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A comparison of RAPD and isozyme analyses for determining the genetic relationships among *Avena sterilis* L. accessions

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Abstract. Isozyme analysis is a valuable tool for determining genetic relationships among breeding lines and populations. The recently developed DNA technologies which can assay a greater proportion of the plant genome are providing a plentiful array of additional genomic markers. The objective of this research was to compare random amplified polymorphic DNA (RAPD) versus isozyme-based estimation of relationships among 24 accessions of a hexaploid wild oat, *Avena sterilis* L. The accessions were evaluated for variation in 23 enzyme systems and by 21 10-mer primers. A total of 77 polymorphic isozyme bands and 115 polymorphic RAPD bands were observed. Two matrices of genetic distances were estimated based on band presence/absence. These matrices were subsequently utilized in cluster analysis and principal coordinate analysis. Both isozymes and RAPDs were proficient at distinguishing between the 24 accessions. The correspondence between the elements of both distance matrices was moderate ($r = 0.36^{**}$). Nevertheless, the overall representation of relationships among accessions by cluster analysis and ordination was in considerable agreement. The two techniques contrasted most notably in pair-by-pair comparisons of relationships. RAPD analysis resulted in a more definitive separation of clusters of accessions. The most significant impact of the DNA-based markers probably will be the more accurate determination of relationships between accessions that are too close to be accurately differentiated by isozymes.

Key words: Cluster analysis – Principal coordinate analysis – Oat – Wild germ plasm – Molecular marker

Introduction

Protein-based polymorphism is a valuable tool in plant research, but the development of DNA-based technologies is providing a plentiful array of additional genomic markers. Restriction fragment length polymorphism (RFLP) analysis is being applied to construct linkage maps in many species (e.g., Bonierbale et al. 1988; Gebhardt et al. 1989; Helentjaris 1987; Heun et al. 1991; Landry et al. 1987a; Liu and Tsunewaki 1991; McCouch et al. 1988; Tanksley et al. 1992), and the practical benefits of its application to plant breeding has been reviewed by Tanksley et al. (1989). Recently Williams et al. (1990) proposed the use of random amplified polymorphic DNAs (RAPDs) as an additional form of molecular marker. The advantages of this technique over RFLPs include faster data production, a protocol that requires less DNA, and no radioactivity. Problems concerning reliability can be eliminated by optimizing the experimental conditions and by following precisely a chosen experimental protocol (Williams et al. 1991; Heun and Helentjaris 1993). RAPD-based linkage maps have been published (Reiter et al. 1992; Tulsieram et al. 1992), and RAPDs have been used to analyze genetic variation in several species (Chalmers et al. 1992; Demeke et al. 1992; Halward

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et al. 1992; Hu and Quiros 1991; Lanham et al. 1992; Vierling and Nguyen 1992).

Given the proliferation of genome marker technologies, a comparison of techniques is timely. The objective of this research was to compare RAPD versus isozyme-based estimates of genetic relationships among accessions of hexaploid wild oat, *Avena sterilis* L.

Materials and methods

A geographically diverse sample of 24 *A. sterilis* accessions was selected from the United States National Small Grains Collection (Table 1). Previously, Phillips et al. (1993) classified the accessions coded A1 through A6, B1 through B6, C1 through C6, and D1 through D6 into four separate clusters based on multivariate analysis of isozymic variation among a sample of 1005 accessions. All isozyme and RAPD analyses were conducted on plants tracing to one greenhouse-grown self-pollinated plant per accession.

Isozyme analysis

Descriptions of the procedures followed for the collection of isozymic data have been outlined in Phillips et al. (1993). Information on the inheritance of isozyme polymorphism in hexaploid oat was limited to esterase (Clegg and Allard 1972) and diaphorase (Souza and Sorrells 1989) when this study was initiated. Thus, isozyme phenotypes were recorded and assumed to be analogous to homozygous genotypes. Twenty-three enzyme systems were assayed, but 3 systems had more than 1 distinct zone of band activity. Esterase gels were divided into 5 zones approximating the descriptions of Clegg and Allard (1972). In addition, flourescent esterase and malate dehydrogenase had 2 zones each, giving a total of 29 zones of band activity across all enzyme systems.

Table 1. Code, *Avena sterilis* accession number, and country and site of accession origin

Code	Accession	Country	Site
A1	PI 412306	Morocco	Tahanoute
A2	PI 367540	Spain	Loja
A3	PI 379944	Israel	Lod airport
A4	PI 367468	Spain	La rabita
A5	PI 380010	Israel	Hadera
A6	PI 411544	Algeria	Oran
B1	PI 411998	Iraq	Hamdaniya
B2	PI 412208	Lebanon	Baalbek
B3	PI 411524	Algeria	El Asnam
B4	PI 411997	Iraq	Hamdaniya
B5	PI 324783	Libya	Al Guarsha
B6	PI 411831	Iran	Gorgan
C1	PI 324747	Sicily	Palma
C2	PI 367484	Spain	Motril
C3	PI 412657	Turkey	Serefilkochisar
C4	PI 393628	Morocco	Rommoni
C5	PI 324789	Libya	Al-Rgaiaat
C6	PI 411662	Ethiopia	Wikro
D1	PI 411972	Iraq	Baghdad
D2	PI 411856	Iran	Kermanshah
D3	PI 411727	Iran	Chalus
D4	PI 411766	Iran	Ghasr-Shrin
D5	PI 220373	Afghanistan	Girbad
D6	PI 411913	Iran	Shah Abad Gharb

For each accession, an isozyme band presence/absence profile was recorded, consisting of a string of 134 1's (presence) or 0's (absence) describing the two alternate states at each of the 134 positions where scorable bands were observed over all 29 zones by Phillips et al. (1993). The 29 zones were divided into three groups based on an estimate of the relative number of loci involved (Clegg and Allard 1972; Souza and Sorrells 1989; Stuber et al. 1988; Yen and Sadanaga 1977): (1) a putative 'one-locus' group (esterases, glycerate-2-dehydrogenase, fluorescent esterase, and amylase); (2) a putative 'two-locus' group (superoxide dismutase, shikimate dehydrogenase, acid phosphatase, aldolase, adenylate kinase, catalase, endopeptidase, fluorescent esterase, triose phosphate isomerase, phosphoglucomutase, aminopeptidase, diaphorase, glutamate oxaloacetate transaminase, isocitrate dehydrogenase, and peroxidase); and (3) a putative 'three-locus' group (leucine aminopeptidase, phosphohexose isomerase, aconitase, malate dehydrogenases, and 6-phosphogluconate dehydrogenase).

For each of the three locus classes, Jaccard's distances (Sneath and Sokal 1973) were estimated between all pairs of the 24 accessions of *A. sterilis*:

$$JD(I,J) = 1 - \frac{M}{(T_I + T_J) - M}$$

where M was the number of band matches between accessions I and J, and T_I and T_J the total number of bands in accessions I and J, respectively. The three distances were weighted according to the purported portion of information contained in each distance estimate. For example, 8 of the zones were assumed to be controlled by a single 'locus', 15 zones by two 'loci', and six zones by three 'loci'. The matrix of combined Jaccard's distances between pairs of accessions were calculated using these weightings (8:30:18) for the three separate estimates.

RAPD analysis

Leaf tissue was harvested from 6-week-old greenhouse-grown plants and lyophilized in preparation for DNA extraction. The dried tissue was ground with a coffee grinder, and 0.3 g was used to extract DNA using the CTAB procedure outlined by Saghai-Maroof et al. (1984). The protocol for detecting RAPDs was similar to that described by Williams et al. (1990) and identical (except for the gel electrophoresis) to that of Heun and Helentjaris (1993). The polymerase chain reaction (PCR) mix (given for a 50-µl total volume) was 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.1 mg/ml gelatin (all included with the 10 × buffer of Boehringer Mannheim, Indianapolis, Ind.), 0.1 m*M* each of dATP, dCTP, dGTP, and dTTP (from Pharmacia, Piscataway, N.J.; mixed fresh when setting up the master mix; see below), 0.25 µ*M* primer (from Operon Technologies, Alameda, Calif.; see below), approximately 130 ng template DNA, and 1.75 units *Taq* DNA polymerase (from Boehringer Mannheim). This mixture was covered with mineral oil (from Perkin Elmer, Norwalk, Conn.) and exposed to the following temperature profiles using the Original DNA Thermal Cycler from Perkin Elmer: (95 °C for 0.5 min; 40 °C for 0.5 min/70 °C for 1.0 min) × 1, (95 °C for 0.5 min/92 °C for 2.5 min/35 °C for 1.0 min/72 °C for 2 min) × 1, (92 °C for 1.0 min/35 °C for 1.0 min/72 °C for 2.0 min) × 44, (72 °C for 10 min) × 1 and then stored at 4 °C. After the addition of 10 µl stopping buffer (70% glycerol, 0.5 × TBE, 20 m*M* EDTA, 0.2% SDS, 0.6 mg/ml bromphenol blue), 10 µl was loaded onto a 1.5% agarose gel and run at high voltage for 5 h, so that the 506/517-bp size marker of the

1-kbp ladder (from Gibco BRL, Gaitherburg, Md.) migrated approximately 9 cm. Sharp bands became visible after staining the gel with ethidium bromide. The negatives of Polaroid film 55 (print area 9 × 11.5 cm) were used to score the bands (see below). Thin-welled tubes (MicroAmp™ Reaction tubes from Perkin Elmer) were used to perform the PCR reactions, and all reactions were performed strictly according to the following scheme: first, the DNA (diluted in 1 µl TE) was put into the tubes, which were then kept at 4 °C. Second, a master mix (buffer, dNTPs) was prepared using ultra-pure water (Fresenius, Bad Homburg, FRG) and vortexed thoroughly. Third, the master mix was divided up, and the primers were mixed in thoroughly. Fourth, *Taq* was added, the mixture was shaken carefully and, finally, 49 µl reaction mix was added into each tube and covered with two drops of mineral oil.

A total of 21 primers (Operon Technologies, Almeda, Calif.) were used. Their sequences read as follows from the 5′ end: A-01 = CAGGCCCTTC, A-02 = TGCCGAGCTG, A-03 = AGTCAGCCAC, A-04 = AATCGGGCTG, A-09 = GGGTAACGCC, A-12 = TCGGCGATAG, A-13 = CAGCACCCAC, A-14 = TCTGTGCTGG, A-16 = AGCCAGCGAA, A-17 = GACCGCTTGT, A-18 = AGGTGACCGT, A-19 = CAAACGTCGG, A-20 = GTTGCGATCC, B-01 = GTTTCGCTCC, B-02 = TGATCCCTGG, B-03 = CATCCCCCTG, B-04 = GGACTGGAGT, B-05 = TGCGCCCTTC, B-06 = TGCTCTGCCC, B-07 = GGTGACGCAG, and B-08 = GTCCACACGG.

Reproducibility of results was evaluated by replicating the RAPD analyses on all 24 accessions with primers A-20, B-01, and B-08; on 12 accessions with primers A-02 and B-02; and on 6 accessions with primers B-03 and B-08.

For each accession, a RAPD band presence/absence profile was recorded, consisting of a string of 177 1's (presence) or 0's (absence) describing the two alternate states at each of the 177 positions where unambiguously scorable bands were observed over all 21 primers. A matrix of Jaccard's distances between pairs of accessions was calculated using the same formula described for isozyme scores. All RAPD bands were given equal weightings.

Distance matrices comparison, clustering, and ordination

The correspondence between the isozyme- and RAPD-based genetic distance matrices was tested using the MXCOMP procedure in NTSYS-pc (Rohlf 1992). If both matrices contain corresponding distance estimates, then the value of the test criterion, Z, would be large compared to chance expectation, when

$$Z = \sum_{i<j}^{n} X_{ij} Y_{ij}$$

and X_{ij} and Y_{ij} are the off-diagonal elements of the distance matrices X and Y (Mantel 1967). The estimated Z was compared with its permutational distribution obtained from 500 random samples of all possible permutations of the matrices. This provided an empirical probability of obtaining a random Z in excess of the estimated Z. A Pearson product-moment correlation (r) between the elements in the two matrices was also computed (Smouse et al. 1986). This correlation, or Normalized Mantel Statistic, is monotonically related to Z.

A clustering procedure [unweighted pair group method algorithm (Sneath and Sokal 1973)] was employed to produce two dendrographs of the 24 accessions based upon the two distance matrices. A Fortran program for computing Jaccard's distance was combined with parts of programs written by D. M. Rodgers (Murphy et al. 1986) and McCammon and Wenninger (1970) to construct the dendrographs.

Two prinicpal coordinate analyses were conducted (Gower 1966) based on the two distance matrices using the DCENTER and EIGEN procedures in NTSYS-pc (Rohlf 1992). Three-dimensional ordination provided an additional representation of genetic relationships among the 24 accessions. Finally, two principal component analyses based on covariance matrices of isozyme and RAPD band frequencies in the four groups of *A. sterilis* accessions was conducted to identify enzyme systems and primers that were important in differentiating among accessions.

Results and discussion

Reproducibility of RAPD analysis was high (Figs. 1, 2). For example, nine unambiguously scorable polymorphic bands were recorded with primer A-20. A

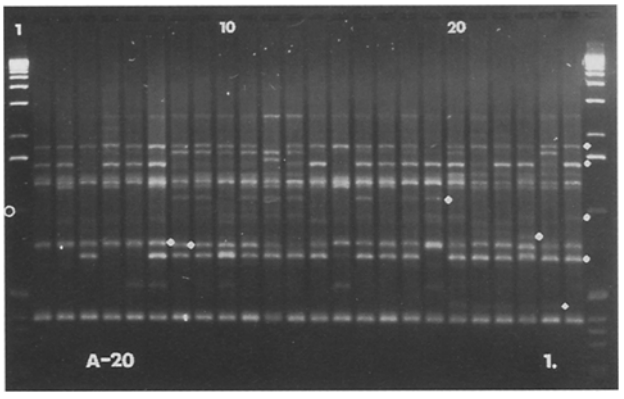

Fig. 1. RAPD patterns of 24 *A. sterilis* accessions with primer A-20. *Lanes 2–7* contain the amplified products from the oat accessions A1–A6, *lanes 8–13* B1–B6, *lanes 14–19* C1–C6, *lanes 20–25* D1–D6. The 1018-bp fragment of the 1-kbp DNA ladder in lane 1 from Gibco/BRL (lanes 1 and 26) is marked by ○. The unambiguously scorable bands are marked by ◆

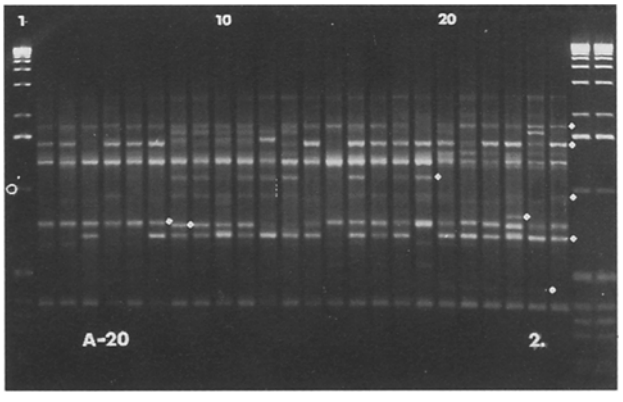

Fig. 2. Replicated RAPD patterns of 24 *A. sterilis* accessions (arranged as described in Fig. 1) with primer A-20. The 1018-bp fragment of the 1-kbp DNA ladder in lane 1 is marked by ○. The unambiguously scorable bands are marked by ◆

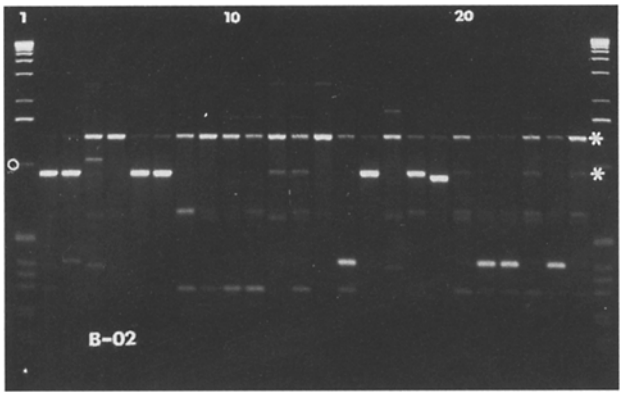

Fig. 3. RAPD patterns of 24 *A. sterilis* accessions (same order as in Fig. 1) with primer B-02. The 1018-bp fragment in lane 1 is marked by ○. Two polymorphic bands showing quantitative intensity variation are marked by *

comparison of the 216 scores over all 24 accessions from the two replicate A-20 amplifications resulted in 97% reproducibility. Results differed for only 7 scores, primarily due to bands scored as absent in one replication and weakly visible in the second. Similar levels of reproducibility were observed in replicated amplifications of the other six primers examined. Quantitative differences in band intensity were observed (Fig. 3). Since the reproducibility of quantitative differences might not be as consistent (Heun and Helentjaris 1993), only band presence versus absence was analyzed in this study. Nevertheless, these quantitative differences probably warrant further investigation since they offer additional differentiation among genotypes.

Both isozyme and RAPD markers were proficient at distinguishing between the 24 *A. sterilis* accessions. Twenty-four unique isozyme and 24 unique RAPD banding patterns were observed. A total of 104 isozyme bands was recorded over all 29 enzyme zones with a mean of 3.6 and a range of 1 to 7 bands per zone. Seventy-seven bands were polymorphic. This compared to 134 total and 125 polymorphic bands recorded by Phillips et al. (1993) in a sample of 1005 *A. sterilis* accessions. Aldolase was the only monomorphic enzyme system. A total of 177 RAPD was observed over all 21 primers with a mean of 8.4 and a range of 2 to 15 bands per primer. One hundred and fifteen RAPD bands were polymorphic. Primer A-02, with 3 bands, was monomorphic. In contrast, 10 of the 11 bands produced by primer B-02 were polymorphic.

The correlation coefficient between the elements of the two distance matrices was 0.36 ($P < 0.01$). In addition, no estimate of Z from 500 random permutations of the matrices was equal to or larger than the observed Z. Thus, both isozyme and RAPD markers provided independent, yet moderately, corresponding estimates of genetic relationships between the 24 accessions. Both matrices resulted in similar dendrographs; the correspondence was most notable in overall dendrograph construction rather than at the level of individual pair-by-pair accession comparisons (Figs. 4, 5). Both dendrographs contained three clusters with almost complete homology in individual cluster membership when compared across dendrographs. The largest cluster contained accessions from the A and C groups of accessions with a trend towards separation of the A and C groups into two subclusters. Accession A6 was the only variant across techniques; it clustered with the B group of accessions in the isozyme analysis and the A group in the RAPD analysis. Otherwise, the separate clusters of the B and C groups of accessions were similar in composition and clearly were separated

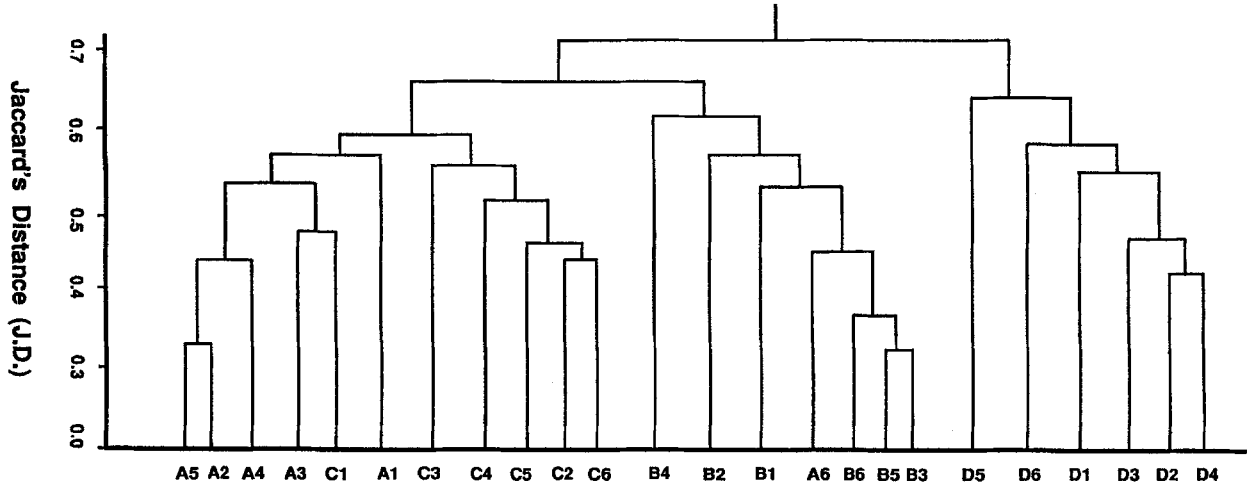

Fig. 4. Dendrograph constructed from matrix of isozyme-based genetic distances between 24 *A. sterilis* accessions

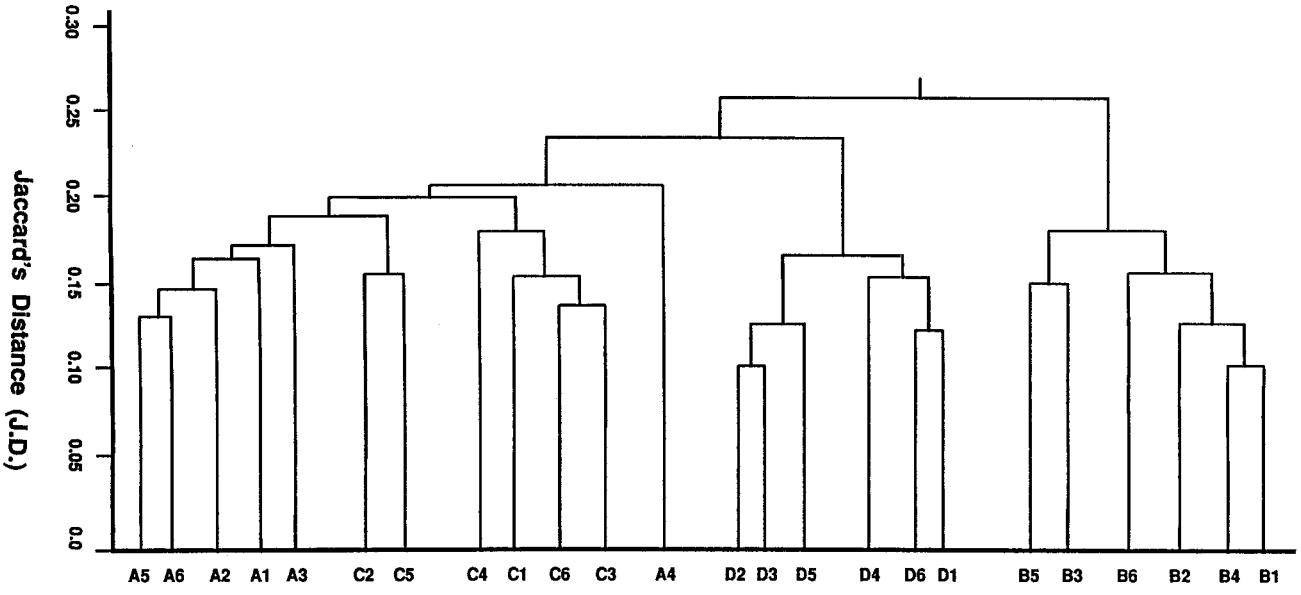

Fig. 5. Dendrograph constructed from matrix of RAPD-based genetic distances between 24 *A. sterilis* accessions

from each other and the larger cluster. The sequence in which these latter two clusters jointed the larger cluster differed for both techniques.

This cluster analysis based upon isozymic data on a subset of 24 accessions closely reflected that of Phillips et al. (1993) based upon a 1005-accession sample. Of the 24 accessions 22 clustered as expected based upon the previous analysis; accessions A6 and C1 were exceptions (Fig. 4). Cluster analysis is affected both by the estimated relationships among the accessions and the particular group of accessions included in the analysis. Two deviants in a subsample of 24 accessions drawn from an original sample of 1005 accessions was not considered irregular. The relatively close relationship

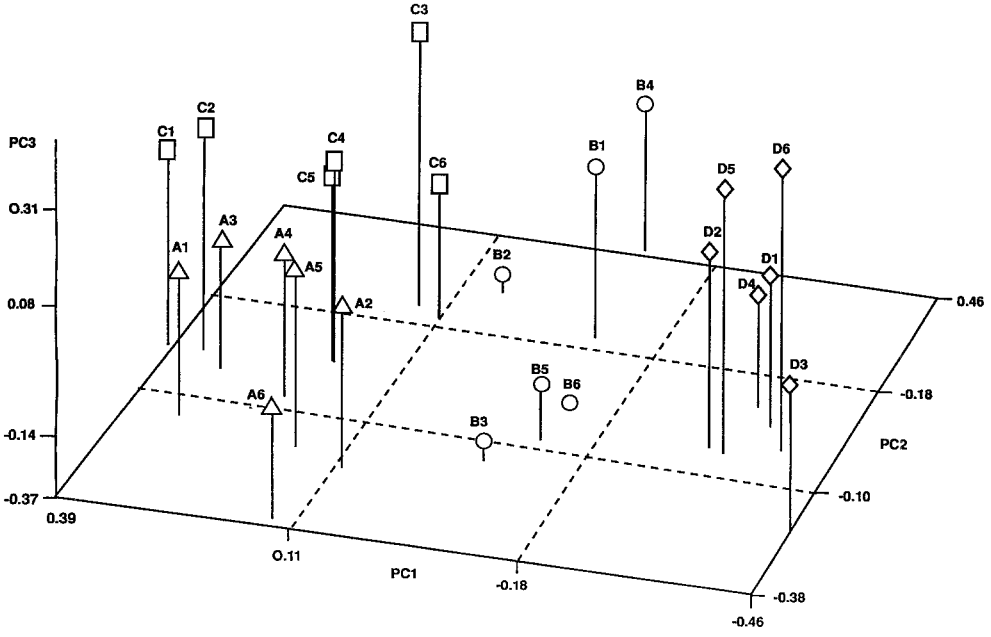

Fig. 6. Plot of the first three principal coordinate scores for 24 *A. sterilis* accessions estimated from the matrix of isozyme-based genetic distances between accessions. The *A* group of accessions represented by *pyramids*, the *B* group by *circles*, the *C* group by squares, and the *D* group by *diamonds*

between the A and C groups of accessions was observed in the previous analysis also (Phillips et al. 1993).

The distributions of the 24 accessions along the first three principal coordinate axes provided some contrast between the two marker techniques (Figs. 6, 7). The first three axes together accounted for 52% and 61% of the variation among accessions in the isozyme and RAPD marker data sets, respectively. Overall, RAPD analysis resulted in tighter, more definitive groupings of accessions. Nevertheless, both three-dimensional plots reflected relationships observed previously in dendrographs. For example, the positioning of the D group of accessions in Figs. 6 and 7 reflected the sequence with which it joined the large A–C cluster of accessions in Figs. 4 and 5. Sokal and Sneath (1973) noted the difficulty in deciding whether clustering or ordination is the most appropriate for a data set. Clustering provides the best estimates of relationships between close relatives and a poorer representation of relationships between major clusters. Representation of relationships by ordination provides the reciprocal levels of accuracy. Although the overall cluster membership was similar for comparable clusters in both dendrographs, the sequence in which accessions joined to form a cluster differed extensively between isozyme- and RAPD-based analyses. Perhaps these differences reflected contrasting estimates of close relationships among accessions by the two techniques. Isozyme analysis resulted in a more clear-cut separation of the A and C group of accessions in the large cluster. RAPD analysis indicated that accessions C2, C5, and A4 did not necessarily conform to that simple classification. In addition, RAPD analysis indicated bifurcation within both the B and D accession clusters – e.g., accession B5 and B3 versus B6, B2, and B4. Given Sokal and Sneath's (1973) assertion that ordination better represents relationships among larger clusters or groups, it is obvious from Figs. 6 and 7 that RAPD-based analysis was more definitive in its separation of clusters than isozyme-based analysis.

Studies comparing marker techniques are beginning to appear in the literature. Landry et al. (1987b) reported only approximate correlations between isozyme and RFLP relationships for a limited number of lettuce (*Latuca sativa* L.) lines. Havey and Muehlbauer (1989) found broad agreement in species relationships in lentils (*Lens* spp.) based on RFLPs and isozymes, but RFLPs detected a greater level of variation. The differentiation of 16 ecospecies of rice (*Oryza sativa* L.) by RAPDs was reported by Fukuoka et al. (1992) to be in accordance with results form prior research using isozymes and RFLP analyses. Prince et al. (1992) reported a significant correlation of 0.44 between isozyme- and RFLP-based estimates of genetic distances between 25 accessions of *Capsicum*. Similar to our research, they found both methods clearly separated major clusters of accessions (northern

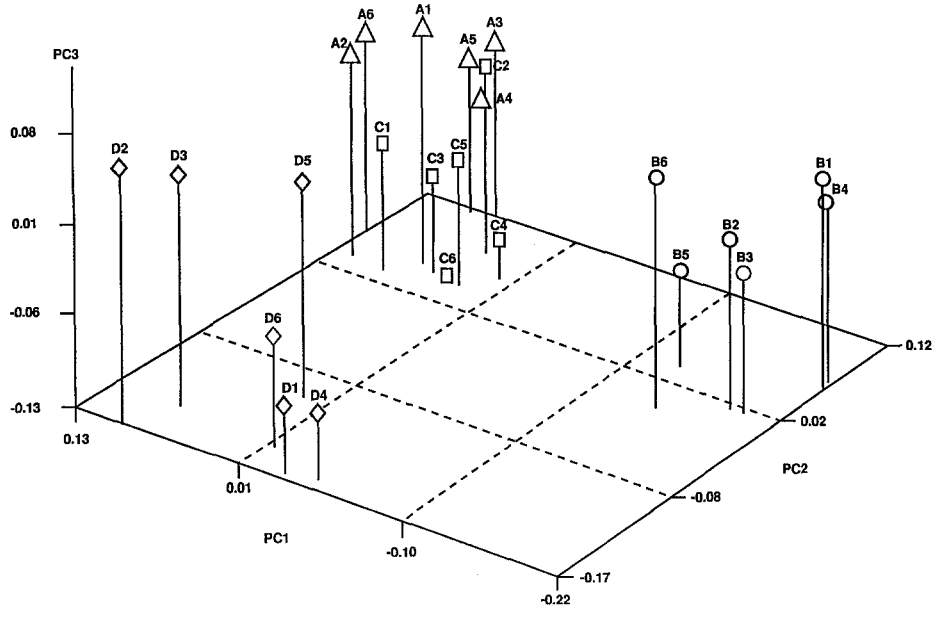

Fig. 7. Plot of the first three principal coordinate scores for 24 *A. sterilis* accessions estimated from the matrix of RAPD-based genetic distances between accessions. The *A* group of accessions represented by *pyramids*, the *B* group by *circles*, the *C* group by squares, and the *D* group by *diamonds*

versus southern Mexican), but the methods varied in depiction of exact relationships between accessions within the major clusters. In *A. sterilis*, Beer et al. (in press) found a low (0.27), significant correlation between isozyme- and RFLP-based estimates of relationship between 177 accessions. When accessions were grouped by country of origin however, the coefficients ranged between 0.02 and 0.76. Correlations between morphological-, isozyme-, and RFLP-based estimates were generally low to moderate.

Although the overall correlation of 0.36 between isozyme- and RAPD-based estimates in this study was only slightly larger than that reported by Beer et al. (in review), the overall representation of relationships among accessions by cluster analysis and ordination was in considerable agreement. The accuracy of pairwise estimates of relationship depends upon the ability of each technique to sample the genome comprehensively. Thirty-three RAPD bands produced by 15 of the 21 primers had eigenvector coefficients greater than 0.10 or less than −0.10 in the first principal component based on analysis of covariances of band frequencies in the four groups in *A. sterilis* accessions. Twenty-two bands provided by 11 of the 23 enzyme systems were similarly weighted by principal component analysis of isozyme-generated data. Forty-eight percent of the variance was accounted for by the first component in both analyses. Based upon the results of this study, it appears that relationships estimated from DNA-based markers won't necessarily be at variance with the broad representation described by isozymes. The most significant impact of the DNA-based markers probably will be more accurate determination of relationships between accessions that are too close to be accurately differentiated by isozymes.

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