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Selecting salt-tolerant clones and evaluating genetic variability to obtain parents of new diploid and tetraploid germplasm in rhodesgrass (*Chloris gayana* K.)

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ABSTRACT

We evaluated survival percentage under salt stress in 46 diploid and tetraploid clones of rhodesgrass (*Chloris gayana* K.) with the aim of obtaining salt tolerant clones. Fifteen clones were selected at 600 mM NaCl under hydroponic conditions. Survival percentage of the selected clones ranged between 50–100% and 50–75% for diploid and tetraploid clones, respectively. Genetic diversity among the 15 salt-tolerant clones was assessed using amplified fragment length polymorphism (AFLP). All tetraploid clones showed genetic diversity, whereas the diploid group included some genetically related clones. Clones tolerant at 600 mM NaCl and showed genetic diversity are proposed as parents for new synthetic varieties of each rhodesgrass ploidy.

1. Introduction

In the last 14 years, agriculture in Argentina has expanded to 12 million acres formerly devoted to livestock production, thus increasing cattle stock in the northeastern and northwestern regions of the country. These areas are naturally subjected to a number of abiotic stresses, especially water and salt stress. In particular, salinity has negative effects on plant growth and development at physiological and biochemical levels (Munns, 2002; Munns and James, 2003). Given the distinctly pastoral characteristic of Argentine livestock (Bazzigalupi et al., 2008; Otondo et al., 2008), selecting salt-tolerant forage plants to be incorporated in a genetic improvement program aimed at obtaining new germplasm is a strategy of economic importance (El-Hendawy et al., 2007; Ribotta, 2011).

Chloris gayana K. (rhodesgrass) is an important forage grass native to Africa and widely grown in tropical and subtropical regions worldwide (Gondo et al., 2009). In Argentina, rhodesgrass has been widely used in stock breeding and rebreeding systems because of its ease of establishment, persistence, high productivity, grazing resistance, and good quality throughout the year (Luna et al., 2002; Pérez et al., 1999). It is a perennial, outbreeding gramineae, with a 1–4% level of self-compatibility (Bogdan, 1977; Nakagawa et al., 1987; Ribotta, 2011; Skerman and Riveros, 1990). Rhodesgrass has diploid and tetraploid forms, which may differ in traits such as growth habit, flowering time, seed production, dry mass production, quality, and tolerance to drought, frosts and salinity (Loch et al., 2004; Pérez, 2005).

Rhodesgrass is naturally tolerant to salt (Kobayashi et al., 2007; Kobayashi and Masaoka, 2008) at high soil Na levels with electrical conductivity (EC) between 6–12 dS/m (Berstein, 1958). Pasternak et al. (1993) reported that dry matter yield was reduced by about 6% per unit increase in soil salinity whit EC between 4 and 12 dS/m. Field assays conducted at EC between 3.94 and 6.48 dS/m did not show yield reduction in diploid cultivars (Toll Vera et al., 2000a,b). Pérez et al. (1999) detected an average 40% reduction in saline plots with an electrical conductivity of 13 dS/m in tetraploid and diploid cultivars.

In Argentina, genetic improvement of rhodesgrass has been poorly developed. Among the available methods, obtaining synthetic varieties is a useful option in allogamous species that have self-incompatibility alleles and in which emasculation is not possible (Cubero, 1999). Moreover, synthetic varieties exhibit some degree of heterosis while preserving genetic variability. The latter depends on the number of parents used and their level of relatedness (Basigalup, 2007). In obtaining salt-tolerant synthetic varieties, the improvement scheme should include rapid selection techniques to evaluate salt tolerance and genetic variability of parent material.

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Salt tolerance is usually measured as the percentage of biomass production in saline soil relative to the biomass production of plants in non-saline soil over a period of time (Munns and Tester, 2008). However, field screening for this characteristic is a laborious task; therefore, plant breeders have explored reliable techniques to assess salt tolerance in germplasm (Arzani, 2008). Fast screening methods in greenhouse environments can be employed to identify potential stress-tolerant parents in a breeding program (Arzani, 2008; Munns and James, 2003).

To assess plant tolerance to salinity stress, survival is one of the typical agronomic selection parameters (Ashraf and Harris, 2004) because it integrates the up- or down- regulation of many physiological mechanisms occurring within the plant (Niknam and McComb, 2000). Moreover, this character used in several forage grasses (De Luca et al., 2001; Griffa, 2010; Luna et al., 2000; Pérez et al., 2009) allows a screening of a large number of genotypes in their ability to survive (Munns and James, 2003). Proportion of dead leaves and leaf Na^+ content have been associated with salinity tolerance (De Luca et al., 2001; Luna et al., 2002).

Salt-tolerant plant selection as parents of new synthetic varieties as well as the knowledge of their genetic diversity are important requirements, because there is a direct correlation between their genetic variability and the probability of achieving valuable germplasm (Griffa et al., 2006). Genetic variation can be evaluated at DNA level using molecular techniques. In rhodesgrass, Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) techniques have been successfully applied (Pérez et al., 1999; Ribotta, 2011; Ubi et al., 2000, 2001, 2003). The objectives of the present work were: I) to evaluate and select salt-tolerant diploid and tetraploid clones, and II) to determine genetic diversity among the tolerant clones for their future use as parents in the generation of synthetic varieties.

2. Materials and methods

2.1. Plant material

The plant material consisted of 46 clones: 27 diploids and 19 tetraploids (Table 1). These clones were obtained from adult plants in non-reproductive state, vegetatively multiplied (cloned) and clipped to an average height of 15 cm.

The diploid material included 12 clones derived from an accession from the south of Africa (Acc. No. 142), 11 clones derived from an accession from the west of Africa (Acc. No. 146), and 2 clones derived from an accession from the north of the province of Córdoba, Argentina (Acc. No. 154). These accessions were previously selected for the good performance of their agromorphological traits (dry weight, deferred forage value, regrowth, height, leaf-stem ratio, and plant persistence). For selection, a field assay was performed comparing accessions and commercial cultivars (Table 2) for two years.

Furthermore, two diploid clones of the Katambora cultivar were incorporated because of their known salt tolerance at 400 mM NaCl at the seedling stage. The tetraploid material comprised 19 clones of the Boma cultivar, which were tolerant to a salt stress of 250–400 mM NaCl at the seedling stage.

2.2. Assessing tolerance to saline stress using NaCl

The experiments were conducted in a greenhouse under natural light conditions (average irradiance 1000 μmol/m^2.s). Day–night temperatures were 30 ± 2 °C and 20 ± 2 °C. Six plants of each diploid clone and eight plants of each tetraploid clone were placed in plastic trays (60 × 40 × 18 cm) with two replicates per clone, containing sand as substrate (De Luca et al., 2001; Luna et al., 2000). The trays were irrigated with Hoagland solution (Hoagland and Arnon, 1950) for 5 days to allow plants to adapt to the experimental system. Salinization started by gradually adding NaCl (100 mM every 48 h) to the Hoagland solution. Salt concentration in the substrate was controlled by measuring Na^+ present in the water drained from the tray using flame photometry (Cole-Parmer Flame Photometer). The final concentration was 600 mM NaCl for 30 days. This concentration was previously found to produce symptoms of toxicity for excess salinity in rhodesgrass (De Luca et al., 2001; Luna et al., 2000; Taleisnik and Grunberg, 1993). After the 30-day period, tolerance to salt stress was evaluated by survival percentage (SP), calculated as the proportion of plants of each clone that tolerated 600 mM NaCl with respect to the total number of plants (100% survival) per replicate and clone. Percentage of dead leaves (PDL) and leaf Na^+ content were determined to assess the physiological state of clones. Leaf Na^+ content was evaluated in fully expanded leaves by flamephotometry, as described by Luna et al. (2002).

2.2.1. Data analysis

Comparisons were made among ploidy and clones for SP using a binomial model. When P values were significant ($P<0.05$), DGC test (Di Rienzo et al., 2001) was employed. PDL was calculated by binomial model, and the variable was defined as the number of plants with PDL > 50% in each tray; differences among clones were estimated by DGC test. The magnitude of the association between the SP variables and the plant physiological state, estimated by PDL, was determined with a Pearson correlation. Statistical analyses were made using InfoStat (Di Rienzo et al., 2012) software program.

2.3. Molecular characterization using AFLP

2.3.1. DNA extraction

Using the CIMMYT (Hoisington et al., 1994) method, genomic DNA was isolated from each of the clones that proved to be tolerant in the screening assay for salt tolerance using survival percentage as selection criterion. Bulks were prepared by pooling equal amounts of fresh leaves from each plant per clone and homogenizing to a fine powder using liquid nitrogen. DNA concentration was determined by visual comparison with a sample of standard λ DNA of known concentration on 0.7% agarose gel stained with 0.5 μg/μl ethidium bromide.

2.3.2. AFLP analysis

AFLP was performed using the technique of Vos et al. (1995) adjusted for rhodesgrass. Digestion and ligation were performed together. For all the samples, a 60 μl enzyme master mix was prepared, which contained 9 U of EcoRI endonucleases, 5 U of MseI endonucleases,

Table 1
Origin of diploid and tetraploid clones of rhodesgrass subjected to salinity tolerance assay under greenhouse conditions.

Material and origin	Clone identification
Diploid clones (D)	
12 clones derived from an accession from the south of Africa	D-I1/D-I3/D-I6/D-I7/D-I8/D-I10/D-I12/D-I13/D-I15/D-I17/D-I18/D-I19
11 clones derived from an accession from the west of Africa	D-II1/D-II2/D-II3/D-II4/D-II5/D-II6/D-II7/D-II8/D-II9/D-II10/D-II11
2 clones derived from an accession from the north of Córdoba-Argentina	D-III1/D-III2
2 clones derived from cultivar Katambora	D-K44/D-K49
Tetraploid clones (T)	
19 clones derived from cultivar Boma	T-29/T-32/T-33/T-35/T-36/T-37/T-38/T-39/T-42/T-43/T-44/T-46/T-47/T-51/T-52/T-53/T-54/T-55/T-56

Table 2
Mean values of the variables of yield components: dry weight (DW) in g, deferred forage value (DFV), regrowth (RG), plant height (PH) in cm, leaf-stem ratio (L/S), and leaf persistence in deferred plant (LP) in %. DFV, RG and L/S were assessed using an arbitrary 1–4 scale in diploid and tetraploid rhodesgrass clones in two years of evaluation. Different letters within a column indicate significant differences (LSD, $P<0.05$).

Origin	Ploidy	DW		DFV		RG		PH		L/S		LP	
cv. Pioneer (Australia)	2x	265.00	bc	3.76	bc	0.83	b	69.50	bc	2.40	b	69.50	b
cv. Katambora (Australia)	2x	318.33	cd	3.53	bc	1.13	bcd	74.00	bcd	2.05	a	79.50	b
cv. Callide (Australia)	4x	153.33	ab	3.40	b	0.00	a	60.00	b	2.40	b	59.17	ab
cv. Samford (Australia)	4x	95.00	a	2.75	a	0.00	a	28.00	a	2.15	a	32.50	a
Acc. (Nº141) from the north of Córdoba-Argentina	2x	257.00	bc	3.66	bc	1.00	bc	74.33	bcd	2.00	a	93.75	b
Acc. (Nº142) from the south of Africa	2x	350.00	cde	3.78	bc	1.57	cde	77.00	bcd	2.35	b	93.75	b
Acc. (Nº146) from the west of Africa	2x	443.33	e	3.85	c	1.67	de	81.00	cd	2.70	b	81.50	b
Acc. (Nº154) from the north of Córdoba-Argentina	2x	390.00	de	3.83	c	1.77	e	78.00	bcd	2.40	b	87.00	b
Acc. (Nº159) from the east of Africa	2x	305.00	cd	3.85	c	1.27	bcde	86.67	d	2.50	b	88.75	b
Acc. (Nº160) from Kenia, Africa	2x	288.33	c	3.68	bc	1.40	bcde	73.00	bcd	3.35	b	86.88	b

10 ng/μl of purified BSA, 0.2 mM ATP, 10 mM Tris Ac pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 5 pmol EcoRI adapter, 50 pmol MseI adapter, 3 U of T4 DNA ligase, and 750 ng of DNA. The reaction was incubated at 37 °C for 4 h. Pre-amplification (PCR + 1) was performed in a master mix with a final volume of 25 μl containing 75 ng of EcoRI and MseI primers with a selective nucleotide extension (EcoRI-A + MseI-A), 50 mM Tris–Cl pH 9, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 1 U *Taq* DNA polymerase, and 4 μl of the ligation reaction. PCR reaction was programmed as follows: a) denaturation at 94 °C for 2 min; b) 30 cycles for 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C; c) extension at 72 °C for 2 min.

For selective amplification (PCR + 3), PCR + 1 product was diluted with 50 μl TE buffer with low EDTA concentration (10 mM Tris–HCl pH 8, 0.1 mM EDTA). An aliquot of 2 μl was mixed with 33 ng of EcoRI and MseI primers with three nucleotide extensions (EcoRI-AAC + MseI-ACG; EcoRI-AAC + MseI-ACC; EcoRI-AAA + MseI-ATG and EcoRI-AAA + MseI-ACG), and with 2.2 μl buffer 10X *Taq* polymerase, 1.6 mM $MgCl_2$, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase, in a final volume of 22 μl. The cycle profile for PCR + 3 was as follows: a) denaturation at 94 °C for 2 min; b) 1 cycle of 94 °C for 30 s, at 65 °C for 30 s and at 72 °C for 60 s; c) for the following 12 cycles the annealing temperature was lowered by 0.7 °C per cycle; d) 25 cycles at 94 °C for 30 s, at 56 °C for 30 s and at 72 °C for 60 s; e) extension for 10 min at 72 °C.

PCR + 3 products were separated by electrophoresis in 6% polyacrylamide sequencing gel with 6 M urea, in 1X TBE buffer. Samples were run at a constant 60 W for 2 h and the gel was stained with NO_3Ag for observation according to Promega (1996).

2.3.3. Data analysis

To evaluate genetic similarity, polymorphic and monomorphic loci were analyzed. Fragments were scored by visual inspection for presence (1) or absence (0) in the output traces, and fragments smaller than 50 and larger than 500 nucleotides were excluded from the analysis. The size of the fragments scored was estimated by comparing with a 25-bp ladder (Promega, 1996).

To study variability between and within ploidies, an analysis of molecular variance (AMOVA) was performed with InfoGen software (Balzarini and Di Rienzo, 2012) based on Euclidean distance as described for AFLP markers (Ubi et al., 2003). The similarity among AFLP patterns was calculated with Jaccard's correlation coefficient (J) for all primer combinations together. The cophenetic coefficient was calculated and the phenogram was constructed according to the unweighted pair-group method using the arithmetic mean (UPGMA; Crisci and López Armengol, 1983) with NTSYS-pc software (Rohlf, 1993).

3. Results and discussion

3.1. Assessing tolerance to saline stress by NaCl

The clones evaluated at 600 mM NaCl differed significantly ($P<0.0001$) in survival percentage (SP), which allowed us to discriminate clones based on their level of salinity tolerance, in agreement with de Luca et al. (2001) and Luna et al. (2000). Of a total of 46 diploid and tetraploid clones, 31 clones exhibited a SP range between 0–38% and were significantly different ($P<0.0001$) from the remaining 15 clones that showed an SP between 50–100% (Table 3). The former group of clones was considered to be sensitive to salt stress. The latter group

Table 3
Mean survival percentage, percentage of dead leaves and tolerance rating of 46 diploid and tetraploid clones of rhodesgrass at 600 mM of NaCl under greenhouse conditions. Different letters within a column indicate significant differences (DGC, $P<0.05$).

Clon	Survival	Dead leaves	Tolerance rating
D-II8	1.00 a	0.00 a	Tolerant
D-K44	1.00 a	0.00 a	
D-II7	1.00 a	0.00 a	
D-II9	0.83 a	0.17 a	
T-33	0.75 a	0.25 a	
T-52	0.75 a	0.50 a	
T-44	0.63 a	0.38 a	
T-53	0.63 a	0.38 a	
T-56	0.63 a	0.38 a	
D-II11	0.50 a	0.50 a	
D-I13	0.50 a	0.00 a	
D-K49	0.50 a	0.50 a	
T-55	0.50 a	0.50 a	
D-I8	0.50 a	0.33 a	
T-32	0.50 a	0.50 a	
T-51	0.38 b	0.75 b	Sensitive
T-46	0.38 b	0.63 b	
T-36	0.38 b	0.63 b	
T-29	0.38 b	0.63 b	
D-III2	0.33 b	0.83 b	
D-I12	0.33 b	0.67 b	
D-II10	0.33 b	0.67 b	
D-III1	0.33 b	0.67 b	
D-II6	0.33 b	0.67 b	
T-47	0.25 b	0.88 b	
T-43	0.25 b	0.75 b	
T-42	0.25 b	0.75 b	
T-39	0.25 b	0.63 b	
T-38	0.25 b	0.75 b	
T-35	0.25 b	0.75 b	
D-I15	0.17 b	0.67 b	
D-II2	0.17 b	0.67 b	
D-I18	0.17 b	0.83 b	
D-I6	0.17 b	1.00 b	
D-I17	0.17 b	0.83 b	
D-I1	0.17 b	0.83 b	
D-I3	0.00 b	1.00 b	
D-I7	0.00 b	1.00 b	
D-II4	0.00 b	1.00 b	
D-I10	0.00 b	1.00 b	
D-II3	0.00 b	1.00 b	
D-II5	0.00 b	1.00 b	
D-II1	0.00 b	1.00 b	
D-I19	0.00 b	1.00 b	
T-54	0.00 b	1.00 b	
T-37	0.00 b	1.00 b	

was salt-tolerant and comprised 8 diploid and 7 tetraploid clones, showing that there were no differences between ploidy levels ($p > 0.05$). Within the latter group, diploid clones D-I13, D-I8, D-II11 and D-K49 had 50% survival, D-II9 had 83% survival and clones D-K44, D-II7 and D-II8, 100% SP. The 7 tolerant tetraploid clones: T-32 and T-55 showed 50% SP, clones T-44, T-53 and T-56 had an intermediate SP value (63%), and clones T-52 and T-33 showed the highest SP (75%) (Table 3). Notably, the 19 tetraploid clones evaluated in this work were previously selected as being NaCl tolerant at the seedling stage; however, only seven of them were found to be tolerant at adult plant using survival as selection criterion. This difference in salt tolerance among ontogenetic stages is consistent with previous results (Foolad, 2007; Foolad and Lin, 1997; Griffa, 2010; Munns et al., 2006; Sabir and Ashraf, 2007) and confirms the need to assess salinity response at different ontogenic states.

Percentage of dead leaves (PDL) (Table 3) discriminated significantly between two groups of clones, one within a range of 0 to 50% and the other, of 63 to 100%. This group formation was consistent with that obtained with SP and a high correlation between those variables was also observed ($r = -0.83$, $P < 0.0001$).

Average leaf Na^+ content determined in the tolerant and sensitive clones was 284.60 μmol $(gFW)^{-1}$ and 470.55 μmol $(gFW)^{-1}$, respectively. Similar results about the accumulation of Na^+ have been reported by Ashraf (2004) for other gramineous species. Likewise, in agreement with findings of de Luca et al. (2001) and Luna et al. (2002), our results showed an association of leaf Na^+ content and PDL with salinity tolerance in rhodesgrass.

Overall, the clones that resulted tolerant at 600 mM NaCl due to a higher SP showed a better physiological state with lower PDL and lower Na^+ content than clones considered sensitive, which exhibited lower SP and higher PDL and Na^+ content.

The results obtained allowed us to identify 8 diploid and 7 tetraploid salt-tolerant clones; those clones are considered potential parents of future synthetic varieties, which are expected to show increased tolerance to salinity. Pérez et al. (2009) obtained Epica INTA-PEMAN tetraploid synthetic variety with increased salinity tolerance by screening of parent clones using survival as selection criterion.

3.2. Molecular characterization using AFLP

The four primer combinations used to evaluate the 15 clones selected as tolerant to salt stress yielded between 27 and 52 AFLP bands, with an average of 38 bands per primer pair (Table 4). Of the total number of bands obtained (152), 111 (73.02%) were polymorphic. Of these, 82 were polymorphic between two or more genotypes, 4 were visualized only in diploid materials, and the remaining 29 corresponded to unique bands. The percentage of polymorphism of the four primer combinations used in this study ranged between 33.33% and 85.24% (Table 4). EcoRI-AAA + MseI-ACG and EcoRI-AAC + MseI-ACC exhibited the highest level of polymorphism (84.62 and 85.24%, respectively).

Unique bands were recorded in all tetraploid clones under study and in two diploid clones (Table 5). These bands detected could be used as clone-specific markers for improving rhodesgrass. Of the four primer combinations used, three yielded clone-specific markers, except for EcoRI-AAA + MseI-ATG, the EcoRI-AAC + MseI-ACC combination and the tetraploid clone T-55 being the ones that yielded highest polymorphism (11.71%).

Table 4
Genetic variability detected with AFLP markers in the 15 salt-tolerant clones of rhodesgrass that were selected.

Primer combinations	Number of amplified fragments	Number of polymorphic bands	Percentage of polymorphism (%)
EcoRI-AAA + MseI-ATG	27	9	33.33
EcoRI-AAC + MseI-ACG	52	40	76.92
EcoRI-AAA + MseI-ACG	39	33	84.62
EcoRI-AAC + MseI-ACC	34	30	85.24
Total	152	111	73.02
Average	38	28	70.03

Table 5
Unique bands detected in salt-tolerant tetraploid and diploid clones of rhodesgrass.

Clones		Number of unique bands	Percentage of polymorphism (%)	Primer combinations
Tetraploid	T-55	13	11.71	EcoRI-AAC + MseI-ACC
	T-32	5	4.50	EcoRI-AAC + MseI-ACG
	T-33	2	1.80	EcoRI-AAC + MseI-ACG/ EcoRI-AAC + MseI-ACC
	T-52	2	1.80	EcoRI-AAC + MseI-ACG
	T-53	2	1.80	EcoRI-AAC + MseI-ACG/ EcoRI-AAA + MseI-ACG
	T-56	2	1.80	EcoRI-AAC + MseI-ACG
	T-44	1	0.90	EcoRI-AAC + MseI-ACG
Diploid	D-II9	1	0.90	EcoRI-AAA + MseI-ACG
	D-K44	1	0.90	EcoRI-AAC + MseI-ACG

Molecular analysis of variance (AMOVA) suggested the existence of genetic variability between ($P < 0.0001$) and within ($P < 0.0001$) ploidies. The components of variance between and within ploidy level were 9.33 and 12.38, respectively. The value of Fst coefficient (0.43) suggests that ploidy factor would generate genetic structure.

Table 6 shows the genetic similarity matrix calculated with four primer combinations, using the total number of bands (mono and polymorphic) for the 15 clones previously selected. Within the diploid clone group, the Jaccard's coefficient (J) ranged from 0.663 to 0.986, whereas in tetraploid clones the J values varied from 0.570 to 0.779 (Table 6). The selection of the least genetically related clone within each group would promote best genetic combinations in the new germplasm obtained (Griffa, 2010).

The phenogram (Fig. 1) of clones selected as tolerant with a cophenetic correlation coefficient of 0.93 revealed two well defined groups (J = 0.54 mean average) corresponding to their ploidy levels. Group I comprised the tetraploid clones and group II, the diploids, showing that the use of four primer pairs was enough to differentiate all clones. This is the first report about differentiation between ploidies in rhodesgrass using the AFLP technique. Nakagawa et al. (1987) studied the relationships between ploidy levels and morphological characters in 12 species of *Chloris*, showing a grouping tendency among those with the same ploidy. Later, Ubi et al. (2000, 2001, 2003) used AFLP to differentiate within and among cultivars but with the same ploidy level.

Group I, consisting of tetraploid clones derived from cultivar Boma, exhibited a genetic variability with a J = 0.570 to 0.779 (Fig. 1; Table 6), considering monomorphic and polymorphic bands. In an analysis that included only the polymorphic bands, Ubi et al. (2000) found high genetic diversity with a lower range (0.73 to 0.82) and without considering the monomorphic bands that would produce a greater reduction of the genetic distance among the materials. In group I, T-44 and T-52 clones presented a J = 0.779 (Table 6) and both clones exhibited a 63 and 75% survival, respectively (Table 3). Except for clone T-55, the remaining clones formed a subgroup with divergence between them (J ranging between 0.63 and 0.73, Table 6) and with high variation in their tolerance to salinity, since SP ranged from 50% for clone T-32 to 75% for clone T-33 (Table 3). Finally, clone T-55 was the most dissimilar by AFLP, with a J average of similarity to the other tetraploid materials of 0.60 and an SP = 50% (Table 3). This clone also had the highest number of unique bands (13), which could be used as clone-specific markers for rhodesgrass improvement.

The results obtained suggest that all selected salt-tolerant tetraploid clones are genetically divergent among them by AFLP, and could be used as future parents in obtaining synthetic tetraploid

Table 6
Genetic similarity among 15 selected salt-tolerant clones of rhodesgrass based on 152 AFLP markers.

	D-I/8	D-I/13	D-II/7	D-II/8	D-II/9	D-II/11	D-K/44	D-K/49	T-32	T-33	T-44	T-52	T-53	T-55	T-56
D-I/8	1.000														
D-I/13	**0.986**	1.000													
D-II/7	0.783	0.774	1.000												
D-II/8	0.691	0.683	0.744	1.000											
D-II/9	0.776	0.766	0.808	0.711	1.000										
D-II/11	0.741	0.731	0.861	0.700	0.718	1.000									
D-K/44	0.735	0.726	0.852	0.695	0.803	0.765	1.000								
D-K/49	0.671	**0.663**	0.702	0.737	0.667	0.722	0.759	1.000							
T-32	0.490	0.485	0.520	0.586	0.478	0.495	0.510	0.551	1.000						
T-33	0.571	0.560	0.605	0.663	0.563	0.560	0.576	0.605	0.694	1.000					
T-44	0.500	0.510	0.546	0.598	0.490	0.520	0.505	0.544	0.747	0.726	1.000				
T-52	0.525	0.520	0.539	0.604	0.500	0.515	0.530	0.537	0.689	0.708	**0.779**	1.000			
T-53	0.541	0.551	0.619	0.659	0.532	0.563	0.561	0.570	0.652	0.689	0.759	0.778	1.000		
T-55	0.472	0.481	0.500	0.573	0.461	0.476	0.477	0.510	**0.570**	0.635	0.629	0.600	0.587	1.000	
T-56	0.542	0.536	0.604	0.607	0.533	0.598	0.563	0.538	0.674	0.733	0.724	0.653	0.635	0.620	1.000

Diploid rhodegrass clones are indicated in light grey. Tetraploid rhodegrass clones are indicated in dark grey. Numbers in bold are maximum and minimum values for each ploidy.

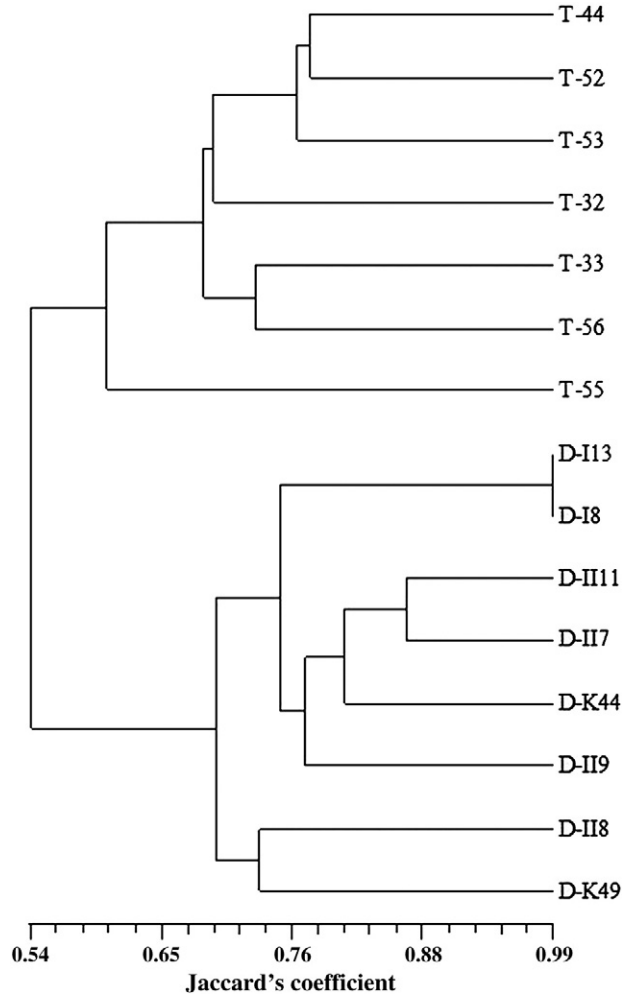

Fig. 1. Phenogram based on AFLP data obtained using UPGMA method with salt-tolerant diploid and tetraploid clones of rhodesgrass.

varieties with increased tolerance to salinity. In turn, a morpho-agronomic assessment, complementary to the molecular evaluation, would be useful to select those materials that exhibit the best forage performance.

In contrast to group I, in group II, diploid clones were found to be more genetically related (J = 0.66 to 0.98, Table 6), despite their different origin. The electrophoretic pattern revealed four bands shared only by the diploid clones and absent in the tetraploids, suggesting quite conserved genomic regions among these materials of different origin. Among the diploid clones (group II) (Fig. 1), D-II8 and D-K49 exhibited the highest genetic diversity (J = 0.74, Table 6) and also the greatest variability in the range of tolerance to salinity (SP = 100% and 50%, respectively; Table 3). The former may be due to their different origin, since D-II8 comes from west Africa and D-K49 is derived from the cv Katambora, from central Africa. Morpho-agronomically, D-II8 was compared to the other clones of western and southern Africa included in this study, and was marked by a greater weight of deferred plant, number of spikes/inflorescence (Ribotta, 2011) and good forage quality (Ribotta et al., 2005). Clones D-II9 and D-K44 showed 80% genetic similarity (Table 6). These clones showed high tolerance to salinity, with an SP = 83 and 100%, respectively (Table 3). In addition, previous studies on the D-II9 clone highlighted agronomically interesting traits such as plant weight, leaf dimensions, leaf/stem ratio (Ribotta, 2011) and crude protein content (Ribotta et al., 2005). The forage performance of clones D-K49 and D-K44 remains to be evaluated. The subgroup including D-II7 and D-II11 from west Africa revealed high genetic similarity, with a J = 0.86 (Table 6). Since they are genetically related, their offspring are expected to show inbreeding depression rather than heterosis (Allard, 1975). Therefore, only one of those clones should be selected to be included in the group of promising parents for obtaining new germplasm. D-II7 clone showed the highest (100%) SP (Table 3) and in field trials greatest plant weight, leaf/stem ratio, leaf width, inflorescence weight (Ribotta, 2011) and crude protein content (Ribotta et al., 2005), being more promising than D-II11. Finally, D-I13 and D-I8 (both derived from south Africa) were the most closely related clones (J = 0.99, Table 6). The high genetic similarity between these clones based on AFLP markers was also reflected in the analysis of

morpho-agronomic characters and by RAPD (Ribotta, 2011). All these results suggest that, to avoid negative effects due to self-incompatibility and inbreeding expression (Ubi et al., 2003), only one of them should be selected to be included along with other clones in the parent group of the synthetic diploid variety. Although both clones were equally tolerant to salt stress (SP = 50%; Table 3), clone D-I8 was found to perform better in the morpho-agronomic features plant height, inflorescen weight, length and number of spikes/inflorescence (Ribotta, 2011), and forage quality, (Ribotta et al., 2005). The results obtained suggest clone D-I8 as a promising parent. All these data indicate that the diploids D-I8, D-II7, D-II8, D-II9, D-K44 and D-K49 are potential parents of new germplasm that will be obtained from their crossings.

In summary, the clones that proved to be salt tolerant and genetically divergent were selected as promising parents to obtain diploid and tetraploid synthetic varieties of rhodesgrass with enhanced salt tolerance.

References

Allard, R.W., 1975. Aspectos generales de la heterosis y de la depresión producida por la consanguinidad. In: Omega, S.A. (Ed.), Principios de la mejora genética de las plantas. Barcelona, España, pp. 226–237.

Arzani, A., 2008. Improving salinity tolerance in crop plants: a biotechnological view. In Vitro Cellular & Developmental Biology–Plant 44, 373–383.

Ashraf, M., 2004. Some important physiological criteria for salt tolerance in plants. Flora 199, 361–376.

Ashraf, M., Harris, P.J.C., 2004. Potencial biochemical indicators of salinity tolerance in plants. Plant Science 166, 3–16.

Balzarini, M.G., Di Rienzo, J.A., 2012. InfoGen Versión 2012. FCA, Universidad Nacional de Córdoba, Argentina. URL http://www.info-gen.com.ar.

Basigalup, D.H., 2007. Mejoramiento genético y desarrollo de variedades. In: Basigalup, D.H., Basigalup, D.H. (Eds.), El cultivo de la Alfalfa en la Argentina. Ediciones INTA, Buenos Aires, Argentina, pp. 81–108.

Bazzigalupi, O., Pistorale, S.M., Andrés, A.N., 2008. Tolerancia a la salinidad durante la germinación de semillas provenientes de poblaciones naturalizadas de agropiro alargado (*Thinopyrum ponticum*). Ciencia e Investigación Agriaria 35, 277–285.

Berstein, L., 1958. Salt tolerance of grasses and forage legumes. Agriculture Information Bulletin, Washington, D.C., pp. 3–7 (Nº 194).

Bogdan, A.V., 1977. Tropical pasture and fodder plants (Grasses and Legumes). Longman, London.

Crisci, J.V., López Armengol, M.F., 1983. Introducción a la Teoría y Práctica de la Taxonomía Numérica. Monografia: Serie de Biologia, Programa de Monografias Científicas, OEA, Washington, D.C., 26.

Cubero, J.I., 1999. Variedades sintéticas. In: Cubero, J.I. (Ed.), Introducción a la Mejora Genética Vegetal. Mundi-Prensa, Madrid, Spain, pp. 145–153.

De Luca, M., García Seffino, L., Grunberg, K., Salgado, M., Córdoba, A., Luna, C., Ortega, L., Rodríguez, A., Castagnaro, A., Taleisnik, E., 2001. Physiological causes for decreased productivity under high salinity in Boma, a tetraploid *Chloris gayana* cultivar. Australian Journal of Agricultural Research 52, 903–910.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, C.W., 2012. InfoStat versión 2012. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. http://www.infostat.com.ar (URL).

Di Rienzo, J.A., Guzmán, A.W., Casanoves, F., 2001. A multiple comparisons method based on the distribution of the Root Node Distance of a Binary Tree. Journal of Agricultural, Biological and Environment Statistics 7, 146–159.

El-Hendawy, S.E., Hu, Y., Schmidhalter, U., 2007. Assessing the suitability of various physiological traits to screen wheat genotypes for salt tolerance. Journal of Integrative Plant Biology 49, 1352–1360.

Foolad, M.R., 2007. Genome mapping and molecular breeding of tomato. International Journal of Plant Genomics 2007, 1–52. http://dx.doi.org/10.1155/2007/64358 (Article ID 64358).

Foolad, M.R., Lin, G.Y., 1997. Absence of a genetic relationship between salt tolerance during seed germination and vegetative growth in tomato. Plant Breeding 116, 363–367.

Gondo, T., Matsumoto, J., Tsuruta, S., Yoshida, M., Kawakami, A., Terami, F., Ebina, M., Yamada, T., Akashi, R., 2009. Particle inflow gun-mediated transformation of multiple-shoot clumps in rhodesgrass (*Chloris gayana*). Journal of Plant Physiology 166, 435–441.

Griffa, S.M., 2010. Caracterización bioquímica y molecular de germoplasma, evaluación de la tolerancia a la salinidad y obtención de híbridos en buffel grass. PhD Thesis. Universidad Nacional de Córdoba, Córdoba, Argentina.

Griffa, S., Díaz, D., Ribotta, A., Lanza Castelli, S., Muñoz, N., López Colomba, E., Luna, C., Grunberg, K., Biderbost, E., 2006. Molecular genetic discrimination of Buffel grass genotypes and F1 hybrids for breeding purposes using amplified fragment length polymorphism analyses. Grass and Forage Science 61, 454–458.

Hoagland, D.R., Arnon, D.I., 1950. The Water-Culture Method for Growing Plants Without Soil: California Agricultural Experiment Station, University of California, Berkley College Agriculture Circular, United States of America, 347, pp. 1–32.

Hoisington, D., Khairallah, M., González de León, D., 1994. Laboratory Protocols: CIMMYT Applied Molecular Genetics Laboratory, Second ed.

Kobayashi, H., Masaoka, Y., 2008. Salt secretion in rhodes grass (*Chloris gayana* Kunth) under conditions of excess magnesium. Soil Science and Plant Nutrition 54, 393–399.

Kobayashi, H., Masaoka, Y., Takahashi, Y., Ide, Y., Sato, S., 2007. Ability of salt glands in Rhodes grass (*Chloris gayana* Kunth) to secrete Na^+ and K^+. Soil Science and Plant Nutrition 53, 764–771.

Loch, D.S., Rethman, N.E.G., Van Niekerk, W.A., 2004. Rhodesgrass. In: Moser, L.E., Burson, B.L., Sollenberger, E.L., Al-Almoodi, L.K., Barbarick, K.A., Roberts, C.A., Dick, W.A. (Eds.), Warm Season (C4) Grasses. American Society of Agronomy/Crop Science Society of America/Soil Science Society of America, Madison, pp. 833–872.

Luna, C., García Seffino, L., Arias, C., Taleisnik, E., 2000. Oxidative stress indicators as selection tolls for salt tolerance in *Chloris gayana*. Plant Breeding 119, 341–345.

Luna, C., De Luca, M., Taleisnik, E., 2002. Physiological causes for decreased productivity under high salinity in Boma, a tetraploid *Chloris gayana* cultivar. II. Oxidative stress. Australian Journal of Agricultural Research 53, 663–669.

Munns, R., 2002. Comparative physiology of salt and water stress. Plant Cell Environmental 25, 239–250.

Munns, R., James, R.A., 2003. Screening methods for salinity tolerance: a case study with tetraploid wheat. Plant and Soil 253, 201–218.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annual Review of Plant Physiology 59, 651–681.

Munns, R., James, R.A., Lauchli, A., 2006. Approaches to increasing the salt tolerance of wheat and other cereals. Journal of Experimental Botany 5, 1025–1043.

Nakagawa, H., Shimizu, N., Sato, H., 1987. Chromosome number, reproductive method and morphological characteristics of *Chloris* species. Journal Japanese Society Grassland Science 33, 191–205.

Niknam, S.R., McComb, J., 2000. Salt tolerance screening of selected Australian woody species—a review. Forest Ecology and Management 139, 1–19.

Otondo, J., Rossi, C.A., Pérez, R.A., Jacobo, E.J., Torrá, E., 2008. Acumulación de materia seca de Poaceas subtropicales en bajos alcalinos-sódicos de la Depresión del Salado. 31ª Congreso Argentino de Producción Animal, Potrero de los Funes, San Luis, Argentina. Vol. 28 (1), pp. 415–416.

Pasternak, D., Nerd, A., De Malach, Y., 1993. Irrigation with brackish water under desert conditions. IX. The tolerance of six forage crops. Agricultural Water Manager 24, 321–334.

Pérez, H., 2005. Características de las especies forrajeras adaptadas a las condiciones del Noroeste del país. Forrajes 2005 Córdoba, Potenciando el desarrollo ganadero sustentable del subtrópico Argentino. Technidea, Córdoba, Argentina, pp. 33–41.

Pérez, H., Bravo, S., Ongaro, V., Castagnaro, A., Garcia Seffino, L., Taleisnik, E., 1999. *Chloris gayana* cultivars: RAPD polymorphism and field performance under salinity. Grass and Forage Science 54, 289–296.

Pérez, H., Taleisnik, E., Pemán, R., 2009. Development of a tetraploid salt-tolerant *Chloris gayana* cultivar. In: Jank, L., Chiari, L., Simeão Resende, R.M. (Eds.), Anais do II Simpósio Internacional sobre Melhoramento de Forrageiras. Embrapa Gado de Corte, Campo Grande. ISBN: 978-85-297-0236-0 (CD-Rom).

Promega, 1996. Silver sequence DNA sequencing system. Technical Manual 023. PROMEGA Corporation Madison, WI, USA.

Ribotta, A.N., 2011. Selección y caracterización de clones parentales diploides para la obtención de nuevo germoplasma con tolerancia incrementada a la salinidad en grama rhodes (*Chloris gayana* Kunth). MSc Thesis. Universidad Nacional de Córdoba, Córdoba, Argentina.

Ribotta, A.N., Griffa, S., López Colomba, E., Grunberg, K., Biderbost, E., 2005. Determinación del contenido proteico en materiales en proceso de selección de *Cenchrus ciliaris* L., *Chloris gayana* K. y *Panicum coloratum* L. Pastos y Forrajes 28, 241–246.

Rohlf, F.J., 1993. NTSYS-pc Numerical taxonomy and multivariate analysis for the IBM PC and compatible. Ver. 1.8 Applied Biostatistics Inc., Exeter Software, New York.

Sabir, P., Ashraf, M., 2007. Screening of local accessions of *Panicum miliaceum* L. for salt tolerance at the seedling stage using biomass production and ion accumulation as selection criteria. Pakistan Journal of Botany 39, 1655–1661.

Skerman, P.J., Riveros, F., 1990. Tropical Grasses. Food and Agriculture Organization of the United Nations: FAO Plant Production and Protection Series, pp. 283–288 (Nº 23).

Taleisnik, E., Grunberg, K., 1993. Tolerancia a la salinidad en plántulas de *Chloris gayana* y *Cenchrus ciliaris*. Revista Argentina de Producción Animal 13, 267–276.

Toll Vera, J.R., Lagomarsino, E.D., Martín, G.O., Nicosia, M.G., Fernández, M.M., 2000a. Evaluación de cultivares de Grama rhodes (*Chloris gayana* Kunth), en distintos tipos de suelos en el oeste de la provincia de Santiago del Estero. 1. Implantación y dinámica de los diferidos. Proceedings 23ª Congreso Argentino de Producción Animal, Corrientes, Argentina, pp. 195–196.

Toll Vera, J.R., Lagomarsino, E.D., Martín, G.O., Nicosia, M.G., Fernández, M.M., 2000b. Evaluación de cultivares de Grama rhodes (*Chloris gayana* Kunth), en distintos tipos de suelos en el oeste de la provincia de Santiago del Estero. 2. Disponibilidad y rebrote primaveral. Proceedings 23ª Congreso Argentino de Producción Animal, Corrientes, Argentina, pp. 196–197.

Ubi, B.E., Fujimori, M., Ebina, M., Mano, Y., Komatsu, T., 2000. AFLP variation in tetraploid cultivars of rhodesgrass (*Chloris gayana* Kunth). Journal of Japanese Society of Grassland Science 46, 242–248.

Ubi, B.E., Fujimori, M., Ebina, M., Komatsu, T., 2001. Amplified fragment length polymorphism analysis in diploid cultivars of rhodesgrass (*Chloris gayana* Kunth). Plant Breeding 120, 85–87.

Ubi, B.E., Kölliker, R., Fujimori, M., Komatsu, T., 2003. Genetic diversity in diploid cultivars of rhodesgrass determined on the basis of amplified fragment length polymorphism markers. Crop Science 43, 1516–1522.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., De Lee, T.V., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23, 4407–4414.