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R. G. Novy · N. Vorsa

Evidence for RAPD heteroduplex formation in cranberry: implications for pedigree and genetic-relatedness studies and a source of co-dominant RAPD markers

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Abstract Silver-stained random amplified polymorphic DNA (ssRAPD) markers have been identified that are always jointly present or absent in the ssRAPD profiles of cranberry varieties. On the basis of segregation data and the ability to re-create these "associated ssRAPDs" through the intermixing of amplified DNA from individuals lacking them, five of the six pairs of associated ssRAPDs analyzed were shown to be consistent with heteroduplex molecules. Heteroduplexes are "hybrid" double-stranded DNAs that are formed following the polymerase chain reaction (PCR) amplification of two DNA segments that have a high degree of homology to one another, yet differ in their nucleotide sequences as a result of base pair deletions, additions, or substitutions. Three of the five putative heteroduplex systems identified are consistent with a one locus, two-allele heteroduplex model. The remaining two systems appeared to be multi-allelic, involving interactions among three and four alleles, respectively. RAPD heteroduplex formation has the potential to confound genetic relatedness and pedigree studies. Heterozygous individuals exhibit heteroduplex RAPDs not seen in either of the two homozygote classes. Genetic estimates under such a circumstance would "inflate" the differences between the heterozygote and the homozygote classes. Heteroduplex formation is also a mechanism for the presence of non-parental RAPDs in progeny of parents homozygous for alternate alleles. While this class of molecular markers can confound RAPD analyses, they also offer a source of co-dominant RAPD markers, which are of value in genetic relatedness estimates and as markers for studying breeding behavior.

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R.G. Novy
Department of Plant Sciences, North Dakota State University, Loftsgard Hall, Fargo, ND 58105, USA

N. Vorsa (✉)
Rutgers Blueberry and Cranberry Research and Extension Center, Rutgers University, Lake Oswego Road, Chatsworth, NJ 08019, USA

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Introduction

Random amplified polymorphic DNA (RAPD) methodology has been used successfully for plant varietal identification and genetic relatedness studies (Hu and Quiros 1991; Stiles et al. 1993; Yang and Quiros 1993; Kresovich et al. 1994), taxonomical classification (Williams and St. Clair 1993; Van Coppenolle et al. 1993; Howell et al. 1994; Stammers et al. 1995), and pedigree studies (Marsolais et al. 1993; Wang et al. 1994). Recently, a unique class of "associated" silver-stained RAPDs has been identified which could potentially have an impact on these types of RAPD analyses (Novy et al. 1994).

These associated RAPDs were always either jointly present or absent in the RAPD profiles of 22 cranberry cultivars, apparently providing redundant genomic information which could inflate genetic dissimilarity/similarity estimates and bias inferences concerning relatedness. While some dissimilarity estimates differed by as much as 27% in comparisons of cranberry RAPD data sets with and without the redundant RAPDs, the clustering of individuals between the two data sets was not substantially different (see Appendix 2, Novy et al. 1994). However, the potential of these associated RAPDs to influence other studies cannot be discounted.

Models that can account for the non-independent nature of these RAPDs include: (1) tightly linked dominant markers in coupling phase, (2) the presence of a primer-recognition site within a larger template sequence, and (3) allelic heteroduplex formation. Heteroduplex formation can occur following the polymerase chain reaction (PCR) amplification of two allelic DNA segments that have a high degree of homology to one another, yet differ in their nucleotide sequences as a result of base pair deletions, additions, or substitutions. Following amplification of both alleles, the final double-stranded DNA products may be formed

from the re-annealing of complementary strands (homoduplexes) or non-complementary strands (heteroduplexes). Two heteroduplex species are possible: (1) the (+) strand of allele *1* re-annealing with the (–) strand of allele *2*, and (2) the (–) strand of allele *1* reannealing with the (+) strand of allele *2*. Heteroduplexes generally migrate at different rates from homoduplexes during electrophoresis due to conformational changes caused by the nucleotide divergence between DNA strands (White et al. 1992). The two heteroduplex species can also be resolved as separate RAPDs in certain instances (Hatcher et al. 1993), therefore having the potential to appear as markers which are jointly present or absent in RAPD profiles. Of the three models proposed to explain the occurrence of associated RAPDs, only the heteroduplex model provides a mechanism for the synthesis of these RAPDs through intermixing of amplified DNA from individuals lacking them. The heteroduplex model can be further divided into intralocus (allelic), or interlocus (non-allelic) submodels. In the allelic model, the heterozygous state results in heteroduplex formation. The homozygous state results in the lack of RAPD formation and gives a null phenotype. In addition, it is possible to recreate the heterozygous phenotype by intermixing DNA of the null homozygous classes.

In the tightly linked dominant-marker loci model and the primer-recognition site within a larger template sequence model, inheritance and segregation would expected to follow a dominant pattern. Alternatively, in the allelic heteroduplex model, the occurrence of associated RAPDs will be a function of heterozygosity. An exception would be heteroduplex formation between tightly linked segments in coupling phase, which would also behave in a dominant fashion.

An understanding of the genetics of these associated RAPD markers would aid in establishing how they should be interpreted and used in genetic relatedness and pedigree studies. If associated RAPDs are the result of tightly linked dominant RAPD markers in coupling phase or a primer-recognition site within a larger template, then removal of all but one RAPD from analysis would eliminate bias in genetic-distance estimates.

However, if the associated RAPDs are heteroduplexes formed between allelic RAPD products that have sequence differences, then heterozygous individuals carrying both alleles would be classified as more divergent from either of the two homozygote classes than the homozygous classes are from one another, even with the removal from analysis of one of the two associated RAPDs. Heteroduplex formation between RAPDs would also confound pedigree analyses in that non-parental RAPDs would be present in the progeny of two parents homozygous for alternate allelic RAPD products. The formation of such heteroduplex RAPD products has been reported in honey bee (Hunt and Page 1992) and flax rust (Ayliffe et al. 1994), and more recently in chickpea and diploid strawberry (Davis et al. 1995).

The segregation patterns of six pairs of associated RAPDs were analyzed in cranberry progenies to determine if these associated RAPDs were jointly inherited. Segregation ratios were tested for a fit to: (1) dominant inheritance expected in tightly linked dominant RAPD markers in coupling phase or a primer-recognition site within a larger template model, and (2) allelic heteroduplex model expectations. Intermixing the amplified DNA of progeny lacking associated RAPDs, null individuals, was also conducted to determine if such RAPDs could be recreated. Formation of associated RAPDs through DNA intermixing would only be expected in the heteroduplex model.

Materials and methods

Plant material

Four cranberry populations (1) 'Wilcox' × 'Stevens' consisting of 30 progeny, and (2) 25 selfed progeny of a cranberry clone WA #2 (Rutgers I. D. #US93-93) (3) 44 selfed progeny of 'Stevens' and (4) 24 selfed progeny of 'Wilcox' were scored for the presence/absence of the six pairs of associated RAPDs indicated in Table 1. These associated RAPDs (AR) were identified in two separate studies in which silver-stained RAPD markers were used to genotype and assess genetic diversity both within and between cranberry varieties (Novy et al. 1994; Novy and Vorsa 1995). 'Wilcox' and 'Stevens' are cranberry cultivars, and WA #2 is a clonal selection from a Washington State cranberry bog. Leaves were collected from field ('Wilcox' × 'Stevens' population) and greenhouse (WA#2, 'Stevens' and 'Wilcox' selfed populations) plants, frozen in liquid nitrogen and stored at –73°C.

DNA extraction and quantification

DNA was extracted from frozen leaf material as described by Stewart and Via (1993). Modification of this procedure was the grinding of liquid nitrogen-cooled leaf material with 1.5 ml of polypropylene mixers (Kontes, Vineland, N.J.) prior to the addition of 65°C extraction buffer [ascorbic acid and diethyldithiocarbamic acid (DIECA) were not included in the buffer]. DNA pellets were dissolved in 25 µl of sterile, distilled water. Ribonuclease A was then added at a final concentration of 10 $\mu g \cdot ml^{-1}$ and tubes were incubated at 37°C for 30 min. DNA concentrations were quantified with a TKO-100 fluorometer (Hoefer Scientific Instruments, San Francisco). Those isolates with readings of greater than 10 $ng \cdot \mu l^{-1}$ were diluted to a standard 5 $ng \cdot \mu l^{-1}$, while those with readings at or below 10 were not diluted further. With 3 µl of DNA added to a PCR reaction, the amount of DNA in the PCR reaction ranged from 3–30 ng. Analogous to observations by Weeden et al. (1992), this DNA template range has been found to provide reproducible RAPD profiles.

Table 1 Associated ssRAPDs in cranberry and the primers responsible for their synthesis. Their presence (+) or absence (–) in the parents of the populations used in the inheritance analysis is also given

Primers[a]	RAPD sizes		Presence/absence of RAPDs in parents		
	(Base pairs)	Designation	Wilcox	Stevens	WA#2
OPA-4	1700, 1800	AR[b]-1 and 2	+	+	+
OPA-7	850, 3100	AR-3 and 4	+	+	+
OPA-9	445, 520	AR-5 and 6	–	+	–
OPA-9	1250, 1350	AR-7 and 8	+	–	–
OPA-11	435, 450	AR-9 and 10	–	–	+
OPA-11	1350, 1360	AR-11 and 12	+	–	–

[a] Operon technologies, Almeda, California
[b] AR is the abbreviation for "Associated RAPDs"

Amplification, scoring, analysis of RAPDs, and DNA intermixing

Polymerase chain reaction (PCR) solutions consisted of 1× buffer (Perkin Elmer Cetus, Norwalk, Conn.), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µm of primer, 1.25 Units of *Taq* polymerase, Stoffel Fragment (Perkin Elmer Cetus), and 3–30 ng of genomic DNA. The final 13-µl reaction volume was placed in a Perkin Elmer Cetus 480 DNA thermal cycler with a DNA amplification program of 7 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. A ramp time of 2 min was included during heating from 36°C to 72°C. At the completion of 40 cycles, reactions were kept at 72°C for 5 min and then cooled to 4°C. Amplified DNA fragments were separated on a 6.75% acrylamide/bis gel in a 0.375 M Tris, pH 8.8 buffer, using a Mini-Protean II gel system (Bio-Rad Laboratories, Hercules, Calif). DNA was visualized using a standard silver-stain (Bio-Rad) protocol, with the exception that fixation was performed in 10% acetic acid for 30 min. To determine if associated RAPDs were examples of heteroduplexes, equal amounts of amplified DNA from individuals lacking the associated RAPDs were intermixed, EDTA at a final concentration of 5 mM was added to inhibit residual Stoffel fragment activity, and the entire volume was heated to 94°C and subsequently allowed to re-anneal at room temperature.

Expected segregation patterns under genetic models

Tightly-linked dominant RAPD markers in coupling phase, or a primer-recognition site within a larger template model

Under these models, the associated RAPDs would be expected to follow a dominant mode of inheritance. Individuals homozygous and heterozygous for RAPD amplification sites would be classified as being RAPD (+), while homozygotes lacking the site would comprise the null (–) class. Under such a model, the selfed progeny of individuals with these RAPDs would be expected to: (1) not segregate for the associated RAPDs if homozygous for the amplification sites, or (2) segregate 3 (+): 1 (–) if heterozygous. Progeny of crosses, e.g., 'Wilcox' × 'Stevens', would be expected to: (1) not segregate if at least one parent was homozygous for the amplification sites, (2) segregate 1(+): 1(–) if one parent was heterozygous (+) and the other (–), and (3) segregate 3 (+): 1(–), if both parents were (+) heterozygotes.

Heteroduplex model

If the associated RAPD markers are heteroduplexes formed between two allelic amplification sites, individuals with associated RAPDs would be the heterozygote class, while the null individuals would comprise one of the two homozygote classes. The associated RAPDs would be expected to segregate 1(+): 1(–) in progeny derived from selfing of (+) individuals, in crosses where one parent was (+) and the other (–), and if both parents are (+).

Based on expected segregation ratios, it is not possible to discriminate the dominant inheritance models from the heteroduplex model with (+) by (–) crosses. Only upon selfing of (+) individuals or intercrossing (+) individuals are the expected segregation ratios between the dominant inheritance models, 3(+): 1(–), versus the heteroduplex model, 1(+): is 1 (–), discriminating. Another method of differentiating the heteroduplex model from the other two models is the intermixing of the amplified DNA of null individuals. If the associated RAPDs are a result of heteroduplex formation, then a proportion of such DNA intermixings (those between the different homozygote classes) should result in heteroduplex formation. The two homozygote classes would only be expected when (+) genotypes are intercrossed or selfed. In (+) by (–) crosses, only one of the two homozygote classes is generated, and intermixing the DNA of null individuals would not be expected to result in re-formation of associated RAPDs in these types of progenies. With the tightly linked dominant RAPD markers in coupling phase or a primer-recognition site within a larger template model, the reconstitution of associated RAPDs by intermixing the DNA of null individuals would not be expected.

In theory, RAPD heteroduplex formation could also result from the interaction of amplification products from different loci (Ayliffe et al. 1994). While this source of heteroduplex formation cannot be entirely ruled out, their occurrence can be explained with a simpler one-locus model as the result of interactions among allelic sequences from a single locus. In the few studies that have analyzed RAPD heteroduplex formation (Hunt and Page 1992; Ayliffe et al. 1994; Davis et al. 1995), their occurrence has been attributed to interactions among alleles of the same locus.

Assignment of genotypes to progeny on the basis of DNA intermixing

Under a two-allele heteroduplex model of inheritance, with *1* and *2* designations given to the RAPD alleles, progeny exhibiting heteroduplex RAPDs would be classified as heterozygotes (genotype=*1,2*). Progeny lacking such markers represent the two homozygote classes (*1,1* and *2,2*). Intermixing of amplified DNA from each of the two homozygote classes (i.e., *1,1* + *2,2*) should produce the heteroduplex RAPDs in question, whereas no heteroduplex formation should be seen when the DNA of homozygous individuals of the same class are combined (i.e., *1,1* + *1,1*, or *2,2* + *2,2*). Through systematic intermixing of the amplified DNA of the null progeny derived from selfing or intercrossing two heterozygous genotypes, it should be possible to assign them to one of the two homozygote classes expected based on the presence/absence of heteroduplex (associated RAPDs) formation. The inability to reconstitute the associated RAPDs following the intermixing of DNA from null individuals would support the dominant inheritance models. In a test cross, (+) by (–), only one null-class genotype is expected and heteroduplex formation would not be expected.

Results

Segregation of associated RAPDs

The segregation data of the six pairs of associated RAPDs in each of the cranberry progenies are given in Table 2. Five of the six pairs segregated for presence/absence in the populations, the exceptions being AR-3 and 4, which were present in all the progenies of 'Wilcox'×'Stevens' and WA#2 selfed progeny. This lack of segregation suggests that AR-3 and 4 are either examples of tightly linked loci in coupling phase or else of a primer-recognition site within a larger template. All associated RAPDs were either jointly present or absent in the segregating progenies.

The observed segregation patterns of AR-1 and 2 and AR-5 and 6 did not allow for the rejection of any model. The AR-1 and 2 segregation ratios in both 'Wilcox' × 'Stevens' and WA#2 selfed progenies were approximately in between the 1:1 and 3:1 ratios expected (Table 2), and neither of the two expected segregation ratios can be rejected at $P<0.05$. The AR-5 and 6 segregation ratio was consistent with the 1: 1 ratio expected with both models in the 'Wilcox' (–) × 'Stevens' (+) cross (Table 2). Segregation of 'Stevens' selfed progeny for AR-5 and 6 also exhibited a ratio approximately intermediate to 1:1 and 3:1 ratios (Table 2).

The observed segregation patterns for AR-7 and 8 and AR-9 and 10 were all consistent with the heteroduplex segregation ratios (Table 2). The segregation ratio of AR-7

Table 2 Segregation of associated RAPDs and fit to expected segregation patterns under dominant versus heteroduplex models in 'Wilcox'×'Stevens' progeny, and selfed progeny from WA#2, 'Wilcox' and 'Stevens'

Markers	Parental phenotype		Obs. ratios (+ : –)	Genetic models						In heteroduplex model, formation of RAPDs through intermixing DNA of null phenotypes[b]	
				Dominant inheritance[a]			Heteroduplex				
	♀	♂		Exp.[c]	χ^{2d}	*P*	Exp.	χ^{2d}	*P*	Exp.	Obs.
'Wilcox' × 'Stevens' progeny											
AR-1 and 2	+	+	19 : 11	3 : 1	1.60	0.21	1 : 1	1.63	0.20	Yes	Yes
AR-3 and 4	+	+	30 : 0	All (+)	–	–	1 : 1	28.00	<0.001	–	–
AR-5 and 6	–	+	14 : 16	1 : 1	0.03	0.86	1 : 1	0.03	0.86	No	No
AR-7 and 8	+	–	13 : 17	1 : 1	0.30	0.58	1 : 1	0.30	0.58	No	No
AR-11 and 12	+	–	6 : 24	1 : 1	9.63	<0.001	1 : 1	9.63	<0.001	No	Yes
WA#2 selfed progeny											
AR-1 and 2	+	+	16 : 9	3 : 1	1.08	0.30	1 : 1	1.44	0.23	Yes	Yes
AR-3 and 4	+	+	25 : 0	All (+)	–	–	1 : 1	23.00	<0.001	–	–
AR-9 and 10	+	+	13 : 12	3 : 1	5.88	0.02	1 : 1	0	1	Yes	Yes
'Stevens' selfed progeny											
AR-5 and 6	+	+	28 : 16	3 : 1	2.46	0.12	1 : 1	2.75	0.10	Yes	Yes
'Wilcox' selfed progeny											
AR-7 and 8	+	+	13 : 11	3 : 1	4.50	0.03	1 : 1	0.04	0.84	Yes	Yes
AR-11 and 12	+	+	14 : 18	3 : 1	15.04	0.001	1 : 1	0.28	0.60	Yes	Yes

[a] Dominant inheritance models include tightly linked dominant markers in coupling phase and/or a primer-recognition site within a larger template sequence model
[b] Two-allele model
[c] If segregation observed, then fit tested to a 3:1 ratio in (+) by (+) crosses, and a 1:1 ratio in (+) by (–) crosses
[d] Yates' correction for continuity used in the calculation of χ^2-values

and 8 in the 'Wilcox' (+) × 'Stevens' (–) cross did not differ significantly from the 1: 1 ratio expected under both dominant inheritance and heteroduplex models. However, based on the segregation ratio in the 'Wilcox' selfed progeny, the dominant inheritance models (3: 1) can be rejected, while the heteroduplex model (1: 1) cannot be rejected (Table 2). The AR-9 and 10 segregation pattern in the WA#2 progeny was consistent with the heteroduplex model, while the 3: 1 ratio, dominant inheritance models, can be rejected (Table 2).

The observed segregation of AR-11 and 12 deviated significantly from the 1: 1 segregation ratio expected in the 'Wilcox' (+) × 'Stevens' (–) progeny (Table 2). AR-11 and 12 markers were under-represented in favor of null (–) types in a 1: 4 ratio. The observed AR-11 and 12 segregation ratio in the 'Wilcox' selfed progeny was consistent with the ratio expected (1:1) in the heteroduplex model, while the dominant inheritance (3:1) models can be rejected.

Intermixing DNA of null types and assignment of progeny to genotypic classes

The formation of RAPDs through the intermixing of amplified DNA from null progeny provides evidence that associated RAPDs are a result of heteroduplex formation. This procedure would be effective in discriminating the heteroduplex model from the dominant inheritance models, particularly in those cases where the observed associated RAPD segregation ratios did not allow for the rejection of either the dominant inheritance or heteroduplex models (i.e., AR-1 and 2, AR-5 and 6 and AR-7 and 8). AR-1 and 2, AR-5 and 6, AR-7 and 8, AR-9 and 10, and AR-11 and 12 were successfully synthesized through the intermixing of DNA of progeny missing such RAPDs (null progeny) suggesting they are examples of heteroduplexes (Table 2 and Fig. 1).

A systematic intermixing of DNA from null progeny, lacking associated RAPDs, for populations segregating for AR-1 and 2, AR-5 and 6, AR-7 and 8, AR-9 and 10, and AR-11 and 12 was conducted. This method allowed for the assignment of the null progeny to one of the two homozygote classes (designated *1,1* or *2,2*) expected with a two-allele heteroduplex model. Progeny with heteroduplex RAPDs were classified as heterozygotes (*1,2*). To maintain a standard classification for the genetic model proposed, these three genotypic designations were used for each of the associated RAPD pairs. The genotype classifications do not correspond across the associated RAPD pairs.

AR-1 and 2

In the 'Wilcox' (+) × 'Stevens' (+) progeny, two null genotypic classes are expected in a two-allele model, and synthesis of associated RAPDs is expected through DNA intermixing (Table 2). The results of DNA intermixing were

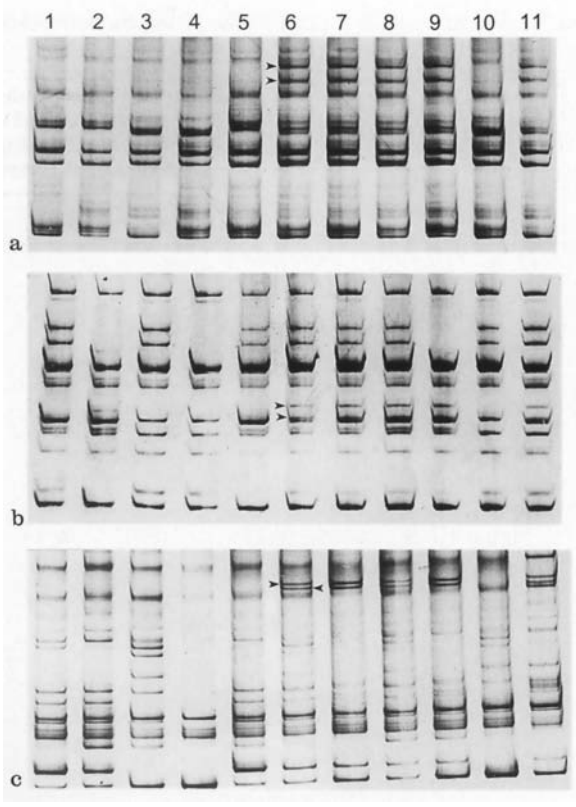


Fig. 1 Synthesis of associated RAPDs AR-1 and 2 (**a**), AR-9 and 10 (**b**), and AR-11 and 12 (**c**) by intermixing the DNA of null individuals (i.e., lacking the associated RAPDs). In each of the three plates, *lanes 1 and 2* are progeny from one null class; *lanes 3 and 4*, progeny from the other null class. *Lane 5*, DNA from clones in *lanes 1 and 2* intermixed (abbreviated 1+ 2); *lane 6*, (1 + 3); *lane 7*, (1 + 4); *lane 8*, (2 + 3); *lane 9*, (2 + 4); *lane 10*, (3 + 4); *lane 11*, heterozygous progeny with heteroduplex RAPDs used as a reference. Intermixing of progeny DNA from different null classes recreates the heteroduplex RAPDs (*lanes 6, 7, 8, 9*) while intermixing of DNA from the same null class does not (*lanes 5 and 10*). *Arrows* indicate the heteroduplex RAPDs of interest

consistent with this hypothesis. The 11 null individuals for AR-1 and 2 separated into two classes of 6 and 5 respectively, based upon the DNA intermixing. The genotypic segregation was 6 (*1,1*): 19 (*1,2*): 5 (*2,2*), which is consistent with an expected genotypic ratio of 1:2:1 (χ^2=2.22, *P*=0.33).

In the WA#2 progeny, the nine null individuals separated into the two genotypic classes having seven and two individuals, respectively. DNA of representative progeny from each of the two homozygote classes was intermixed with *1,1* or *2,2* genotypes from the 'Wilcox' × Stevens' population in order to assign consistent genotypic classes across populations. The final segregation ratio was 7(*1,1*):16(*1,2*):2(*2,2*) which is consistent with the expected genotypic ratio of 1:2:1 (χ^2=3.93, *P*=0.14).

AR-5 and 6

The synthesis of AR-5 and 6 was not expected, nor observed, when the DNA of null progeny from 'Wilcox' (–) × 'Stevens' (+) cross was intermixed (Table 2). However, it was observed that the 'Wilcox' × 'Stevens' progeny segregated into four distinct classes based on their RAPD profiles in the proximity of AR-5 and 6. Two of the four classes were parental, in that these RAPD profiles were observed in 'Wilcox' or 'Stevens'. The remaining RAPD profiles, however, were non-parental, and one contained a RAPD not previously observed in either parent. Assignment of progeny to one of these four RAPD profile classes gave a segregation ratio of 8:6:9:7. This ratio is consistent with a three-allele heteroduplex model, where 'Wilcox' is assigned the genotype *2,3* and 'Stevens' the genotype *1,2*. Intercrossing these two parents would produce four genotypic classes of progeny (*1,2*, *1,3*, *2,3*, and *2,2*) (Fig. 2), with classes *1,2* and *2,3* corresponding to the parental genotypes. The observed segregation showed a good fit to the expected ratio (χ^2=0.67, *P*=0.88), under the three-allele model. In this model, the two upper bands observed in the profiles of *1,2* and *1,3* would be heteroduplexes between the alleles (observed as the two lower bands) comprising that genotype; allele *1* is seen as the smaller-sized fragment i.e., the lowest band (Fig. 2). The association observed between AR-5 and 6 is noteworthy in that it would not be between two heteroduplex RAPDs, but instead between a heteroduplex (migrating as a 520-bp fragment) and a homoduplex (allele *1*, migrating as a 445-bp fragment) (Fig. 2). The non-parental RAPD observed in six of the progeny was postulated to be a heteroduplex formed between alleles *1* and *3*. These two alleles, not present together in either of the parents, are a result of "non-parental" heteroduplexes observed in the six progeny with the *1,3* genotype.

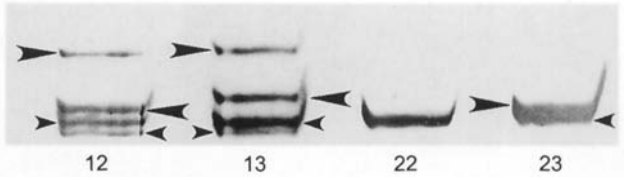


Fig. 2 The four ssRAPD profiles observed in 'Wilcox' × 'Stevens' progeny near associated RAPDs 5 and 6. Genotypic designations represented by each ssRAPD profile are given below each lane. Genotype *1, 2* has a ssRAPD profile consisting of two homoduplex RAPDs (*small arrows*) and two heteroduplexes (*large arrows*). The ssRAPD profile of genotype *1, 3* also consists of two homoduplexes (*small arrows*) and two heteroduplexes (*large arrows*). Genotype *2, 2*, as expected, is represented by one RAPD. Genotype *2, 3* has a ssRAPD profile consisting of two RAPDs. The upper RAPD (*large arrow*) is thought to represent both heteroduplexes (not resolved as separate RAPDs), and the lower RAPD represents the homoduplexes of alleles *2* and *3* (not resolved as separate RAPDs)

The upper heteroduplex RAPDs of genotypes *1,2* and *1,3* migrated similarly during electrophoresis and were initially thought to be the same RAPD (i.e., a 520-bp fragment). On the basis of this model, however, this 520 RAPD is represented by two heteroduplexes. An increase in the duration of electrophoresis by 7 min did indicate two distinct RAPDs.

The three-allele heteroduplex model is also consistent with the inability to synthesize AR-5 and 6 by the inter-

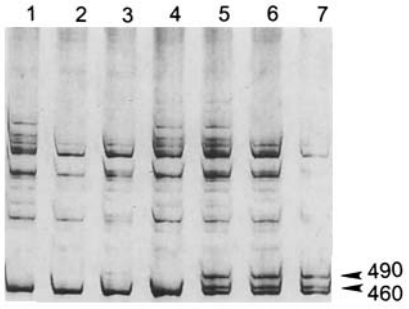


Fig. 3 Heteroduplex RAPDs observed following the intermixing of DNA of null individuals from the 'Wilcox' × 'Stevens' and WA#2 populations. *Lane 1*, amplified DNA of WI × ST #4 (genotype *2,2)*; *lanes 2 and 3*, amplified DNA of WA#2–3 (genotype *2,2*) and WA#2–16 (genotype *4,4*) – representatives from each of two null classes identified in the WA#2 population. Heteroduplex RAPDs migrating as 460- and 490-base pair fragments were recreated when DNA of WI×ST #4 was intermixed with DNA of WA#2–16 (*lane 5*), but not when intermixed with DNA of WA#2–3 (*lane 4*). Intermixing of the two WA#2 progeny also recreates the heteroduplex RAPDs (*lane 6*). *Lane 7* contains amplified DNA of WA#2–20, a progeny with the presence of the heteroduplex RAPDs (genotype *2,4*)

mixing of null individuals from the 'Wilcox' × 'Stevens' population. Genotypes *2,2* and *2,3* would have been the null individuals, both lacking allele *1*. Without this allele, AR-5 and 6 would not be observed following DNA intermixing.

RAPDs 445 and 520, (i.e., AR-5 and 6) were not present within WA#2 progeny. However, in the proximity were RAPDs with approximate fragment sizes of 460 and 490. These previously unidentified RAPDs segregated together and in a ratio of 11(+):14(–). The observed segregation did not fit the 3:1 ratio expected under a dominance model of RAPD inheritance (χ^2=11.21, P=0.00), but did fit a 1: 1 mode of inheritance expected with a two-allele heteroduplex model (χ^2=0.16, P=0.69). Random intermixing of the amplified DNA of the 14 null progeny resulted in the synthesis of the 460 and 490 RAPDs. Based on the intermixing results, the null progeny separated into two genotypic categories of nine and five individuals respectively, giving a final genotypic segregation ratio of 9:11:5. This observed ratio fits the 1:2:1 genotypic ratio expected under a simple two-allele heteroduplex model of inheritance (χ^2=1.64, P=0.44). This finding, together with the presence in all of the progeny of a RAPD similar in size to alleles *2* and *3* of the 'Wilcox' × 'Stevens' progeny, suggested that these RAPDs might relate to those heteroduplex RAPDs observed in the 'Wilcox' × 'Stevens' progeny. The differential migration of the 460 and 490 heteroduplexes from those observed in the 'Wilcox' × 'Stevens' progeny suggested that additional alleles might exist within the WA#2 population.

To determine if the heteroduplex RAPDs observed between the two populations were products of different alleles, amplified DNA from genotype *2,2* of the 'Wilcox' × 'Stevens' population was intermixed with amplified DNA from each of the two genotypic classes in the WA#2 population. The genotypic class consisting of nine individuals showed no heteroduplex formation following intermixing, whereas RAPDs 460 and 490 were formed using representatives from the other class (Fig. 3). On the basis of these intermixing results, the class consisting of nine individuals was given the genotypic designation *2,2*, while the other class was given a new genotypic classification, *4,4*. Formation of heteroduplex RAPDs 460 and 490 was therefore dependent upon the presence of alleles *2* and *4* within a heterozygous individual.

Within the 'Stevens' selfed progeny, a maximum of two alleles should be present. Intermixing the DNA of null individuals resulted in the synthesis of AR-5 and 6, and two homozygote classes having approximately equal numbers were identified 9(*1,1*): 28(*1,2*):7(*2,2*). The segregation pattern did not differ significantly from a 1:2:1 ratio (χ^2=3.45, P=0.18).

In summary, it appears that four alleles are present across the two populations. Three of the alleles migrate similarly during electrophoresis (alleles *2*, *3*, and *4*), but can be distinguished from one another based on their RAPD patterns of heteroduplex formation. Allele *1* could be distinguished from the other three alleles based on faster migration during electrophoresis. It was not known how alleles *1* and *3* may interact in the presence of allele *4* because of a lack of homozygous *1,1* and *3,3* individuals. DNA from the heterozygotes *1,2* and *1,3* was intermixed with DNA of *4,4* in order to better assess what might be expected under such circumstances (similar to a multi-allelic state encountered in polyploids). The intermixing of DNA from genotypes *1,2* and *4,4* did not produce any distinct RAPDs that could be attributed to heteroduplex formation between alleles *1* and *4*. Such an outcome would be expected if the heteroduplex complexes co-migrated with the homoduplexes, or with other RAPDs that were monomorphic in the progeny. The heteroduplex complexes of alleles *1,2* and *2,4* were observed as expected.

The intermixing of genotypes *1,3* and *4,4* did produce a RAPD not previously seen in the either of the two genotypes. The presence of this RAPD could be attributed to the formation of a heteroduplex complex between alleles *3* and *4*, since alleles *1* and *4* had previously shown no unique RAPD formations.

AR-7 and 8

Consistent with the heteroduplex model, AR-7 and 8 were not recovered through systematic DNA intermixing of the 'Wilcox'(+) × 'Stevens'(–) null progeny (Table 2). Also consistent with this model, was the synthesis of these RAPDs when the DNA from 'Wilcox' selfed progeny lacking AR-7 and 8 was intermixed (Table 2). Two homozygote classes of 7 (*1,1*) and 4 (*2,2*) individuals were identified. The observed segregation pattern of 7(*1,1*):13(*1,2*): 4(*2,2*) did not differ significantly from the expected 1:2:1 ratio (χ^2=0.92 , P=0.63), with the heteroduplex model.

AR-9 and 10

Systematic DNA intermixing of the WA#2 selfed progeny lacking AR-9 and 10 identified two homozygote classes of 5 (*1,1*) and 7 (*2,2*) individuals. The observed genotypic ratio of 5:13:7 is consistent with the 1:2:1 genotypic ratio (χ^2=0.35 , *P*=0.84) expected with the heteroduplex model.

AR-11 and 1 2

Synthesis of AR-11 and 12 was possible through the systematic intermixing of DNA from 'Wilcox'(+) × 'Stevens'(–) progeny lacking AR-11 and 12 which is unexpected in a two-allele heteroduplex model (Table 2). An alternative three-allele heteroduplex model can be proposed which is consistent with both the DNA intermixing results as well with the significant deviation from the 1: 1 segregation in the two-allele heteroduplex model discussed earlier. Under such a model, the parent 'Wilcox' with AR-11 and 12 present, was assigned genotype *1,2*, while 'Stevens' which lacked these RAPDs was classified as genotype *2,3*. Four genotypic classes, *1,2*, *1,3*, *2,2*, and *2,3*, would be expected in the progeny. Under the assumption that AR-11 and 12 are examples of heteroduplex complexes between alleles *1* and *2*, only 25% of the progeny would be expected to display these RAPDs. Under the three-allele model, the 6(+):24(–) segregation of RAPD positive to null individuals is accepted (χ^2=0.18, *P*=0.67).

The 24 progeny lacking AR-11 and 12 should be composed of genotypes *1,3*, *2,2*, and *2,3*. Intermixing of the amplified DNA of genotypes *1,3* with either *2,2* or *2,3* should produce the expected heteroduplex RAPDs, whereas no RAPDs would be expected if genotypes *2,2* and *2,3* are intermixed. Therefore, the 24 progeny should separate into two classes based on the intermixing results (i.e., class A=genotypes *2,2* and *2,3*, class B=genotype *1,3*). Class A would be expected to have twice the number of individuals of class B. Intermixing of the amplified DNA of the 24 null individuals identified two groups of 6 and 18 individuals, approximating the expected outcome under a three-allele model.

It is still not known, however, which genotypes actually represented which class, i.e., did genotypes *2,2* and *2,3* comprise the 18-individual class and genotype *1,3* the 6-individual class or should their representations be reversed? To answer this question, progeny from each of the two classes had their amplified DNA intermixed with that of the 'Stevens' parent (genotype *2,3*). Associated RAPDs 11 and 12 were observed in the intermixing study when 'Stevens' was combined with representatives from the 6-individual class, but not when combined with representatives of the 18-individual class. Such an outcome would be expected if genotype *1,3* represented the 6-individual class (due to heteroduplex formation between alleles *1* and *2*) and genotypes *2,2* and *2,3* represented the other class (no contribution of allele *1*, therefore no heteroduplex formation). Based on these results, a genotypic segregation of 6 (*1,2*):6 (*1,3*):18 (*2,2* and *2,3*) was observed which is in agreement with the expected 1:1:2 ratio of the three-allele heteroduplex model (χ^2=1.20, *P*=0.55).

In selfed progeny of a heterozygous individual, the expected outcome is the same as in a two-allele model. The systematic DNA intermixing of the 'Wilcox' selfed progeny lacking AR-11 and 12 identified two homozygote classes of 10 (*1,1*) and 8 (*2,2*) individuals. The observed genotypic ratio of 10:14:8 does not differ significantly from the 1:2:1 ratio (χ^2=0.75 , *P*=0.69) expected under the heteroduplex model.

Discussion

RAPD technology generally produces dominant molecular markers (Williams et al. 1990), while co-dominant allelic products have been reported infrequently (Hunt and Page 1992; Ayliffe et al. 1994; Shoemaker et al. 1994). Heteroduplex formation may offer a source of RAPD co-dominant markers, where heterozygous individuals carrying both alleles form heteroduplex complexes (Hunt and Page 1992; Ayliffe et al. 1994). Depending upon sequence divergence and the gel system used, heteroduplexes may migrate more slowly during electrophoresis than the homoduplexes from which they were derived (White et al. 1992).

In the present study, five of the six associated RAPDs identified previously in cranberry (Novy et al. 1994) appear to be examples of heteroduplex formation; the intermixing of the amplified DNA of null individuals produced the associated heteroduplex RAPDs. Neither tightly-linked loci in coupling phase nor primer recognition sites within a larger template sequence can account for these DNA intermixing results. On the basis of the DNA intermixing results and the segregation of the associated RAPDs, all five examples of associated RAPDs are consistent with a single-locus heteroduplex model with interactions among the RAPD alleles.

It should be noted that a two-locus (unlinked) heteroduplex model would segregate in a 9 (+):7 (–) ratio, which would be difficult to discriminate from the 1 (+):1 (–) ratio in a one-locus model. In addition, DNA intermixing would be expected to reconstitute these bands. However, one of seven null progeny would not be expected to form any heteroduplex bands through DNA intermixing. This result was not observed in this study. The presence of heteroduplexes should have no impact on the DNA fingerprinting of varieties as long as they can be consistently reproduced. It has been reported that heteroduplex formation can be sensitive to PCR reaction conditions (Hatcher et al. 1993; Ayliffe et al. 1994) in that heterozygous individuals sometimes do not manifest the expected heteroduplex complexes. A second replicate of DNA amplification for each of 77 null progeny examined in this study identified only two instances in which heterozygous individuals did not exhibit the expected heteroduplex RAPDs in the first amplification, indicating a misclassification rate of 2.6% (unpublished data). Both instances of misclassification in-

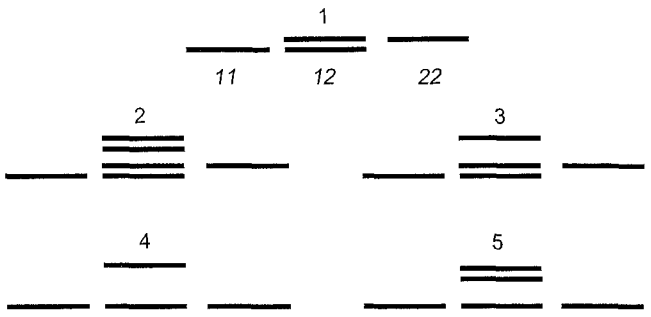


Fig. 4 Banding patterns possible with DNA amplification of two ssRAPD alleles (*1* and *2*) – heterozygote individual (*1,2*) in center lane, homozygotes on either side. (*1*) Both alleles distinguishable on gel – no heteroduplex formation. (*2*) Same as (*1*), but with heteroduplex formation. (*3*) Same as (*2*), but two heteroduplexes migrate as one RAPD. (*4*) Alleles not distinguishable from one another, dual heteroduplexes seen as one RAPD. (*5*) Alleles not distinguishable – two heteroduplexes observed

Table 3 Calculated RAPD differences among genotypes comprising co-dominant genetic system based on the ssRAPD profiles of Fig. 4

ssRAPD profile	RAPD differences among genotypes	
	1,2 -vs- (*1,1* or *2,2*)	*1,1* -vs- *2,2*
1	1	2
2	3	2
3	2	2
4	1	0
5	2	0

volved AR-9 and 10 which stained the lightest of the associated RAPDs examined in the study. The use of the silver-staining technique, with its increased sensitivity for DNA visualization (2–5 fold more sensitive than ethidium bromide) and 40 cycles of PCR amplification [exceeding the 30 cycles of PCR amplification suggested by Hatcher et al. (1993) for consistent heteroduplex formation], ensured high levels of accuracy in the identification of heterozygotes.

The presence of heteroduplex RAPDs could potentially have an impact on pedigree or genealogical studies in that the intercrossing of two parents with alternate alleles could produce non-parental (heteroduplex) RAPDs in the heterozygous progeny (Hadrys et al. 1992). Such an outcome was reported in an F1 strain of flax rust (Ayliffe et al. 1994), and also in the present study when a non-parental RAPD was observed in the progeny of 'Wilcox' and 'Stevens' when screened with primer OPA-9. Heteroduplex formation can also explain the presence of a non-parental RAPD found in a cocoa hybrid and half of its backcross progeny (Ronning et al. 1995). While the authors had no explanation for the presence of the non-parental RAPD, its segregation and the description of proximal RAPDs are completely consistent with a heteroduplex model (Appendix 1).

Heteroduplex formation would not be unique to cranberry (Davis et al., 1995). At this time it is difficult to ascertain the frequency of this class of RAPD markers in plant species because their aberrant segregation has most likely led to their exclusion in mapping and inheritance studies. For example, individuals with the presence of heteroduplex RAPDs (i.e., heterozygous genotypes carrying both RAPD alleles) would when selfed segregate 1:1 rather than 3:1 for the presence/absence of the RAPDs.

Heteroduplex formation may also complicate the interpretation of relationships among clones by confounding genetic-distance estimates. In a simple two-allele heteroduplex model with heteroduplexes distinguishable from homoduplexes, four different RAPD patterns are possible (Fig. 4). The differences in RAPD profiles are strictly a consequence of the ability/inability to distinguish between the homoduplexes (alleles) and the two possible heteroduplex products using a given gel system. The phenotypic variance in RAPD profiles can have an impact on the calculation of genetic dissimilarity estimates among individuals (Table 3), in most cases by overestimating genetic distances between the homozygote and heterozygote classes, and underestimating distances between the homozygote classes. Distance estimates can become even more confounded as the number of heteroduplex alleles increases, due to the potential formation of a larger number of heteroduplex complexes.

The degree to which heteroduplexes confound genetic-relatedness studies in plant species will most likely be influenced by the mating system and the ploidy level of the organism. Cranberry has protandrous flowers which promote outcrossing, although self-pollination is also tolerated (Sarracino and Vorsa 1991). Outcrossing species, such as cranberry, might be expected to have higher frequencies of heteroduplex formation than autogamous crops, due to a higher frequency of heterozygous loci with the potential to form heteroduplex complexes. This hypothesis is supported in that eight pairs of associated RAPDs (i.e., 16 RAPDs) were identified among a total of 66 RAPDs in cranberry (Novy et al. 1994). Therefore, potentially 24% of the total RAPDs in the study could be examples of heteroduplex formation. Of those eight pairs of associated RAPDs, one pair in the present study was shown to be an example of heteroduplexes (AR-1 and 2), while another pair proved to be an association between an allele and one of its heteroduplexes (AR-5 and 6).

Outcrossing polyploid crops, such as blueberry or potato, might be expected to have even higher levels of heteroduplex formation than diploid species since multiple alleles at a given amplification site or locus are possible. A tetraploid individual with four co-dominantly expressed alleles at a locus could potentially form ten heteroduplex complexes.

Hatcher et al. (1993), with primers designed to flank a *B*-globin gene, observed heteroduplex formation between amplified alleles only with >30 cycles of amplification. They hypothesized that in later PCR amplification cycles, the concentration of the amplified alleles is greater than that of the remaining unincorporated primer. Heteroduplex formation subsequently occurs when the primer is outcompeted by the allelic fragments during the annealing phase.

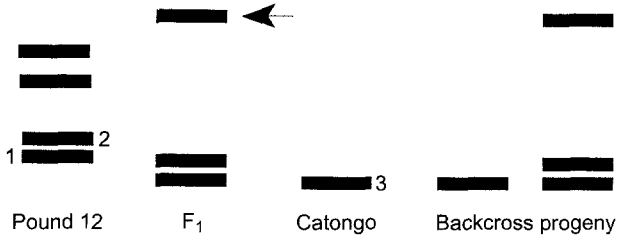
An informal review of the RAPD literature indicates that most researchers have found 35–45 cycles of DNA amplification to be optimal. Theoretically, a reduction in this number of amplification cycles could protect against heteroduplex formation and negate their impact on genetic-relatedness studies.

Based upon the findings of Hatcher et al. (1993), the DNA of heterozygote progeny with the heteroduplex RAPDs AR-1 and 2, AR-9 and 10, and the described heteroduplex RAPDs associated with AR-5 and 6, were amplified for 20, 25, and 30 cycles (unpublished data). This was conducted so as to evaluate the impact of cycle number on RAPD heteroduplex formation. Twenty cycles was not sufficient for the visualization and scoring of amplified DNA. Twenty-five and 30 cycles of DNA amplification did produce RAPD profiles which were scorable, although substantially lighter than our standard 40 cycles of amplification. The heteroduplexes associated with AR-5 and 6 were not seen after 25 cycles of amplification, but were observed after 30 cycles. AR-1 and 2, and AR-9 and 10 were present after both 25 and 30 cycles of amplification. Therefore, it appears that decreasing the number of amplification cycles to less than 30 does not eliminate heteroduplex formation in all instances, and therefore cannot be considered as a solution for preventing heteroduplex formation.

While heteroduplex RAPDs have the potential to confound genetic-distance estimates and pedigree studies, they also offer a source of co-dominant RAPD markers. Co-dominant markers are more informative than dominant markers in population and pedigree studies. The presence of "associated RAPDs", as observed in cranberry, or non-parental RAPDs in hybrid progeny populations, may be indicative of heteroduplex formation. Once confirmed that these RAPDs are heteroduplexes (by the intermixing of amplified parental or null progeny DNA) these associated RAPDs can be excised from the gel and jointly re-amplified using the same primer as described by Weaver et al. (1994). Alternatively, if in high enough concentrations, excised heteroduplexes could be jointly de-natured and allowed to re-anneal without the need for further amplification. In either of the two protocols, the presence of both heteroduplex RAPDs and the de-naturing and re-annealing of their DNA strands should allow for the identification of the homoduplex RAPDs (i.e. the two co-dominantly expressed RAPD alleles) following gel electrophoresis. This has been successfully done with heteroduplexes identified in this study (unpublished data). Following identification, the co-dominant alleles can be cloned, sequenced to determine how they differ, and oligonucleotide primers specific for their amplification can then be constructed.

Appendix 1

Ronning et al. (1995) produced an F1 hybrid between two cultivars of cocoa, 'Pound 12' and 'Catongo', this F1 was then backcrossed using 'Catongo' as the recurrent parent. The authors noted the appearance of a non-parental RAPD in the hybrid and half of the backcross progeny when primer H15 was used. The observed RAPD patterns are shown below and were taken from Fig. 3 of Ronning et al. (1995):



The non-parental RAPD in the F1 (arrow) could not be explained by the authors. However, based upon the observed RAPD patterns and segregation data, this non-parental RAPD could be an example of a heteroduplex RAPD. 'Pound 12' can be assigned the genotype *1,2*, with the RAPD alleles evident as the two lower bands – the two upper bands being heteroduplexes formed between the two alleles (viewed as a single band in agarose gels, the two bands were only separated in acrylamide gels). 'Catongo' can be assigned the genotype *3,3*, indicating it is homozygous for a third allele not present in 'Pound 12'. The resulting F1, based upon its RAPD pattern, is the genotype *1,3* with its two heteroduplex species migrating as one band, a band which would not be seen in either of the two parents. Subsequent backcrossing of the hybrid to 'Catongo' produces two genotypes in the backcross progeny, *3,3* which lacks the non-parental RAPD, and *1,3* with the presence of the non-parental RAPD. The backcross progeny would therefore segregate for the non-parental RAPD in a 1:1 ratio, as reported by the authors.

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