Morphological and molecular characterization of italian ryegrass populations

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ABSTRACT

Italian ryegrass is the most important temperate grass in Rio Grande do Sul, Brazil. Despite its overall importance, there are no breeding programs for this species in this State. The purpose of this study was to evaluate the existing variability within and between four Italian ryegrass populations, three from Rio Grande do Sul, Brazil, and one from Uruguay. The populations were characterized based on morphological traits such as: number of tillers, canopy diameter, heading date, total leaf area, number of leaves, area/leaf, leaf dry matter, stem dry matter, leaf/stem ratio and dry matter yield. Molecular variation was also characterized using RAPD markers. A large variability was found within the populations (98.41%), which limited the complete separation of populations. However, significant differences were found among populations for traits of great forage interest, such as the number of tillers, canopy and heading date, indicating that the present variability is suitable for the initiation of a breeding program.

KEY WORDS: Variability, forage, breeding.

INTRODUCTION

A large area in the state of Rio Grande do Sul (southern Brazil) is covered by natural pastures composed mainly of species with spring-summer growth, and with a large decrease in production during the cold season (Mota et al., 1981). Among the species that were introduced to supply food during winter, the Italian ryegrass (*Lolium multiflorum* Lam.) is the most important economically speaking (Maia, 1995), cultivated in more than one million hectares.

The classical breeding of forage species is based on the generation of synthetic populations (Vogel and Pedersen, 1993). They are expected to produce under a wide range of climatic, edaphic and management conditions (Breese and Hayward, 1972). Therefore, retention of a degree of heterogeneity in selected populations is fundamental for the adaptation of new cultivars. The success of the British perennial ryegrass cultivar S23 was based on its genetic heterogeneity, resulting in a buffering ability and high phenotypic performance over a wide range of soil types (Valentine and Charles, 1975).

The most important temperate forages, such as the Italian ryegrass, are all cross-pollinated; consequently, both natural ecotypes and synthetic populations are likely to be highly genetically heterogeneous (Forster et al., 2001). The measurement of genetic variability present in germplasm collections is an important factor to maintain and use these collections in a better way (Casler, 1995), as well as to plan strategies to develop synthetic populations.

Molecular marker technologies have been successfully applied in the characterization of germplasm. The RAPD marker technology, where a unique primer allows the amplification of multiple loci in the genome (Tingey and del Tufo, 1993), has proved to be a good alternative to investigate the genetic variability in forage species (Huff et al., 1993; Sweenwey, et al., 1996; Huff, 1997; Chai and Sticklen, 1998; Kölliker et al., 1999).

The comparison of molecular and morphological characterizations is of paramount importance when studying forage crops, since they can be used as complementary tools in the development of breeding programs that are highly dependent on the quality of the used germplasm (Loos, 1994a). Traits such as dry matter yield and forage quality are described as essential in germplasm evaluation programs (Wilkins, 1991).

This study aimed at evaluating the variability within and among Italian ryegrass populations grown in Rio

Grande do Sul, Brazil. A molecular characterization was performed using RAPD markers. A morphological evaluation was performed based on the following traits: number of tillers/plant, canopy, heading date, total leaf area, number of leaves, average leaf area, leaf dry weight, stem dry weight, leaf/stem ratio and dry matter yield.

MATERIAL AND METHODS

Plant Materials

Three Italian ryegrass populations from Rio Grande do Sul, named "Dom Pedrito" (DP), "Pantano Grande" (PG) and "Pedro Osório" (PO), and one from Uruguay, cultivar "La Estanzuela-284" (LE-284) were used in this study. Selection criteria for the Brazilian populations were their good seed quality and performance, based in the information provided by Fonsêca (1997). The cultivar "La Estanzuela-284" was chosen because it is largely cultivated in Rio Grande do Sul.

Morphological Characterization

Each population consisted of 300 plants, planted at a distance of 0.30 m from each other, laid out in the field in a randomized complete block design with three replications containing plots of 100 plants each. A random sample of 30 individuals per replication was used for measuring most morphological traits. The number of tillers/plant and the canopy diameter were measured in all plants 98 days after sowing, at the beginning of the elongation stage (Moore and Moser, 1995). The canopy diameter was determined as the average between two diameter/plant measurements. The heading date was measured by the number of days after sowing until the first tiller anthesis. The leaf area and the number of leaves per plant were measured in the laboratory in an area meter (Model LI-3100, LI-COR, Lincoln, NE) one week after anthesis. Leaves and stems were dried separately at 65°C with forced air until a constant weight. Dry matter yield was obtained by adding leaf and stem weights. The leaf/steam ratio was obtained by dividing the leaf weight by the stem weight.

Molecular Characterization

A minimum of 95 plants chosen by chance from each population was evaluated with molecular markers. The DNA analysis was optimized by standardizing DNA concentrations through ethidium bromide staining compared to lambda/*Hin*d III markers and using two independent DNA extractions from each plant. Two grams of plant tissue were extracted using the CTAB method (Saghai-Maroof et al., 1984). Further steps in DNA extraction were performed as previously described (Yang et al., 1996).

Thirty primers from University of British Columbia, UBC 1 to 30, were screened to find those most effective for producing polymorphic bands in these populations. The six primers showing the best amplification products (UBC 2: CCTGGGCTTG; 3: CCTGGGCTTA; 4: CCTGGGCTGGG; 9: CCTGCGCTTA; 12: CCTGGGTCCA; 13: CCTGGGTGGA) were chosen. RAPD reactions were performed in 25 µl, with 2.5 ng genomic DNA; 0.2 mM dNTPs, 0.2 mM cresol red; 0.5 mM primer; 2.5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl pH 8.0; 1 mL/mL tritonX-100 and 1 U Taq polymerase (Pharmacia Biotech Inc.).

The amplifications were performed on a thermocycler (MJ Research, Inc.) consisting of denaturation at 94 °C (180s), followed by 44 cycles at 94 °C (60s), 38 °C (60s) and 72 °C (90s). In the end, samples were submitted to 5 min at 72 °C for a final extension. Amplified fragments were separated in 1.4 % (w/v) agarose gels, stained with ethidium bromide and visualized on a UV light.

Statistical Analysis

The morphological data were subjected to a Levene's test (Snedecor and Cochran, 1980) to verify the homogeneity of phenotypic variance, and then analysed using the GLM procedure of the SAS statistical package (SAS Institute, 1990). When the differences between populations were significant, the average values of each population for each trait were tested with the Least Significant Difference test (LSD, $P \leq 0.05$).

The populations were also compared pair wise in relation to the proportion of genotypes found in the interval above the general mean of the trait within a population. For this procedure, a chi-square analysis was performed according to Snedecor and Cochran (1980), with a 2x2 contingency table as:

	Population A	Population B	Total
Plants above the general mean	$f_{i=1}$	$f_{i=2}$	X
Plants below the general mean	$f_{i=3}$	$f_{i=4}$	Y
Total	W	K	Z

$$\chi^2 = (f - F)^2 \sum_{i=1}^{4} \frac{1}{Fi}$$

where f was the observed frequence and F was the expected frequence obtained by the formula:

$$F1 = \frac{(W.X)}{Z} \qquad F2 = \frac{(K.X)}{Z} \qquad F3 = \frac{(W.Y)}{Z} \qquad F4 = \frac{(K.Y)}{Z}$$

For the molecular analysis, only bands present in both extractions were scored as present (1) or absent (0) on a given genotype and recorded in a binomial matrix. Based on the molecular data, the genetic distance between individuals was estimated as Euclidean distance (E) according to Excoffier et al. (1992) and Huff et al. (1993),

$$E = n\left[\frac{(1-2n_{xy})}{2n}\right]$$

where n is the total number of polymorph bands, and n_{xy} is the number of bands shared by the individuals x and y.

The pair wise comparison of populations was obtained with the help of the Arlequin software (Schneider et al., 1996). Starting from a matrix obtained from the square of the Euclidean distance, an estimate of the genetic variability among and within populations was calculated through an analysis of molecular variance (AMOVA). The AMOVA calculates the ϕ_{st} value that is equivalent to the proportion of the total variation shared between the two populations (Excoffier et al., 1992). The genetic distance between any two populations was represented by its value ϕ_{st} and referred to as inter-populational distance.

Both morphological and molecular data were separately submitted to a principal component analysis with the help of the statistical package SAS (SAS Institute, 1990).

RESULTS

All variances for morphological data showed homogeneity by the Levene's test and did not require data transformation. Significant differences were found through the analysis of variance in the number of tillers, canopy diameter and heading date traits.

The "DP" population had more tillers per plant (59.52) than "LE-284" at 48.29 (Table 1). These populations were also different from the other two populations in the study. "PG" and "PO" populations did not differ, with 53.76 and 52.45 tillers/plant, respectively. The "PG" population had a significantly larger canopy (61.75 cm in diameter) than the other three populations. The four populations showed significant differences for the heading date. The earliest was "LE-284", with an average flowering time at 120 days after sowing, followed by "PG" (124 days), "PO" (126 days) and "DP" (127 days).

Significant differences were found in the number of tillers, canopy diameter, heading date, number of leaves and leaf/stem ratio when populations were compared based on the proportion of genotypes within populations that were above the general mean for each trait (Table 1). Population "DP" presented 83 % of the genotypes with more than 50 tillers/plant, differing from all other populations. Population "PG" seemed intermediate between populations "LE-284" and "PO", although it didn't significantly differ from them, with 66 % of genotypes with more than 50 tillers/plant. The "LE-284"and "PO" populations had 60 % and 73 % of individuals with more than 50 tillers/plant, respectively.

The "PG" population had the largest number (84.18 %) of genotypes above the 60 cm mean for canopy diameter. For heading date, "LE-284" had fewer plants (28.63 %) flowering after 124 days, and "DP" had the most genotypes flowering after 124 days (92.75 %). For the number of leaves, significant differences were only found between population "PG"

Table 1. Averages, ranges, standard deviation and proportion of genotypes within each of four annual ryegrass populations evaluated that were above the general mean for number of tillers, canopy diameter, heading date, total leaf area, number of leaves, average leaf area, leaf dry matter, stem dry matter, dry matter yield, and leaf/stem ratio traits.

Population†	Average	Minimum	Maximum	Standart deviation	Proportion above general mean
Number of tillers					%
"LE-284"	48.3a‡	14	172	21.2	60a¦
"PG"	53.8b	14	132	24.5	66ab
"DP"	59.5c	21	173	21.2	83c
"PO"	52.5b	13	120	19.15	73b
General mean	50				
Canopy diameter		(cm)			%
"LE-284"	57.5a‡	32.5	104.5	12.4	52.7a¦
"PG"	61.8b	32.5	120.0	11.8	84.2b
"DP"	57.1a	34.0	100.0	11.6	54.1a
"PO"	55.2a	24.0	95.0	13.0	47.4a
General mean	60				
Heading date		(days)			%
"LE-284"	120.2a‡	100	136	5.4	28.6a¦
"PG"	124.2b	101	137	5.1	63.3b
"DP"	126.8c	107	141	4.5	92.8c
"PO"	125.7d	103	137	5.0	88.0c
General mean	124				
Total leaf area §		(cm^2)			%
"LE-284"	1163.4	280.2	3806.6	782.3	31.0a¦
"PG"	1464.3	364.8	2931.3	766.4	51.6a
"DP"	1085.3	180.4	3092.0	687.1	29.0a
"PO"	1342.8	185.5	3519.5	1110.4	33.3a
General mean	1250				
Number of leaves §					%
"LE-284"	194.3	75	508	97.6	41.4ab¦
"PG"	241.7	76	479	86.5	61.3a
"DP"	215.5	92	473	95.5	35.5b
"PO"	240.1	80	546	122.5	40.0ab
General mean	220				
Average leaf area §		(cm^2)			%
"LE-284"	5.7	3.34	10.7	1.8	48.3a¦
"PG"	6.0	1.75	10.3	2.1	54.8a
"DP"	4.8	1.3	8.2	1.8	32.3a
"PO"	5.3	2.0	10.3	1.8	43.3a
General mean	5.5				
Leaf dry matter §		(g)			%
"LE-284"	6.8	1.7	16.7	3.8	37.9a¦
"PG"	8.4	1.7	17.2	4.1	45.2a
"DP"	6.4	1.5	18.3	3.7	29.0a
"PO"	8.4	1.4	26.9	6.5	33.3a
General mean	7.5				
Steam dry matter §		(g)			%
"LE-284"	18.6	5.7	40.0	8.0	75.9a¦
"PG"	25.6	9.3	48.5	11.5	77.4a
"DP"	21.7	7.8	54.0	11.6	74.2a
"PO"	23.2	7.9	52.2	12.2	70.0a
General mean	20.0				
Dry matter yield §		(g)			%
"LE-284"	25.2	7.4	47.7	10.7	48.3a¦
"PG"	32.4	11.0	62.8	11.4	67.7a
"DP"	26.3	9.3	53.2	13.8	46.7a
"PO"	31.1	10.8	71.5	16.7	56.7a
General mean	30.0				
Leaf/steam ratio §		(ratio)			%
"LE-284"	0.37	0.15	0.61	0.13	65.5a¦
"PG"	0.35	0.17	0.57	0.11	54.8ab
"DP"	0.30	0.07	0.61	0.10	36.7b
"PO"	0.33	0.13	0.71	0.13	44.7ab

† "LE-284", population La Estanzuela-284; "PG", population Pantano Grande; "DP", population Dom Pedrito; "PO", population Pedro Osório; ‡ Within columns, means followed by the same letter are not significantly different to LSD (p=0.05); § Traits that did not differ significantly at the 0.05 probability level by the ANOVA were not submitted to a LSD test; ¦ Within columns, means followed by the same letter are not significantly different according to Chi-square test (p=0.05).

– which was the highest with 61.30 % - and "DP", which was the lowest with 35.48 % of the genotypes with more than 220 leaves.plant^{-1}. "LE-284" had 65.51 % of its plants with the leaf/stem ratio > 0.35 and "DP" was the lowest at 36.66 %. The analysis of the main components of the morphological trait data showed that the two first components explained 72.82 % of the total variation (Figure 1). In the first component all traits showed similar loadings. On the other hand, the second component was strongly influenced by the heading date, which was responsible for the separation among the "LE" and "DP" populations, and the decreasing influences of number of tillers/plant, stem weight and dry matter yield.

In the molecular analysis, six RAPD primers amplified 72 polymorph bands. Results of the AMOVA showed that 98.41 % of total variation was present within the four populations, and only 1.59 % was found among populations (Table 2). The pair wise comparison of populations (Table 3) showed significant differences in all cases: the highest difference between "LE-284" and "PO" (2.84 %), and the lowest between "LE-284"

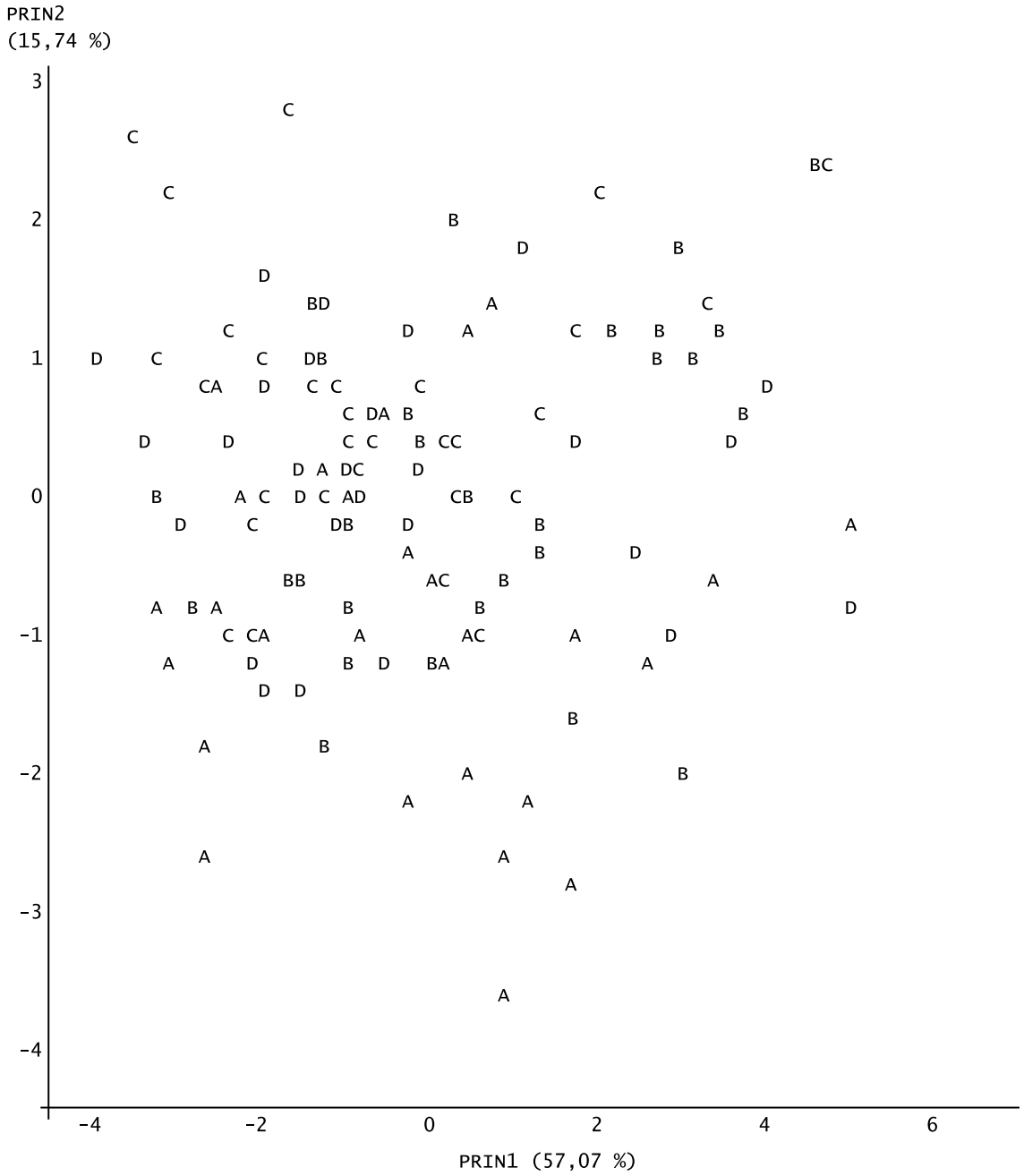

Figure 1. Dispersion by the main components analysis from four Italian ryegrass populations based on the number of tillers, canopy, heading date, total leaf area, number of leaves, average leaf area, leaf dry matter, stem dry matter, dry matter yield and leaf/stem ratio morphological traits. A: Population "LE" (La Estanzuela-284); B: Population "PG" (Pantano Grande); C: Population "DP" (Dom Pedrito); D: Population "PO" (Pedro Osório).

and "PG" (0.44 %). Based on molecular markers, the two first principle components explained only 15.05 % of the total variation and a major overlap among populations was observed (Figure 2). The first component is a measurement of overall molecular markers. The first eigenvector shows approximately equal loading on all markers. The second eigenvector has positive and similar values for approximately all markers, except for two markers out of nine produced by primer UBC2, six markers out of 16 produced by primer UBC4, two markers out of seven produced by primer UBC9, eight markers out of 14 produced by primer UBC13 and all 12 markers produced by primer UBC12, which contributed with negative loadings.

DISCUSSION

The results of the present study show that either on morphological or molecular data the largest portion of genetic variability in ryegrass is found within populations. However, the populations significantly differed in important traits in forage breeding. The long period of cultivation in distinct environments probably gave rise to mutations in these populations which affected their fitness value, resulting in the differences revealed in this study.

The yield of a forage is a direct function of the number of tillers/plant and production/tiller (Nelson, et al., 1977; Wilkins, 1991). The canopy is an estimate of the available forage yield (Coser et al., 1989). The populations evaluated were significantly different in the number of tillers/plant, canopy and heading date, and they also differed in the proportion of individuals within populations that are above the general mean for these traits as well as for the number of leaves/plant and leaf/steam ratio (Table 1). These results indicate that these populations can be improved, or be used as sources of improvement for forage quantity and quality (Casler and Vogel, 1999).

The "DP" population presented the highest number of tillers/plant, differing from all other populations, whereas "PG" showed the largest canopy diameter, also differing from the others (Table 1). Based on these results, we can predict that "PG" has a greater yield/tiller than "DP", which is confirmed by the fact that these two populations also differed in the proportion of genotypes above the general mean for the number of leaves/plant (Table 1), one of the main traits responsible for yield/tiller.

The selection of individuals from both populations can enhance forage yield. Traits as number of tillers/plant and yield/tiller are negativelly correlated (Nelson et al., 1977). However, it is desirable to maintain the heterogeneity in a forage cultivar, to obtain a population rich in both types of genotypes. In this sense, the number of tillers/plant is the main responsable for forage yield until the moment that plants reach the tiller density equilibrium, when addition of new tillers offsets death of older ones. After this point, forage yield is associated mainly with yield/tiller (Zarrough et al., 1983), as well as during early stages of regrowth, when tiller density commonly does not increase (Zarrough and Nelson, 1980).

Plant maturity is the main factor that influences forage quality with all traits that are positively correlated with quality decrease with the advance in plant maturity (Buxton and Mertens, 1995). A delay in the flowering date could maintain for a longer period of time a higher nutritive value, digestibility and palatability of the forage (McLean and Watson, 1992). All populations differed significantly in heading date as assessed by analysis of variance, showing the possibility of selecting genotypes with late flowering, mainly in population "DP".

Leaf/steam ratio also has influence on forage quality. Population "LE-284" showed significant differences from "DP" in the proportion of genotypes with more than 35 % of leaf/steam ratio. Therefore, the selection of individuals from these two populations, with opposite values for leaf/stem ratio and time of flowering, can enhance quality as a whole in the resulting selected population.

The high variability found within populations based on morphological data was reflected in the analysis based on molecular data. There were no bands unique to one population. High variability within populations is consistent with those described by Loos (1994b), Huff (1997) and Kölliker et al. (1999). Huff et al. (1993) showed that while AMOVA (Excoffier at al., 1992) was developed initially to be used with RFLP data, this analysis can be easily accommodated to RAPD data. In the present study, the AMOVA indicated that all populations differed significantly, with small differences found between them despite their origin as landraces (not breed) or breed populations. Other studies have shown that high variability is retained within breed ryegrass populations, comparable to that found in natural populations (Huff, 1997; Forster et al., 2001). It has

Table 2. Analysis of molecular variance (AMOVA) based on 72 RAPD markers of 375 individuals of *Lolium multiflorum* belonging to four populations.

Source of variation	DF	SS	Variance components	Percentage of total variation
Among populations	3	102.97	0.22	1.59[1/]
Within populations	371	5062.45	13.65	98.41
Total	374	5165.42	13.87	100

[1/]Significant at the 0.05 probability level.

Table 3. Summary analysis of the 2x2 comparison among Italian ryegrass populations obtained by AMOVA. The percentage of total molecular variation existing between populations (ϕ_{st}) is a measurement of genetic distance between populations (below diagonal). The test of significance of each value ϕ_{st} was calculated as the probability that a found value ϕ_{st} could be higher than the observed value (above diagonal).

Population†	LE-284	PG	DP	POR
LE-284	-	0.44	1.64	2.84
PG	0.02	-	0.84	1.94
DP	0.00	0.00	-	1.61
PO	0.00	0.00	0.00	-

† "LE-284", population La Estanzuela-284; "PG", population Pantano Grande; "DP", population Dom Pedrito; "PO", population Pedro Osório.

been shown that more than 60 years of intensive breeding in ryegrass did not reduce genetic variability (Casler, 1995).

In the analysis of molecular variance, population "PO" showed the higher number of differences when compared to the others (Table 3). Additionally, based on some morphological traits such as leaf dry matter and dry matter yield, genotypes with maximum values for these traits were present in this population and not found in any other population (Table 1). Population "PG" was less divergent from the others based in molecular data (Table 3), but significant differences were found between this population and the others based in morphological data (Table 1). Population "DP" showed similar divergence from populations "LE-284" and "PO" with the molecular data (Table 3). However, the morphological data indicated the largest differences between populations "DP"and "LE-284" (Table 1 and Figure 2). This divergence of results between molecular and morphological data agrees with the random distribution of RAPD markers in the genome, that shows potential to evaluate genetic variability in forage species (Chai and Sticklen, 1998), as well as in other crops (Yang et al., 1996), but is not necessarily well correlated with morphological traits.

The majority of ryegrass molecular studies to date have involved RAPDs and RFLPs. These analyses have given valuable information on the genetic structure of populations, however, RAPD is now relatively desfavoured due to problems with reproducibility (Jones et al., 1997). Our study tried to overcome these problems analysing two independent DNA extractions from each genotype.

Molecular analyses using AFLP (Roldan et al., 2000) and SSR markers (Kubik et al., 1999; Jones et al., 2001) have shown capability in detecting variation between closely related genotypes, being powerfull molecular markers to evaluate genetic distintictiveness of new cultivars as well as the assessment of genetic stability over time (Forster et al., 2001). In a future step, when cultivars will be released, these analyses will be probably performed to ensure the breeders rights. At this time, RAPD, joined to morphological data, furnished the necessary data to allow the start of a breeding program.

The principal component analysis based on morphological traits detects a small separation of populations "LE-284" and "DP" by the second component (Figure 1). Variation of the heading date is the main parameter responsible for this separation. The first component shows all populations

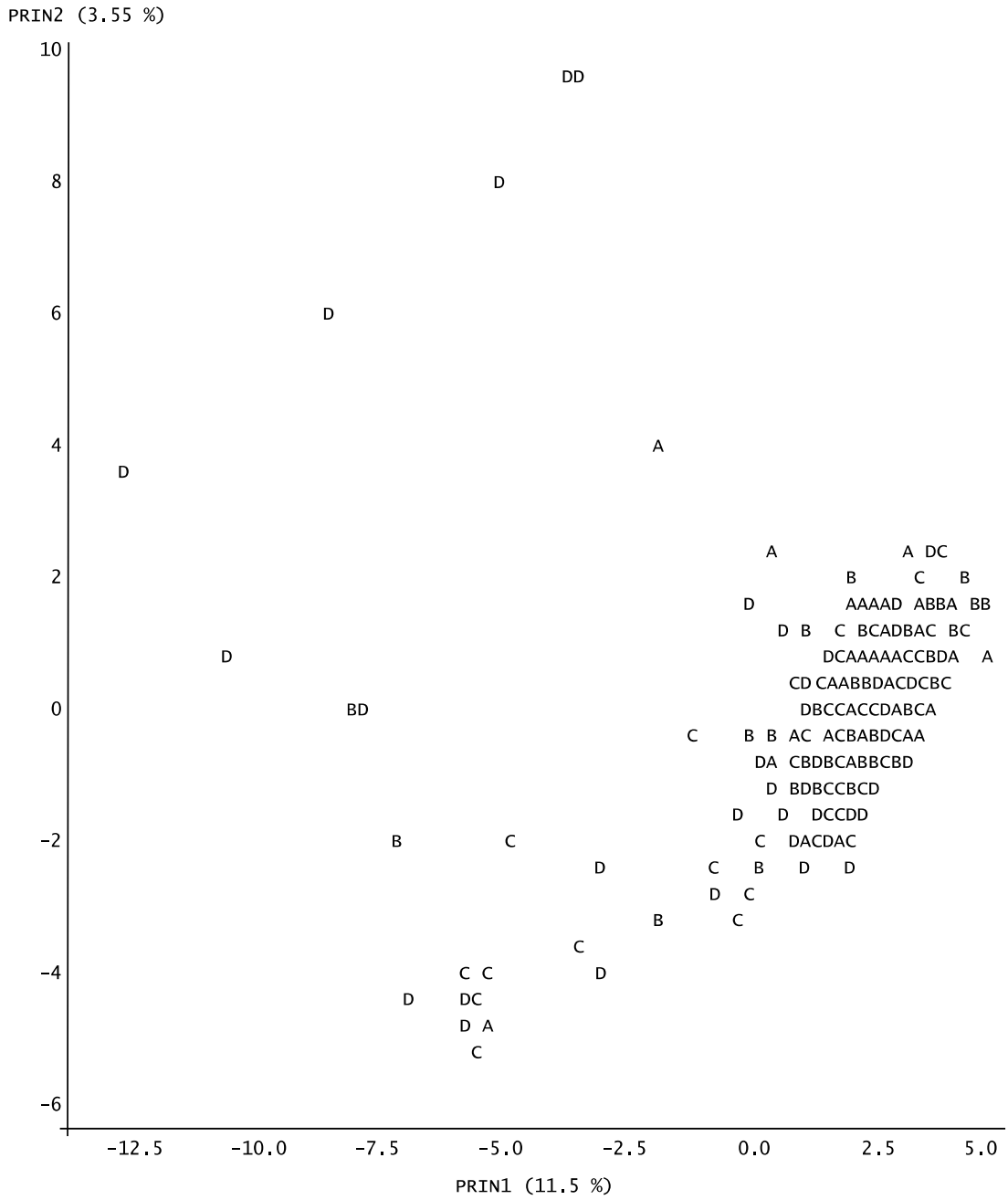

Figure 2. Dispersion by main components analysis from four Italian ryegrass populations based on 72 RAPD markers. A: Population "LE" (La Estanzuela-284); B: Population "PG" (Pantano Grande); C: Population "DP" (Dom Pedrito); D: Population "PO" (Pedro Osório).

† 225 genotypes are not visible on the graphic.

overlapping. This component is a measure of overall morphological traits. In the analysis based on molecular data just a few genotypes from "PO" are separated from a large group composed by individuals from all populations (Figure 2). All primers employed had basically equal loadings in both, first and second eigenvectors. A little distinction may be attributed to primer UBC12, wich all bands contributed negatively in the second component. However, other primers also resulted in bands with negative loadings. It supports the absence of a band unique to each population, that in the case of presence would have a strong influence in the components.

Our study showed that a considerable amount of variability is found even when dealing with four Italian ryegrass populations. Regardless the nature of the data, molecular or morphological, the variability measures obtained can aid the choice of

the best combinations between parent populations and breeding strategies.

Besides the variability present within populations, they also show differences in traits of paramount importance, agreeing with Breese and Hayward (1972), that as a consequence of the relatively short history of forage grasses and legumes as intensively cultivated crops, a large variability is found in the populations, allowing one to achieve a rapid improvement just by the exploitation of ecotypes that occur naturally.

RESUMO

Caracterização morfológica e molecular de populações de azevém anual

O azevém é a gramínea anual forrageira de maior utilização no Rio Grande do Sul, Brasil. Apesar de sua importância, pouco tem sido feito para melhorar esta espécie desde sua introdução. Os objetivos deste trabalho foram avaliar a variabilidade existente dentro e entre quatro populações de azevém anual, três do Sul do Brasil, e uma do Uruguai. As populações foram caracterizadas molecularmente, com o uso de RAPD e morfologicamente com base nos caracteres: número de afilhos, cobertura do solo, ciclo, área foliar total, número e peso de folhas, área/folha, peso de colmos, relação folha/colmo e matéria seca total. Grande variabilidade foi encontrada dentro das populações (98.41%), limitando a separação completa das mesmas. Entretanto, diferenças significativas foram encontradas entre as populações para características de grande interesse forrageiro, como número de afilhos/planta, cobertura do solo e ciclo, indicando que a variabilidade presente é apropriada para o início de um programa de melhoramento.

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