Theor Appl Genet (1994) 89:323–328

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Relationships among genetic distance, forage yield and heterozygosity in isogenic diploid and tetraploid alfalfa populations

Received: 6 August 1993 / Accepted: 2 February 1994

Abstract Isogenic diploid and tetraploid alfalfa (*Medicago sativa* L.) was studied with molecular markers to help understand why diploid performance and breeding behavior does not always predict that of tetraploids. In a previous study of partially heterozygous alfalfa genotypes, we detected a low correlation between yields of isogenic diploid (2*x*) and tetraploid (4*x*) single-cross progenies, and genetic distances were more highly correlated with yields of tetraploids than diploids. These differences may be related to the level of RFLP heterozygosity expected among progenies derived from heterozygous parents at the two ploidy levels. The objectives of this study were to determine the relationships among genetic distance, forage yield and heterozygosity in isogenic 2*x* and 4*x* alfalfa populations. Four diploid genotypes were chromosome doubled to produce corresponding isogenic autotetraploids, and these genotypes were mated in 4 × 4 diallels to produce 6 single-cross families at each ploidy level for field evaluation. Allele compositions of parents were determined at 33 RFLP loci by monitoring segregation of homologous restriction fragments among individuals within progenies, and these were used to estimate RFLP heterozygosity levels for all single-cross progenies at both ploidy levels. RFLP heterozygosity rankings were identical between progenies of isogenic diploid and tetraploid parents; but significant associations ($P < 0.05$) between estimated heterozygosity levels and forage yield were detected only at the tetraploid level. Since tetraploid families were nearly 25% more heterozygous than the corresponding diploid families, inconsistencies in the association between molecular marker diversity and forage yields of isogenic 2*x* and 4*x* single crosses may be due to recessive alleles that are expressed in diploids but masked in tetraploids. The gene action involved in heterosis may be the same at both ploidy levels; however, tetraploids benefit from greater complementary gene interactions than are possible for equivalent diploids.

Key words Heterozygosity · Genetic diversity · RFLPs · Alfalfa · *Medicago sativa* L.

Introduction

Molecular markers are valuable for studying the relationship between genetic distances and heterosis. Isozymes (Price et al. 1986; Lamkey et al. 1987) and restriction fragment length polymorphisms (RFLPs) (Lee et al. 1989; Godshalk et al. 1990; Smith and Smith 1991; Boppenmaier et al. 1992) have been used to estimate genetic distances among inbreds of maize, and in most cases, correlations between these estimates and heterosis of F_1 hybrids were too low to be of predictive value. Researchers have suggested that inadequate genome coverage, the use of randomly dispersed markers that are unlinked to quantitative trait loci, and/or differences in levels of dominance among hybrids may be responsible for the low associations detected in these studies.

High levels of RFLP have been detected within and among accessions of *Medicago* (Brummer et al. 1991; Kidwell et al. 1994a). In a recent study, we investigated the relationship between molecular marker diversity and forage yields of isogenic diploid and tetraploid alfalfa hybrids derived from partially heterozygous parents (Kidwell et al. 1994b). Molecular marker diversity was correlated with hybrid yield of tetraploid alfalfa populations but not for their isogenic diploid counterparts. These differences may be related to

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Communicated by A. R. Hallauer

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greater RFLP heterozygosity in tetraploids due to tetrasomic segregation.

Depending on the allele composition of the parents, different percentages of heterozygous individuals within progenies are expected in single-crosses of isogenic 2*x* and 4*x* genotypes (Bingham 1980). Tetraploid progenies theoretically contain a higher percentage of heterozygous individuals than progenies of corresponding crosses at the diploid level (Table 1). However, the level of heterozygosity within a family cannot be estimated unless the genotypes of the parents are known. The objectives of the study presented here were (1) to determine the RFLP genotypes of isogenic diploid and tetraploid parents by monitoring segregation in their single-cross progenies; (2) to estimate the level of RFLP heterozygosity in diploid and corresponding tetraploid families from their parental genotypes; (3) to determine the relationship between molecular marker diversity of parents and the level of RFLP heterozygosity in diploid and tetraploid single-cross progenies; and (4) to correlate the estimated levels of heterozygosity with forage yield.

Materials and methods

Plant materials

Tetraploid alfalfa genotypes containing a maximum of 2 alleles per locus were created by chromosome doubling four diploid hybrids using methods described by Pfeiffer and Bingham (1983). Two of the progenitor diploids, L and V, were extracted from a population derived from 'Iroquois' and 'Vernal'. *P* was obtained from a population developed by intercrossing haploids from 'Perry', 'Vernal', and 'Saranac', and *R* was selected from a 2*x* Regen-S population that was developed from 'DuPuits' and 'Saranac'. Diploid genotypes L, P, and V were chromosome doubled with colchicine, and *R* doubled spontaneously in tissue culture. Tetraploid genotypes and their isogenic diploid progenitors were mated in diallel fashion at each ploidy level. Seeds from reciprocal crosses were bulked to produce 6 single-cross progenies at each ploidy level for field evaluation.

Field evaluations and forage yield data analyses

Forage yields of single-cross progenies were evaluated in spaced-plant and microplot field trials over 2 and 3 years, respectively. Seeds of all single crosses were sown in the greenhouse in 1989, and the seedlings were later transplanted to spaced-plant plots in a randomized complete block (RCB) design, Each plot consisted of 5 transplants per 2-m row with 40 cm between plants and 1 m between rows. Four to six replications were evaluated per entry. Two harvests were taken in 1990 and three were taken in 1991. Progenies were also seeded in microplots. Microplots consisted of three or four replicates of each entry in 1-m rows sown at a rate of 50 seeds per row with 0.5 m between rows in a RCB design. A total of seven harvests were taken over 3 years. Dry weights of herbage were combined over harvests for each entry in both field trials. Complete descriptions of field evaluations are reported elsewhere (Kidwell et al. 1994b).

Data were analyzed as a split-plot design with ploidy level as the whole plot factor and single-crosses as the sub-plot factor using the GLM procedure in SAS (SAS Institute 1988) for unbalanced designs (Steel and Torrie 1980). Ploidy level was a fixed effect in the model in both field trials, whereas single-crosses and replications were considered random effects. For spaced-plant evaluations, plants were nested within replications, single-crosses, and ploidy level. Mean squares from analysis of variance were used to calculate least significant difference (LSDs) among forage yields of single-cross progenies at both ploidy levels for each field trial.

RFLP analysis and genetic distance calculations

Remnant seed was available for 10 of the 12 single-crosses evaluated in the field trials, and this seed was sown in individual Jiffy peat pots in 1991. Individuals from these single-crosses, along with the parents, were maintained in the greenhouse for tissue collection. The procedures for genomic DNA isolation, restriction enzyme digestion, gel electrophoresis, Southern transfer, probe labeling by random-hexamer priming, hybridization, autoradiography, and recording of banding profiles were as described previously (Kidwell et al. 1994b). *Eco*RI-digested plant genomic DNAs were hybridized to 30 of the 61 alfalfa genomic DNA clones used in a previous study (Kidwell et al. 1994b). DNAs were evaluated for 11 individuals from each of 5 diploid single-crosses (2*x* PXR, VXR, LXP, PXV, and LXV). This is the number of progeny needed to recover either homozygous class ($P > 95\%$) from a cross between parents heterozygous for the same alleles (Sedcole 1977). In order to confirm diploid segregation results, RFLP patterns were monitored for 19 individuals each of 4*x* LXR, PXR, LXP, and LXV, and 10 of 4*x* VXR.

Table 1 Theoretical percentages of homozygous (Hom) and heterozygous (Het) individuals expected in single-cross progenies of two diploid genotypes and their corresponding isogenic tetraploids derived via chromosome doubling for seven possible parental genotypes at a given locus

Ploidy level	Number of alleles per locus	Locus state[a]	Parental genotype[b] 1	2	3	4	5	6	7
			% Hom or Het progeny						
2*x*	1	Hom	100	0	50	0	50	25	0
2*x*	2	Het	0	100	50	100	50	75	100
4*x*	1	Hom	100	0	17	0	6	3	0
4*x*	2	Het	0	100	83	33	94	31	11
4*x*	3	Het	0	0	0	67	0	66	44.5
4*x*	4	Het	0	0	0	0	0	0	44.5

[a] Heterozygous loci may carry 2, 3, or 4 alleles in tetraploid genotypes

[b] 1, parents homozygous for the same alleles; 2, parents homozygous for different alleles; 3, one parent homozygous, the other heterozygous, and they have a common allele; 4, one parent homozygous, the other heterozygous, and they have different alleles; 5, parents heterozygous for the same alleles; 6, parents heterozygous with one allele in common; 7, parents heterozygous for different alleles

Allele relationships among RFLPs were determined by evaluating the segregation patterns of restriction fragments among individuals from the various single-cross progenies at both ploidy levels. RFLP alleles identified in each parent were combined over all loci and were used to calculate Nei's genetic distances (NGDs; Nei 1972) using the following equation in NTSYS-pc (Rohlf 1992):

$$\mathrm{NGD}_{ij} = -\ln\frac{\Sigma|x_{ki}x_{kj}|}{\sqrt{\Sigma x_{ki}^2 x_{kj}^2}}$$

where x_{ki} represents the frequency of the kth RFLP allele in parent i, x_{kj} represents the frequency of the kth RFLP allele in parent j, and the summation is for the total number of RFLP loci evaluated. We also used genetic dissimilarity values (GDs), reported previously (Kidwell et al. 1994b) as the compliment of Dice's coefficient, for comparisons to NGD values. GD and NGD values of 0 indicate that no genotypic differences exist between two lines, whereas values of 1.0 indicate that genotypes differ for all fragments on RFLP alleles detected by this set of probes. Phenotypic correlations were calculated between GD and NGD using the PROC CORR procedures in SAS.

Heterozygosity estimation

Genotypes of parents at RFLP loci were determined by monitoring segregation in their single-cross progenies. On the basis of Mendelian expectations (see Allard 1960 for review), parental RFLP genotypes were used to estimate the percentage of progeny expected to be heterozygous at each RFLP locus for each single-cross at both ploidy levels (Table 1). In addition, the effect of accumulating multiple RFLP alleles at a locus was evaluated at the tetraploid level. Heterozygosity estimates for tetraploid families were partitioned into percentage of individuals per single-cross expected to carry a single RFLP allele (monoallelic), 2 different RFLPs (diallelic), 3 different RFLPs (triallelic), and 4 different RFLPs (tetra-allelic) across RFLP loci (Bingham 1980). The following assumptions were made: (1) strict chromosome assortment occurred with no numerical nondisjunction; (2) no selfing, selection, linkage, or maternal effects affected the segregation of RFLP alleles among progenies (Dunbier and Bingham 1975). Values were averaged over loci to estimate the level of RFLP heterozygosity in each $2x$ and $4x$ single-cross family. Phenotypic correlation coefficients were calculated for NGD and estimated RFLP heterozygosity level with forage yields of $2x$ and $4x$ single-crosses in microplot and spaced-plant field trials. Phenotypic correlations were calculated between the average percentage of individuals within tetraploid progenies having 1, 2, 3, or 4 RFLP alleles and the NGD, the number of RFLP allele differences between parents, and the forage yields of single-cross families.

Results

RFLP evaluation of parental genotypes

The 30 recombinant DNA clones used in this study hybridized to 102 RFLP alleles at 33 loci, with 3 clones identifying 2 different loci each. An average of 89 RFLP alleles were identified per single-cross combination. Genotypes L and R had the largest number of RFLP allele differences (68), whereas only 32 were detected between L and V (Table 2). A maximum of 5 RFLP alleles were identified among all parental genotypes at any 1 locus. Of the 33 RFLP loci, 6 segregated for the presence of null (non-hybridizing) alleles. Homologous restriction fragments were not detected at these loci; however, the null phenotype segregated consistently among individuals within all $2x$ and $4x$ progenies evaluated. For 3 of the 6 loci, the null allele was detected in only one of the four genotypes. Null alleles detected in two or more genotypes were assumed to represent identical states for heterozygosity estimation. Between 56 and 60 RFLP alleles were detected in each parent (average of 1.8 RFLP alleles per parent per locus). Twenty-seven RFLP alleles were unique to one of the four genotypes, with 15, 8, 3 and 1 present in R, L, P and V, respectively. Six, 8, 9 and 10 RFLP loci were homozygous in genotypes L, V, R and P, respectively; however, alleles at these loci were not unique to any individual. NGDs calculated from RFLP allele data ranged from 0.285 to 0.685 (Table 2) and were highly correlated ($r = 0.91$, $P < 0.01$) with GDs (Kidwell et al. 1994b).

For the 33 RFLP loci, the average level of heterozygosity estimated in single-cross progenies ranged from 54% to 70% for diploid families, and 82% to 93% for $4x$ families (Table 2). Estimated levels of RFLP heterozygosity for single-cross progenies were highly correlated ($r = 0.91$, $P < 0.01$) between the two ploidy levels. However, estimated heterozygosity levels differed by 18

Table 2 Total number of RFLP alleles detected, number of RFLP allele differences, Nei's genetic distance (NGD), percentages of single-cross (SC) progenies estimated to be heterozygous (Het) based on the genotypes of isogenic $2x$ and $4x$ parents for 33 RFLP loci, and forage yields of single-cross progenies from microplot and spaced-plant field trials

SC	Number of RFLP alleles	Number of allele differences	NGD[a]	Estimated % het loci[b]		Forage yield[c]			
						Microplots		Spaced- plants	
				$2x$	$4x$	$2x$	$4x$	$2x$	$4x$
LXR	98	68	0.685	69.7	87.9	491a	720c	65b	147a
PXR	90	54	0.601	70.4	93.0	486a	952a	46d	142a
VXR	91	54	0.575	65.9	89.8	393b	852b	52c	107b
LXP	90	49	0.502	64.4	89.6	431b	958a	92a	151a
PXV	81	36	0.336	55.3	83.6	277c	491e	47d	86b
LXV	84	32	0.285	53.8	82.1	464a	624d	52c	90b
Mean	89	49	0.497	63.3	87.7	424	766	59	121

[a] NGDs between parental genotypes were equivalent at the $2x$ and $4x$ ploidy levels

[b] Loci containing 2, 3, or 4 RFLP alleles were considered heterozygous at the $4x$ level

[c] Microplot yields reported as grams of dry matter per row, and spaced-plant yields reported as grams of dry matter per plant averaged over all harvests. Values within columns having the same letter did not differ significantly ($P < 0.05$) based on LSDs

to 28% between corresponding 2*x* and 4*x* families. The most heterozygous diploid single-cross family (PXR) was 12% less heterozygous than the least heterozygous tetraploid single-cross family (LXV).

Relationships among genetic distance, estimated RFLP heterozygosity and forage yield

Significant correlations were detected at the diploid ($r = 0.97$, $P < 0.01$) and tetraploid ($r = 0.81$, $P < 0.05$) levels between NGD of the parents and the estimated percentage of heterozygous RFLP loci in the progenies. The correlation between NGD and the number of RFLP allele differences detected between parents also was highly significant ($r = 0.98$, $P < 0.01$). The NGDs and estimated percentages of heterozygous RFLP loci for diploid progenies were not significantly correlated ($P > 0.05$) with either spaced-plant or microplot forage yield (Table 3). At the tetraploid level, significant correlations ($P < 0.05$) were detected between NGD and forage yield of spaced-plants, and between the estimated percentage of heterozygous RFLP loci and forage yield of spaced-plants and microplots (Table 3).

Relationship between RFLP genotypes of 4*x* progenies and forage yields

The percentages of individuals within tetraploid progenies having 1, 2, 3 or 4 RFLP alleles were estimated on the basis of parental RFLP genotypes and averaged across RFLP loci (Table 4). LXV had the highest percentage (17.9) of individuals with monoallelic RFLP loci, and PXR had the lowest percentage (7). Most of the individuals (average of 56%) within all progenies were diallelic, and 22 to 31% were triallelic. Tetra-allelic percentages per single cross ranged from 0 for PXV to 14.8 for LXR.

Correlations among estimated percentages of individuals having different numbers of RFLP alleles (Table 4), NGD (Table 2), the number of RFLP allele differences (Table 2), and forage yields (Table 2) were determined for tetraploid progenies (Table 5). NGD, number of RFLP allele differences, and forage yields were negatively correlated with the percentages of mono- and diallelic individuals and positively correlated with percentages of tri- and tetra-allelic individuals. Forage yields were significantly correlated ($P < 0.05$) with monoallelic percentages in both field trials and with triallelic percentages in the microplot trial. The triallelic percentage was also highly correlated ($r = 0.74$, $P = 0.06$) with forage yield of spaced plants. The di- and tetra-allelic percentages were not significantly correlated ($P > 0.05$) with forage yield in either trial.

Table 3 Phenotypic correlations of microplot and spaced-plant forage yield with Nei's genetic distance (NGD) between parents and the estimated percentage of heterozygous RFLP loci among 2*x* and 4*x* progenies of alfalfa

Field trial	Ploidy level	NGD	Estimated % heterozygosity
Microplots	2*x*	0.50	0.56
	4*x*	0.63	0.89**
Spaced-plants	2*x*	0.20	0.20
	4*x*	0.79*	0.76*

*,** Significant at $P < 0.05$ and $P < 0.01$, respectively

Table 4 Percentages of individuals within tetraploid single-cross (SC) progenies estimated to have 1, 2, 3 or 4 RFLP alleles averaged for 33 RFLP loci

SC	Number of RFLP alleles			
	1	2	3	4
LXV	17.9	57.1	23.6	1.4
PXV	16.4	61.4	22.2	0
LXR	12.1	44.1	29.0	14.8
LXP	10.4	57.3	28.3	4.0
VXR	10.2	55.5	30.3	4.0
PXR	7.0	59.3	31.0	2.7
Mean	12.3	55.8	27.4	4.5

Table 5 Phenotypic correlations between the average percentage of individuals within 4*x* progenies having 1, 2, 3 or 4 RFLP alleles and Nei's genetic distance (NGD), number of RFLP allele differences detected between genotypes, and forage yield of 4*x* single-cross progenies from microplot and spaced-plant field trials

Number of RFLP alleles	NGD	Allele differences	Field trial	
			Microplots	Spaced-plants
1	−0.81*	−0.70	−0.89**	−0.76*
2	−0.63	−0.75*	−0.04	−0.45
3	0.89**	0.81*	0.87**	0.74
4	0.56	0.85**	0.15	0.61

*,** Significant at $P < 0.05$ and $P < 0.01$, respectively

Discussion

Segregation analysis of RFLP alleles

In a previous study, we were concerned that molecular marker diversity may not accurately reflect the level of RFLP heterozygosity present within single-cross families derived from heterozygous alfalfa genotypes (Kidwell et al. 1994b). Allelic relationships between homologous restriction fragments were not known, and RFLPs in the heterozygous state in parents would segregate among progeny. In this study, RFLP genotypes of isogenic 2*x* and 4*x* parents were determined by monitoring the segregation of RFLPs among their single-cross progenies, and these data were used to estimate the level of heterozygosity in the progenies.

Of the DNA clones evaluated in this study, 10% (3/30) hybridized to 2 RFLP loci. This was not unexpected since gene duplication has been detected in other studies of alfalfa (Quiros and Morgan 1981; Brummer et al. 1993; Kiss et al. 1993; Echt et al. 1994). Of the 33 RFLP loci, 18% (6/33) segregated for the presence of a null allele. Null alleles also have been detected in other studies using RFLPs, and the nonhybridizing alleles are thought to result from deletion events or sequence divergence that decreases the level of homology between the target sequence and the probe (McCouch et al. 1988; Slocum et al. 1990; Chyi et al. 1992). Among four heterozygous diploid genotypes and their isogenic 4*x* counterparts, up to 8 different alleles could exist per RFLP locus. However, no more than 5 RFLP alleles were detected at any 1 locus in this study, and in each parent a large percentage of RFLP loci (18–30%) were homozygous. Although these values may reflect overall levels of homozygosity in the parents, polymorphisms were detected based on the use of only 1 restriction enzyme. Additional polymorphism may be revealed by using other enzymes.

NGDs calculated from allelic data in this study were highly correlated with GDs calculated from fragment data in a previous study ($r = 0.91$), and both genetic distance estimates were correlated with estimated RFLP heterozygosity levels of single-cross progenies at both ploidy levels. Thus, prior knowledge of allele relationships between RFLPs may not be required to obtain genetic distance estimates that accurately reflect heterozygosity levels in segregating progenies.

Estimated RFLP heterozygosity levels of progenies and correlations with forage yields for isogenic 2*x* and 4*x* single crosses

Tetraploid families derived from partially inbred genotypes were estimated to have higher percentages of heterozygous individuals across RFLP loci than diploid families (Table 2). In spite of this, estimated RFLP heterozygosities were significantly correlated ($P < 0.01$) between 2*x* and 4*x* families. Thus, 2*x* and 4*x* forage yields should also be highly correlated if maximizing heterozygosity has a similar effect at both ploidy levels. However, the only significant associations between estimated RFLP heterozygosity levels and forage yield were detected at the tetraploid level.

Among 4*x* progenies, negative correlations ($P < 0.05$) were detected between estimated percentages of individuals with monoallelic RFLP loci and forage yield (Table 5). Positive correlations ($P < 0.05$) were detected only between forage yield and the estimated percentage of individuals carrying 3 different RFLP alleles (Table 5). This suggests that tetraploid families with the lowest levels of RFLP homozygosity or the highest levels of RFLP heterozygosity were the highest yielding. The low correlations detected between the percentage of tetra-allelic RFLP loci expected in progenies and forage yield were probably due, in part, to the low percentage (an average of 4.5) of loci estimated to carry 4 RFLP alleles (Table 4). Low percentages of tetra-allelic loci would be expected in single crosses of autotetraploids because the frequency of tetra-allelic loci is not maximized until the double cross or even later generations (Bingham 1983).

Differences in the relationships among genetic distance, forage yield and heterozygosity in isogenic diploid and tetraploid progenies can be explained by greater complementary gene interactions with tetrasomic segregation than are possible at the diploid level (Bingham et al. 1994). The ability to maintain multiple alleles at a locus or multiple chromosome segments in a region permits higher proportions of recessive alleles to be maintained within populations. It also provides the greatest opportunity for acquiring at least one favorable dominant allele at each locus (Bingham et al. 1994). In general, diploid progenies should be less heterozygous than progenies of isogenic tetraploid parents. However, the difference in levels of heterozygosity depends on the genotypes of the parents (Table 1). Based on the segregation of restriction fragments at RFLP loci, we estimated that the diploid progenies used in this study were about 25% less heterozygous on average than tetraploid progenies (Table 2). The larger proportion of homozygous recessive loci within diploid progenies may have negated the beneficial effects of favorable dominant alleles at other loci. It is well-known that deleterious recessive traits such as dwarfism can be epistatic over favorable alleles for height. Furthermore, if recessives were present at some homozygous loci, a smaller proportion of the total population would be affected at the tetraploid level than at the diploid level. Thus, the gene action involved in forage yield could be the same at both ploidy levels; however, the frequency of homozygous loci is greater at the diploid level. Tetraploids benefit from greater complementary gene interactions than are possible for equivalent diploids (Bingham et al. 1994).

The associations between the molecular markers evaluated in this study and genes conditioning forage yield were not determined. However, a positive relationship between genetic distance and forage yield was found among tetraploid genotypes, and the accumulation of more than 1 RFLP allele (or chromosome segment) at RFLP loci was related to improved yield. These results suggest that molecular markers may be useful for identifying diverse genotypes to hybridize in heterozygous, heterogeneous polyploid species.

Acknowledgements The authors express their sincere thanks to Jim Coors, Mike Casler and an anonymous reviewer for critically evaluating this manuscript. Support for this work was provided by the University of Wisconsin College of Agriculture and Life Sciences, USDA Competitive Grant No. 91-37301-6378 (to E.T.B.), and a Pioneer Graduate Fellowship (to K.K.K.).

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