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An examination of the ability of RAPD markers to determine the relationships within and between *Rubus* species

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Abstract RAPD markers were generated from 13 different *Rubus* species in order to assess the degree of similarity between species from the important subgenera. All ten primers revealed scorable polymorphisms within both the closely related and the genetically diverse individuals. Three hundred and seventy-two markers were generated and scored from the material analysed. Estimates of similarity, dendrograms and principle co-ordinate analysis were calculated, with the results generally being in agreement with previous classifications of the species studied, confirming the validity and usefulness of the RAPD method. However, amongst the species studied, *R. macraei* of the *Idaeobats* proved more diverse and grouped in with both the *Idaeobats* and *Eubats* at only 26% similarity.

Key words *Rubus* · Random primer PCR · Genetic diversity

Introduction

The genus *Rubus* is one of the most diverse in the plant kingdom, comprising a highly heterozygous series of some 500 species with a ploidy range from diploid to dodecaploid (Jennings 1988). Few plant genera are as confused as to nomenclature and identity (Ourecky 1975).

The genus has been subdivided into 12 subgenera of which only a few have been domesticated. Of the domesticated subgenera, 1 contains the raspberries, another the blackberries, 2 contain the Arctic fruits and 1 contains the flowering raspberries that have been used in breeding programmes.

Further studies may well show that some of the subgenera contain hybrids between species of diverse subgenera. The *Idaeobatus* subgenus containing the raspberries has a northerly distribution, principally Asia, Africa, Europe and North America. It has some 200 species showing considerable differentiation, of which the most important are the European red raspberry (*R. idaeus* subsp. *vulgatus* Arrhen) designated *R. idaeus*, the North American red raspberry (*R. idaeus* subsp. *strigosus* Michx) designated *R. strigosus* and the black raspberry (*R. occidentalis* L.). The subgenus *Eubatus* is extremely variable and complex. It contains all the blackberries and dewberries and has several sections in South America, a very prominent one in Europe and another in North America. Thousands of taxonomic units have been given specific rank, and it is often not possible to assign cultivars to individual species. The *Anoplobatus* contain six species of flowering raspberries.

The development of molecular biology has resulted in DNA based marker procedures that should lead to a greater understanding of relationships between species, and more accurate taxonomic classification. These techniques should also lead to a more effective understanding and utilisation of genetic diversity by the breeder and an ability to identify species and cultivars by means other than morphological characteristics.

The accurate classification of species and cultivars based on morphological characters can be difficult, and fingerprinting techniques based on paper chromatography (Haskell and Garrie 1966) and isozyme techniques (Cousineau and Donnelly 1989) have proved unsuccessful. Chloroplast DNA probes have also been unable to detect variation between raspberry cultivars (Waugh et al. 1990), and minisatellite DNA and other oligonucleotides used as probes (Nybom et al. 1990; Parent and Page 1992) have proved to be time consuming and require the use of radioisotopes. Recently, DNA fingerprinting of ten closely related red raspberry cultivars has been achieved (Graham et al. 1994) using a novel technique, reported by both Welsh and McClelland (1990) and by Williams et al. (1990), based on the amplification of random DNA sequences by the polymerase chain reaction (PCR) with arbitrary primers. In this study each raspberry cultivar could be conclusively identified using a minimum of three arbitrary primers. As

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random primers and PCR could differentiate between closely related cultivars, it was decided to use this form of molecular markers to determine if random amplified polymorphic DNA (RAPDs) could estimate relatedness between species and construct groups between and within species. This initial small-scale study was carried out on a number of *Rubus* accessions from 3 of the subgenera commonly used in breeding and hybrids between two species.

Materials and methods

Plant material

Twenty-four different accessions belonging to 13 species from the *Idaeobats*, *Eubats* and *Anoplobats* and two hybrids between the *Idaeobats* and *Eubats* were studied (Table 1).

DNA extraction technique

From each species, 1 g leaf material from a number of young, i.e. 'just unfolded', glasshouse-grown plants was ground in liquid nitrogen. Hexadecyltrimethylammoniumbromide (CTAB) solution (5 ml) was added and incubated at 65 °C for 30 min prior to the addition of 4 ml chloroform/isoamyl alcohol. The mixture was agitated for 15 min followed by a spin of 6 min at 5000 *g*. The aqueous layer was filtered through sterile muslin, and an equal volume of ice cold propan-2–o l was added, mixed and incubated at room temperature for 15 min to precipitate the DNA. The DNA was either hooked out, or pelleted by spinning at 500 *g* for 10 min and resuspended in 1 ml TRIS-EDTA buffer. Rnase 5–10 µl was added to the DNA, which was then incubated at 37 °C for 1 h, and stored at –70 °C until required.

Table 1 *Rubus* genotypes studied

Genotype	Subgenus
1. *R. deliciosus*	*Anoplobatus*
2. *R. leucodermis*	*Idaeobatus*
3. *R. distractiformis*	*Eubatus*
4. *R. occidentalis*	*Idaeobatus*
5. *R. gelertii*	*Eubatus*
6. *R. caesius*	*Eubatus*
7. *R. fruticosus* L. agg cv. 'Loch Ness'	*Eubatus*
8. *R. coreanus*	*Idaeobatus*
9. *R. macraei*	*Idaeobatus*
10. *R. nepalensis*	*Idaeobatus*
11. *R. occidentalis* cv. 'Black River'	*Idaeobatus*
12. *R. idaeus* × *R. strigosus* cv. 'Golden Queen'	*Idaeobatus*
13. *R. loganobaccus* cv. 'Tayberry'	*Eubatus* × *Idaeobatus*
14. *R. loganobaccus* cv. 'Sunberry'	*Eubatus* × *Idaeobatus*
15. *R. idaeus* cv. 'Williamette'	*Idaeobatus*
16. *R. idaeus* cv. 'Spinefree Williamette'	*Idaeobatus*
17. *R. idaeus* cv. 'Glen Moy'	*Idaeobatus*
18. *R. idaeus* cv. 'Glen Clova'	*Idaeobatus*
19. *R. strigosus* cv. 'Latham'	*Idaeobatus*
20. *R. idaeus* cv. 'Heritage'	*Idaeobatus*
21. *R. idaeus* cv. 'Malling Jewel'	*Idaeobatus*
22. *R. idaeus* cv. 'Glen Garry'	*Idaeobatus*
23. *R. idaeus* cv. 'Glen Prosen'	*Idaeobatus*
24. *R. idaeus* cv. 'Malling Delight'	*Idaeobatus*

DNA amplification (RAPD)

For PCR reactions the DNA was precipitated with 5×volume of 100% ethanol, and the DNA hooked out and dried under vacuum and resuspended in 200 µl TRIS-EDTA buffer. PCR reactions were carried out in 50-l volumes containing per reaction, 40 ng genomic DNA, 5 µl dNTP solution (2 m*M*), 5 µl primer (2 M), 5 µl of 10 Taq buffer and 0.08 µl Taq DNA polymerase (5 U/µl). Each reaction was overlaid with 40 µl mineral oil to prevent evaporation. PCR reactions were carried out in a Hybaid OmniGene thermal cycler (Middlesex, England) under the following programme conditions: 45 cycles at 92 °C for 1 min, 35 °C for 3 min and 72 °C for 2 min, followed by one cycle at 72 °C for 5 min. Random primers were obtained from Pharmacia. Amplification products were resolved on a 1.5% agarose gel run in 0.5×TBE buffer, stained with ethidium bromide and visualised by illumination with ultraviolet radiation (312 nm).

Data analysis

Estimates of similarity, dendrograms and principle co-ordinate analysis were calculated by the method of Nei and Li (1979) and Powell et al. (1991) using the Genstat 5 statistical package.

Results and discussion

All PCR reactions were carried out in triplicate, and the markers generated in all three reactions were analysed. From the ten arbitrary primers, used (Table 2), 372 RAPD amplification products (markers) were stably generated (Fig. 1), all of which were polymorphic, reflecting the genetic diversity in *Rubus*, and were therefore included in the analysis. The data were analysed as a whole and also in groups reflecting the subgenus to which they belong. The similarity matrix (Fig. 2), dendrogram (Fig. 3) and principle co-ordinate analysis (Fig. 4) obtained from analysis of the whole data set are shown.

Two red raspberry cultivars, (*R. idaeus*) 'Williamette' and 'Spinefree (Sf) Williamette', were included in the study to determine what degree of similarity RAPDs would show. As the only difference between the two cultivars is a mutation in a single gene, it was regarded by the authors as being unlikely that RAPDs would be able to detect this single mutation, hence 100% similarity was expected. As anticipated, 'Williamette' and 'Sf Williamette' generated identical markers.

Table 2 Random primers used for the detection of polymorphism in *Rubus*

Primers	No. markers generated
GGTAGCAGTC	20
GGTCCTCAGG	40
CAGTTCGAGG	45
TACCGACACC	37
TCGGAGTGGC	23
ACTCAGGAGC	49
CCACCGCCAG	36
AGAGATGCCC	29
AGCCAGCGAA	42
AATCGGGCTG	51

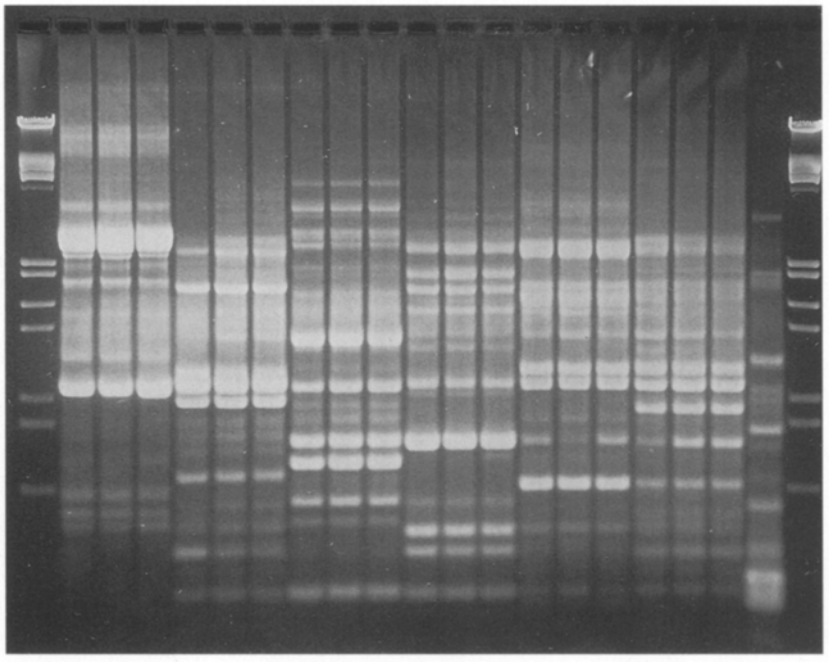

Fig. 1 Products amplified from *Rubus* DNA. *Lane 1* Mr standards, *2–4* Loch Ness, *5–7 R. coreanus*, *8–10 R. macraeii*, *11–13 R. nepalensis*, *14–16* Black River, *17–19* Golden Queen, *20* non-specific priming, *21* Mr standards

	R. deliciosus	*R. distractiformis*	Loch Ness	*R. gelertii*	*R. caesius*	*R. occidentalis*	*R. leucodermis*	Tayberry	Sunberry	Williamette	SF Williamette	Heritage	Glen Moy	Glen Clova	Glen Garry	Glen Prosen	Malling Delight	Malling Jewel	Latham	Black river	Golden Queen	*R. coreanus*	*R. nepalensis*	*R. macraeii*
R. deliciosus	100																						
R. distractiformis	22	100																					
Loch Ness	26	39	100																				
R. gelertii	23	30	28	100																			
R. caesius	26	34	37	44	100																		
R. occidentalis	26	22	19	45	37	100																	
R. leucodermis	25	27	28	42	35	50	100																
Tayberry	22	33	31	35	27	34	42	100															
Sunberry	23	33	25	30	29	24	28	57	100														
Williamette	28	25	32	37	31	41	44	59	40	100													
SF Williamette	28	25	32	37	31	41	46	59	40	100	100												
Heritage	25	26	25	35	32	36	41	56	40	76	76	100											
Glen Moy	23	23	27	40	30	39	42	60	43	68	68	62	100										
Glen Clova	28	27	30	44	36	44	48	57	41	69	69	66	83	100									
Glen Garry	25	29	31	34	30	41	48	58	48	76	76	68	69	73	100								
Glen Prosen	27	31	30	41	33	44	48	58	54	71	71	64	73	70	84	100							
Malling Delight	23	29	25	37	31	39	43	59	47	68	68	69	71	76	83	79	100						
Malling Jewel	27	26	31	38	30	42	44	60	51	74	74	69	72	68	75	81	72	100					
Latham	29	25	26	40	34	42	43	56	43	57	57	63	53	60	58	60	56	58	100				
Black river	20	27	28	37	35	41	34	46	37	50	50	42	40	41	48	47	44	47	42	100			
Golden Queen	26	28	24	40	34	34	34	38	35	40	40	43	33	35	37	43	39	39	41	61	100		
R. coreanus	20	25	23	35	30	34	36	42	35	37	37	39	37	42	37	37	39	37	34	48	41	100	
R. nepalensis	23	27	20	34	27	31	33	28	26	34	34	35	28	28	32	36	30	37	33	37	41	42	100
R. macraeii	22	17	22	32	27	30	32	22	14	28	28	28	28	32	20	25	26	26	29	30	33	24	29	100

Fig. 2 Similarity matrix based on 372 polymorphic markers

This study included species from the 2 most commercially important subgenera, the *Idaeobats* and *Eubats,* as well as 1 species from the *Anoplobats,* the flowering raspberries. The RAPD data correctly separated the species into the 3 subgenera with the possible exception of *R. macraei*, the rare tropical species (Gardner 1983). *R. macraei* has been placed in the *Idaeobatus* subgenus, but on the basis of the RAPD data it has only 26% similarity to other species within the *Idaeobats* and a 26% similarity to species in the *Eubats*.

Included from the *Idaeobats* were a number of red raspberry cultivars from the European and North American types, which due to morphological and geographical dis-

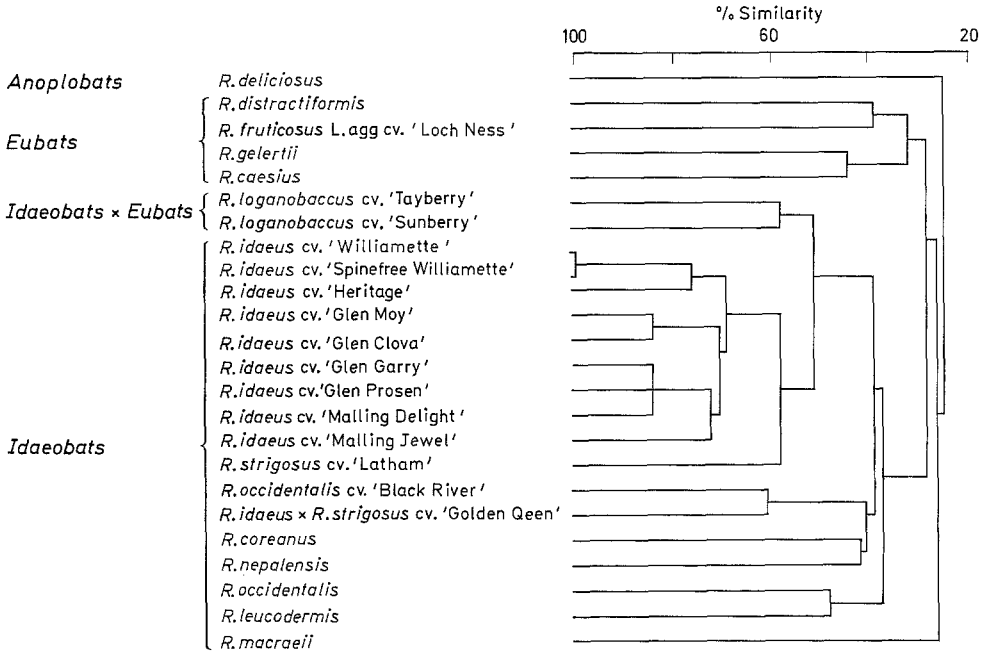

Fig. 3 Dendogram of *Rubus* species based on 372 polymorphic markers

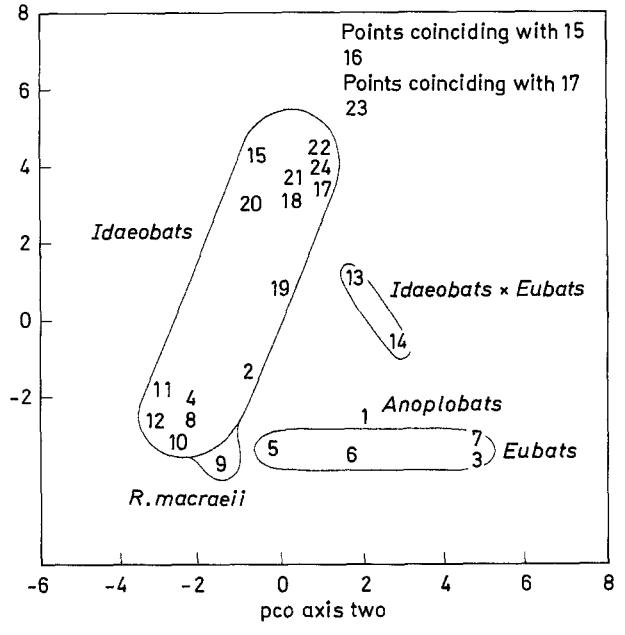

Fig. 4 Principle co-ordinate analysis of the 24 *Rubus* genotypes analysed using 372 polymorphic markers

tribution are regarded as subspecies and known as *R. idaeus* (European) and *R. strigosus* (N. American). Within this group, as anticipated, and as shown in the dendrogram and other data, the *R. idaeus* cultivars, 'Williamette Spinefree', 'Williamette', 'Heritage', 'Glen Moy', 'Glen Clova', 'Glen Garry', 'Glen Prosen', 'Malling Delight' and 'Malling Jewel', were very closely related, having common founding clones. 'Latham' was the most distinct of the red raspberry cultivars in this grouping, being the only *R. strigosus* type.

Also included were 2 wild black raspberries, *R. occidentalis*, a species only indigenous to North America, and *R. leucodermis*, similar to *R. occidentalis*, but which only occurs from British Columbia to California. *R. occidentalis* and *R. leucodermis*, with their slightly differing geographical locations, grouped together on the dendrogram with 50% similarity. The dark fruited cultivar 'Black River', thought to be a *R. occidentalis* selection from the wild (R. Daubeny 1981 personal communication), had only 41% similarity with wild *R. occidentalis* and between 41–50% similarity with any of the cultivated red raspberries. It appears to be most closely related to 'Golden Queen' with 61% similarity, suggesting it may be a hybrid between *R. occidentalis* and either *R. strigosus* or *R. idaeus* × *R. strigosus*. 'Golden Queen' originated as a bud sport from the old cultivar 'Cuthbert', a *R. idaeus* × *R. strigosus* cross (Ourecky 1975).

R. coreanus, a Korean species closely related to the red raspberry and which has been used in breeding both red and black raspberries (Jennings 1978), was also included, as was *R. nepalensis*, which was collected wild in Nepal. *R. coreanus* and *R. nepalensis* grouped together at 42%, similarity and with the other *Idaeobats* at approximately 40% similarity. One species of tropical raspberry or Akala berry, *R. macreaei* from Hawaii, completed the study of the *Idaeobats*. This species appears to be much more diverse than any of the other *Idaeobats* and is as similar to the *Idaeobats* as it is to the *Eubats*.

Four *Eubats* or blackberries were included in the study. The *Eubats* are a diverse subgenera, and this is reflected in the results, with an overall similarity of approximately 32%. *R. caesius*, which can be distinguished morphologically from other blackberries by some unique characters and some characters more typical of the *Idaeobats*, or the Arctic berry (sungenus *Cylactis*), had a 44% similarity with

R. gelertii. 'Loch Ness' designated *R. fruticosus* L. agg., grouped with *R. distractiformis* at approximately 40% similarity. *R. fruticosus* L. agg. is a species description used to denote blackberries in the *Moriferi* complex (European and eastern parts of North America) that cannot be assigned to any one species.

Two hybrids between red raspberry and blackberry (cvs 'Tayberry' and 'Sunberry'), known as *R. loganobaccus*, completed the study. These cultivars grouped together on the dendrogram with 57% similarity. 'Sunberry' was obtained by crossing a tetraploid mutant of 'Malling Jewel' with *R. ursinus*, and 'Tayberry' was obtained from a cross between the blackberry cv 'Aurora' (*R. macropetalis* × *R. canadensis*) and an unnamed tetraploid raspberry selection of very similar ancestry to 'Malling Jewel'. This explains the level of similarity.

The genetic data generated by RAPDs in this study was in general agreement with existing knowledge of the origins of the germ plasm. However, *R. macraei*, the tropical berry, was more diverse than other *Idaeobats*, and the cultivar 'Black River' thought to be *R. occidentalis* is probably a hybrid between species. RAPDs will therefore provide an exciting and valuable tool for a large-scale study on *Rubus* species, with the advantages of being easy to generate and only requiring small amounts of DNA.

RAPD data, in conjunction with other information on the species and cultivars, can be used to improve taxonomic classifications, determine identity and improve utilisation of genetic resources.

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