Use of PCR-based Markers for Differentiating Elite Broccoli Inbreds

Anna L. Hale¹ and Mark W. Farnham¹

USDA/ARS U.S. Vegetable Laboratory, Charleston, SC 29414

Monica A. Menz²

Institute for Plant Genomics and Biotechnology, Department of Soil and Crop Science, Texas A&M University, College Station, TX 77843

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ABSTRACT. Breeders of cole crops (*Brassica oleracea* L.) have an interest in utilizing current and emerging PCR-based marker systems to differentiate elite germplasm. However, until efficiency and cost-effectiveness are determined, most breeders are hesitant to change methods. In this study, our goal was to compare simple sequence repeat (SSR), amplified fragment-length polymorphism (AFLP), and sequence-related amplified polymorphism (SRAP) marker systems for their effectiveness in differentiating a diverse population of 24 elite broccoli (*B. oleracea* Italica Group) inbreds. Published SSR primer sequences for *Brassica* L. species were used along with AFLP and SRAP primer combinations. Several SSR primers failed to amplify DNA in the broccoli population, but all AFLP and SRAP primer combinations produced multiple bands. Twenty-nine percent of the SSR primers were monomorphic, while most of the remaining primers detected only one or two differences among inbreds. AFLP and SRAP methods produced multiple differences per primer in almost every case. Phenetic analysis revealed that the type of marker affected the classification of the genotypes. All three marker systems were able to successfully differentiate between the 24 elite inbreds, however, AFLPs and SRAPs were more efficient, making them better alternatives than SSRs over other established methods for fingerprinting *B. oleracea* inbreds.

Commercial and public broccoli breeders have an ongoing interest in utilizing current and emerging PCR-based marker systems to differentiate elite germplasm in their vegetable improvement programs. Any potential new marker systems must be more efficient and cost-effective alternatives to older techniques already in use by the respective programs. Until efficiency and cost-effectiveness are determined, most breeders are hesitant to change their methods.

The first DNA-based marker system widely used by *Brassica* breeders was restriction fragment length polymorphisms (RFLPs). These markers have been used to study genetic similarity, taxonomy, and genome evolution (dos Santos et al., 1994; Neinhuis et al., 1993; Song et al., 1988). While RFLPs have proven to be useful co-dominant markers, they have two major limitations. First, RFLPs are time consuming, and results are limited only to the DNA sequence used as a probe, and thus, a small area of the genome. Additionally, most effective RFLP methods require the use of radioactivity.

Random amplified polymorphic DNA (RAPD) markers were the first widely used alternative to RFLPs (Williams et al., 1990). Using RAPDs, large numbers of samples could be analyzed economically and quickly. The method required small quantities of DNA, and most of the genome could be sampled with a potentially unlimited number of markers. RAPDs have been used for cultivar identification (Hu and Quiros, 1991; Lee et al., 1996), studies on genetic diversity among populations (Farnham, 1996; Kaminski et al., 2003; Lanner-Herrera et al., 1996), gene bank management (Divaret et al., 1999; Kresovich et al., 1992; Phippen

et al., 1997) and identification and mapping of markers (Camargo et al., 1997; Giovannelli et al., 2002). Based on the number of past and recent publications on RAPDs and the frequent use of them by private companies, they may still be the most common PCR-based marker system used in *B. oleracea* breeding programs. Despite the advantages RAPDs have over RFLPs, they tend to be dominant markers, have low multiplex ratios (meaning they produce few markers per primer) and poor consistency between different breeding programs. These disadvantages have put them out of favor by many geneticists.

Following wide usage of RAPDs in *Brassica* breeding, new PCR-based techniques have been developed. Each of these techniques has its advantages and disadvantages. AFLPs have a high multiplex ratio, are less sensitive to the template DNA concentrations, and are highly reproducible (Becker et al., 1995; Breyne et al., 1999; Vos et al., 1995). The primary disadvantages of this method include the multiple steps involved in the process, the time required for optimization, potentially uneven marker distribution (Haanstra et al., 1999), and the difficulty in isolating specific bands for sequencing. AFLPs have been used in *B. oleracea* to identify quantitative trait loci and for generating linkage maps (Holme et al., 2004; Sebastian et al., 2000). Previous work with a multitude of species has shown that SSRs, while lacking the high multiplex ratio seen in AFLPs and requiring prior sequence knowledge to design primers, are co-dominant and exhibit a much higher degree of polymorphism than do any other markers (Bowers et al., 1996). While SSRs have not been used extensively on *B. oleracea*, they have been utilized in related *Brassica* species for genome identification and to study genome evolution (Lowe et al., 2002; Plieske and Struss, 2001; Saal et al., 2001). A relatively new PCR-based technique, SRAPs, is gaining popularity due to its simplicity, reliability, a higher frequency of co-dominant markers, moderate through-put ratio, and their tendency to map in close proximity to expressed regions of the genome (Li and Quiros, 2001). Despite their advantages and successful use in

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¹Research Geneticist.

²Assistant Professor.

related *Brassica* species, AFLPs, SRAPs, and SSRs have not been as widely utilized as RAPDs in *B. oleracea* research.

The objective of the current study was to compare the informativeness of SSR, AFLP, and SRAP markers for their ability to discriminate between a set of broccoli inbreds and to determine the feasibility of using these markers in place of older techniques, such as RAPDs, that are typically used in *B. oleracea* breeding programs.

Materials and Methods

PLANT MATERIALS AND DNA EXTRACTION. A population of 24 elite broccoli inbreds was selected to represent a diverse phenotypic and genotypic array of parental broccoli material that might typically occur in a breeding program aimed at developing elite cultivars. Fourteen of the lines are doubled haploids developed using standard techniques for anther culture (Farnham, 1998). These are designated USVL012, USVL089, and USVL013 derived from 'Everest'; USVL047, USVL048, and USVL073 derived from 'Marathon'; USVL105 from 'Arcadia'; USVL032 from 'Green Valiant'; USVL070 from 'Futura'; USVL066 from 'Viking'; USVL039 from 'High Sierra'; USVL107 from 'Patriot'; USVL106 from 'Major'; and USVL093 from 'Sultan'. The population also included 10 inbreds developed through conventional pedigree selection. These include USVL110, USVL111, USVL059, USVL060, and USVL064 (derived from 'Green Comet', 'Paragon', 'Packman', 'Southern Comet', and 'Symphony', respectively) developed at the U.S. Vegetable Laboratory (USVL) in Charleston, S.C.; USVL112 and USVL113 selected at the USVL from heterozygous lines obtained from M. Dickson (Cornell Univ.); S240-5-20 and S440 obtained from J. Myers (Oregon State Univ.), and USVL109 developed at the USVL from a heterozygous line from Syngenta (Boise, Idaho).

Genomic DNA was isolated from leaves using DNeasy plant mini-kits (Qiagen, Valencia, Calif.). DNA was quantified using a TKO 100 fluorimeter (Hoefer Scientific Instruments, San Francisco) in conjunction with a Hoechst dye-based protocol.

SSR ANALYSIS. The test population of 24 broccoli inbreds was screened with 44 previously identified SSR primers. Fifteen primers were identified by Suwabe et al. (2002), seven by Lowe et al. (2002), seven by Kresovich et al. (1995), and 15 by Szewc-McFadden et al. (1996). Of the 44 SSRs used in this study, 15 were identified in *B. rapa* L., 24 in *B. napus* L., three in *B. nigra* L., and two in *B. oleracea* (Table 1). PCR was performed in 10- μ L reactions containing 1X PCR buffer (Promega, Madison, Wis.), 2.5 mM MgCl₂, 100 μ M dNTPs (Sigma, St. Louis), 0.25 μ M of each primer pair (Sigma Genosys, Houston), 0.2 U Taq DNA polymerase (Promega), and 25 ng of DNA. The PCR conditions were identical to that of Saal et al. (2001), and annealing temperatures ranged from 49 to 68 °C (Table 1). The PCR products were separated on a 7% native polyacrylamide gel with ethidium bromide in the running buffer and visualized on a ultraviolet transilluminator (Shi et al., 2001).

SRAP ANALYSIS. Twenty-four SRAP primer combinations were evaluated on the test population using seven forward primers and eight reverse primers (Table 2). The seven forward primers and three of the reverse primers were previously described by Li and Quiros (2001), and the remaining reverse primers were developed according to the rules for primer design as outlined in the same paper. For visualization purposes, the forward primers were all labeled with one of three WellRED dyes (D2, D3, or D4; Prologo, Boulder, Colo.), and to ensure proper dye balance, the amount of

Table 1. SSR primers, the source of the sequence, and the respective annealing temperatures (TA) used to analyze 24 elite broccoli inbreds.

Primer	Source	Species	TA
BRMS001	Suwabe et al. (2002)	<i>Brassica rapa</i>	54
BRMS005	Suwabe et al. (2002)	<i>B. rapa</i>	52
BRMS007	Suwabe et al. (2002)	<i>B. rapa</i>	51
BRMS008	Suwabe et al. (2002)	<i>B. rapa</i>	53
BRMS017	Suwabe et al. (2002)	<i>B. rapa</i>	55
BRMS019	Suwabe et al. (2002)	<i>B. rapa</i>	49
BRMS020	Suwabe et al. (2002)	<i>B. rapa</i>	48
BRMS025	Suwabe et al. (2002)	<i>B. rapa</i>	65
BRMS002	Suwabe et al. (2002)	<i>B. rapa</i>	59
BRMS003	Suwabe et al. (2002)	<i>B. rapa</i>	51
BRMS006	Suwabe et al. (2002)	<i>B. rapa</i>	56
BRMS014	Suwabe et al. (2002)	<i>B. rapa</i>	57
BRMS015	Suwabe et al. (2002)	<i>B. rapa</i>	60
BRMS016	Suwabe et al. (2002)	<i>B. rapa</i>	64
0109-A03	Lowe et al. (2002)	<i>B. oleracea</i>	50
0109-A06	Lowe et al. (2002)	<i>B. oleracea</i>	59
NA10-B10	Lowe et al. (2002)	<i>B. napus</i>	59
NA10-F06	Lowe et al. (2002)	<i>B. napus</i>	63
NI2-B02	Lowe et al. (2002)	<i>B. nigra</i>	58
NI2-C12	Lowe et al. (2002)	<i>B. nigra</i>	58
NI-F02	Lowe et al. (2002)	<i>B. nigra</i>	56
BRMS044	Suwabe et al. (2002)	<i>B. rapa</i>	60
BN72A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	61
BN38A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	65
BN12A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	62
BN6A2	Szewc-McFadden et al. (1996)	<i>B. napus</i>	65
BN6A3	Szewc-McFadden et al. (1996)	<i>B. napus</i>	62
BN9A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	55
BN16A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	66
BN18A1	Szewc-McFadden et al. (1996)	<i>B. napus</i>	65
BN20A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	56
BN25A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	60
BN19A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	63
BN25C2	Szewc-McFadden et al. (1996)	<i>B. napus</i>	62
BN26A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	61
BN72B2	Szewc-McFadden et al. (1996)	<i>B. napus</i>	68
BN40C1	Szewc-McFadden et al. (1996)	<i>B. napus</i>	59
BN50F	Szewc-McFadden et al. (1996)	<i>B. napus</i>	52
BN59A1	Szewc-McFadden et al. (1996)	<i>B. napus</i>	64
BN68/1	Szewc-McFadden et al. (1996)	<i>B. napus</i>	64
BN75A	Szewc-McFadden et al. (1996)	<i>B. napus</i>	60
BN80/3	Szewc-McFadden et al. (1996)	<i>B. napus</i>	63
BN83B1	Szewc-McFadden et al. (1996)	<i>B. napus</i>	59
BN92A1	Szewc-McFadden et al. (1996)	<i>B. napus</i>	60

dye-labeled primer added to the PCR reaction varied depending on which dye was used. Each 10- μ L PCR reaction contained 1X PCR buffer (Promega), 2.5 mM MgCl₂, 200 μ M dNTPs (Sigma), 30 ng of reverse primer (IDT, Coraville, Iowa), 0.4 U Taq DNA polymerase (Promega) and 25 ng of DNA. In addition, WellRED dye labeled forward primers were added in the following amounts: 15 ng for a D4 primer; 30 ng for a D3 primer; or 75 ng for a D2 primer. PCR conditions were identical to Li and Quiros (2001), except 40, as opposed to 35, cycles were performed. The PCR products were prepared for running on a CEQ8800 capillary sequencer (Beckman Coulter, Fullerton, Calif.) by pooling 0.3 μ L of the D4 reaction, 0.3 μ L of the D3 reaction, 0.5 μ L of the D2 reaction, 0.28 μ L of D1 size standard, and 30 μ L of de-ionized formamide in a 96-well PCR plate. Samples were run on the

Table 2. Primer sequences for SRAP reactions.

Primer	Direction	Sequence
ME1	Forward	TgAgTCCAAACCggATA
ME2	Forward	TgAgTCCAAACCggAgC
ME3	Forward	TgAgTCCAAACCggAAT
ME4	Forward	TgAgTCCAAACCggACC
ME5	Forward	TgAgTCCAAACCggAAG
AB2	Forward	AgTgATTCAACCggATA
AB3	Forward	AgTgATTCAACCggAgC
EM1	Reverse	gACTgCgTaCgAATTAAT
EM5	Reverse	gACTgCgTACgAATTAAC
EM6	Reverse	gACTgCgTACgAATTgCA
BA1	Reverse	gTCgAgCTgCCAATTATA
BA2	Reverse	gTCgAgCTgCCAATTAAT
BA3	Reverse	gTCgAgCTgCCAATTgC
BA4	Reverse	gTCgAgCTgCCAATTTT
BA5	Reverse	gTCgAgCTgCCAATTTAA

CEQ8800 capillary sequencer using the fragment 3 parameters and analyzed using the built-in fragment analysis software that was bundled with the system. A relative peak height threshold of 10% was used for calling the peaks. The fragments between 75 and 400 bp were scored as present (1) or absent (0).

AFLP ANALYSIS. AFLP analysis was conducted according to Vos et al. (1995) with minor modifications. Genomic DNA was restricted with 5 U of *EcoRI* (rare cutter) and 5 U of *MseI* (frequent cutter) (New England Biolabs, Beverly, Mass.) and the double stranded adapters were ligated to the fragment ends. Preselective amplification was carried out according to Klein et al. (2000) with minor modifications. 25- μ L reactions contained 1X PCR buffer, 1.5 mM $MgCl_2$, 160 μ M dNTPs, 0.56 μ M *Eco*+A and *Mse*+C pre-amplification primers, 1 U Taq DNA polymerase, and 5 μ L of restricted adapter-ligated, diluted DNA. The primer sequences were previously given by Vos et al. (1995), and the PCR profile was identical to that of Klein et al. (2000), except 25, as opposed to 20, cycles were performed. The resulting product was diluted 1:10 with water and run on a 1% agarose gel to verify amplification.

Following preselective amplification, selective amplification was performed using primers with three selective bases on the 3' end (Vos et al., 1995). For visualization purposes, the *EcoRI* selective primer was labeled with one of three WellRED dye labels (D2, D3, or D4). Twenty-four selective amplification primer combinations were tested on the test population using three WellRED dye labeled forward primers and eight unlabeled reverse primers. The primer combinations included AGA(D4)-CCA, CGA, CGG, CGC, CGT, CCC, CCT, CCG, and CTC; ACC(D3)-CCA, CGA, CGG, CGC, CGT, CCC, CCT, CTC, and CCG; ACT(D2)-CCA, CGT, CCC, CCT, CCG, and CTC. Reactions are the same as those described above for SRAP analysis. The PCR conditions were as follows. Cycle 1 began with a 2-min 20-s denaturation step at 94 °C followed by a 30-s annealing step at 65 °C and a 2-min extension at 72 °C. Cycle 2 was similar to the first cycle; however, the denaturing step was for 2 min only. Cycle 3 began at 94 °C for 20 s followed by an annealing step at 63 °C for 30 s, and a 2-min extension at 72 °C. The annealing step was reduced by 1 °C per cycle for the next seven cycles, giving a touch down phase of eight cycles. Thirty-five cycles were then performed with a 20-s denaturing step at 94 °C, a 30-s annealing step at 56 °C and a 2-min extension at 72 °C. Following these 35 cycles, an additional extension step was performed at 60 °C for 30 min. Samples were pooled, visualized, and analyzed as described above for SRAPs.

DATA ANALYSIS. AFLP and SRAP markers were scored as present (1) or absent (0), and percent polymorphism of the markers were determined. For SSRs differences in band size were scored as different allelic forms of a given locus, and the data were transformed to binary presence (1) vs. absence (0). The genetic similarity between each pair of lines was calculated according to the Dice similarity coefficient (Nei and Li, 1979). Values of genetic similarity range from 0 (no peaks in common) to 1 (identical peak patterns for all markers). Monomorphic peaks were not included in the analysis.

Four similarity matrices were generated for comparing the marker systems. These matrices were generated using the following data sets: 1) SSR data only, 2) SRAP data only, 3) AFLP data only, and 4) a concatenated dataset of SSR, AFLP, and SSR data together. Dendrograms were created from each similarity matrix by the unweighted pair group method with arithmetic average [UPGMA (Sneath and Sokal, 1973)]. A cophenetic matrix was generated for each of the dendrograms and then compared to its corresponding similarity matrix using the Mantel matrix correspondence test (Mantel, 1967). Dendrograms created using different marker systems were compared by estimating the correlation among their corresponding cophenetic matrices and similarity matrices using the same Mantel test. The cophenetic correlation generated by the Mantel test is used as a measure of goodness of fit for a cluster analysis; 1000 permutations were used in each of our Mantel tests. Genetic similarities, similarity matrices, dendrograms, and Mantel tests were all performed by the appropriate routines in NTSYS-pc version 2.0 (Exeter Software, Setauket, N.Y.). A bootstrap analysis was performed using the WinBoot software (Yap and Nelson, 1996) to estimate the confidence limits for the groupings produced by each dendrogram; 5000 bootstrap resamplings were used for each dendrogram presented.

Results and Discussion

LEVELS OF POLYMORPHISM. Each of the three marker types evaluated were able to successfully differentiate the diverse broccoli inbreds in the test population; however, the levels of polymorphism detected varied between the marker systems.

No amplification was observed for five of the 44 SSR primer pairs, and three others were either noisy or had poor amplification. The remaining 36 primer pairs produced a total of 87 apparent loci, 55 of which were polymorphic. Fifteen SSR primers (29%) produced a monomorphic marker phenotype. A high level of monomorphic bands was also reported in a SSR study on *B. napus* where only one-third of the SSR primers tested were polymorphic (Sobotka et al., 2004). Of the 36 primer pairs analyzed, an average of two loci per SSR primer pair was observed and 63% of these loci were polymorphic. The SSRs were not analyzed on the Beckman CEQ8800 capillary sequencer because it was not cost efficient to label individual primers that yield so few bands per primer.

At the beginning of the study, eight SRAP primer combinations, eight AFLP primer combinations, and eight SSRs were run in duplicate to test for reproducibility. Since no problems were observed, and previous reports indicated they were highly reproducible, the methods were deemed robust and the remaining primers were run only once.

The 24 SRAP primer combinations produced a total of 312 scoreable peaks, with an average of 13 per primer combination; 255 (82%) of these peaks were polymorphic. Between 4 and 24

fragments were produced per primer combination. The number of polymorphic fragments per primer combination ranged from one to 23, with an average of 11.

AFLPs produced more peaks than SRAPs or SSRs with a total of 510 for the 24 primer combinations. This averaged to 21 peaks per primer combination, with a polymorphism rate of 61%. Between 10 and 40 fragments were produced per primer combination. The number of polymorphic fragments per primer combination ranged from three to 26 with an average of 13.

GENETIC SIMILARITY. Genetic similarity over all marker systems among the 24 lines ranged from 0.32 to 0.91. SSRs had genetic similarities ranging from 0.32 (between USVL070, and USVL111, derived from 'Futura' and 'Paragon', respectively) and 0.91 (between S440 and S240-5-20, both from Oregon State Univ.). The range of similarity using SRAP markers varied between 0.43 (between USVL032 and USVL059 derived from 'Green Valiant' and 'Packman', respectively), and 0.84 (between USVL012 and USVL089, both derived from 'Everest'). AFLP markers had a range of similarity between 0.57 (between USVL105 and USVL106, derived from 'Arcadia' and 'Major', respectively) and 0.87 (between S440 and S240-5-20, both from Oregon State Univ.; between USVL047 and USVL073, both derived from 'Marathon'; and between USVL032 and USVL039, derived from 'Green Valiant' and 'High Sierra', respectively). Overall, AFLPs had the highest similarity values, and SRAPs had the lowest, while SSRs had the largest range in genetic similarities (Table 3). Differences in rates of polymorphism were expected since the SRAPs preferentially amplify expressed regions, AFLPs amplify both expressed and nonexpressed regions, and SSRs tend to amplify nonexpressed regions (Ferriol et al., 2004).

Dendrograms were generated based on each marker type and a combination of all three (Fig. 1A–D). While the topology of each tree is unique, there are evident similarities among them. First, the derivatives of 'Everest' (USVL012, 089, and 013) always grouped together with USVL070 (derivative of 'Futura') with all three marker sets. Second, the inbreds from Oregon State Univ. (S440 and S240-5-20) grouped together regardless of the marker system used as did USVL111 and USVL110, two lines selected for adaptation to hot summer conditions. In addition to the above commonalities, USVL047 and 048 (derived from 'Marathon'), USVL032 (derived from 'Green Valiant'), USVL039 (derived from 'High Sierra'), and USVL105 (derived from 'Arcadia') grouped together with all three markers. USVL066 (derived from 'Viking') and USVL073 (derived from 'Marathon') were also positioned within the latter group by AFLPs, and SRAPs, but SSRs clustered them differently. USVL106 and USVL109 were also found clustered together regardless of marker type, however, their positions with respect to the other lines varied, based on marker type. Discrepancies in formation of subgroups within major groups were observed as well as in the positioning of the remaining inbred lines. Inbred lines derived from the Cornell Breeding Program germplasm (USVL112 and USVL113) as well as USVL093 exemplify genotypes that always grouped differently depending on the marker type used.

COMPARISON BETWEEN MARKER SYSTEMS. Mantel test comparisons of dendrograms created using cophenetic and similarity matrices based on different sets of markers were all highly significant, indicating that there was a correlation between matrices. Despite the significance of the Mantel tests, the three marker systems showed slightly different groupings of the 24 genotypes. Correlation coefficients between cophenetic and similarity matrices were low to medium. Among the cophenetic matrices, the highest

Table 3. Mean, minimum, and maximum values of Dice similarity coefficient among 24 broccoli inbreds using different PCR-based markers.

Marker set	Loci (no.)	Genetic similarity		
		Maximum	Minimum	Mean
SSR	55	0.91	0.32	0.64
AFLP	311	0.87	0.57	0.69
SRAP	255	0.84	0.43	0.62
AFLP-SRAP-SSR	621	0.87	0.54	0.66

correlation was found between SSR and SRAP (0.58) followed by AFLP and SRAP (0.45) and AFLP and SSR (0.35). The correlation coefficients of the similarity matrices were somewhat different, with the highest coefficient between SSR and SRAP (0.5) followed by AFLP and SSR (0.35) and SRAP and AFLP (0.2) (Table 4). In general, those groupings with relatively high bootstrap confidence limits based on the WinBoot program tended to group the same regardless of the marker system, and those with lower confidence limits grouped differently. The confidence limits were much lower for SSRs than for any other type of marker, presumably because of the low number of bands generated in this evaluation.

The cophenetic correlation coefficient is used as a measure of goodness of fit for a cluster analysis. It indicates how well the clustering of genotypes accurately represents the estimates of genetic similarity obtained with each respective marker system (Pejic et al., 1998). Overall, the cophenetic correlation coefficients showed intermediate values that were not extremely different between the three marker systems. SRAPs had the highest correlation (0.78) followed by SSRs (0.77) and AFLPs (0.74). When all three sets of markers were combined into a single data set and analyzed together, the cophenetic correlation was 0.81, which is generally considered to be a high value (Rohlf, 2002). This indicates that a combination of the three marker systems gives a better estimate of similarity than any single marker type alone.

A majority of inbred lines used in this study were derived from commercial hybrids and the pedigree information for these hybrids is considered a trade secret and not available. Thus, the true relationships among the inbreds at the pedigree level are not known. Lacking this information, at least one assumption can be made regarding the similarities among lines. Mainly, inbreds derived from the same hybrid are more similar than those derived from different hybrids. Despite differences in the dendrograms, all three marker systems reflected this assumption.

The low correlation between the groupings obtained with different marker systems probably reflects the differential sampling of the genome provided by each of the markers. SRAP markers preferentially target expressed regions of the genome and a previous study by Li and Quiros (2001) on *B. oleracea* showed that ~45% of gel isolated bands matched known genes in the Genebank database. In contrast to SRAPs, AFLP markers obtained using *EcoRI*/*MseI* enzymes amplify both coding and noncoding regions of the genome, and can be unevenly distributed across the genetic map. Studies carried out with tomato (*Lycopersicon pennelli* L.) and sorghum [*Sorghum bicolor* (L.) Moench] have shown that these markers are mainly clustered in centromeric regions (Bonnema et al., 2002; Menz et al., 2002). Thus, it is probable that the centromeric regions are overrepresented with AFLP markers in *B. oleracea* as well, weighing these regions more heavily when grouping germplasm. Menz et al. (2004) suggest that *PstI*/*MseI* AFLP genetic markers might be a better marker choice on crops where saturated genetic maps do not yet

Fig. 1. Dendrograms showing relationships among 24 elite broccoli inbreds using (A) AFLP, (B) SSR, (C) SRAP, and (D) a combination of all three marker types based on Dice similarity coefficient and UPGMA method. Bootstrap values are shown to the left of the branch and are based on 5000 resamplings of the data sets. The cultivars from which the inbreds were derived follow the USVL (U.S. Vegetable Laboratory, Charleston, S.C.) designation. EV = 'Everest', FU = 'Futura', NY = Cornell Univ. breeding line, OSU = Oregon State Univ. breeding line, PT = 'Patriot', SYM = 'Symphony', SC = 'Southern Comet', PA = 'Packman', PG = 'Paragon', GC = 'Green Comet', MA = 'Marathon', GV = 'Green Valiant', HS = 'High Sierra', AR = 'Arcadia', VI = 'Viking', MJ = 'Major', SYG = derived from a Syngenta line, and SU = 'Sultan'.

Table 4. Correlation between cophenetic matrices (above diagonal) and similarity matrices (below diagonal) obtained with different marker sets. Cophenetic correlation coefficients for the dendrograms are in bold on the diagonal.

	SSR	AFLP	SRAP
SSR	0.77	0.35	0.58
AFLP	0.35	0.74	0.45
SRAP	0.5	0.2	0.78

exist since these markers amplify largely unmethylated gene-rich regions of the genome. All SSR primers were publicly available sequences which were chosen at random, and their distribution across the *B. oleracea* genome is not known. It is probable that the modest number of SSR markers were not evenly distributed and possible that entire chromosomes were not represented with the SSR data. This, along with the relatively low number of markers obtained with this system could explain the differences between this and the other marker types. It was suggested by Almanza-Pinzon et al. (2003) that since SSRs evolve more quickly than other marker types, they may provide a more accurate measurement of similarity in highly related lines, and decreased accuracy as the lines become less related.

Each of these marker systems was able to discriminate between the 24 elite inbreds for fingerprinting purposes, however, when determining relatedness, the confidence levels could be increased by combining marker systems and/or using more of each type of primers. Each of these techniques has some obvious advantages over the older marker systems. The high multiplex ratio of AFLPs along with the reported repeatability make them very efficient at generating large numbers of markers. The possibly uneven distribution of *EcoRI/MseI* could lead to some difficulties when trying to map under-represented regions, but switching to AFLPs generated from enzymes such as *PstI/MseI*, which are more evenly distributed in some species, could overcome this problem. SRAP markers are reported to be tightly linked to coding regions and a linkage map in *B. oleracea* showed that they were evenly distributed across the nine major linkage groups (Li and Quiros, 2001). Moreover, it has been reported that 20% of the SRAP markers are co-dominant whereas almost all AFLP primers are dominant (Li and Quiros, 2001). Although SSRs have the advantage of being co-dominant, they were the least efficient and most time-consuming of the marker systems analyzed. AFLPs and SRAPs yielded higher confidence limits and required fewer primers to discriminate among the inbreds making them better choices to replace older techniques. However, the results of this

study demonstrate that using more than one marker system increases the amount of the genome that is sampled and probably gives a more accurate estimate of genetic similarity.

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