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Randomly Amplified Polymorphic DNA in Bulb Onion and Its Use to Assess Inbred Integrity

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Abstract. **Commercial bulb-onion *(Allium cepa* L.) growers often complain that hybrids they have grown successfully for a few years fail to perform at the expected level. Inbreds used to produce hybrid-onion seed rarely have been self-pollinated for more than two generations and retain a high level of heterozygosity. Over time, selection, drift, or contamination of inbreds may contribute to disappointing hybrid performance. We identified randomly amplified polymorphic DNA (RAPD) between two inbred onion lines, demonstrated their Mendelian inheritance, and tried to distinguish among and examine changes in independently maintained, publicly released inbred lines of onion. We observed poor agreement between data sets based on genetically characterized and uncharacterized RAPD markers. Our analyses used only genetically characterized RAPD markers and revealed that contamination, in addition to-drift and/or selection, likely contributed to differences among independently maintained, publicly released inbreds. However, RAPD markers were not able to distinguish confidently among four related inbreds. RAPD markers will be useful in *Allium* genetics and breeding, but identifying and characterizing reliable polymorphisms is critical.**

Commercial bulb-onion growers often complain that a hybrid they have grown successfully for a few years fails to perform at the expected standard. Presumably, changes in hybrid performance may be attributed to changes in the inbreds used to generate the hybrid. Because of severe inbreeding depression, inbred onion lines are generally developed by self pollination of a single bulb for one or two generations followed by cross-pollination among a few selected bulbs (Pike, 1986). The inbred line remains quite heterozygous, allowing changes in genetic constitution over time by drift or selection.

Randomly amplified polymorphic DNA (RAPD) has been proposed as a substitute for or a complement to isozymes and restriction fragment-length polymorphisms (RFLPs) for many applications in plant genetics and breeding (Williams et al., 1990). RAPD markers require only small amounts of DNA, are potentially cheaper and faster, and require less technical expertise than RFLPs. Disadvantages of RAPD markers include dominance (Williams et al., 1990), difficulties with repeatability, and skewed segregation ratios (Echt et al., 1992; Heun and Helentjaris, 1993; Reiter et al., 1992; Riedy et al., 1992; Thormann et al., 1994; Tulsieram et al., 1992; Weeden et al., 1992). There have been two reports of RAPD markers in *Allium,* assessing variability among species (Wilkie et al., 1993) and cultivars (Roxas and Peffley, 1992). However, neither presented inheritance data. In this study, we identified 12 normally segregating RAPD markers and evaluated changes in independently maintained, publicly released inbreds due to contamination, drift, or selection.

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Materials and Methods

Plant material and DNA extraction. 'Alisa Craig' (AC) is an European cultivar grown as a show onion in the United Kingdom that has been selected for large size and possesses correlated traits of low pungency, low soluble solids, and poor longevity in storage (Bedford, 1984; Hosfield et al., 1977a, 1977b). 'Brigham Yellow Globe' (BYG) is a cultivar grown in the eastern United States primarily as a storage onion and is characterized by high pungency, high soluble solids, and good longevity in storage (Magruder et al., 1941). AC43 and BYG 15-23 are inbreds developed by self-pollinating single bulbs for one and two generations, respectively. These two inbreds and an F_2 family from self pollination of a single F, plant were the gift of J.F. Watson, SunSeeds, Brooks, Ore.

Green leaf tissue was harvested from at least 20 seedlings each of BYG15-23, AC43, 59 F_3 families from self pollination of F_2 bulbs from BYG15-23 x AC43 (seed lots listed by Bradeen, 1994), and four publicly released inbred lines (Table 1) maintained at five separate locations [U.S. Dept. of Agriculture (USDA) and one company in Wisconsin, two companies in Idaho, and one in Oregon]. Harvested tissue was frozen in liquid nitrogen and lyophilized. DNA was extracted and purified through CsCl gradients (Murray and Thompson, 1980). Concentrations were determined spectrophotometrically and adjusted to 1 ng DNA/µl with sterile distilled water.

RAPD amplification conditions and procedures. Amplifications were carried out in a 15-µl volume with 50 mM KCl, 20 mM Tris-HCl (pH 9.0), 0.1% Triton X- 100, 2 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, and dTTP (dNTPs), 5 ng template DNA, 0.33 µM decamer primer, and 0.5 unit Taq polymerase (Promega,. Madison, Wis.). A total of 580 decamer primers (Operon, Alameda, Calif.) was examined, sets A-E and AA-AX (each set contains 20 numbered primers; sequences listed by Bradeen, 1994). Thermal cycler (9600 PCR machine; Perkin-Elmer, Norwalk, Conn.) con-

Table 1. Publicly released inbred lines selected from open-pollinated populations.

Inbred	Original population"	Release date
MSU611B	Downing Yellow Globe	12 Oct. 1979
MSU826B	Downing Yellow Globe-Trapp's Strain	23 Apr. 1982
MSU4535B	Downing Yellow Globe-Trapp's Strain	1 Feb. 1974
MSU5718B	Iowa Yellow Globe 53	23 Apr. 1982

zOpen-pollinated population from which inbred was selected. In all cases, the inbred was developed from progeny from self pollination of a single bulb from the open-pollinated population. Downing Yellow Globe (DYG) is an open-pollinated population historically grown in Michigan. The Trapp's strain of DYG is a specific selection grown near Beulah, Mich. Iowa Yellow Globe 53 is a synthetic population developed by C.E. Peterson in 1953 by crossing among Iowa strains of storage onions. Bark and Havey (1995) demonstrated thatpopulations of storage onions grown in the north-eastern and north-central United States are closely related.

ditions were one cycle of 2 min at 94C; 40 cycles of 2 sec at 94C, 1 min at 40C, 2 min at 72C; and hold at 4C. To minimize experimental variation between replications and across primers, bulk mixes containing the reaction buffer—$MgCl_2$, dNTPs, and sterile distilled water-were prepared and stored at -20C until use. PCR-amplification products were electrophoresed through a 1.4%-agarose gel with 75 µg ethidium bromide in lx TBE (Sambrook et al., 1989) at 90 V for 3 h. After electrophoresis, gels were photographed (Eagle-Eye System, Stratagene, La Jolla, Calif.) and polymorphic bands were scored as present (1) or absent (0).

Primers detecting putative polymorphisms were first identified using nonreplicated reactions with DNA of AC43 and BYG15-23 and then verified with two additional replications comparing a control (sterile distilled water) with DNA of AC43 and BYG15-23. For 53 primers (A12, A20, B01, C09, C15, C17, D03, D05, D07, D10, D12, D17, E14, AA12, AB11, AB14, AB16, AB19, AB20, AC09, AC13, AC19, AD06, AD19, AD20, AE04, AE09, AF07, AF12, AF16, AG19, AH05, AH08, AH15, AI15, AI17, AI20,AJ02,AJ05,AJ09,AJ12,AJ14,AJ17,AK02,AK13,AK20, AO13, AP12, AQ20, AS10, AU05, AU08, and AW07), the polymorphic bands appeared in a minimum of three replicate reactions and were absent from the control. Ten F_3 families were then evaluated to determine whether the polymorphism was segregating. Those primers consistent across a minimum of two replications of the 10 F_3 family DNAs were used to examine segregations in a minimum of two replications of all 59 F_3 families. Primers (C15, D03, D10, D12, AB16, AB20, AD19, AE09, AF12, AG19, AK20, and AP12) revealing RAPD markers fitting the expected 3: 1 ratio for the replications were used to evaluate AC43, BYG15-23, the inbred x location accessions, and one control, using a minimum of two replications. All polymorphic bands, including those not segregating in the BYG15-23 x AC43 F_3 families, were scored as present or absent for the 20 inbred x location accessions, BYG15-23, and AC43.

Cytoplasmic determinations. Conditions and procedures for detecting polymorphic restriction-enzyme sites in the chloroplast genome were as previously described (Havey, 1991). Orchid chloroplast clones 3, 4, and 6a (Chase and Palmer, 1989) were hybridized to blots of *Bgl II* (GIBCO-BRL, Gaithersburg, Md.) digested DNA from each of the 20 inbred x location accessions and from one normal male-fertile (N; B3350B) and one sterile (S; YB986A) cytoplasmic check (Havey, 1993). Each inbred x location accession was classified as possessing N, S, or a mixture of cytoplasms as described by Havey (1993).

Data analysis. Chi-square tests for goodness-of-fit to expected 3:1 ratios and two-point linkage analysis were conducted using Linkage-1 version 3.5 (Suiter et al., 1987). Two-point linkage analyses were also completed using Mapmaker version 4.0 (Lander et al., 1987) with LOD of 3.0 and maximal recombination of 0.40.

For RAPD analysis of the inbreds, two data sets were generated. The first consisted only of bands segregating at the expected 3: 1 ratio (subsequently referred to as genetically characterized). The second set consisted of polymorphisms not segregating in BYG15-23 **x** AC43 (referred to as genetically uncharacterized). The matrix correlation was calculated for the simple matching (SM; Euclidean distance) matrices of the two data sets (NTSYS-pc 1.70, Exeter Software, Setauket, N.Y.). The SM matrix of the genetically characterized data set was used for UPGMA cluster analysis (NTSYS) and the raw genetically characterized data set for principal component analysis (PCA) (SAS Institute, Cary, N.C.).

Results and Discussion

Identification and segregation of RAPD markers. Of 580 primers examined, 527 failed to amplify reliably a polymorphism between BYG15-23 and AC43. Of the 527 primers, 22 did not amplify any clearly defined bands, 30 amplified a polymorphism that was ambiguous or could not be repeated, and 475 amplified distinct, repeatable bands but detected no polymorphisms. This low level of putative polymorphism may be due, in part, to the highly heterozygous nature of onion and the dominance of RAPD markers. Of the remaining 53 primers that revealed repeatable polymorphisms among the parents (9.1% of the total number evaluated), 10 amplified putative RAPD markers that did not segregate, possibly due to heterozygosity in one parent, and 28 were eliminated due to ambiguities in the presence of polymorphic bands among F_3 families; 15 primers (2.6% of the original 580) revealed one storable segregating RAPD each. Twelve of the RAPD markers segregated at the expected 3: 1 ratio ($P > 0.05$) for a dominant marker (Fig. 1, Table 2). No codominant RAPD markers were detected. Linkage analysis of these 12 polymorphisms revealed three associations: DO3 and D12 at a recombination level of 0.18, AD19 and AE09 at 0.09, and Cl5 and AF12 at 0.07.

Decamer primers were less efficient at detecting polymor-

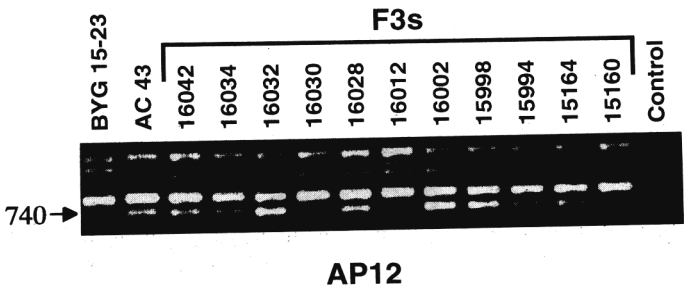

Fig. 1. Segregation of randomly amplified polymorphic DNA using primer AP12 for parental inbreds (AC43 and BYG15-23), 11 F_2-derived F_3 families, and control (no DNA).

Table 2. Observed segregation ratios and chi-square goodness-of-fit values to expected 3:1 segregation ratios for 1.5 randomly amplified polymorphic DNA markers.

Primer	A-:aa	χ^2	P
C15	46:13	0.28	0.60
D03	41:18	0.95	0.33
D10	42:17	0.46	0.50
D12	40:19	1.63	0.20
AB14	36:23	6.15	0.01*
AB16	50:9	2.99	0.08
AB20	50:9	2.99	0.08
AD19	43:16	0.14	0.71
AE09	46:13	0.28	0.60
AF07	51:8	4.12	0.04*
AF12	42:17	0.46	0.50
AG19	42:17	0.46	0.50
AJ14	54:5	8.59	0.00*
AK20	45:14	0.05	0.82
AP12	46:13	0.28	0.60

*Significantly deviating from expected ratios at $P < 0.05$.

phisms in onion than hybridizations of random cDNAs. Only 9.1% of the primers detected a reliable polymorphism between AC43 and BYG15-23 and 2.1% of all primers revealed polymorphisms segregating at expected Mendelian ratios. Bark and Havey (1995) observed that 67% of cDNA probes detected RFLPs between AC43 and BYG15-23 digested with four restriction enzymes. Although we have not established segregations for all of the RFLPs, their frequency in onion suggests that RFLPs may provide more numerous and useful codominant markers for map development. However, to identify RAPD markers more efficiently in a highly heterozygous crop such as onion, a better approach would be to evaluate F_3 family DNAs to reveal segregating RAPD markers that appear monomorphic between heterozygous parents.

Inbred integrity. For this study, we specifically requested N-cytoplasmic lines as determined by pedigree. Chloroplast clones differentiating N and S cytoplasms (Havey, 1993) confirmed that all USDA accessions possessed only N cytoplasm. S cytoplasm was detected in MSU611B fromcompanies 1, 2, and 4; in MSU826B from companies 1 and 2; and trace amounts in MSU4535B and MSU5718B from company 1 (Fig. 2). Tatebe (1968), Corriveau and Coleman (1988), and Havey (1995) established maternal inheritance of the chloroplast DNA in *A. cepa.* Therefore, contamination of the N-cytoplasmic inbreds with S cytoplasm most likely occurred via seed or bulb mixtures due to human error in maintaining the inbred lines. Because the S-cytoplasmic lines are male-sterile, it is not possible for S cytoplasm to be transferred to the N-cytoplasmic maintainer line by infrequent paternal or biparental inheritance of the cpDNA.

Each of the 12 primers generated at least one polymorphism among or within inbred lines and revealed 11 genetically characterized and 11 uncharacterized RAPD markers [C15, D03, D12, AB16, AB20, AD19, AE09 (two polymorphisms), AF12, and AG19 (two polymorphisms)] (Fig. 3; Table 3). All primers except AB16 detected polymorphisms among inbred lines for the geneti-

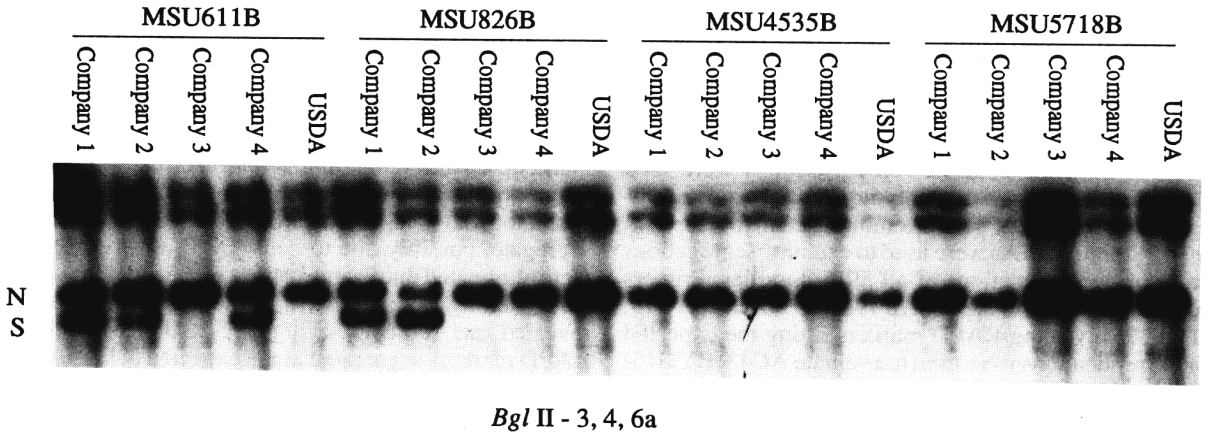

Fig. 2. Autoradiograph of inbred DNA digested with *Bgl II* and hybridized with orchid chloroplast clones 3, 4, and 6a differentiating male-fertile (N) and male-sterile (S) cytoplasms.

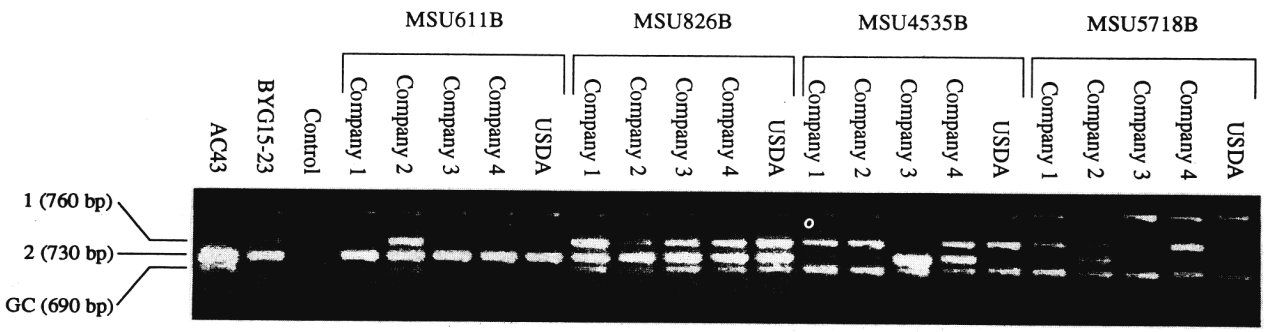

Fig. 3. Randomly amplified polymorphic DNA for AC43, BYG15-23, and inbred lines generated using primer AG19. The genetically characterized band (GC) is polymorphic within and across inbred lines. Additionally, other bands that were not genetically characterized (numbers 1 and 2) were polymorphic within and among inbred lines.

Table 3. Size and presence (1) or absence (0) of genetically characterized (C[z]) and uncharacterized (U[y]) randomly amplified polymorphic DNA (RAPD) markers for parents of segregating family and inbred lines.

		Primer[x]								
		C15	C15	D03	D03	D10	D12	D12	AB16	AB16
		C	*U*	*C*	*U*	*C*	*C*	*U*	*C*	*U*
Inbred	Source	750[w]	780	720	670	650	530	570	1160	1070
AC43		1	1	1	1	1	1	1	0	0
BYG15-23		0	1	0	1	0	0	1	1	0
MSU611B	Company 1	0	1	0	1	1	0	1	1	0
	Company 2	0	1	0	1	1	0	0	1	0
	Company 3	0	0	0	1	1	0	1	1	0
	Company 4	0	0	0	1	1	0	1	1	0
	USDA	0	0	0	1	1	0	1	1	0
MSU826B	Company 1	0	1	1	1	1	0	0	1	1
	Company 2	0	1	1	1	1	0	1	1	0
	Company 3	1	1	1	1	1	0	0	1	0
	Company 4	1	1	1	1	1	1	0	1	1
	USDA	0	1	0	1	0	1	0	1	1
MSU4535B	Company 1	0	1	1	0	0	0	0	1	0
	Company 2	0	1	1	0	1	0	0	1	0
	Company 3	0	1	1	0	1	0	1	1	0
	Company 4	0	1	1	0	1	0	0	1	0
	USDA	0	1	1	0	0	0	0	1	0
MSU5718B	Company 1	0	1	1	1	1	0	1	1	0
	Company 2	0	1	1	1	1	0	1	1	0
	Company 3	0	1	1	1	1	0	1	1	0
	Company 4	0	1	1	1	1	0	1	1	0
	USDA	0	0	1	1	1	0	1	1	0

		Primer							
		AB20	AB20	AD19	AD19	AE09	AE09	AE09	AF12
		C	*U*	*C*	*U*	*C*	*U*	*U*	*U*
		840[w]	790	880	840	700	860	770	800
AC43		0	1	0	1	0	0	1	0
BYG15-23		1	1	1	1	1	1	0	1
MSU611B	Company 1	1	1	1	0	0	1	1	0
	Company 2	0	1	1	0	0	1	0	1
	Company 3	0	1	1	0	0	1	1	0
	Company 4	0	0	1	0	0	1	1	0
	USDA	0	0	1	0	0	1	1	0
MSU826B	Company 1	0	0	1	1	1	0	1	1
	Company 2	1	0	0	1	0	1	1	1
	Company 3	0	0	1	1	1	0	1	1
	Company 4	0	0	1	1	1	0	1	1
	USDA	0	0	1	1	1	0	1	1
MSU4535B	Company 1	0	1	1	1	0	1	0	0
	Company 2	0	1	1	1	0	1	0	0
	Company 3	0	1	1	1	0	1	0	0
	Company 4	1	1	1	1	0	1	1	1
	USDA	0	1	1	1	0	1	0	0
MSU5718B	Company 1	0	1	1	1	0	1	0	0
	Company 2	0	1	0	1	0	1	0	1
	Company 3	0	1	0	1	0	1	0	0
	Company 4	0	1	1	1	0	1	0	0
	USDA	0	1	0	1	0	1	0	0

cally characterized markers; however, the genetically characterized polymorphism from primer AF12 could not be unambiguously scored for all inbreds and was excluded. Two genetically characterized (AB14 and AP12) and three uncharacterized (D03, AD19, and AF07) RAPD markers were polymorphic among inbred lines but monomorphic within an inbred line (Table 3). Differences were detected among sources of the same inbred lines for pairs of linked genetically characterized RAPD markers, e.g., MSU5718B from companies 1 and 4 for primers AD 19 and AE09 (Table 3).

The matrix correlation between the genetically characterized and uncharacterized SM matrices was only 0.38, indicating substantial disagreement between the two data sets. The 11 genetically characterized and 11 uncharacterized polymorphisms identified

Table 3 (continued).

		Primer				
		AG19	AG19	AG19	AK20	AP12
		C	*U*	*U*	*C*	*C*
		690[w]	760	730	910	740
AC43		1	0	1	1	1
BYG15-23		0	0	1	0	0
MSU611B	Company 1	0	0	1	1	0
	Company 2	1	1	1	0	0
	Company 3	0	0	1	1	0
	Company 4	0	0	1	1	0
	USDA	0	0	1	1	0
MSU826B	Company 1	1	1	1	1	1
	Company 2	1	1	1	1	1
	Company 3	1	1	1	1	1
	Company 4	1	1	1	1	1
	USDA	1	1	1	1	1
MSU4535B	Company 1	1	1	0	1	0
	Company 2	1	1	0	1	0
	Company 3	1	1	0	1	0
	Company 4	1	1	1	1	0
	USDA	1	1	0	1	0
MSU5718B	Company 1	1	1	0	1	0
	Company 2	1	1	1	1	0
	Company 3	1	0	0	1	0
	Company 4	1	1	0	1	0
	USDA	1	0	0	0	0

[z]C = genetically characterized; segregations established.
[y]U = genetically uncharacterized; segregations not demonstrated. For AE09 and AF12, the uncharacterized RAPD was polymorphic between AC43 and BYG15-23 but did not segregate.
[x]Primers refer to Operon kit by letter and number, see Materials and Methods.
[w]Sizes in base pairs estimated according to Schaffer and Sederoff (198 1) using equal mixture of bacteriophage λ singly digested with Hind111 and doubly digested with *Ava* l and *Bam* HI.

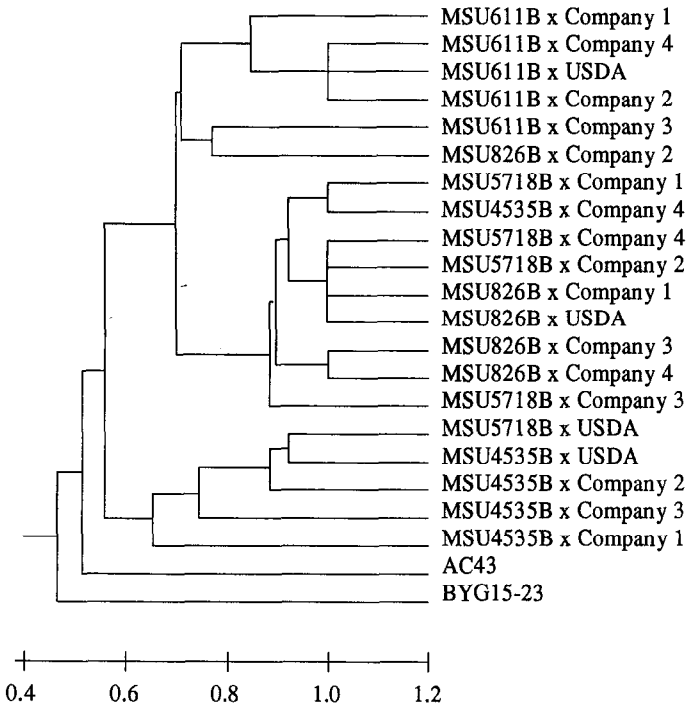

Fig. 4. UPGMA cluster analysis of independently maintained, publicly released inbreds using the simple matching (SM) matrix from genetically characterized randomly amplified polymorphic DNA markers. Scale shows SM coefficients.

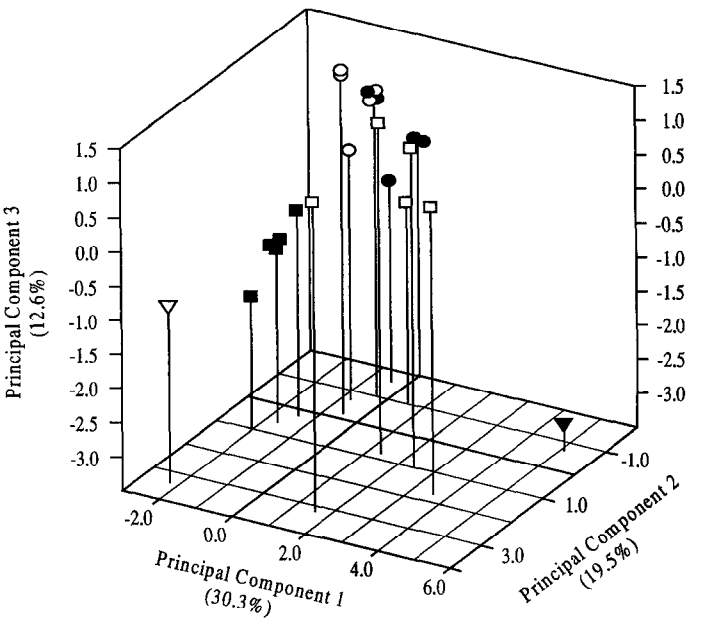

Fig. 5. Plot of the first three principal components generated from genetically. characterized randomly amplified polymorphic DNA markers among independently maintained, publicly released inbreds. (■) MSU611B; (❑) MSU826B; (❍) MSU4535B; (●) MSU5718B; (▼) AC43; and (▽) BYG15-23. Numbers in parentheses under axis labels refer to the percent variation explained by the corresponding principal component.

among inbreds is a limited number, and the matrix correlation may have been greater if more data points were available. The literature is divided on the use of genetically uncharacterized RAPD markers in population studies. Demeke et al. (1992), Fukuoka et al. (1992), Heusden and Bachmann (1992), and Santos et al. (1994) support their use, while Hu and Quiros (1991) used segregation data and Thormann et al. (1994) completed Southern (1975) analyses to verify that polymorphic bands of similar size were homologous before using them for diversity studies. Because of the potential risk of misinterpreting relationships using the genetically uncharacterized RAPD markers, we omitted these data from our analyses. All inferences to population changes and relationships among inbreds were made solely on genetically characterized polymorphisms.

If a genetically characterized RAPD were present in all but one accession of an inbred, its loss could be due to drift or selection [MSU826B from Company 2 (Fig. 3)]. However, the appearance of a genetically characterized polymorphism in one accession and its absence in all other accessions could be evidence of contamination. This scenario occurred with MSU611B from Company 2; primer AG19 failed to amplify the genetically characterized polymorphic band for all other accessions of MSU611B (Fig. 3). Other examples of potential contamination were observed for inbreds MSU611B from Company 1, MSU826B from Company 2, and MSU4535B from Company 4 with primer AB20 (Table 3). As more RAPD markers become available and more genotypes are evaluated, we will be able to assess more confidently changes in independently maintained inbreds. Nevertheless, it is clear from this study that genetically characterized RAPD markers were able to reveal differences within a narrow genetic background.

We tried to use the genetically characterized RAPD markers to distinguish among the four inbreds. UPGMA cluster analysis of the SM matrix failed to confidently separate the inbreds (Fig. 4). PCA explained 62.4% of the observed variation with the first three principal components (Fig. 5). There was a tendency for different sources of the same inbred line to cluster, e.g., accessions of MSU611B formed a loose group (Figs. 4 and 5). However, MSU826B **x** Company 2 was weakly placed in the MSU611B cluster (SM coefficient of 0.697). For UPGMA cluster analysis and PCA, AC43 and BYG15-23 were distant from each other (Figs. 4 and 5) as expected because we selected RAPD markers segregating in this cross. The inability of our 11 genetically characterized RAPD markers to separate the four groups of inbreds clearly may be due to the limited data set, close relationships among the inbred lines, contamination as evidenced by the presence of S cytoplasm (Fig. 2) or the appearance of genetically characterized fragments in one inbred (e.g., AB20 in Table 3), selection or drift [loss of genetically characterized band in MSU826B (Fig. 3)], or the inefficiency of dominant markers in a highly heterozygous crop.

Because of the poor agreement between the genetically characterized and uncharacterized data sets, we chose to omit the uncharacterized polymorphisms from our analyses. As the numbers of genetically characterized RAPD markers increase, it will be possible to answer more sophisticated questions. Eventually, enough codominant RAPD markers or other PCR-based markers may be available to allow the seed industry to characterize inbreds quickly and easily and document changes in their constitution. However, there are serious caveats associated with the use of RAPD markers. Before any application, it is crucial that RAPD markers be characterized, either by segregation analysis or band sequence homology.

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