

# Within- and Among-Cultivar Genetic Variation in Alfalfa: Forage Quality, Morphology, and Yield

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## ABSTRACT

Alfalfa (*Medicago sativa* L.) cultivars are synthetic populations formed from 8 to 200 parents and thus have a broad genetic base. Within-cultivar variation was compared with among-cultivar variation for energy value traits, morphological traits, and dry matter yield. Eleven cultivars, each represented by 15 clones of 7 to 20 genotypes, were evaluated in field plots simulating a dense canopy at INRA (National Institute of Agronomic Research), Lusignan in France. Six harvests spanning 3 yr were analyzed. Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), enzymatic digestibility, leaf-to-stem ratio (LSR), number of stems, stem height, and dry matter yield were measured. Within-cultivar variance accounted for 31 to 70% of the genetic variance for LSR and quality traits and 57 to 100% for morphological traits and dry matter yield. Large within-cultivar variation for yield-related traits could impart yield stability across environments, as a result of competition in alfalfa canopies. Phenotypic and genetic correlations were relatively low in each harvest for dry matter yield and NDF content, but high for NDF content and LSR. Within-cultivar variation could be exploited as an additional source of genetic variation in breeding programs for quality traits to achieve a higher genetic gain per breeding cycle.

ALFALFA CULTIVARS have a complex genetic structure. Landraces are populations bred by farmers, and registered cultivars are synthetic populations with various numbers of parents and various numbers of generations of multiplication before commercial seeds are sold.

The allogamy of this species and its autotetraploidy contribute to large within-population or within-variety genetic variation. Even though valuable individuals or alleles can generally be found in every population, this within-population variation can hinder the rate of improvement for polygenic traits such as forage yield, resistance to diseases caused by *Phytophthora medicaginis* E.M. Hans. & Maxwell and *Colletotrichum trifolii* Bain & Essary (Hill et al., 1988; Mackie and Irwin, 1998), resistance to stem nematode, lodging, or the initial rate of digestion measured in the rumen of fistulated cows (Goplen et al., 1993)

Current selection procedures often include feeding-value characters (digestibility and fiber contents) to improve the energy value of alfalfa forage. Genetic variation among cultivars for digestibility or fiber contents has been described (Heinrichs et al., 1969; Buxton et al., 1987; Lenssen et al., 1991; Julier et al., 1996; Julier and Huyghe, 1997), but the identification and development of high yielding, highly digestible cultivars is complicated by the negative relationship between digestibil-

ity and forage yield (Julier and Huyghe, 1997). A wide range of variation for digestibility could be found at the individual level, as for other traits. Depending on the importance of within-cultivar variation compared with among-cultivar variation, and on the genetic correlations when including this additional source of variation, breeding programs could include the analysis of individual plant digestibility.

The objective of this study was to measure within-cultivar and among-cultivar variances for alfalfa traits related to the energy value, forage yield, and several morphological traits, and to assess the phenotypic and genetic correlations when including the within-cultivar variation.

## MATERIALS AND METHODS

During the spring of 1992, 56 alfalfa cultivars were established in a spaced-plant nursery at INRA at Lusignan (France). Among them, 11 cultivars (Table 1), including four French landraces (Julier, 1996), were chosen in the fall of 1994 to represent a wide range of genetic variation and different eras of breeding. Twenty plants (i.e., genotypes) per cultivar were grown in sand in a greenhouse in early spring 1995 and cloned via cutting to obtain 15 clones. Seven to 20 genotypes per cultivar produced the 15 clones. The other genotypes were discarded because of their poor ability to be cloned. These clones were transplanted to the field on 4 May 1995 at INRA Lusignan in a deep clay silt soil in randomized complete blocks with three replicates. Five clones per genotype comprised each block. Clones were transplanted on 0.10-m centers to simulate a dense canopy, with a plot size of 0.05 m<sup>2</sup>. Each block was surrounded by a border row planted with the lodging-resistant cultivar Orca. The trial was irrigated. Despite cloning, the plants appeared vigorous, with an erect growth habit.

Ten harvests were performed on 1 June 1995, 12 July 1995, 17 Aug. 1995, 29 Sept. 1995, 2 Nov. 1995, 31 May 1996, 4 July 1996, 13 Aug. 1996, 10 Oct. 1996, and 21 May 1997. The six highest yielding harvests were analyzed: two in 1995 (12 July and 29 September), three in 1996 (30 May, 4 July, 10 October), and one in 1997 (21 May). The other harvests were discarded because not all genotypes could be analyzed because of low forage yields. Each plot was cut, number of stems was counted, and the longest stem was measured. On 30 randomly chosen plots per harvest, leaves and stems were separated to calculate the leaf-to-stem ratio (LSR). Forage was dried, weighed, and ground to pass a 1-mm grid. On all samples with dry weight higher than 4 g, near infrared spectra (NIRS) were collected (NIRSystems 6500, NIRSystems Inc., Silver Spring, MD) between 1100 and 2500 nm at every 2 nm. Enzymatic digestibility (Lila et al., 1986), NDF, ADF, and ADL by the Van Soest method (Goering and Van Soest, 1970), and LSR were predicted, by equations based on samples from this experiment and from previous experiments on alfalfa (including Julier

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**Abbreviations:** ADF, acid detergent fiber; ADL, acid detergent lignin; ADL<sub>w</sub>, lignin in the cell wall; LSR, leaf to stem ratio; NDF, neutral detergent fiber; RAPD, random amplified polymorphic DNA.

**Table 1. Names, origin, year of registration of 11 alfalfa cultivars, and number of genotypes cloned in each cultivar.**

Cultivar	Origin	Cloned genotypes
		no.
Flamande	French landrace	15
Marais de Luçon	French landrace	20
Poitou	French landrace	12
Provence	French landrace	20
Europe	French cultivar (1961)	18
Orca	French cultivar (1966)	20
Luzelle	French cultivar (1993)	20
Luisante	French cultivar (1997)	11
Alfagraze	U.S. cultivar	17
AuPX	French experimental variety	7
ProtG3	French experimental variety	7

and Huyghe, 1997). Twenty-five to 50 samples per harvest (chosen to represent spectra variations) were analyzed for digestibility, NDF, ADF, and ADL by wet chemistry. The prediction equations were tested for accuracy by the standard error of cross validation (SECV) and the coefficient of determination ( $R^2$ ). These values were 0.24 and 0.71 for LSR, 1.62 and 0.90 for enzymatic digestibility, 1.14 and 0.96 for NDF, 1.13 and 0.95 for ADF, and 0.40 and 0.87 for ADL. Lignin in the cell wall (ADLcw) was calculated as  $100 \times \text{ADL}/\text{NDF}$ .

For each harvest, analyses of variance were performed on all traits, assuming fixed block effects and random cultivar and genotype-within-cultivar effects by the GLM procedure of SAS and the RANDOM statement (SAS Institute, 1988). Cultivar effects accounted for among-cultivar variation ( $\sigma_c^2$ ), and genotype-within-cultivar effects accounted for within-cultivar variation ( $\sigma_w^2$ ). These variances were calculated with the VARCOMP procedure of SAS by the REML method. Standard error of the variance for Effect  $g$  was calculated (Becker, 1975) as

$$SE(\sigma_g^2) = \sqrt{\frac{2}{k^2} \sum_i \frac{MS_i}{f_i + 2}}$$

where  $MS_i$  is the mean square of Effect  $i$  used to estimate variance component  $g$ ,  $f_i$  is the degrees of freedom for  $MS_i$ , and  $k$  is the coefficient for  $\sigma_g^2$  in the expectation of  $MS_g$ .

A broad-sense heritability was calculated as

$$H^2 = (\sigma_c^2 + \sigma_w^2)/(\sigma_c^2 + \sigma_w^2 + \sigma_R^2)$$

where  $\sigma_R^2$  is the residual variance

Phenotypic correlations among dry matter per square meter, NDF, ADF, ADL, ADLcw contents, digestibility, and LSR were calculated from genotype means across the three blocks for each harvest. Genetic correlations were estimated at the genotype-within-cultivar level, from the variance-covariance matrices given by the MANOVA statement of the GLM procedure of SAS. Significance value for the genetic correlation  $r$ , with  $n$  observations (i.e., genotypes), was calculated as

$$r \sqrt{\frac{n-2}{1-r^2}}$$

and tested to Pearson's value with  $n-2$  degrees of freedom.

An analysis of variance was performed across harvests assuming random effects for blocks, cultivars, genotypes within cultivars, and fixed effects for harvest dates. Broad-sense heritability was calculated as

$$H^2 = (\sigma_c^2 + \sigma_w^2)/(\sigma_c^2 + \sigma_w^2 + \sigma_{cH}^2 + \sigma_{wH}^2 + \sigma_R^2)$$

where  $\sigma_{cH}^2$  is the among-cultivar  $\times$  harvest interaction variance, and  $\sigma_{wH}^2$  is the within-cultivar  $\times$  harvest interaction variance.

## RESULTS

In the analysis of variance within harvest dates (not shown), cultivar effects were not significant for dry matter yield when each harvest date was analyzed separately, nor were they significant for ADL content in the 12 July 1995 harvest. Cultivar and genotype-within-cultivar effects were highly significant for all other harvest date  $\times$  trait combinations. For quality traits and LSR, within-cultivar variance was similar to or higher than among-cultivar variance (Table 2), except for LSR in two harvests. For morphological traits and for yield, the within-cultivar variance was higher than the among-cultivar variance (Table 2). Heritabilities were similar among morphological traits, dry matter yield, and quality traits; however, large residual variances for the 4 July 1996 harvest led to lower heritabilities for quality traits.

Figure 1 illustrates the large variation within cultivars relative to the among-cultivar variation for NDF. Some cultivars, such as ProtG3 and Luzelle, showed less variation than the overall variation. Overall differences for NDF content ranged from 10 to 15 percentage units among genotypes depending on the harvest. Average values for each trait varied with harvest date (Table 2), but the level of within-cultivar variation also depended on the harvest date. For example, the within-cultivar variance for NDF content was lower for the harvest on 29 Sept 1995 than for the harvest on 12 July 1995 (Table 2).

Phenotypic and genetic correlation coefficients for digestibility, NDF, ADF, and ADL contents exceeded 0.90 (not shown). Correlations for each harvest between forage yield and NDF content (Table 3) were low but significant except for the 21 May 1997 harvest. Genetic correlations at the genotype-within-cultivar level were similar to phenotypic correlations. Correlations between LSR and NDF content were highly negative in each harvest, indicating that a large part of the genetic variation for NDF content was associated with variation for LSR. Correlations between LSR and forage yield were significant but low. ADLcw was weakly correlated with both NDF content and forage yield.

The harvest date  $\times$  cultivar interaction and the harvest date  $\times$  genotype-within-cultivar interaction were significant for all traits (data not shown). These variances contributed to reduced heritabilities relative to those calculated for each individual harvest (Table 3). In the combined analysis of variance, the cultivar effect was not significant for dry matter yield. Across the six harvest dates, correlations between forage yield and NDF content were significant but low (Table 3), especially at the genotype level. LSR was strongly correlated to NDF content.

## DISCUSSION

Large within-cultivar genetic variation was observed for quality traits, dry matter yield, and morphological traits. The within-cultivar variation for quality traits was generally as high as the among-cultivar variation, though this sample of cultivars represented a wide range of geographic origins and breeding eras.

**Table 2.** Mean, among-cultivar ( $\sigma_c^2$ ), within-cultivar ( $\sigma_w^2$ ) and residual ( $\sigma_R^2$ ) variances and their standard errors, and heritability ( $H^2$ ) for morphological (leaf to stem ratio [LSR], forage yield per m<sup>2</sup>, number of stems per m<sup>2</sup> and stem height) and quality traits (NDF and ADL contents, digestibility [Dig] and lignin in the cell wall [ADLcw]) in six harvest dates, and across all harvest dates. Units are indicated for the means.

Harvest date	NDF	ADL	Dig	ADLcw	LSR	Yield	No. stems	Height
	%					g/m <sup>2</sup>	no. m <sup>-2</sup>	cm
<b>12 July 1995</b>								
Mean	40.4	7.0	65.0	17.3	1.15	347		52.6
$\sigma_c^2$	2.05 (0.68)	0.08 (0.03)	1.39 (0.45)	0.04 (0.02)	0.020 (0.006)	1 652 (1 544)		16.0 (8.7)
$\sigma_w^2$	4.40 (0.55)	0.17 (0.02)	2.79 (0.37)	0.11 (0.02)	0.019 (0.003)	27 712 (3 560)		82.6 (12.0)
$\sigma_R^2$	3.12 (0.27)	0.16 (0.01)	2.36 (0.20)	0.14 (0.01)	0.020 (0.002)	11 664 (904)		69.6 (2.3)
$H^2$	0.67	0.61	0.64	0.51	0.66	0.72		0.59
<b>29 Sept. 1995</b>								
Mean	31.2	4.5	74.1	14.5	1.88	197	582	31.7
$\sigma_c^2$	1.44 (0.56)	0.07 (0.02)	1.16 (0.40)	0.14 (0.05)	0.007 (0.003)	0 (215)	77 (1 460)	20.0 (8.8)
$\sigma_w^2$	1.68 (0.26)	0.07 (0.01)	1.18 (0.20)	0.13 (0.03)	0.008 (0.002)	7 840 (1 136)	42 572 (6 164)	26.1 (4.5)
$\sigma_R^2$	2.04 (0.19)	0.09 (0.01)	1.90 (0.18)	0.29 (0.03)	0.018 (0.002)	5 984 (463)	34 268 (2 652)	38.1 (2.9)
$H^2$	0.60	0.61	0.55	0.48	0.45	0.57	0.55	0.55
<b>30 May 1996</b>								
Mean	44.1	7.6	61.5	17.1	0.70	541	432	78.0
$\sigma_c^2$	2.29 (0.69)	0.11 (0.03)	1.56 (0.47)	0.08 (0.03)	0.014 (0.005)	751 (3 928)	0 (1 056)	23.4 (12.6)
$\sigma_w^2$	2.29 (0.36)	0.10 (0.01)	1.62 (0.27)	0.09 (0.01)	0.014 (0.003)	115 864 (14 488)	36 780 (5 036)	120.2 (16.9)
$\sigma_R^2$	3.03 (0.26)	0.10 (0.01)	2.58 (0.22)	0.11 (0.01)	0.030 (0.003)	41 876 (3 268)	21 918 (1 700)	91.2 (7.1)
$H^2$	0.60	0.68	0.55	0.60	0.48	0.74	0.63	0.61
<b>4 July 1996</b>								
Mean	37.7	6.3	66.7	16.6	0.95	366	636	59.2
$\sigma_c^2$	1.35 (0.52)	0.07 (0.03)	1.30 (0.46)	0.06 (0.03)	0.011 (0.004)	983 (2 216)	2 687 (3 856)	45.9 (22.5)
$\sigma_w^2$	2.27 (0.51)	0.07 (0.02)	1.45 (0.37)	0.08 (0.02)	0.007 (0.002)	58 175 (7 212)	94 136 (12 224)	112.5 (15.8)
$\sigma_R^2$	6.13 (0.56)	0.29 (0.03)	4.69 (0.42)	0.28 (0.03)	0.034 (0.003)	22 525 (1 740)	41 941 (3 260)	92.2 (7.2)
$H^2$	0.37	0.33	0.37	0.34	0.35	0.72	0.70	0.63
<b>10 Oct. 1996</b>								
Mean	37.1	5.7	66.4	15.4	1.23	341	583	52.6
$\sigma_c^2$	1.43 (0.65)	0.09 (0.04)	1.25 (0.54)	0.15 (0.06)	0.009 (0.003)	953 (1 468)	4 198 (2 892)	22.0 (10.9)
$\sigma_w^2$	1.54 (0.28)	0.07 (0.01)	1.13 (0.22)	0.13 (0.03)	0.012 (0.002)	27 490 (3 880)	39 116 (6 248)	30.5 (4.6)
$\sigma_R^2$	2.95 (0.27)	0.14 (0.01)	2.38 (0.22)	0.27 (0.03)	0.022 (0.002)	23 457 (1 876)	50 720 (4 040)	34.3 (2.7)
$H^2$	0.50	0.53	0.50	0.51	0.49	0.55	0.46	0.60
<b>21 May 1997</b>								
Mean	43.1	7.1	63.6	16.5	0.69	528	445	74.4
$\sigma_c^2$	3.34 (1.38)	0.15 (0.06)	2.63 (1.07)	0.14 (0.05)	0.018 (0.008)	6 504 (5 560)	13 208 (5 908)	33.6 (15.9)
$\sigma_w^2$	3.31 (0.55)	0.12 (0.02)	2.46 (0.42)	0.12 (0.02)	0.008 (0.003)	77 156 (12 388)	44 376 (6 848)	69.0 (14.1)
$\sigma_R^2$	5.00 (0.47)	0.19 (0.02)	4.11 (0.39)	0.19 (0.02)	0.037 (0.003)	100 079 (8 252)	55 417 (4 492)	157.5 (12.6)
$H^2$	0.57	0.59	0.50	0.58	0.41	0.46	0.51	0.39
<b>All harvest dates</b>								
Mean	39.1	6.4	66.1	16.3	1.09	385	537	57.8
$\sigma_c^2$	1.44 (0.36)	0.08 (0.02)	1.12 (0.26)	0.07 (0.02)	0.009 (0.003)	0 (1 348)	1 264 (3 280)	17.8 (8.8)
$\sigma_w^2$	1.89 (0.24)	0.07 (0.01)	1.28 (0.17)	0.07 (0.01)	0.010 (0.001)	42 468 (5 080)	50 221 (5 560)	66.2 (7.5)
$\sigma_R^2$	4.36 (0.16)	0.18 (0.01)	3.71 (0.13)	0.23 (0.01)	0.033 (0.001)	33 560 (1 080)	40 656 (1 440)	85.5 (2.7)
$H^2$	0.38	0.39	0.35	0.31	0.34	0.48	0.53	0.45

The within-cultivar variance accounted for 31 to 70% of the total genetic variance ( $\sigma_c^2 + \sigma_w^2$ ) for quality traits (including LSR), depending on the trait and harvest date. The within-cultivar contribution was 92 to 100%

for dry matter yield, 77 to 100% for stem number, and 57 to 84% for stem height. Similarly, Heinrichs et al. (1969), in a greenhouse experiment with five populations and 41 to 92 plants per population, found within-

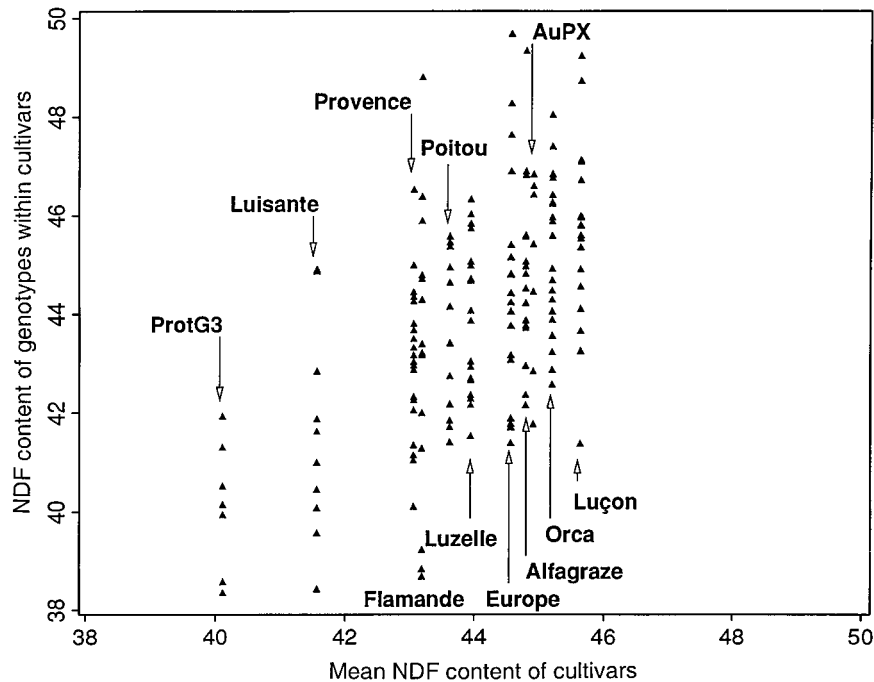


Fig. 1. Within- and among-cultivar variation for NDF content (%) observed for the harvest on 30 May 1996, for 7 to 20 genotypes per cultivar and 11 cultivars. Each value represents the average of three plots of five clones per genotype.

population variances ranging from 73 to 88% of the total genetic variance for leaf and stem dry matter and from 51 to 97% for digestibility and crude fiber.

For non-directly selected traits such as digestibility or fiber content, the large within-cultivar variation is consistent with calculations made with random amplified polymorphic DNA (RAPD) markers by Crochemore et al. (1996) that the within-population variance accounted for 50% of the total genetic variance among 26 alfalfa populations and 30 plants per population. Similarly, Dehghan-Shoar et al. (1997) and Ghérardi et al. (1998) found large within-population variation using RAPD markers in alfalfa. For the selected morphological and yield traits, within-cultivar variation was greater than for quality traits. High yielding cultivars are those that perform well in all environments and across all harvests. Rotili (1979) stated that breeding for higher yield in alfalfa required higher homogeneity of cultivars. On the contrary, our experiment showed that recent cultivars are highly heterogeneous. In fact, the genotypes contributing to forage yield may not be the same under all conditions. Indeed competition in an alfalfa

canopy selects genotypes which are expected to be the most vigorous (Rotili, 1979) and to contribute the most to forage yield. Large within-cultivar variation for yield and morphological traits may be needed to achieve high yield under various environmental conditions. This may relate to the need to avoid inbreeding depression for growth and yield by incorporating a broad genetic base in cultivars (Gallais, 1992). Conversely, breeding for high quality would appear to require a high proportion of plants improved for a specific quality trait.

The large within-cultivar variation for digestibility and fiber content should be used in breeding programs to maximize genetic progress per breeding cycle. This implies individual evaluation and selection of plants. In alfalfa, three possibilities can be considered: (i) individual evaluation of forage quality on spaced plants in a nursery, (ii) cloning of individual plants by stem cuttings and evaluation of the clones planted in a replicated experimental design in a nursery, and (iii) progeny tests. The first two possibilities require a high correlation between quality traits measured on spaced plants and those measured in a dense canopy. This has been ob-

Table 3. Range of correlation coefficients between NDF content, cell wall ADL (ADLcw), leaf-to-stem ratio (LSR) and forage yield, for 6 harvest dates (12 July 1995, 29 Sept. 1995, 30 May 1996, 4 July 1996, 10 Oct. 1996, 21 May 1997), and correlations across harvest dates (*italic*). Phenotypic correlations appear above the diagonal, and genetic correlations appear below.

	NDF	ADLcw	LSR	Yield
NDF		0.23**/0.48*** <i>0.74***</i>	-0.64***/-0.78*** <i>-0.89***</i>	0.14 NS/0.55*** <i>0.47***</i>
ADLcw	0.10 NS/0.50*** <i>0.22***</i>		-0.00NS/-0.40*** <i>-0.67***</i>	-0.06 NS/0.41*** <i>0.35***</i>
LSR	-0.62***/-0.82*** <i>-0.68***</i>	-0.12 NS/0.33*** <i>-0.02 NS</i>		-0.09 NS/-0.44*** <i>-0.47***</i>
Yield	0.01 NS/0.60*** <i>0.31***</i>	-0.09 NS/0.47*** <i>0.23***</i>	-0.15 NS/-0.62*** <i>-0.36***</i>	

\*\* , \*\*\* Significant at the 0.01 and 0.001 probability levels, respectively.

served on a cultivar-mean basis (B. Julier, 1997, unpublished data). The first possibility also implies a low within-nursery variation. The second possibility delays observations by 1 yr, the time required for cloning. It could also introduce bias against genotypes recalcitrant to cloning. For the third possibility, progeny evaluation is delayed by the time required to produce and multiply progeny. Progeny evaluation can be accomplished with dense stands, i.e., under conditions closer to common agronomic practices. However, depending on the inheritance of the character, differences between progenies may be smaller than among parental plants because of common pollinator effects. These differences may then become non-significant. Shenk and Elliot (1970, 1971) showed the high efficiency of clonal selection on a digestibility trait. However, breeding for feeding value must be accompanied by other traits such as forage yield, seed yield, disease and pest resistances, winter resistance, and fall dormancy. Coors et al. (1986) proposed a breeding scheme taking into account forage quality and agronomic traits where healthy vigorous individual plants in a nursery were identified, and their protein content measured. On the plants with the highest protein content, ADF content was also measured. This method should enable the identification of superior parents for synthetic cultivars.

Choice of the most efficient method requires further investigation of the environmental effect on digestibility, the relationships between spaced plants and dense stands, and the inheritance of quality traits. Rates of success of breeding for highly digestible cultivars would be greater if the trait is additively inherited. Gil et al. (1966) found that general combining ability effects were much more important than specific combining ability effects for digestibility, indicating mainly additive inheritance.

Correlations between dry matter yield and NDF were moderately positive, ranging from 0.14 to 0.55 at the phenotypic level, and from 0.01 to 0.60 at the genetic level, when analyzed by harvest date. These values indicate the possibility of combining high yield and high quality in one genotype.

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