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Rapid estimation of genetic relatedness among heterogeneous populations of alfalfa by random amplification of bulked genomic DNA samples

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Abstract. A procedure which involves the use of RAPD markers, obtained from bulked genomic DNA samples, to estimate genetic relatedness among heterogeneous populations is demonstrated in this study. Bulked samples of genomic DNA from several alfalfa plants per population were used as templates in polymerase chain reactions with different random primers to produce RAPD patterns. The results show that the RAPD patterns can be used to determine genetic distances among heterogeneous populations and cultivars which correspond to their known relatedness. The results also indicate that, by using ten primers with bulked DNA samples from ten individuals, 18–72 populations or cultivars can be distinguished from each other on the basis of at least one unique RAPD marker. We anticipate that DNA bulking and methods for comparing RAPD patterns will be very useful for identifying cultivars, for studying phylogenetic relationships among heterogeneous populations and for selecting parents to maximize heterosis in crosses.

Key words: Polymerase chain reaction – Random primers – Genetic distance – Random amplified polymorphic DNA – *Medicago sativa*

Introduction

Information about germplasm diversity and relationships among elite breeding materials is of fundamental importance in plant breeding (Hallauer et al. 1988). This is especially true for a species like alfalfa (*Medicago sativa* ssp.) which is an outcrosser and suffers severe inbreeding depression. Ideally, methods for elucidating genetic relationships in alfalfa should be based on comparisons of plants using monogenic traits whose expression is not affected by plant development or growth environment (Quiros and Bauchan 1988).

Biochemical methods, such as isozyme analysis, have been used to distinguish between homozygous and heterozygous individuals and to determine the degree of genetic variability in plant populations (Melchinger et al. 1991). However, isozyme analyses are limited by the small number of marker loci that are available, a general lack of polymorphism for these loci in elite breeding materials, and the variability in banding patterns due to plant development (Tanksley 1983; Tanksley and Orton 1983; Tanksley et al. 1989). Restriction fragment length polymorphisms (RFLPs) are available in almost unlimited numbers for an increasing number of plant species and are very powerful tools for exploring genetic relationships among species and populations (Saghai-Maroof et al. 1984; Tanksley et al. 1989). Because RFLP patterns are DNA-based, they are little affected by plant development. However, the detection of RFLPs by Southern-blot hybridization is expensive, time consuming and technically complex. These are particularly important factors with a species like alfalfa which is genetically complex both at individual and population levels.

Recently, DNA polymorphisms obtained with the polymerase chain reaction (PCR) were used as genetic markers to tag genes Martin et al. 1991), to fingerprint viruses, bacteria, fungi, plants and humans (Welsh and McClelland 1990; Caetano-Anollés et al. 1991; Goodwin and Annis 1991) as well as to determine genetic relationships among peanut (*Arachis hypogaea* L.)

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species (Halward et al. 1992). For these applications a random amplified polymorphic DNA (RAPD) assay was used in which genomic DNAs from individuals were amplified with primers which had arbitrary sequences of 5–10 nucleotides in length to generate DNA fragment polymorphisms (Williams et al. 1990). This procedure eliminates the need for prior DNA sequence information and relies on screening a set of random primers to identify those that result in polymorphisms among the samples being compared.

Although the RAPD procedure is relatively simple and rapid, its practical application to genetic studies of heterogeneous populations is still limited in cases where large numbers of individuals need to be examined. One approach to overcome this limitation is to bulk genomic DNA samples. Bulked DNA samples from segregating populations have been used as templates to rapidly identify RAPD markers linked to disease-resistance genes in lettuce (Michelmore et al. 1991).

In the present study a method using bulked genomic DNA samples of alfalfa to represent a heterogeneous population for RAPD analysis is described. We show that this method is rapid and useful for distinguishing among different cultivars in the *Medicago sativa* complex as well as for estimating genetic relatedness among breeding populations of *Medicago sativa* L.

Materials and methods

Materials

Three cultivars (Dupuits, Peace and Anik) and four breeding populations (V0, V3, P0 and P3) of alfalfa were used in this study. All plant materials were grown in a growth cabinet set to a 16 h light (approximately 500 $\mu E\,m^{-2}\,s^{-1}$)/8 h dark photoperiod with a 23 °C light/18 °C dark temperature regime. The plants were watered daily. The random primers (Table 1), *Taq* DNA polymerase, and dNTPs were purchased from the University of British Columbia Biotechnology Lab, Promega, and Pharmacia, respectively.

DNA isolation and bulking

Genomic DNA was extracted from an alfalfa leaflet by homogenizing it in 400 µl of extraction buffer (made by mixing 10 ml of TRIS-HCl pH 7.4, 2.5 ml of 5 *M* NaCl, 2.5 ml of 0.5 *M* EDTA pH 8.0, 2.5 ml of 10% SDS and 32.5 ml of double-distilled H_2O) in a sterile Eppendorf tube. The tissue was homogenized in the buffer with a pestel that fitted snugly into the tube and was attached to a motor revolving at aproximately 500 rpm. The homogenate was centrifuged for 1 min in a microfuge and the DNA was precipitated from 300 µl of the supernatent, in a new Eppendorf tube, by adding 300 µl of isopropanol. The DNA was pelleted by centrifugation. After air drying, the pellet was dissolved in 100 µl of distilled water without agitation. The sample was centrifuged for 1 min and the supernatent was used for PCR.

Equal amounts of genomic DNA from five or seven individual plants were mixed for the bulked RAPD analyses.

Table 1. Twenty-two primers used in this study and their sequences

Primer#	Sequence
1	CGTCTGCCCG
2	GAAGGCTCTG
3	TCCATGCCGT
4	CTGGCGGCTG
5	TAGCCCGCTT
6	AGTAGAAGGG
7	TGTCGGGAAC
8	GTGCGTCCTC
9	TGACCCCTCC
10	CGTGATTGCT
11	CAAACGGCAC
12	GTGCGTCGCT
13	AGAATCCGCC
14	GATCTCAGCG
15	GCTTGTGAAC
16	TGTCCACCAG
17	AGCAGCGTGG
18	GCTGTAGTGT
19	CAGGCGGCGT
20	GCTGGACATC
21	GCAAGTCACT
22	GTATTGCCCT

DNA amplification and separation

Each 25 µl reaction contained 1 µl of the DNA extract (approximately 20 ng), 10 mM TRIS-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 0.36 µM of random primer (Biotechnology Lab UBC) and 1 unit of *Taq* polymerase (Promega). Amplification was performed in a MJ Research Programmable Thermal Controller (PTC-100) programmed for 45 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C. The RAPD fragments were separated by agarose-gel electrophoresis, using a 1.4% gel, and visualized with ethidium bromide.

Data analysis

The genetic identity index (I) was determined from RAPD patterns of individual plants in each population according to the equation:

$$I = 1/N \sum_{i=1}^{N} \frac{2Vi^{(1)} \cdot Vi^{(2)}}{[Vi^{(1)}]^2 + [Vi^{(2)}]^2}$$

where N is the number of different bands scored for all primers in the two populations being compared; $Vi^{(1)}$ and $Vi^{(2)}$ are the frequencies of occurrence of an individual band i in populations 1 and 2, respectively. The index of genetic distance (IGD) between two populations was calculated as:

$IGD = -\ln(I)$ (Apuya et al. 1988).

The genetic distance (D) between two populations was calculated from RAPD patterns of bulked DNA samples using:

$D = -\ln(F)$ (Swafford and Olson 1990).

F is an estimate of similarity and was calculated with the formula:

$F = 2X_{1,2}/X_1 + X_2$ (Packer et al. 1991);

where $X_{1,2}$ is the number of amplified DNA fragments with the same molecular weight found in both populations, X_1 is the total number of fragments found in one population and X_2 is the total number found in the other.

To determine a phylogeny for the three cultivars Dupuits, Peace and Anik as well as the breeding population V0, DNA samples from seven plants per cultivar or population were bulked and the PCR reactions were individually primed with ten different primers. The RAPD patterns were used to calculate F and D values and the latter were clustered with an unweighted pair group method using arithmetic averages (Kuhnlein et al. 1989).

The percentage of population-specific bands (PSB %) was determined according to:

$$PSB\,(\%) = [1 - AveF_{(n)}] \times 100,$$

where $AveF_{(n)}$ is the mean $F_{(n)}$ value for all possible comparisons of n populations among a collection of populations and:

$$F_{(n)} = \frac{\sum_{i=0}^{n-2} (n-i)\, c_{1\ldots n}^{(n-i)}}{\sum_{a=1}^{n} Xa}$$

where n is the number of populations being compared:

$n = 2 \ldots \infty$;

$i = 0 \ldots (n-2)$;

$c_{1\ldots n}^{(n-i)}$ is the number of common bands in all possible camparisons of n populations in $(n-i)$ groups [note $(n-i)$ is not an exponent]; Xa is the total number of bands per population;

$a = 1 \ldots n$.

Results

Screening for population polymorphic primers

Out of 100 primers tested, 25 effectively primed the amplification of alfalfa genomic DNA and resulted in polymorphic banding patterns both within a population and between (at least) two populations. On average, approximately five discrete DNA products per primer (with a range of 1–12 bands) were generated (Fig. 1). The size of the amplified fragments that could be scored ranged from 0.3 to 3.5 kb. All amplification reactions were repeated three to five times for the three cultivars (Dupuits, Peace, Anik) with primers #1 to #4. Since the results for these primers were consistent among replicated amplifications with respect to the presence of bands that could be scored (data not shown), amplifications with primers #5 to #22 were repeated only when obvious problems with the PCR procedure occurred. Occasionally, amplifications products were observed in control reactions that did not have the template DNA. However, these products were always absent when template DNA was included (data not shown).

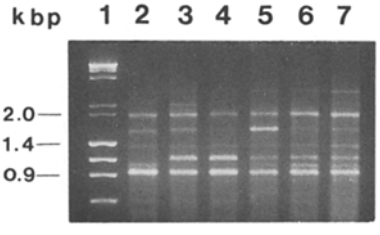

Fig. 1. RAPD patterns obtained from individual (*lanes 2–6*) and bulked (*lane 7*) genomic DNA samples of Dupuits with primer #6

A comparison between individual and bulked methods

Figure 1 shows the RAPD patterns obtained from five individuals in a populations as well as the patterns obtained from a bulked DNA sample of those individuals. Of the seven different DNA fragments obtained from the five plants, only one 2.3-kb fragment, which was unique to individual 3, was missing in the pattern obtained with the bulked DNA sample. The order of the genetic distance (D) values, calculated from RAPD patterns obtained from bulked DNA samples of Dupuits, Peace and Anik populations, was the same as the order of the index of genetic distance (IGD) values calculated from RAPD patterns obtained from five individuals per population (Table 2). For both estimates of genetic distance the maximum value occurred between Anik and Peace and the percentages of the maximum value for the Dupuits/Anik and Dupuits/Peace comparisons were similar for the D and IGD values.

Determination of appropriate sample size for the bulked method

Comparisons among RAPD patterns obtained from bulked samples containing DNA from seven, ten, or 15 individuals of the alfalfa breeding population V0 or P3

Table 2. Values for the index of genetic distance (IGD) and the genetic distance (D) calculated from RAPD patterns of three alfalfa cultivars using individual and bulked genomic DNA samples, respectively. Values in brackets are the percent of the maximum values of IGD and D. Five individuals and four primers (#1–#4) were used for each cultivar

Cultivar		Dupuits	Anik	Peace
Dupuits	IGD	0.00	1.06 (83%)	0.32 (25%)
	D	0.00	0.60 (84%)	0.20 (28%)
Anik	IGD		0.00	1.28 (100%)
	D		0.00	0.71 (100%)
Peace	IGD			0.00
	D			0.00

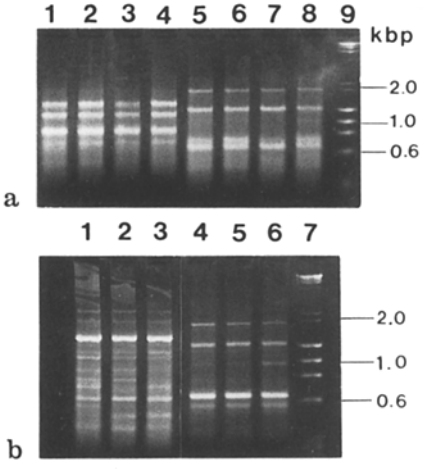

Fig. 2a, b. RAPD patterns obtained from bulked genomic DNA samples containing DNA from different numbers of V0 plants: *lanes 1a, 5a, 1b and 4b* from 15 plants; *lanes 2a, 6a, 2b and 5b* from ten plants; lanes *3a and 7a* from seven plants and *lanes 4a, 8a, 3b and 6b* from another random sample of seven plants. The patterns were obtained with primer #12 (*lanes 1a–4a*), primer #15 (*lanes 5a–8a*), primer #14 (*lanes 1b–3b*), and primer #8 (*lanes 4b–6b*)

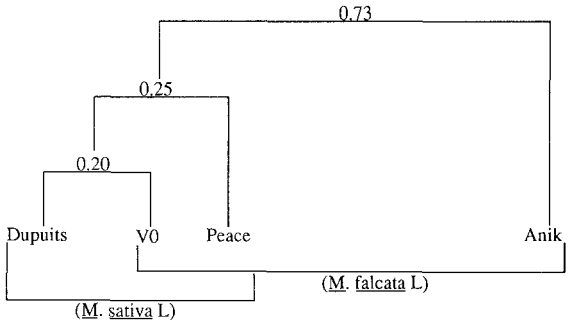

Fig. 3. Phylogeny for three cultivars (Dupuits, Peace, Anik) and one breeding population (V0) based on genetic distances calculated from RAPD patterns obtained from bulked samples containing DNA from seven plant per cultivar or population and ten different PCR primers (#5–#14). The known sources of the materials are indicated on the bottom

indicated that, in most cases, the RAPD patterns obtained from the bulks of seven individuals could represent the patterns seen in larger samples of the population (Fig. 2a). Only with one primer (#14) of the ten that were tested, and then only for the V0 population, was a band seen in the patterns obtained from the bulked DNA samples of ten or 15 individuals that was not present in the sample containing DNA from seven individuals (Fig. 2b).

Applications

To test the usefulness of the RAPD patterns from bulked DNA for determining genetic relatedness, the F values (which give a measure of the fraction of shared bands between two populations) were calculated for comparisons among three cultivars (Dupuits, Peace, Anik) and a breeding population (V0). The relationships among these populations are known and Fig. 3 shows that the genetic distance values obtained from the F values ($D = -\ln F$) reflect the fact that Anik (which is 100% *Medicago falcata*) is a greater distance from Dupuits (which is 100% Flemish *Medicago sativa*) than are Peace or V0 (which have both *M. falcata* and *M. sativa* in their backgrounds). The discrepancy in the relative distances between Dupuits and Anik versus Peace and Anik given in Table 1 and Fig. 3 can probably be attributed to the fact that the former analysis (Table 1) was based on RAPD pattern obtained from five plants per bulk and four primers, whereas the latter distances were based on data from seven plants per

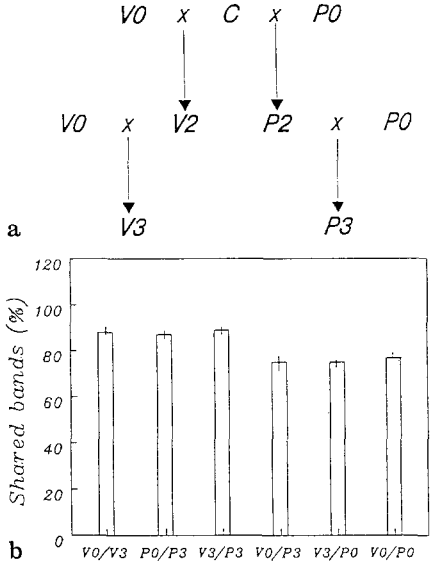

Fig. 4. Relationships among four breeding populations of alfalfa (V0, V3, P0, P3). **a** *V0* contains 50% Shield and 50% Ultra. *P0* contains 50% Sure and 50% Surpass. *C*, the common parent in the crosses P0 × C and V0 × C, is A70-34 alfalfa, an embryogenic genotype selected from Rangelander (D. C. W. Brown, Agriculture Canada). *V3* and *P3* were screened for their ability to produce somatic embryos (S. Bowley, personal communication). **b** Comparisons of percent shared bands (% SB) in RAPD patterns between pairs of alfalfa breeding populations (V0, V3, P0, P3). Genomic DNAs from seven plants per population were bulked and separate PCR reactions were primed with 20 different primers (#3–#22). The mean % SB between two populations and their standard errors are shown. According to paired t-tests at the 5% level, the values for comparisons V0/V3, P0/P3, and V3/P3 were significantly different from the V0/P3, V3/P0 and V0/P0 comparisons

bulk and ten primers. The latter is considered to be more accurate by virtue of the larger number of RAPD bands that were considered in the calculation.

The test of the procedure was extended to comparisons among breeding populations in *M. sativa*. Figure 4 shows that the percent of shared bands in bulked RAPD profiles for related populations was significantly higher than for unrelated populations. In particular, the comparisons V0/V3 and P0/P3 had approximately 88% shared bands compared to 75% for the V0/P0, V3/P0 and V0/P3 comparisons. The relatively high value obtained for the V3/P3 comparison (89%) can probably be attributed to the fact that all of the individuals sampled from the V3 and P3 populations had been selected for their ability to form somatic embryos (S. Bowley, personal communication), a trait that they had obtained from their common parent C.

Figure 5 plots the dependence of the percent of population-specific bands in RAPD profiles from bulked samples containing DNA from seven alfalfa plants per population on the number of populations being compared. A comparison of V0, P0, V3, P3, Dupuits and Peace showed that on average 20% of the bands in the RAPD patterns were specific to a population when any two of the populations were compared, but only 11%, 8.4%, and 7.3% of the bands were specific to a population when three, four, five populations were compared, respectively. The addition of a population like Anik, which is genetically remote from the other populations (see Fig. 3), to the pool of populations that were compared increased the average percent of population-specific bands in all of the comparisons to between 8–5% relative to the comparisons without Anik (Fig. 5a).

In some instances it is of interest to distinguish a particular variety or population from others within a group. Figure 5b shows the percent of RAPD markers that were specific to a population for only those comparisons containing V0 when two to five populations in the group including V0, P0, V3, P3, Dupuits and Peace were compared. The results show that on average 17.2%, 11.1%, 8.5% and 7.2% of the bands were specific to a particular population in these comparisons, respectively (Fig. 5b). Analogous analysis comparison for only those comparisons containing Anik in the group Anik, V0, P0, V3, P3, Peace and Dupuits indicated that 48.3%, 24%, 17%, 15.2%, and 13.2% of the RAPD markers were specific to a population for 2, 3, 4, 5 and 6 population comparisons, respectively (Fig. 5b).

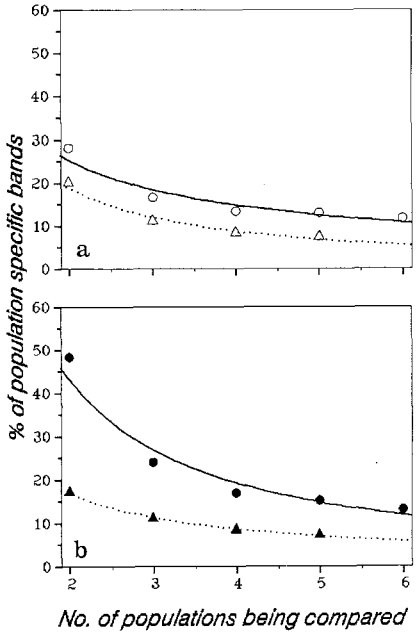

Fig. 5a, b. Dependence of the percentage of population-specific bands (% PSB; as defined in the experimental protocol) on the number of populations being compared. DNA samples from seven plants per population were bulked in equal proportions and separate PCR reactions were primed with ten different primers (#5–#14). **a** PSB% values obtained from means of all possible $F_{(n)}$ values calculated from sub-samples of the seven populations including Anik (○), or six populations without Anik (△), are shown. For the comparisons the values represent means of $c_n^a = n!/a!(n-a)!$ $F_{(n)}$ values or means of $c_7^2 = 21$, $c_7^3 = 35$, $c_7^4 = 35$, $c_7^5 = 21$ and $c_7^6 = 7$, respectively, of $F_{(2)}$ to $F_{(6)}$ values for those including Anik or $c_6^2 = 15$, $c_6^3 = 20$, $c_6^4 = 15$, and $c_6^5 = 6$, respectively, of $F_{(2)}$ to $F_{(5)}$ values for those without Anik. The equation of the line fit to the former is $y = 43.17x^{-0.77}$ ($R^2 = 0.92$) and to the latter is $y = 40.72x^{-1.11}$ ($R^2 = 0.97$). **b** Only $F_{(2)}$–$F_{(6)}$ combinations including Anik (●) or $F_{(2)}$–$F_{(5)}$ combinations with V0 but excluding Anik (▲) are shown. The equation of the line fit to the former data is $y = 96.99x^{-1.17}$ ($R^2 = 0.95$) and to the latter is $y = 32.72X^{-0.96}$ ($R^2 = 0.99$)

Discussion

The genetic diversity seen in populations of outcrossing plant species complicate many aspects of plant breeding and cultivar identification. The present report indicates that RAPD patterns that are characteristic of plant populations or cultivars can be obtained by using bulks of genomic DNA from several individuals of heterogeneous population in PCR reactions with random primers. The utility of the DNA bulking procedure was illustrated by applying it to an analysis of alfalfa populations and cultivars. Because of severe inbreeding depression, alfalfa cultivars are synthetics based on many parents; modern varieties have approximately 100 parents on average. Therefore, comparisons among cultivars must take into account intracultivar variability. Our results indicate that the RAPD patterns obtained by using mixtures of equivalent amounts of DNA from approximately ten individ-

uals give a good sample of the variety of RAPD markers that exists for a particular cultivar or population.

The most important advantage of the DNA bulking procedure is the increase in efficiency that it affords. In the procedure, approximately ten individual PCR reactions with DNA samples from ten different plants in a population are replaced by a single PCR reaction with a bulk of the different DNA samples. Thus, the bulking procedure makes RAPD analysis particularly well suited for genetic studies of heterogeneous populations. The RAPD patterns obtained from bulked DNA samples generally consisted of superpositions of the patterns seen for individual lines with the exception that some faint bands that were present in the patterns from individual DNA samples were absent from the bulked DNA pattern. Thus, the PCR patterns obtained from the bulked DNA samples represented consensus patterns for the common RAPD markers in the populations under test. Perhaps, this is because DNA sequences that were common to several individuals provided relatively high concentrations of template to the bulk and so resulted in intense bands in the PCR pattern, whereas those sequences that were found in only a few individuals made up too small a proportion of the templates in the mixtures to be effectively amplified. Michelmore et al. (1991) also found that rare RAPD markers could not be detected in bulks when the DNA sample they were derived from represented less than 10% of the total DNA.

Another important finding of the present study was that the RAPD patterns from bulked DNA samples can be analyzed to estimate relatedness among alfalfa populations. This conclusion was based on the demonstration that an F value calculated from RAPD patterns of bulked samples gave a measure of relatedness that was very similar to the index of genetic distance (IGD) calculated from comparisons of individuals. Previous estimates of relatedness have been based on comparisons of RFLP or RAPD patterns obtained from individual plant, animal or fungal samples (Nei 1987; Goodwin and Annis 1991; Packer et al. 1991; Halward et al. 1992). The intra-cultivar variability seen in alfalfa makes a comparison based on an analysis of individuals an arduous task. As indicated previously, the workload can be decreased to a tenth by bulking. The bulking procedure should also be suited to comparisons among wild species represented by several accessions or heterogeneous natural populations.

The validity of the relatedness calculations carried out with alfalfa in this study was illustrated by the correspondence of the distance values obtained from comparisons of the RAPD patterns to the known relationships among varieties in the *Medicago sativa* complex. The procedure was also shown to be useful for estimating relatedness among breeding populations of *Medicago sativa* L. Questions of relatedness arise in plant breeding when parents for crosses are being selected. A potential application of the analysis described above in alfalfa breeding (or the breeding of any other species that suffers inbreeding depression) would be to determine the relatedness among potential parents so that combinations that maximize genetic distance and heterosis can be made. This suggestion is supported by previous findings that RFLP-based genetic distances between maize inbreds are good predictors of heterosis in their F_1 hybrids (Smith et al. 1990).

Methods for determining the identify of cultivars and populations are important for protecting plant breeders rights and for assessing seed purity. The current results indicate that for groups of 5–6 alfalfa cultivars or populations, 7.2 to 15.2% of the bands in RAPD profiles from bulked DNA samples of seven individuals can be expected to be unique to an alfalfa cultivar or population. The number of populations that can be distinguished from each other on the basis of at least one RAPD marker difference can be estimated from the equations of the lines fitted to the data. The number of populations at 2% (1 marker/50 markers in total) for the cmparison without Anik is 18.4 and for the comparison with Anik is 72.3. Previous estimates have indicated that, for an outcrossing species, ten RFLP loci tested against ten individuals per cultivar should suffice to separate one cultivar from another (Soller and Beckman 1983). The advantages of the RAPD procedures for fingerprinting are that they do not require prior knowledge of DNA sequences or a library of hybridizable clones. Also, no radioactivity is used in the procedure.

Although the complete range of applications of the procedures described in this report has yet to be tested we believe that they will also be useful for identity testing, pedigree analysis, and comprehensive phylogenetic studies of heterogeneous plant and animal populations.

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Appendix

When three populations are compared using $F_{(n)}$, then: $n = 3$; $i = 0, 1$; $a = 1, 2, 3$ and

$$F_{(3)} = \frac{3c^3_{1,2,3} + 2c^2_{1,2,3}}{x_1 + x_2 + x_3},$$

where $c^3_{1,2,3}$ is the number of bands common to all three populations and $c^2_{1,2,3}$ is the total number of bands found in only two populations when all possible pairwise comparisons are made among the three populations.

When four populations are compared using $F_{(n)}$, then n = 4, i = 0, 1, 2; a = 1, 2, 3, 4 and

$$F_{(4)} = \frac{4c^4_{1,2,3,4} + 3c^3_{1,2,3,4} + 2c^2_{1,2,3,4}}{x_1 + x_2 + x_3 + x_4}$$

$c^4_{1,2,3,4}$ is the number of bands common to all four populations; $c^3_{1,2,3,4}$ is the number of bands found in only three of the four populations and $c^2_{1,2,3,4}$ is the number of bands found in only two of the populations. $F_{(n)}$ expands further according to the formula described in the Materials and methods.

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