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Variation in vigour and in RFLP-estimated heterozygosity by selfing tetraploid alfalfa: new perspectives for the use of selfing in alfalfa breeding

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Abstract The effect of selfing on vigour and heterozygosity was analysed in six independent families obtained by recurrent cycles of self-fertilization and selection until the S_3 generation. The heterozygosity level, estimated by means of 11 homologous probes, decreased from the S_0 to S_3 generations and was partially restored in $S_2 \times S_2$ polycrosses. The decreasing trend was influenced by the inbreeding level of the chosen mother plant in each self generation to advance in subsequent selfing. Plant vigour estimated by Dry Matter Yield (DMY) decreased during inbreeding, and phenotypic selection of S_2 individuals seemed to prove effective as the differences in DMY between vigorous and weak plants were maintained in $S_2 \times S_2$ crosses. No correlation between plant vigour and heterozygosity was found between subgroups of vigorous and weak plants selected within the same S_2 family. Results are discussed with a view to selecting the best performing and least heterozygous plants during inbreeding to isolate useful genes and linkats in superior partially inbred parental lines.

Key words Alfalfa · Inbreeding depression · Heterozygosity · Plant vigour · Molecular markers

Introduction

Progress in increasing alfalfa (*Medicago sativa* L.) yield has been slower than in other grain crops for a number of reasons, most of which are related to the genetics of the species itself with tetrasomic inheritance being the most important. Insect-mediated allogamy, a reproductive system with hermaphrodite flowers, plant architecture and meadow conditions may represent additional obstacles to successful breeding for yield.

Several studies have proved that yield in a series of progressively more heterozygous genotypic structures derived from a common genetic background is correlated with heterozygosity (Demarly 1963; Dunbier and Bingham 1975; McCoy and Rowe 1986). On the basis of these results, modifications in current methods for alfalfa variety development aimed at maximizing heterozygosity have been suggested (Bingham 1980). However, the emphasis on heterozygosity has overshadowed what remains the most important objective of geneticists and breeders: improvement of the genetic value of mother plants (Rotili et al. 1999).

In the 1970s, Demarly (1979) proposed and expanded the concept of linkat as a basis for heterosis to overcome some inconsistencies of multiple allelic models in alfalfa. This author suggested that the alleles of genes for the best selective values may interact epistatically and are clustered into protected chromosome segments that tend to be inherited together (linkats). Bingham et al. (1994) coined the phrase "complementary gene interaction" to define the gene action for yield in Demarly's linkats. According to Bingham's interpretation, the best allelic condition for yield is when favourable dominant alleles are accumulated in the same linkat and linked in repulsion phase. The loss of complementary gene interaction which occurs upon selfing is responsible for the abrupt inbreeding depression noted in alfalfa by Busbice and Wilsie (1966), which cannot be explained by the common theories of inbreeding in autotetraploids (Bartlett and Haldane 1934; Bennett 1976). Thus, linkats as functional, segregational and selective units can be the object of selection in alfalfa, as proposed by Rotili (1976). In this new context, inbreeding and selection for yield are powerful tools for alfalfa forage yield improvement. Significant reduction in inbreeding depression was obtained through a selection for vigour over three generations of selfing by Rotili (1970). The same author (Rotili 1976)

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showed that the mean of some single cross families increased when selection in selfing phase was practised; this increase was transmitted to the Syn 2 generation of synthetics. Therefore, intentional selection of vigorous plants, grown at agronomical density, in selfing generations increases the frequency of favorable genes and linkats, which is the parental plant breeding value, making the selection successful.

The breeding scheme adopted for direct vigour improvement in alfalfa set up at the Lodi Institute (Rotili et al. 1999) includes a phase of reduction of heterozygosity through selfing, with selection for vigour among and within selfed progenies in dense stands, and a phase of heterozygosity reconstruction by intercrossing the best performing partially inbred clones.

A crucial aspect of selfing and selection in autotetraploid alfalfa is that the starting point, represented by the average heterozygosity levels of the original populations, the homozygosity level of selected plants at the end of the selfing phase and the speed of the selfing process are unknown. For example, inadvertent selection for heterozygotes, already reported when performing phenotypic selection for vigour (Rotili 1976; Woodfield and Bingham 1995) may slow the reduction of heterozygosity. This highlights the need for a method to measure both the heterozygosity level in individuals under selfing and the genetic distance of the parental lines selected at the end of the selfing process. RFLP (Restriction Fragment Length Polymorphism), which is caused by mutations at the DNA level, may reveal codominant marker loci suitable for the purpose.

The objectives of the study reported here were therefore: (1) to investigate the suitability of RFLP for estimating the heterozygosity level in genotypes under selection,(2) to evaluate variations in heterozygosity during the successive steps of selfing and selection within selfed families and (3) to test if within-family groups differing in vigour also differ in their heterozygosity level.

Materials and methods

Plant material

All families studied belong to cv. Lodi (I.S.C.F., Lodi, Italy), a synthetic variety consisting of eight S_2 constituents. Six S_0 plants chosen for vigour represented the origin of the selfed progenies S_1–S_3 (Table 1). Each selfed family was derived by plant selection for vigour in the previous generation. Single plants were grown in 5-cm-diameter PVC tubes filled with a sandy-loamy soil (density of about 500 plants/m^2) in a hangar in Lodi, with an unlimited water supply and in the absence of the mineral N. This culture technique made it possible to reproduce the density condition that is essential for an effective phenotypic selection in alfalfa, without interference among plants; in addition, the setting up of selfing and polycrosses of the chosen plants was extremely easy. Cutting schedule was every 30 days (May-September). S_1 and S_2 families represented by 50 plants per family were grown in 1991 (sowing year; 3 cuts) and 1992 (1st production year; 5 cuts) together with polycross families $S_0 \times S_0$; S_3 families were grown in 1995 (sowing year; 2 cuts) and 1996 (1st production year; 3 cuts) together with polycross families $S_2 \times S_2$. Fifteen plants randomly chosen within each S_1 family, were used for molecular analysis; the S_2 plants analysed consisted of two subgroups of opposite vigour (vigorous and weak plants) selected within the same family. S_3 progenies were formed by the total number of viable plants in the first production year. S_2 plants were also polycrossed within the same subgroup of vigor. Both selfing and polycrossing were hand-performed without emasculation.

RFLP analysis

The CTAB method of Saghai-Maroof et al. (1984) was used for DNA extraction. Ten micrograms of DNA was digested with the six cutter *Hin*dIII (New England Biolabs) according to the supplier's instructions, electrophoresed on 1% agarose gels and vacuum-blotted onto Hybond-N$^+$ (Amersham) membranes using a Vacugene XL apparatus (Pharmacia). The procedures followed for probe labelling, blot hybridization, washing and exposure were those of Businelli et al. (1993). Eleven alfalfa genomic clones selected for their low-copy number (Businelli et al. 1993) and capacity to detect highly polymorphic loci in alfalfa genome (Pupilli et al. 1996) were used in this study.

Table 1 Plant material used for RFLP analysis

S_1		S_2			S_3		$S_2 \times S_2$
Family	Number of plants	Family	Number of plants		Family	Number of plants	Number of plants
			Vigorous	Weak			
225	15	225.1	8	9			
		225.2	7	9			
227	15	227.8	10	10	227.8.2083+[a]	3	15
					227.8.3976–	2	8
231	15	231.1	10	10			
233	15	233.4	9	10	233.4.3342–	11	
		233.7	7	10			
235	15	235.1	10	10	235.1.1823+	6	15
					235.1.1828–	6	15
268	15	268.1	7	10			
		268.5	10	10	268.5.3876+	13	
Total	90		78	88		41	53

[a] +, Vigorous; –, weak

Data analysis

Inbreeding effect (InE%) on vigour, estimated by Dry Matter Yield (DMY), was calculated by the formula: $100\times(S_nDMY-S_{n-x}DMY)/S_{n-x}$ DMY, where S_n is the inbreeding generation in which the effect was calculated and S_{n-x} the generation taken as a reference point (x represents the generation gap). Similarly, an estimate of heterozygosity (Heterozygosity Index=HI) was calculated for each plant by summing up the restriction fragments present at all loci; for each individual belonging to selfed progenies an inbreeding effect (InE%) was computed comparing its heterozygosity index with that of the corresponding Mother Plants (MP) in the previous generations using the formula: $100\times(S_nHI-S_{n-x}HI)/ S_{n-x}HI$. RFLP and agronomic data were statistically analysed using the GLM and CORR procedures in the SAS (1987) programme.

Results

The average inbreeding effect occurring in the first generation of selfing was –9.55% (Table 2), a much lower value than was expected considering that all of the loci had tetra- or three-allelic structures (–25% and –17.6% respectively) and slightly lower (–11.5%) than was calculated assuming an identical frequency of all possible genotypic structures (Scotti et al. 1992). With respect to the progenies for which three selfing generations could be obtained, the observed mean inbreeding effect ranged from –9.82% (S_1) to –16.55% (S_2) and to –19.46% (S_3) relative to the S_0 MPs (Table 2) – a progressive reduction in the amount of inbreeding effect through selfing generations is evident (–9.82%, -6.73% and –2.91%). The 9 S_1 plants chosen as MPs for subsequent S_2 generations were the most vigorous within their families but not necessarily the most heterozygous; in fact, these MPs were situated in the $x\pm1s$ class of their own S_1 family. The same holds true for the six S_2 MPs with the exception of two cases, one (235.1.1828) exceeding x+1s and the other (233.4.3342) which was lower than x–1s.

No significant relationship between inbreeding effect on vigour and on estimated heterozygosity was detected. When vigour is considered, the S_0 generation is not represented by the individual MPs but by their polycross families $S_0\times S_0$; polycross may have affected the level of heterozygosity and therefore vigour, thus influencing the

Table 2 Heterozygosity index: absolute values (HI) and percentage variation with respect to S_0 MP (InE%) in selfing generations

S_0		S_1			S_2			S_3		
MP	HI	Family	HI	InE%	Family	HI	InE%	Family	HI	InE%
225	37	225	33.80	– 8.65	225.1	31.35	–15.26			
					225.2	32.19	–13.01			
227	44	227	38.67	–12.12	227.8	35.65	–18.98	227.8.2083+[a]	33.00	–25.00
								227.8.3976–	36.50	–17.05
231	39	231	35.33	– 9.40	231.1	32.00	–17.95			
233	43	233	38.53	–10.39	233.4	36.58	–14.93	233.4.3342–	32.91	–23.47
					233.7	37.18	–13.54			
235	44	235	40.73	– 7.42	235.1	38.15	–13.30	235.1.1823+	37.00	–15.91
								235.1.1828–	37.83	–14.02
268	40	268	36.27	– 9.33	268.1	33.94	–15.15			
					268.5	32.40	–19.00	268.5.3876+	31.46	–21.35
Mean	41.17		37.22	– 9.55		34.38	–15.68		34.78	–19.46

[a] +, Vigorous; –, weak

Table 3 DMY: absolute values (g/plant, 1st production year) and percentage variation (InE%) of S_1 and S_2 generations with respect to $S_0\times S_0$ polycross families and of the S_3 generation with respect to $S_2\times S_2$ polycross families

S_1			S_2			S_3		
Family	DMY	InE%	Family	DMY	InE%	Family	DMY	InE%
225	22.83	–28.41	225.1	15.80	–50.45			
			225.2	16.28	–48.95			
227	24.84	–33.94	227.8	24.74	–34.20	227.8.2083+[a]	13.91	–52.38
						227.8.3976–	21.71	+ 3.43
231	30.30	– 9.31	231.1	21.82	–34.69			
233	26.10	–22.25	233.4	18.59	–44.62	233.4.3342–	21.30	–18.55
			233.7	17.23	–48.67			
235	24.83	–26.10	235.1	18.65	–44.49	235.1.1823+	18.60	–38.92
						235.1.1828–	14.90	–42.36
268	20.18	–32.67	268.1	16.53	–49.40			
			268.5	17.93	–45.12	268.5.3876+	21.70	–26.69
Mean	24.85	–26.46		18.62	–44.89		18.69	–30.89

[a] +, Vigorous; –, weak

Table 4 Within-family correlations between vigour (DMY) and estimated. Heterozygosity Index in sowing year (A) and sowing year +1st production year (B)

A

S_1 (DMY, sum of 3 cuts)		S_2 (DMY, sum of 3 cuts)			S_3 (DMY, sum of 2 cuts)	
Family		Family	Vigorous	Weak	Family	
225	–0.11ns	225.1	–0.33ns	–0.33ns		
		225.2	–0.01ns	–0.75*		
227	0.15ns	227.8	0.34ns	0.66*	227.8.2083+	0.54ns
					227.8.3976–	–
231	0.38ns	231.1	–0.35ns	0.48ns		
233	0.19ns	233.4	–0.29ns	–0.62ns	233.4.3342–	0.29ns
		233.7	0.09ns	–0.21ns		
235	0.03ns	235.1	0.18ns	0.31ns	235.1.1823+	0.84*
					235.1.1828–	0.89*
268	–0.04ns	268.1	0.62ns	0.13ns		
		268.5	–0.47ns	0.37ns	268.5.3876+	0.38ns

B

S_1 (DMY, sum of 8 cuts)		S_2 (DMY, sum of 8 cuts)			S_3 (DMY, sum of 5 cuts)	
Family		Family	Vigorous	Weak	Family	
225	0.04ns	225.1	–0.12ns	–0.26ns		
		225.2	–0.60ns	0.08ns		
227	0.08ns	227.8	–0.34ns	–0.09ns	227.8.2083+	0.95ns
					227.8.3976–	–
231	–0.22ns	231.1	–0.35ns	0.63ns		
233	0.15ns	233.4	–0.27ns	–0.64*	233.4.3342–	0.32ns
		233.7	0.55ns	–0.11ns		
235	0.19ns	235.1	0.17ns	0.61ns	235.1.1823+	0.81*
					235.1.1828–	0.24ns
268	0.10ns	268.1	0.45ns	–0.09ns		
		268.5	–0.40ns	0.15ns	268.5.3876+	0.20ns

* $P<0.05$; ns, not significant
[a] +, Vigorous; –, weak

comparison of S_1 with S_0. Nevertheless, the S_1 and S_2 progenies of the most (227) and among the less (231) heterozygous S_0 MPs showed some of the highest DMY values (Table 3). In the case of 227 selfed series, high performances seem to be due to residual heterozygosity in a greater proportion than in the case of 231: the trend of inbreeding effect on heterozygosity of the two series seems to confirm this hypothesis (Table 2). The S_3 progeny of the weak S_2 MP 227.8.3976, represented by only 2 plants, showed a slightly higher DMY (+3.43%, Table 3) than its corresponding $S_2 \times S_2$ family; similarly, the same progeny showed an increase in HI over the average HI value of the corresponding S_2 progeny (Table 2). This suggests that beyond a certain heterozygosity threshold, probably different for each family, selection favouring heterozygotes may occur.

In the S_2 generation, 7–10 plants at the opposite ends of DMY distribution (1st production year) for each family were grouped (Table 1), and the relationship between HI and DMY was examined within and between subgroups of the same family. Correlation analysis between HI and DMY performed within subgroups of opposite vigour showed an overall absence of the relationship (Table 4). The only three significant cases of the S_2 families occurred in the weak groups: in two of them the correlation was negative (families 233.4 and 225.2 only for the sowing year); in the third case a positive correlation between HI and vigour was found in the sowing year, but not in the total DM production (family 227.8). In the S_3 generation, families 235.1.1823 and 235.1.1828, derived from S_2 MPs plants of opposite vigour, showed a positive significant correlation between HI and DMY in the sowing year and, in the case of 235.1.1823, also between HI and DMY of the 1st production year. The significant cases suggest a possible relation, as inbreeding progresses, between HI and the early stages of growth (1st production cycles of the sowing year).

The effect of selection for vigour on HI within each family is reported in Table 5. With the exception of one case the two subgroups did not differ significantly in their estimated heterozygosity levels; only in family 268.1 did the vigorous group show a distinctly higher HI than the weak group. Thus, the different genotypic structures seem to be distributed randomly in the two subgroups, while plant phenotype is clearly different. Comparison of DMY among S_3 progenies of vigorous and weak plants within the same S_2 families was only possible for families 235.1.1823(+) and 235.1.1828(–). The two mother plants differed widely in vigour but not in HI; a difference in DMY, though not a significant

Table 5 Effect of phenotypic choice for DMY on. Heterozygosity Index in S_2 families: vigorous vs. weak subgroups (*F*-test values)

Family	DMY	HI
225.1	168.12***	0.39ns
225.2	103.21***	0.84ns
227.8	51.34***	0.10ns
231.1	56.32***	1.76ns
233.4	155.86***	0.03ns
233.7	63.86***	0.39ns
235.1	153.30***	1.72ns
268.1	106.70***	9.26**
268.5	280.51***	1.91ns

**$P \leq 0.01$; ***$P \leq 0.001$; ns, not significant

Table 6 $S_2 \times S_2$ families: DMY (sum of 6 cuts of the 1st production year) and percentage variation of HI relative to the respective S_2 mother plants

$S_2 \times S_2$ family	DMY	HI		
		Mean	Minimum	Maximum
227.8.2083+[a]	38.55	+6.79	–8.93	+16.07
227.8.3976-	30.70	–2.66	–8.20	3.28
235.1.1823+	37.81	+1.40	–8.77	8.77
235.1.1828-	31.50	–0.79	–15.25	6.78

[a] +, Vigorous; –, weak

one, was maintained in their respective S_3 families, (Table 3).

To verify the degree of heterozygosity restoration in $S_2 \times S_2$ polycross families, we measured HI in four progenies derived from full-sib S_2 plants of opposite vigour (Table 6).The average HI of polycross families slightly increased (vigorous progenies) or decreased (weak progenies) relative to their respective S_2 mother plant. In theory, both behaviours are possible depending upon the inbreeding coefficient of the plants polycrossed and on the allelic diversity present at the loci analysed. In fact, S_2 MPs of low vigour showed a higher HI than their respective vigorous S_2 counterparts (Table 2), and this residual heterozygosity caused segregation in $S_2 \times S_2$ polycross families. Furthermore, all the plants in the $S_2 \times S_2$ polycrosses belonged to the narrow-base cv. Lodi, meaning a reduced potential of genetic diversity. The possibility of estimating genetic diversity by molecular markers seems therefore of crucial importance to direct the rate of heterozygosity recovered in crossing partially inbred parental plants (Rotili et al. 1985). Again, no intra-family correlation was found between vigour and estimated HI.

Discussion

To the authors' knowledge, this is the first study in which molecular markers have been used to monitor heterozygosity while inbreeding tetraploid alfalfa breeding lines. The effectiveness of HI in estimating the heterozygosity levels in alfalfa breeding lines under selfing was confirmed by the constant decrease in the estimated average heterozygosity level detected along the S_0–S_3 series and its partial restoration in $S_2 \times S_2$ polycrosses. Heterozygosity decline was slightly slower than expected assuming an identical frequency of the five possible genotypic structures of a tetraploid. Similar observations have been reported by Brower and Osborn (1997) while inbreeding diploid alfalfa; these authors attributed their findings to inadvertent selection in favour of heterozygous individuals. Since the unmasking of deleterious allele combinations during inbreeding in tetraploid alfalfa is buffered by a higher heterozygosity level than in diploids, the gap between detected and expected decline in heterozygosity was narrower in our material than reported for diploids. Nevertheless self-sterility in our material showed a dramatic increase in the third selfing generation, suggesting that beyond a certain heterozygosity threshold further inbreeding is counterbalanced by self-sterility (Rotili 1976); this behaviour may in part account for the lower inbreeding depression in HI in the S_3 generation than in earlier generations.

Numerous studies have reported correlations between heterozygosity and both fertility and vigour in alfalfa (Dunbier and Bingham 1975; Groose et al. 1989; Bingham et al. 1994; Kidwell et al. 1994). In this study, no significant correlation were detected either within or between families; the few cases of positive correlations between these two parameters were detected in the S_3 generation and were related mainly to the sowing year production, suggesting a possible relation between HI and the early stages of stand growth in advanced inbreeding generations.The lack of noticeable correlations between vigour and heterozygosity within S_2 families was found in an unbiased sample where the effect of genotype is negligible. The same result was recently reported in a similar genetic context in potato where the performance of subpopulations of First and Second Division Restitution (FDR and SDR) 2n gametes derived from the same 2x–4x cross was compared. (Douches and Maas 1998). More heterozygosity is usually transmitted in FDR than in SDR gametes, but in this case the performance of the two subpopulations was not significantly different, indicating that heterozygosity may not be as critical on yield as previously reported.

In our breeding method the number of gene and linkat interactions due to the original heterozygosity level was lowered by selfing, as indicated by the decreasing trend of HI. In this situation, redistribution of the genes and linkats implicated in vigour which were originally present in S_0 MPs seems to be the most important factor acting on vigour itself. Intrafamily variation in the estimated heterozygosity index seems, in general, sufficient to select vigorous individuals with comparatively lower heterozygosity levels: these individuals should be of greater interest than parental plants because vigour would rely more on the additive value of genes and linkats than on heterozygosity levels.

Similarly to HI, plant vigour ranked as expected with inbreeding $S_0 > S_1 > S_2 > S_3$. Two orders of evidence dem-

onstrate the effectiveness of phenotypic selection for vigour: (1) $S_2 \times S_2$ test progenies of plants belonging to the vigorous groups had a better yield than their weak counterparts in the first production year and (2) in the only family for which comparison between weak and vigorous S_3 progenies was possible the difference in DMY, though not significant, was maintained. This is further evidence (Rotili 1976) that phenotypic selection during inbreeding acts on the additive part of vigour by increasing the frequency of genes and linkats favourable to vigour that are transmitted to polycross progenies.

After we improved the breeding value of the partially inbred clones, the positive effect of overall heterozygosity on vigour was exploited by crossing the selected clones. In our material, the level of heterozygosity reached in the first polycross $S_2 \times S_2$ progenies was only partially restored. The reasons for this may be attributed either to the presence of residual heterozygosity in the S_2 parents or, as previously reported (Demarly 1963; Rotili 1976), to the fact that the frequency of tetra-allelic loci is not maximized until double cross or even later generations, or, even to insufficient genetic diversity between the parental lines (Rotili et al. 1985).

Taken together these results highlight the need for improving the effectiveness of the inbreeding process by estimating the heterozygosity level of the phenotypically chosen plants; the lower this level, with comparable DMY, the faster the heterozygosity reduction phase. But more important, a comparatively lower heterozygosity content associated with high vigour would mean an improvement in the breeding value of the parental plants. This study can thus be treated as a baseline from which to start further studies to verify the effectiveness of the procedures based on phenotypic selection for vigour and molecular marker-assisted selection for low heterozygosity during successive cycles of selfing – in the subsequent phase of variety construction (Syn 1–Syn 4 generations of synthetics or free-hybrids). The question of how to improve the efficiency of the heterozygosity estimates in alfalfa is still open and is essentially a matter of the number, kind and genomic coverage of the markers being used.

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