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## Nuclear Ribosomal ITS Region Sequences for Differentiation of *Rubus* Genotypes

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### Abstract

Previous molecular investigations into *Rubus* species diversity have yielded significant knowledge about species relatedness. However, little work has been focused at the cultivar level. Random amplified polymorphic DNA (RAPD)-PCR studies have successfully differentiated closely related cultivars. The ability to definitively distinguish blackberry and red raspberry cultivars based on other molecular methods could prove useful in many aspects of breeding and proprietary protection. In this study, the nuclear ribosomal DNA internal transcribed spacer regions (ITS) of six *Rubus* cultivars were sequenced. DNA sequencing revealed little genetic variation among blackberry cultivars, but revealed distinctions between blackberry and red raspberry cultivars. Analysis by maximum-parsimony and maximum-likelihood confirmed the small variation among blackberry cultivars, although cultivars Apache, Brazos, and APF-12 did organize a weak sub-cluster within the blackberry genotypes. However, ITS region sequences do not appear to differentiate among closely related blackberry genotypes for purposes of cultivar discrimination or plant patent protection.

### Introduction

Blackberries (*Rubus* subgenus *Rubus* Watson; subgenus *Eubatus*) and red raspberries (*R. idaeus* L.; subgenus *Idaeobatus*) are economically and ecologically important fruiting plants which belong to the diverse genus *Rubus*. Most modern Eastern American blackberry cultivars were derived from diploid blackberry species *R. allegheniensis* Porter and *R. argutus* L. (Hall, 1990). Morphological differentiation between blackberries and raspberries is fairly well delineated with distinct fruiting characteristics being the primary recognition, but these identifiers are not present throughout the year (Parent and Pagé, 1992). Also, raspberry species have been used in blackberry breeding, thus donating significant traits which were not previously known in blackberry germplasm (e.g., fall-fruiting habit). Breeding of new *Rubus* cultivars has resulted in a narrowing of the genetic diversity, thus making definitive cultivar identification difficult (Jennings, 1988). However, little is known about the actual relationships among cultivars to their progenitors. Therefore, improvement in cultivar identification could be of significant benefit to verify identity and assist in confirming proprietary rights.

Since the internal transcribed spacer (ITS) region is repeated within plant nuclear genomes, amplification and sequencing of the nuclear ribosomal DNA is easily achieved (Baldwin et al., 1995). Previous studies on *Rubus* involving nuclear ribosomal DNA ITS region sequences have focused entirely at the species level (Alice, 2002; Alice and

Campbell, 1999; Alice et al., 2001). Alice and Campbell (1999) reported that *Rubus* ITS sequences are mainly informative among subgenera, but variability is low between closely related species. Since the lineages of most blackberry cultivars include several distinct species, it may be possible to discriminate among the cultivars based on ITS sequences.

The objectives of this study were to distinguish blackberry and raspberry genotypes with the use of ITS sequences and also to ascertain if the results obtained would be useful for incorporation into a *Rubus* breeding program and plant patent protection.

### Materials and Methods

Actively growing leaf material was sampled from six *Rubus* cultivars (Table 1) growing in the field during summer 2001 at the Univ. of Arkansas Fruit Research Substation, Clarksville or at the Agricultural Research and Extension Center in Fayetteville. All plant material was stored in a freezer (-20°C) until the DNA extraction procedure was initiated, which was generally within 24 hours. Actively growing leaf tissue (100 mg) was ground to a powder with a mortar and pestle in the presence of liquid nitrogen. DNA extraction followed manufacturer's protocols (Qiagen Dneasy Plant Mini Kit, Valencia, CA). 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

Nuclear Ribosomal ITS Region Sequences for Differentiation of *Rubus* GenotypesTable 1. Parentage and program of origination of six *Rubus* genotypes studied.

Genotype	Parentage	Program location
Apache	(SIUS 68-6-15 x Comanche) x Navaho	Fayetteville, Arkansas
APF-12 <sup>Z</sup>	Arapaho x A-830	Fayetteville
Arapaho	(A-550 x Cherokee) x A-883	Fayetteville
Brazos	Lawton x Nessberry	College Station, Texas
Heritage <sup>Y</sup>	(Milton x Cuthbert) x Durham	Geneva, New York
Illini Hardy	NY95 x Chester Thornless	Carbondale, Illinois

<sup>Z</sup>Unreleased breeding selection, not an item of commerce.<sup>Y</sup>Raspberry cultivar.Table 2. Pairwise genetic distances among nine *Rubus* genotypes and two outgroup taxa.

No.	Genotype	1	2	3	4	5	6	7	8	9	10	11
1	APF-12			-								
2	Apache			0.009	-							
3	Brazos			0.009	0.003	-						
4	Heritage			0.039	0.036	0.038	-					
5	<i>R. idaeus</i>		0.040	0.035	0.036	0.003	-					
6	Illini Hardy		0.014	0.007	0.009	0.028	0.030	-				
7	<i>R. allegheniensis</i>	0.012	0.004	0.006	0.027	0.028	0.000	-				
8	<i>R. argutus</i>		0.012	0.006	0.008	0.030	0.032	0.001	0.000	-		
9	Arapaho			0.014	0.004	0.006	0.031	0.033	0.003	0.000	0.001	-
10	<i>A. arguta</i> <sup>Z</sup>		0.702	0.697	0.699	0.696	0.699	0.699	0.699	0.696	0.697	-
11	<i>V. corymbosum</i> <sup>Z</sup>	0.705	0.708	0.706	0.703	0.705	0.703	0.703	0.705	0.704	0.287	-

<sup>Z</sup>Outgroup taxa.

resulting DNA pellet was dried and then washed with 70% ethanol and re-suspended in 50  $\mu$ L of TE buffer (pH 7.4).

The PCR reaction mixtures for each sample were comprised of 5  $\mu$ L of 10X Taq buffer with MgCl<sub>2</sub> (Promega, Madison, WI), 4  $\mu$ L dNTP (Pharmacia Biotechnology, Piscataway, NJ), 1  $\mu$ L primer ITS 4 (5' -

TCCCTCCGCTTATTGATATGC- 3'), 1  $\mu$ L ITS 5 (5' - GGAAGTAAAAGTCGTAACAAGG- 3') (White et al., 1990), 0.4  $\mu$ L Taq polymerase (Promega), and 40  $\mu$ L double distilled water all of which were master mixed to ensure uniformity among reagents. An aliquot of 48  $\mu$ L was added to a 0.5 ml thin-walled microcentrifuge tube (Sarstedt, NC),

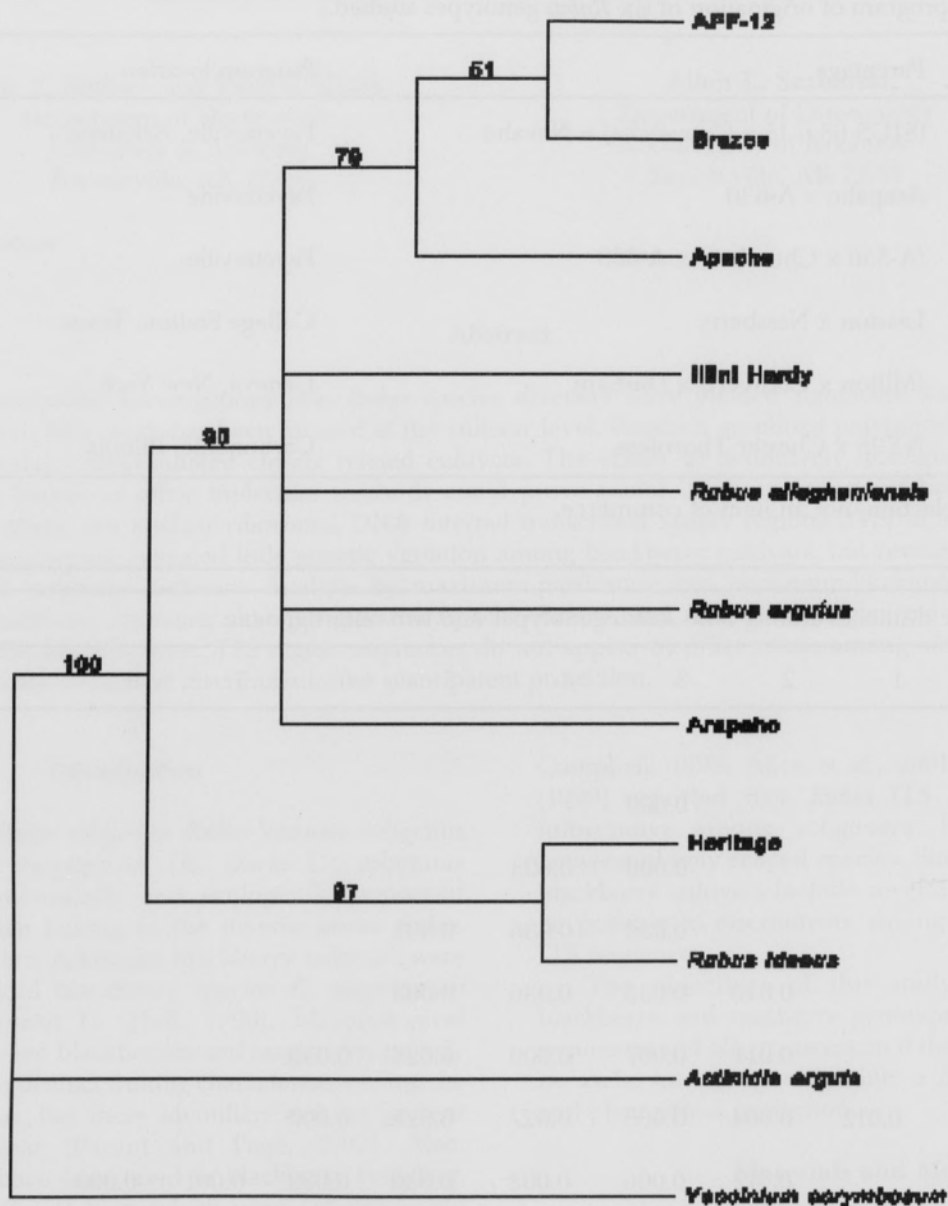


Fig. 1. Consensus parsimony tree with nine *Rubus* genotypes and two outgroups based on ITS region DNA sequences. Bootstrap values greater than 50% are indicated at nodes.

then 2  $\mu\text{L}$  of template DNA was added to comprise a total reaction volume of 50  $\mu\text{L}$ . A Techne Touchgene 40 x 0.5 ml (Techne Corp., Princeton, NJ) was used to perform the PCR. The thermocycling program used for this study consisted of an initial denaturation of 94 °C for 2 min., then 40 cycles of 94 °C for 45 s, 46 °C for 1 min, and 72 °C for 1 min, followed by a final extension for 5 min at 72 °C.

A 1% agarose solution (Fisher Scientific, St. Louis, MO), was placed into the gel box (Owl Scientific, Cambridge,

MA) filled with 0.5X TBE buffer solution. The PCR product and dye buffer totaled 10  $\mu\text{l}$ . Ten  $\mu\text{l}$  of 100 bp DNA ladder (Promega) was added in the first well. Ethidium Bromide was added to the buffer solution and the voltage was set at ~165 V. The electrophoresis ran for 50 min. The gel was removed and photographed in the BioDoc-It imaging system (UVP, Inc., Upland, CA).

Amplified DNA of six sample genotypes (five blackberry and one raspberry) was purified as described in



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Szalanski et al. (2000). The purified DNA samples were sent to the DNA core sequencing lab at the Univ. of Arkansas (Fayetteville) to be sequenced in both directions with an ABI Prism 377 DNA sequencer. An amplified fragment of DNA between 700 and 800 bp in size was sequenced for all samples. However, to understand genetic relationships with existing Genbank DNA sequences, the sequences were reduced to 643 bp.

Sequences derived from ribosomal DNA were aligned with the Genetics Computer Group (GCG, Madison, WI) PILEUP program (gap weight = 5.0, gap length weight = 1.0) and used *Vaccinium corymbosum* L. and *Actinidia arguta* (Sieb. & Zucc.) Planch. ex Miq. as outgroups. Maximum likelihood and maximum parsimony analyses were performed with PAUP\* 4.0b10 (Swofford, 2002). Bootstrap testing (100 re-sampled datasets) was used to determine tree robustness (Felsenstein, 1985).

## Results and Discussion

Bases were revealed at the frequency of 0.20967, 0.28372, 0.27825, and 0.22836 for A, C, G, and T, respectively. Existing Genbank sequences for *A. arguta* (AF323836; Li et al., 2002) and *V. corymbosum* (AF419778; Powell and Kron, unpublished data) were used as outgroups. *R. idaeus* (AF0557552), *R. allegheniensis* (AF055772), and *R. argutus* (AF0557742) were existing Genbank DNA sequences that were included to compare species with the modern cultivars included in the study (Alice and Campbell, 1999). Parsimony analysis resulted in 643 total characters consisting of 147 constant characters (22.8%), 176 variable characters that were parsimony-uninformative (27.3%), and 320 parsimony-informative characters (49.7%). Pairwise genetic distances (Tajima and Nei, 1984) ranged from 0.00 ('Illini Hardy'/*R. allegheniensis* and *R. allegheniensis*/*R. argutus*) to 0.04 (APF-12/*R. idaeus*) within the *Rubus* cultivars (Table 2). Alice and Campbell (1999) reported that ITS sequence divergence among species of the blackberry subgenus averaged only 1.2%, and this appears also be a close representation of the data obtained in this study.

Only one parsimonious tree was derived from the data set (Fig. 1) with a length of 603 and a consistency index (CI) of 0.982. Bootstrap analysis indicated little divergence among blackberry cultivars and species, but suggested a weak sub-cluster of APF-12, 'Brazos', and 'Apache'. The value of 51% at the APF-12/'Brazos' node implies a very weak support for that separation. The maximum-likelihood analysis starting with the parsimonious tree found 3 trees, but all were similar and none of the changes resulted in a significant change in topology. The resulting tree was similar in topology to that of the consensus parsimony tree (data not shown). Blackberry and red raspberry genotypes were separated as distinct clusters by both the maximum-parsimony and maximum-likelihood tests. The raspberry

genotypes displayed informative deletions at sites 75 and 442, whereas the blackberry genotypes had a deletion at 515 (data not shown). These are likely strong indicators for differentiating the two groups.

The phylogenetic maximum likelihood tree derived from PAUP\* differentiated the subgenera *Idaeobatus* and *Eubatus* (red raspberry and blackberry, respectively), but did not recognize any notable differences within the blackberry cultivars. Thus, *Rubus* cultivar differentiation, which has been done with other molecular techniques (Stafne et al., 2003), does not appear to be viable with ITS region sequences. Blackberry cultivars containing more divergent species have yet to be tested and may show distinction from the other blackberry cultivars tested in this study.

## Conclusions

The main benefit for *Rubus* appears to be if morphological ambiguity exists. ITS sequence analysis may distinguish between red raspberry and blackberry genotypes, but probably not within cultivars of those groups unless they contain highly divergent progenitor species. Since ITS sequences cannot distinguish between similar cultivars, its utility for plant patent protection is limited.

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