Cross-species transfer of SSR markers in *Setaria sphacelata* and *Trichloris crinita* sp.

Transferencia cruzada de marcadores SSR en *Setaria sphacelata* y *Trichloris crinita* sp.

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ABSTRACT

Setaria sphacelata and *Trichloris crinita* are subtropical forage species that are important for livestock breeding in Argentina. Genomic information is scarce for these species, and there are no molecular markers designed for them; this limits the development of genetic improvement programs. We performed a cross-species transfer of SSR markers from several Poaceae species. In *S. sphacelata*, 8 SSR markers were transferred from *Setaria italica* (40% transfer rate), exhibiting 83% polymorphism. Kazungula, Splenda and Narok cultivars were genetically differentiated and the experimental material "Selección INTA" was separated from Narok, from which it was derived. For *T. crinita*, 19 microsatellites were transferred from 5 Poaceae species (7.3% transfer rate), with 69% polymorphism. The results obtained in this study show the potential of the transferred SSR markers for assessing genetic variation and for expanding the genetic resources available for these species.

Key words: microsatellites, polymorphism, feed crops, genetic variation, plant genetic resources.

RESUMEN

Setaria sphacelata y *Trichloris crinita* son especies forrajeras subtropicales, estratégicas para el desarrollo de la actividad ganadera argentina. Para estas especies, la información genómica es escasa y no existen marcadores moleculares desarrollados en las mismas, por lo cual el desarrollo de programas de mejoramiento genético se ve limitado. En este contexto, realizamos una transferencia de marcadores SSR de varias especies de poáceas. En *S. sphacelata*, se transfirieron 8 marcadores desarrollados en *Setaria italica* (tasa de transferencia del 40%), mostrando un 83% de polimorfismo. Los cultivares Kazungula, Splenda y Narok se diferenciaron genéticamente y el material experimental "Selección INTA" se separó de Narok, del cual se deriva. Para *T. crinita*, se transfirieron 19 microsatélites de 5 especies poáceas (tasa de transferencia del 7.3%), con 69% de polimorfismo. Todos los individuos se pudieron difrenciar genéticamente. Los resultados obtenidos en este trabajo muestran la capacidad de los marcadores SSR transferidos para evaluar la variabilidad genética, expandiendo los recursos genéticos disponibles para estas especies.

Palabras clave: microsatélites, polimorfismo, plantas forrajeras, variación genética, recursos genéticos vegetales.

Introduction

Setaria sphacelata (Poaceae) is a perennial pasture grass native to tropical Africa (Hacker, 1991). It is a cross-pollinating, tetraploid species (2n=4x=36) (Hacker and Jones, 1969; Le Thierry d´Ennequin *et al*., 1998) of summer growth. It is cultivated in regions with rainfall exceeding 750 mm; but it also exhibits resistance to drought, flooding, and nutrient-deficient soils (Borrajo and Pizzio, 2006). In Argentina, *S. sphacelata* is cultivated in the northern regions, and the most agronomically important cultivars are Splenda, Kazungula, and Narok (Pensiero, 1999; Borrajo *et al*., 2006; Burghi *et al*., 2014).

Trichloris crinita (Lag.) Parodi (Poaceae) is a perennial grass species native to arid regions of the American continent (Kozub *et al.,* 2017). It is a warm-season species with good forage quality due to its protein content and palatability (Nicora and Rúgolo de Agrasar, 1987). It is an autogamous tetraploid (2n=4x=40) (Fedorov, 1969), tolerant to drought and low-salinity (Aronson, 1989; Greco and Cavagnaro, 2003), which is used for protection against soil erosion (Dalmasso, 1994). In Argentina, *T. crinita* integrates the grasslands of arid and semi-arid regions (Cano, 1988). In 2012, the genus *Trichloris* was embedded in *Leptochloa* and the species *T. crinita* was renamed *L. crinita* (Snow and Peterson, 2012).

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Both species are used as forage in Argentina and are strategic for the development of regional and national livestock breeding. The accessibility of molecular markers could complement the genetic improvement programs for these species. Although there are no microsatellites specifically designed for these grass species to date, a few studies have been reported using molecular markers. On the one hand, Li *et al.* (1998) applied random amplification of polymorphic DNA (RAPD) analysis to assess the intraspecific and interspecific variation in several species of genus *Setaria*, including *S. sphacelata*. Moreover, simple sequence repeat (SSR) markers were developed in *S. italica* and transferred to *S. sphacelata* (Gupta *et al*., 2012; Gupta *et al.,* 2013; Kumari *et al*., 2013; Pandey *et al*., 2013). Cavagnaro *et al.* (2006) assessed the genetic diversity in *T. crinita* varieties using amplified fragment length polymorphism (AFLP) markers. More recently, SSR markers were developed in *T. crinita* using sequence data from related grass species (Kozub *et al*., 2018).

In order to expand the set of molecular markers available for genetic improvement programs of *S. sphacelata* and *T. crinita*, we conducted an SSR cross-amplification from related species of the Poaceae family. For this, we first optimized the DNA extraction for *T. crinita*.

Materials and methods

Plant materials

We studied three *S. sphacelata* cultivars in this research: Narok, Splenda, and Kazungula. We also included an experimentally improved population derived from Narok, called "Selección INTA" (EEA INTA Mercedes, Corrientes), in the analysis. Twenty individuals from each cultivar were analyzed in bulk.

We collected *Trichloris crinita* material from four sites in Argentina: one individual from La Pampa (36°35'34.4" S and 64°41'47.6" W), eight individuals from Catamarca (29º18'22" S and 65º08'40" W), seven individuals from Cordoba (three individuals from 29º57-59' S, 64º28-29' W, and 300 m a.s.l., and four individuals from 29º49-54' S, 64º27-28' W, and 310 m a.s.l.), and seven individuals from La Rioja (three individuals from 31º24-25' S, 66º46-47' W, and 490 m a.s.l. and four individuals from 31º30-32' S, 66º48-49' W, and 450 m a.s.l.) (Quiroga *et al*., 2010).

Plants were grown at 25°C in 20 cm pots filled with a 3:1 mixture of fertilized soil and vermiculite in a greenhouse at the Ewald A. Favret Institute of Genetics, National Institute of Agricultural Technology (IGEAF-INTA). Pots were watered approximately every 3 d. The photoperiod was 16 h light and 8 h dark.

DNA extraction

Different protocols were attempted for DNA extraction from *S. sphacelata* without successful results. Therefore, in order to obtain high-purity genomic DNA, we modified the extraction protocol described by Dellaporta *et al.* (1983). Fresh young leaves were ground in liquid nitrogen and 30-40 mg were resuspended in 800 µl of extraction buffer (50 mM Tris-HCl, 0.5 mM EDTA, 50 mM NaCl, 10% SDS, 10 mM β-mercaptoethanol, pH 8.0). Tubes were incubated at 65ºC for 30 min. Then, 200 µl of potassium acetate (5 M) was added, and the tubes were incubated in ice for 20 min. The suspension was centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatants were transferred into clean tubes. Next, 800 µl of chloroform:isoamyl alcohol 24:1 (v/v) was added, and the tubes were centrifuged again at 12,000 rpm for 10 min. Isopropanol (1 volume) was added to the supernatants for DNA precipitation, and the tubes were spun at 12,000 rpm for 30 min at 4ºC. The supernatants were discarded and the DNA pellets resuspended in 250 µl of TE1X buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0). Then, 2.5 µl of RNase (10 mg/ml) was added, and the tubes were incubated for 30 min at 37ºC. Later, phenol (1 volume) was added, and the tubes were centrifuged at 12,000 rpm for 10 min at 4ºC. The supernatants were washed with TE1X buffer and spun. One volume of chloroform:isoamyl alcohol 24:1 (v/v) was added to the supernatants, and the tubes were centrifuged again. The supernatants were transferred to new tubes, and the DNA was precipitated with isopropanol (1 volume). The tubes were incubated for 30 min at 4°C and then spun at 12,000 rpm for 40 min at 4ºC. The supernatants were discarded, and the pellets were washed with 500 µl of 70% (v/v) ethanol. Finally, the tubes were centrifuged at 12,000 rpm for 2 min, and the DNA pellets were resuspended in HPLC water.

Genomic DNA from *T. crinita* was extracted using the Saghai-Maroof method, modified by Pérez de la Torre *et al.* (2008). The DNA extracted from both species was verified by 0.8% agarose gel electrophoresis.

SSR markers

We selected a set of 29 SSR primers for cross-amplification in *S. sphacelata*: 20 pairs from *S. italica* (www.ncbi.nlm.nih.gov, Jia *et al*., 2007), two from *Bromus tectorum* (Ramakrishnan *et al*., 2004), one from *Lolium perenne* (Jones *et al*., 2001), three from *Triticum aestivum* (Röder *et al*.,

1998), and three from *Zea mays* selected from http//www.agron.missouri.edu. We labeled all forward primers for *S. sphacelata* with FAM (6-carboxyfluorescein) and HEX (hexachloro-6-carboxyfluorescein) fluorescent dyes (Alpha DNA).

For cross-amplification in *T. crinita*, we selected a set of 260 SSR primers: six pairs from *B. tectorum* (Ramakrishnan *et al*., 2004), 50 from *Cenchrus ciliaris* (Jessup, 2005), seven from *Eleusine coracana* (Dida *et al*., 2007; Arya *et al*., 2009), 20 from *Festuca arundinacea* (Saha *et al*., 2003), 10 from *L. perenne* (Jones *et al*., 2001), 12 from *Panicum maximum* (Ebina *et al*., 2007; Chandra and Tiwari 2010), 20 from *S. italica* (Jia *et al*., 2007; www.ncbi.nlm.nih.gov), 66 from *T. aestivum* (Röder *et al*., 1998; http://wheat.pw.usda.gov), and 69 designed in *Z. mays* (http//www.agron.missouri.edu).

PCR amplification

We performed PCR reactions in a total volume of 20 µl, which contained 75 ng of DNA, 1X PCR buffer (Invitrogen), 0.5 U of Taq DNA polymerase, 0.125 mM of each primer, 0.15 mM of dNTPs, and 2.5 mM of $MgCl_2$. The amplification reactions were performed in a thermal cycler (Mastercycler Epgradient S, Eppendorf), under the following conditions: initial denaturation temperature of 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 30 s, and amplification at 72°C for 2 min, with a final extension at 72°C for 10 min. We applied each PCR four times.

In *S. sphacelata*, we analyzed the individuals from each cultivar in bulk. We detected SSR fragments by ABI PRISM 3130 Genetic Analyzer using Genemapper 3.4 software (Applied Biosystems). In *T. crinita*, we separated PCR products by 6% non-denaturing polyacrylamide gels and stained them with ethidium bromide. The fragment size for each product was determined by 100, 50, and 10 bp standard size markers (Invitrogen).

Data analysis

We estimated genetic distances among the materials using Dice and Jaccard coefficients for *S. sphacelata* and *T. crinita*, respectively. We conducted clustering between species using the Unweighted Pair-Group Method with the arithmetic Average (UPGMA). In the dendrogram, we used *S. italica* and *C. ciliaris*as external controls (outgroups) for *S. sphacelata* and *T. crinita*, respectively. We quantified the level of polymorphism for each SSR with the Polymorphism Index Content (PIC) (Botstein *et al*., 1980). We performed Principal Coordinates Analysis, along with the rest of the statistical analyses described in this section, using InfoGen software (Balzarini and Di Rienzo, 2016). We estimated genetic variability within and among species via an analysis of molecular variance (AMOVA) using Gen AIEx 6.2 software (Peakall and Smouse, 2006).

Results and discussion

Eight out of the 20 markers designed in *S. italica* were transferable to *S. sphacelata*. None of the primer pairs from the other species, *B. tectorum* (2), *L. perenne* (1), *T. aestivum* (3) and *Z. mays* (3), generated amplicons.

We obtained 21 different PCR products with an average of 2.62 bands per marker and with sizes ranging from 120 to 411 bp. The average transfer rate of SSR markers from *S. italica* to *S. sphacelata* was 40% (Tab. 1). Six of the eight transferred microsatellites could amplify polymorphic bands, which represents a 75% level of polymorphism. The polymorphism index content (PIC) varied from 0.27 to 0.36.

Transferability between *S. italica* and *S. sphacelata* was consistent with the results obtained by Barbará *et al*. (2007) for amplification within genera in monocots (40%). However, the polymorphism obtained in this study for the

TABLE 1. Characteristics of the SSR markers transferred from *Setaria italica* to *Setaria sphacelata*. bp: base pairs; nd: not determined.

SSR	Forward primers (5´- 3´)	Reverse primers (5´- 3´)	Size (bp)		Repeat motif	Reference
			Expected	Observed		
2	AGAAAGTTGTAGATTGGGAAGA	AATAATGTGGAAAGACCCTGT	346	346	nd	GI: 29123369
3	ACTATGTGGTGGAGGGCGGT	GATGATAAGAGGCAGGAGTG	279	186- 393	nd	GI: 30351051
4	TACTTGACTGCTCACACCTTC	TTGTCCTTGTAATCCACTCC	376	375- 411	nd	GI: 22002467
9	TATGCCTCAAACAACATCC	ACTCCCTTCCAATGATACAAC	333	273-357	nd	GI: 62318483
15	AGAAAGTTGTAGATTGGGAAGA	GAGAGCGACTGAGACACC	286	270- 285	nd	GI: 15558946
P2	CCAACACGCAATCGCAGAA	AGGCAGTGGGTTTGAGCAT	120-127	120	CT	Jia *et al*., 2007
P5	TTGCCTTGAGCTCTTTGATG	GCTGATACTGATATGTCTGATGAGGA	300-307	300-310	CAT	Jia *et al*., 2007
P13	GGAGAGATTCCGGGCTCTAGT	ACGGTTCCGACATTTTAACG	166-170	160- 170	CA	Jia *et al*., 2007

transferred markers to *S. sphacelata* (75%) was higher than the 26% observed by Jia *et al*. (2007) with microsatellites developed and evaluated in *S. italica*. It is also higher than the polymorphism obtained by Wang *et al*. (2005) between Poaceae species (67%) and between self-incompatible species (57%).

In order to assess the applicability of the transferred SSR markers, we performed a genetic diversity analysis in *S. sphacelata* cultivars (Fig. 1). The genetic distance of materials ranged from 0.18 to 0.33. The dataset computed a high cophenetic correlation between matrixes (0.892). "Selección INTA" was separated from Narok cultivar at a genetic distance of 0.18, which is expected considering that the former derives from the latter (Borrajo *et al*., 2009). The genetic distance between *S. sphacelata* and *S. italica* obtained in this study was similar to that reported by Kumari *et al.* (2013).

Previous research on *S. sphacelata* reports significant variability in agronomically relevant, morphological characteristics among populations, lines, and cultivars (Hacker and Cuany, 1997; Jank *et al*., 2007). In this context, the availability of molecular markers for the species could contribute to a production of more proficient genetic improvement programs. Furthermore, Hacker and Cuany (1997) studied variation in seed production between cultivars of *S. sphacelata* and found that Kazungula and Narok are the most contrasting ones. Similar results are reported by Hacker (1991), Hacker and Cuany (1997), Jank and Hacker (2004) and Jank *et al.* (2007), coinciding with the molecular classification obtained in this study (Fig. 1).

For *T. crinita*, 19 (out of the 260 evaluated markers) SSR were successfully transferred from five Poaceae species (a 7.3% transfer rate). Microsatellites from *C. ciliaris* and *Z. mays* showed the highest transfer rates of 18% and 10%, respectively (Tab. 2). These results are in full agreement with reports by Barbará *et al.* (2007) on transfer rates between genera of approximately 10% for eudicots and even lower for monocots.

PIC varied from 0.11 to 0.36. Sixty-one different PCR products were obtained with an average of 3.21 bands per marker and sizes ranging from 85 to 450 bp. In total, 69% of the transferred SSR were polymorphic. These are high levels of polymorphism in comparison with the 34-46% obtained by Saha *et al.* (2006) with transferred SSR markers from *F. arundinacea* to Poaceae species. Our results are similar to those obtained by Wang *et al. (*2005) for *Cynodon* with SSR transferred from major cereal crops.

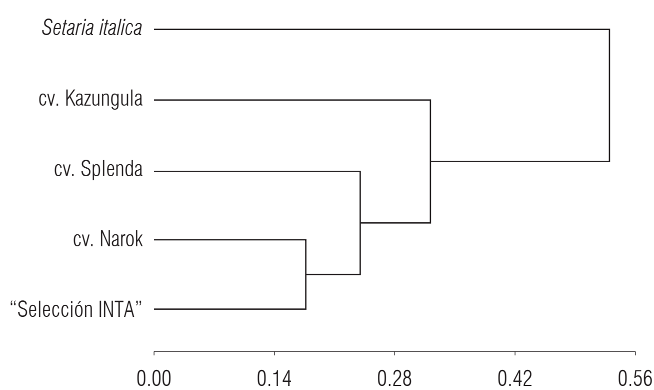

FIGURE 1. Genetic variability among *Setaria sphacelata* cultivars based on the 8 SSR markers transferred from *S. italica*. The clustering analysis was performed using the UPGMA and Dice coefficient. *S. italica* was used as an outgroup.

Kozub *et al.* (2018) also transferred SSR markers to *T. crinita*. Whereas the level of polymorphism obtained in our work (69%) is higher than that obtained by the authors (37.5%), our transfer rate (7.3%) is lower than theirs (15.2%). This could be explained by the use of highly phylogenetically related species by Kozub *et al.* (2018).

We assessed genetic diversity among *T. crinita* individuals through cluster analysis. We grouped materials in four clusters, with genetic distances ranging from 0.13 to 0.37 and a cophenetic correlation coefficient of 0.905 (Fig. 2). Although the distribution of the individuals into the four clusters was not completely consistent with the collection sites, the SSR markers used for this study exhibited a potential for discriminating the individuals from Catamarca and Cordoba. Individuals from La Rioja were scattered between the groups. The sample collected in La Pampa (cluster A) was the first to separate, with a genetic distance of 0.37. Cluster B, formed by four individuals collected in Cordoba, separated at a genetic distance of 0.34. Finally, clusters C and D separated at 0.31. The first was composed of three individuals from Cordoba and two from La Rioja, and the latter consisted mainly of individuals from Catamarca.

Additionally, we performed a principal coordinates analysis (PCoA) to better visualize the variability among the individuals (Fig. 3). The distribution obtained with the PCoA is analogous to the classification obtained with the cluster analysis. Moreover, an AMOVA was computed, and we observed that 79% of the molecular variance belonged to differences within populations, whereas 21% belonged to differences among them.

TABLE 2. Summary of the SSR markers transferred to *Trichloris crinita* from five Poaceae species.

SSR	Forward primers (5´- 3´)	Reverse primers (5´- 3´)	Size (bp)		Repeat motif	Donor species	References
			Expected	Observed			
LPSS-RH01A07	TGGAGGGCTCGTGGAGAAGT	CGGTTCCCACGCCTTGC	350	225-400	nd	*L. perenne*	Jones *et al*., 2001
NFFa019	GCTCGTGTATGGCCTTCAAT	TGGATTTGCAATTAGCCTCA	190	180-350	nd	*F. arundinacea*	Saha *et al*., 2003
2D03b	CAATGGGAGCTCAAATTAGCA	CGGGGAAGAAGTTTGTTCTTT	250	160-200	AT	*C. ciliaris*	Jessup, 2005
2D09	CAAATCGGAGCAAATCGG	AGGAAAGCCTCGGGAAAC	358	115-205	AAC	*C. ciliaris*	Jessup, 2005
6E10	ACTCCACTGCTGCCTCCT	CTTCCACCACCATACCCT	389	180-200	GCC	*C. ciliaris*	Jessup, 2005
7B11	CTCCATTCGCCTCCCTAC	GTTTCGTCTCTCCCATCAG	391	200	GA	*C. ciliaris*	Jessup, 2005
7E09	GGAGGTAGATGTTGATGTTGA	CCCTTTGTCCCGCCATAC	360	85-320	CGG	*C. ciliaris*	Jessup, 2005
7H12	TCTTATTCCTCCGAGCCGTA	GGAAAATTGGGACCCTTTGT	182	170-450	CT	*C. ciliaris*	Jessup, 2005
10E12	CTCTTGAACCCCGAGGCTAT	ATCTCGCGTCATCGTTTAGG	196	105-350	GT	*C. ciliaris*	Jessup, 2005
10G10a	AAGAAGAAGAAGAAGAAGAAGGA	GGAAGAGGAGACCAACAAA	193	350-380	TGAC	*C. ciliaris*	Jessup, 2005
10H10	CGACTCAGACCACCTCTC	GGCTCCCAGTTCTTCATC	307	160	CGA	*C. ciliaris*	Jessup, 2005
xgwm190	GTGCTTGCTGAGCTATGAGTC	GTGCCACGTGGTACCTTTG	201-253	165-350	CT	*T. aestivum*	Röder *et al*., 1998
bnlg0439	TTGACATCGCCATCTTGGTGACCA	TCTTAATGCGATCGTACGAAGTTGTGGAA	nd	200-220	nd	*Z. mays*	http//www.agron.missouri.edu
bnlg1014	CACGCTGTTTCAGACAGGAA	CGCCTGTGATTGCACTACAC	nd	160-190	AG	*Z. mays*	http//www.agron.missouri.edu
bnlg1016	CCGACTGACTCGAGCTAACC	CCGTAACTTCCAAGAACCGA	nd	120-180	AG	*Z. mays*	http//www.agron.missouri.edu
bnlg1811	ACACAAGCCGACCAAAAAAC	GTAGTAGGAACGGGCGATGA	nd	165-185	AG	*Z. mays*	http//www.agron.missouri.edu
bnlg2057	CAGCAGAACCTGTGGACAGA	TGCATACTTGAGGATCGGAG	nd	125-195	AG	*Z. mays*	http//www.agron.missouri.edu
umc1220	ATCTTTTTCCTTCCGAGCTGTACG	GTGGACGAGTCCGTGCTCAG	nd	130-140	AG	*Z. mays*	http//www.agron.missouri.edu
umc1331	TTATGAACGTGGTCGTGACTATGG	ATATCTGTCCCTCTCCCACCATC	nd	145-170	GGT	*Z. mays*	http//www.agron.missouri.edu

bp: base pairs; nd: not determined.

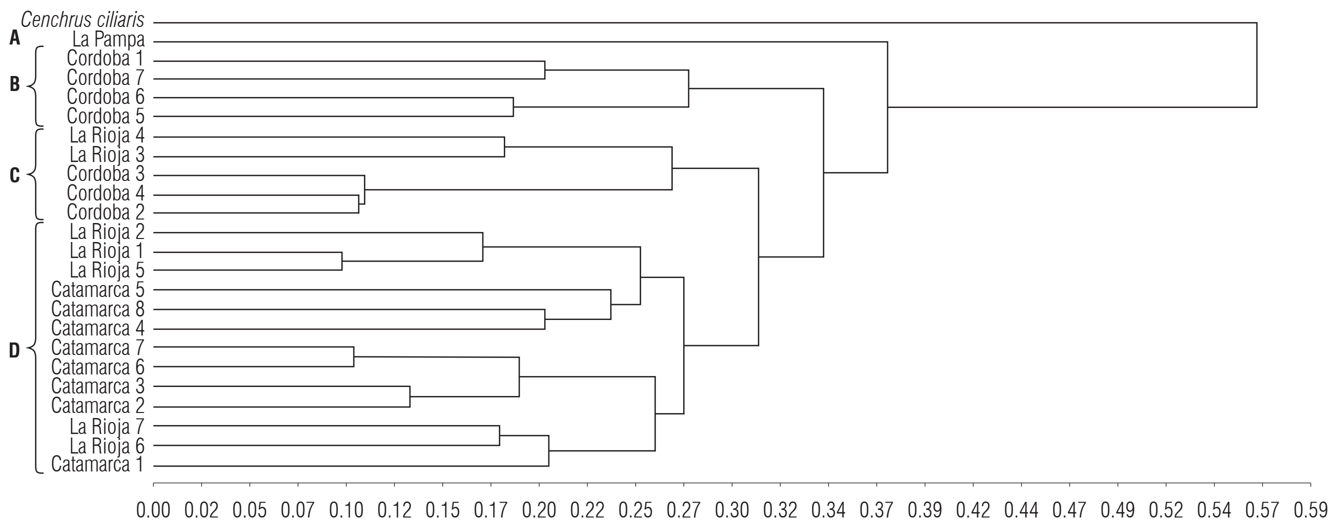

FIGURE 2. Dendrogram showing the genetic relationships among 23 *Trichloris crinita* individuals based on the transferred SSR markers. The clustering analysis was performed using the UPGMA and Jaccard coefficient. Letters indicate groups discriminated at 50% of the maximum estimated genetic distance. We used *C. ciliaris* as an outgroup.

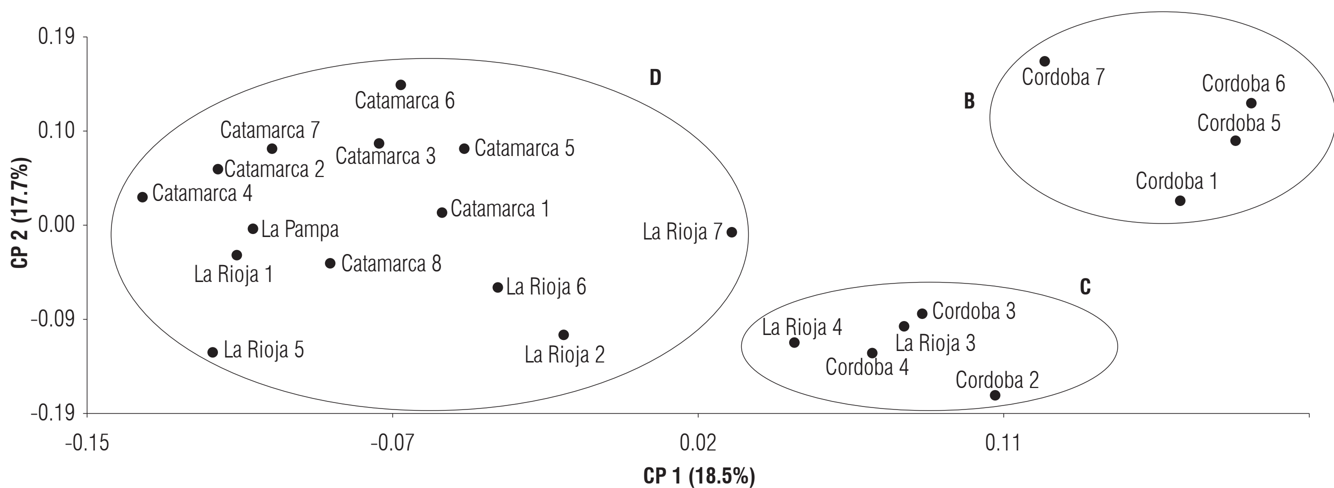

FIGURE 3. Principal coordinates analysis plot. Variability among 23 *Trichloris crinita* individuals based on the transferred SSR markers. Circles delimit the individuals grouped into clusters B, C, and D according to the clustering analysis showed in Figure 2.

Conclusions

The goal of this study was to generate a molecular tool for *S. sphacelata* and *T. crinita* that could be used to make more efficient genetic improvement programs in these species. The strategy was to exploit the available genetic resources of agronomically relevant crops to identify markers in grass species with limited genomic information.

We successfully transferred eight polymorphic SSR markers to *S. sphacelata* and 19 to *T. crinita*. These markers widen the available molecular resources for these forage crops, especially in *T. crinita* for which this study constituted the second report of transferred microsatellites in the species. As for *S. sphacelata*, these findings not only expanded the available genetic resources but also increased the number of methodological tools for the species with the optimization of the DNA extraction protocol. We expect the results obtained in this study will be valuable for the development of new molecular breeding programs and novel strategies to assess genetic diversity in the species.

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