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Development and characterization of SSR markers for *Trichloris crinita* using sequence data from related grass species

Desarrollo y caracterización de marcadores moleculares SSR para *Trichloris crinita* usando secuencias de gramíneas filogenéticamente cercanas

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Abstract

Trichloris crinita is among the most important native forage grasses in arid regions of America. Despite its importance, molecular resources and sequence data are extremely scarce in this species. In the present study, SSR markers were developed using available DNA sequences from grass taxa phylogenetically-related to *Trichloris* (*Eleusine coracana*, *Cynodon dactylon* and '*Cynodon dactylon* x *Cynodon transvaalensis*'). Marker transferability was evaluated in a panel of eight *T. crinita* accessions and five closely-related species. Of the 105 SSR primer pairs evaluated, 16 amplified products of expected size in *T. crinita*, whereas transferability to other grass species ranged from 12 (in *Chloris castilloniana*) to 28 SSRs (in *Eleusine coracana*). Six of the 16 SSR markers successfully transferred to *T. crinita* (37.5%) were polymorphic, and were further used to assess genetic diversity in eight *T. crinita* accessions. The analysis revealed a total of 23 SSR alleles (3.83 alleles/locus), allowing the discrimination of all *T. crinita* accessions, with pair-wise genetic similarities ranging from 0.35 to 0.81 (Jaccard coefficient). Mean (and range) values for observed (*Ho*) and expected heterozygosity (*He*) were 0.53 (0.0-1.0) and 0.63 (0.48-0.79), respectively.

Keywords
Chloridoideae • forage grass • genetic diversity • marker transferability • microsatellites

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Resumen

Trichloris crinita es una importante gramínea forrajera, nativa de regiones áridas del continente americano. A pesar de su importancia, no existen herramientas moleculares ni secuencias nucleotídicas disponibles para esta especie. En este estudio, se desarrollaron marcadores moleculares SSR ("simple sequence repeats") a partir de secuencias nucleotídicas de especies filogenéticamente cercanas a *Trichloris* (*Eleusine coracana*, *Cynodon dactylon* y '*Cynodon dactylon* x *Cynodon transvaalensis*') y se evaluó su transferibilidad en ocho accesiones de *T. crinita* y cinco especies de gramíneas cercanamente emparentadas. De los 105 pares de cebadores evaluados, 16 amplificaron productos del tamaño esperado en *T. crinita,* mientras que la transferibilidad a otras especies varió entre 12 (en *Chloris castilloniana*) y 28 SSRs (en *Eleusine coracana*). De los 16 SSRs transferibles a *T. crinita*, seis fueron polimórficos y se utilizaron para analizar el grado de diversidad genética en ocho accesiones de esta especie. El análisis reveló 23 alelos, los cuales permitieron diferenciar todas las accesiones de *T. crinita,* con valores de similitud genética entre pares de accesiones de 0,35 a 0,81 (Jaccard). Se obtuvieron valores medios de heterocigosidad observada y esperada de 0,53 y 0,63 respectivamente.

Palabras clave

Chloridoideae • gramínea forrajera • diversidad genética • transferibilidad • microsatélites

Introduction

Trichloris crinita (Chloridoideae, Poaceae) is one of the most important native grass species in arid regions of South America, due to its extensive area of distribution (25), good forage quality (7, 27), and resistance to drought (13), trampling and grazing by wild and domestic animals (4). These characteristics and its competing aggressiveness among other native grasses (22), have led to a widespread utilization of *T. crinita* in range grazing and revegetation projects in arid environments (24).

Despite its importance, advances in genetic research and breeding of *T. crinita* have been limited, mainly due to the scarcity of molecular resources and the fact that the species mode of reproduction was unknown until very recently. The main approach used to improve *T. crinita* has been the selection of interesting phenotypes from natural populations, followed by their characterization based on traits of interest, such as biomass production (5), forage quality (7, 27), and drought resistance (12). Data from a recent study (16), demonstrating that *T. crinita* is a sexually-propagated autogamous species, encourages the practice of conventional breeding strategies in the species. Along with this, the possibility of developing molecular markers for assisting selection may accelerate *T. crinita* breeding goals.

Molecular resources in *T. crinita* are very scarce. To date, only two studies regarding the use of molecular markers in this species have been published (5, 16). The first study, published in 2006, used AFLP markers for characterizing genetic diversity in a *T. crinita* germplasm collection (4), whereas a more recent study by Kozub *et al.* (2017) used simple

sequence repeats (SSR) markers for inferring about the mode of reproduction of this species. The present work reports on the development and evaluation, at various levels, of the SSR markers used in the latter report.

SSRs are robust and informative PCR-based markers and they are generally favored over AFLPs due to their higher level of polymorphism, reproducibility and codominant inheritance (18). These markers have been successfully used for multiple genetic and breeding purposes in many crop species, including grapevine (19), carrot (6), alfalfa (12) and soybean (3). However, the development of SSR markers generally requires DNA sequence data to search and detect SSR motifs, and design primers flanking the SSR. In the case of *T. crinita*, using sequence databases to identify SSRs is not feasible since very few sequences are available for the species (only 34 *T. crinita* sequences are available at the NCBI database).

The use of sequence data from related taxa for developing SSR markers in a species lacking sequence information has been widely used in plants, with variable degrees of success, generally increasing success rate with the phylogenetic proximity between SSR donor and target species (15). This approach has been particularly successful in the Poaceae family, where SSR markers were developed for numerous orphan species, using sequences from economically important cereal crops (34, 35). This strategy may also be effective for developing SSR markers for *T. crinita* using the available sequence data from phylogenetically related genera, such as *Eleusine* and *Cynodon.*

Objectives

1) To develop SSR markers for *Trichloris crinita* using sequence data from *Eleusine* and *Cynodon* species.

2) To evaluate marker transferability across *T. crinita*-related taxa.

3) To assess SSR polymorphism and genetic diversity in *T. crinita* accessions.

Materials and methods

Source of sequence data, microsatellites search and primer design

All the available genomic sequences (GSS) of *Eleusine coracana* (633 sequences; 0.4 Mbp), *Cynodon dactylon* (404 sequences; 0.2 Mbp) and '*Cynodon dactylon* x *Cynodon transvaalensis*' (92 sequences; 0.04 Mbp) were downloaded from the NCBI database on April 15, 2016. These species were selected based on their phylogenetic proximity to *T. crinita* and the availability of sequences in the database.

SSR motifs were identified using the program MISA (31). Only perfect microsatellites, with a basic motif of 2-6 nt, and a minimum length of 12 nt (for di-, tri-, and tetranucleotides), 15 nt (for pentanucleotides), and 18 nt (for hexanucleotides) were considered.

The position of the detected SSRs in the genomic sequence was recorded, and primers pairs flanking 105 SSRs were designed. These SSR loci were selected because they had larger number of repeat units, as this feature has been associated with higher polymorphism rate (6).

For primer design, the software Primer3 v.4.0.0 (32) was used, using parameters to generate amplicons of 200-500 bp, and primer length of 22-30 nt with Tm of 55-62°C and GC content of 30-50 %. Other parameters used the program default values.

Plant materials

Eight *Trichloris crinita* accessions and five related grass species (*Cynodon dactylon, Eleusine coracana, Eleusine indica, Chloris gayana*, and *Chloris castilloniana*) from the Germplasm Bank of Native Grasses (GBNG) at the Argentine Institute for Research in Arid Regions (IADIZA) (Mendoza, Argentina), were used in this study. Characteristics of these plant materials -used for marker development, evaluation of marker transferability across species, and analysis of genetic diversity within *T. crinita*- are presented in table 1 (page 5).

DNA extraction and PCR and gel electrophoresis conditions

For DNA extraction, fresh young leaves of a single plant from each accession were harvested and immediately ground in liquid nitrogen with a mortar.

The resulting powder (~20 mg) was used for DNA isolation according to Murray and Thompson (1980). DNA concentration and purity were determined with a Picodrop spectrophotometer (PicoPet 01), and DNA integrity was evaluated by 0.9% agarose gel-electrophoresis. DNA samples were diluted to a final concentration of ~30 ng/µl and used as template in PCR amplifications.

PCR reactions were performed in 20 µl final volume containing 11.6 µl water, 2 µl 10 × DNA polymerase buffer, 1.6 µl dNTPs (2.5 mM each), 1 µl 5µM of each primer, 0.3 µl Taq polymerase at 3 U/µl and 2.5 µl of genomic DNA. Thermocyclers were programmed as follows: initial denaturation at 94°C for 30 sec, followed by 40 cycles of 94°C for 45 sec, appropriate annealing temperature for 30 sec, and 72°C for 1 min; and a final step of 72°C for 4 min.

Agarose (3%) gel electrophoresis used ethidium bromide(4ul/100ml of TAE buffer) for visualization of the amplicons. A 100 bp ladder (Invitrogen) was used as size marker. Denaturing polyacrylamide (6%) gel electrophoresis was run at 1500 V, 60 W and 40 mA for 3 hours. Silver staining was performed and the gels were photographed for later genotype analysis. SSR allele sizes were estimated by comparisons with a 100 bp DNA ladder (Invitrogen).

SSR marker analysis

For analysis of marker transferability across species, 105 SSR primer pairs were evaluated using as template for PCRs genomic DNA from eight *T. crinita* accessions and five phylogenetically-related grass species (table 1, page 5). PCR products were resolved by agarose gel electrophoresis. An SSR marker was considered transferable to a target species when positive amplifications with amplicons of expected size, were observed in that species. For the accessions and SSRs that failed to produce amplicons of expected size or yielded no PCR products at all, a second round of PCRs was performed, and if the same results were observed, they were recorded as "negative PCR reactions".

Table 1. Species and accessions used for SSR marker development, and analyses of marker transferability and genetic diversity

Tabla 1. Especies y accesiones utilizadas para el desarrollo de los marcadores SSR, análisis de transferibilidad y diversidad genética.

Species	Accession Number (IADIZA-GBNG)	Comments / remarks	References
Trichloris crinita	Tc-3	Medium biomass production. Medium drought resistance.	(5, 9)
Trichloris crinita	Tc-4	High biomass production. Medium drought resistance.	(5, 9)
Trichloris crinita	Tc-7	High biomass production.	(9)
Trichloris crinita	Tc-8	Low biomass production.	(9)
Trichloris crinita	Tc-9	Low biomass production. Good forage quality.	(2, 9)
Trichloris crinita	Tc-12	High biomass production. High drought resistance.	(5, 9)
Trichloris crinita	Tc-17	Medium biomass production. Medium drought resistance.	(5, 9)
Trichloris crinita	Tc-24	High biomass production. Medium drought resistance.	(5, 9)
Chloris castilloniana Lillo & Parodi	Chc-01	South American native grass. High forage quality. No available SSRs for this species.	(32)
Chloris gayana Khunt (Rhodes grass)	Chg-01	Important forage grass native to tropical and subtropical Africa. No available SSRs for this species.	(33)
Eleusine coracana (finger millet)	Ec-01	SSR donor species / Native to Africa. Used as cereal crop in arid/semi-arid regions.	-
Eleusine indica (goosegrass)	Ei-01	African native C4 grassweed of rain-fed agriculture. Very few SSRs available for this species.	(34, 35)
Cynodon dactylon (Bermuda grass)	Cd-01	SSR donor species / Important perennial grass, widely cultivated in warm regions.	-
***C. dactylon* x *C. transvaalensis* ***	-	SSR donor species.	-

Species used for marker development (*i.e.*, SSR donor species) are indicated in bold. * '*C. dactylon* x *C. transvaalensis*' was only used as a source of DNA sequence for marker development but was not included in the analyses of marker transferability and/ or genetic diversity.

IADIZA-GBNG: Argentine Institute for Research in Arid Regions- Germplasm Bank of Native Grasses.

Las especies utilizadas para el desarrollo de marcadores están indicadas en negrita. * '*C. dactylon* x *C. transvaalensis*' fue utilizada para el desarrollo de marcadores, pero no fue incluida en los análisis de transferibilidad y/o diversidad genética.

IADIZA-GBNG: Instituto Argentino de Investigación en Regiones Áridas - Banco de Germoplasma de Pastos Nativos.

SSRs with positive amplifications (*i.e.*, SSRs that produced amplicons of expected size) in all *T. crinita* accessions and the related grass species were selected for further analysis of marker polymorphism and genetic diversity. For this, SSR amplification products were generated and resolved by denaturing polyacrylamide gel (6%) electrophoresis, run at 1500V, 60W and 40 mA for 3 hours. The gels were stained with silver nitrate and photographed for later analysis. SSR allele sizes were estimated by comparison with a DNA size ladder.

The genetic diversity analysis was performed on eight *T. crinita* accessions and five other grass species phylogenetically-related to *Trichloris*. The latter species were included in the analysis as putative out-groups. Marker data for each locus and accession were recorded, and a binary matrix with information on the presence or absence of SSR alleles was constructed and used for estimating pair-wise genetic similarities among the accessions (Jaccard coefficient) (28), using the software XLSTAT v.2016.05.33324.

A dendrogram reflecting genetic similarities among the taxa was constructed using the Unweighted Pair Group Method with arithmetic Average (UPGMA) procedure with the XLSTAT software.

Based on the SSR band patterns, and considering *T. crinita* as a tetraploid species (26), observed (*Ho*) and expected (*He*) heterozygosity values were estimated for each marker, according to Bever and Felber (1992) with the program ATETRA 1.0 (33) using 10,000 Monte Carlo simulations. He was used as a measure of the level of polymorphism in each locus**,** despite the absence of Hardy-Weinberg equilibrium. Three categories of *He* values [low (He ≤ 0.3), moderate (0.3 < He ≤ 0.5) and high (0.5 < He)] were established to describe the polymorphism level at each SSR locus. It must be noted that analyses of *Ho* and *He* was restricted to the *T. crinita* germplasm.

Results

A total of 105 primer pairs were designed flanking SSRs detected in genomic sequence of three grass species phylogenetically-related to *Trichloris* [*Eleusine coracana* (65 SSRs), *Cynodon dactylon* (21 SSRs) and '*C. dactylon* x *C. transvaalensis*' (19 SSRs)]. The selected SSRs corresponded to di- (37%), tri- (27%), tetra- (29%) and pentanucleotides (7%).

SSR marker transferability across grass species

The number and percentage of transferable SSR markers from each donor species to *T. crinita* accessions and 5 other grasses are presented in table 2 (page 7). In total, 16 markers, representing 15.2% of the SSRs tested, were transferable to *T. crinita* (*i.e.*, 16 markers amplified products of expected size in *T. crinita* accessions). Of these, seven SSRs were developed from *E. coracana*, six from *C. dactylon*, and three from '*C. dactylon* x *C. transvaalensis*'. Table 3 (page 8-9), presents further information on the 16 SSRs that were successfully transferred to *T. crinita*, including primer sequence, annealing temperature, repeat motif, expected and observed amplicon lengths, number of alleles, and observed (*Ho*) and expected (*He*) heterozygosity values for each marker.

Table 2. Number (and percentage) of transferable SSR markers from each donor species to *Trichloris crinita* accessions and five other grasses.

Tabla 2. Número (y porcentaje) de marcadores SSR transferibles de cada especie donante a las accesiones de *Trichloirs crinita* y otras cinco gramíneas.

Number (%) of transferable SSRs															
Donor species	**Positive / total SSRs (%)**	***Eleusine indica***	***Eleusine coracana***	***Chloris gayana***	***Chloris castilloniana***	***Cynodon dactylon***	***Trichloris crinita***								**Mean (%)** ζ
							Tc-3	**Tc-4**	**Tc-7**	**Tc-8**	**Tc-9**	**Tc-12**	**Tc-17**	**Tc-24**	
E. coracana	24/65 (36.9)	15 (23.1)	24 (36.9)	7 (10.8)	5 (7.7)	6 (9.2)	5 (7.7)	6 (9.2)	5 (7.7)	6 (9.2)	7 (10.8)	5 (7.7)	6 (9.2)	6 (9.2)	8 (12.1)
C. dactylon	10/21 (47.6)	3 (14.3)	2 (9.5)	6 (28.6)	4 (19)	10 (47.6)	4 (19)	5 (23.8)	4 (19.0)	5 (23.8)	6 (28.6)	4 (19.0)	5 (23.8)	5 (23.8)	5 (23.1)
C. dactylon x *C. transvaalensis*	19 *	1 (5.3)	2 (10.5)	1 (5.3)	3 (15.8)	10 (52.6)	2 (10.5)	2 (10.5)	3 (15.8)	2 (10.5)	3 (15.8)	3 (15.8)	3 (15.8)	3 (15.8)	3 (15.4)
Total (%) †		19 (18)	28 (26.6)	14 (13.3)	12 (11.4)	26 (24.7)	11 (10.5)	13 (12.4)	12 (11.4)	13 (12.4)	16 (15.2)	12 (11.4)	14 (13.3)	14 (13.3)	

* '*C. dactylon* x *C. transvaalensis*' was only used as a source of sequence data for developing SSR markers, but was not included in the transferability assay, therefore, the number and percentage of positive SSRs were not calculated for this species.

ζ Mean and percentage values calculated on the basis of the SSR markers developed from the donor species *E. coracana* (65), *C dactylon* (21) and '*C. dactylon* x *C. transvaalensis*' (19).

† The percentage of positive SSRs amplified in each species was calculated on the basis of 105 total SSRs tested.

*'*C. dactylon* x *C. transvaalensis*' fue utilizada para el desarrollo de marcadores pero no fue incluida en los análisis de transferibilidad, por lo tanto, el número y porcentaje de SSRs positivos no fue calculado para esta especie.

ζ Valores medios y porcentajes calculados sobre la base de número de SSR desarrollados a partir de cada una de las especies donantes *E. coracana* (65), *C dactylon* (21) and '*C. dactylon* x *C. transvaalensis*' (19).

† El porcentaje de SSRs con amplificación positiva en cada especie fue calculado sobre la base de un total de 105 SSRs evaluados.

Table 3. Information for 16 genomic SSR markers (GSSRs) successfully transferred to *Trichloris crinita*, and number of alleles (A), and observed (*Ho*) and expected (*He*) heterozygosity values estimated for each SSR locus in a collection of *Trichloris crinita* accessions.

Tabla 3. Información de los 16 marcadores SSR genómicos (GSSRs) que fueron exitosamente transferidos a *Trichloris crinita,* número de alelos (A), y valores de heterocigosis observada (*Ho*) y esperada (*He*) para cada locus SSR en accesiones de *Trichloris crinita.*

SSR marker	Motif	Donor species	PCR product size (bp)		Primer sequence (5'- 3')	Tm (°C)	A	*He*	*Ho*
			Exp.	Obs.					
GSSR-2	(TG)42	C. dactylon x *C. transvaalensis*	243	110	F: AAAACGGGTCCATCCATGTTGATGC R: TAGGCTTCGTCACAGAATTTTATCTGCCT	59.8	1	0	0
GSSR-8	(AC)28	*C. dactylon* x *C. transvaalensis*	179	128-140	F: CAGAAAATCACAGTTCAGATTACTG R: AGTTCTTTTGTACCCTTTATAAGACATCT	53.2	5	0.59	0.45
GSSR-11	(TG)23	*C. dactylon* x *C. transvaalensis*	191	139-165	F: AATATGGTATGCCTGAGATTCAAGCTCA R: GTTCCAATAATTGGTGGGTTCTGTAG	56.8	6	0.79	1
GSSR-44	(TGA)11	*Eleusine coracana*	303	270	F: ACTCGAATGAGGGAGGCATTGCTACA R: CTTGTTTCTCAAGTAGCTCCTCTTGCC	60.5	1	0	0
GSSR-51	(TGA)8	*Eleusine coracana*	240	250	F: ACCATCATAGAGTCATGAGATGTAACCTT R: ATAGCTAGCTGAGGTCGATGTAGAAGCT	58.6	1	0	0
GSSR-52	(TGA)8	*Eleusine coracana*	197	300	F: ATGAGATGTAACCTTTGATGAAACAAACT R: TTTGACCCTCTTCTGTAGTGGTGGAAGCA	59	1	0	0
GSSR-53	(TGA)8	*Eleusine coracana*	230	400	F: CTAACACCATCATAGAGTCATGAGATGT R: CGATGTAGAAGCTTTTTGACCCTCTTCT	57.2	1	0.22	0

Polymorphic markers in the *T. crinita* collection are denoted in bold. SSRs that amplified successfully across all the taxa of the transferability panel (*i.e.*, all *T. crinita* accessions, *Eleusine indica, Eleusine coracana, Chloris castilloniana, Chloris gayana, Cynodon dactylon*) are denoted in italics.

Los marcadores polimórficos en *T. crinita* se indicaron en negrita. Los SSRs que amplificaron exitosamente en todos los materiales del panel de transferibilidad (*i.e.*, todas las accesiones de *T. crinita* y las demás gramíneas) se indicaron con letras itálicas.

Table 3 (cont.). Information for 16 genomic SSR markers (GSSRs) successfully transferred to *Trichloris crinita*, and number of alleles (A), and observed (*Ho*) and expected (*He*) heterozygosity values estimated for each SSR locus in a collection of *Trichloris crinita* accessions.

Tabla 3 (cont.). Información de los 16 marcadores SSR genómicos (GSSRs) que fueron exitosamente transferidos a *Trichloris crinita,* número de alelos (A), y valores de heterocigosis observada (*Ho*) y esperada (*He*) para cada locus SSR en accesiones de *Trichloris crinita.*

SSR marker	Motif	Donor species	PCR product size (bp)		Primer sequence (5'- 3')	Tm (°C)	A	*He*	*Ho*
			Exp.	Obs.					
GSSR-72	(GCA)6	*Eleusine coracana*	294	540-550	F: TAGGAATTCGCCGCCGAATCTTTCGAT R: TTGCCATTTGAAATAGGCTCCATCT	59.5	4	0.66	0.61
GSSR-76	(CATC)4	*Cynodon dactylon*	298	350	F. TATGAATCACGAGGTCATCGCAGCA R: TTTTGCTTTCTGCAAGTCCTCATCAGGCG	61.5	1	0	0
GSSR -77	(GATC)4	*Cynodon dactylon*	642	600-610	F: ATCGCCGAACATTGAGATGGACGA R: ACGTCATGTAGTGCGTGTATCGGTTACA	60.7	3	0.70	0.83
GSSR-80	(GCGT)4	*Eleusine coracana*	180	170-180	F: CTCTGACTTGTTTAGGCCTAGTAGC R: TAGTTACCGGCGTGTGTGCTTGTATCGTT	59.8	1	0	0
GSSR-89	(AGCT)3	*Cynodon dactylon*	164	400	F: ACTGCCTACTTACTGTCAGCAAGGCAA R: GAGCAGGGGACTCAACAATATCTTA	58.9	1	0	0
GSSR-90	(CGGC)3	*Cynodon dactylon*	187	170-180	F: AAATGCTTGATTAGCTAAGGGGAGA R: TACGGTTCCCAGTTCGACACATTGTT	57.6	2	0.49	0.50
GSSR-93	(TTGC)3	*Cynodon dactylon*	184	180-190	F: TTGATTGATTCGTCTTACCATTTCCGC R: GAAACAAAACCATCCTCTACAGGT	56.4	3	0.56	0.31
GSSR-94	(TTGC)3	*Cynodon dactylon*	181	180	F: TTGATTGATTCGTCTTACCATTT R: ACAAAACCATCCTGTACAGGTCACA	54.5	1	0	0
GSSR-100	(CACG)3	*Eleusine coracana*	187	187-190	F: TATGAGAGAAACGAACCGGTAGGAGACT R: TACGGTTCCCAGTTCGACACATTGTT	60.2	2	0.48	0

Polymorphic markers in the *T. crinita* collection are denoted in bold. SSRs that amplified successfully across all the taxa of the transferability panel (*i.e.*, all *T. crinita* accessions, *Eleusine indica, Eleusine coracana, Chloris castilloniana, Chloris gayana, Cynodon dactylon*) are denoted in italics.

Los marcadores polimórficos en *T. crinita* se indicaron en negrita. Los SSRs que amplificaron exitosamente en todos los materiales del panel de transferibilidad (*i.e.*, todas las accesiones de *T. crinita* y las demás gramíneas) se indicaron con letras itálicas.

The extent of marker transferability across grass species was associated with the phylogenetic proximity between donor and target species (table 2, page 7). Thus, markers developed from *E. coracana* sequences were more transferable to *Eleusine* species (~ 23-37% of transferable SSRs) than to *Chloris* (5-7%) and *Cynodon* (6%). Similarly, SSRs developed from *Cynodon* sequence data (*i.e.*, from *C. dactylon* and '*C. dactylon* x *C. transvaalensis*') had highest transferability to *C. dactylon* (~ 48-53% of transferable SSRs), with reduced success rate observed in *Chloris* (~ 5-29%) and *Eleusine* (~5-14%).

Overall, regardless of sequence source, 19 SSR markers amplified successfully in *Eleusine indica*, 28 in *Eleusine coracana*, 14 in *Chloris gayana*, 12 in *Chloris castilloniana*, and 26 in *Cynodon dactilon*. Further information on these SSR markers is presented in Electronic Supplementary Material-Tables S1-S5, including primer sequence, annealing temperature, SSR motifs, amplicon lengths, and sequence source (donor species and sequence IDs at NCBI).

Four SSR markers, namely GSSR-51, GSSR-52, GSSR-53, and GSSR-93 (table 3, page 8-9), amplified products of expected size in all the accessions and species evaluated (table 1, page 5), suggesting that they may be useful for comparative analysis among Chloridoideae species.

SSR polymorphism and genetic diversity in Trichloris crinita

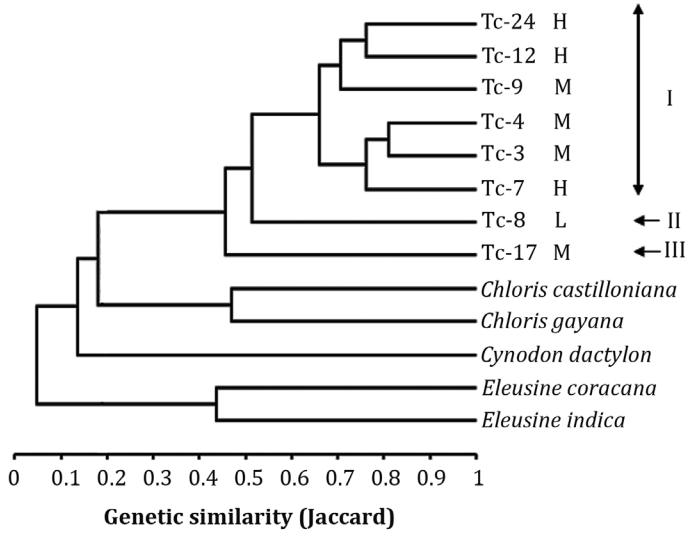
Six of the 16 SSRs (37.5%) that were successfully transferred to *T. crinita* were polymorphic -as resolved by polyacrylamide gel electrophoresis- in a sample set of eight *T. crinita* accessions varying in forage biomass production and morphological traits (table 1, page 5). These polymorphic SSRs yielded a total of 23 alleles. The number of alleles per SSR locus (A) varied from two to six, with a mean of 3.8. Expected heterozygosities (*He*) ranged from 0.48 to 0.79, with mean of 0.63, whereas observed heterozygosities (*Ho*) ranged from 0.0 to 1.0, with mean of 0.53 (table 3, page 8-9). GSSR-11 (A = 6, *He* = 0.79) and GSSR-100 (A = 2, He = 0. 48) were the most and the least polymorphic markers, respectively. Analysis of these types of data, including *He*, *Ho* and the inbreeding coefficient (F_{IS}) were used in a previous study with these SSRs to infer about the reproductive mode of *T. crinita*.

Considering all the markers (*i.e.*, polymorphic and monomorphic SSRs), the mean number of alleles per locus was 2.12, with a range of one to six. Mean *He* was 0.28 and ranged from 0.0 to 0.79, whereas mean *Ho* was 0.23 and ranged from zero to one (table 3, page 8-9). Nine SSRs (56%) had *He* values lower than 0.3 (low polymorphism), two SSRs (12%) exhibited values between 0.3 and 0.5 (moderate polymorphism) and five SSRs (31%) had *He* values higher than 0.5 (high level of polymorphism).

Genetic diversity and relatedness among *T. crinita* accessions was evaluated by estimating pair-wise genetic similarities (Jaccard coefficient) among the accessions. Genetic similarity (GS) values ranged from 0.35 (between Tc-3 and Tc-17, indicating that these were the two most genetically different accessions) to 0.81 (between Tc-3 and Tc-4, the genetically closest accessions). The mean GS among all *T. crinita* accessions was 0.57.

A phenogram, constructed on the basis of six polymorphic SSRs depicted genetic relations among *T. crinita* accessions and five related grass species (figure 1). All *T. crinita* accessions were clearly separated from the other grasses (used as outgroups), including two close relatives of *Trichloris* (*Chloris castilloniana* and *Chloris gayana*), indicating a coherent genetic clustering of the taxa.

Within *T. crinita*, all the accessions were clearly discriminated. By clustering the accessions with more than 60% genetic similarity (GS=0.6), three groups of accessions were revealed. Cluster I grouped 75% of the accessions, whereas groups II and III had one accession each.



Roman numbers indicate groups discriminated at 60% genetic similarity. For *Trichloris crinita*, taxa are indicated by the accession number, followed by a letter indicating whether they have high (H, > 120 g dry matter/plant), medium (M, between 60 and 120 g DM/plant), or low (L, less than 60 g DM/plant) biomass yield, according to Cavagnaro *et al.* (5).

Los números romanos indican grupos de accesiones con ≥ 60% de similitud genética. Para *Trichloris crinita*, se indica el número de accesión seguido de una letra que indica si es de alta (H, > 120 g materia seca/planta), media (M, entre 60 y 120 g MS/planta) o baja (L, menos de 60 g MS/planta) productividad de biomasa forrajera, de acuerdo con Cavagnaro *et al.* (5).

Figure 1. Phenetic relations among eight *Trichloris crinita* accessions and 5 phylogenetically-related grass species (outgroups) obtained from SSR marker data, using UPGMA cluster analysis of the Jaccard similarity coefficient.

Figura 1. Relaciones fenéticas entre ocho accesiones de *Trichloris crinita* y 5 especies de gramíneas filogenéticamente-relacionadas con *Trichloris*, obtenidas sobre la base de datos de marcadores SSRs. El análisis de agrupamiento usó la metodología UPGMA y el coeficiente de similitud genética de Jaccard.

No clear association was found between the clustering of the taxa and the accessions biomass production, as reported previously using AFLP markers (5), nor between clustering and the geographical origin of the taxa (data not presented).

Discussion

The present study is the first report on the development of SSR markers in *Trichloris crinita*, an important forage grass from arid and semi-arid regions of America. Due to the lack of sequence data for this species, the markers developed herein used available genomic sequences (GSS) from other phylogenetically-related grasses of the Chloridoideae subfamily, namely *Eleusine coracana*, *Cynodon dactylon* and '*C. dactylon* x *C. transvaalensis*'. Nearly 15% (16/105) of the total SSRs evaluated were successfully transferred to *T. crinita*, with transfer rates ranging from 10.8% (for markers developed from *E. coracana* sequences) to 28.6 % (for *C. dactylon* markers). These values are seemingly low, as compared with previously reported cross-genera SSR transfer rates within Poaceae subfamilies. For example, transferability of EST-SSRs between two Chloridoideae species, tef (*Eragrostis tef*) and bermuda grass (*Cynodon dactylon*), was ~ 32% (35). Similarly, transferability of EST-SSRs in the Pooideae subfamily was also higher than in the present study, with transfer rates from 27% [from wheat (*Triticum aestivum*) to harding grass (*Phalaris aquatica*)] to 68% [from tall fescue (*Festuca arundinacea*) to Kentucky bluegrass (*Poa pratensis*)], as well as in Panicoideae, with 79-100% of transferred markers among maize, sorghum and earl millet (35).

However, these comparisons are not surprising, since EST-derived SSRs are known to present higher transferability, because coding regions are more conserved among related species, than SSRs developed from genomic sequence (GSSRs) (8, 9). Thus, transfer rates of the GSSRs developed in this study, although still relatively low, are comparable with cross-genera transferabilities of GSSRs reported previously in Apiaceae (22-42%) (6), Rosaceae (20%) (29), Asteraceae [17.6% between sunflower (*Helianthus annuus*) and safflower (*Carthamus tinctorius*)] (11), and Fabaceae [~ 23-24% among mungbean (*Vigna radiata*), common bean (*Phaseolus vulgaris*), and soybean (*Glycine max*)] (30).

Six of the 16 SSRs successfully transferred were polymorphic in a collection of eight phenotipically-diverse *T. crinita* accessions (table 1, page 5), revealing two to six alleles per SSR, with a mean of 3.83.

The level of polymorphism at each locus was estimated by means of the expected heterozygosity (*He*), instead of the 'polymorphism index content' (PIC) traditionally used for estimating SSR polymorphism in diploid species, because estimation and interpretation of the PIC in polyploids, such as the tetraploid *T. crinita*, is particularly complex, as discussed by Liu *et al.* (2007). These and other authors (23) have reported strong and significant correlation ($r = 0.9917$, $p < 0.01$) between *He* and PIC in tetraploids, suggesting that *He* can be used effectively for estimating the level of polymorphism in SSR loci in tetraploids. Thus, for the six polymorphic markers of our study, the degree of polymorphism ranged from moderate (*He* =0.48), in one SSR, to high (*He* = 0.56-0.79), in five SSR loci, with a mean *He* of 0.63 (table 3, page 8-9). It must be noted that these polymorphic SSRs were used in a previous study for calculating inbreeding

coefficient (F_{IS}) values in *T. crinita* accessions, as a means for inferring about the species reproductive behavior (16).

The relatively low number of polymorphic loci observed (6/16) may be partially due to the autogamous nature of *T. crinita* (16). Also, the sample set of accessions selected for marker analysis in this study (table 1, page 5) may have limited the detection of more polymorphic SSRs.

In other words, if larger and more genetically diverse *T. crinita* germplasm were evaluated (*e.g*., in natural populations, or using germplasm collections from different geographical origins), additional polymorphic markers and new alleles may be revealed.

The SSR analysis revealed considerable genetic variation among *T. crinita* accessions, with pair-wise GS values ranging from 0.35 to 0.81. These results are in full agreement with those reported by Cavagnaro *et al.* (2006), indicating GS values of 0.31 to 0.92 among 20 *T. crinita* accessions using AFLP markers. It must be noted that the same GS coefficient (Jaccard) was used in both studies, and that the eight accessions used in the present study were among the plant materials used by Cavagnaro *et al*. (2006).

The SSR-based cluster analysis clearly separated all the grass genera into distinct groups (figure 1, page 11), and depicted their genetic relatedness to *Trichloris*, and among themselves, in full agreement with current phylogenetic and taxonomic relationships in the Chloridoideae (10, 14). Thus, in decreasing order of phylogenetic proximity to *Trichloris*; *Chloris*, *Cynodon* and *Eleusine* were associated with the *Trichloris* cluster at GS values of 0.18, 0.13, and 0.05, respectively (figure 1, page 11).

Chloris and *Trichloris* are close relatives and the two genera share many morphological features. This has led to controversies of whether *Chloris* and *Trichloris* are the same genus (1) or whether they should be regarded as different genera (21).

The results from the SSR cluster analysis, demonstrating a clear separation of all *Trichloris* accessions from the two *Chloris* species, at GS of 0.18 (figure 1, page 11), and the fact that GS values between all the *Trichloris* taxa and *C. castilloniana* (mean GS = 0.15; range = 0.12-0.17) and *C. gayana* (GS mean = 0.21; range = 0.18-0.30) were very low, support the separation of *Chloris* and *Trichloris* as two distinct genera.

In addition to the 16 SSRs transferred to *T. crinita*, 12 and 14 SSR markers could be transferred to *Chloris castilloniana* and *Chloris gayana*, respectively (Electronic Supplementary Material-Tables S1-S5). These represent the first SSR markers developed for these orphan grass species. Additionally, these markers were polymorphic -and they were able to discriminate- between the two *Chloris* species (figure 1, page 11).

The high resolution observed for these SSRs in separating the two *Chloris* species (at a GM value < 0.50) suggest that they may be useful for fingerprinting and characterizing genetic diversity in intraspecific plant collections of *C. gayana* and *C. castilloniana*.

The positive SSRs in *Cynodon dactylon* (26 SSRs), *Eleusine coracana* (28 SSRs) and *E. indica* (19 SSRs), adds potentially new informative markers to the molecular toolkit of these species.

Conclusions

It is expected that the SSR markers developed in this study will be instrumental in a number of genetic research projects of *Trichloris crinita* and -perhaps- related grass species. Successful applications of these markers for progeny testing and estimation of inbreeding coefficients were recently reported in a study aimed at elucidating the reproductive system of *T. crinita* (16). In addition, these SSRs can be used for assisting breeding programs of this species.

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