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# Comparison of RAPD and RFLP genetic markers in determining genetic similarity among *Brassica oleracea* L. genotypes

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**Abstract.** Genetic similarity among 45 *Brassica oleracea* genotypes was compared using two molecular markers, random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLPs). The genotypes included 37 broccolis (var. *italica*), five cauliflowers (var. *botrytis*) and three cabbages (var. *capitata*) which represented a wide range of commercially-available germplasm, and included open-pollinated cultivars, commercial hybrids, and inbred parents of hybrid cultivars. Fifty-six polymorphic RFLP bands and 181 polymorphic RAPD bands were generated using 15 random cDNA probes and 62 10-mer primers, respectively. The objectives were to compare RFLP and RAPD markers with regard to their (1) sampling variance, (2) rank correlations of genetic distance among sub-samples, and (3) inheritance. A bootstrap procedure was used to generate 200 random samples of size n ($n = 2, 3, 5, \ldots 55$) independently from the RAPD and RFLP data sets. The coefficient of variance (CV) was estimated for each sample. Pooled regressions of the coefficient of variance on bootstrap sample size indicated that the rate of decrease in CV with increasing sample size was the same for RFLPs and RAPDs. The rank correlation between the Nei-Li genetic similarity values for all pairs of genotypes (990) based on RFLP and RAPD data was 0.745. Differences were observed between the RFLP and RAPD dendrograms of the 45 genotypes. Overlap in the distributions of rank correlations between independent sub-samples from the RAPD data set, compared to correlations between RFLP and RAPD sub-samples, suggest that observed differences in estimation of genetic similarity between RAPDs and RFLPs is largely due to sampling error rather than due to DNA-based differences in how RAPDs and RFLPs reveal polymorphisms. A crossing algorithm was used to generate hypothetical banding patterns of hybrids based on the genotypes of the parents. The results of this study indicate that RAPDs provide a level of resolution equivalent to RFLPs for detemination of the genetic relationships among genotypes.

**Key words:** *Brassica oleracea* var. *italica* – *B. oleracea* var. *botrytis* – B. *oleacea* var. *capitata* – Genetic distance – Genetic similarity – Crossing algorithm – Cluster analysis

## Introduction

Knowledge of genetic similarity (distance) between genotypes is useful in a breeding program because it facilitates efficient sampling and utilization of germplasm resources. The breeder can use genetic similarity information to make informed decisions regarding the choice of genotypes to cross for the development of populations, or to facilitate the identification of diverse parents to cross in hybrid combinations in order to maximize the expresion of heterosis (Smith et al. 1990; Nienhuis and Sills 1992).

Estimates of genetic similarity based on restriction fragment length polymorphisms (RFLPs) have been shown to be consistent with expectations based on known breeding behavior and pedigrees in numerous crops, including maize (Lee et al. 1989; Melchinger et al. 1990a, b; Smith et al. 1990; Messmer et al. 1992) and among *Brassica oleracea* genotypes (Nienhuis et al. 1992).

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In 1990 two groups of scientists independently described a new technique for detecting polymorphism at the DNA level using PCR with single random oligonucleotide primers of arbitrary sequence (Welsh and McClelland 1990; Williams et al. 1990). Such polymorphisms can occur either as a result of base pair or positional changes in the restriction sites (RFLP) or primer sites (RAPD) which flank a chromosomal location. RFLPs can be detected by hybridization of labeled DNA clones containing sequences homologous to a portion of the chromosomal fragment; whereas, RAPDs are detected by differential amplification of DNA fragments. The principal advantage of RAPDs compared to RFLPs is the technical simplicity of the methodology (Williams et al. 1990; Caetano-Anolles et al. 1991; Paran et al. 1991; Welsh et al. 1991). The principal disadvantage of RAPDs compared to RFLPs is that they are usually dominant rather than codominant markers. In addition, the reproducibility of RAPD banding patterns can be affected by different concentrations of reaction components and cycle conditions (Weeden et al. 1992).

In order for plant breeders to make informed decisions regarding the choice of molecular marker technology, comparisons of the inheritance and reliability of RAPDs and RFLPs are needed. The objectives of this study were to: (1) compare the sampling variance of RFLP vs RAPD markers, (2) compare estimates of genetic similarity based on RFLP and RAPD data, and (3) compare the inheritance of RAPD and RFLP banding patterns within and among three cultivated subspecies of *Brassica oleracea*, var. *botrytis* (cauliflower), var. *capitata* (cabbage) and var. *italica* (broccoli).

## Materials and methods

### *Germplasm*

Forty-five *B. oleracea* L. genotypes, previously described and characterized in a companion publication by Nienhuis et al. (1992) using RFLPs, were employed in this study. The choice of genotypes was influenced by the desire to focus on broccoli genotypes and also by the quantity and quality of remnant DNA samples. The genotypes included 37 broccolis (var. *italica*), five cauliflowers (var. *botrytis*) and three cabbages (var. *capitata*), which represented a wide range of commercially-available germplasm, and included open-pollinated cultivars, vintage cultivars, commerical hybrids, and inbred parents of hybrid cultivars. In addition, by identiying "off-type" plants in spaced plantings of commerical hybrid plants, the possible female parents corresponding to several hybrid cultivars were identified (Nienhuis et al. 1992).

### *RFLP and RAPD procedures*

Plant DNA was isolated from lyophilized leaf tissue and a restriction endonuclease (*EcoR1*) was used to digest the crude DNA samples. The procedures for DNA isolation, restriction endonuclease digestion, electrophoresis, Southern blotting, hybridization and autoradiography have all been previously described (Song et al. 1988; Slocum et al. 1993). A total of 16 random *Brassica* genomic clones containing low-copy- number inserts were individually hybridized to *EcoRl*-digested total genomic DNA samples. The set of random genomic clones utilized as probes in this study included EW1G03, EW1D02, EW2D03, EW2A06, EW2A07, EW1D03, EW1F08, EW4A05, EW3D07, EW5F07, EW4G11, EW2B12, EW4G08, EW3C10, EW2E07 and EW1E04 (Pioneer Hi-Bred International, Johnston, Iowa). Frequently the probes hybridized to multiple restriction fragments within individual samples, which resulted in complex banding patterns. From one to seven bands, which were polymorphic among this sample of genotypes, were scored for each of the 16 probes resulting in a total of 56 scored fragments.

The same DNA samples used in the previous RFLP analyses were used as templates for RAPD reactions. The DNA was recovered from lyophilized remnant samples that had been stored for 3 years in a $-70\,^{\circ}C$ freezer at Agridyne Incorporated, Salt Lake City, Utah. RAPD reactions were mixed in volumes of 10 µl using the following reagents: 20 ng of genomic template, 100 µM of dNTP, 0.4 µM of primer, 0.6 units of *Taq* polymerase (Promega, Madison, WIS.), 2.0 mM of $MgCl_2$, 50 mM of Tris pH 8.5, 20 mM of KCl, 5 µg/µl of BSA, 2.5% of Ficoll 400, and 0.002% xylene cyanole. The reactions were performed in glass capillary tubes in a thermal cycler (Idaho Technology, Idaho Falls, Idaho) programmed to cycle 40 times under the following conditions: for the first two cycles, denaturation for 60 s at 91 °C, annealing for 7 s at 42 °C and elongation for 70 s at 72 °C; the subsequent 38 cycles were run with the denaturation time reduced to 1 s at 91 °C. After amplification the reaction products were separated by electrophesis in 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light with Polaroid 667 film.

The number of polymorphic primers used from sets A, B, C, D, E, F, G, L, M and N (Operon Technologies, Alameda, Calif.) were 8, 10, 8, 4, 3, 4, 8, 10, 4 and 3, respectively. Information on specific primers is available from the authors. Polymorphic bands were classified as intense, medium or faint, based on resolution and degree of amplification (Weeden et al. 1992). Only bands classified as intense or medium were included in the analysis. From one to six bands, which were polymorphic among this sample of genotypes, were scored for each of the 62 probes, resulting in a total of 181 scored bands.

### *Genetic similarity estimates*

Polymorphic RFLP or RAPD bands across all genotypes were each assigned numbers $(1, 2, 3, \ldots n)$ according to decreasing molecular weights. For both RAPD and RFLP data, each band was treated as a unit character, and the genotype was scored for the presence or absence of band and coded as 1 or 0, respectively. Genetic similarities were calculated between pairs of genotypes based on the method similar to that reported by Nei and Li (1979), i.e.,

$$GS(XY) = C(XY)/[N(XY)]$$

where (GS(XY) is the measure of genetic similarity between a pair of lines, C(XY) is the number of concordant bands (both present or absent) between lines X and Y, and N(XY) is the total number of bands scored for lines X and Y, respectively. The rank correlation between the RFLP and RAPD genetic similarity matrices was calculated.

### *Sampling variance*

To compare the variance of genetic similarity estimates based on RFLP and RAPD data, 200 random bootstrap samples each of

size n (n = 3, 5, 7, 12, 33 and 55) were drawn independently from the RAPD and RFLP data sets (Enfron and Tibshirani 1986). The genetic similarity between all pairs of genotypes (1990) was calculated for each bootstrap sample. The variance among the 200 bootstrap samples for each pair of ecotypes was standardized to the coefficient of variance (CV) by dividing the variance by the bootstrap sample mean. Natural log transformations were used to linearize the relationship between the coefficient of variation (CV) and the number of bands in each sample (sample size).

*Cluster analysis and correlation between independent estimates of genetic similarity*

Cluster analyses were done independently for the RFLP and RAPD matrices of genetic similarity using the Euclidean distance and the single linkage clustering options of SYSTAT (Wikinson 1989).

One-hundred pairs of independent samples of 56 bands were drawn from the RAPD data set and between the RFLP and RAPD data sets. The genetic similarity among all genotype pairs (990) was calculated for each independent sample. The rank correlation between pairs of samples was computed, and the mean and variance of the distribution of rank correlation coefficients was calculated for the 100 pairs of independent RAPD samples and for the 100 pairs of RFLP and RAPD samples.

*Crossing algorithm*

The following algorithm was used to predict the inheritance of RFLP fragments in hybrids: $1 \times 1 = 1$, $1 \times 0 = 1$ and $0 \times 0 = 0$. This algorithm is interpreted as follows: if either parent has a band, RAPD or RFLP, then that band will be observed in their progeny, and only if both parents lacked a band will it be absent in their progeny (Quinn et al. 1987). The algorithm is predictive if the observed band represents a homozygous parent. If the fragment represents an allele of a heterozygous parent, then the probability of that allele being transmitted to any one progeny is 1/2; however, if the analysis is based on a pooled sample of DNA from several progeny, then the probability of that allele being observed is increased ($1-1/2^n$, where n = the number of progeny sampled). Thus, although heterozygosity in the parents can be a potential source of error in the algorithm, this error is minimized by sampling pooled leaf material from several individuals from each entry.

## Results

*Sampling variance of RFLP and RAPD data*

Comparisons between RFLPs and RAPDs may be confounded because different numbers of bands (56 RFLP vs 181 RAPD) were used in the calculations of genetic similarity, or because more, or different types of, information may be provided by one type of marker band compared to the other. Bootstrap estimates of variance using samples of equal numbers of RFLP and RAPD bands were used to provide a band-for-band comparison of RFLP vs RAPD bands. The pooled regression indicated a significant reduction in the coefficient of variance (CV) with increasing numbers of bands sampled; moreover, there was no heterogeneity observed for either the intercept or slope between the regression lines for RFLPs and RAPDs (Fig. 1).

*Comparison of cluster analyses*

The genetic relationships, including sub-species classification, pedigrees, parent-hybrid combinations, and breeding behavior, among many of the 45 genotypes used in this study were known (Nienhuis et al. 1992). Both the RFLP and RAPD dendrograms of the relationships among genotypes based on genetic similarity values were in general agreement with known pedigrees; however, inconsistencies between clustering based on the RFLP and RAPD dendrograms were observed (Fig. 2). Both the RFLPs and RAPDs identified distinct initial clusters representing the three subspecies, cabbage, cauliflower and broccoli. However, RFLPs had an initial clustering of the late-season broccolis ('Shogun' and 'Cruiser' types) with the cauliflowers. Both RFLPs and RAPDs included four of the Oregon State University (OSU) breeding lines and the cultivar 'Tendan' from the Kyowa Seed Company in the same cluster. RAPDs identified separate clusters for the "Shogun" and "Cruiser" groups, whereas these two groups were combined in the RFLP clustering. The possible female parents of several hybrid cultivars, including 'Shogun', 'Crusier', 'Sunseeds 7190', 'Sunseeds 7183' and 'Sunseeds 7195', tended to closely cluster with their corresponding hybrid cultivar. In contrast, the possible female inbred and the corresponding hybrid of the cultivar 'Nancy', which were closely clustered based on RFLP markers, were separated into separate clusters based on RAPD marker information. Based on RAPDs, Harris-Moran breeding lines '87-0873', '97-0152' and '87-0766' all clustered with the late-season broccoli cultivar 'Shogun' and it's female parent; however, based on RFLPs none of these breeding lines were included in the 'Shogun' cluster.

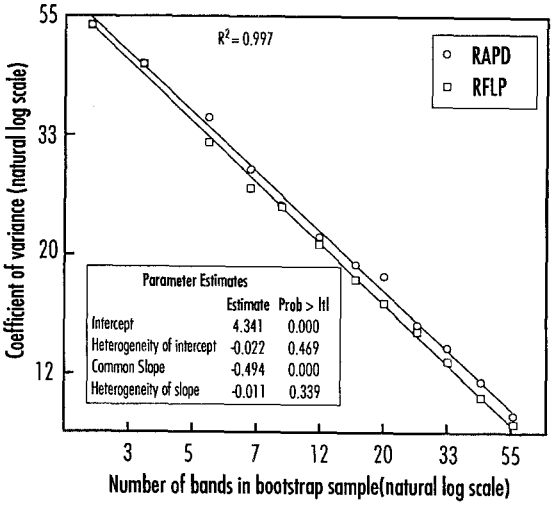


**Fig. 1.** Linear regressions of coefficients of variance on bootstrap sample size for RFLP and RAPD molecular markers

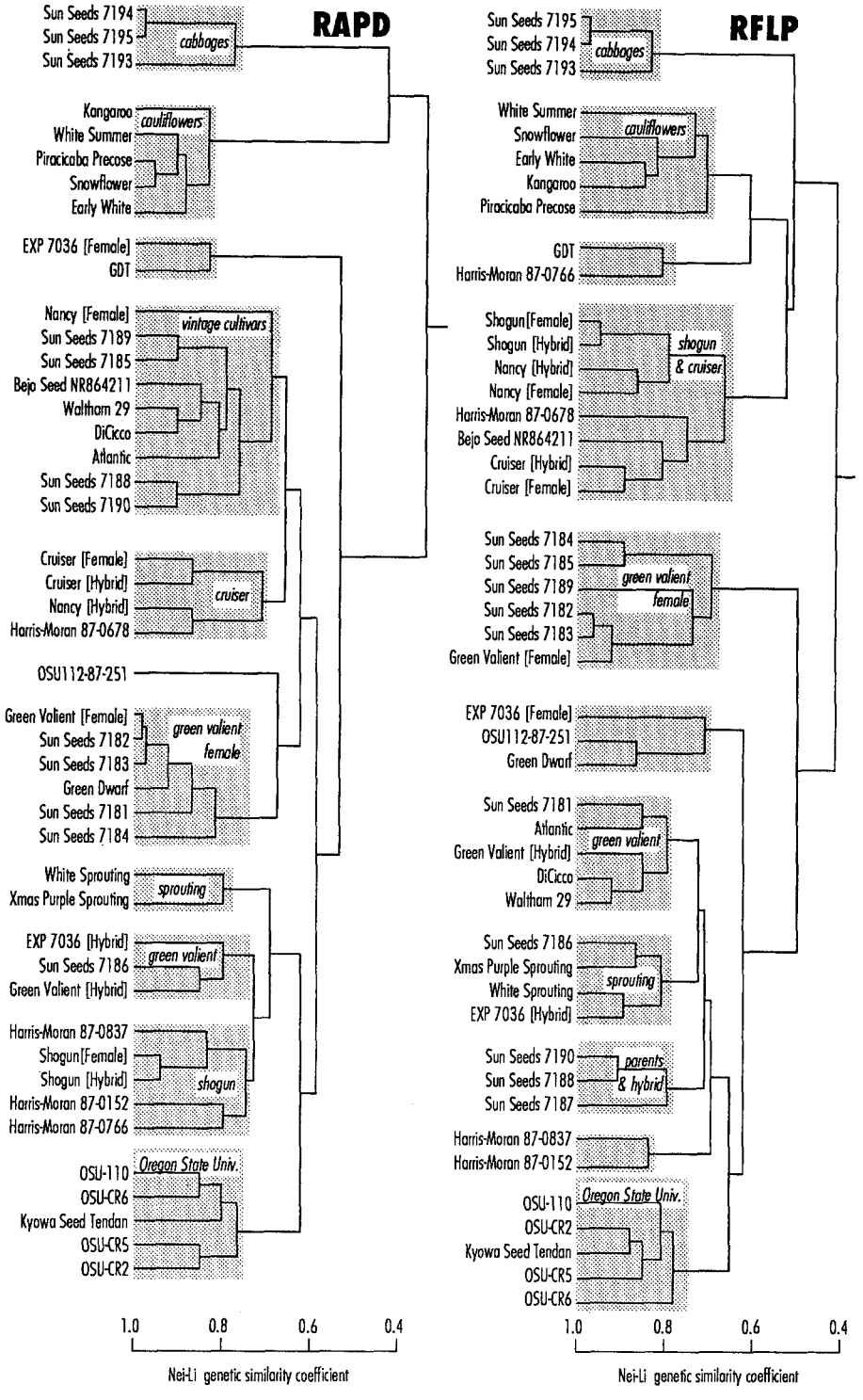


**Fig. 2.** Comparison of cluster analyses of *B. oleracea* genotypes based on RAPD and RFLP molecular marker data. *Shaded areas* represents clusters of related gentoypes. *Names* within the shaded areas are arbitrarily based on the predominant cultivar or relationship within each cluster

The magnitude of the rank correlation between the full RFLP and RAPD genetic similarity matrices was 0.745, using 56 and 181 bands of information, respectively. The distribution of rank correlation coefficients among 100 independent paired samples of 56 bands of RAPD data had a mean of 0.645 (Fig. 3). The distribution of rank correlation coefficients between 100 independent paired samples of 56 bands of RFLP and RAPD data had a mean of 0.587. A pooled T-test indicated that the means were significantly different;

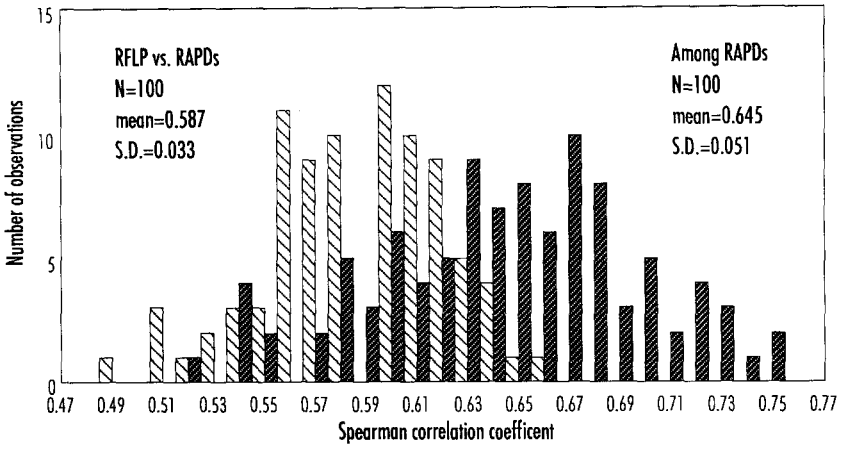


**Fig. 3.** Distribution of rank correlation between 100 pairs of independent samples of 56 RAPD bands used to estimate genetic among 45 *B. oleracea* genotypes (Among RAPDs), and distribution of rank correlation coefficients between 100 pairs of samples between 56 RAPD bands and 56 RFLP bands used to estimate genetic similarity among 45 *B. oleracea* genotypes (RFLP vs RAPDS)

nevertheless, the probability of an observation occurring in either distribution (overlap) was 0.61.

*Inheritance of RAPD and RFLP bands*

Three combinations, two broccoli and one cabbage, of parents and their corresponding experimental $F_1$ hybrids were provided by Sunseeds, Salem, Oregon. These combinations were originally provided as "unknowns", which were later correctly identified using predicted and observed RFLP banding patterns (Nienhuis et al. 1992). Because of approximately 8.5% missing data, '7187' was not included in previous analyses with the other 45 genotypes, but was included in this inheritance study as an inbred parent of a hybrid cultivar. The genetic similarity between the predicted and actual hybrid in the two broccolis was 0.967 and 0.934. In contrast, the genetic similarity between the predicted and actual hybrid in the cabbage was lower, 0.869. The frequency of expected (e) and unexpected (u) genotypic classes in hybrids was consistent for both RFLPs and RAPDs (Table 1).

## Discussion

The corresponding reductions in the CV of genetic similarity with increasing band numbers for both RAPD and RFLP markers indicates that, on a band-for-band basis, the sampling errors ar equivalent. Moreover, inspection of the regression line indicates that CVs as low as 10% for estimating genetic similarity among the genotypes in this study could be achieved by sampling as few as 55 RFLP or RAPD bands. Nevertheless, differences between RFLPs and RAPDs in estimation of genetic similarity are reflected in inconsistencies between dendrograms of the genetic relationships among the 45 *B. oleracea* genotypes. Chase et al. (1991) observed general agreement between dendrograms of *Phaseolus vulgaris* accessions based on isozyme and RFLP loci, and concluded that the two markers were correlated. Overlap in the distributions of rank correlations between independent sub-samples from the RAPD data set compared to correlations between RFLP and RAPD sub-samples indicates that over 60% of the observed ranks of genetic similarities could have come from either distribution. Thus, although differences in estimation of genetic relationships based on RFLP and RAPD data were observed in this study, the analyses suggest that the differences were primarily due to sampling errors rather than due to fundamental differences in how RAPDs and RFLP polymorphisms measure genetic similarity. This may reflect the similar manner in which RFLP and RAPD polymorphisms are revealed by either base-pair or positional changes in the restriction sites (RFLP) or primer sites (RAPD) which flank a chromosomal region.

In interspecific comparisons in the genus *Glycine* (Williams et al. 1992) and intraspecific comparisons within *Brassica* (Thormann and Osborn 1992) the presence of a RAPD band in both genotypes indicated a high level of sequence homology. These results suggest that RAPD bands which are shared between individuals represent homologous characters in a manner analogous to RFLP bands. Moreover, in both RFLPs and RAPDs, comparisons involving the presence of a band in one genotype and its absence in the other, as well as comparisons involving the coincident absence of bands in both genotypes, provide limited information on the relative magnitudes of sequence differences. Thus, although the informativeness may vary depending on the type of comparison (Skroch et al. 1992), the shared presence of either RFLP or RAPD bands between genotypes does reflect sequence homology.

One assumption in the interpretation of this data is that the RFLP and RAPD bands are randomly distributed over the genome is such a way that a subsample will reflect sequence differences between genomes.

**Table 1.** Comparison of inheritance of RFLP and RAPD bands in three hybrid combinations

| Combination | | | Genotype of parents[a] | | Genotype of hybrid[b] | Frequency of genotypic class[c] | |
|---|---|---|---|---|---|---|---|
| Parent-1 | Parent-2 | Hybrid | 1 | 2 | | RAPD (%) | RFLP (%) |
| '7184' | '7189' | '7185' | 0 | 0 | 0 (e) | 39.8 | 45.6 |
| | | | | | 1 (u) | 0.6 | 1.8 |
| | | | 0 | 1 | 1 (e) | 9.4 | 10.5 |
| | | | | | 0 (u) | 1.1 | 1.8 |
| | | | 1 | 0 | 1 (e) | 6.1 | 12.3 |
| | | | | | 0 (u) | 1.1 | 0.0 |
| | | | 1 | 1 | 1 (e) | 40.9 | 26.3 |
| | | | | | 0 (u) | 1.1 | 0.0 |
| | | | 1 | . | 1 (e) | 0.0 | 1.8 |
| | | | | | 0 (u) | 0.0 | 0.0 |
| '7187' | '7190' | '7188' | 0 | 0 | 0 (e) | 26.7 | 35.1 |
| | | | | | 1 (u) | 0.6 | 0.0 |
| | | | 0 | 1 | 1 (e) | 16.4 | 10.5 |
| | | | | | 0 (u) | 1.8 | 5.3 |
| | | | 1 | 0 | 1 (e) | 7.9 | 7.0 |
| | | | | | 0 (u) | 2.4 | 1.8 |
| | | | 1 | 1 | 1 (e) | 34.5 | 38.6 |
| | | | | | 0 (u) | 1.2 | 0.0 |
| | | | . | 1 | 1 (e) | 8.5 | 0.0 |
| | | | | | 0 (u) | 1.2 | 0.0 |
| '7193' | '7194' | '7195' | 0 | 0 | 0 (e) | 39.7 | 36.8 |
| | | | | | 1 (u) | 0.0 | 1.8 |
| | | | 0 | 1 | 1 (e) | 1.6 | 5.3 |
| | | | | | 0 (u) | 0.0 | 0.0 |
| | | | 1 | 0 | 1 (e) | 0.1 | 0.0 |
| | | | | | 0 (u) | 16.7 | 12.3 |
| | | | 1 | 1 | 1 (e) | 41.3 | 43.8 |
| | | | | | 0 (u) | 0.0 | 0.0 |

[a] . = missing data

[b] Letters in parenthesis indicate the expected (e) and unexpected (u) inheritance assuming that if either parent has a band it will be observed in their hybrid pogeny, and if both parents lack a band it will be absent in their progeny

[c] Percentages within columns for a specific cross may not add to 100% due to rounding errors

Sampling error, especially for small samples, could be reduced by using uniformly-dispersed markers. Of the RFLP probes used in this study, those which were polymorphic in an unrelated mapping population are known to be widely dispersed about the genome (Slocum et al. 1993). Construction of a linkage map in *B. oleracea* based upon RAPD markers is therefore anticipated.

The crossing algorithm does not utilize the allelic information which is often available with RFLP markers, and assumes that both RFLP and RAPD bands are inherited as dominant markers (Nienhuis et al. 1992). Thus, at a given locus, heterozygotes will have the same banding pattern as one of the parents. Nevertheless, the correspondence in "errors" associated with the use of RAPDs vs RFLPs in the crossing algorithm further suggests that both reveal informative DNA polymorphisms. The failure to more accurately predict the genotype of a hybrid given the genotypes of it's parents is reflected in an unexpected inheritance of bands in a manner inconsistent with the crossing algorithm, e.g., the appearance of bands in an $F_1$ hybrid when no band was present in either parent, or the absence of a band in the $F_1$ hybrid when one or both parents possess a band. The source of "errors" in the crossing algorithm are unknown, but are likely to be due to sampling errors associated with heterozygous loci. The correspondence between RFLPs and RAPDs in the frequency of expected and unexpected genotypic classes suggests that the errors associated with both molecular markers are equivalent. Moreover, the general correspondence in the frequency of expected and unexpected genotypic classes suggests that errors in the scoring of RFLP and RAPD bands may be similar in magnitude.

For both RFLPs and RAPDs the frequency of unexpected inheritance of bands is usually well below 5%, except for one genotypic class in the cabbage parental $F_1$-hybrid combination, '7193' × '7194' = '7195'. The genotypic class in which parent '7193' possesses a band (1), parent '7193' lacks a band (0), and the band is present in the $F_1$ hybrid (1), occurs with a frequency of 0.1 and 0.0 percent of the total bands for

RAPDs and RFLPs, respectively. However, the unexpected absence of a band in the $F_1$ hybrid, even though parent '7193' possesses a band, occurs with a frequency of 16.7 and 12.3 percent of the total bands in this parental $F_1$-hybrid combination for RAPDs and RFLPs, respectively. When this unexpected inheritance was first observed in the RFLP data the source of this "error" was unknown; however, the concordance of this unexpected inheritance in the RAPD data suggests that it is a genuine biological phenomenon rather than an experimental error. In addition, the calculated genetic similarity between one of the parents ('7193') and the hybrid is 0.968, whereas the genetic similarity between the other parent and the hybrid is much lower (0.651). This data is consistent with the hypothesis that '7195' is not a pure hybrid, but rather is the result of self-pollination by its female parent (probably '7193'). This, hypothesis could be tested by sampling individuals within each population, rather than using bulk DNA.

Relatively few RAPD bands, less than 55, could distinguish among all the *B. oleracea* cultivars included in this study. This is in agreement with Hu and Quiros (1991) who distinguished between 26 broccoli and cauliflower cultivars using 40 RAPD bands (markers). The results of our study indicate that RAPDs are equivalent to RFLPs in the estimation of genetic similarity among 45 B. oleracea genotypes; moreover, because of their relative simplicity and lower cost, RAPDS are considered more practical than RFLPs for studies on germplasm organization and characterization.

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