Identification of Raspberry Cultivars by Nonradioactive DNA Fingerprinting

Jean-Guy Parent and Daniël Pagé

Service de phytotechnie de Québec (MAPAQ), Complexe scientifique, 2700, rue Einstein, Sainte-Foy, Qué G1P 3W8, Canada

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Abstract. Characterization and identification of 13 red raspberry (*Rubus idaeus* L.) and two purple raspberry (*R. × neglectus* Peck) cultivars were obtained by nonradioactive genetic fingerprinting. DNA from leaves was digested with *Hae* III and *Hinf* I restriction enzymes and probed with alkaline phosphatase-labeled oligonucleotide. All tested cultivars could be identified by a unique band pattern. No differences were noted within cultivars when the reproducibility of the fingerprints was evaluated by analyzing the effects of age of the raspberry plantation, developmental stage during the growing season, or position of the sampled leaf on stem. These results suggest that simple nonradioactive DNA fingerprinting can be routinely used to identify raspberry cultivars.

Up to now, red raspberry and purple raspberry cultivar identification for the production of certified plants has relied mostly on morphological traits. Fruit and flower characteristics have been most commonly used but are often absent, making authentication difficult for plants during vegetative growth, including plants micropropagated in vitro. This lack of a permanent and objective test sometimes leads to cultivar mislabeling (J.C. Cousineau and D.J. Donnelly, personal communication). Raspberry cultivars are propagated by asexual reproduction and hence can be considered to be clones with no or with low levels of genetic variation. This genetic uniformity has allowed the development of simple laboratory tests for cultivar identification based on electrophoretic analysis of isoenzymes (Cousineau and Donnelly, 1989). Unfortunately, despite the analysis of several enzymes, some cultivars cannot be distinguished from one another. These findings preclude the use of isoenzyme analysis as the sole test in certification programs.

Recently, a powerful DNA fingerprinting test, initially described for humans (Jeffreys et al., 1985), was found to be relevant to the analysis of Rosaceae species (Nybom, 1990; Nybom and Hall, 1991; Nybom and Schaal, 1990; Nybom et al., 1989, 1990). The test allowed identification of individuals or clones through the analysis of hypervariable minisatellite DNA present in many *Rubus* spp. Applications included the capacity to distinguish a few raspberry cultivars from each other by using ³²P-labeled probes. Safety

considerations and waste disposal problems associated with the use of radioisotopes impeded the practical use of the test. Our objectives were to demonstrate the ability of the DNA fingerprint test to identify numerous cultivars of red and purple raspberries and to develop a reproducible, simple, and nonradioactive version of this test for routine use.

Plant material. In vitro plantlets were aseptically cultured in Murashige and Skoog medium (1962) supplemented with 1.0 mg 6-benzylaminopurine (BA)/liter, 0.01 mg gibberellic acid (GA₃)/liter, and 175 mg NaH₂PO₄·H₂O/liter (H. Rousseau, unpublished). Outdoor plants used in this study were grown within a perimeter of 50 km of Quebec City. Red raspberry cultivars included 'Anelma', 'Bonanza', 'Boyne', 'Comet', 'Festival', 'Gatineau', 'Heritage', 'Killarney', 'Madawaska', 'Nova', 'Pathfinder', 'Titan', and 'Willamette'; purple raspberry cultivars were 'Brandywine' and 'Royalty'. These are the cultivars acknowl-

edged by the certification program of the province of Quebec. Except where indicated, young but fully expanded leaves were collected and kept on ice during transport to the laboratory. Midribs were removed, and the remaining leaf material was cut in 3- to 5-mm squares, and 1-g portions were frozen at - 80°C until DNA extraction.

DNA isolation and analysis of fingerprints. DNA was extracted and purified according to the hexadecyltrimethylammonium bromide (CTAB) method for lyophilized plant leaves of Saghai-Marroof et al. (1984) with the modifications of Doyle and Doyle (1987) for fresh tissue. To estimate DNA concentration, samples of raspberry DNA were compared with dilutions of known concentrations of salmon sperm DNA after agarose gel electrophoresis and ethidium bromide staining. Levels of contamination by proteins and by polyphenols/carbohydrates were assessed, respectively, using A₂₆₀:A₂₈₀ and A₂₆₀:A₂₃₀ ratios measured by UV spectrophotometry (Logemann et al., 1987). For each fingerprint analysis, two DNA samples were digested with restriction enzymes *Hae* III and *Hinf* I. Seven micrograms of digested DNA was deposited in wells of 0.8% or 1.4% (w/v) agarose gels (20 × 25 cm) and subjected to electrophoresis in a Tris-acetate buffer system (Maniatis et al., 1982). Gels were treated and blotted onto nylon membranes, after which the DNA was W-bound to the membranes (Khandjian, 1987).

Transferred DNA was hybridized with enzyme-labeled DNA probes (SNAP DNA fingerprinting kit, Molecular Biosystems, San Diego). Such probes are alkaline phosphatase-linked, single-stranded, 22-mer oligonucleotides (Edman et al., 1988) corresponding to the core sequence of a human myoglobin minisatellite (Jeffreys et al., 1985). The protocol for chemiluminescent detection recommended by the manufacturer was followed except that LumiPhos 530 (Lumigen, Detroit, Mich.) was used as chemiluminescent substrate. For each gel, a sheet of Kodak X-

Fig. 1. Fingerprints of raspberry cultivars from *Hinf* I fragments separated in 0.8% agarose gel. Letters correspond to 'Anelma' (A), 'Bonanza' (B), 'Boyne' (C), 'Brandywine' (D), 'Comet' (E), 'Festival' (F), 'Gatineau' (G), 'Heritage' (H), 'Killarney' (I), 'Madawaska' (J), 'Nova' (K), 'Pathfinder' (L), 'Royalty' (M), 'Titan' (N), and 'Willamette' (O). Numbers correspond to the position of molecular weight markers ($\times 10^3$ base pairs). Open and closed arrowheads indicate the position of fragments with slightly different lengths (see text for details).

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Table 1. Similarity index values^a for all pairwise comparisons of the raspberry cultivars.

BON ^b	0.60															
BOY	0.44	0.54														
BRA	0.33	0.37	0.33													
COM	0.47	0.51	0.42	0.34												
FES	0.59	0.50	0.40	0.34	0.64											
GAT	0.55	0.46	0.46	0.42	0.63	0.66										
HER	0.48	0.49	0.36	0.38	0.38	0.48	0.42									
KIL	0.36	0.47	0.62	0.41	0.37	0.35	0.40	0.37								
MAD	0.58	0.53	0.45	0.60	0.60	0.62	0.61	0.48	0.42							
NOV	0.56	0.70	0.56	0.38	0.48	0.44	0.45	0.41	0.50	0.52						
PAT	0.34	0.42	0.38	0.34	0.38	0.34	0.44	0.43	0.47	0.40	0.38					
ROY	0.54	0.44	0.38	0.52	0.49	0.54	0.56	0.38	0.39	0.61	0.58	0.32				
TIT	0.61	0.54	0.46	0.40	0.56	0.64	0.65	0.43	0.38	0.67	0.51	0.36	0.61			
WIL	0.67	0.49	0.46	0.31	0.52	0.50	0.53	0.33	0.28	0.51	0.53	0.24	0.54	0.66		
AVE	0.51	0.50	0.45	0.39	0.49	0.50	0.52	0.42	0.41	0.54	0.50	0.37	0.49	0.53	0.47	
ANE		BON	BOY	BRA	COM	FES	GAT	HER	KIL	MAD	NOV	PAT	ROY	TIT	WIL	

^aValues correspond to the mean of two separate experiments with 0.8% or 1.4% agarose gels. Results from *Hae* III and *Hinf* I fingerprints were combined in each experiment.

^bNames of cultivars are abbreviated by their first three letters. AVE = average index value.

Fig. 2. Fingerprints of raspberry cultivars from *Hae* III fragments separated in 0.8% agarose gel. Letters and numbers are the same as for Fig. 1

Omat SB-5 was exposed to emitted light during 5 h at room temperature before photographic development. When some bands or patterns were too faint, another film was immediately exposed for a longer duration (maximum of 2 days). Standards (1 kb DNA Ladder, Gibco BRL, Gaithersburg, Md.) were included in the outer two lanes of all gels to allow determination of molecular weight and correspondence between gels.

Cultivar comparisons. Fingerprints of the raspberry cultivars were obtained from plants of a demonstration plantation at the Saint-Augustin Research Station, Que. (Figs. 1 and 2). Two separate experiments were performed to obtain the best electrophoretic separation for different ranges of DNA molecular weights, one with 0.8% agarose gels and the other with 1.4% gels. We analyzed 40 bands from patterns of *Hae* III fragments of 500 to 3000 base pairs (bp) (with an average of 12.4 bands for each cultivar) and 24 bands from patterns of *Hinf* I fragments with a range of 1000 to 6000 base pairs (average of 9.7 bands). Smaller fragments could probably also reveal polymorphism but were not resolved well enough in our gel system to allow adequate analysis. Bands representing larger fragments were sometimes observed; these correspond to DNA fragments with restriction sites that are resistant to

digestion and must not be considered of diagnostic value. We used only bands that were not present in all cultivars for the analysis. To compare banding patterns between cultivars, two sets of gels were used, one with an alphabetical arrangement of the cultivars and the other with a random disposition. Thus, four gels, of 0.8% and 1.4% agarose with two cultivar arrangements, were used to determine similar and dissimilar bands between cultivars. Banding patterns between cultivars varied greatly, but similar and dissimilar bands could be distinguished when all four gels were compared. For example, in Fig. 1, a 2.5-kbp band (marked with closed arrowheads) was present in patterns of cultivars in lanes B, D, G, I, J, L, and M, whereas a band of slightly lower molecular weight (open arrowheads) was present in lanes A and N. The chance occurrence that any two cultivars shared an identical fingerprint was calculated to be a maximum of 2.6×10^{-3} using the method of Nybom et al. (1989) and a single gel/enzyme combination. Thus, it is highly improbable that two cultivars would have the same banding pattern, especially since two experiments involving two restriction enzymes were performed. However, chance occurrence values depend on the number of bands analyzed and on the relationship of the cultivars used in the determination. Even if

the probability of having two indistinguishable cultivars is low, it must not be completely rejected because the genetic base of cultivated raspberry is narrow (Dale et al., 1989).

Our fingerprint patterns were different from, and could not be compared with, those previously obtained for raspberry (Nybom and Hall, 1991; Nybom and Schaal, 1990; Nybom et al., 1989), as we did not use the same probe (M13 probe vs. myoglobin minisatellite DNA). However, DNA restriction patterns of ornamental plants obtained with *Hinf* I had useful bands of 2000 to 5000 base pairs when the same type of probe as ours was used (Tzuri et al., 1991).

Similarity index values (Nybom et al., 1989) were calculated for each pair of cultivars by combining results from *Hae* III and *Hinf* I fingerprints. These index values are for comparative purposes, as their results depend on the number of chosen bands, the number of cultivars, resolution of the gels, etc. Every cultivar had its own specific fingerprints and the resulting index values ranged from 0.24 to 0.70, with a mean of 0.47 (Table 1). Values could often be related to the degree of interrelatedness as deduced from pedigrees as observed for some *Rubus* cultivars (Nybom and Hall, 1991; Nybom et al., 1989). For example, 'Killarney' has low similarity index values with all cultivars except 'Boyne', which originated from a 'Chief' $\times$ 'Indian Summer' cross, like 'Killarney'. In addition, pairs of cultivars, like 'Titan' and 'Royalty', that could not be separated by isoenzyme analysis (Cousineau and Donnelly, 1989) were resolved effectively by analysis of their fingerprints. However, a high index value was not always related to close pedigrees. The purple raspberry 'Brandywine' ('NY631' $\times$ 'Hilton') and the red raspberry 'Madawaska' ('Lloyd George' $\times$ 'Newman 23') have distinct parentage but share a similarity index value of 0.60, especially high for 'Brandywine' (average of 0.39).

Average index values (Table 1) indicate the degree of relationship of one cultivar with others. Most cultivars have similar average index values (LSD: 0.058; *P*: 0.10), except 'Brandywine', 'Pathfinder', 'Heritage', and

'Killarney', which have lower values and could be considered less related to the others. The three former cultivars are different, as they are, respectively, a purple raspberry and two primocane-fruiting red raspberries. Genetic divergence could result from these characteristics, although the purple raspberry 'Royalty' does not have a low index value. For 'Killarney', a comparison with isoenzyme analysis of Cousineau and Donnelly (1989) was helpful. This cultivar had its own patterns for two out of five enzymes, reflecting its genetic distance as calculated from our fingerprints. Similar relations were observed for 'Brandywine' purple raspberry.

Fingerprint reproducibility. Fingerprint reproducibility was first checked with plants cultivated from the same original plant stock but differing in the duration of cultivation. In vitro plantlets, plants from the certification program (Foundation and Certified) and certified plants grown by a commercial grower for up to 6 years, represented a range of 11 years of culture. Seven of the cultivars were checked, and we could not find any fingerprint difference within cultivar. Fingerprints of all cultivars were obtained from leaves collected at three periods of the growing season (28 May and 10 and 29 July). Again, we detected no intracultivar variation. The same result was obtained when we compared leaves collected from the top, middle, or bottom of floricanes or from primocanes.

While the fingerprints are reproducible during the growing season, some technical aspects were affected by the physiological stage of the raspberry plants. DNA digestion by restriction enzymes is known to be inhibited by contaminants. By using the extraction method described, the level of contamination by polyphenols/carbohydrates of DNA was constant ($A_{260} : A_{230}$ 1.70; SD, 0.16; n, 50) up to the end of June (beginning of fruit formation), after which a sharp increase in contaminants was observed ($A_{260} : A_{230}$ 1.25; SD, 0.19; n, 66). Protein contamination followed the same pattern, but variations were less pronounced. These high

levels of contaminants in DNA preparations are typical of plants of the Rosaceae family (Manning, 1991). Thus, large quantities of restriction enzyme coupled with long incubation periods were required to achieve complete digestion of many DNA extracts. Reduction of enzyme concentration or incubation time was successful with May or June samples but failed with many of those from late summer (unpublished). Extracts from primocane leaves were slightly less contaminated than those from floricanes. The number of years of field cultivation had no effect on contamination levels. However, contamination by polyphenols/carbohydrates of in vitro plants was high ($A_{260} : A_{230}$ 1.34; SD, 0.18; n, 8). Nevertheless, these DNA contaminants were not sufficient to impede the experiments, and we were able to digest the raspberry DNA and to obtain fingerprints with all kinds of plants.

We have shown that nonradioactive DNA fingerprinting is a reliable technique to identify raspberry cultivars considerably quicker than radioactivity procedures, reducing 5- to 10-day autoradiographs to chemiluminescent exposures of < 2 days. In addition, reliability of the technique is independent of the plant age, developmental stage of the plant, and position of the leaf on the stem. Verification of identity is easily performed by direct comparisons of fingerprints with those from known standard cultivars. The reproducibility of the procedure provides a high level of confidence when used in the authentication of raspberry certification programs.

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