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Pedigree assessment using RAPD-DGGE in cereal crop species

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Summary. The introduction of molecular biology methodologies to plant improvement programs offers an invaluable opportunity for extensive germplasm characterization. However, the detection of adequate DNA polymorphism in self-pollinating species remains on obstacle. We have optimized a denaturing-gradient-gel electrophoresis (DGGE) system which, when used in combination with random amplified polymorphic DNA (RAPD) analysis, greatly facilitates the detection of reproducible DNA polymorphism among closely related plant lines. We have used this approach to estimate pedigree relationships among a spectrum of plant materials in wheat, barley and oat. Based on analysis with one or two primers, we were able to distinguish soft from hard winter wheat, and 2-rowed from 6-rowed barley. Further analysis with additional primers allowed resolution of polymorpisms even among closely related lines in highly selected populations. We placed 17 cultivars of oat into two distinct clusters that differed significantly from previous oat pedigree assessments. We believe that DGGE-RAPD is a superior method for detecting DNA polymorphism when compared to RFLP, agarose-RAPD, or polyacrylamide-RAPD methods.

Key words: DNA polymorphism – DNA fingerprinting – Oat – Barley – Wheat

Introduction

Assessment of the degree of relatedness among lines or populations of a crop species provides a useful parameter for evaluating the effects of selection over time. Accurate pedigree evaluation also serves as a basis for the selection of appropriate parents in the development of crossing schemes. Methods for the evaluation of relatedness have in the past been based primarily on plant breeding records and plant phenotype (St. Martin 1982; Delannay et al. 1983; Rodgers et al. 1983; Cox et al. 1985a) or on biochemical markers (Wrigley 1970; Price et al. 1984; Cox et al. 1985b; Landry et al. 1987; Bonierbale et al. 1988; Tanksley et al. 1988). Here we present a DNA fingerprinting method that offers greatly enhanced resolution of genotypic variation even among closely related lines of self-pollinating plant species.

Diversity among 167 cultivars of North American spring barley was previously evaluated by a coefficient of parentage method (Martin et al. 1992a). Cluster analysis based on a coefficient of parentage matrix produced 30 distinct clusters. Similarly, Souza and Sorrells (1988, 1989) performed a predigree analysis of oat cultivars released from 1951 to 1985, grouping them into seven major groups. Using soft and hard winter wheat cultivars in cluster analysis produced two major groups with 13 subgroups (Murphy et al. 1986). Because of the extent of available pedigree information, we have selected these three crop species for a comparative pedigree assessment using a DNA-based method.

One of the most common biochemical marker methods used for genetic fingerprinting and the estimation of genetic diversity in plants utilizes restriction fragment length polymorphism (RFLP) analysis. In combination with the development of a well-defined RFLP map, one is then able to identify the regions of the genome that account for the greatest genetic diversity. RFLP-based mapping efforts are ongoing in oat, barley and wheat. The development of such a map in self-pollinated crops is often complicated by the lack of polymorphism detected among closely related plant lines. The introduction of polymerase chain reaction (PCR) technologies (Saiki

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et al. 1988) has offered alternative methods for enhancing polymorphism detected and, therefore, for applying these mapping methodologies to plant breeding. Williams et al. (1990) and Welsh and McClelland (1990) have described a random amplified polymorphic DNA (RAPD) analysis technique based on the amplification of multiple, random segments of the genome using arbitrary primers. RAPD analysis has proven valuable in plant genotype fingerprinting (Welsh and McClelland 1990; Caetano-Anolles et al. 1991; Hu and Quiros 1991), population and pedigree analyses (Van Heusden and Bachman 1992), phylogenetic studies (Halward et al. 1992), and genetic mapping (Williams et al. 1990; Martin et al. 1991 b; Michelmore et al. 1991; Reiter et al. 1992). Many of these studies utilized RAPD analysis in combination with agarose- or polyacrylamide-gel electrophoresis. However, for studies involving some self-pollinating species, this approach does not yield levels of polymorphism adequate for distinguishing closely related lines. Recently, we have adapted a denaturing-gradient-gel electrophoresis (DGGE) system (Fisher and Lerman 1983) for use in combination with RAPD analysis to efficiently detect unusually high levels of DNA polymorphism in wheat (He et al. 1992). Here we extend the use of this system for high resolution pedigree assessment of wheat, barley and oat.

Materials and methods

Plant materials

The spring oat, spring barley, and red winter wheat lines used in this study were selected based on previous pedigree assessment studies (Murphy et al. 1986; Souza and Sorrells 1988, 1989; Martin et al. 1991 a) and are listed in Table 1. Spring barley lines were kindly provided by Dr. Tom Blake (Montana State University) and Dr. J. Franckowiak (North Dakota State University).

DNA isolation

Leaf tissues from 2-week-old seedlings grown in a growth chamber were collected and immediately frozen in liquid nitrogen. DNA was isolated from 1 gm of fresh tissue by the modified procedures of Gawel and Jarret (1991). The tissues were ground in liquid nitrogen and suspended in 15 ml of extraction buffer (100 mM Tris-HCl, pH 8.0, 3.0% hexadecyltrimethylammonium bromide, 20 mM EDTA, 1 mM 1,10 o-phenanthroline, 1.4 M NaCl and 0.1% beta-mercaptoethanol). The slurry was incubated for 60 min at 60 °C. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and the aqueous fraction mixed with an equal volume of isopropanol. Precipitated DNA was removed from solution using a glass rod, washed in 80% ethanol and 15 mM ammonium acetate, and dissolved in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0.

PCR conditions

Oligonucleotide primers (10-mers) were purchased from Operon Technologies (Alameda, Calif.). The PCR reaction conditions were optimized for this study. Reaction mixtures (75 µl total volume) consisted of 10 mM Tris-HCl, pH 8.8 at 25 °C, 50 mM KCl, 1.5 mM $MgCl_2$, nucleotides dATP, dTTP, dCTP, and dGTP (200 µM each), 0.2 µM primer, 100 ng template DNA, and 1.5 units of *Taq* DNA polymerase (Promega). The reaction mixture was overlain with 80 µl of light mineral oil. Amplifications were carried out in a Genetic Thermocycler (Precision) programmed for 45 cycles of 1 min at 94 °C, 30 s at 36 °C, 1 min at 72 °C, and ending with 10 min at 72 °C. The primers were used individually or in combinations of two (see Table 2).

Table 1. List of lines from wheat, oat and barley used for RAPD-DGGE analysis

Crop	Type	Lines
Wheat (*Triticum aestivum* L)	Soft red winter	Arthur, Abe, Oasis, Beau, Adder, Monon, Benhur, Fillmore, Caldwell, Omega, Pioneer 2550, Ruler, Knox, Blueboy
	Hard red winter	Parker 76, Newton
Barley (*Hordeum vulgare* L)	2-rowed	Gallatin, Harrington, Klages, Betzes, Bowman, Bonus, Bearpaw, Hector, Clark, Lewis, CI2376
	6-rowed	Bonanza, Morex, Larker, Atlas 68, Steptoe, Chevron
Oats (*Avena sativa* L)		Tippecanoe, Stout, Clintland 64, Putnam 61, Ogle, Tyler, Clinton, Clinton 59, Hazel, Clintford, Dubois, Norline, Allen, Noble, Albion, Dal, Porter
		One line of *Avena strigosa* included

Gel electrophoresis

The PCR products (25 µl) were fractionated by denaturing-gradient-gel electrophoresis on a Hoefer vertical-gel apparatus (SE600). Gels consisted of 12% acrylamide (37.5:1 acrylamide:bisacrylamide) in TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA; pH 7.7). The gel denaturant gradient of 10–50% (100% denaturant concentration of 7 M urea, 40% formamide) was prepared with a gradient maker (Hoefer). Gels were 0.75 mm in thickness. Electrophoresis conditions were 130 V for 5 h at 60 °C. To maintain 60 °C conditions, the circulating water bath temperature was set at 65 °C. Gels were then stained in ethidium bromide (1 µg/ml) for 10 min, destained in deionized water for 1 h, and photographed over a long wave UV light source.

Agarose-gel electrophoresis consisted of 1.2% agarose in 0.5 TBE buffer (0.045 M Tris-borate and 1.0 mM EDTA pH 8.0), at 4 V/cm for 3 h. Polyacrylamide gel conditions were identical to those for DGGE excluding the denaturant. Gels were run at room temperature.

Data analysis

RAPD profiles were scored visually. Variation among samples was evaluated from pair-wise comparisons of the proportion of shared fragments among samples, i.e., two times the number of shared fragments divided by the total number in the profiles

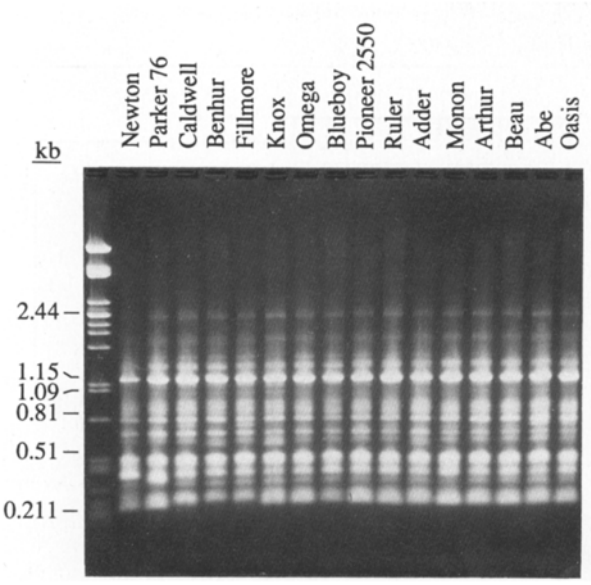

Fig. 1. PCR amplification of genomic DNA from 16 lines of winter wheat with primer A17 and agarose-gel electrophoresis. The DNA samples were fractionated on 1.2% agarose using 0.5 × TBE and run for 3 h at 4 V/cm. The gel has been stained with ethidium bromide

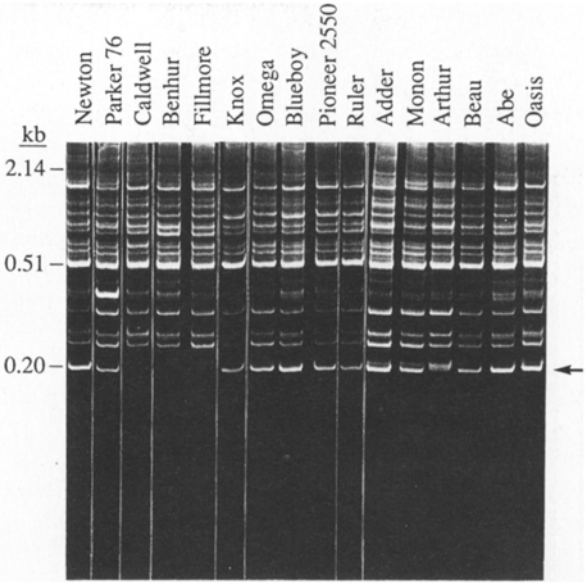

Fig. 2. PCR amplification of genomic DNA from 16 lines of winter wheat with primer A17 and polyacrylamide-gel electrophoresis. The DNA samples were fractionated on 12% polyacrylamide using 1 × TAE pH 7.4 at 25 °C. The gel, 0.75 mm in thickness, was run for 5 h at 150 V and stained with ethidium bromide. The *arrow* indicates enhanced resolution of DNA fragment polymorphism over that observed in agarose

being compared. This method was equivalent to calculating Nei and Li's (1979) index of genetic similarity (S_{xy}) for RFLP comparisons. Relationships among samples were evaluated with a phenetic cluster analysis, using the unweighted pair group method for arithmetic averages (UPGMA), and plotted in a phenogram. Statistical confidence intervals for the branching arrangements of phenograms were determined using a bootstrap procedure (Efron and Gong 1983) of 1,000 repetitive samplings of the RAPD data and computing the 95% range of the branch point values in the resultant UPGMA phenograms (modified after Nei et al. 1985). All computations and statistical analyses were performed using SAS (version 6) programs.

Results

Comparisons of agarose- (1.2%), polyacrylamide- (12%), and denaturing-gradient-gel electrophoresis of identical RAPD products are shown in Fig. 1, 2, and 3. Little or no visible polymorphism among wheat and barley lines was detected in agarose (data not shown). Polyacrylamide improved resolution, but both gel systems were inferior to the DGGE system for the enhanced detection of DNA polymorphism. Figures 1–3 demonstrate results from the three gel systems for the analysis of wheat.

Differentiation among wheat cultivars

Analysis of 1,056 reproducible amplified DNA sequences was possible from a total of eight gels and eight primers

Table 2. Oligonucleotide (10-mer) primers used and the fragment polymorphism resolution achieved with RAPD-DGGE

Crop	Primer	Sequence	Resolution[a]
Wheat	A05	AGGGGTCTTG	3
	A09	GGGTAACGCC	3
	A11	CAATCGCCGT	3
	A15	TTCCGAACCC	3
	A17	GACCGCTTGT	2
	A07/A16	GAAACGGGTG/ AGCCAGCGAA	1
	A16/A17		1, 3
	A07/A18	/AGGTGACCGT	3
Barley	A05		3
	A11		1, 3
	A15		1
	A17		1, 2
	A16/A17		1
Oats	A05[b]		3
	A09		3
	A11		2, 3
	A05/A18		1, 3
	A12/A18	TCGGCGATAG/	3
	A16/A17		3

[a] 1: distinguishes primary clusters
2: distinguishes subclusters within primary groupings
3: distinguishes between individual lines within a cluster

[b] All primers tested with oats distinguish *A. sativa* from *A. strigosa*

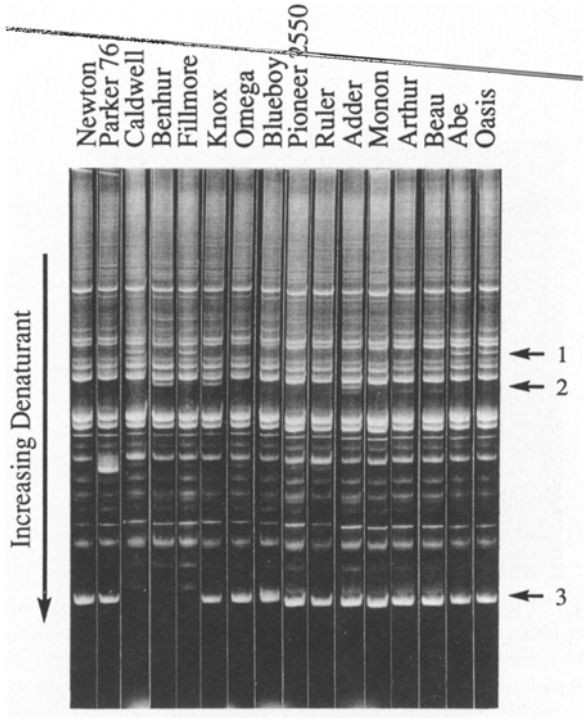

Fig. 3. PCR amplification of genome DNA from 16 lines of winter wheat with primer A17 and DGGE. The DNA samples were fractionated in 12% polyacrylamide and a 10–50% denaturant concentration gradient. The gel was run in 1 × TAE buffer pH 7.4 at 60 °C for 5 h and stained with ethidium bromide. *Arrows 1 and 2* indicate increased resolution of DNA polymorphisms over standard polyacrylamide-gel electrophoresis. *Arrow 3* indicates a fragment polymorphism that differentiates subgroup A″ in soft winter wheats

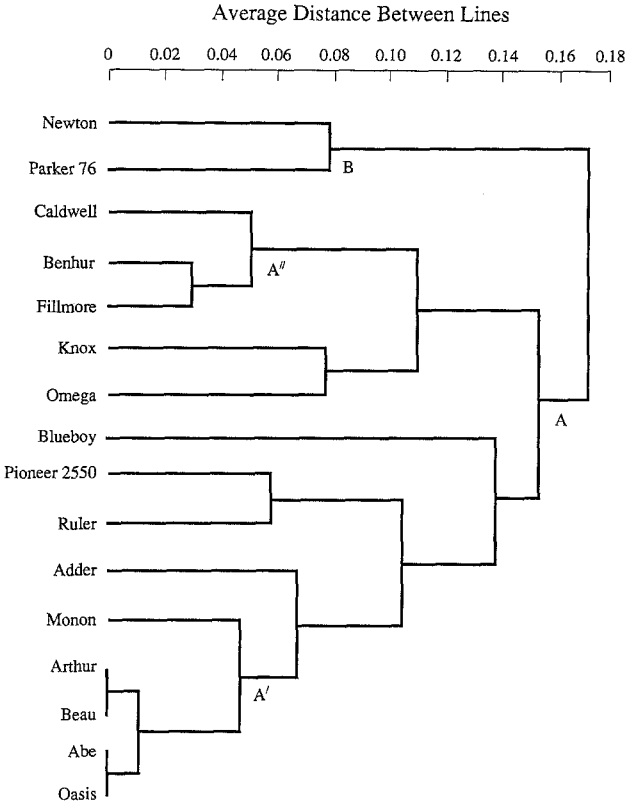

Fig. 4. Wheat phenogram generated using UPGMA and demonstrating the relationships among winter wheat lines based on a compiled data set from DGGE-based DNA fragment polymorphisms. This analysis indicates two main wheat groups, A (soft winter wheats) and B (hard winter wheats) with two subgroups within A

(Table 2) applied to 14 cultivars of soft red winter wheat and two cultivars of hard red winter wheat (see Fig. 3). Among the DNA bands scored, 32% were polymorphic. Our study indicated that soft winter wheats comprise a heterogenous group with an average similarity of 85%, differing collectively ($P<0.08$) from the hard winter wheat cultivars (Fig. 4). Among the soft winter wheats, two significantly different ($P<0.05$) subgroups (each with >95% average similarity) were observed. Cultivars Oasis, Abe, Beau, Arthur and Monon form one cohesive subgroup (A′) and cultivars Fillmore, Benhur and Caldwell form the other (A″). Not all primers or primer combinations were equally effective in differentiating these groups (Table 2).

Differentiation among oat cultivars

Analysis of 1,170 reproducible amplified DNA sequences obtained from 17 cultivars of oat (*Avena sativa*) and one line of *Avena strigosa* obtained from six gels and eight primers showed that *A. sativa* cultivars cluster into two significantly different ($P<0.05$) groups (Figs. 5 and 6). Group A comprises six cultivars with an average similarity of 86% and with no significant subclustering; i.e., statistically, no two cultivars are more closely related to each other than to other members of the group. Group B contains 11 cultivars with greater average similarity, 92%, and with one subcluster. Cultivars Clinton, Clinton 59, Clintford and Hazel (group B′) form a cohesive subgroup that is significantly distinct from cultivar Porter (group B″). Both *A. sativa* groups are more similar to each other than either is to the sample from *A. strigosa* (group C). Certain primers or primer combinations allowed the differentiation of distinct groups (Fig. 5), while all primers tested distinguished between *A. sativa* and *A. strigosa* (Table 2). The level of polymorphism among *Avena sativa* was 38% using agarose-gel electrophoresis but increased to 56% using RAPD-DGGE.

Differentiation among barley cultivars

Of the 17 lines of spring barley used in this study, six are 6-rowed and ten are 2-rowed cultivars. Figure 7 demonstrates the obvious distinction in RAPD patterns pro-

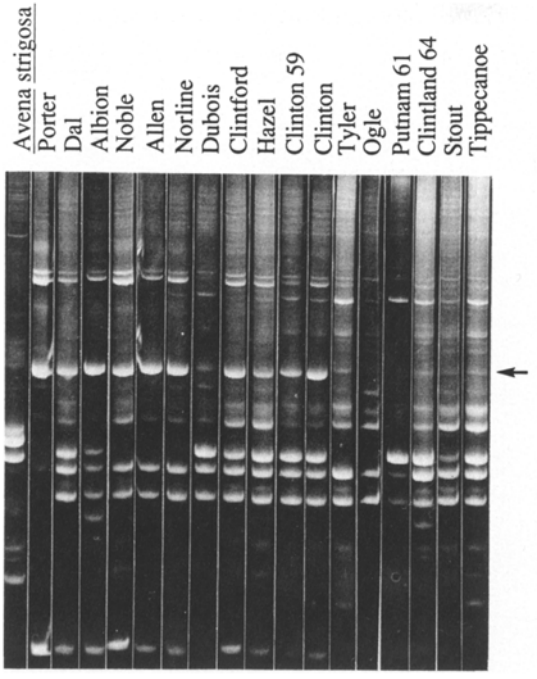

Fig. 5. Amplification products from genomic DNA of 17 lines of oat, *A. sativa*, and one line of diploid oat, *A. strigosa*, using primer combination A05/A18. The amplification products were fractionated by DGGE. The *arrow* indicates a DNA polymorphism associated with group B and absent from group A or C

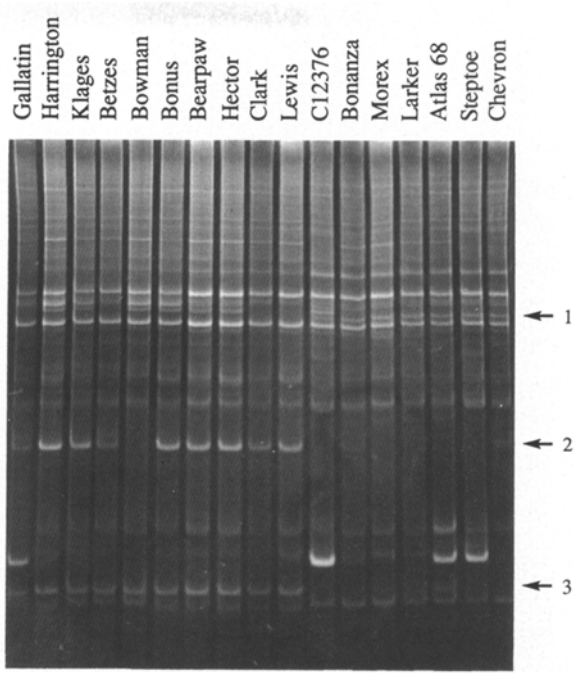

Fig. 7. PCR amplification of genomic DNA from 17 lines of barley using primer combination A16/A17. *Arrows 1, 2, and 3* indicate polymorphisms that distinguish 2-rowed types from 6-rowed varieties

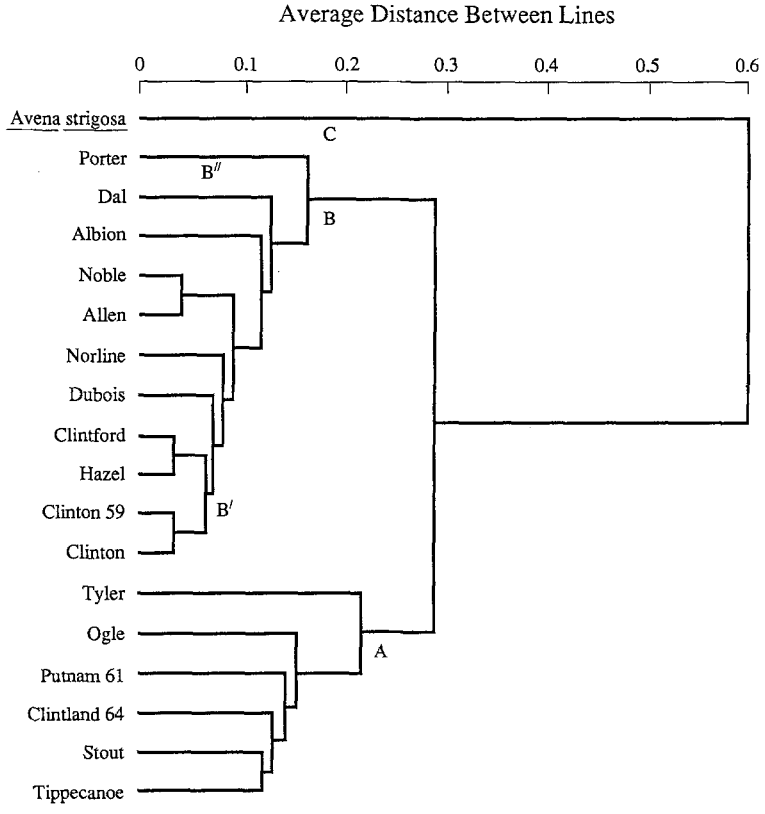

Fig. 6. Oat phenogram demonstrating the relationships among oat lines based on a compiled data set recorded from DNA fragment polymorphisms with DGGE. This analysis places 18 lines of oat into three groups, A, B, and C. Two subgroups were distinguished within group B

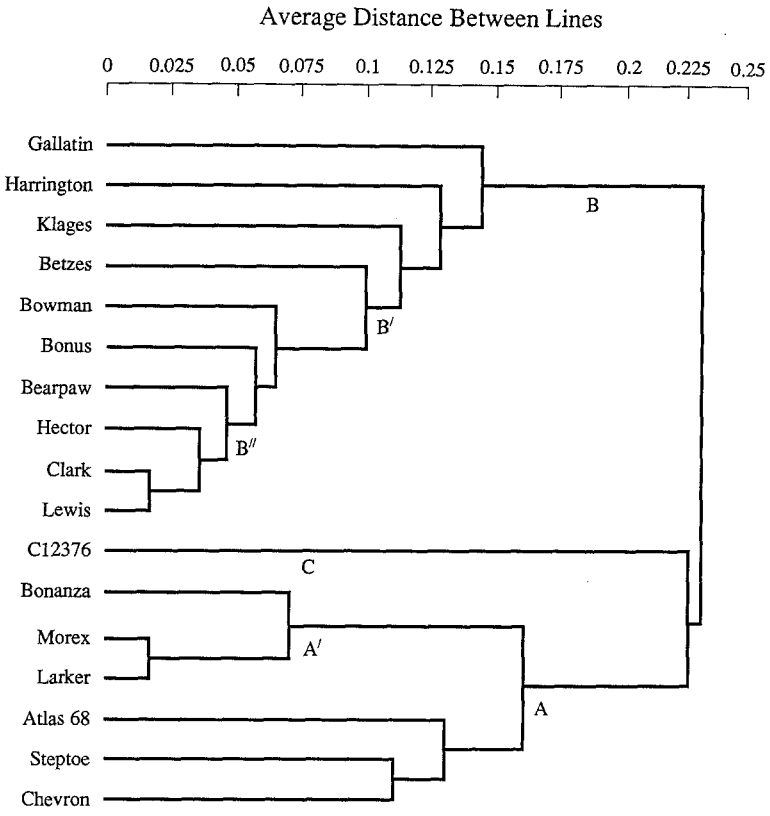

Fig. 8. Barley phenogram demonstrating the relationships among spring barlery lines based on compiled DGGE-based fragment polymorphism data. The analysis distinguished 6-rowed barley varieties into group A and 2-rowed barleys to groups B and C. One subgroup was identified within group A and two subgroups within group B

duced by these two groups. Cluster analysis of 986 DNA amplified sequences obtained from five gels and five primers (Table 2) indicated that all the barley cultivars tested comprise a single heterogenous group with substantial subgrouping (Fig. 8). Most of the 2-rowed cultivars (subgroup B′, >95% average similarity) are significantly different from all of the 6-rowed cultivars (group A) and from the 2-rowed landrace CI 12376. Among the 6-rowed cultivars, Larker, Morex and Bonanza form a cohesive subgroup (A′) that also differs from CI 12376. Treating all of the 2-rowed cultivars collectively, and comparing to CI 12376, the two groups are differentiated from one another only marginally ($P<0.20$). Among the 2-rowed cultivars, Lewis, Clark, Hector, Bearpaw and Bonus form a cohesive subgroup (B″) that differs from cultivar Gallatin. The level of polymorphism observed among the barley lines was 41%.

All DNA bands scored in this study were reproduced two to three times. The level of reproducibility was 100% for all intense bands visualized as described previously (He et al. 1992).

Discussion

The most commonly used gel matrix for the resolution of RAPD-based polymorphism is agarose. The complexity of patterns obtained with DGGE suggests that a large number of different DNA species are contained within a single band resolved on agarose. Consequently, the use of polyacrylamide greatly enhances resolution of the multiple amplification products (Caetano-Anolles et al. 1991). With the addition of a gradient of denaturant to the gel system, added resolution of DNA sequence polymorphisms is achieved (He et al. 1992; demonstrated in Figs. 2 and 3).

Denaturing-gradient-gel electrophoresis is designed to allow the resolution of sequence differences among fragments of similar or identical size (Fisher and Lerman 1983; Myers et al. 1987). The procedure takes advantage of the fact that even single base-pair differences will alter fragment melting properties (Tm), thus resulting in altered gel migration rate. DGGE as an alternative method for detecting DNA polymorphism has been successfully used in a number of plant and animal systems (Riedel et al. 1990; Burmeister et al. 1991; Gray et al. 1991; Lessa 1992). It should be pointed out, however, that other difficulties inherent in the PCR technique that influence reproducibility (Saiki et al. 1988) are not necessarily reduced by modifying the gel system.

A comparison of the derived wheat pedigree presented by Murphy et al. (1986) and that based on RAPD-DGGE indicated good agreement (Table 3). The high degree of similarity (85%) among the soft winter wheat

Table 3. A comparison of pedigree assessment using coefficient of parentage (CP) estimates versus RAPD-DGGE

Line	Cluster	
	CP	RAPD-DGGE
Wheat		
Oasis	A[a]	A′
Abe	A	A′
Beau	A	A′
Arthur	A	A′
Monon	A	A′
Adder	A	A
Fillmore	B	A″
Benhur	B	A″
Caldwell	B	A″
Omega	?	A
Pioneer 2550	C	A
Ruler	D	A
Knox	E	A
Blueboy	F	A
Newton	I	B
Parker	L	B
Barley		
Clark	1[b]	B″
Lewis	1	B″
Hector	1	B″
Bearpaw	1	B″
Bowman	1	B′
Harrington	1	B
Klages	1	B
Gallatin	17	B
Bonanza	8	A′
Morex	7	A′
Larker	7	A′
Atlas 68	4	A
Steptoe	14	A
Oat		
Tippecanoe	Central[c]	A
Stout	Central	A
Clintland 64	Landhafer	A
Putnam 61	Kherson	A
Ogle	Central	A
Tyler	Canadian	A
Clinton	Kherson	B′
Clinton 59	Kherson	B′
Hazel	Central	B′
Clintford	Central	B′
Dubois	Red rustproof	B
Norline	Red rustproof	B
Allen	Central	B
Noble	Central	B
Albion	Kherson	B
Dal	Central	B
Porter	Central	B

[a] Pedigree data from Murphy et al. (1986)
[b] Pedigree data from Martin et al. (1991 a)
[c] Pedigree data from Souza and Sorrells (1988, 1989)

lines is due to the predominance of a relatively small number of cultivars used as parents in most winter wheat breeding programs (Murphy et al. 1986). The subclustering we observed in group A allowing us to differentiate A′ from A″ reflects the predominant parent giving rise to the cultivars in each subcluster, with cultivar Arthur and related lines predominant in A′ and Benzur-related lines predominant in A″. The overlapping of germplasm between soft and hard winter wheats may be the reason for the average degree of similarity (83%) between the A and B groups.

A recent classification of barley (Martin et al. 1991 a) divided 167 spring barley lines into two major groups based on the number of rows per spike. We observed a similar division based on RAPD-DGGE, although the 17 lines used in our study represented a single heterogenous group. Subcluster B″ is significantly different from groups A and C (Fig. 8). The four lines within the B″ subgroup were obtained from Montana State University, where Clark and Lewis are sister lines derived from a Hector/Klages cross, and Bearpaw was derived from a TR440/Clark cross. The modest variability among barley lines sampled here may be a consequence of intensive selection for malting quality, resulting in a significant reduction in germplasm diversity (Martin et al. 1991 a).

We observed a number of differences between the oat classifications based on the pedigrees (Souza and Sorrells 1988, 1989) and relationships indicated by RAPD-DGGE (Table 3). The observed symmetry between previously established pedigrees of wheat and barley and those established by RAPD-DGGE suggests that the RAPD-DGGE system provides a useful method for the determination of genetic relationship. The apparent discrepancies observed between the previous analysis of oat and that by RAPD-DGGE may reflect the difference in resolution achieved by the two methods of pedigree assessment used. The previous studies of oat relied primarily on estimates of coefficient of parentage, dependent on the accuracy of plant breeding records and the accurate estimation of genetic contributions from various ancestors of a cultivar. The genetic contribution from each parent in a cross is assumed to be 50%. However, selection for particular genes or gene combinations in a segregating population may alter this genetic ratio and, consequently, also alter estimates of genetic relatedness.

The RAPD-DGGE method provides a reasonably simple means for verification of genetic relationships without the dependence on additional sources of information that may be subject to human error over time. Use of DNA-based methodology for estimation of genetic relatedness also allows the incorporation of influences such as genetic drift and selection, not possible with previous approaches. The advantages of DNA-based pedigree assessment have been demonstrated previously in maize (Marsan et al. 1992).

The primers used in this study provided different levels of resolution. Some primers allowed the distinction between major groups, while others allowed detection of subgroups, and some allowed identification of genetic differences between very closely related lines. It is important to note that, using this approach, we were able to resolve DNA polymorphisms among every line in our study. Our investigation was not extensive enough to determine those features of primers critical to success in detection of polymorphism. However, it is clear that use of the RAPD-DGGE system is effective at allowing the genetic distinction between even the most closely related lines such as wheat cultivars Abe and Arthur, presumed to share over 94% of their genomes in common. The RAPD-DGGE system, therefore, provides an opportunity not previously available in certain self-pollinated species to directly apply DNA-based technologies toward germplasm evaluations, gene mapping, and marker-assisted selection in plant improvement programs.

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