; *c* 'Red Lake'; *d* 'Redstart'; *e* 'Rolan'; *f* 'Rotet'; *g*, 'Rovada'; *h*, 'Versailles'; *i*, 'Jonkheer van Tets'; *j R. petraeum*; *k R. sativum*; *l R. multiflorum*; *m* 'Augustus'; *n* 'Werdavia'; *o* 'White Pearl'; *p* 'Zitavia'

Table 3 The number of bands and polymorphisms detected by each of the anchored microsatellite primers

Primer	816	888	891	Total
Number of bands detected	39	60	85	184
No. of polymorphisms detected	20	28	32	80
% Of bands which were polymorphic	51	47	38	43

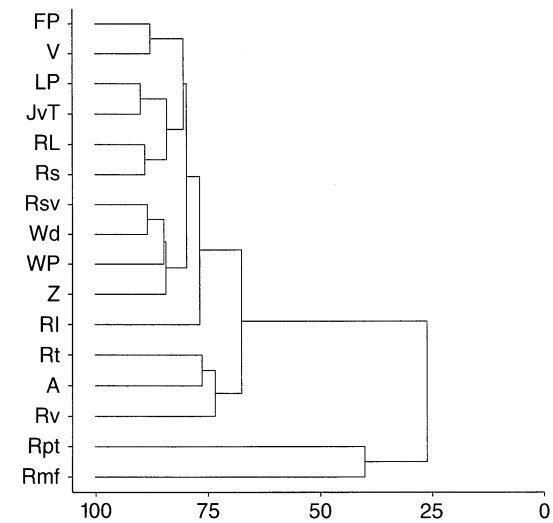

Fig. 2 Dendrogram of the redcurrant genotypes based on the Nei-Li estimate of similarity. *FP* 'Fay's Prolific'; *LP* 'Laxton's Perfection'; *RL* 'Red Lake'; *Rs* 'Redstart'; '*Rl* Rolan'; *Rt* 'Rotet'; *Rv* 'Rovada'; *V* 'Versailles'; *JvT* 'Jonkheer van Tets'; *Rpt R. petraeum*; *Rsv R. sativum*; *Rmf R. multiflorum*; *A* 'Augustus'; *WP* 'White Pearl'; *Wd* 'Werdavia'; *Z* 'Zitavia'

genetic backgrounds: 'Jonkheer van Tets' is a derivative of 'Fay's Prolific', 'Red Lake' is from unknown parentage, and 'Redstart is from the cross 'Red Lake' × [*R. multiflorum* × *R. sativum*]. In addition, their geographical origins are also diverse [New York (USA), Minnesota (USA), France, The Netherlands, and the UK; Table 1].

The second group consists of the three whitecurrant varieties, 'Werdavia', 'White Pearl', and 'Zitavia' along with *R. sativum*. The cultivar 'Rolan', a derivative from 'Heinnemann's Rote Spatlese' which has *R. multiflorum* in its parentage, was ungrouped but had the greatest affinity with groups 1 and 2 at the 80% level. The cultivar 'Rovada' is of similar derivation to 'Rolan'.

The third group is composed of three cultivars from the Netherlands 'Augustus', 'Rotet' and 'Rovada'. The latter two cultivars are derivatives of 'Heinnemann's Rote Spatlese'.

Table 4 Similarity matrix based on the Nei-Li estimate of similarity. FP 'Fay's Prolific'; LP 'Laxton's Perfection'; RL 'Red Lake'; Rs 'Redstart'; 'Rl Rolan'; Rt 'Rotet'; Rv 'Rovada'; V 'Versailles'; JvT 'Jonkheer van Tets'; Rpt *R. petraeum*; Rsv *R. sativum*; Rmf *R. multiflorum*; A 'Augustus'; WP 'White Pearl'; Wd 'Werdavia'; Z 'Zitavia'

FP	–															
LP	86	–														
RL	87	90	–													
Rs	79	85	89	–												
Rl	77	77	74	68	–											
Rt	67	72	63	62	71	–										
Rv	70	68	64	61	70	74	–									
V	88	77	79	70	78	66	74	–								
JvT	84	90	84	77	80	77	68	80	–							
Rpt	23	19	18	24	21	29	37	22	14	–						
Rsv	81	86	81	77	82	70	68	83	82	22	–					
Rmf	26	20	22	24	21	45	51	28	21	40	17	–				
A	69	67	68	70	62	76	72	68	69	32	65	50	–			
WP	73	82	79	81	78	61	59	72	79	21	85	13	64	–		
Wd	80	80	74	73	81	69	67	82	78	31	88	25	69	84	–	
Z	83	80	83	82	71	66	69	82	78	30	83	26	71	84	85	–
	FP	LP	RL	Rs	Rl	Rt	Rv	V	JvT	Rpt	Rsv	Rmf	A	WP	Wd	Z

The fourth group contains *R. petraeum* and *R. multiflorum* which join together at the 40% level only.

Discussion

Molecular markers have been used to characterise germplasm from a range of plant species (Ayad et al. 1997). In the present report the characterization of redcurrant germplasm using anchored microsatellite markers is described.

The cluster analysis of the data revealed that all the cultivated varieties grouped together at the 68% level (Fig. 2). It is noteworthy that group 1 from the cluster analysis includes genotypes which have come from very different geographic origins, implying that there is little variation between American and European germplasm at the molecular level.

Although whitecurrants are a colour variant of redcurrant, rather than a separate taxonomic group, all three whitecurrant varieties grouped tightly together in this study. These three genotypes are of varying origins, however; 'White Pearl' is a cultivar of unknown provenance introduced into Belgium in about 1850. 'Zitavia' (origin unknown) and 'Werdavia' (a selection from the old variety 'White Dutch', first described over 200 years ago) both originate from Germany, and were introduced commercially in 1976. All three, in addition, show varying agronomic characteristics (Brennan, unpublished).

The analysis of the progenitor species *R. sativum* and *R. petraeum* gave very different results, with *R. sativum* being closely clustered among the cultivars, especially the whitecurrants, and *R. petraeum* having little in common with the cultivated varieties (Fig. 2). This is also evident in the similarity matrix where the highest similarity that *R. petraeum* has with a cultivar is with 'Rovada' (37% only), a derivative of *R. multiflorum*. This indicates that *R. sativum* has been utilized far more than *R. petraeum* in the development of redcurrant germplasm worldwide. Available evidence suggests that *R. petraeum* was used in some of the oldest cultivars, such as 'Gonduin' (Keep 1975), but its use in more recent times has become restricted. However, there is clear potential for its future use as a backcross parent for specific characters, since it is a very variable species in terms of fruit colour and distribution, occupying many mountainous habitats in Europe, Siberia and North Africa (Keep 1995).

R. multiflorum material has not to date been integrated into redcurrant germplasm to the same degree as the other species mentioned, although its use in breeding offers considerable potential (Brennan 1996).

The differences between *R. petraeum*, *R. multiflorum* and the rest of the redcurrant germplasm (Fig. 2, Table 4) indicate that they are valuable sources of genetic variation for potential incorporation into redcurrant breeding programmes in the future. Other species, such as *R. longeracemosum*, still await widespread use in redcurrant breeding, although a few initial hybrids have been developed (Keep 1995).

There is a paucity of cloned sequences from *Ribes* species in genebank databases; therefore the development of single-locus microsatellite markers for redcurrant germplasm will require library screening, cloning, sequencing and PCR primer design and evaluation. Such procedures would necessarily be labour intensive and time consuming. The development of anchored microsatellite markers (Wu et al. 1994; Zietkiewicz et al. 1994), which do not require prior sequence information, potentially offers a very useful alternative. Other plant species, e.g. *Brassica napus* (Charters et al. 1996), *Poncirus trifoliata* (Fang et al. 1997), and *Citrus* spp. (Fang and Roose 1997), have been fingerprinted

with these markers. The redcurrant germplasm utilised here was readily fingerprinted using anchored microsatellites, with any of the three primers used being sufficient to generate fingerprints of all the genotypes. In all the three primers generated a total of 80 markers. This is in contrast to the use of RAPDs to fingerprint *R. nigrum* L. genotypes, where a total of 23 primers was required to amplify 54 markers (Lanham et al. 1995).

The anchored microsatellite primers used in this study all targeted $(CA)_n$ repeats. Although $(CA)_n$ and $(TG)_n$ repeats are complementary to each other, the 5′-anchor ensures that primers 888 and 889 amplify different target loci. Primer 816 which has only a single base anchor at the 3′-end also proved to be useful in detecting polymorphism. The variation identified in this study indicates that $(CA)_n$ motifs are common in the redcurrant genome and, if single-locus microsatellites were to be developed, $(CA)_n$ repeats should provide a useful source of markers.

In conclusion, redcurrant germplasm was characterised using anchored microsatellite markers. The markers proved efficient in fingerprinting the germplasm and in a subsequent analysis of the genetic relatedness of the various genotypes.

Acknowledgements The authors thank Mr. J. McNicol, Biomathematics and Statistics Scotland, Scottish Crop Research Institute, for the use of the 'Bandmap' program and L. Ross, Scottish Crop Research Institute, for technical assistance. Funding from the Scottish Office Agriculture, Environment and Fisheries Department is also gratefully acknowledged.

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