

Discrimination of *Rubus* Cultivars Using RAPD Markers and Pedigree Analysis

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Abstract

Few studies with random amplified polymorphic DNA (RAPD) markers have focused on blackberry genotypes. In this study we attempted to differentiate several blackberry and raspberry cultivars for genetic identification by pedigree and RAPD analyses. *Rubus allegheniensis* was present in 14 of 16 cultivars and had a mean genetic contribution of 15.9%. The cultivar 'Brewer' and *R. argutus* were also recurrent founding clones. The raspberry cultivars in this study ('Dormanred' and 'Heritage') participated as the outlier group and were identified as such in relation to the blackberry cultivars both in terms of RAPD and pedigree analysis. One hundred fifty-seven RAPD loci were used to identify 10 of 16 genotypes with unique banding patterns. Blackberries averaged 55% similarity amongst themselves, and 22% to raspberries. Overall, pedigree and RAPD data should be interpreted separately, with the RAPD data likely being more reliable.

INTRODUCTION

The breeding of new *Rubus* cultivars has led to a narrowing of the genetic diversity, with most being closely related and difficult to differentiate morphologically (Jennings, 1988). The lack of genetic variability in *Rubus* can lead to erroneous identification by purely phenotypic or morphological evaluation. Phenotypic plasticity within the genus can cause confusion as to taxonomy as well (Nybom and Hall, 1991). Another problem is that most cultivars are certified according to morphological traits such as fruit or flower characteristics that are not continuously present (Parent and Pagé, 1992). Thus, improvement in the area of cultivar identification must be a high priority for *Rubus* breeders in order to verify identity and assist in confirming proprietary rights.

Several studies using RAPD markers from *Rubus* have been reported. Parent et al. (1993) found stability in markers even though they used plant material from different propagation methods, seasons, and sources. Another study (Graham et al., 1994) had similar results in identifying cultivars of red raspberry but found that reliability was a problem. Graham and McNicol (1995) stated that RAPD data can identify cultivars, species, and interspecific crosses. Trople and Moore (1999) studied 42 *Rubus* genotypes including two red raspberry cultivars and found that 'Tulameen' and 'Meeker' had a high genetic similarity. The major limitation of RAPD markers is the inconsistent reproducibility of banding patterns, and therefore, comparisons cannot be made between studies. However, RAPD markers have proven to be adequate in cultivar similarity studies.

Pedigree studies for *Rubus* have been restricted to red raspberries. The first study evaluated pedigrees of 91 cultivars released since 1960 (Dale et al., 1989). The cultivars were grouped according to their geographic origin based on the genetic contribution of founding clones. The second study was an expanded version of the first, this time evaluating 137 red raspberry cultivars (Dale et al., 1993).

The objectives of this study were to differentiate *Rubus* cultivars via RAPD markers and pedigree analysis and compare the results of the two analyses to determine if a relationship exists between the two methods.

MATERIALS AND METHODS

Actively growing shoot tips were collected from 16 *Rubus* cultivars (Table 1) growing in the field during summer 2001 at the University of Arkansas Agricultural Research and Extension Center, Fayetteville or the Fruit Research Substation, Clarksville.

Shoot tip tissue (100 mg) was ground to a fine powder in liquid nitrogen with a mortar and pestle. DNA was extracted according to manufacturer's protocols with a Qiagen DNeasy Plant Mini Kit (Valencia, California). The DNA was re-precipitated with ammonium acetate (one-half of the original extraction volume) and 100% ethanol (three times the extraction volume plus the ammonium acetate). The sample DNA pellet was allowed to dry and then washed with 70% ethanol and re-suspended in 50 μ L of TE buffer (pH 7.4).

The Bio-Rad Fluorescent DNA Quantitation Kit (Hercules, California) was used to stain the DNA found in a 5- μ L sample of the template stock solution with a Hoechst dye, and the DNA was then quantified with a Bio-Rad Versafluor fluorometer (Hercules, California)

The PCR reaction mixtures were comprised of reagents from the PCR Core System II kit from Promega (Madison, Wisconsin). The reaction mixture consisted of Promega's 1X thermophilic DNA polymerase reaction buffer (500 mM KCL, 100 mM Tris-HCL at pH 9.0, and 1% Triton® X-100), 1.5 mM MgCl₂, 200 μ M dNTP, 1.25 ng- μ L⁻¹ of template DNA, 1.5 units per reaction volume of Novagen Novataq DNA polymerase (Madison, Wisconsin), and 0.2 μ M primer from Operon (Alameda, California) which comprised a total reaction volume of 50 μ L. One hundred random ten-base oligonucleotide primers were screened for the presence of consistent bands with the cultivar Apache. The PCR reactions were conducted twice to insure reproducibility of bands. The primers that gave reproducible bands were then evaluated against all cultivars. These primers were OPA (2, 13), OPB (5, 6, 7), OPC (2, 5), and OPD (2, 3, 8). Components were mixed in PGC Scientifics (Gaithersburg, Maryland) 0.65-mL thin-walled microcentrifuge tubes. A positive control supplied with the Promega PCR Core System II kit was run in all experiments. The PCR reactions were carried out in a Hybaid PCR Sprint thermocycler (Middlesex, England) programmed to cycle through a modified version of the temperature regime outlined by Levi et al. (1993) of one cycle of 94 °C for 240 s, 48 °C for 70 s, and 72 °C for 120 s, followed by 45 cycles of 94 °C for 45 s, 48 °C for 70 s, and 72 °C for 120 s, and lastly one cycle of 72 °C for 300 s with a final holding temperature of 4 °C.

After the PCR reaction, each sample was mixed with 8 μ L of loading buffer (bromophenol blue and orange G added to 25% Ficoll™). Then, 15 μ L of the loading buffer/PCR product mixture was loaded into the wells of a 1% agarose gel immersed in 1 x TBE running buffer. The 1 Kb Plus DNA ladder (Life Technologies, Rockville, Maryland) was run alongside the samples so that the size (in base pairs) of DNA fragments could be estimated. The gel was run in an Owl horizontal electrophoresis system (Portsmouth, New Hampshire) at 120 V for ~3 h. Following electrophoresis, the gels were stained with ethidium bromide and digitally photographed with an Alpha Innotech ChemImager gel documentation system (San Leandro, California)

The banding pattern matrix was generated by hand, with 1 (present) or 0 (absent) to score each band. Genetic similarities were calculated by the Dice (1945) formula in the Numerical Taxonomy and Multivariate Analysis System for PC (NTSYS-pc, version 2.1) (Rohlf, 2000). Construction of dendrograms was derived from the application of the unweighted pair-groups method average (UPGMA) to the similarity matrices in NTSYS-pc in the TREE program. A cophenetic correlation was used to determine the goodness of fit of the RAPD similarity matrix to the UPGMA cluster dendrogram using the Mantel test (Mantel, 1967) in the COPH and MXCOMP programs.

All pedigree information was obtained from a variety of sources (Hedrick, 1922; Darrow, 1967; Jennings, 1988; Hall, 1990); however, if the lineage was in question, the original release paper was used as the definitive source when available. The pedigrees were traced back to the founding clones. In some cases, no information was found on parentage; therefore, the particular clone was considered a founding clone. Genetic contribution (GC) was determined according to the method of Dale et al. (1993). Open-pollinated genotypes were considered to be self-pollinated by the female parent as described in Dale et al. (1989). Since parental contribution is only an estimate of actual contribution, the founding clones were scored in a matrix of 1 (present) or 0 (absent) to obtain a relative relationship ranking among the cultivars. A similarity index was calculated by the Dice formula and the dendrogram and goodness of fit test were conducted as mentioned previously.

RESULTS AND DISCUSSION

The founding clones among the *Rubus* cultivars in this study were a diverse group of genotypes; however, only 10 of the 27 clones contributed more than three times in the lineage of the 16 cultivars tested. *Rubus allegheniensis* Porter had the highest frequency of occurrence among all founding clones and was found in the background of 14 of the 16 cultivars, with a mean genetic contribution (GC) of 15.9% (data not shown). This was followed in GC by 'Brewer', an old variety, with 10.9%. The second highest frequency was *R. argutus* Link, which was found in 13 of the 16 cultivars. *R. parvifolius* L. had a relatively high mean GC (4.7%), but it was only found in one cultivar, where it was a donor of 75% to the raspberry cultivar 'Dormanred' (data not shown).

'Black Butte', 'Dormanred', and 'Heritage' were the most dissimilar of the cultivars tested due to the high amount of raspberry germplasm in their backgrounds. Alternately, 'Apache', 'Arapaho', 'Chickasaw', 'Choctaw', 'Kiowa', 'Navaho', and 'Shawnee' were similar because they all shared the same founding clones, especially 'Apache', 'Choctaw', 'Navaho', and 'Shawnee'. This was not surprising since all of the cultivars were released from the University of Arkansas blackberry breeding program.

Subjecting the founding clone results to the Dice test was meant to yield approximate figures for the pedigree analysis. It was expected that some calculations would overestimate the actual results and some would be underestimated. Pedigree similarity values underestimated percentage relationships for nearly all estimates involving 'Black Butte', 'Brazos', 'Darrow', 'Dormanred', and 'Heritage' (data not shown). This was due to the low number of shared founding clones with the other genotypes, especially the raspberries, which shared few or no founding clones with the blackberries in this study. 'Dormanred' had founding clones in common only with 'Heritage' and 'Black Butte'. 'Heritage' shared *R. argutus* with most blackberries except for 'Brazos' and 'Rosborough'.

Cluster analysis of the pedigree data yielded results that were more or less expected (Fig. 1a). The raspberry cultivars 'Dormanred' and 'Heritage' did not join, but 'Heritage' did join 'Black Butte', which also has raspberry germplasm in its background. 'Navaho' and 'Apache', 'Arapaho' and APF-12, 'Chester' and 'Illini Hardy', and 'Brazos' and 'Rosborough' were all joined together. This is not surprising since the former cultivars are all parents of the latter genotypes. The Mantel matrix correlation test generated a value of $r = 0.95$, suggesting a very good fit of the data to the resulting dendrogram.

Overall, 157 RAPD loci were used to calculate the similarity estimates, a number that Fu et al. (2002) deemed to be within an acceptable range. Of this number, most were polymorphic due to the inclusion of the raspberry germplasm. There was only one monomorphic band for all genotypes and five monomorphic bands across all blackberries. Ten of the 16 genotypes were identified specifically within this set of primers.

When compared amongst themselves, the blackberries displayed from 47% ('Black Butte') to 60% (APF-12) similarity, with the average at 55% for RAPD marker data. The blackberry genotypes averaged 22% similarity with the raspberries. The Arkansas blackberry genotypes averaged 54% similarity to non-Arkansas blackberry genotypes and 60% similarity to other Arkansas blackberries.

Only one other molecular marker study has included Arkansas blackberries to test for relatedness (Nybom et al., 1989). In their work, random fragment length polymorphisms (RFLP) were used to distinguish several blackberries as well as two raspberries. Our similarity results were parallel to some of their percentages. For example, the RAPD data for 'Darrow' paired with 'Brazos', 'Choctaw', and 'Rosborough' (55, 61, 53%, respectively) were very consistent with the results from the RFLP analysis (53, 67, 56%, respectively). Also, 'Rosborough' and 'Shawnee' displayed a 70% similarity in both studies, denoting a degree of accuracy. All other RFLP results were higher than the RAPD results for the remaining pairwise comparisons.

Pedigree and RAPD similarity matrices produced results that were comparable. For example, 'Apache' and 'Brazos' had results of 57% similarity in both matrices (data not shown). Although that pairing was the only one with exact percentage similarity, several others were within 5% of one another and some as close as 1%. This suggests that in certain

instances RAPD and pedigree data can correlate, however in most cases pedigree data should be approached with caution as to its degree of certainty.

RAPD marker data suggests that even though raspberry genotypes that share one ('Heritage') or zero ('Dormanred') founding clones with the other genotypes in this study, they do indeed exhibit genomic similarities. In this study, 'Brilliant' was considered distinct from *R. strigosus* Michx., even though it is a botanical variety of that species. This is deemed a likely point of similarity in the raspberries with the blackberries.

The RAPD cluster analysis was not as clear-cut as the pedigree dendrogram (Fig. 1b).

The Mantel test revealed a correlation of $r = 0.94$, a very good fit of the similarity matrix data to the cluster analysis. In the RAPD dendrogram, 'Dormanred' and 'Heritage' was a distinct cluster from the blackberry genotypes; however, 'Black Butte' clustered with the blackberries instead of the raspberries as seen in the pedigree dendrogram. This result is likely more accurate than that of the pedigree dendrogram because of the taxonomic designation of 'Black Butte' as a blackberry. 'Chester' and 'Illini Hardy' also joined as expected. However, some discrepancies became evident. Surprisingly, 'Brazos' and 'Rosborough' did not join together, and were not in the same cluster. Yet, APF-12 and 'Rosborough' did join together, and had the highest similarity percentage of any pair of genotypes (75%). This is comparable to the result in the pedigree similarity matrix (71%), but is still an unexpected result. APF-12 has 'Arapaho' as a female parent, thus one would conclude that APF-12 shows more similarity to 'Arapaho' than to 'Rosborough'. 'Rosborough' has 'Brainerd' as a female parent, a cultivar that is not in the background of APF-12. However, 'Brazos' is the male parent of 'Rosborough' and also prominent in both sides of the pedigree of APF-12. Thus, the contribution of 'Brazos' to both APF-12 and 'Rosborough' could account for their degree of similarity detected in this study.

When the matrices of the pedigree data and the RAPD data were tested with the Mantel matrix correlation the result was poor correlation ($r = 0.30$), therefore the pedigree and RAPD matrices should not be considered as relating to one another in a meaningful way. Each has its own merits, but they should be interpreted separately.

CONCLUSIONS

From the results gained in this study, there seems to be a modicum of relationship between the pedigree and RAPD data. When compared, the pedigree and RAPD data did not correlate for genotypes that shared many of the same founding clones, as the results tended to overestimate relatedness. However, there was evidence of the pedigree and RAPD results being close. If a more accurate assessment of pedigree relatedness among cultivars was used, then the results may have been more precise.

In general, RAPD marker data proved to be a good method of assessing genetic relatedness among different *Rubus* genotypes. Ten of the 16 genotypes displayed a single band which distinguished them from all other genotypes in this study. This number could likely be expanded if more primers were tested. Therefore, RAPD markers can effectively differentiate closely related blackberry genotypes, as well as more distantly related raspberry genotypes.

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Tables

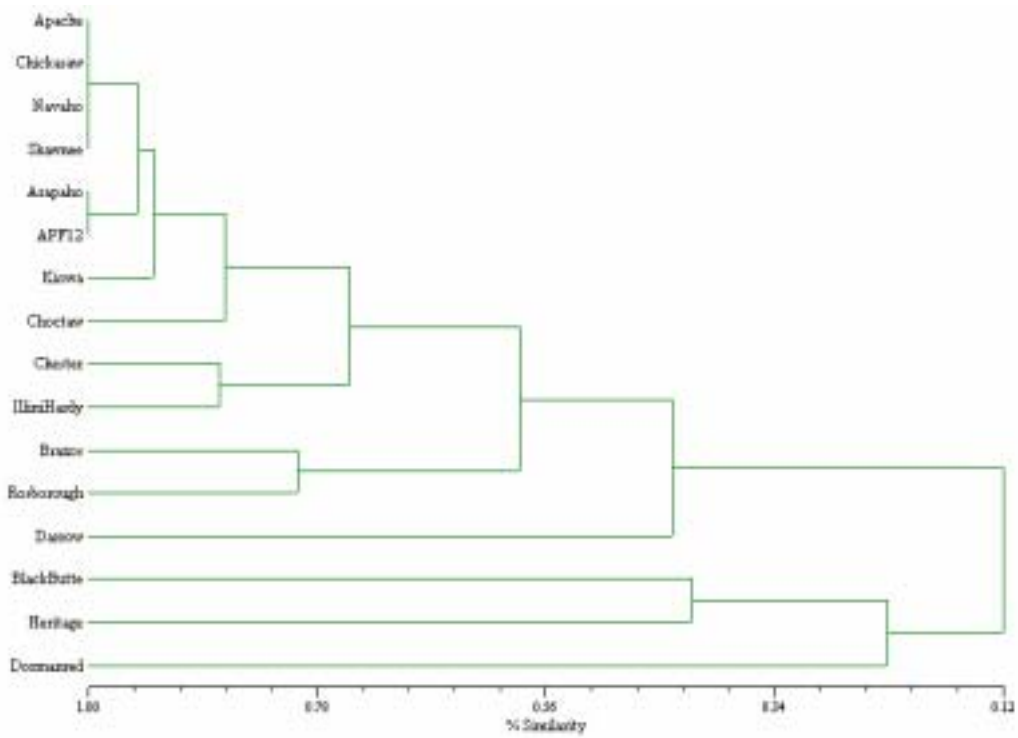
Table 1. Parentage and program location of 16 *Rubus* genotypes studied.

Genotype	Parentage	Program location
Apache	(SIUS 68-6-15 x Comanche) x Navaho	Fayetteville, Arkansas
APF-12 ¹	Arapaho x A-830	Fayetteville
Arapaho	(A-550 x Cherokee) x A-883	Fayetteville
Black Butte	ORUS 830-4 x ORUS 728-3	Corvallis, Oregon
Brazos	Lawton x Nessberry	College Station, Texas
Chester	SIUS 47 x Thornfree	Beltsville, Maryland
Chickasaw	(Comanche x A-516) x A-1246	Fayetteville
Choctaw	(Darrow x Brazos) x Rosborough	Fayetteville
Darrow	(Eldorado x Brewer) x Hedrick	Geneva, New York
Dormanred	Dorsett x <i>R. parvifolius</i>	Starkville, Mississippi
Heritage	(Milton x Cuthbert) x Durham	Geneva, New York
Illini Hardy	NY95 x Chester Thornless	Carbondale, Illinois
Kiowa	(A-586 x Comanche) x (A-628 x Rosborough)	Fayetteville
Navaho	(Thornfree x Brazos) x (A-550 x Cherokee)	Fayetteville
Rosborough	Brainerd x Brazos	College Station
Shawnee	Cherokee x (Thornfree x Brazos)	Fayetteville

¹Unreleased breeding selection, not commercially available.

Figures

a.



b.

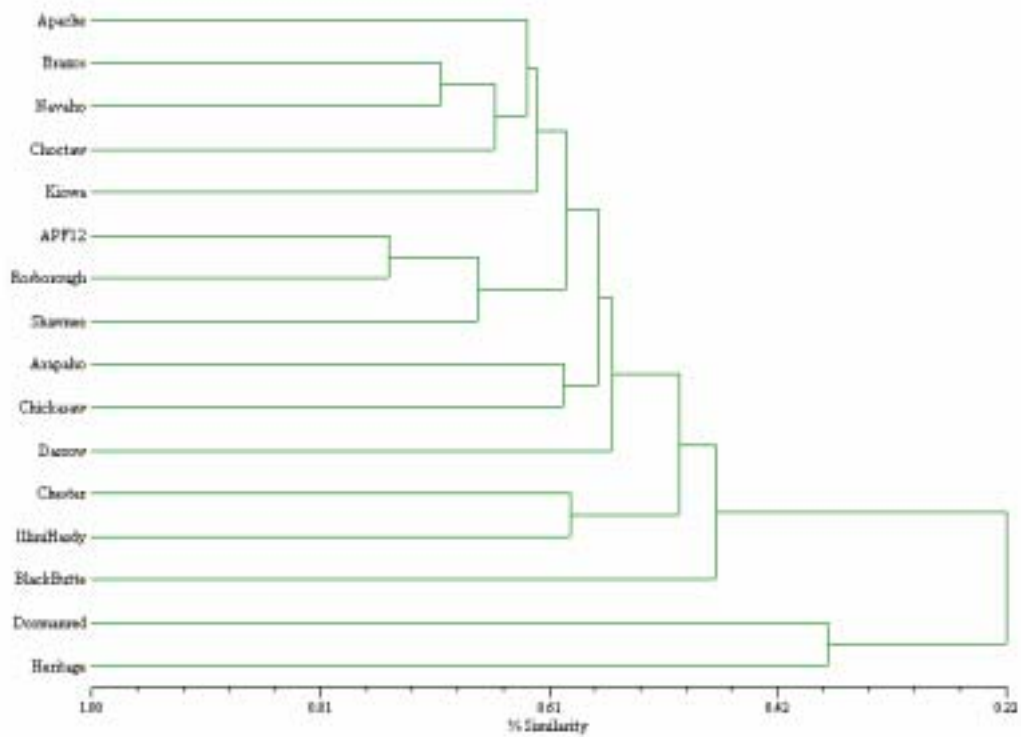


Fig. 1. Pedigree (a) and RAPD (b) cluster analysis dendrograms