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Identifying Blueberry Cultivars and Evaluating Their Genetic Relationships Using Randomly Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat- (SSR-) anchored Primers

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Abstract. Fifteen highbush (or highbush hybrid) blueberry cultivars (*Vaccinium corymbosum* Linnaeus), two rabbiteye blueberry cultivars (*V. ashei* Reade), and one southern lowbush (*V. darrowi* Camp) selection from the wild were examined using seventeen 10-base RAPD and seven 15- to 18-base SSR-anchored primers (primers comprised of SSR motifs) in polymerase chain reactions (PCRs). Fifteen RAPD and three SSR markers resulting from these reactions were chosen to construct a DNA fingerprinting table to distinguish among the genotypes included in this study. Similarity values were calculated based on 132 RAPD and 51 SSR bands, and a dendrogram was constructed based on the similarity matrix. The *V. ashei* cultivars and *V. darrowi* selection grouped out separately from the *V. corymbosum* cultivars as expected. However, estimates of relative genetic similarity between genotypes within the *V. corymbosum* group did not agree well with known pedigree data and, thus, indicated that RAPD and SSR data did not accurately assess the genetic relationships of cultivars within this species.

The blueberry (*Vaccinium* spp.) is the most recent major fruit crop to be cultivated, having been domesticated during the twentieth century. It comprises three major types: the lowbush blueberries of Maine and eastern Canada, the rabbiteye blueberries of the deep southern United States, and the highbush blueberries native to the eastern United States (for a thorough review, see Galletta and Ballington, 1996). Recently, breeders have released several blueberry cultivars comprised of diverse species and from widely different geographical areas (Ballington, 1990; Lyrene, 1990).

Similar to many perennial, outcrossing plant species, breeding programs and genetic analysis in blueberry have been constrained by a paucity of genetic markers. Until recently, only a few distinct inherited traits, albino seedling and albino fruit (Draper and Scott, 1971; Hall and Aalders, 1963), and a few isozyme genetic markers (Krebs and Hancock, 1989; Vorsa et al., 1988) have been characterized for blueberry. Recently, RFLP (restriction fragment-length polymorphism) and RAPD markers have been identified and analyzed in blueberry. RFLP analysis has been performed for the chloroplast and mitochondrial genomes of the blueberry species *V. corymbosum, V. angustifolium* Aiton, *V. darrowi*, and *V. ashei*. No substantial polymorphism was detected in the chloroplast genome, while high levels of polymorphism were observed in the mitochondrial genome (Haghighi and Hancock, 1992). The recent development of the PCR-based arbitrarily primed genetic assay termed RAPD (Williams et al., 1990) has furnished an efficient means to produce large numbers of molecular markers that permit the estimation of relatedness among breeding materials, distinguish cultivars, and allow construction of genetic linkage maps (Tingey and del Tufo, 1993; Tingey et al., 1993). Aruna et al. (1993, 1995) have examined the extent of genetic relatedness among cultivars and wild selections of rabbiteye blueberry (*V. ashei* Reade) using RAPD markers. Their analyses were consistent with phylogenetic data provided for rabbiteye blueberries. In recent studies, we have optimized RAPD methodology (Levi et al., 1993) and constructed a low-density genetic linkage map (RAPD-based) using a *V. darrowi* x *V. elliottii* Chapman (2X) derived population (Rowland and Levi, 1994). Qu and Hancock (1995) and Vorsa and Rowland (1997) have used RAPD markers to determine the mode of 2n gamete formation in a diploid *V. darrowi* clone. In addition, Qu and Hancock (1995) have used RAPD markers to establish a tetrasomic mode of inheritance in interspecific hybrids of diploid *V. darrowi* and tetraploid *V. corymbosum*.

Like most higher eukaryotes, plant genomes are comprised of a variety of repetitive DNA sequences (Dow et al., 1995; Lagercrantz et al., 1993). A subclass of repeated sequences containing iterations of very short motifs (1 to 5 base pairs) are commonly referred to as SSRs or microsatellites (Weber and May, 1989). SSRs are highly abundant in plant genomes and their loci are polymorphic (Wang et al., 1994). PCR primers comprised of SSR motifs (in this study designated as SSR-anchored primers) have been shown to be an effective tool for the production of molecular markers in animals and plants (Zietkiewicz et al., 1994).

In the present study the objectives were to 1) identify polymorphic RAPD and SSR markers that would be useful for distinguishing blueberry cultivars and 2) examine the efficacy of these DNA markers in the assessment of genetic diversity among twelve northern and three southern highbush blueberry (*V. corymbusm*) cultivars, two rabbiteye (*V. ashei*) cultivars, and one southern evergreen lowbush (*V. darrowi*) selection from the wild.

Materials and Methods

Plant material. The following blueberry cultivars and selections were examined: 'Tifblue' and 'Climax' (rabbiteye, *V. ashei*, 6x), Florida 4B (southern evergreen lowbush selection, *V. darrowi*, 2x), 'Berkeley', 'Bluecrop', 'Bluegold', 'Bluejay', 'Blueray', 'Duke', 'Nelson', 'Patriot', 'Sierra', 'Sunrise', 'Toro', 'Weymouth' (northern highbush or highbush hybrids, *V. corymbosum*, 4x), and 'Georgiagem', 'Gulfcoast', and 'Cooper' (southern highbush, *V.*

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Table 1. SSR-anchored and RAPD primers used and the number of scorable (usable) polymorphic marker fragments resulting from each.

Primer	Sequence	Scorable markers (no.)
UBC-818	5′ACACACACACACACACY[z]G	11
UBC-841	5′ACACACACACACACYA	11
UBC-854	5′GACAGACAGACAGACA	14
UBC-856	5′CACACACACACACACAG	5
UBC-857	5′GGGTGGGGTGGGGTG	6
UBC-873	5′GAGAGAGAGAGAGAGAYC	2
UBC-881	5′TCTCTCTCTCTCTCTCR[y]G	2
UBC-105	5′CTCGGGTGGG	14
UBC-149	5′AGCAGCGTGG	12
UBC-169	5′ACGACGTAGG	8
UBC-171	5′TGACCCCTCC	9
UBC-181	5′ATGACGACGG	4
UBC-559	5′GAGAACTGGC	7
UBC-584	5′GCGGGCAGGA	6
OP-B05	5′TGCGCCCTTC	9
OP-B06	5′TGCTCTGCCC	9
OP-B14	5′TCCGCTCTGG	8
OP-P01	5′GTAGCACTCC	4
OP-P09	5′GTGGTCCGCA	4
OP-V07	5′GAAGCCAGCC	10
OP-V14	5′AGATCCCGCC	9
OP-V15	5′CAGTGCCGGT	10
OP-V17	5′ACCGGCTTGT	3
OP-X03	5′TGGCGCAGTG	6

[z]Y = (C or T).
[y]R = (A or G).

corymbosum and *V. darrowi* hybrids, 4x). In addition, the original parents, F_1s, and testcross parents of our mapping populations (Rowland et al., 1995) were included in some of the analyses. These plants are Florida 4B (listed previously), US799, and NJ88-13-15 (*V. darrowi*), 'Knight' (*V. elliottii*), W85-20 (*V. caesariense* Mackenzie), Florida 4B x W85-20 F_1 (#5), Florida 4B x W85-20 F_1 (#6), and Florida 4B x 'Knight' F_1 (US388).

DNA EXTRACTION. Young leaves (5 g) were collected from greenhouse-grown plants. After collection, leaves were ground with dry ice in a coffee grinder and stored at –70 °C. DNA was extracted using a CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle and Doyle, 1987) modified by Rowland and Nguyen (1993), except that the polyethylene glycol precipitation was omitted. Concentrations of DNA extracts were determined by analysis of DNA samples on 0.9% agarose gels (containing 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide) alongside uncut lambda DNA standards (50 to 2000 ng).

DNA AMPLIFICATION AND ELECTROPHORESIS CONDITIONS. DNA amplification reactions were performed as we have described previously (Levi et al., 1993) using 0.2 μM of 10-base RAPD primers (Operon Technologies, Inc., Alameda, Calif.) and 10-base RAPD or 15- to 18-base SSR-anchored primers (Biotechnology Laboratory, Univ. of British Columbia). Primers are listed in Table 1. Optimal DNA amplification was obtained through 45 cycles of 40 s at 94 °C, 70 s at 48 °C (for 10-base primers) or 59 °C (for 15- to 18-base primers), and 120 s at 72 °C (Levi et al., 1993). For analysis, amplified products were resolved via agarose gel (1.4%) electrophoresis as previously described (Levi et al., 1993; Rowland and Levi, 1994). Reactions with each primer were repeated two or three times. Only distinct bands, consistent from experiment to experiment, were used for fingerprinting and calculating similarity values as described below.

DATA ANALYSIS. The fraction of bands common to each pair of genotypes (F) was determined using the formula of Nei and Li (1979), i.e., $F = 2M_{xy}/(M_x + M_y)$, where M_{xy} is the number of bands shared by two genotypes (x and y) and M_x and M_y are the total number of bands in each genotype. Similarity values were calculated as $F \times 100$. Using the numerical taxonomy and multivariate analyses system program package for personal computer (NTSYS-pc, version 1.70, Rohlf, 1993) and unweighted pair-group method with arithmetic averages (UPGMA), cluster analyses were performed on each similarity data matrix, and dendrograms were constructed.

Results and Discussion

Amplification using the seventeen 10-base RAPD primers (typical RAPD reaction shown in Fig. 1) and the seven 15- to 18-base SSR-anchored primers (typical SSR-anchored PCR reaction shown in Fig. 2) resulted in 132 and 51 scorable polymorphic DNA fragments, respectively. A fragment was judged to be scorable if it was intense enough and different enough in size from neighboring bands to be scored with confidence, as well as being reproducible from experiment to experiment. Of 100 SSR-anchored primers that we initially screened, 15 resulted in good amplification of blueberry DNA. Of these 15 primers, 7 produced polymorphisms among genotypes and were used in our analysis.

Of the 183 fragments that were used for calculation of similarity coefficients, 18 of the most easily scorable fragments were identified that would distinguish among the cultivars or selections in this study. These markers are listed in Table 2 along with how each cultivar or selection was scored for the presence (+) or absence (–) of each of them. With as few as six of these primers (an example is the combination of primers UBC-169, UBC-181, UBC-841, OP-P09, OP-V07, and OP-X03) all cultivars and selections could be distinguished. Aruna et al. (1995) found a combination of four RAPD primers that could uniquely identify 19 rabbiteye blueberry cultivars.

The 183 RAPD and SSR fragments were used to calculate similarity values and estimation of relatedness among cultivars

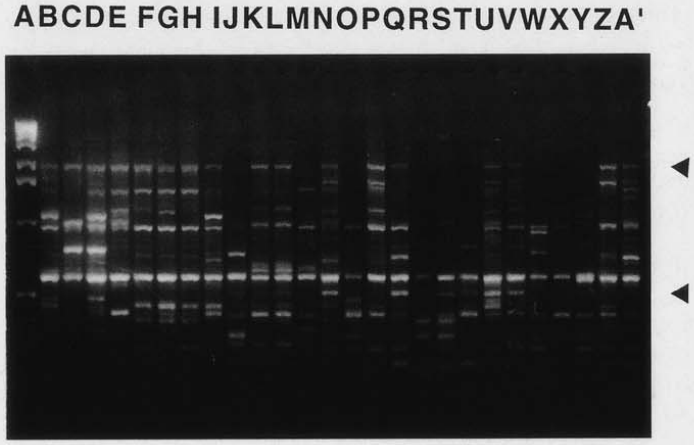

Fig 1. RAPD patterns of blueberry cultivars and selections produced by primer OP-X03: (**A**) 1 kb ladder(Gibco, BRL), (**B**) Florida 4B, (**C**) 'Knight', (**D**) US388, (**E**) US799, (**F**) W85-20, (**G**) Florida 4B x W85-20 hybrid #5, (**H**) Florida 4B x W8520 hybrid #6, (**I**) NJ88-13-15, (**J**) 'Tifblue', (**K**) 'Premier', (**L**) 'Climax', (**M**) 'Berkeley', (**N**) 'Bluecrop', (**O**) 'Bluegold', (**P**) 'Bluejay', (**Q**) 'Blueray', (**R**) 'Cooper', (**S**) 'Duke', (**T**) 'Georgiagem', (**U**) 'Gulfcoast', (**V**) 'Nelson', (**W**) 'Patriot', (**X**) 'Sierra', (**Y**) 'Sunrise', (**Z**) 'Toro', (**A′**) 'Weymouth'. Note: so called 'Premier' cultivar had an identical banding pattern to 'Climax' with all primers used and was later discovered, in fact, to be 'Climax', mistakenly sold to us as 'Premier'. Thus, it was excluded from subsequent analyses. Arrows to the right of the gel indicate markers listed in Table 2.

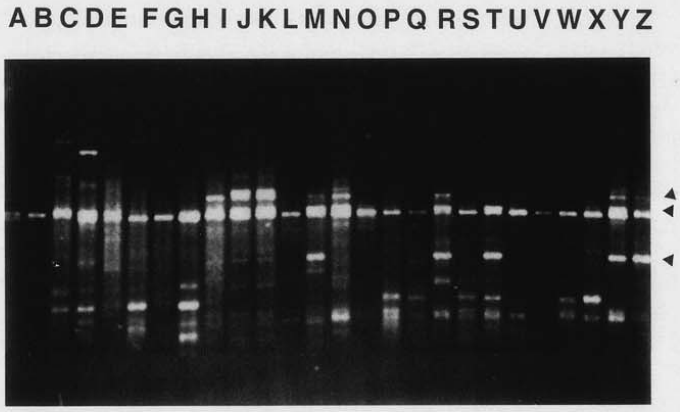

Fig. 2. SSR patterns of blueberry cultivars and selections produced by primer UBC-841: (**A**) Florida 4B, (**B**) 'Knight', (**C**) US388, (**D**) US799, (**E**) W85-20, (**F**) Florida 4B x W85-20 hybrid #5, (**G**) Florida 4B x W85-20 hybrid #6, (**H**) NJ88-13-15, (**I**) 'Tifblue', (**J**) 'Premier', (**K**) 'Climax', (**L**) 'Berkeley', (**M**) 'Bluecrop', (**N**) 'Bluegold', (**O**) 'Bluejay', (**P**) 'Blueray', (**Q**) 'Cooper', (**R**) 'Duke', (**S**) 'Georgiagem', (**T**) 'Gulfcoast', (**U**) 'Nelson', (**V**) 'Patriot', (**W**) 'Sierra', (**X**) 'Sunrise', (**Y**) 'Toro', (**Z**) 'Weymouth'. Arrows to the right of the gel indicate markers listed in Table 2.

(Table 3). In a parallel experiment similarity values were compared between original parent plants of our mapping populations, Florida 4B (*V. darrowi*), 'Knight' (*V. elliottii*), and W85-20 (*V. caesariense*), and three of their F_1 progeny, using 15 of the same primers used in the study with cultivars. In this case, we found that the similarity values between the parents and the F_1 progeny ranged from 62% to 78% (data not shown). These values are comparable to many of the similarity values among *V. corymbosum* cultivars (Table 3). The average similarity value among all *V. corymbosum* cultivars in our study is 56.3% compared to 70.6% calculated among 15 rabbiteye cultivars (Aruna et al., 1993). However, in our study the similarity value between the rabbiteye cultivars Climax and Tifblue is 59.6%, while, according to Aruna et al. (1993), it is 76.1%. None of the primers used in our study was used by Aruna et al. (1993); thus, it is evident that different sets of primers may result in differences in similarity values since they reveal different random parts of the genome.

The similarity matrix was used to generate a dendrogram (Fig. 3). As expected, relatively low similarity values were observed between the cultivars or selections of different species, differentiating the *V. darrowi*, *V. ashei*, and *V. corymbosum* representatives into separate clusters. The product-moment correlation between the similarity coefficients (calculated from the RAPD and SSR markers) and relationship coefficients [calculated as the probability that each pair of cultivars/selections carry the same allele (Wright, 1922)] of all possible pairwise combinations is $r = 0.48$ ($P < 0.01$); and, thus, is highly significant.

The similarity values within the *V. corymbosum* group alone, on the other hand, generally are not consistent with the parentage information available (Ehlenfeldt, 1994; Galletta and Ballington, 1996). For instance, the highest similarity values were found between 'Sierra' and 'Patriot' (78%) and 'Sunrise' and 'Sierra' (76%) that originated from different immediate parents. 'Sierra', in fact, is a hybrid of four different species (*V. corymbosum*, *V. darrowi*, *V. constablaei* Gray, and *V. ashei*). The two sets of full-sibs in this study, 'Gulfcoast'/'Cooper' and 'Bluecrop'/'Blueray', also do not have greater similarity values than these cultivars have with some much less related cultivars. In addition, an examination of the overall similarity values among *V. corymbosum* cultivars indicates that 'Weymouth' is the most divergent with an average similarity value of 49%. However, 'Weymouth' is a parent of 'Earliblue', and 'Earliblue' is an ancestor of many of the cultivars. Indeed, the product-moment correlation between the similarity coefficients and the relationship coefficients of the *V. corymbosum* group by itself is $r = 0.18$ ($0.05 < P < 0.1$); thus, it is not statistically significant.

In conclusion, RAPD and SSR-anchored primers are useful tools for identifying blueberry cultivars or selections. Amplification with SSR-anchored primers yields fewer bands than with RAPDs but analysis of the gels is simplified. With regard to evaluation of genetic diversity among germplasm, RAPD and SSR data effectively separate out the different species examined in this study but do not effectively assess the genetic relationships of cultivars within the *V. corymbosum* species. A weak positive correlation between similarity coefficients calculated from RAPD

Table 2. SSR and RAPD markers (size in kb) most useful for distinguishing among the blueberry cultivars and selections: Florida 4B (FL), 'Tifblue' (TB), 'Climax' (CX), 'Berkeley' (BK), 'Bluecrop' (BC), 'Bluegold' (BG), 'Bluejay' (BJ), 'Blueray' (BR), 'Cooper' (CP), 'Duke' (DK), 'Georgiagem' (GG), 'Gulfcoast' (GC), 'Nelson' (NL), 'Patriot' (PT), 'Sierra' (SA), 'Sunrise' (SR), 'Toro' (TR), and 'Weymouth' (WT).

RAPD marker		FL	TB	CX	BK	BC	BG	BJ	BR	CP	DK	GG	GC	NL	PT	SA	SR	TR	WT
UBC-169	(1.2 Kb)	+	−	+	−	+	−	−	−	+	−	+	−	−	−	−	+	−	−
	(0.9 Kb)	+	+	−	−	−	+	−	−	+	−	−	+	−	−	−	−	−	+
UBC-171	(0.6 Kb)	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
UBC-181	(1.1 Kb)	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	+
	(0.2 Kb)	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−
UBC-559	(0.7 Kb)	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
UBC-841	(0.8 Kb)	−	+	+	−	+	+	−	−	−	+	−	+	+	−	−	−	+	+
	(0.7 Kb)	+	−	+	−	+	+	−	−	−	+	−	+	−	−	−	−	+	−
	(0.5 Kb)	−	−	+	−	+	−	−	−	−	+	−	+	−	−	−	−	+	+
OP-B06	(1.3 Kb)	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+
OP-P09	(0.8 Kb)	+	+	−	+	+	−	−	−	+	−	+	+	−	−	+	+	−	−
OP-V07	(0.9 Kb)	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
	(0.7 Kb)	−	+	−	+	−	+	−	−	+	+	−	−	+	+	+	+	−	−
OP-V15	(1.1 Kb)	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
	(0.8 Kb)	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	(0.7 Kb)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
OP-X03	(2.0 Kb)	+	−	+	−	+	−	+	+	−	−	−	+	+	−	−	−	+	+
	(0.5 Kb)	−	+	+	−	+	−	−	+	−	+	+	+	+	−	−	−	−	−

Table 3. Similarity values for each pairwise combination of blueberry genotypes.

	FL	TB	CX	BK	BC	BJ	BG	BR	CP	DK	GG	GC	NL	PT	SA	SR	TR	WT
FL	0																	
TB	40	0																
CX	38	59	0															
BK	31	38	26	0														
BC	49	42	40	56	0													
BJ	34	35	33	51	67	0												
BG	35	37	40	45	66	70	0											
BR	36	33	40	53	61	59	63	0										
CP	43	41	28	50	60	53	43	60	0									
DK	29	42	35	52	62	68	58	60	48	0								
GG	40	30	24	63	62	55	49	64	70	52	0							
GC	44	37	36	53	60	51	47	61	61	50	63	0						
NL	33	33	33	54	55	59	53	70	58	54	64	68	0					
PT	38	35	20	61	47	51	47	52	64	53	66	54	61	0				
SA	35	40	23	58	46	48	37	54	70	56	63	51	54	78	0			
SR	30	44	29	63	59	59	49	61	63	65	62	51	59	67	76	0		
TR	38	43	36	54	57	65	59	52	44	64	50	62	56	48	47	50	0	
WT	33	39	38	33	53	59	53	53	42	60	45	49	60	39	44	46	56	0

and SSR data and relationship coefficients calculated from parentage information for the V. *corymbosum* group was found, but it was not significant at $P < 0.05$. Likewise, a similarity matrix based on RAPD data from 27 strawberry cultivars grown in the eastern United States was only weakly correlated with parentage information (data not shown). On the contrary, Hancock et al. (1994) found a significant product-moment correlation between number of shared RAPD profile phenotypes and pairwise coefficient of coancestry for eight strawberry cultivars or advanced selections from the Univ. of California, Davis, breeding program. Basically good agreement between RAPD data and parentage information has been reported for cultivars of a number of other crops, including celery (Yang and Quiros, 1993), apple (Dunemann et al., 1994), and rhododendron (Iqbal et al., 1995).

A possible explanation for this limitation of RAPD and SSR-marker data from populations of fairly closely-related *V. corymbosum* individuals is that a RAPD or SSR marker is determined to be shared by two cultivars if the same size (or very similar size) fragment is amplified from both cultivars. Actual DNA:DNA hybridizations generally are not carried out, in contrast with RFLP markers, to confirm that the fragments are homologous, i.e., represent similar sequences from the genome. A fairly large percentage of the fragments judged to be shared by some of the cultivars could, in fact, not be the same fragments but just fragments fortuitously of the same size. Other possibilities include the effect of a high percentage of heterozygous markers in these cultivars combined with small sample sizes, linkage of some markers to traits for different regional adaptations and/or to traits for horticultural value, Florida 4B (the *V. darrowi* clone used in this study and grandparent of southern highbush cultivars) genes being selected against in subsequent generations, and competition between primer sites in different genotypes. Our results suggest that caution should be taken in drawing conclusions regarding genetic relationships of cultivars or selections within a species if using RAPD or SSR markers alone.

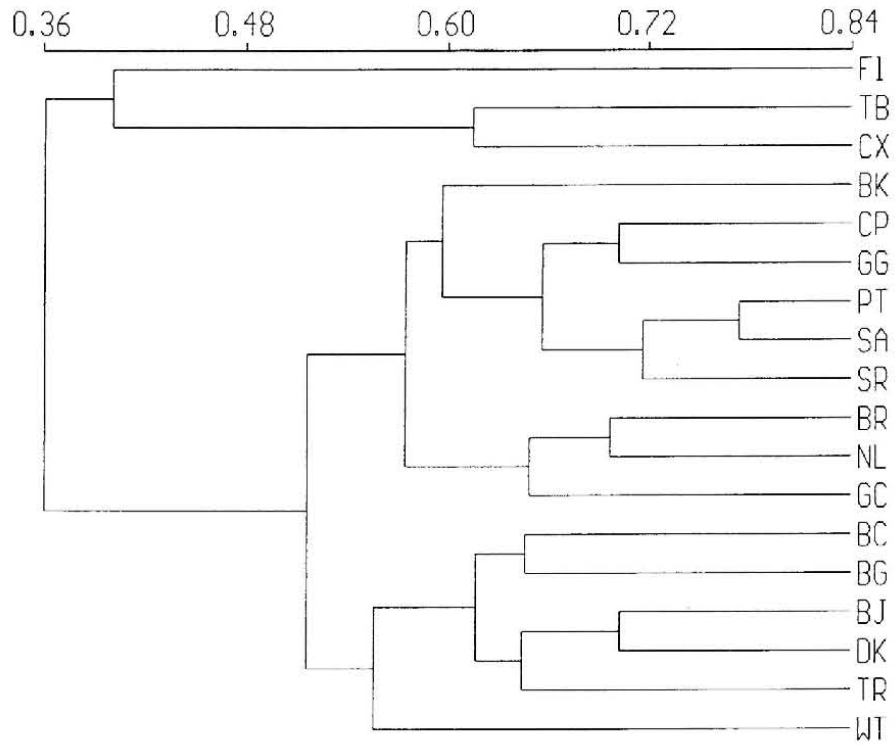

Fig. 3. Dendrogram of blueberry genotypes derived from UPGMA analysis of genetic similarity values.

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