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RESEARCH ARTICLE

Assessing the genetic diversity of *Panicum coloratum* var. *makarikariense* using agro-morphological traits and microsatellite-based markers

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Abstract

Panicum coloratum var. *makarikariense* is a perennial C_4 grass native to South Africa with relatively good forage production under limited-resource conditions. Genetic characterisation and breeding efforts have been scant, thus limiting its use in cattle raising systems. The goal of the present study was to assess the genetic diversity of a collection of *P. coloratum* var. *makarikariense* using agro-morphological traits and molecular markers, in comparison with one accession of var. *coloratum* and one population of *Panicum bergii*. Agro-morphological variability between and within accessions of var. *makarikariense* in a common garden setting was observed, showing that there is still opportunity for selection. Some accessions performed better than the commercialised material in relation to potential forage production. A total of 117 ISSR bands and 48 SSR alleles allowed the detection of genetic variability between and within accessions. The presence of accession-specific bands suggested distinctness and limited gene flow. The genetic variability encountered in the commercialised material suggested that it is a stabilised population which has not undergone a strong selection process. Low correlation between agro-morphologic and molecular variability was observed indicating that both approaches provide complementary information. Both morphological and molecular markers reveal genetic differentiation between varieties and species. This study provides a set of new SSR markers available for diversity assessment and valuable information that can be applied directly in collection management for breeding and conservation programmes.

Introduction

Panicum coloratum (Fam. Poaceae) is a warm-season perennial grass native from South Africa, introduced as a pasture species in USA, Australia, Japan and South America (Tischler & Ocumpaugh, 2004). It is usually described as a cross-pollinated polyploid species (Hutchison & Basha, 1964; Pritchard & De Lacy, 1974) and comprises two botanical varieties: var. *makarikariense* Gooss. (Goossens, 1934) and var. *coloratum* (Bogdan, 1977). The existence of different biotypes in the species has hampered sub-specific classification (Tischler & Ocumpaugh, 2004).

In Argentina, the advance of crop farming over recent decades has displaced livestock production to less favourable areas, where pasture resources are subjected to drought, waterlogging, salinity or thermal stress (Rearte, 2007; Manuel-Navarrete *et al.*, 2009). In addition, the grasslands of the world face a range of climate changes that includes elevated atmospheric carbon dioxide, increasing temperatures and modified patterns of rainfall (Humphreys *et al.*, 2011). *P. coloratum* has been recommended to help overcome forage shortages in areas with climatic and edaphic constraints (Stritzler, 2008; Ferri, 2011). In particular, var. *makarikariense* is

especially well adapted to soils that experience periods of drought and seasonal flooding (Lloyd, 1981). Forage yield and adequate nutritive value as deferred forage are good attributes of this species compared with other C_4 grasses (Stritzler *et al.*, 1996; Ferri *et al.*, 2006; Ruiz *et al.*, 2008). Despite these advantages, the use of *P. coloratum* for forage in Argentina has not spread throughout the country. Shattering hinders seed harvest; consequently, it diminishes quality of available commercial seed (Lloyd, 1981; Young, 1986). Other problems are related to low seedling vigour and excessive mesocotyl elongation, making establishment difficult (Petruzzi *et al.*, 2003; Lodge *et al.*, 2010). There is only one accession of var. *makarikariense* currently available from seed companies, commercialised as cv. 'Bambatsi'. Also, adjusted management techniques are needed to take advantage of its potential.

The genetic diversity of the species has scarcely been characterised and breeding efforts have been scant (Hussey & Holt, 1986; Young, 1986; Tischler & Voigt, 1995). So far, most of the studies on *P. coloratum* have involved morphological or physiological traits (Tischler *et al.*, 1989; Taleisnick *et al.*, 1998; Boschma *et al.*, 2010; Lodge *et al.*, 2010) with a few exceptions that document molecular variability (Komatsu *et al.*, 2007). Within the PCR-based class, inter-simple sequence repeat (ISSR) (Zietkiewicz *et al.*, 1994) and simple sequence repeat (SSR) markers (Powell *et al.*, 1996) have been used successfully to assess genetic polymorphisms in grass forage species (Azevedo *et al.*, 2011; Che *et al.*, 2011; Chen *et al.*, 2012; Li *et al.*, 2013). As DNA sequence data are scarce for *P. coloratum*, the use of heterologous SSR markers from related species represents a suitable strategy for molecular variability assessment (Chandra & Tiwari, 2010; Huang *et al.*, 2011).

The *P. coloratum* collection is maintained at the National Institute of Agricultural Technology (INTA), Rafaela, Argentina, comprising var. *makarikariense* and var. *coloratum* which have clear morphological differences between them (Armando *et al.*, 2013). Plants of var. *makarikariense* were introduced to Argentina by a low number of recent events; therefore, local adapted populations might contain low variability. The objectives of the present study were (a) to assess agro-morphological and molecular variation in a *P. coloratum* var. *makarikariense* collection, (b) to establish the within and between components of accession variability in comparison to the commercialised cultivar, and (c) to examine the correlation between phenotypic and molecular variation. One accession of var. *coloratum* and one population of *Panicum bergii* were included for comparison. The ultimate aim of this study is to provide data applicable for conservation and breeding.

Materials and methods

Plant materials

A screening of *P. coloratum* var. *makarikariense* was performed in 2006 through several locations in Argentina varying in soil types and precipitation regimes. Collected plants were transplanted in a common garden at INTA Rafaela Experiment Station (31°11′41″ S; 61°29′55″ W; Armando *et al.*, 2013). The collection composed by six accessions comprising 32 plants each. The plants were placed at 0.6 m intervals in one 8 × 4 plot. Additionally, 15 IFF materials clonally propagated into eight ramets each were also arranged linearly at a distance of 0.6 m (Table 1). A previously analysed accession of var. *coloratum* (CH) with desirable attributes related to forage production was included. For the molecular analysis, one population of *P. bergii* (PB), a species closely related to *P. coloratum* that grows as a spontaneous weed at the experimental station, was also included.

Agro-morphological evaluation

Phenotypic evaluation was conducted during two growing seasons: September to December 2008 (Spring) and January to April 2009 (Summer). A total of 97 plants were evaluated: 12 individuals per accession and one ramet of each of the 15 IFF clonal materials (Table 1). Eighteen agro-morphological traits were recorded (Table 2). All observations and measurements were performed on adult plants growing at the centre of the plots.

To calculate leaf elongation rate (LER) (Berone *et al.*, 2007), the plants were cut to a height of 15 cm and one emerging leaf from three different tillers per plant was tagged. Leaf length was measured every 2 days from 28 October to 24 November 2008 (Spring) and from 12 January to 5 February 2009 (Summer). Phyllochron (Phy) was calculated as the difference in growing degree days between the appearances of successive leaves (Gallagher, 1979). Daily growing degree units (GDU) were calculated as follows:

$$\text{GDU} = [(\text{Daily maximum temperature} - \text{Daily minimum temperature})/2] - \text{Tb}$$

where Tb: 10°C base temperature (Ferri *et al.*, 2008).

Accession means and standard errors were used as descriptive measures for all evaluated variables. A relative performance (*Rp*) to compare each accession with the commercial material cv. 'Bambatsi' (CM) was obtained for each variable as:

$Rp_i = \left[\frac{\overline{x}_i}{\overline{x}_{CM}} - 1\right] \times 100$, where $\overline{x}_i$ is the mean value of the accession i and $\overline{x}_{CM}$ is the mean value of 'Bambatsi' cultivar.

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Table 1 Accessions of *P. coloratum* and their collection site description

Variety	Accession Code	Description	Site	Province
P. coloratum var. *makarikariense*	DF	12-year-old pasture. Under heavy cattle grazing.	Dean Funes (150 km North West from Cordoba city).	Córdoba
	UCB	Pasture not grazed. Introduced from Africa.	University Catholic of Cordoba.	Córdoba
	MR	Pasture not grazed. Introduced from Africa.	University Catholic of Cordoba.	Córdoba
	BR	10-year-old pasture under cattle grazing. Introduced from Brazil.	Mercedes Experiment Station (INTA).	Corrientes
	ER	5-year-old pasture under cattle grazing.	Private farm near Mercedes.	Corrientes
	IFF 1-15	Clonal materials	Institute of Phytopathology and Plant Physiology (IFFIVE-INTA).	Córdoba
	CM	Seeds commercially distributed by a private company.	cv. 'Bambatsi'	–
P. coloratum var. coloratum	CH	Under cattle grazing. Intersown in native rangeland.	Farm near Chacharramendi.	La Pampa

Table 2 Agro-morphological traits and sampling methods used for the characterisation of *P. coloratum* accessions

Abbreviation	Trait Descriptor and Sampling Method
PH	Plant height (cm, at reproductive stage): mean height reached by the leaves.
*LLS**	Leaf length in Spring (cm from the collar of the elongated leaf to the tip): 1 leaf tiller^{-1}, 3 tillers plant^{-1}.
*LLSu**	Leaf length in Summer (cm from the collar of the elongated leaf to the tip): 1 leaf tiller^{-1}, 3 tillers plant^{-1}.
FLL	Flag leaf length (cm): 3 tillers plant^{-1}.
*LWS**	Leaf width in Spring (cm at the widest portion of the elongated leaf): 1 leaf/tiller, 3 tillers/plant.
*LWSu**	Leaf width in Summer (cm at the widest portion of the elongated leaf): 1 leaf/tiller, 3 tillers/plant.
FLW	Flag leaf width (cm): 3 tillers plant^{-1}.
*LDWS**	Leaf dry weight in Spring (mg of fully formed leaves): 1 leaf/tiller, 3 tillers/plant. Samples oven-dried at 70°C for 72 h.
*LDWSu**	Leaf dry weight in Summer (mg of fully formed leaves): 1 leaf/tiller, 3 tillers/plant. Samples oven-dried at 70°C for 72 h
FLDW	Flag leaf dry weight (mg of fully formed leaf): 3 tillers/plant. Samples oven-dried at 70°C for 72 h.
PL	Panicle length (cm from the crown to the base of the flag leaf, rachis not included): 3 tillers/plant.
PN	Number of panicles/plant.
1000-SW	1000-seed weight (mg plant^{-1}). Average of three samples of 20 mature seeds.
DW	Dry weight/plant (total gr/aerial biomass plant harvested at the end of the growing season). Samples oven-dried at 70°C for 72 h.
LERS	Leaf elongation rate per tiller in Spring (cm.tiller^{-1}.day^{-1}, at vegetative stage): 3 tillers plant^{-1}.
LERSu	Leaf elongation rate per tiller in Summer (cm.tiller^{-1}.day^{-1}, at vegetative stage): 3 tillers/plant.
PhyS	Phyllochron in Spring (°C day, at vegetative stage). 10°C base temperature: 3 tillers plant^{-1}.
PhySu	Phyllochron in Summer (°C day, at vegetative stage). 10°C base temperature: 3 tillers plant^{-1}.

*Length, width and dry weight measured in the third leaf completely expanded per tiller.
Tillers were randomly selected.
Seeds refer to caryopsides of Poaceae.

Principal component analysis (PCA) based on the standardised Euclidean distance was performed. Phenotypic diversity per accession was estimated using the mean Euclidean distance (MED), obtained as the average of the Euclidean distances among individuals in each accession. All computations of morphological data were carried out using Infostat program (Di Rienzo *et al.*, 2008).

Molecular evaluation

The 97 plants of *P. coloratum* var. *makarikariense* and var. *coloratum* previously evaluated for agro-morphological traits were studied using ISSR and SSR markers. Ten plants of *P. bergii* population (PB) were included for comparison.

DNA extraction was carried out using a modified SDS method (Edwards *et al.*, 1991). To approximately 150 mg of homogenised leaf tissue, 700 µL of extraction buffer containing: 50 mM Tris (pH 8), 10 mM EDTA (pH 8), 100 mM NaCl, 10 mM β-mercaptoethanol and 10% sodium dodecyl sulphate (SDS), was added, and incubated at 65°C for 20 min. After adding 200 µL of 5 M potassium acetate (pH 4.8), the sample was incubated on ice for at least 20 min, and then centrifuged at 13,000 g for 20 min. This was followed by precipitation with 700 µL of iso-propanol incubated at −20°C

Table 3 Polymorphic inter-simple sequence repeats (ISSR) and simple sequence repeats (SSR) markers used in this study

ISSR		SSR			
Primer	Sequence (5′→3′)	Primer	Repeat Motif	Sequence (5′→3′)	Source
1	$(GA)_9$ T	1	$(AG)_8$ T$(AG)_7$	F: TGTATGAGCTGAGTCGC R: TGGTAATCTAGTTGATATTC	EST- *P. maximun*
2	$(CT)_8$TG	2	$(TC)_{16}$	F: GCTGAAACCAGGAAACGAAA R: CACCACACATCTGGCTTCTG	EST- *P. virgatum*
3	$(CAA)_5$	3	$(AG)_8$	F: CCCGAGGCGATCCGATTCGTT R: TACGCCGACGACGAGGACGA	EST- *P. maximun*
4	$(CAC)_4$GC	4	$(AG)_{23}$	F: GGCCATCAAGGTAACTCA R: GAAATCCGGCTGCTGGTTATA	$_g$SSRs- *P. maximun*
5	$(GACA)_4$	5	$(AT)_{13}$	F: TCCAGATGACTCCCAGGAAC R: TCATCACTCGATTCCTCAAGC	EST- *P. virgatum*
6	$(GATA)_2(GACA)_2$	6	$(GT)_{11}$-$(GA)_{12}$	F: TCAGGTTCAGGCTTCAGATG R: TAGCCGAACTGATAAGGGCT	$_g$SSRs- *P. virgatum*
7	$(ACTG)_2$ACCGACTG)	7	$(CGT)_n$	F: TCTCCAACACGCCACGAC R: CAATCCACTACAAGAAACCAC	EST- *E. curvula*
		8	$(CGA)_n$	F: CACTTGGTGCCCGATGAC R: CTTATTACGCTGGTGAACAAA	EST- *E. curvula*
		9	$(GTT)_n$	F: GCTGACAAGATGGTGAAGA R: CACTGAGACGAAGATGATG	EST- *E. curvula*
		10	$(AAG)_n$	F: ATAAAGTAAAGGTAGGCGGG R: CTGCGGAATAAACCAACT	EST- *E. curvula*
Tm: **1, 3–7**: 52 °C; **2**: 42 °C		*TD/Tm*: **1**, **4**, **6–10**: 63-53/58 °C; **2**: 70-60/65 °C; **3**: 65-55/60 °C; **5**: 50-40/45 °C			

SSR **1**, **3** and **4** (Ebina *et al.*, 2007); **2** and **5** (Tobias *et al.*, 2006); **6** (Wang *et al.*, 2011); **7–10** (Cervigni *et al.*, 2008).

for 10 min, and centrifugation at 13,000 g for 4 min. The resulting pellet was washed with ethanol 70% and dissolved in 80 µL of 1× TE buffer. DNA quality was evaluated in agarose gel and the quantity was determined by spectrophotometry.

Twenty ISSR primers (Garayalde *et al.*, 2011) were tested for amplification. After a preliminary screening, seven primers with clear and reproducible patterns were selected for further analyses (Table 3). Amplification reactions were performed in 25 µL volume containing: 20 ng of DNA template, 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 350 ng of primers and 1.5 U of Taq DNA polymerase in 1× buffer. Negative controls (no DNA template) were also included. PCR reactions were carried out in MJ Research Thermal Cycler (PTC-100, MJ Research, Inc., Watertown, MA). Initial denaturation of 94°C for 1 min 30 s, followed by 40 cycles of 94°C for 40 s, 45 s at annealing temperature (Table 3), 1 min 30 s at 72°C and a final 5 min extension at 72°C. Amplification products were resolved on 2.5% agarose gels, run at 70 V in 1× TAE for 180 min, and then visualised by staining with ethidium bromide and photographed under ultraviolet light. Each gel contained the individuals of one accession of *P. coloratum* var. *makarikariense* along with reference samples consisting of two individuals of var. *coloratum*, two of *P. bergii* and a 100-pb ladder in order to facilitate profile comparisons between the gels. ISSR bands were visually determined on the photographs.

In an attempt to increase the number of markers available for the species, 42 heterologous SSR loci were tested for amplification. They comprised 10 genomic (gSSRs) and genic (EST-SSRs) SSR from *Panicum maximum* (Ebina *et al.*, 2007; Chandra & Tiwari, 2010), nine from *P. virgatum* (Tobias *et al.*, 2006; Wang *et al.*, 2011), two from *P. miliaceum* (Hu *et al.*, 2009), and 19 from *Eragrostis curvula* (Cervigni *et al.*, 2008). A total of two gSSRs and eight EST-SSRs loci with clear and reproducible polymorphic fragments were selected for further analysis (Table 3). Amplification reactions were performed in 20 µL volume containing: 30 ng of DNA template, 2.5 mM $MgCl_2$, 0.125 mM of each dNTPs, 10 pmol of each primer and 1 U of Taq DNA polymerase in 1.6× buffer. Negative controls (no DNA template) were also included. PCR reactions were carried out in an MJ Research Thermal Cycler. The optimum annealing temperature (Tm) was determined for each locus (Table 3). By touchdown PCR (TD), the annealing temperature was decreased to 1°C starting with 5°C over the set annealing temperature. Initial denaturation step of 95°C for 3 min, followed by 10 touchdown cycles of 94°C for 30 s, touchdown annealing temperature for 30 s and 72°C for 45 s. PCR products were subsequently amplified for 34 cycles at 94°C for 30 s, annealing temperature for 30 s, and 72°C for 45 s with a final extension at 72°C for 20 min. PCR products were analysed on 6% denaturing polyacrylamide gel, with a TBE 1× electrophoresis buffer at 50 W for 1 h and 45 min to

2 h. Forty-five individuals and five positive and negative controls were loaded onto each gel. Bands were visualised by silver staining (Benbouza *et al.*, 2006) and scanned.

ISSR and SSR profiles were scored as binary presence/absence data. Only unambiguous bands across all the samples were considered. Genetic diversity was estimated in each accession using the percentage of polymorphic loci (%P), number of accession specific bands and mean genetic distances (MGD). This last measure was obtained as the average of the genetic distances among individuals in each accession. Dissimilarity between pairs of individuals was estimated through genetic distances (GD) (Huff *et al.*, 1993). Total genetic variation was partitioned into three levels via analysis of molecular variance (AMOVA): between individuals within accessions, between accessions within varieties or species and between varieties or species (Excoffier *et al.*, 1992; Peakall *et al.*, 1995). Variation was expressed both as the proportion of the total variance and as φ-statistics (F-statistic analogues): φ_{PR}, φ_{RT} and φ_{PT}. φ_{PR} represents the correlation between individuals within an accession relative to that of individuals from the same variety or species, the correlation of individuals from the same variety or species, relative to that of individuals of the total, and the correlation between individuals within an accession, relative to that of individuals of the total, respectively.

Pairwise φ_{PT} values between all pairs of accessions were calculated. Statistical significance was tested through 1000 random permutations. Principal coordinate analyses (PCO) and cluster analyses (UPGMA) were performed based on GD genetic distance. All molecular data were analysed using GenAlEx 6 (Genetic Analysis in Excel; Peakall & Smouse, 2006, 2012) and Infostat programs (Di Rienzo *et al.*, 2008).

Correlation analysis

Correspondence between phenotypic and molecular matrices containing Euclidean and GD, respectively, was investigated through a Mantel test (Mantel, 1967). Statistical significance was determined using 1000 random permutations, with the Infostat program (Di Rienzo *et al.*, 2008). In this test, a correspondence measure (r_{xy}) is calculated between the elements of two matrices X and Y.

Results

The collection of var. *makarikariense* contains considerable morphological variation and differs from the commercial cultivar in some traits

The mean values for 18 agro-morphological traits in accessions of *P. coloratum* are shown in Table 4. Some trends in variation could be observed. Based on the MED, the IFF and CM accessions showed the highest intra-accession variability, whereas the lowest values were observed in MR and BR.

PCA biplot based on phenotypic variability of var. *makarikariense* is shown in Fig. 1. The first two axes explained 43% of the variation and most variables attained a percentage of reconstruction of more than 50%, except for LWSu, PL, PN, LER and Phy. Individuals were widely spread and did not congregate according to the accessions. In accordance with the MED values, the IFF clonal materials and CM accession were the most diverse within the collection, whereas MR and BR individuals tended to form more cohesive groups. Regarding accession characterisation, BR showed the lowest values in the majority of the evaluated characters, while DF and MR involved individuals with greater width and length of leaf, dry leaf weight and height per plant.

When the agro-morphological means of each accession were compared to cv. 'Bambatsi' (CM), DF and MR showed at least 10% better performance than CM in four traits related to forage production (Table 4). On the other hand, the DF, UCB and MR accessions showed higher rates of leaf elongation in relation to CM and all accessions required comparatively fewer degree days for leaf appearance. On the contrary, these accessions all presented lower values than CM in traits related to seed production.

Fig. 2 shows the PCA analysis with the inclusion of one accession of var. *coloratum*. The first two axes explained 53.4% of the variation; most variables were represented in this two dimensional plane with a reconstruction over 50%, except PN, LER and Phy. Individuals of var. *coloratum* (CH) were clearly distinguishable from those of var. *makarikariense*, mainly for their lower values in length, width and weight of leaves, 1000-seed weight, aerial biomass dry weight and LER.

Molecular markers reveal genetic differentiation between accessions, varieties and species

We first evaluated the transferability of SSR markers across species and genera. Out of 21 gSSRs and EST-SSRs developed for *P. maximum*, *P. virgatum* and *P. miliaceum*, six (28.6%) were successfully amplified in *P. coloratum* (Table 3), 12 (57.2%) rendered ambiguous PCR profiles, and 3 (14.3%) failed amplification. Out of 19 EST-SSRs belonging to *E. curvula*, 4 (21.1%) showed clear banding patterns, and 1 (5.3%) and 14 (73.7%) produced a low-quality profile or lack of amplification, respectively.

The molecular analysis of 87 individuals of *P. coloratum* var. *makarikariense* produced 117 ISSR reproducible bands of which 103 were polymorphic (88%). The number of amplified bands per primer ranged from 4 to 22 and the

Table 4 Mean (M), standard error (SE), relative performance (*Rp*) and mean Euclidean distance (MED) in *P. coloratum* accessions for 18 agro-morphological traits

Accession	DF		UCB		MR		BR		ER		CM	CH	
Trait	M ± SE	*Rp*	M ± SE	*Rp*	M ± SE	*Rp*	M ± SE	*Rp*	M ± SE	*Rp*	M ± SE	M ± SE	Grand means[a]
PH	65.0 ± 2.7		66.9 ± 1.6		75.8 ± 1.4	+13	34.8 ± 1.4	−48	71.6 ± 2.6		67.4 ± 3.3	68.9 ± 0.9	63.58
LLS	38.2 ± 2.5		32.3 ± 1.2	−15	36.5 ± 1.0		27.0 ± 1.2	−29	28.8 ± 1.8	−24	38.0 ± 2.0	24.7 ± 1.0	33.47
LLSu	23.6 ± 1.2		25.3 ± 1.3		26.8 ± 1.4		19.9 ± 0.4	−22	22.7 ± 1.2	−11	25.6 ± 1.2	16.3 ± 0.9	23.98
FLL	25.0 ± 0.8		22.6 ± 0.8		24.2 ± 0.5		20.1 ± 0.9	−15	21.9 ± 1.1		23.7 ± 1.3	12.8 ± 0.6	22.92
LWS	1.01 ± 0.03	+12	0.98 ± 0.02	+10	0.89 ± 0.02		1.00 ± 0.02	+11	0.92 ± 0.04		0.90 ± 0.04	0.67 ± 0.03	0.95
LWSu	1.06 ± 0.04	+14	0.96 ± 0.03		0.90 ± 0.02		0.93 ± 0.02		0.96 ± 0.03		0.93 ± 0.05	0.55 ± 0.04	0.96
FLW	1.06 ± 0.04	+19	0.94 ± 0.03		1.03 ± 0.01	+16	0.96 ± 0.03		0.92 ± 0.05		0.89 ± 0.04	0.56 ± 0.03	0.97
LDWS	162.2 ± 9.4		143.5 ± 8.8	−10	149.7 ± 5.5		123.0 ± 7.8	−23	104.8 ± 9.3	−35	160.0 ± 11.3	57.3 ± 4.6	140.53
LDWSu	111.6 ± 8.3	−11	114.5 ± 8.8		96.0 ± 28.5	−24	83.9 ± 4.0	−33	106.9 ± 7.3	−15	125.6 ± 7.9	39.7 ± 4.6	106.42
FLDW	118.9 ± 9.2	+14	96.4 ± 5.8		109.6 ± 8.2		79.6 ± 5.7	−24	89.3 ± 9.1	−14	104.2 ± 6.4	28.0 ± 3.2	99.67
PL	60.2 ± 1.2		61.1 ± 3.4		72.7 ± 1.3	+16	67.5 ± 3.4		67.7 ± 2.5		62.8 ± 2.3	46.0 ± 2.2	65.33
PN	98.0 ± 10.8	−35	144.0 ± 12.2		151.7 ± 16.5		129.0 ± 15.2	−15	122.7 ± 13.1	−19	151.8 ± 26.1	245.3 ± 31.9	132.87
1000-SW	1196.4 ± 12.4		1134.7 ± 22.0		1099.4 ± 4.8		1053.6 ± 6.0	−13	1169.0 ± 37.8		1204.3 ± 20.9	978.8 ± 28.1	1142.90
DW	210.2 ± 13.7	−12	204.3 ± 15.9	−14	389.2 ± 19.9	+63	238.6 ± 32.2		202.5 ± 31.1	−15	238.6 ± 40.0	90.5 ± 7.8	247.23
LERS	3.8 ± 0.2	+23	4.3 ± 0.3	+39	4.0 ± 0.2	+29	3.3 ± 0.2		2.7 ± 0.3	−13	3.1 ± 0.4	2.6 ± 0.1	3.53
LERSu	3.9 ± 0.4	+15	3.9 ± 0.3	+15	4.1 ± 0.2	+21	3.4 ± 0.2		3.4 ± 0.3		3.4 ± 0.2	1.9 ± 0.2	3.68
PhyS	97.8 ± 8.2	−27	89.9 ± 7.5	−33	89.5 ± 5.9	−33	82.9 ± 6.2	−38	107.9 ± 11.2	−20	134.2 ± 15.3	112.1 ± 6.7	100.37
PhySu	82.7 ± 6.2		70.3 ± 7.5	−22	77.5 ± 6.6	−14	69.4 ± 7.9	−23	79.5 ± 8.1	−12	89.8 ± 8.9	109.3 ± 12.2	78.20
MED	25.1		23.0		13.9		15.5		32.8		38.4	36.0	

n = 12 individuals per accession.
Rp, positive and negative numbers indicate higher or lower values than CM, respectively. *Rp* values lower than 10% are not shown.
MED from IFFIVE = 41.3.
See table 1 and 2 for a fuller description of the accessions and variables evaluated.
[a]Mean across var. *makarikariense* accessions.

percentage of polymorphic loci per primer varied from 70.6 to 100.0%. All analysed SSR loci were polymorphic and produced a total of 48 alleles ranging from two to nine alleles per locus. When one accession of *P. coloratum* var. *coloratum* (CH) and one population of *P. bergii* (PB) were added to the analysis, a total of 157 reproducible ISSR bands (97.5% polymorphic) and 81 SSR alleles were recorded (data not shown).

Differences in genetic diversity were observed between accessions within var. *makarikariense* (Table 5). According to the percentage of polymorphic loci and the MGD estimated with ISSR and SSR markers, IFF, CM and DF were the most variable accessions, the opposite of MR and BR. Accession-specific ISSR bands and SSR alleles were found in all accessions, particularly in MR and BR.

In the whole analysis, var. *coloratum* (CH) and *P. bergii* (PB) accessions appeared to contain more genetic variation than var. *makarikariense* (Table 5). Those materials showed high values for the percentage of polymorphic loci, total number of alleles and number of accession specific alleles using both ISSR and SSR makers, most of them being higher than in the var. *makarikariense* accessions.

PCO analysis based on the ISSR distance matrix is shown in Fig. 3. The first two axes explained 52.9% of the variation. Individuals of BR, MR and IFF grouped rather cohesively according to their provenance whereas individuals of CM and ER accessions and of DF and UCB accessions were intermixed. All individuals separated according to provenance in the dendrogram based on the individual GD ISSR distance matrix (Fig. 4, $r = 0.972$). Fig. 5 shows the PCO analysis based on individual SSR distance matrix. The first two axes explained 52.5% of the variation. Individuals in the SSR-PCO diagram showed a more widespread arrangement in comparison to ISSR-PCO (Fig. 3) with only BR and MR individuals tending to group together. Similar associations were observed from a dendrogram based on the individual GD SSR distance matrix (Fig. 6, $r = 0.958$). Concerning the inter-accession differences, all the pair comparisons displayed significant φ_{PT} ($P < 0.01$) ranging from 0.61 to 0.94 for ISSR and 0.12–0.68 for SSR markers. BR and MR accessions showed the largest φ_{PT} values within the var. *makarikariense*, estimated with both markers (Table 6).

AMOVA performed on var. *makarikariense* ISSR data showed that 77% of the molecular variability is attributable to accession differences. All seven ISSR initiators were coincident and consistently revealed that between-accession differences accounted for most of the total genetic variation, ranging from 62% to 100%. In contrast, AMOVA performed on SSR data detected 37% of molecular variability attributable to differences between accessions. However, the partition of variance

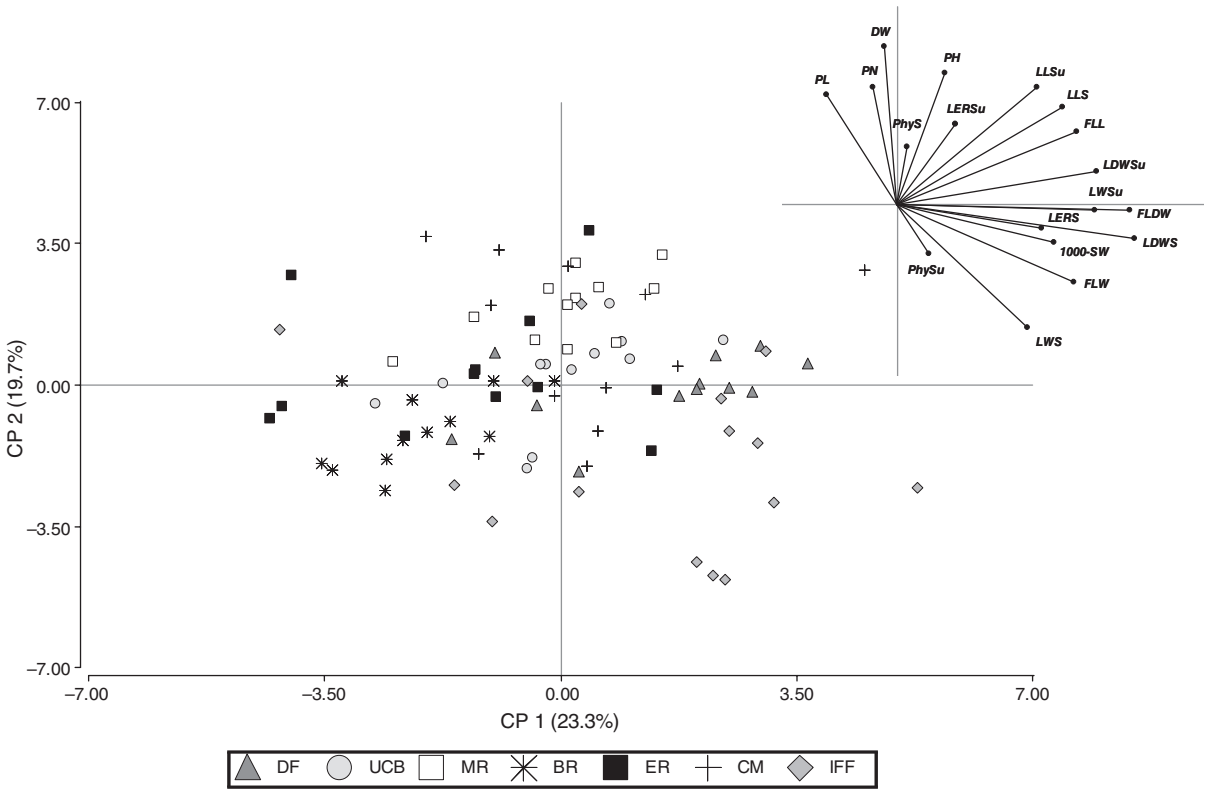

Figure 1 Individual and variable plots of principal component analyses (PCA) based on the individual distance matrix of *P. coloratum* var. *makarikariense* calculated from 18 agro-morphological traits. See Tables 1 and 2 for a fuller description of the accessions and variables evaluated.

widely differed across the SSR loci. Loci 1, 2, 4, 6 and 8 detected most of the genetic diversity (>67%) within accessions whereas for locus 7 the major component of variance resided between the accessions (70%). SSR loci 3, 5 and 9 showed a similar partitioning of variance into within and between accessions.

PCO analysis performed on ISSR and SSR data including var. *coloratum* (CH) and *P. bergii* (PB) are shown in Figs 7 and 8, respectively. The first two axes explained 67.3 and 75.9% of the total variability. Individuals of *P. coloratum* var. *makarikariense*, var. *coloratum* and *P. bergii* formed three definite groups using both types of molecular markers. In line with these results, all the pair-accession comparisons between var. *makarikariense* with var. *coloratum* and *P. bergii* displayed significant φ_{PT} ($P < 0.01$, Table 6), ranging from 0.64 to 0.85 for ISSR and 0.36–0.83 for SSR markers. In addition, AMOVA identified significant partitioning ($P < 0.001$) of molecular variation between varieties (35% for ISSR and 50% for SSR markers) and between species (33% for ISSR and 51% for SSR markers, Table 7). This is in accordance with the presence of 49 accession specific ISSR bands in var. *makarikariense*, six in var. *coloratum* (CH) and 15 in *P. bergii* (PB), and 26 accession specific SSR alleles in var. *makarikariense*, 18 in var. *coloratum* (CH) and 14 in *P. bergii* (PB).

The correspondence analysis between phenotypic and molecular distances among individuals of var. *makarikariense* showed a significant positive low correlation between the agro-morphological and ISSR matrices ($r = 0.26$, $P < 0.0001$), and between the agro-morphological and SSR matrices ($r = 0.13$, $P = 0.012$). In addition, a significant positive moderate correlation was found between the ISSR and SSR matrices ($r = 0.48$, $P < 0.0001$).

Discussion

Wide morphological diversity was found in this *P. coloratum* var. *makarikariense* collection. This may be considered an unexpected result given the fact that this variety was introduced to cattle production systems in Argentina not long ago, through few known events (Petruzzi *et al.*, 2003), but it otherwise agrees with limited selection operating since it was introduced. Phenotypic variation has previously been mentioned for *P. coloratum* at the species level (Bryant, 1967; Pritchard & De Lacy, 1974; Young, 1994). Morphological features that show

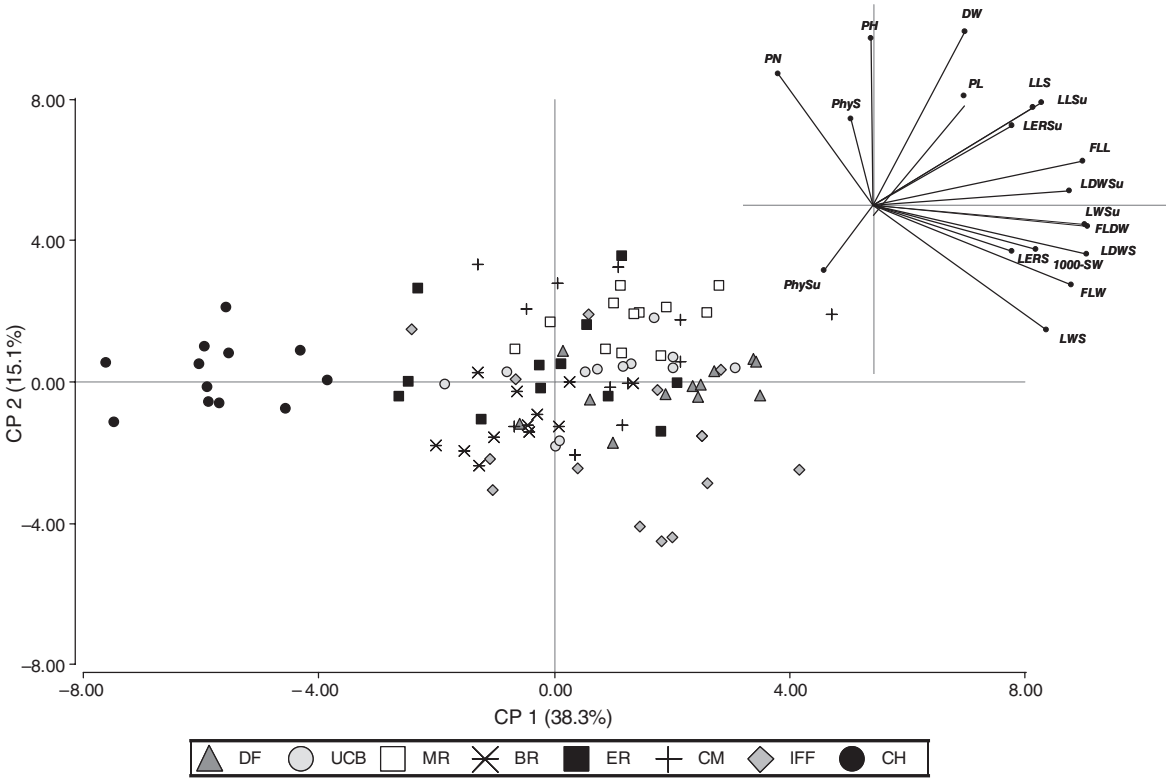

Figure 2 Individual and variable plots of principal component analyses (PCA) of *P. coloratum* var. *makarikariense* and var. *coloratum* calculated from 18 agro-morphological traits. See Tables 1 and 2 for a fuller description of the accessions and variables evaluated.

Table 5 Genetic diversity parameters per accession for ISSR and SSR markers

		DF	UCB	MR	BR	ER	CM	IFF	CH	PB
ISSR	Nl	65	61	64	58	65	66	66	74	84
	%P	16.6	14.0	7.6	3.8	15.3	18.5	23.6	28.0	33.8
	MGD	9.00	8.64	4.61	1.27	10.68	11.65	11.56	17.39	26.67
	NlSp	2	2	8	6	6	5	9	–	–
	NlSp*	49							6	15
SSR	Na	26	30	26	18	24	28	20	41	24
	%P	90	90	50	40	70	90	60	90	60
	MGD_a	8.61	7.32	3.05	1.49	6.38	7.42	5.11	12.36	6.89
	NaSp	0	4	7	1	2	3	1	–	–
	NaSp*	26							18	14

Total number of loci and alleles (Nl and Na, respectively). Percentage of polymorphic loci (%P), mean genetic distances (MGD), number of accession specific loci and alleles (NlSp and NaSp, respectively), number of accession specific loci and alleles in var. *makarikariense*, var. *coloratum* (CH) and *P. bergii* (PB) (NlSp* and NaSp*). See Table 1 for a fuller description of the accessions evaluated.

the difference between var. *makarikariense* and var. *coloratum* have recently been described (Armando *et al.*, 2013). Among the traits evaluated in the present study, the greatest variation was found in characteristics related to forage biomass and seed production (dry weight per plant and number of panicles per plant, CV = 52.2 and 51.5%, respectively). As the field study was conducted under common garden conditions, the results indicate that the genetic variation contained in the collection is useful for breeding. In contrast, the leaf width and 1000-seed weight were quite constant (CV = 13 and 8%, respectively), the latter being described as a fairly stable yield component (Sadras & Slafer, 2012). Despite the relatively low variability observed for 1000-seed weight within the var. *makarikariense* collection, an increase of 17% after two cycles of recurrent phenotypic selection could be obtained for this trait (Giordano *et al.*, 2013). This genetic gain in seed weight has improved

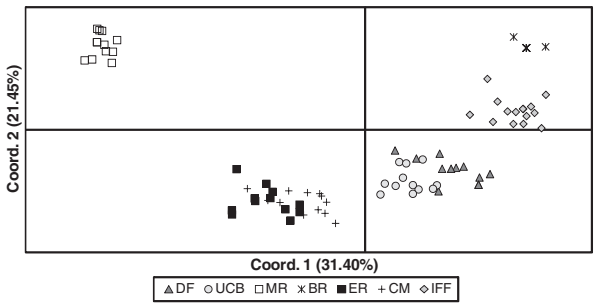

Figure 3 Principal coordinate analysis (PCO) plot based on the individual ISSR distance matrix of *P. coloratum* var. *makarikariense*. See Table 1 for a fuller description of the accessions evaluated.

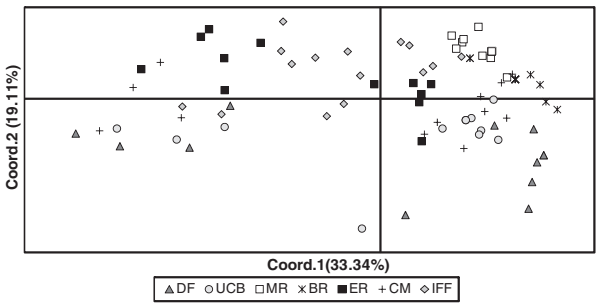

Figure 5 Principal coordinate analysis (PCO) plot based on the individual SSR distance matrix of *P. coloratum* var. *makarikariense*. See Table 1 for a fuller description of the accessions evaluated.

the establishment of pasture lots, a trait frequently claimed by farmers. It is worth noting that leaf width, a morphological trait with very little variation within var. *makarikariense*, showed a significant contribution to the differentiation between var. *makarikariense* and var. *coloratum* (Armando *et al.*, 2013).

Agro-morphological variability was observed both within and between accessions of var. *makarikariense*. As accessions were in general quite variable, separation between them was frequently not sharp causing a certain degree of phenotypic overlapping. In contrast, the BR accession revealed a particular morphotype characterised

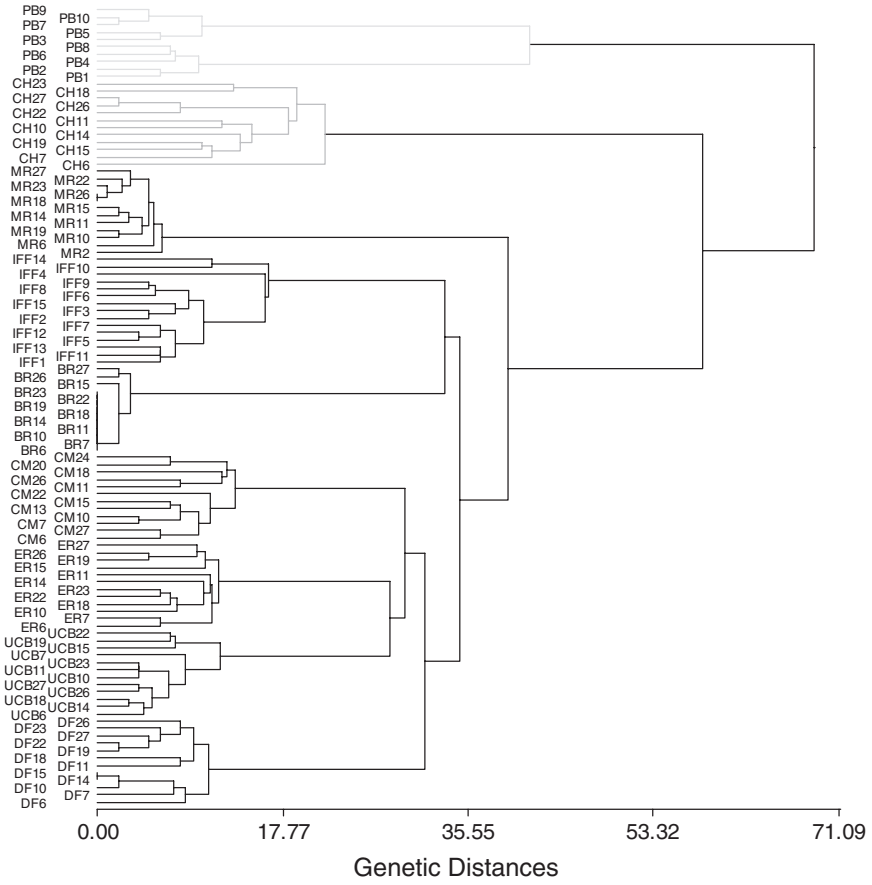

Figure 4 Unweighted pair-group method with arithmetical average (UPGMA) dendrogram based on the GD ISSR distance matrix of *P. coloratum* var. *makarikariense* and var. *coloratum* and *P. bergii*. See Table 1 for a fuller description of the accessions evaluated.

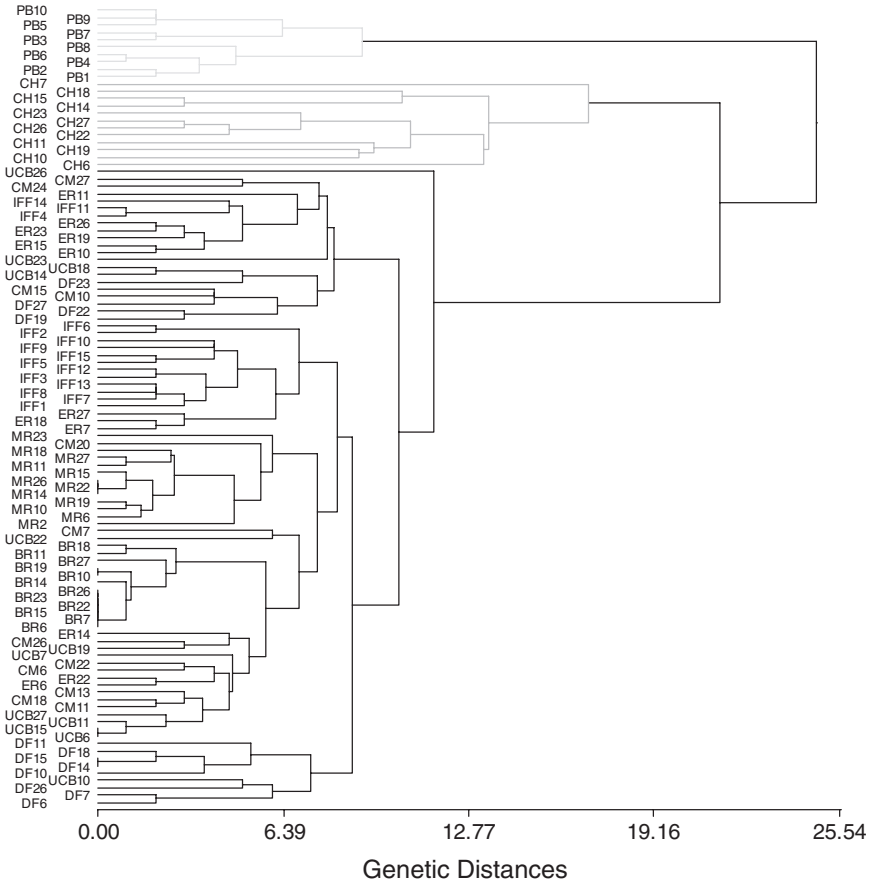

Figure 6 Unweighted pair-group method with arithmetical average (UPGMA) dendrogram based on the GD SSR distance matrix of *P. coloratum* var. *makarikariense* and var. *coloratum* and *P. bergii*. See Table 1 for a fuller description of the accessions evaluated.

Table 6 Pairwise φ_{PT} values between all pairs of accessions.

Coefficients	DF	UCB	MR	BR	ER	CM	IFF	CH	PB
DF	–	0.147	0.429	0.452	0.264	0.252	0.347	0.505	0.701
UCB	0.703	–	0.449	0.422	0.171	0.120	0.326	0.566	0.729
MR	0.833	0.827	–	0.681	0.445	0.452	0.535	0.648	0.809
BR	0.847	0.854	0.935	–	0.518	0.443	0.573	0.648	0.834
ER	0.712	0.656	0.783	0.847	–	0.181	0.240	0.566	0.724
CM	0.660	0.670	0.785	0.828	0.605	–	0.359	0.359	0.359
IFF	0.677	0.657	0.803	0.792	0.693	0.684	–	0.589	0.732
CH	0.747	0.763	0.812	0.845	0.770	0.752	0.763	–	0.623
PB	0.638	0.753	0.769	0.801	0.798	0.756	0.737	0.729	–

All values are significantly different from zero ($P < 0.01$).
φ_{PT} ISSR values are shown below diagonal and φ_{PT} SSR values are shown above diagonal.
Probability values were based on 999 permutations.

by plants of low height (almost crawling), shorter leaves and smaller seeds (Fig. 1; Table 4). Conversely, the DF and MR accessions comprised taller individuals with larger leaf size, even larger that the cultivar commercialised in Argentina, indicating there might be an opportunity for selection to improve characters such as forage production. The DF and MR accessions also showed a faster rate of regrowth after defoliation (higher LER and lower Phy) compared to the commercial material. As the rates of leaf and tiller appearance are closely associated (Skinner & Nelson, 1994), one might expect that accessions with shorter Phy would eventually be good forage producers. Knowledge of the leaf development rate in tillers of a perennial grass accession provides useful information for

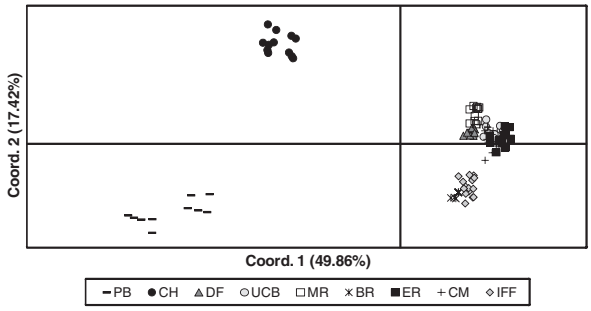

Figure 7 Principal coordinate analysis (PCO) plot based on the individual ISSR distance matrix of *P. coloratum* var. *makarikariense* and var. *coloratum* and *P. bergii*. See Table 1 for a fuller description of the accessions evaluated.

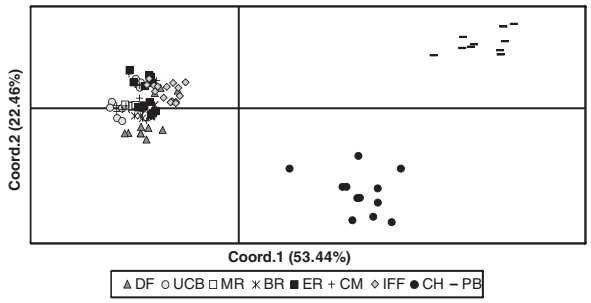

Figure 8 Principal coordinate analysis (PCO) plot based on the individual SSR distance matrix of *P. coloratum* var. *makarikariense* and var. *coloratum* and *P. bergii*. See Table 1 for a fuller description of the accessions evaluated.

determining the timing of management practices, such as defoliation, fertilisation and the use of growth regulators (Moore & Moser, 1995).

The *coloratum* and *makarikariense* varieties could be easily distinguished by their phenotypical traits. Only a few individuals of var. *coloratum* were analysed in this study, nonetheless the results agree with a previous report (Armando *et al.*, 2013) in which 72 individuals of var. *makarikariense* performed better as a forage and seed producer in comparison to 80 individuals of var. *coloratum*. Other features, such as higher sensitivity to low temperatures and/or the limited availability of certified seeds, might be possible reasons to explain why it is not as widespread as var. *coloratum* in Argentina, even though var. *makarikariense* shows superior performance as a forage crop.

The molecular analysis demonstrated SSR transferability of ~24% (10/42) between *Panicum* species and *E. curvula*. This level of SSR transference was comparable with a previous report (Chandra & Tiwari, 2010), although it differed from other studies that found percentages of transferability of 10% (Huang *et al.*, 2011) and 46% (Hu *et al.*, 2009). Within the 10 selected SSR loci, 80% corresponded to EST-SSRs and 20% to gSSRs. A higher transferability of SSR from conserved transcribed regions in comparison to random genomic SSR is a common observation reported in the literature (Cordeiro *et al.*, 2001; Kantety *et al.*, 2002).

By combining ISSR and SSR markers, an extensive genetic variation in *P. coloratum* var. *makarikariense* was revealed, with 88% of ISSR bands and 100% of SSR loci being polymorphic. The presence of unique bands in all accessions, in particular BR and MR, suggests distinction and limited gene flow between accessions while emphasising the importance of preserving dissimilar materials in the collection. Commercial accession (CM), currently commercialised as cv. 'Bambatsi' in Argentina, showed high molecular variability. High levels of variation were also reported in a previous morphological study (Armando *et al.*, 2013), in all suggesting that this variety could still be significantly modified by selection.

The ISSR and SSR marker systems differed in the between-accession component of variance (77% and 37%, respectively). First, both classes of markers scan different portions of genomes, varying in levels of polymorphisms. Moreover, the higher genetic differentiation shown between individuals using SSR markers rather than ISSR markers is undoubtedly related to the application of more sensitive methods of separation and detection of alleles. An additional effect might originate in the binary codification system used for SSR alleles at polyploid level given that it is not possible to register the amplification profile in terms of co-dominant alleles at each locus, as typically occurs in diploid species. Despite these differences, the two marker systems detected significant genetic variation in var. *makarikariense* at the accession level.

The whole genetic analysis showed that the var. *coloratum* accession Chacharramendi (CH) and the *P. bergii* population (PB) presented greater genetic variability than var. *makarikariense*. The presence of more developed rhizomes in var. *makarikariense* compared to those in var. *coloratum* (Bogdan, 1977; Armando *et al.*, 2013), might favour vegetative propagation, thus reducing within-accession variance. Moreover, the comparatively lower genetic variability in var. *makarikariense* could be due to a lower number of introductions in comparison to var. *coloratum*, which was the first variety adopted as forage and it has spread through the semiarid regions of Argentina (Ruiz *et al.*, 2008; Ferri, 2011).

Molecular analyses defined var. *makarikariense*, var. *coloratum* and *P. bergii* as genetically differentiated groups. Interestingly, the magnitude of the difference between var. *makarikariense* and var. *coloratum* was comparable to that encountered between *P. coloratum* and *P. bergii*. In the same line of evidence, high genetic differentiation and

Table 7 Analysis of molecular variance (AMOVA) and sources of variation

	Var. *makarikariense*		*P. coloratum*		*Panicum* species	
% Variance	ISSR %	SSR %	ISSR %	SSR %	ISSR %	SSR %
Between varieties or species	–	–	35	50	33	51
Between accessions/varieties or species	77	37	49	17	49	22
Individuals/ accessions	23	63	16	34	18	26
φ_{RT}	–	–	0.350	0.495	0.329	0.513
φ_{PR}	–	–	0.748	0.334	0.738	0.461
φ_{PT}	0.769	0.370	0.836	0.664	0.824	0.737

ISSR, inter-simple sequence repeat; SSR, simple sequence repeats.

$\varphi_{RT}\varphi_{PR}$ and φ_{PT} are defined in the Materials and Methods. All φ values were significant ($P < 0.001$).

some grade of reproductive isolation between *P. coloratum* varieties has been observed (Komatsu *et al.*, 2007).

The low correspondence between morphological and molecular variation (Morph. *vs* ISSR = r = 0.26 year Morph. *vs* SSR = $r = 0.13$) is a common observation that can be addressed to different forces shaping both classes of traits. Variability in quantitative morphological traits is the rough material for both natural and artificial selection to shape populations by means of adaptation. Most of the DNA markers constitute a sample of random genomic sites in which polymorphism has no effect on fitness and they are therefore the outcome of neutral genetic variation (Holderegger *et al.*, 2006). On the other hand, significant positive correlations ($r = 0.50$) between different marker systems, as reported in this study, are common in the literature (Budak *et al.*, 2004; de Lima *et al.*, 2011; Huang *et al.*, 2012). This analysis showed that ISSR and SSR molecular markers do not vary in terms of comparability with morphological traits and that the data from these three systems provide complementary information.

Identification and differentiation of accessions within the collection of *P. coloratum* var. *makarikariense* was possible using the phenotypic traits and molecular markers combined. The number of accession specific bands and alleles does not seem to support a common origin for the currently commercialised cultivar and the rest of the accessions. A more extensive sampling is necessary to confirm this point. Additionally, the set of SSR defined in this study is of great importance in a species such as *P. coloratum*, in which molecular variability has scarcely been studied and sequence information is almost absent.

Knowledge of genetic variation and the relationships between accessions of *P. coloratum* is an important step towards germplasm management and preservation. The collection at Rafaela was set up with materials collected from different sites in Argentina that have been exposed to selective pressures for several years, mainly driven by severe grazing and extreme weather conditions. The variability encountered in the collection opens new perspectives for genetic improvement.

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