Evolution of the Avocados as Revealed by DNA Restriction Fragment Variation

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Individuals representing the genus *Persea*, subgenus *Persea* were assayed for restriction fragment length polymorphisms in their chloroplast genome, nuclear ribosomal DNA, and the genes coding for the enzyme cellulase. The subgenus *Persea* appears to consist of *P. schiedeana* and a separate taxon containing the remaining species. *P. americana* does not appear to be a monophyletic group. If *P. americana* is to be maintained as a species containing var. *americana*, var. *drymifolia*, and var. *guatemalensis*, then our data suggest that it should also contain varieties corresponding to *P. floccosa*, *P. nubigena*, and *P. steyermarkii*. *P. americana* var. *guatemalensis* appears to have originated as a hybrid between *P. steyermarkii* and *P. nubigena*. The root-rot-resistant cultivar G755A is a hybrid progeny of *P. schiedeana* and *P. americana* var. *guatemalensis*. The three varieties of *P. americana* were all distinguished by mutations.

Persea is a large genus within the family Lauraceae. As in other genera of this family, the phylogenetic relationships among *Persea* species have been difficult to determine.[23] The genus is composed of two distinct subgenera. The subgenus *Eriodaphne* is the larger, with over 40 species, but the subgenus *Persea* has received more attention because it contains the commercially important avocado.[1,24] Archaeological evidence suggests that the avocado may have been under cultivation for thousands of years.[19,20]

The currently planted cultivars of avocado are members of the three varieties of *P. americana* (var. *americana*, var. *drymifolia*, and var. *guatemalensis*). Because they have been under cultivation for such a long time, the origins and relationships among the three varieties are unclear. Some taxonomists have suggested that *P. nubigena* is actually a fourth variety of *P. americana* (var. *nubigena*),[11] whereas others have suggested that *P. americana* var. *guatemalensis* is actually a variety of *P. nubigena* (var. *guatemalensis*).[24] Bergh et al.[2] have suggested that *P. floccosa* also may actually be a variety of *P. americana*.

Recently, DNA restriction fragment length polymorphisms (RFLPs) have proved to be useful characters in determining the phylogenetic relationships among various plant taxa.[13] We have examined RFLPs in the chloroplast DNA (cpDNA) and nuclear genes coding for ribosomal RNA (rDNA) and cellulase in species of the subgenus *Persea*. Included in our sample were a large number of cultivars of *P. americana*. Our objectives were to clarify the phylogenetic relationships among these taxa and to determine whether RFLPs would be useful for determining the identity of plant material in a breeding program.

Materials and Methods

Leaf tissue was collected from plants on the campus of the University of California, Riverside, and at the university's South Coast Field Station. Each of the taxa (listed in Table 2) was represented by one individual. In addition, we sampled one individual each from *P. borbonia* and *P. indica*, members of the subgenus *Eriodaphne* that were used as outgroups. Total cellular DNA was prepared from individual trees by the following modifications of the method of Rawson et al.[15] Approximately 100 g (fresh weight) leaves were ground in a blender with 500 ml of a cold grinding buffer containing 0.1 M Tris-HCl (pH 8.0), 0.05 M KCl, 0.025 M EDTA, 0.35 M sucrose, 5.0% polyvinylpyrrolidone, 0.01 M diethyldithiocarbamic acid, and 0.01 M 2-mercaptoethanol. The lysing buffer was 0.05 M Tris-HCl (pH 8.0) and 0.1 M EDTA. A proteinase K (50 mg/ml) digest was done for 1 h at 37°C immediately after the lysing buffer was added. Aliquots of approximately 2 μg DNA

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Table 1. Descriptions of mutations observed in *Persea* species and cultivars

Mutation	Enzyme	Region[a]	Present[b]	Absent[b]	Taxa[c]
1	*Eco*RI	cpDNA SSC	2.3, 3.0	1.6	*
2	*Eco*RI	cpDNA LSC	2.4	2.3	*
3	*Bgl*II	cpDNA LSC	30 bp deletion		*
4	*Eco*RI	cpDNA SSC		restriction site	*
5	*Hind*III	cpDNA SSC		2.0, 5.2	*
6	*Hind*III	cpDNA LSC	3.7	4.4	*
7	*Bgl*II	cpDNA LSC		restriction site	*
8	*Eco*RI	cpDNA LSC	3.2		*
9	*Hind*III	cpDNA SSC	4.7	5.2	B, I
10	*Eco*RI	cpDNA SSC	1.4	1.3	B, I
11	*Bam*HI	cpDNA SSC	1.3, 2.7	2.6	B, I
12	*Eco*RI	cpDNA LSC	2.8		B, I
13	*Eco*RI	cpDNA LSC	1.9, 2.1, 2.8, 3.5, 18.6	2.5, 3.2, 21.9	I
14	*Eco*RI	cpDNA LSC	0.9, 3.2	1.0, 3.1	B
15	*Hind*III	cpDNA LSC	1.4	0.9, 3.8	B
16	*Hind*III	cpDNA LSC	1.9, 2.1, 4.5, 7.2	2.0, 7.7	B
17	*Bgl*II	cpDNA LSC	1.8, 5.7	6.1	B
18	*Sst*I	cpDNA LSC	2.1, 9.4	10.0	B
19	*Xmn*I	cpDNA LSC or IR	4.4	0.5, 4.7	B
20	*Eco*RI	cpDNA SSC	2.6	2.5	B
21	*Eco*RI	cpDNA SSC	3.5	2.5	I
22	*Eco*RI	rDNA	2.8		*
23	*Eco*RI	rDNA	3.3		*
24	*Eco*RI	rDNA	3.8		*
25	*Eco*RI	rDNA	4.4		*
26	*Eco*RI	rDNA	2.5		*
27	*Eco*RI	rDNA	2.7		B
28	*Sst*I	rDNA	0.9		*
29	*Sst*I	rDNA	1.5		*
30	*Xmn*I	rDNA		4.9	B, I
31	*Eco*RI	rDNA		0.4	I
32	*Eco*RI	cellulase	3.2		B, *
33	*Eco*RI	cellulase	2.6		*
34	*Eco*RI	cellulase	2.9		*
35	*Eco*RI	cellulase	9.1		*
36	*Eco*RI	cellulase	10.9		*
37	*Eco*RI	cellulase	5.8		B, *
38	*Eco*RI	cellulase	8.5		B, *
39	*Eco*RI	cellulase	5.2		*
40	*Eco*RI	cellulase		2.9	*
41	*Eco*RI	cellulase		3.2	*
42	*Eco*RI	cellulase	2.4, 2.8, 10.7, 12.7		*
43	*Eco*RI	cellulase		2.6	*
44	*Eco*RI	cellulase	7.2		*
45	*Eco*RI	cellulase	4.4, 5.5, 19.6		B
46	*Eco*RI	cellulase	2.1		B
47	*Hind*III	cellulase	16.5		*
48	*Hind*III	cellulase	5.2		*
49	*Hind*III	cellulase	6.7		*
50	*Hind*III	cellulase	3.5	3.4	*
51	*Hind*III	cellulase	1.9, 2.1	3.4	B
52	*Hind*III	cellulase	6.7	8.4	I

[a] IR = inverted repeat; LSC = large single copy region; SSC = small single copy region.

[b] Numbers refer to fragment sizes in kilobases.

[c] B = *P. borbonia*; I = *P. indica*; * = see subgenus *Persea* taxa in Table 2.

were digested with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Sst*I, and *Xmn*I. DNA fragments were size fractionated by electrophoresis in 0.7% agarose gels and transferred to nylon membranes (Zetabind or GeneScreen) using 20 × SSC as a transfer buffer.

Each of a complete set of cloned cpDNA fragments from *Petunia hybrida*[21] was used as a probe to identify restriction fragments of the chloroplast genome of the *Persea* samples. Because two of these fragments overlap the borders of the small single copy region and the inverted repeats, an 18.8 kbp cpDNA *Sac*I fragment spanning the small single copy region of *Lactuca sativa*[10] was used to localize *Persea* fragments. Variation in nuclear ribosomal RNA coding regions was assayed using rDNA clones pSR1.1 and pSR1.2 from *Glycine max*.[5] The nuclear genes encoding cellulase enzymes were assayed using a 2.1 kbp cDNA clone.[22]

Probes were labeled with radioactive ^{32}P by the random primer method.[7,8] Hybridizations and washes of nylon membranes were conducted by the methods of Palmer.[12] Fragments were visualized by autoradiography. Labeled probe was removed prior to rehybridization by washing the membranes in 0.4 M NaOH at 42°C for 30 min, followed by 30 min at 42°C in 0.2 M Tris-HCl (pH 7.5), 0.1 × SSC, 0.5% SDS.

The precise nature of many of the mutations could not be determined. In the case of the cpDNA, this is likely due to extensive rearrangements between the *Persea* cpDNA and the *Petunia* and *Lactuca* cpDNA used as probes. In the case of the cellulase gene probe, results were difficult to interpret for three reasons. Since a genomic clone was not available, a cDNA

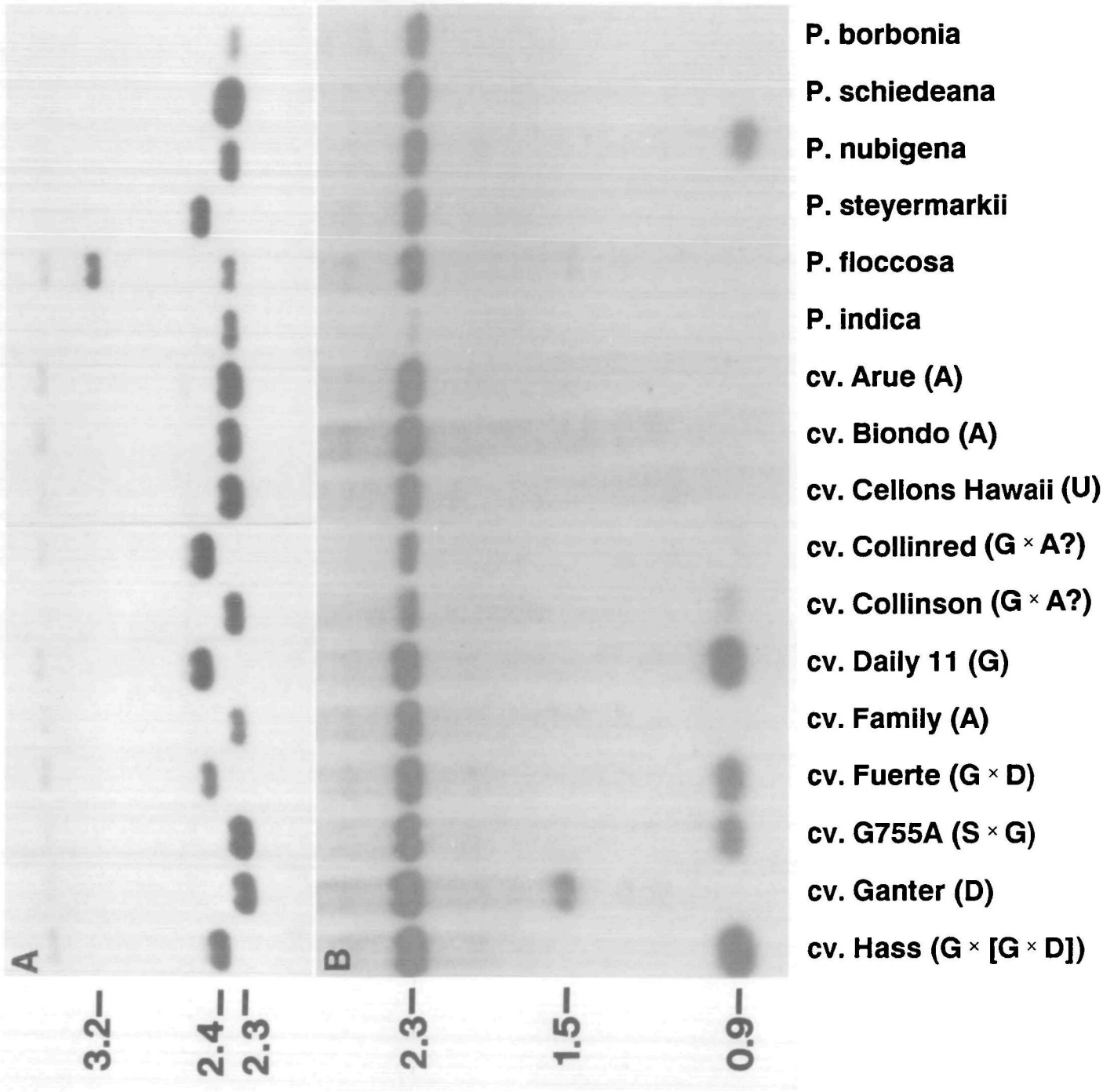

Figure 1. Southern blots of some of the *Persea* taxa assayed. **(A)** *Eco*RI digest hybridized to cpDNA probe P16 showing mutations 2 and 8. **(B)** *Sst*I digest hybridized to nuclear rDNA probe pSR1.2 showing mutations 28 and 29. A = *P. americana* var. *americana;* D = *P. americana* var. *drymifolia;* G = *P. americana* var. *guatemalensis;* S = *P. schiedeana,* U = *P. americana* var. unknown.

probe was used. Lack of knowledge of the number and sizes of introns complicates interpretation of the band patterns. The likelihood that cellulase is encoded by a multigene family[22] adds to the difficulty. Finally, because avocados are an outbreeding crop, the potential presence of heterozygotes further complicates interpretations. In assigning mutations, we attempted to use a conservative approach, counting related fragment changes as single events.

Parsimony phylogenetic analyses were conducted using the program Hennig86 (ver. 1.5, from J. S. Farris, State University of New York, Stony Brook). Only phylogenetically informative characters were included in the analyses. Taxa of known intervarietal and interspecific hybrid origin were excluded from these analyses.

Results

Members of the subgenus *Eriodaphne* differed from members of the subgenus *Persea* by many mutations (Table 1). Twenty-three phylogenetically informative mutations were observed within the subgenus *Persea* (Table 2; Figure 1). In the cpDNA assay, two mutations linked all var. *guatemalensis* cultivars with *P. steyermarkii*

Table 2. Distribution of observed mutations in species and cultivars of the genus *Persea*, subgenus *Persea*

Species	Mutations[a]																					
	cpDNA								rDNA							Cellulase						
	1	2	3	4	5	6	7	8	22	23	24	25	26	28	29	32	33	34	35	36	37	38	39
P. schiedeana				+		+																+	
P. floccosa								+		+	+	+			+	+	+	+	+	+			
P. steyermarkii	+	+								+			+			+	+			+	+		
P. nubigena									+					+			+	+	+		+		
P. americana[b]																							
cv. Arue (A)					+				+							+	+	+	+				
cv. Biondo (A)					+				+							+	+	+	+				
cv. Family (A)					+				+							+	+	+	+		+		
cv. Pollock (A)					+				+							+	+	+	+				
cv. Cellons Hawaii (U)									+							+	+	+	+				
cv. Ganter (D)			+							+	+	+			+	+	+	+	+	+			+
cv. Mexicola (D)			+							+	+	+			+	+	+	+		+			
cv. Topa-Topa (D)			+							+	+	+				+	+	+		+			
cv. Ygnaccio (D)			+							+	+	+				+	+	+		+			
cv. Daily 11 (G)	+	+							+					+		+	+	+	+				
cv. Linda (G)	+	+							+					+		+	+	+	+				
cv. Nabal (G)	+	+							+					+		+	+	+	+				
cv. Nimlioh (G)	+	+							+					+		+	+	+	+				
cv. Queen (G)	+	+							+					+		+	+	+	+				
cv. Fuerte (G × D)	+	+												+		+	+	+	+	+			+
cv. Hass (G × [G × D])	+	+							+					+		+	+	+	+				+
cv. Collinred (G × A?)	+	+							+							+	+	+	+		+		
cv. Collinson (G × A?)	+	+					+		+					+		+	+	+	+		+		
cv. G755A (S × G)				+					+					+		+	+	+	+				

[a] See Table 1 for description of mutations and their distributions in *P. borbonia* and *P. indica*.

[b] A = *P. americana* var. *americana*; D = *P. americana* var. *dymifolia*; G = *P. americana* var. *guatemalensis*; S = *P. schiedeana*; U = *P. americana* var. unknown.

(mutations 1, 2), one event linked all var. *drymifolia* cultivars together (mutation 3), one event linked all var. *americana* cultivars together (mutation 5), and one event linked the cultivar G755A with *P. schiedeana* (mutation 4).

A number of rDNA variants were observed (Table 1). All individuals of var. *drymifolia* possessed three variants not found in other *P. americana* but found in *P. floccosa* (mutations 23–25). Another mutation linked two of the four var. *drymifolia* cultivars with *P. floccosa* (mutation 29). All members of var. *americana*, var. *guatemalensis*, and *P. nubigena* shared a variant not found in var. *drymifolia* (mutation 22). One mutation linked all var. *guatemalensis* cultivars except the intervarietal hybrid cultivar Collinred with *P. nubigena* (mutation 28). That mutation also linked cultivar G755A with the other var. *guatemalensis* cultivars.

In the cellulase assay, *P. schiedeana* was distinct from most other subgenus *Persea* taxa at a number of mutations (mutations 32–35, 38, 40–43, 48–49). All var. *drymifolia* cultivars shared a mutation with *P. floccosa* (mutation 36), and two of the four var. *drymifolia* cultivars shared an additional mutation with *P. floccosa* (mutation 47). All var. *americana* and var. *guatemalensis* cultivars and one var. *drymifolia* cultivar also shared a mutation with *P. floccosa* (mutation 35). Two mutations linked *P. schiedeana* and cultivar G755A (mutations 42, 48).

Discussion

Much speculation has centered on the origin of *P. americana* var. *guatemalensis*. The presence of a number of morphological similarities has led to the belief that it arose from *P. nubigena*,[24] although Schieber and Zentmeyer[17] have suggested that *P. steyermarkii* may also have been involved in its origin. Our data suggest that both of these species gave rise to var. *guatemalensis*. The cpDNA data support a relationship to *P. steyermarkii*, whereas the rDNA data support a relationship to *P. nubigena*. These data are best explained by a hybridization event. Chloroplasts are inherited maternally in the majority of angiosperms surveyed.[9,18] A cross between a maternal *P. steyermarkii* and a paternal *P. nubigena* would yield progeny showing the cpDNA genotype of *P. steyermarkii* and the rDNA genotypes of both parents. Since *P. nubigena* is characterized by the presence of an additional rDNA *Ssfl* restriction fragment beyond those found in *P. steyermarkii*, the F_1 heterozygote would show the restriction fragment profile of *P. nubigena*. This is precisely the pattern shown by our data. These species are morphologically rather similar and are both native to the montane forests of western Guatemala.[24]

The parsimony analysis placed var. *guatemalensis* in a trifurcation on a tree with var. *americana* and *P. nubigena*. However, algorithms for inferring phylogenies are unable to include hybridization events. We combined the results of the parsimony analyses with the inferred hybridization event to derive an inferred phylogeny for the subgenus *Persea* (Figure 2). Phylogenetic topologies typically have large standard errors. Owing to the presumed reticulate evolution in the subgenus *Persea*, it is impossible to estimate standard errors in this case. This phylogeny indicates that *P. americana* as currently defined is not a monophyletic group. The subgenus appears to consist of *P. schiedeana* and a large taxon combining the remaining species and varieties. *P. floccosa* was embedded within the group of var. *drymifolia* cultivars, supporting the suggestion of Bergh et al.[2] that *P. floccosa* may be a variety of *P. americana*. If *P. americana* is to be maintained as a taxon containing var. *americana*, var. *drymifolia*, and var. *guatemalensis*, then our data suggest that it should also contain varieties corresponding to *P. floccosa*, *P. nubigena*, and *P. steyermarkii*. Our placement of *P. schiedeana* agrees well with its being the most morphologically distinct member of the subgenus *Persea*.[11,23]

The intervarietal hybrid cultivars Collinred and Collinson pose a pit of a puzzle. Rounds[14] states that these two cultivars

Table 2. Continued

Species	Mutations[a] (continued)								
	Cellulase (continued)								
	40	41	42	43	44	47	48	49	50
P. schiedeana	+	+	+	+			+	+	
P. floccosa						+			
P. steyermarkii	+								
P. nubigena		+							
P. americana[b]									
cv. Arue (A)									
cv. Biondo (A)									
cv. Family (A)									
cv. Pollock (A)									
cv. Cellons Hawaii (U)									
cv. Ganter (D)									
cv. Mexicola (D)						+			+
cv. Topa-Topa (D)						+			
cv. Ygnaccio (D)									
cv. Daily 11 (G)									
cv. Linda (G)									
cv. Nabal (G)									
cv. Nimlioh (G)									
cv. Queen (G)									
cv. Fuerte (G × D)						+			
cv. Hass (G × [G × D])									
cv. Collinred (G × A?)									
cv. Collinson (G × A?)									
cv. G755A (S × G)			+		+		+		

are siblings from the same cross of a maternal var. *guatemalensis* parent and a paternal var. *americana* parent, yet Collinson lacks the var. *guatemalensis* cpDNA genotype (mutations 1, 2) while Collinred lacks the extra rDNA *Sst*I fragment (mutation 28) common to the other var. *guatemalensis* sources. The absence of the var. *guatemalensis* rDNA fragment in Collinred may be due to selection, drift, or other random processes.[4] The absence of the var. *guatemalensis* cpDNA genotype in Collinson is more difficult to explain, possibly involving a case of biparental cpDNA inheritance followed by loss of the maternal cpDNA. It is also possible that the presumed parentage of these two cultivars is in error.

We had no prior knowledge of the varietal identity of cultivar Cellons Hawaii. Although it does not show complete identity with any of the other taxa assayed, it appears to be most closely related to members of var. *americana*.

The cultivar G755A is gaining in importance as a rootstock for avocado breeding programs because of its superior root-rot resistance.[3] Morphological characteristics suggest that it may be the result of hybridization between *P. americana* and *P. schiedeana*.[16] Ellstrand et al.[6] found G755 genotypes all combined allozyme alleles unique to *P. americana* with alleles unique to *P. schiedeana*, suggesting a hybrid origin. Our data support that hypothesis. G755A shares cpDNA (mutation 4) and cellulase (mutations 42, 48) mutations in common with *P. schiedeana* as well as the extra rDNA *Sst*I fragment (mutation 28) common to *P. americana* var. *guatemalensis* sources, indicating that it was the result of a cross between a maternal *P. schiedeana* and a paternal *P. americana* var. *guatemalensis*.

The combination of mutations observed in this study was sufficient to distinguish many of the taxa and could be useful in the identification of germ plasm. The limits of our germ plasm collection permitted us to sample only one individual per cultivar, preventing us from assessing the degree of variation within cultivars. However, we expect little variation within cultivars given that they are vegetatively propagated. All of the species in this study could be distinguished. Within *P. americana*, members of the three varieties could be distinguished, and within var. *drymifolia* all cultivars were unique. The var. *americana* and var. *guatemalensis* cultivars were less variable, although the cultivar Family could be distinguished from other var. *americana* cultivars. The hybrid cultivars Fuerte, Hass, Collinred, Collinson, and G755A were all uniquely identifiable. The use of additional probes should allow the unique identification of all cultivars, thus providing a powerful tool for plant breeders.

This study again illustrates the utility of molecular data in understanding the phylogenetic relationships among plant taxa. Unlike many other studies, it also points out the dangers of basing phylogenetic interpretations solely on variation in organelle genomes. Based on cpDNA data alone, *P. steyermarkii* would be identified as a progenitor of var. *guatemalensis*, but *P. nubigena* would not. A similar situation occurs in understanding the origin of cultivar G755A. This is an important consideration in many plant taxa, but particularly in crop species, where hybridization events may have been important during domestication.

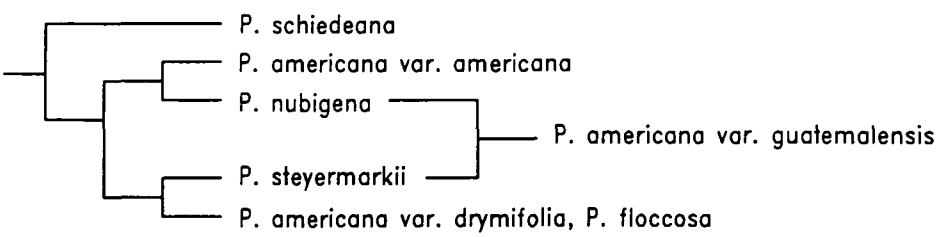

Figure 2. Inferred phylogeny of the genus *Persea*, subgenus *Persea* based on DNA RFLPs in cpDNA, nuclear rDNA, and genes encoding cellulase. The phylogeny was constructed by parsimony methods with the addition of the hybridization event inferred by separate examination of cpDNA and nuclear data. *P. borbonia* and *P. indica* were used as outgroups for rooting the phylogeny.

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