

## Diversity, genetic structure, distinctness and agronomic value of Italian lucerne (*Medicago sativa* L.) landraces

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### Summary

Eleven landraces belonging to the seven commercial ecotypes of northern Italy, and seven elite varieties, were evaluated with the aims of: (i) comparing landrace and variety germplasm for dry weight (DW, recorded across three harvests spanning over two years) and morphophysiological characteristics; (ii) estimating among-population ( $s_A^2$ ) and within-population ( $s_W^2$ ) genetic variances of landrace and variety germplasms; (iii) investigating the inter-relationships among traits at the plant and the population levels; (iv) exploring patterns of phenotypic diversity, and the relationships between diversity and environmental variables at landrace collecting sites; and (v) assessing the distinctness of ecotypes. Some 256 or 512 genotypes per landrace, and 128 genotypes per variety, were grown in dense stand in an unreplicated design together with a replicated clone. Compared with varieties, landrace material showed higher total DW (+9%) and DW at the last harvest (+43%), lower (2.5-fold) summer mortality and autumn dormancy ( $P < 0.01$ ), and similar winter mortality. Landraces exhibited a greater  $s_W^2$  value than varieties for all traits except autumn dormancy and number of florets per inflorescence ( $P < 0.01$ ), with  $s_W^2$  always exceeding  $s_A^2$  ( $P < 0.01$ ). The ratio of  $s_W^2$  to  $s_A^2$  averaged 23.1 for landraces and 6.5 for varieties across eight traits. Frequency of mowing at collecting sites was associated with higher total DW, better persistence, lower summer and winter mortality, larger leaflets and more stems per plant ( $r \geq 0.66$ ,  $P < 0.05$ ). Cluster analysis showed that landraces geographically close or belonging to the same commercial ecotype tended to greater similarity. However, one landrace that evolved under very frequent mowing differed greatly from other landraces of the same ecotype. Based on discriminant analysis, two ecotypes may be sufficiently distinct for registration in a *sui generis* system, even though they may not meet the criteria for registration under a homegenity requirement.

### Introduction

Cultivated lucerne (*Medicago sativa* L. subsp. *sativa*,  $2n = 4x = 32$ ) was introduced from the Middle East to Italy around the 2nd century BC, where it became the main fodder crop during the Imperial Age (Columella, 1846). After disappearing during the Barbarian invasions, lucerne was reintroduced to Italy from Spain in the 16th century (Prosperi et al., 1995). Currently, lucerne is largely grown in northern Italy, where it is the second most-grown crop after maize. Its importance in Europe is expected to continue, owing to its contribution to sustainable agriculture and its productivity of

feed proteins per unit area that is the greatest among the forage or grain legumes (Huyghe, 2003).

Until recently, most of the Italian seed market of lucerne was represented by landraces (Sommovigo et al., 1999) because of their low seed price and their allegedly good agronomic performance. Depending on their area of production, farm landraces were grouped into 14 commercial ecotypes, seven of which were located in northern Italy (Figure 1). A limited number of farms were initially identified for each ecotype as donors of the basic seed. However, farms within each production area were subsequently allowed to multiply any seed of the local ecotype obtained from the market,

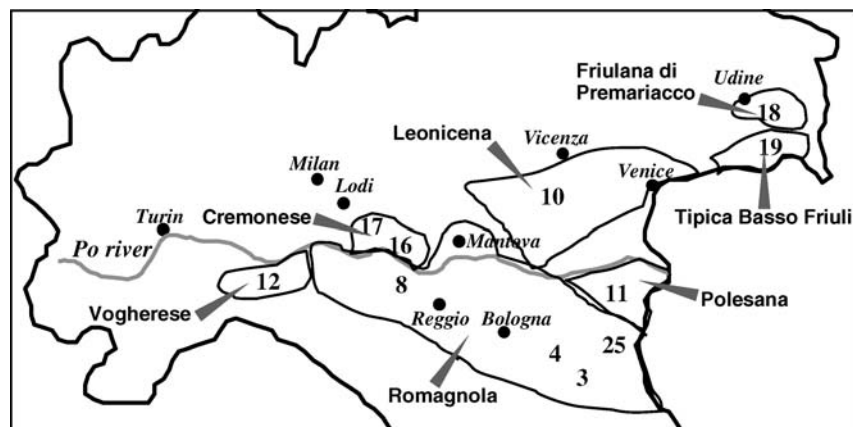


Figure 1. Site of origin of eleven landraces and production area of seven commercial ecotypes of lucerne.

to meet the seed demand. The lack of effective control on the seed origin introduced by this system contributed to the decision to ban the commercialization of any ecotype seed from 2003. Concurrently, it was recommended to register each individual ecotype as a variety in case it satisfied the requirements of distinctness and homogeneity that are contemplated by legislation (UPOV, 1988). However, crop landraces may fail to meet these requirements, especially the latter one, justifying the adoption of a *sui generis* registration system that contemplates only the distinctness requirement (Leskien & Flitner, 1997). For lucerne, one landrace from central Italy showed distinctness and homogeneity comparable with that of some varieties and was proposed, therefore, for variety registration (Falcinelli et al., 1994), but a very large number of morphophysiological traits was needed to make various landraces distinguishable from each other by discriminant analysis (Russi & Falcinelli, 1997). It is unknown whether Italian ecotypes of lucerne are sufficiently distinct and homogeneous according to standard descriptors for variety registration (UPOV, 1988), or whether they are sufficiently distinct for the possible registration in a *sui generis* system.

The ban to commercialization of lucerne landraces has also entrained a need for collecting and preserving these genetic resources, which still represent the backbone of Italian breeding (Russi & Falcinelli, 1997). A recent assessment of landraces from northern Italy across artificial environments confirmed their value especially when pursuing a specific adaptation strategy (Annicchiarico & Piano, 2005). The same study revealed the specific adaptation of these landraces to

drought stress levels and soil type that are close to those of their environment of origin. However, the relationship between morphophysiological variation and environmental variables at collecting sites has not been investigated, though it should assist in the understanding of the evolutionary aspects underlying the patterns of diversity and provide guidelines for the collection of germplasm possessing specific and desirable attributes (Frankel, 1989).

Lucerne is a basically outcrossing species, owing to various mechanisms that strongly limit the self-fertilization (Viands et al., 1988). Information on the genetic structure of populations for adaptive and yield traits has noticeable implications on the strategies for germplasm collection, conservation and exploitation (Frankel & Hawkes, 1975). Estimates of within-population and among-population variances have been reported by Julier et al. (2000) for forage yield and quality and by Bolaños-Aguilar et al. (2000) for seed yield traits of lucerne varieties, highlighting a trend towards larger variation within populations. Evidence of larger within-population than among-population variation has also emerged for landraces in studies based on molecular markers although only few populations have been studied (Pupilli et al., 1996, 2000; Zaccardelli et al., 2003).

In the present study, a set of landraces belonging to the seven commercial ecotypes of northern Italy and several elite commercial varieties were evaluated with the objectives of: (i) comparing landrace vs. variety germplasm for forage yield and morphophysiological characteristics; (ii) investigating the genetic structure of landraces by estimating among-population

and within-population genetic variances, and comparing these variances with those relative to variety germplasm; (iii) investigating the interrelationships among traits at the plant and the population levels; (iv) exploring the patterns of phenotypic diversity among populations, and the relationship between diversity and environmental variables at collecting sites of landraces; and (v) assessing the distinctness of commercial ecotypes, concurrently verifying the discriminant ability of each of several morphophysiological descriptors.

### Materials and methods

The study comprised 11 landraces and seven varieties (Table 1) collected across northern Italy (Figure 1). Landraces 3 to 19 were collected in 1995 and 1996 from farms that had been registered as donors of the basic seed for the relevant ecotype and had been multiplying their seed for at least 20 years. Three landraces of 'Romagnola' are included due to the larger geographical production area and commercial importance of this ecotype (Figure 1). Two accessions of 'Cremonese' are included to further verify the extent of variation between farm landraces of the same ecotype. Accession 25 of 'Romagnola' was derived by pooling equal proportions of seed from two farms located 15 km apart that occasionally shared seed for multiplication. The control varieties were chosen among the best-performing entries in recent trials for admission to the Italian Register of Varieties and include locally recommended varieties (Tabaglio et al., 2004).

Some 512 genotypes for each of the landraces 8, 17 and 19, 256 genotypes for each of the other landrace accessions, and 128 genotypes for each control variety were randomly obtained from seed of the populations. The seedlings were grown in jiffy pots for two months before transplanting into a field in May 2002. Some 128 clones, obtained from vegetative multiplication of a single genotype and previously rooted in jiffy pots, were concurrently transplanted. This material, used for estimating the environmental variance, was selected for homogeneity prior to transplanting and showed for all traits a response in the range of those recorded for the test material during the trial. The experiment lay-out is described by Annicchiarico (2005). The 4480 unreplicated genotypes and 128 clonal controls were randomly subdivided into 128 different units (grids) of 36 plants each. Each unit included a fixed number of genotypes from each population, namely: one clonal control, four genotypes for each of the

landraces 8, 17 and 19, two genotypes for each of the other eight landraces, and one genotype for each of the seven varieties. The units had a square shape, with plants grown at 10 cm spacing between and within rows. This design allows plant competition, and aims to increasing the reliability of the agronomic assessment, given the moderate to low consistency of genotype response for yield traits between spaced and dense planting (Rotili & Zannone, 1975; Riday & Brummer, 2004). In the field, the 128 units were arranged in four parallel columns of 32 adjacent units each. The relative homogeneity of plant material between units allowed for investigating major spatial trends for quantitative traits and readjusting the data for the effects of: i) column of units; ii) row of units (by covariance analysis, using row number as the covariate); and iii) column of plants within units (Annicchiarico, 2005).

The experiment was carried out in Lodi (northern Italy) on a sandy-loam soil under rainfed conditions. It was mown three times in 2002 and four times in 2003, at intervals of six weeks between harvests 1 and 2 and four weeks between other harvests. The first harvests took place on July 11 in the first year and May 7 in the second year. All traits were recorded on individual plants. Dry weight (DW) was measured at harvests 2, 4 and 7. Lateness of flowering was assessed at harvest 2 by the visual score of first reproductive nodes proposed by Kalu and Fick (1981), modified as follows: 1 = ripe seed pods; 2 = unripe seed pods; 3 = open flowers; 4 = floral buds; 5 = vegetative. Length of the main stem, number of stems per plant, length and width of the central leaflet (observed if possible on the third leaf below the first reproductive node), and number of florets per inflorescence were also recorded at harvest 2. Plant height was recorded in autumn 2002 (25 days after clipping) and at the onset of spring 2003 to estimate of the autumn dormancy (UPOV, 1988; Rotili et al., 1999). Flower colour, observed at harvest 2 according to Barnes (1972), was used to assess the proportions of variegated and deep purple flowers of populations (UPOV, 1988). Plant mortality of populations was assessed from the difference in plant number between spring 2002 and autumn 2002 for summer mortality, and between autumn 2002 and early spring 2003 for winter mortality. The level of cold stress in winter 2003 was near the long-term site average, with 54 frost days and  $-7.8^{\circ}\text{C}$  as the absolute minimum temperature.

Leaflet dimension as provided by leaflet length  $\times$  width, and autumn-winter growth as average of autumn

Table 1. Mean value of twelve morphophysiological traits for landraces belonging to seven commercial ecotypes and for varieties of lucente

Ecotype/Variety	Landrace accession no.	Total dry weight (g/plant)	Dry weight last harvest (g/plant)	Lateness of flowering (score 1-5)	Autumn-Winter growth (cm)	Main stem length (cm)	No. stems/plant	Leaflet length $\times$ width (cm <sup>2</sup> )	No. florets/inflorescence	Winter mortality (%)	Summer mortality (%)	Variiegated flowers (%)	Deep purple flowers (%)
Romagnola	3	6.89 b	0.45 b	2.6 a	11.7	56.2 b	4.50 b	4.32 b	16.4 ab	16.0 a	25.1	11.9 a	5.9 b
Romagnola	4	7.71 b	0.53 b	2.6 a	11.8	57.7 ab	4.75 b	4.52 b	16.8 ab	19.9 a	22.4	9.4 a	15.3 a
Romagnola	8	9.36	0.62 b	2.0 b	12.5	59.6 a	5.45	4.62 b	17.7 ab	15.6 a	12.7	10.0 a	7.3 ab
Leonicensa	10	10.67 a	1.35 a	1.7 b	14.1	57.1 ab	7.08 a	5.16 a	19.0 a	3.9 b	2.4 b	9.8 a	5.6 b
Polesana	11	8.72 b	0.77	2.2 a	12.4	57.4 ab	6.02	4.82 a	16.9 ab	9.0 ab	6.0 b	2.4 b	9.4 a
Vogherese	12	9.52 a	0.81	2.0 b	12.8	57.1 ab	6.12	4.44 b	16.9 ab	15.2 a	6.0 b	5.6 ab	11.9 a
Cremonese	16	10.29 a	1.01	1.8 b	13.7	58.8 a	6.00	5.07 a	17.0 ab	6.2 b	2.1 b	8.9 ab	7.5 ab
Cremonese	17	10.22 a	1.16 a	1.8 b	12.7	57.0 ab	6.76	4.91 a	17.3 ab	4.5 b	3.7 b	6.4 ab	7.5 ab
Friulana di P.	18	9.29	0.70	2.5 a	11.9	57.9 ab	4.99 b	4.73 b	16.5 ab	9.4 ab	12.9	15.0 a	2.0 b
Tipica Basso Friuli	19	11.02 a	0.80	2.2 a	12.2	59.3 a	6.50	4.87 a	17.2 ab	11.3 a	11.2	6.1 ab	6.5 ab
Romagnola	25	10.91 a	1.21 a	2.2 a	12.4	54.5 b	7.98 a	4.80 ab	17.9 ab	2.3 b	4.0 b	8.1 ab	9.0 a
Classe	-	10.02 a	0.63 b	2.2 a	12.5	60.0 a	5.84	4.81 ab	18.4 ab	7.8 b	13.6	0.0 b	15.5 a
Colosseo	-	7.46 b	0.51 b	1.8 b	16.1 a	61.9 a	5.55	4.74 ab	17.2 ab	18.0 a	19.0	0.0 b	0.0 b
Eietta	-	6.64 b	0.42 b	2.8 a	7.7 b	56.9 b	6.76	4.07 b	17.9 ab	11.7 a	63.7 a	8.3 ab	0.0 b
Gamma	-	11.04 a	0.98	2.1 ab	11.4	58.0 ab	8.02 a	4.58 b	15.1 b	4.7 b	9.0 b	1.5 b	4.5 b
PR57N02	-	7.67 b	0.55 b	2.5 a	10.8	56.4 b	6.70	3.94 b	17.0 ab	9.4 ab	30.2	0.0 b	4.0 b
PR58N57	-	8.75 b	0.56 b	1.8 b	13.0	59.1 a	6.56	4.31 b	15.3 b	9.4 ab	17.2	2.5 b	1.2 b
Prosementi	-	9.66 a	0.53 b	2.2 ab	10.7	59.4 a	5.87	4.43 b	19.1 a	17.2 a	19.8	11.5 a	9.6 a

Means followed by letter 'a' do not differ from the top-ranking, and means followed by letter 'b' do not differ from the bottom-ranking, based on Bonferroni's test at  $P < 0.05$ .

and late winter plant height values, were analysed in place of their individual component traits in all cases except discriminant analysis, in which they were considered as independent descriptors according to current recommendations (Rotili et al., 1999). Lateness of flowering was analysed as quantitative, since its distribution for the individual populations revealed just a slight trend towards a left-skewed and flatter distribution than the normal one. Percent data relative to plant mortality, variegated flowers or deep purple flowers were submitted to the angular transformation prior to statistical analyses.

Multiple mean comparisons between populations were performed by Bonferroni's test following a one-way analysis of variance (ANOVA), holding the variation among genotypes within population as the error term. A linear contrast compared landrace vs. variety germplasm. A one-way, random-model ANOVA was performed separately on landrace and variety germplasm groups for assessing the genetic variation among populations and within populations. The former coincided with the among-population variance component ( $s_A^2$ ), estimated from the among-population ( $M_A$ ) and the within-population ( $M_W$ ) mean squares by the formula (Snedecor & Cochran, 1967 p. 290):

$$s_A^2 = (M_A - M_W)/n',$$

where

$$n' = (1/(p - 1))(\sum n_i - \sum n_i^2 / \sum n_i)$$

where  $p$  is the number of populations and  $n_i$  is the total number of genotypes for the population  $i$ . Its significance was tested by the  $F$  ratio of  $M_A$  to  $M_W$ . The average genetic variation within population coincided with the within-population variance component ( $s_W^2$ ), estimated as the difference between  $M_W$  (average phenotypic variance within populations) and the environmental variance (estimated by the variance among the values of clones,  $s_E^2$ ). Its significance was tested by the  $F$  ratio of  $M_W$  to  $s_E^2$ . Comparisons between  $s_A^2$  and  $s_W^2$  values within germplasm groups, or between  $s_A^2$  or  $s_W^2$  values of the two groups, were performed by Fisher's bilateral  $F$  test. The within-population variance of each population was estimated as the difference between the variance among genotypes (phenotypic variance) and the environmental variance. Its value was expressed as genetic coefficient of variation ( $CV_g$ ), dividing the variance by the population mean for the trait.  $CV_g$  values of the different traits were averaged to provide

a synthetic measure of the within-population genetic variation.

Coefficients of simple correlation between traits were computed for values of populations and individual genotypes. Multivariate patterns of variation among populations were investigated by a principal components analysis performed on the correlation matrix of all the traits except plant DW (which was excluded because of the concurrent presence in the analysis of its component traits). Classification of populations was performed by cluster analysis using the scores of populations on the principal component (PC) axes as input variables. The analysis adopted the average linkage method and a squared Euclidean distance as the dissimilarity measure, as recommended by Crossa et al. (1995).

The relationship of seven environmental variables recorded at collecting sites with PC scores on the first two axis (expressing the main patterns of phenotypic diversity), individual traits or the average  $CV_g$  of landraces was assessed by simple correlation analysis. The environmental variables were: (i) the level of summer drought stress, estimated as the difference between potential evapotranspiration (ETP) and average rainfall plus irrigation over June, July and August according to long-term values of the nearest meteorological station; (ii) three soil variables, i.e. clay content, sand content and pH (in water); (iii) three management variables, i.e. average meadow duration, average no. harvests per year, and year of the crop cycle chosen more frequently to produce seed.

The distinctness of the individual ecotypes or varieties was verified by a discriminant analysis, assessing the value of discriminant functions by the proportion of misclassified observations according to a jack-knife cross-validation procedure (Afifi & Clark, 1984, p. 266). Landraces of the same commercial ecotype were pooled together in the analysis (with the exception of one accession that proved very different from all other entries of the same ecotype). The univariate comparison of varieties recommended by UPOV (1979) is based on four replicates per population that are mean values of many individual plants per plot. Likewise, the four columns of the experiment lay-out acted as replicates for computation of winter and summer mortality, proportions of deep-purple and variegated flowers, and values of the other characters (averaged across the relevant genotypes) for each material. The analysis excluded plant DW, as well as no. florets per inflorescence (which could be recorded only on a distinctly smaller subset of genotypes relative to the other traits).

A preliminary stepwise discriminant analysis was performed to verify the discriminant ability of each character. The improvement of the discriminating function caused by the progressive addition of each trait was verified by Rao's approximate  $F$  test (Afifi & Clark, 1984, p. 269).

The Statistical Analysis System (SAS, 1999) software was used for all statistical analyses.

## Results

### *Comparison of landrace vs. variety germplasm for morphophysiological traits*

Variation among populations was apparent for all traits ( $P < 0.05$ ). The mean value of total DW per plant showed by the best-yielding varieties 'Gamma', 'Classe' and 'Prosementi' was also attained by several landraces (Table 1). Landraces '10', '17' and '25' outperformed any other material for DW per plant at the last harvest (Table 1), which could be considered as an indicator of persistence. These landraces and the top-ranking variety 'Gamma' were characterized by low winter and summer mortality, intermediate autumn-winter growth, long main stem (except accession '25'), many stems per plant (except accession '17'), and large leaflets (except 'Gamma') (Table 1). The excellent agronomic performance of the landrace material was confirmed by its superiority over the variety group ( $P < 0.01$ ) for total DW (+9%) and DW at the last harvest (+43%) (Table 2). This superiority was mainly associated with much lower (2.5-fold) summer mortality (Table 2). Further, the landrace germplasm had fewer stems per plant (-7%), larger leaflets (+8%), slightly shorter main stem (-2%) and lower autumn dormancy as indicated by higher autumn-winter growth, as well as higher proportions of variegated and deep purple flowers, compared with the variety group (Table 2). No difference ( $P < 0.05$ ) between germplasm groups was noted for mean values of winter mortality, no. florets per inflorescence and lateness of flowering.

The most winter-active population was variety 'Colosseo' (Table 1), classified into Barnes et al.'s (1995) dormancy class 7 by Rotili et al. (2000), which also showed high winter mortality (Table 1). Landraces would fall into classes 4-6 of the same classification, considering that they were distinctly less winter-active than 'Colosseo' and at least as winter-active as 'Gamma' (which belongs to class 4). 'Eletta' showed

Table 2. Comparison of landrace vs. variety germplasm of lucerne for mean value of twelve morphophysiological traits

Trait	Landraces (no. = 11)	Varieties (no. = 7)	$P$ level
Total dry weight (g/plant)	9.51	8.75	**
Dry weight last harvest (g/plant)	0.86	0.60	**
Lateness of flowering (score 1-5)	2.2	2.2	NS
Autumn-Winter growth (cm)	12.6	11.7	**
Main stem length (cm)	57.5	58.8	*
No. stems/plant	6.01	6.46	**
Leaflet length $\times$ width (cm <sup>2</sup> )	4.75	4.41	**
No. florets/inflorescence	17.2	17.1	NS
Winter mortality (%)	10.3	11.2	NS
Summer mortality (%)	9.9	24.6	**
Variegated flowers (%)	8.5	3.4	**
Deep purple flowers (%)	8.0	5.0	**

NS, \*, \*\* = difference not significant and significant at  $P < 0.05$  and  $P < 0.01$ , respectively.

distinctly greater autumn dormancy and summer mortality than any other material (Table 1).

### *Among-population and within-population genetic variance of germplasm groups*

The among-population variance component ( $s_A^2$ ) was different from zero at  $P < 0.05$  within each germplasm group for all traits except number of florets per inflorescence in landraces ( $P < 0.08$ ). The within-population variance component ( $s_W^2$ ) was different from zero at  $P < 0.05$  in all cases except lateness of flowering in both germplasm groups and DW at the last harvest in varieties.

The two groups differed in  $s_A^2$  ( $P < 0.05$ ) only for number of florets per inflorescence and autumn-winter growth, for which landraces revealed narrower among-population variation (Table 3). Landraces showed relatively narrow genetic variation also within population for these two traits, for which they did not differ from varieties in  $s_W^2$  ( $P < 0.05$ ). For any other trait, landraces had a greater  $s_W^2$  than varieties (Table 3). The ratio of  $s_W^2$  to  $s_A^2$  in landraces ranged from 3.9 (DW at the last harvest) to 70.5 (no. florets per inflorescence), averaging 23.1 across traits (Table 3). The ratio was always lower in varieties, where it never exceeded 15.1 and averaged 6.5 across traits. In conclusion, the genetic variation of the landrace germplasm tended to be narrower for autumn dormancy and no. florets per inflorescence, and wider for the other traits but only at

Table 3. Comparison of landrace vs. variety germplasm of lucerne for variance among populations ( $s_A^2$ ), average variance within populations ( $s_W^2$ ) and ratio of  $s_W^2$  to  $s_A^2$ , for eight morphophysiological traits

Trait	$s_A^2$ a,b			$s_W^2$ a,c			$s_W^2/s_A^2$ ratio <sup>d</sup>	
	Landraces	Varieties	<i>P</i> level	Landraces	Varieties	<i>P</i> level	Landraces	Varieties
Total dry weight (g/plant) <sup>2</sup>	1.00	1.92	NS	25.63	21.37	**	25.6**	11.1**
Dry weight last harvest (g/plant) <sup>2</sup>	0.07	0.04	NS	0.28	0.02	**	3.9**	0.6 NS
Lateness of flowering (score 1–5) <sup>2</sup>	0.06	0.06	NS	0.46	0.19	**	7.5**	3.1 NS
Autumn-Winter growth (cm <sup>2</sup> )	0.35	3.92	**	6.79	6.75	NS	19.5**	1.7 NS
Main stem length (cm <sup>2</sup> )	2.05	1.72	NS	39.43	26.07	**	18.8**	15.1**
Stems/plant (no. <sup>2</sup> )	0.74	0.83	NS	7.61	5.55	**	10.3**	6.6*
Leaflet length × width (cm <sup>2</sup> ) <sup>2</sup>	0.04	0.08	NS	1.09	0.71	**	28.8**	9.0**
Florets/inflorescence (no. <sup>2</sup> )	0.15	2.05	**	10.58	9.31	NS	70.5**	4.5*

<sup>a</sup>NS, \*, \*\* = Difference between germplasm groups not significant and significant at  $P < 0.05$  and  $P < 0.01$ , respectively, based on Fisher's bilateral test.

<sup>b</sup>DF are 10 for landraces and 6 for varieties.

<sup>c</sup>DF are 1303 for no. florets/inflorescence and  $\geq 2858$  for other traits of landraces, and 318 for no. florets/inflorescence and  $\geq 591$  for other traits of varieties.

<sup>d</sup>NS, \*\* = Difference between  $s_W^2$  and  $s_A^2$  not significant and significant at  $P < 0.05$  and  $P < 0.01$ , respectively, based on Fisher's bilateral test.

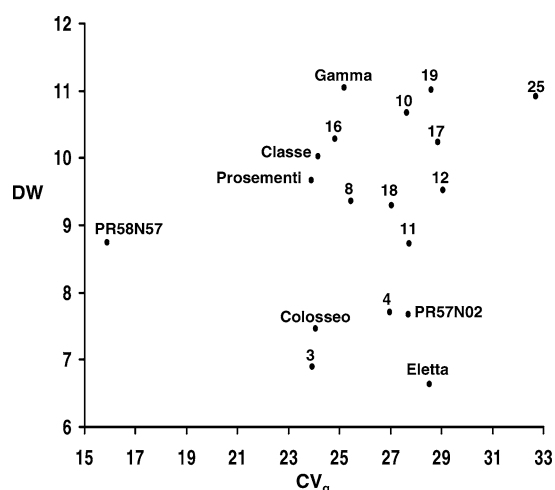


Figure 2. Scatter plot of total dry weight (DW, g/plant) and genetic coefficient of variation averaged across eight traits ( $CV_g$ ) for eleven landraces (indicated by accession number) and seven varieties of lucerne (see Figure 1 for site of origin of landraces).

the within-population level, compared with the variety germplasm.

For each population, the average value of eight genetic coefficients of variation (relative to the traits listed in Table 3) is reported in Figure 2 together with the mean value of total DW per plant. Both the landrace and the variety groups varied widely in within-population variation. A few landraces, such as the accessions '3' and '16', showed intra-population variation in the range

of those of most varieties, whereas accession '25' revealed outstanding variation.

Figure 2 could be used for locating germplasm that combines high DW per plant with wide intra-population variation, which is of special interest as genetic resource in breeding programs. The most interesting material in this respect was represented by some landraces, i.e., the accessions '25', '19', '10' and '17'.

#### Interrelationships among traits

Total DW and DW at the last harvest were correlated both across populations and across individual genotypes ( $r \geq 0.67$ ,  $P < 0.01$ ), and were closely related to winter and summer mortality of populations ( $r \geq 0.64$ ,  $P < 0.01$ ; Table 4). Greater total and last-harvest yields of genotypes were associated ( $P < 0.01$ ) with lower autumn dormancy (greater autumn-winter growth), more stems per plant, longer main stem, larger leaflets, and earlier flowering (Table 4). These relationships tended to be confirmed, at least as a trend, at the population level, with the exception of those of forage yield traits with main stem length (Table 4). No trade-off across populations or across genotypes emerged either among the components of forage yield (main stem length, no. stems per plant and leaflet size), or between no. florets per inflorescence and any forage yield trait or trait component (Table 4).

Populations with fewer stems per plant showed a distinct trend towards higher winter mortality, whereas

Table 4. Correlation coefficients for morphophysiological traits of populations (upper triangle) and individual genotypes (lower triangle) of lucerne

Trait	Total dry weight	Dry weight last harvest	Lateness of flowering	Autumn-Winter growth	Main stem length	No. stems/plant	Leaflet length × width	No. florets/inflorescence	Winter mortality	Summer mortality
Total dry weight	–	0.78**	–0.56*	0.29	0.00	0.54*	0.68**	0.13	–0.64**	–0.78**
Dry weight last harvest	0.67**	–	–0.55*	0.36	–0.36	0.62*	0.73**	0.14	–0.81**	–0.80**
Lateness of flowering	–0.38**	–0.26**	–	–0.74**	–0.41	–0.32	–0.59**	–0.03	0.32	0.69**
Autumn-Winter growth	0.59**	0.41**	0.35**	–	0.43	–0.10	0.64**	–0.03	–0.08	–0.67**
Main stem length	0.49**	0.26**	–0.42**	0.38**	–	–0.38	0.19	0.03	0.45	0.03
No. stems/plant	0.52**	0.42**	–0.19**	0.24**	0.10**	–	0.10	0.00	–0.76**	–0.23
Leaflet length × width	0.28**	0.24**	–0.05**	0.21**	0.21**	0.06**	–	0.25	–0.45	–0.80**
No. florets/inflorescence	0.21**	0.13**	–0.09**	0.12**	0.24**	0.04	0.18**	–	0.00	0.01
Winter mortality	–	–	–	–	–	–	–	–	–	0.50*

\*, \*\* = Significant at  $P < 0.05$  and  $P < 0.01$ , respectively. DF are 16 for populations, 1633 for no. florets/inflorescence of genotypes, and  $\geq 3465$  for other traits of genotypes.

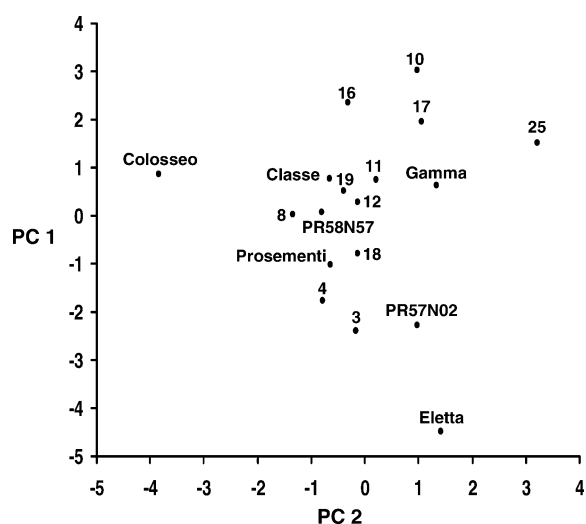


Figure 3. Ordination of eleven landraces (indicated by accession number) and seven varieties of lucerne in the space of the first two axes (PC) of a principal components analysis performed on ten morphophysiological traits (see Figure 1 for site of origin of landraces).

those more dormant or with smaller leaflets tended to higher summer mortality (Table 4).

#### Ordination and classification of populations

In Figure 3, PC 1 accounted for 35% of the total variation and, based on its largest trait eigenvectors (reported into parentheses), was mainly an indicator of lower summer mortality (–0.50), greater autumn and winter

activity (0.41), reduced lateness of flowering (–0.45) and larger leaflets (0.46). PC 2, accounting for 21% of the total variation, mainly indicated lower winter mortality (–0.49), shorter main stem (–0.60), more stems per plant (0.45), and lower autumn and winter activity (–0.35). As implied by the meaning of these main patterns of diversity and by previous correlation results for populations (Table 4), PC 1 was correlated positively ( $P < 0.01$ ) with mean values of total DW ( $r = 0.77$ ) and DW at the last harvest ( $r = 0.80$ ) of populations, and PC 2 was correlated with DW at the last harvest ( $r = 0.54$ ,  $P < 0.05$ ). Two varieties, ‘Eletta’ and ‘Colosseo’, and accession ‘25’, were quite distinct from any other entry (Figure 3). Accession ‘25’ was also markedly different from any other landrace belonging to its commercial ecotype ‘Romagnola’ (i.e., the accessions ‘3’, ‘4’ and ‘8’), as well as from any variety known to derive from landraces of ‘Romagnola’ (i.e., ‘Classe’ and ‘Prosementi’). The expected similarity between the two accessions ‘16’ and ‘17’ of the ecotype ‘Cremonese’ was confirmed by the analysis (Figure 3).

Classification analysis (Figure 4) confirmed the phenotypic distance of ‘Eletta’ and ‘Colosseo’ from any other population, and suggested the classification of the remaining entries into three groups based on their similarity. The first group included the Pioneer varieties ‘PR57N02’ and ‘PR58N57’ and variety ‘Gamma’. The second comprised the landraces of the two ecotypes originated from the Po Valley north to the Po river, i.e. ‘Cremonese’ (accessions ‘16’ and ‘17’) and ‘Leonicena’ (‘10’), as well as the accession ‘25’ of



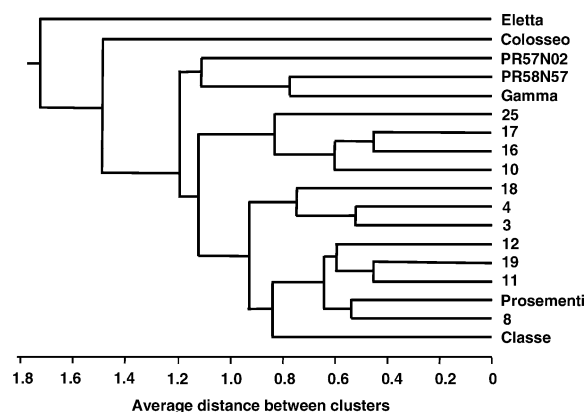


Figure 4. Classification of eleven landraces (indicated by accession number) and seven varieties of lucerne based on cluster analysis of eight morphophysiological traits (see Figure 1 for site of origin of landraces).

'Romagnola'. The last group included all the remaining accessions of 'Romagnola' ('3', '4' and '8'), the two varieties derived from this ecotype, the other ecotypes originated in the Po Valley south to the Po river, i.e. 'Polesana' ('11') and 'Vogherese' ('12'), and the accessions of the north-eastern ecotypes 'Friulana di Premariacco' ('18') and 'Tipica Basso Friuli' ('19').

#### Relationships between diversity and collecting variables of landraces

Significant ( $P < 0.05$ ) correlations for environmental variables at collecting sites can be summarized as follows. The landrace ordination on PC 1 was associated only with the average no. harvests ( $r = 0.77$ ,  $P < 0.01$ ). Landraces that evolved under more frequent mowing also tended to greater agronomic value as indicated by higher total DW ( $r = 0.66$ ,  $P < 0.05$ ), better persistence ( $r = 0.68$ ,  $P < 0.05$ ), lower summer ( $r = -0.75$ ,  $P < 0.01$ ) and winter ( $r = -0.73$ ,  $P < 0.05$ ) mortality, larger leaflets ( $r = 0.82$ ,  $P < 0.01$ ) and more stems per plant ( $r = 0.70$ ,  $P < 0.05$ ). Materials evolved in soils with higher sand content tended to higher plant DW ( $r = 0.69$ ,  $P < 0.05$ ) and lower winter mortality ( $r = -0.69$ ,  $P < 0.05$ ) in the sandy-loam soil of the evaluation environment. Finally, landraces from drier collecting sites tended to smaller size of leaflets ( $r = -0.61$ ,  $P < 0.05$ ). No relationship with collecting variables emerged for landrace ordination on PC 2 or the measure of intra-population variation.

Table 5. Approximate  $F$  test and average squared canonical correlation (ASCC) for morphophysiological traits selected in a stepwise fashion by a discriminant analysis applied to lucerne materials

Trait	$F$ value	$P$ level	ASCC
Autumn growth	31.54	0.01	0.064
Summer mortality	19.36	0.01	0.124
Deep purple flowers	5.07	0.01	0.166
No. stems/plant	5.68	0.01	0.203
Winter growth	5.76	0.01	0.225
Variegated flowers	4.75	0.01	0.257
Leaflet length	2.84	0.01	0.289
Lateness of flowering	2.36	0.05	0.318
Winter mortality	1.87	0.10	0.338
Leaflet width	1.61	0.15	0.361
Main stem length	1.12	0.40	–

Lucerne materials are listed in Table 6; observations are four replicates per material.

#### Discrimination of ecotypes and varieties

The ecotype 'Romagnola' was represented by landraces '3', '4' and '8' in the discriminant analysis, keeping accession '25' separated because of its sharp dissimilarity from the other 'Romagnola' entries as revealed by ordination and classification results.

The order of selection of the traits in the stepwise discriminant analysis (Table 5) provides an indication of the value of each trait for discrimination of the fifteen populations (seven ecotypes, seven varieties and accession '25'). Autumn and winter growth, summer mortality, no. stems per plant and proportions of deep purple and variegated flowers were the most useful characters. Main stem length made the smallest contribution to discrimination, improving it only at  $P < 0.40$  in the presence of the other traits. Therefore, it was excluded from the final analysis. Table 5 also reports the effect of variable selection on the average squared canonical correlation value (which approaches the unity value when all groups are well-separated in the discriminant space).

The cross-validation results relative to discriminant functions are given in Table 6. Two varieties, 'Colosseo' and 'PR58N57', were perfectly distinct (0% misclassification), whereas 'Gamma' and 'Prosementi' showed rather modest distinctness (50% misclassification). Among the ecotypes, 'Cremonese' was perfectly distinct and 'Vogherese' moderately distinct (25% misclassification), whereas the other material revealed

Table 6. Percent of misclassification of discriminant functions for commercial ecotypes or varieties of lucerne based on ten descriptors, according to a jackknife cross-validation procedure

Material <sup>a</sup>		Observations classified into groups															Misclassified (%)
No.	Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1	Romagnola	2	0	0	0	0	0	1	1	0	0	0	0	0	0	0	50
2	Landrace no. 25	0	3	0	0	1	0	0	0	0	0	0	0	0	0	0	25
3	Cremonese	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
4	Leonicena	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	50
5	Polesana	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	75
6	Vogherese	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	25
7	Friulana di P.	1	0	0	0	1	0	2	0	0	0	0	0	0	0	0	50
8	Tipica Basso Friuli	1	0	0	0	2	0	1	0	0	0	0	0	0	0	0	100
9	Classe	0	0	0	0	1	0	0	0	3	0	0	0	0	0	0	25
10	Colosseo	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0
11	Eletta	0	0	0	0	0	0	0	0	0	0	3	0	1	0	0	25
12	Gamma	0	0	0	0	1	0	0	0	0	0	0	2	1	0	0	50
13	PR57N02	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	25
14	PR58N57	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
15	Prosementi	0	0	0	0	0	1	0	1	0	0	0	0	0	0	2	50

<sup>a</sup>'Romagnola' includes landraces no. 3, 4 and 8; 'Cremonese' includes landraces no. 16 and 17. Selected descriptors are listed in Table 5; observations are four replicates per material.

modest ('Romagnola', 'Leonicena', 'Friulana di Premariacco') to low distinctness ('Polesana' and 'Tipica Basso Friuli'). Poorly distinct material also complicated the classification of the other groups. For instance, seven observations from other entries were classified as 'Polesana' (Table 6). The exclusion from discriminant analysis of 'Polesana' and 'Tipica Basso Friuli' allowed for reducing the rate of misclassification for various entries, leading it to 0% also for the ecotype 'Vogherese' (data not reported).

## Discussion

An earlier multi-environment study indicated the remarkable agronomic value of lucerne landraces from northern Italy relative to modern varieties in terms of forage yield (Annicchiarico & Piano, 2005). This study, mainly focusing on the assessment of intra- and inter-population variation, confirms those findings for the smaller inference space provided by one evaluation environment. It suggests, in addition, that the yield advantage of the best-yielding landraces compared with variety germplasm mainly depends on two characteristics: (i) the somewhat lower autumn dormancy (allowing for longer growth) associated with no penalty in winter mortality; and (ii) the lower summer

mortality. The value of the former characteristic is well-recognized in temperate countries, where substantial effort is being devoted to break the correlation of low dormancy with high winter mortality that exists across contrasting gene pools (Volenc et al., 2002). Although the present correlations revealed that better yield and persistence were closely related to low winter mortality, the correlation of winter mortality with dormancy was elusive (Table 4) as some landraces could combine relatively low dormancy with low winter mortality. Winter hardiness in low-dormancy material is reportedly associated with high content of total sugars in roots (Weishaar et al., 2005). The other positive characteristic of best-performing landraces, namely the lower summer mortality, may descend from better drought tolerance, better tolerance to pests and diseases, or better ability to withstand plant competition (whose importance is emphasized by the dense planting: Rotili & Zannone, 1975). The visually modest spread of pests and diseases suggests that the second factor is likely to be of minor importance.

The trend of varieties towards smaller leaflets than landraces may imply lower leaf-to-stem ratio and does not support the hypothesis that modern germplasm may possess better forage quality. The smaller frequency of variegated flowers in varieties than in landraces was expected as a consequence of selection against this trait

in some varieties. The similarity of landrace and variety germplasm, and the limited among- and within-landrace variation, for number of florets per inflorescence suggest the modest occurrence of natural and anthropic selection for this trait, possibly because of its modest impact on the seed yielding ability. Indeed, seed yield was mainly associated with number of inflorescences per plant, and only moderately related to other seed yield components, in lucerne varieties (Bolaños-Aguilar et al., 2000). The current focus on number of florets per inflorescence is justified by its lower cost compared with other seed yield components, since its evaluation is compatible with the timing of a forage yield assessment. The substantial independence of this trait from forage yield and persistence suggested by correlation results encourages its use as indirect selection criterion even if its correlation with seed yield is only moderate.

Total DW per plant tended to correlate with its component traits at the genotype and population levels (with the only exception of main stem length in populations). The general lack of significant inverse correlations among components of forage yield suggests that the simultaneous improvement of these traits could be possible. However, at the population level some negative correlations (e.g., that between main stem length and no. stems per plant: Table 4) may not be significant due to the small sample size, whereas at the genotype level the dominating plants in the stand may have assembled higher values of all forage yield components (and the dominated plants lower values of these traits) as a result of the greater vigour allowed for by competitive ability. The association of more stems per plant with lower mortality in the following winter may be related to the better ability of populations with larger plants to recover from winter injury by greater accumulation of root reserves.

The ratio of within-landrace to among-landrace genetic variance, averaging about 23 across traits, is intermediate between the earlier estimates of about 59 and 7 that were based, respectively, on RFLP (Pupilli et al., 2000) and AFLP (Zaccardelli et al., 2003) genetic markers. The large within-population variation of this germplasm may be attributed to the difficulty and the low impact of directional selection for agronomic traits performed by farmers on an outcrossing, autotetraploid and densely-grown species, and to the positive effect of genetic diversity on the adaptability of populations (Brown & Burdon, 1987). A similar genetic structure was also reported for ecotypes from northern Italy of another outcrossing forage legume, namely

white clover (Annicchiarico & Piano, 1995). The small genetic variation among and within lucerne landraces that emerged for winter dormancy may be explained by the close association of this crucial adaptive trait with the level of winter-hardiness that is locally required (Melton et al., 1988) and the limited variation for winter cold stress that exists across sites of the collecting region (Brunetti et al., 1993).

For collection and conservation activities, the extent of among- and within-population variation suggest that the Italian landrace germplasm may be represented by relatively few populations belonging to different commercial ecotypes and a relatively large sample of individuals per population (e.g., by collecting and regenerating seed bulks). Likewise, breeding may conveniently be directed to a few populations of which the genetic variation is mainly exploited by phenotypic selection among large numbers of individuals. These indications on exploitation of genetic resources may largely be extended to modern varieties. Although tending to lower within-population variation than landraces, this germplasm revealed the larger extent of this variance component relative to the among-population variance, in agreement with earlier findings by Julier et al. (2000) and Bolaños-Aguilar et al. (2000) for varieties mostly of French origin. Again, this may be due to the wide heterozygosity that descends from allogamy and autotetraploidy, as well as to the heterogeneity associated with the wide genetic base that characterizes most varieties (Hill et al., 1988). However, the present results do not suggest a strict relation between intra-population variation and number of components of the synthetic variety. Of the four varieties that derived from over 150 parental genotypes, namely 'Eletta', 'PR57N02', 'Classe' and 'PR58N57', only the first and the second revealed wide variation (Figure 2), whereas the fourth was less variable than any other variety (including 'Colosseo' and 'Gamma', derived from 12 and 10 parents, respectively).

This study confirms the wider intra-population variation of landraces relative to varieties that is expected as a consequence of their wider genetic base and the limited anthropic selection. The similarity of within-population variation between landrace and variety germplasm, reported by Falcinelli et al. (1994) for one landrace from central Italy, may well occur (Figure 2) but is likely to be infrequent. Therefore, individual landraces may only occasionally satisfy the homogeneity requirement in the UPOV's registration system (which is assessed in relation to a set of control varieties: UPOV, 1979), whereas commercial ecotypes

(which comprise several landraces) are hardly expected to ever satisfy this requirement. Especially for ecotypes, these findings highlight the interest of adopting a *sui generis* system in which the distinctness is the only requirement for registration.

Landraces which were geographically closer to each other or which belonged to the same commercial ecotype tended to be more similar, with the noticeable exception of accession '25' of 'Romagnola'. The difference between this entry and the other landraces of 'Romagnola' may partly be explained by differences in frequency of mowing at the collecting site and its impact on the morphophysiological characteristics. Accession '25' (derived from pooling the seed of two farms that were geographically close, exchanged seed and adopted similar agronomic practices) evolved under far more frequent mowing (average: 5.5 harvests/year) than the other 'Romagnola' landraces (average: 2.5 to 4 harvests/year). The possible introgression of genes as a consequence of accidental gene flow from nearby varieties may have contributed to this difference, although it is unlikely that this event may have occurred at both farms of origin of this entry. The substantial intra-population variation of this accession may be due to its origin from two farms or, again, to accidental gene flow from other material. In conclusion, accession '25' confirmed its greater agronomic value relative to other 'Romagnola' landraces reported by Rotili et al. (2000), but it may hardly represent the "true" ecotype of 'Romagnola' based on its overall characteristics. For registration as a variety in the UPOV system, this population may satisfy the distinctness requirement (according to discriminant analysis results) but not the homogeneity requirement (owing to its very wide intra-population variation).

The positive correlation of frequency of mowing at collecting sites with summer survival, forage yield and persistence has implications on collecting strategies, suggesting to preferably collect intensely-mown landraces as genetic resources for breeding. It also confirms indirectly the interest of plant selection under frequent mowing put forward by Rotili et al. (1996) as a means to improve the forage yielding ability and the persistence. Material selected under frequent mowing tends to better growth and survival by accumulating more rapidly in taproots the nitrogen and carbon reserves that are needed for regrowth (Avicé et al., 1997).

The trend of landraces evolved under more sandy soils towards greater forage yield and lower winter mortality in the sandy-loam soil of the evaluation

environment reflected the specific adaptation to soil type that is known to characterize the lucerne landraces from northern Italy (Annicchiarico & Piano, 2005). The relationship of smaller leaflets with greater drought stress at collecting sites may imply a positive effect of this trait on drought tolerance. Smaller leaves generally contribute to drought resistance by reducing the water loss from shoots (Turner, 1979). However, material selected for smaller leaflet showed higher transpiration rate besides lower growth rate per unit area compared with the larger-leaflet selection in a relatively cool environment (Estill et al., 1993).

Ordination and classification results for the accessions of 'Romagnola' highlighted the need to verify the homogeneity among landraces of the same ecotype prior to assess, for a subset of landraces that faithfully represent the basic ecotype seed for future multiplication and commercialization, the requirement of distinctness from other material. A greater homogeneity among landraces is expected for ecotypes that evolved in a geographically restricted area, as confirmed by results for the two landraces of 'Cremonese'. Most of the current ecotypes originated from such areas (Figure 1), suggesting that results of distinctness based on just one landrace may largely be valid for the ecotype. In the case of 'Leonicena', its rather large area of production actually results from only a small number of farm landraces (Rotili et al., 2000).

The use of multivariate methods such as discriminant analysis for verifying the distinctness of varieties has been envisaged as a means to increase the impact and the reliability of the information provided by individual descriptors (Loch, 1998). This study included all the descriptors recommended by Rotili et al. (1999) for assessing the distinctness of lucerne varieties, with the exception of growth habit, leaf colour and stem colour (which were visually little variable among genotypes) and the shape of the central leaflet (reportedly useless for cultivar discrimination; Russi & Falcinelli, 1997). It comprised, in addition, summer mortality and no. stems per plant, which are not listed also among the internationally-recommended descriptors (UPOV, 1988). These two traits, as well as all other descriptors with the exception of main stem length, were shown to be of some value for cultivar discrimination on a multivariate basis. Only two ecotypes, 'Cremonese' and 'Vogherese', showed a level of distinctness that may be compatible with a *sui generis* registration system. Some commercial varieties showed an insufficient distinctness, but the lower sample size of these entries (32 genotypes per replication) relative to commercial

ecotypes (at least 64 genotypes per replication) made the assessment of distinctness less reliable for this material. The use of molecular markers, despite the complexity implied also for these methods by the tetrasomic inheritance and the complex genetic structure of landraces (Pupilli et al., 2000; Zaccardelli et al., 2003), or the adoption of very large numbers of morphophysiological descriptors (Russi & Falcinelli, 1997), may be needed to increase the discrimination among ecotypes. The high agronomic value, wide intra-population variation and poor distinctness of most ecotypes and landraces suggest, anyway, that the main interest of this germplasm lies in its utilization as a genetic resource for plant breeding. The seed of the evaluated material is available to potential users.

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