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Genetic and morphological characterization of *Acroceras macrum* Stapf

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Abstract

Acroceras macrum is a warm-season grass used for pastures, and it has high nutritive value. The scarce genetic and reproductive information about this grass is limiting its breeding for forage. The objective of this research was to determine the number of genotypes and cytotypes present in a collection established in Argentina, and to estimate the diversity of this species based on molecular markers, and morphological and agronomic characteristics. The number of genotypes among forty-seven accessions was determined using ISSR markers. The identified genotypes were further characterized for ploidy levels. In addition, the genetic distances among them were estimated with ISSR markers. They were also propagated and planted into the field following a randomized complete block design with four replications, and a group of sixteen morphological and agronomic variables were evaluated. A total of twenty-seven genotypes were identified in this collection; twenty-two genotypes were tetraploid ($2n = 4x = 36$) and five were hexaploid ($2n = 6x = 54$). These two cytotypes were found clearly separated by both genetic and phenotypic characteristics. Above-ground biomass, internode length, initial growth and number of spikelets per inflorescence were the most variable traits. The high diversity present in this collection will allow for the initiation of a breeding programme for *A. macrum*.

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Introduction

Acroceras macrum Stapf, Nilegrass, is a warm-season grass native to tropical and subtropical Africa and is cultivated as a species of permanent pastures in grazing systems around the world (Skerman and Riveros, 1992). It grows well in humid areas with poorly drained soils (Rhind and Goodenough, 1979; Yang *et al.*, 2007). The species has a C_3 leaf anatomy (Oliveira *et al.*, 1973; Zuloaga *et al.*, 1987), which is unusual among cultivated warm-season forage grasses (Moser *et al.*, 2004). This characteristic of *A. macrum* seems to be associated with the high nutritive value observed at different stages of maturity. Typically, C_3 grasses have greater concentrations of crude protein and soluble carbohydrates, but lesser concentrations of cell-wall components such as cellulose and hemicellulose, when compared with C_4 grasses at similar stages of maturity (Coleman *et al.*, 2004). Moreover, it has been observed that *A. macrum* also maintains a good nutritive value in swards when grazed during the winter as stockpiled forage (Rhind and Goodenough, 1979).

Acroceras macrum is a rhizomatous and stoloniferous species that is vegetatively propagated (Skerman and Riveros, 1994). Although this mode of propagation limits its adoption by farmers, very little information is available about its mode of reproduction and the potential causes for the observed low seed yield and seed germination (Rhind and Goodenough, 1979). It is known that different ploidy levels exist for *A. macrum*, including tetraploid ($2n = 4x = 36$), pentaploid ($2n = 5x = 45$) and hexaploid ($2n = 6x = 54$) cytotypes (Moffett and Hurcombe, 1949; Rhind and Goodenough, 1976).

Germplasm of *A. macrum* was introduced to Argentina between 1970 and 1997 with the objective of

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evaluating its performance under local conditions. Its adaptation has been proved to be excellent, especially in north-eastern Argentina. However, the lack of information related to chromosome numbers, breeding system and plant diversity has not allowed for the initiation of a programme for the genetic improvement of this species.

Perennial-grass breeding involves several phases, including germplasm collection and evaluation, breeding and selection, small-plot evaluation trials that are often conducted at multiple locations and grazing trials for performance evaluation of advanced strains before they are released as cultivars (Vogel and Burson, 2004). The time needed for the first phase of germplasm characterization depends on the available information for a particular species. Previous research in South Africa attempted to characterize this species for its agronomic performance, ploidy levels and mode of reproduction (Rhind and Goodenough, 1976, 1979). These authors reported that although they were not able to establish the mode of reproduction present in *A. macrum*, high variation was observed among hybrids generated by crossing different strains, indicating that a sexual process was involved. They also indicated, however, that the occurrence of apomixis should not be disounted.

Considering the limited availability of information, the germplasm of *A. macrum* introduced in Argentina needs to be first characterized for its diversity and ploidy levels, and then the mode of reproduction needs to be defined for this species before a breeding programme can be initiated. Thus, the objective of this research was to identify the genotypes and cytotypes present in a collection of *A. macrum* and evaluate the diversity in this germplasm using Inter-Simple Sequence Repeat (ISSR) markers, and morphological and agronomic traits.

Materials and methods

Plant material

The germplasm of *A. macrum* used for this research was originally planted at the INTA EEA Corrientes experimental station (27°40′S, 58°45′W) near the City of Corrientes, Argentina. An area of approximately 150 m^2 was planted in 1995 with fifty breeding lines imported from the Cedara Agricultural Research in South Africa. These fifty lines were hybrids and ecotypes that represented the collection kept in South Africa for a now-terminated breeding programme for *A. macrum*. The area in Corrientes was abandoned a few years after planting, and subsequently used for grazing. In 2011, the area was mown and plants of *A. macrum* were identified covering several small patches. With the objective of recovering this germplasm, forty-seven small cuttings were gathered from each remaining patch and planted in a greenhouse at the University of Northeastern Argentina.

Identification of genotypes

Genomic DNA was obtained using 50 mg of the basal portion of young fresh leaves from each of the forty-seven plants, following the protocol described by Brugnoli *et al.* (2014). A genotype of *Paspalum notatum* (accession V14327 from Capivari, RS, Brazil) was used as a control. The genomic DNA was quantified by visual comparison to a known standard by electrophoresis in agarose 1% gels in 1× TAE buffer (40 mM Tris-HCl; 5 mM NaOAc; 0·77 mM EDTA; pH 8·0) at 40 V for 2 h. Genomic DNA was visualized under UV light and photographed with the GelDoc-It® Imaging System (UVP LLC, Upland, CA, USA) after staining with ethidium bromide (10 mg mL^{-1}). Each DNA sample was adjusted to 10 ng μL^{-1} for their use in PCR amplifications.

The number of genotypes in the collection was identified using ISSR marker analysis. Primers used were generated with repeat motif of eight dinucleotides, five trinucleotides or four tetranucleotides, plus one or two 3′selective nucleotides and PCR was performed according to the methodology described by Cidade *et al.* (2008). Ten primers were tested, and five were selected for further analysis based on polymorphism and good band resolution (Table 1).

The PCR mix (25 μL) for the ISSR analysis included 10 μg templated genomic DNA, 1× PCR buffer (Promega®, Madison, WI, USA), 0·25 mM of each dNTPs, 0·2 mM primer and 1 U of *Taq* polymerase (Promega®). Amplification was performed in a MyCycler Thermal Cycler (Bio-Rad®, Hercules, CA, USA) programmed with an initial denaturation at 94°C for 5 min and final extension at 72°C for 5 min with the intervening 40 cycles of 94°C for 1 min, 48–60°C (depending of the primer; Table 1) for 1 min, and 72° for 1 min. PCR products were supplemented with 5 μL of loading buffer (40% w/v sucrose, 0·25% bromphenol blue, 0·25% w/v, xylencianol, 0·25% w/v). Electrophoretic separation of the PCR products was performed on 2% agarose gels at 55 V for 4 h in 1× TAE buffer, and the gels were stained with ethidium bromide. The molecular profiles were visualized under UV light, photographed and stored for further analysis with the GelDoc-It® Imaging System.

Identification of cytotypes

Once the different genotypes were identified, ploidy levels were determined through chromosome counting

Table 1 Inter-simple sequence repeat molecular characterization of twenty-seven genotypes of *Acroceras macrum*: analysed primers, annealing temperature, number of amplified loci and polymorphism index obtained with each primer. The information is provided for the tetraploid and hexaploid cytotypes.

Primer		Number of *loci*			Polymorphism index (%)		
	Ta (°C)	Total	4×	6×	Total	4×	6×
$(ATG)_5GA$	42	25	24	16	96	92	81
$(GA)_8T$	46	14	10	11	85	90	73
$(AG)_5T$	46	24	24	18	100	100	56
$(GA)_8C$	48	14	12	11	100	92	64
$(CA)_8G$	48	23	22	11	96	100	82
$(GT)_8C$	48	14	12	7	85	100	57
$(GA)_8TC$	50	20	17	14	95	88	57
$(AG)_8GC$	52	33	31	21	97	100	86
$(AGAC)_4GC$	52	20	19	15	100	100	53
$(CTC)_6AC$	60	28	26	20	93	100	85
Total		215	197	144	95	97	71

in meristematic cells of roots. Root tips of young plants grown in pots in the greenhouse were collected in the morning (between 08.00 and 09.00), washed and pre-treated in a saturated solution of α-bromonaphthalene for four and a half hours.

After pre-treating, the solution was discarded and the roots were softened with a solution of 1N HCl at 60°C for 10 min. The root tips were transferred to a new tube containing Feulgen's staining solution and stained until the meristematic region turned purple. The meristematic portion of the root tip was dissected and separated from the root cap using a small knife. The dissection was carried out using an EZ4 (Leica®, Wetzlar, Germany) stereo microscope. One drop of acetocarmine was placed on a new glass slide and the portion of root dissected was transferred to it and macerated. The rest of the tissue was removed, a cover slip was then placed on the macerated tissue, and cells were spread by pressing the cover slip firmly by hand. The preparation was observed with a light microscope (Leica DM 2500 ®) at 20 and 100× magnification. The chromosome number was counted in the mitotic prometaphase of meristematic cells.

Additional molecular characterization

The genetic distances between the identified genotypes were determined using a group of additional ISSR markers. Eight more primers were tested, and five of them that showed high polymorphism and good resolution of bands were selected for further analysis (Table 1). The PCR and the electrophoretic separation were performed following the same method described for the genotype identification.

Morphological and agronomic characterization

The identified genotypes were propagated in a greenhouse and planted in the field as spaced plants at 2 m apart, following a randomized complete block design with four replications. The experiment was planted on 1 November 2012 at an experimental station near the city of Corrientes (27°40′S, 58°45′W). The soil at this location is classified as Argiudoll. Plants were irrigated every other day after planting during the first 2 weeks. Once they were well established, a group of 16 morphological and agronomic variables were determined, with measurements made between December 2012 and May 2013 (Table 2).

Statistical analyses

Products of ISSR were scored for the presence (1) and absence (0) of homologous DNA bands. Pair-wise genetic distances among the accessions and genotypes was estimated using the Jaccard's dissimilarity coefficient (1-S). A dendrogram was constructed using a cluster analysis (UPGMA: Unweighted Pair Group Method with Arithmetic Mean). The number of different genotypes was determined using the Genotype and Genodive software (Meirmans and van Tienderen, 2004), to detect possible mutation events. This analysis also provided the minimum genetic distance used for separation of genotypes.

Means, Pearson's correlation coefficient, and coefficients of variation for morphological and agronomic traits were calculated. Analysis of variance (ANOVA) and mean separations using the Tukey test were calculated using INFOSTAT software (Di Rien-

Table 2 Group of sixteen morphological and agronomic characteristics measured on twenty-two genotypes of *Acroceras macrum*.

Code	Character	Description
VP	Vegetative phase (day)	Number of days from planting to the emergence of the first panicle
FP	Flowering peak (day)	Number of days from the emergence of the first panicle to the flowering peak
PH	Plant height (cm)	Measured from the soil surface to the top of the canopy
CD	Crown diameter (cm)	Average value of two diameters
IG	Initial growth	Estimated 45 days after planting with a visual scale from 1 to 5, where five was the most vigorous plant
GC	Ground cover (%)	Per cent ground cover, measured with the software of analysis of digital image Cobcal® on digital images taken at 1·5 m above the ground level
PB	Plant biomass (g)	The above-ground biomass of individual plants harvested 180 day after planting and dried at 60°C for 48 h
LL	Leaf blade length (mm)	Measured on the first fully expanded leaf. Average of three tillers per plant
LW	Leaf blade width (mm)	Measured in the widest portion of on the first fully expanded leaf. Average of three tillers per plant
IL	Internode length (mm)	Length of the first visible internode. Average of three tillers per plant
PL	Panicle length (mm)	Measured from the insertion of the basal raceme in the axis to the tip of the panicle. Average of three panicles per plant
BRL	Basal raceme length (mm)	Measured from the axis to the base of the spikelet on the tip. Average of three racemes per plant
SN	Number of spikelets per panicle	Counted when the inflorescence was completely expanded. Average of three panicles per plant
SL	Length of the spikelet (mm)	Measured from the base of the spikelet to the tip of the lemma. Average of three spikelets per raceme, three racemes per panicle and three panicles per plant
SW	Width of the spikelet (mm)	Measured in the widest portion of the spikelet. Average of three spikelets per raceme, three racemes per panicle and three panicles per plant
FOD	Degree of flower opening (%)	Percentage of flowers where anthesis occurred. Measured when the spikelets of the basal raceme ended flowering. Average of three panicles per plant

zo *et al.*, 2002). Principal coordinates analysis was performed to estimate the diversity among genotypes using Info-Gen software (Balzarini and Di Rienzo, 2004). The relation between the molecular and phenotypic data was evaluated using the Mantel's test.

Results

Identification of genotypes and cytotypes

A total of ten ISSR primers were tested, and five were used for the identification of different genotypes among the forty-seven accessions of *A. macrum* tested. A total of 101 ISSR loci were amplified, with ninety-nine being polymorphic. A total of twenty-seven genotypes were identified using a minimum genetic distance of 0·2 (Jaccard's dissimilarity coefficient or 1-S) (Figure 1). Twenty-two of these were identified as tetraploid ($2n = 4x = 36$), and the other five were hexaploid ($2n = 6x = 54$) (Figure 2).

Diversity

Molecular characterization

One example of the obtained electrophoretic pattern can be observed in Figure 3. A total of 215 ISSR loci were amplified, and 204 (95%) were identified as polymorphic. The number of amplified loci varied between fourteen and thirty-three among the different primers used for this analysis (Table 1). The proportion of polymorphic loci varied between 85 and 100% among the evaluated primers. The number of amplified and polymorphic loci was higher in the tetraploid group as compared to the hexaploid genotypes (Table 1).

Genetic distances among genotypes varied from 0·22 to 0·71 (Figure 4). Genotypes appeared to form two clusters depending on their ploidy level (i.e. one cluster for tetraploids and another for hexaploids). The genetic distance varied within the tetraploid cluster from 0·28 to 0·64, and from 0·22 to 0·63 within the hexaploid. The maximum distance (0·71) was observed between the tetraploid and hexaploid clusters.

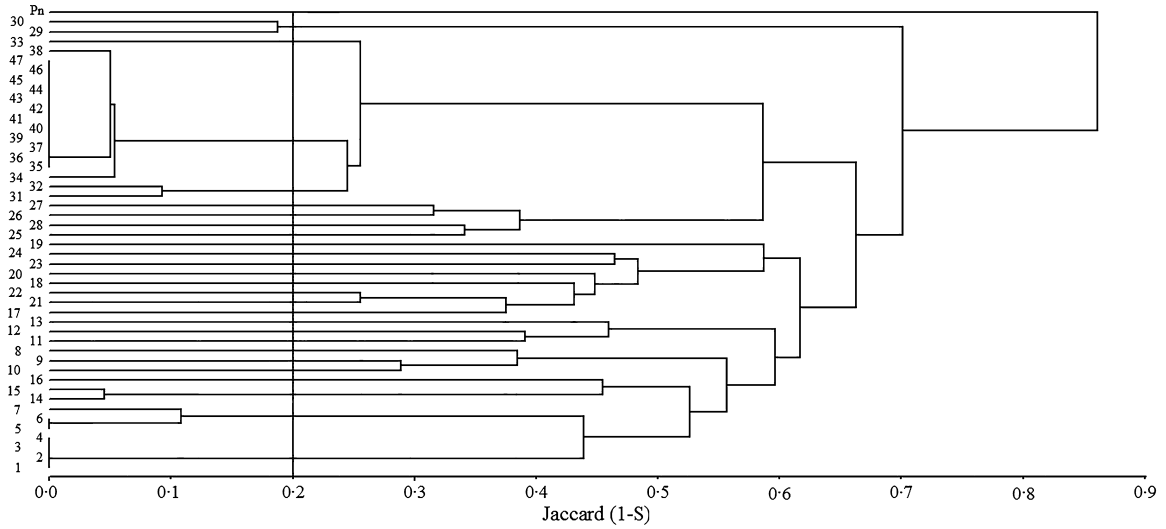

Figure 1 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrograms constructed using the Jaccard's dissimilarity coefficient (1-S) for forty-seven accessions of *Acroceras macrum* based on ISSR molecular profiles. A genotype of *Paspalum notatum* (Pn) appears as a control.

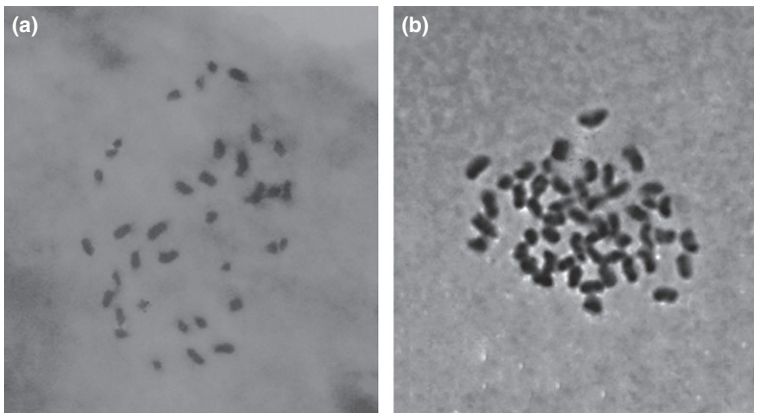

Figure 2 Root tip cells of *Acroceras macrum* treated with the squash method and the Feulgen staining technique. (a) Cell from a tetraploid genotype, with thiry-six chromosomes. (b) Cell with fifty-four chromosomes from a hexaploid genotype.

Morphological and agronomic characterization

Significant differences were observed for all evaluated morphological and agronomic variables among the twenty-seven genotypes (Table 3). Above-ground biomass, internode length, initial growth and number of spikelets per inflorescence were the most variable traits, whereas spikelet length and width, and per cent ground cover were the least variable.

The hexaploid cytotype was significantly superior for plant height, leaf length and width, internode length, spikelet length and width, and time to maximum flowering, while the tetraploid genotype was superior for per cent ground cover, number of spikelets and per cent of opened flowers. There were no significant differences between the two cytotypes for duration of the vegetative phase, crown diameter, initial growth, plant biomass, panicle length and basal raceme length.

Some of the most variable traits were correlated with other traits. Above-ground biomass was correlated with initial growth ($r = 0{\cdot}61$; $P < 0{\cdot}001$) and plant diameter ($r = 0{\cdot}50$; $P < 0{\cdot}001$). In addition, initial

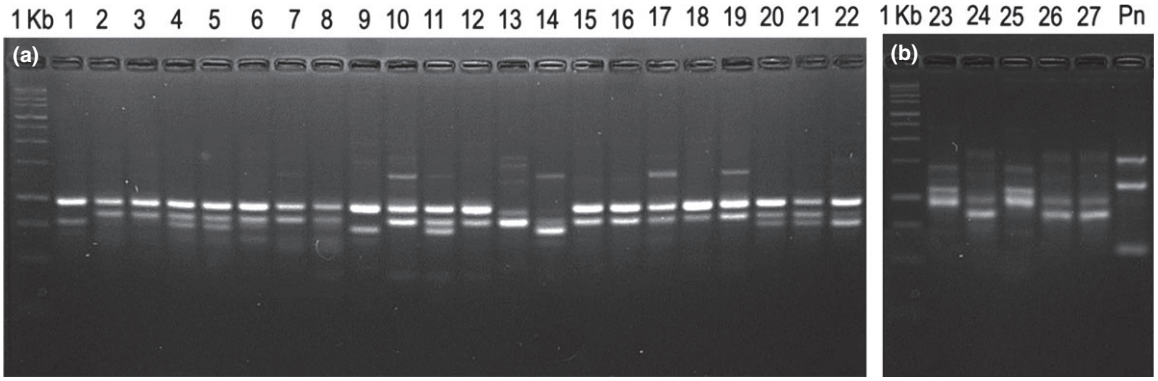

Figure 3 Electrophoretic pattern obtained with the $(GA)_8G$ ISSR primer from twenty-seven genotypes of *Acroceras macrum*. (a) Tetraploid genotypes ($2n = 4x = 36$). (b) Hexaploid genotypes ($2n = 6x = 54$) plus one genotype of *Paspalum notatum* (Pn). 1 kb is one-kilo-base molecular weight marker, from down to above the bands are 250 base pairs (bp), 500 pb, 750 pb, 1000 pb, 1500 pb, 2000 pb, 2500 pb, 3000 pb.

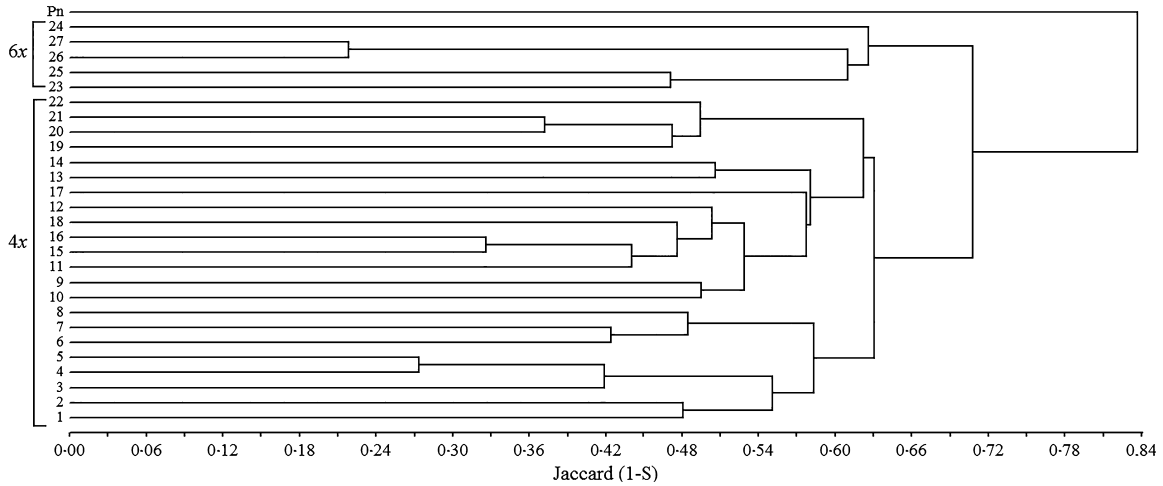

Figure 4 Dendrogram constructed using the Jaccard's dissimilarity coefficient (1-S) and the average linkage method (UPGMA, Unweighted Pair Group Method with Arithmetic Mean) for twenty-seven lines of *Acroceras macrum* based on ISSR profiles. A genotype of *Paspalum notatum* (Pn) appears as a control.

growth was correlated with plant height ($r = 0{\cdot}53$; $P < 0{\cdot}001$).

When the diversity was estimated, based on a multivariate data analysis considering all the evaluated traits, marked differences were observed among genotypes. The Euclidean distance varied from 0·46 to 2·37. The hexaploid genotypes formed a distinct cluster. The range of Euclidean distances varied from 0·46 to 0·99 for the group of hexaploid plants and from 0·49 to 2·10 for the tetraploids. The diversity observed in the hexaploid cytotypes for this group of traits represented only a fraction of the diversity contained in the tetraploid group (Figure 5). A significant correlation was observed between the data obtained based on the two techniques ($r = 0{\cdot}18$; $P = 0{\cdot}011$).

Discussion

The initial step in the development of a breeding programme for any forage species is to assemble germplasm that may be utilized as a resource for breeding (Poehlman and Sleper, 1995). The sources from which seed or vegetative material can be obtained may be local ecotypes, introductions from the area where the species is indigenous, improved cultivars, improved populations or hybrids. In this research, we have

Table 3 Mean and variation coefficients of sixteen morphological and agronomic traits measured on a group of genotypes of *Acroceras macrum*.

Genotype	Ploidy level	VP (day)	FP (day)	PH (cm)	CD (cm)	IG*	GC (%)	PB (g pl^{-1})	IL (mm)	LL (mm)	LW (mm)	PL (mm)	BRL (mm)	SN	FOD (%)	SL (mm)	SW (mm)
1	4×	40^{bcde}	69^{bcdef}	44·3^{bcde}	90^{ab}	2·8^{abc}	81·5^{a}	908·7^{abcd}	43^{cde}	165^{defgh}	5·9^{bcdef}	166^{cde}	67^{ij}	103^{abcde}	90·6^{a}	4·5^{fghi}	1·7^{c}
2	4×	56^{a}	93^{a}	45·8^{bcde}	110^{ab}	2·5^{abcd}	84·8^{a}	1030·6^{abc}	57^{abcde}	163^{efgh}	5·6^{bcdefgh}	203^{bcd}	92^{abcdefgh}	93^{cdef}	86·1^{a}	4·9^{abcdefg}	1·9^{abc}
3	4×	59^{a}	93^{a}	28·8^{ef}	90^{ab}	1·0^{b}	80·6^{a}	614·3^{bcd}	39de	117^{a}	4·7^{hijk}	186^{bcd}	70^{ghij}	98^{bcde}	84·0^{a}	4·7^{cdefghi}	1·85^{abc}
4	4×	58^{a}	93^{a}	44·0^{bcde}	100^{ab}	2·5^{abcd}	80·7^{a}	792·4^{abcde}	38de	133^{ha}	7·7^{a}	181^{bcd}	97^{abcde}	133^{a}	94·1^{a}	4·6^{defghi}	1·95^{abc}
5	4×	61^{a}	93^{a}	22·0^{f}	100^{ab}	1·0^{b}	52·5^{c}	266·5^{d}	39de	144^{gha}	6·2^{bcde}	278^{a}	94^{abcdef}	85^{defg}	86·4^{a}	4·7^{bcdefgh}	2·1^{a}
6	4×	37de	51^{fgh}	45·8^{bcde}	100^{ab}	4·0^{a}	81·9^{a}	1044·5^{abcd}	57^{abcde}	162^{efgh}	5·9^{bdefg}	201^{bcde}	99^{abcde}	129^{ab}	77·6^{a}	4·5^{efghi}	2·1^{ab}
7	4×	33^{e}	40^{h}	56·3^{abc}	70^{b}	3·5^{ab}	74·1^{ab}	797·1^{abc}	61^{abcde}	163^{efgh}	4·1^{k}	221^{b}	100^{ab}	87^{defg}	88·8^{a}	4·9^{abcdefg}	1·95^{abc}
8	4×	38^{cde}	59^{bcdefgh}	58·3^{ab}	80^{ab}	3·5^{ab}	81·6^{a}	861·2^{abcd}	57^{abcde}	178^{abcdef}	5·2^{efghijk}	194^{bcde}	95^{abcdef}	123^{abc}	72·1^{ab}	4·4^{ghij}	1·85^{abc}
9	4×	40^{bcde}	61^{defg}	47·3^{bcd}	90^{ab}	3·5de	85·9^{a}	1042·3^{abcd}	45^{bcde}	191^{abcde}	6·0^{bcdef}	186^{bcde}	69^{hij}	117^{abcde}	91·7^{a}	4·5^{ghij}	1·7^{c}
10	4×	40^{bcde}	82^{abc}	58·3^{ab}	90^{ab}	4·0^{a}	77·4^{ab}	1431·9^{abc}	57^{abcde}	202^{ab}	6·4^{bcd}	215^{b}	100^{abc}	128^{ab}	91·2^{a}	4·6^{defghi}	1·9^{abc}
11	4×	40^{bcde}	85^{ab}	50·8^{abcd}	110^{ab}	3·8^{ab}	83·9^{a}	1293·5^{ab}	70^{a}	201^{abc}	5·3^{defghij}	210^{bcde}	95^{abcdef}	92^{cdef}	81·6^{a}	5·1^{abcde}	2·05^{a}
12	4×	42^{bcd}	82^{abc}	52·5^{abcd}	110^{ab}	4·0^{a}	84·8^{a}	1298·9^{ab}	69^{ab}	186^{abcdef}	5·5^{cdefghi}	187^{bcde}	78^{cdefghij}	75^{efg}	79·7^{a}	4·5^{efghi}	1·85^{abc}
13	4×	38^{cde}	74^{abcd}	57·5^{ab}	110^{ab}	3·8^{ab}	78·4^{ab}	1286·6^{ab}	56^{abcde}	209^{a}	6·4^{bcd}	211^{bcd}	95^{abcde}	97^{bcde}	74·1^{ab}	4·3^{hij}	1·9^{abc}
14	4×	59^{a}	88^{ab}	40·0^{cde}	80^{ab}	1·5^{cb}	79·3^{a}	586·3^{bcd}	39de	139^{gha}	5·4^{cdefghi}	204^{bcd}	94^{abcdef}	72^{efg}	81·1^{a}	5·2^{ac}	2·0^{abc}
15	4×	40^{bcde}	66^{cdefg}	42·3^{bcde}	70^{ab}	2·5^{abcd}	83·5^{a}	762·2^{abcd}	37^{e}	141^{gha}	4·4^{ijk}	164de	61^{j}	79^{efg}	92·1^{a}	4·0^{j}	1·9^{abc}
16	4×	40^{bcde}	64^{cdefg}	42·8^{bcde}	70^{ab}	2·8^{abc}	77·5^{ab}	464·1^{cd}	45^{bcde}	155^{fgh}	4·7^{ghijk}	164de	68^{ij}	88^{bef}	84·2^{a}	4·1^{ij}	2·0^{abc}
17	4×	38^{cde}	47^{gh}	48·0^{abcd}	70^{ab}	3·0^{abc}	79·2^{a}	811·6^{abcd}	74^{a}	179^{abcdef}	4·1^{jk}	216^{b}	85^{bcdefghi}	62^{fg}	96·5^{a}	4·6^{defghi}	1·9^{abc}
18	4×	40^{bcde}	55^{efgh}	49·3^{abcd}	110^{ab}	3·8^{ab}	78·7^{a}	1293·0^{ab}	55^{abcde}	199^{abcd}	4·9^{fghijk}	214^{bc}	93^{abcdefg}	84^{defg}	87·6^{a}	5·1^{abc}	2·05^{ab}
19	4×	38^{cde}	77^{abcd}	53·3^{abcd}	90^{ab}	3·3^{ab}	82·7^{a}	1125·0^{abc}	74^{a}	171^{bcdefg}	4·9^{fghjk}	212^{bcd}	111^{a}	100^{abcde}	72·7^{ab}	4·5^{ghij}	1·85^{abc}
20	4×	40^{bcde}	89^{a}	36·3^{def}	110^{ab}	2·3^{bcd}	79·1^{a}	1029·4^{abc}	43^{cde}	166^{defgh}	4·7^{hijk}	210^{bcd}	113^{a}	98^{bcde}	87·9^{a}	4·7^{bcdefgh}	1·85^{abc}
22	4×	40^{bcde}	74^{abcde}	42·5^{bcde}	80^{ab}	3·5^{ab}	80·0^{a}	628·1^{bcd}	65^{abc}	169^{cdefg}	4·5^{hijk}	174^{bcde}	75^{efghij}	54^{g}	49·6^{b}	4·9^{abcdefg}	1·8^{bc}
23	6×	47^{b}	93^{a}	58·3^{ab}	70^{b}	3·3^{ab}	65·8^{b}	662·1^{bcd}	73^{a}	172^{bcdefg}	6·3^{bcde}	154^{e}	60^{j}	59^{fg}	76·7^{ab}	5·3^{a}	2·05^{ab}
24	6×	46^{bc}	90^{a}	64·5^{a}	100^{ab}	3·5^{ab}	73·9^{ab}	1010·6^{abc}	73^{a}	181^{abcdef}	6·4^{bcd}	193^{bcde}	66^{ij}	76^{efg}	79·4^{a}	5·1^{abcd}	2·0^{abc}
25	6×	42^{bcd}	93^{a}	55·0^{abc}	110^{ab}	3·0^{abc}	79·4^{a}	800·5^{abcd}	72^{a}	184^{abcdef}	6·8^{ab}	194^{bcde}	75^{defghij}	86^{defg}	76·5^{ab}	5·0^{abcdef}	2·0^{abc}
26	6×	40^{bcde}	92^{a}	49·5^{abcd}	110^{a}	3·3^{ab}	80·4^{a}	899·9^{abcd}	67^{abc}	179^{abcdef}	6·4^{bcd}	197^{bcde}	72^{fghij}	73^{efg}	82·0^{a}	5·2^{ab}	2·0^{abc}
27	6×	38^{cde}	93^{a}	54·8^{abc}	100^{ab}	2·5^{abcd}	76·0^{ab}	698·6^{bcd}	65^{abc}	168^{cdefg}	6·6^{abc}	199^{bcde}	68^{ij}	81^{defg}	72·4^{ab}	5·2^{abc}	2·1^{a}
Mean		43	77	48·0	100	3·0	78·6	901·9	57	170	5·6	197	84	91·2	82·2	4·7	1·9
CV		19	23	22·8	21·3	34·7	10	41	35·6	18·2	22	14·7	20·7	26	16	8·2	8·2

VP, vegetative phase; FP, flowering peak; PH, plant height; CD, crown diameter; IG, initial growth; GC, ground cover; PB, plant biomass; IL, internode length; LL, leaf length; LW, leaf width; PL, panicle length; BRL, basal raceme length; SN, spikelet number; FOD, flower opening degree; SL, spikelet length; SW, spikelet width. *Measured with a visual scale, where 1 represents the lowest and 5 the highest initial growth. Genotype means followed by different letters are different ($P \leq 0{\cdot}05$).

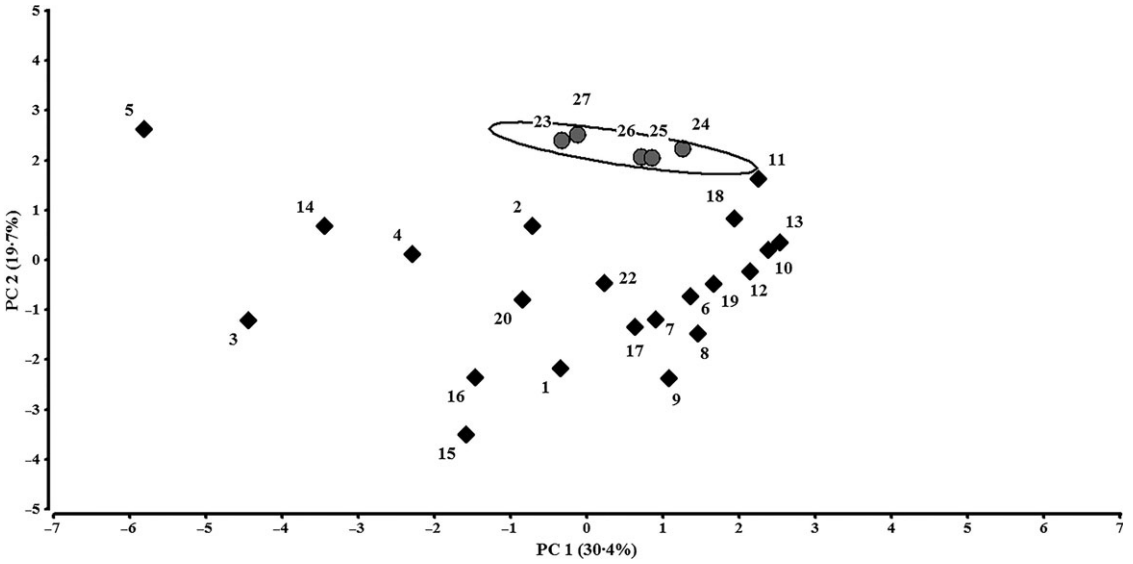

Figure 5 Principal coordinates graph for twenty-seven genotypes of *Acroceras macrum* based on sixteen morphological and agronomic traits. The circle indicates the group of hexaploid genotypes.

attempted to characterize a collection from an old plant-introduction nursery of *A. macrum* that was expected to be diverse, based on historical records of the origin of the material planted at the nursery.

DNA markers have provided valuable data for the identification of suitable material for germplasm preservation with maximum diversity and for the detection of undesirable duplicates (Weising *et al.*, 2005). This is especially true for vegetatively propagated material because of the high cost of maintaining live germplasm banks. In this research, we have found information that supports this idea. A low number of genotypes of *A. macrum* were found among a collection (twenty-seven genotypes of forty-seven accessions). This could be related to the loss of some of the original introductions from South Africa or because some accessions were imported as duplicates. These results indicate the importance of identifying distinct genotypes in a collection for a more efficient utilization of available resources. This is especially important for stoloniferous or rhizomatous grass species as they become easily mixed when conserved in live germplasm banks. Identification of these twenty-seven genotypes allowed for molecular, cytogenetic and agronomic characterization of the introduced germplasm, which was part of this work. In addition, it will allow for a future characterization of the mode of reproduction of *A. macrum*, considering seed set on self- and cross-pollination, megasporogenesis, megagametogenesis and pollen viability.

Most genotypes (81·5%) from the analysed collection were identified as tetraploid and some as hexaploid (18·5%). Considering that tetraploids, pentaploids and hexaploids have been reported for this species (Moffett and Hurcombe, 1949; Rhind and Goodenough, 1976), the collection is lacking the pentaploid cytotype. This odd ploidy level could, however, be created by crossing the tetraploid and hexaploid to complete the three ploidy levels reported. The identification of these two cytotypes also allowed for evaluating the importance of the ploidy level on plant morphology and the performance of each genotype for agronomic traits. It will also allow for considering the effect of the ploidy level on seed fertility and pollen shape and viability as part of future research.

The molecular evaluation of the genetic distances indicated that most genotypes were not closely related. When the proportion of distances between pairs of genotypes is considered, it is possible to observe that 93% of them are above 0·5 (Jaccard dissimilarity). These results indicate that although the number of genotypes is not large, the diversity among genotypes should allow, assuming sexual hybridization, for the generation of an adequate population to initiate a breeding programme for this species. The introduction of additional genotypes from other sources should also be considered to enrich the available germplasm for breeding purposes. Furthermore, the cytotypes were separated using ISSR markers, and most of the diversity was present within the tetraploid (Figure 4). In a different investigation, the cytotypes of Bermudagrass (*Cynodon dactylon* L.), which is also sexual and clonally propagated, were also successfully separated using ISSR markers (Farsani *et al.*, 2012).

The different genetic distances observed between genotypes in this study would also allow for the evaluation of heterosis once hybrids are created using combinations of parents with different degrees of relationship.

The morphological and agronomic characterization indicated that this group of twenty-seven genotypes contained a rich diversity for most of the evaluated traits. The results indicate that it would be possible to directly select genotypes that could be used as vegetatively propagated clones or aim to breed for any of this group of characteristics. Of special interest are genotypes that are able to quickly cover the soil and are able to produce more forage. Genotypes identified with numbers 12 and 13 are the most promising as potential forage crops considering initial growth, forage yield and ground cover. There remains a need for further evaluations that will better define the performance of these genotypes as forages. There was no clear superiority of one cytotype over the other considering the group of evaluated traits. These results indicate that an increment in ploidy level does not necessarily imply an incremental change in plant morphology or performance in *A. macrum*. Breeding efforts should be concentrated on the tetraploid group, as the tetraploid cytotype is more diverse. A population could be created with the aim of initiating a breeding programme for this species. A complete characterization of the species' mode of reproduction should be carried out with the objective of defining the most appropriate breeding technique for use in genetic improvement of the tetraploid form of *A. macrum*.

Both of the techniques used to estimate the diversity contained in this collection (i.e. ISSR markers and the morphological and agronomic characterization) were correlated and both proved to be useful for this purpose. A positive correlation ($r = 0{\cdot}23$) between these two methods was also observed by Brugnoli *et al.* (2014) when analysing a large group of populations of *Paspalum simplex* Morong. In both cases, the correlation was significant and positive, but it was also low. This could be related to the nature of the DNA markers used, which randomly explore both coding and non-coding regions, while the morphological and agronomic characterization is related only with the coding regions, Furthermore, the phenotypic expression of these characters is influenced by the environment.

In conclusion, a genetic characterization of a collection of *Acroceras macrum* was accomplished. This collection contains a group of twenty-seven genotypes with a high diversity among them. Most of this diversity is present within a group of twenty-two tetraploid genotypes, and the rest identified as hexaploid. These results will allow for a reproductive characterization of the species and also for the initiation of a breeding programme.

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