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IDENTIFICATION OF A FRAGMENT OF THE INTERNAL TRANSCRIBED SPACER REGION OF RDNA IN *CENCHRUS PURPUREUS* (POACEAE)

IDENTIFICACIÓN DE UN FRAGMENTO DE LA REGIÓN DEL ESPACIADOR INTERNO TRANSCRITO DEL ADNR EN *CENCHRUS PURPUREUS* (POACEAE)

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The internal transcribed spacer (ITS) region is easy to amplify due to its numerous copies present in the plant genome, but there in not researchers of this region in the forage species *Cenchrus purpureus*. This study aimed to identify by Polymerase Chain Reaction (PCR) a fragment of the ITS region of the ribosomal DNA of the nucleus in *C. purpureus* using the primers ITS1/ITS4 and compare it with two samples of *Urochloa* spp. The molecular study used DNA samples from 62 *C. purpureus* accessions from the grass and forage germplasm bank belonging to Instituto de Ciencia Animal and two samples of *Urochloa* spp. accessions from the Genetic Resources Program of the Alliance of International Bioversity and the International Center for Tropical Agriculture. The DNA from the 62 *C. purpureus* accessions and *Urochloa* spp. controls were amplified with ITS1/ITS4 primers. The amplification products revealed a clear polymorphic band in the gels for each accession and an approximate size of 1000 bp in *C. purpureus* and 850 bp in *Urochloa* spp. The primers determined differences in amplifications between samples of *C. purpureus* and *Urochloa* spp. although they did not show variability between accessions of the same species. These molecular markers can be used to verify PCR amplification of *C. purpureus* DNA samples and to differentiate species in Poaceae genera.

Key words: *forage, genome, ITS, molecular marker, PCR*

La región del espaciador interno transcrito (ITS), es fácil de amplificar por sus numerosas copias presentes en el genoma vegetal, pero no se reportan investigaciones de esta región en la especie forrajera *Cenchrus purpureus*. El presente estudio tuvo como objetivo identificar mediante Reacción en Cadena de la Polimerasa (PCR) un fragmento de la región ITS del ADN ribosomal del núcleo en *C. purpureus* mediante los cebadores ITS1/ITS4 y compararlo con dos muestras de *Urochloa* spp. En el estudio molecular se emplearon muestras de ADN de 62 accesiones de *C. purpureus* del banco de germoplasma de pastos y forrajes, pertenecientes al Instituto de Ciencia Animal y dos muestras de accesiones de *Urochloa* spp. del Programa de Recursos Genéticos de la Alianza de Bioversity International y el Centro Internacional de Agricultura Tropical. El ADN de las 62 accesiones de *C. purpureus* y muestras de *Urochloa* spp., amplificaron con los cebadores ITS1/ITS4. Los productos de amplificación revelaron una banda polimórfica nítida en los geles para cada accesión y tamaño aproximado de 1000 pb en *C. purpureus* y 850 pb en *Urochloa* spp. Los cebadores determinaron diferencias en las amplificaciones entre las muestras de *C. purpureus* y *Urochloa* spp. aunque no demostraron variabilidad entre accesiones de la misma especie. Estos marcadores moleculares se pueden utilizar para comprobar la amplificación, mediante PCR, de muestras de ADN de *C. purpureus* y en la diferenciación de especies en géneros de Poaceae.

Palabras clave: *forraje, genoma, ITS, marcador molecular, PCR*

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Introduction

Cenchrus purpureus (Schumach.) Morrone, formerly *Pennisetum purpureum*, is one of the most important forage grasses in tropical livestock systems and its use in ruminants feeding through cutting or grazing is notable. In addition, other uses are reported as an ornamental and medicinal plant, in the industry its fiber is used for paper manufacturing and as biofuel (Nguyen *et al.* 2021, Tan *et al.* 2022, Wessapak *et al.* 2023 and POWO 2025).

At the Instituto de Ciencia Animal (ICA) of the Republic of Cuba, accessions of *C. purpureus* developed through the genetic improvement program of this genus aimed at increasing biomass production, nutritional quality and tolerance to abiotic stresses such as salinity and drought are conserved (Herrera 2022, Fortes *et al*. 2023 and Álvarez *et al.* 2024). This collection was evaluated for forage production and response to grazing using morphological and quality indicators (Herrera *et al.* 2019) and molecular markers such as isozymes, Simple Sequence Repeat (SSR) and regions between simple sequence repeat (ISSR) (Cruz *et al.* 1993, González and Martínez 2019 and Álvarez 2021).

Molecular markers are fundamental tools for characterizing genetic diversity, optimizing germplasm conservation, and designing assisted selection strategies (Özbek 2024). Currently, specific primers exist for studying genetic diversity in plants, such as the plant DNA barcode markers *rbcL* and *matK*, which are used in phylogenetic studies of flowering plants and conifers (de Vere *et al.* 2012 and Jones *et al.* 2021). However, other markers derived from the ITS (Internal Transcribed Spacer) region of the nuclear ribosomal DNA are notable for their utility in identifying different evolutionary levels such as fungi, bacteria, and plants; in the latter, their use stands out in phylogenetic studies at the genus and species level due to their high nucleotide substitution rate and wide availability in databases (Alaklabi 2021).

In Poaceae, this region has clarified taxonomic relation in genera such as *Sorghum* (Sun *et al.* 1994), *Urochloa* (González and Morton 2005), *Chloris* (Liao *et al.* 2020), *Phalaris* (Al Rahbawi *et al*. 2021) and the species *Cenchrus americanus* (L.) Morrone (Almutairi 2021). However, there is a not study reported in the literature where ITS primers are used in *C. purpureus*, which limits its genomic information, which in turn restricts its application in phylogenetic and intraspecific diversity studies, and its management in genetic improvement programs.

In Cuba, studies using molecular markers in *C. purpureus* are scarce and have focused on traditional markers, limiting the understanding of its genomic diversity. Therefore, the objective of this study was to identify a fragment of the ITS region using the universal primers ITS1/ITS4 in *C. purpureus* and contribute to the molecular genetics knowledge for future phylogenetic and assisted selection analyses.

Materials and Methods

This research was conducted in the DNA Laboratory, belonging to Semillas del Futuro building, at the Alliance of Bioversity International and the International Center for Tropical Agriculture (CIAT), Cali, Valle del Cauca, Colombia.

Plant material: the samples under study were obtained from 62 accessions of *C. purpureus*, with similar regrowth age and cultivation conditions, preserved in the germplasm bank of grasses and forages, belonging to the Experimental Center of Grasses and Forages Miguel Sistachs Naya from Instituto de Ciencia Animal, San José de las Lajas, Mayabeque, Cuba, located at 22º 53 NL and 82º 02 WL at 80 m.o.s.l. Additionally, two samples of *Urochloa decumbens* (Stapf) R. D. Webster - CIAT 606 and *Urochloa ruziziensis* (R. Germ. & C. M. Evrard) Crins - CIAT 6713 were included from the Genetic Resources Program of the Alliance of Bioversity International and the International Center for Tropical Agriculture, Colombia.

DNA extraction and amplification: for the extraction of genomic DNA, the modified MATAB method was used (Risterucci *et al.* 2000). The DNA amplification was performed by polymerase chain reaction (PCR), using the ITS1/ITS4 direct and reverse primer combination described by White *et al.* 1990 (table 1).

The PCR mixture was performed in a final volume of 12 µL, using 4 µL of 2X Promega buffer (GoTaq® Green Master Mix), 0.2 µL of ITS1 and ITS4, with a final concentration of 0.2 µM in each primer and 6.6 µL of ultrapure water (UltraPure™ DNase/RNase-Free Distilled Water, Catalog number: 10977015-Invitrogen) and 1 µL of genomic DNA with a concentration of 10 ng.

Amplification was performed in an Eppendorf Mastercycler Nexus Gradient Thermal Cyclers Cole-Parmer® USA. The PCR reaction was carried out following a program lasting approximately 2 hours. The thermal profile consisted of an initial denaturation at 95 °C for 2 minutes, followed by 35 cycles consisting of: denaturation at 95 °C for 30 seconds,

Table 1. List of primers and their sequences used in the study

Primer	Direction	Sequence 5'---- 3'	Source
ITS1	Direct	5'TCCGTAGGTGAACCTGCGG3'	(White *et al*. 1990)
ITS4	Reverse	3'TCCTCCGCTTATTGATATGC5'	

hybridization at 55 °C for 1 minute, and extension at 72 °C for 45 seconds. Finally, a final extension was performed at 72 °C for 10 minutes to complete the synthesis of the amplified fragments.

Separation of PCR products: the amplified products were analyzed by electrophoresis on a 1.5 % agarose gel prepared with GelRed™ (Biotium) as an intercalating agent. The run was performed in 0.5X TBE buffer at 100 V for approximately 2 hours. The 1Kb DNA Ladder molecular weight markers, INVITROGEN®, was used.

Visualization of PCR products: visualization and analysis of the amplified DNA fragments was performed by photography, using the BIO-RAD ChemiDoc MP Imaging System Universal Hood III Photodocumenter, USA.

Results and Discussion

The DNA from the 62 *C. purpureus* accessions and the controls *U. decumbens* and *U. ruziziensis* were amplified with the ITS1/ITS4 primers. The amplification products revealed, in most samples, a clear polymorphic band in the gels. However, in some cases amplification of double bands could be observed in accessions 6, 28, 31, 42, 47, 49, 51, 52, 59 and 61 as well as weak bands in accessions 29, 30, 46, 50 and 53 or diffuse bands in accessions 5, 17, 32, 40, 47 and 49. The amplified fragments had an approximate size of 850 bp in *Urochloa* spp and 1000 bp in the *C. purpureus* accessions (figure 1).

The molecular differences, between species and genera, found in this study are in agreement with Ghosh *et al.* (2017), who state that ITS are considered a useful source of characters for the identification of different groups of Angiosperm plants, since they generate important polymorphisms within the same taxonomic entity, particularly for genus and species. Also, the ITS region does not encode amino acids and therefore are subject to high variability due to its ubiquitous nature, rapid evolution, high representation in the genome, and the ability to be amplified with minimal amounts of DNA.

The amplification of 1000 and 850 bp ITS primers in *C. purpureus* and *Urochloa* spp. respectively, differ from those reported by Baldwin *et al.* (1995) who stated that the ITS region is small and uniform in flowering plants and its total length is between 600 and 700 bp. In contrast, Liston *et al.* (1996) describe shorter lengths of this region for Angiosperms, ranging from 565 to 700 bp; in contrast, the ITS region of gymnosperms is considerably longer and has a greater range of variation, from 750 to 3125 bp.

Studies of the ITS region in the species *C. purpureus, U. decumbens* and *U. ruziziensis* are scarce. However, Chen *et al.* (2010) in a research on 15 species of the *Cenchrus* genus from areas such as Fujian, Jiangsu and Hainan in China, reported amplification of 573 to 586 bp for *C. purpureus x C. americanus* hybrids. In addition, in the species *C. americanus* 2n=2x=14 chromosomes and AA genome, genetically homologous to *C. purpureus* 2n=4x=28 chromosomes and A'A'BB genome, Almutairi (2021) found a length of 772 to 774 bp for the amplification of the ITS region, in a study of six local cultivars of pearl millet from Saudi Arabia and their similarity to 31 accessions from India and France.

On the other hand, in a molecular phylogenetic study in 22 species from *Urochloa* genus it was determined that the amplification of the ITS region varied from 582 bp in *U. decumbens*, to 587 bp in *Urochloa eruciformis* (Sm.) Nelson & Fern. Casas, *Urochloa xantholeuca* (Hack. ex Schinz) H. Scholz, *U. ruziziensis*, and *Urochloa mosambicensis* (Hack.) Dandy (González and Morton 2005).

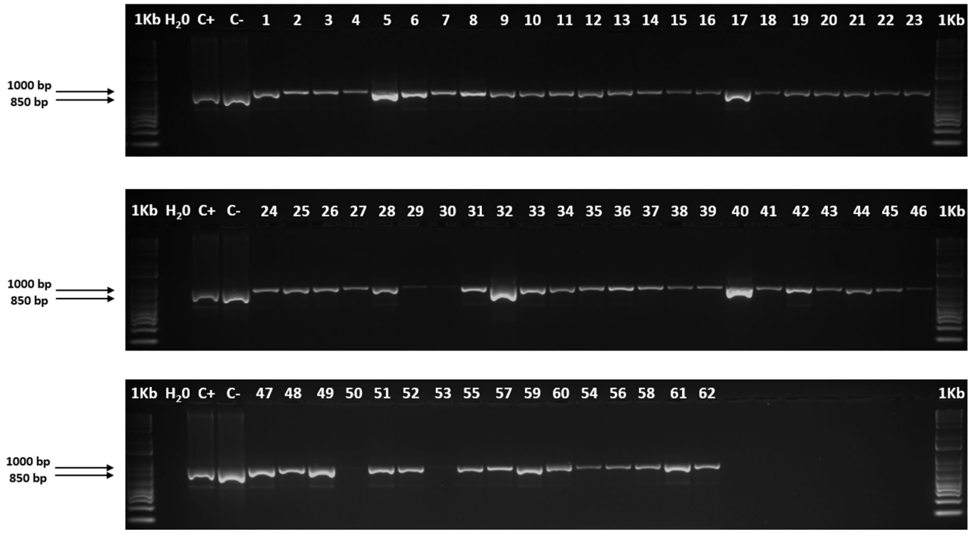

Figure 1. Horizontal electrophoresis in 1.5 % agarose gel of the PCR products with the amplification of the 62 accessions of *C. purpureus* with the primers ITS1/ITS4, and samples of *U. decumbens* - CIAT 606 (Ud) and *U. ruziziensis* - CIAT 6713 (Ur)

The results regarding base pair number in *C. purpureus* and *Urochloa* spp. can be explained according to those reported by Baldwin *et al.* (1995), who stated that approximately 300 bp is estimated for each ITS spacer, but depending on the plant family, ITS 1 may be larger or smaller and potentially more informative. Thus, Bult and Zimmer (1993) indicated that ITS spacers are more variable compared to the coding regions that are conserved in the genome.

The variability in the amplification of the ITS region in Angiosperms, compared to the results obtained in *C. purpureus* and *Urochloa* spp., can be observed in other species of Poaceae such as the study carried out by Hsiao *et al.* (1995) in 26 species of grasses. In the research, the authors found that the ITS region ranged from 585 to 602 bp among the tribes Oryzeae, Aveneae, Brachypodieae, Bromeae, Meliceae, Poeae, Stipeae, and Triticeae.

Also, Hsiao *et al.* (1998) continued the study and expanded the sample to 200 grass species. The results of the study revealed variations ranging from 584 to 633 bp for the ITS region in six subfamilies: Bambusoideae, Pooideae, Arundinoideae, Centothecoideae, Chloridoideae, and Panicoideae. Both researchers showed differences in this region in Poaceae and confirmed its ubiquitous nature in the plant genome.

The ITS markers have bi-paternal inheritance, allowing to reveal cases of hybridization and polyploidy in genera and species of Poaceae (Wang *et al.* 2022). However, in *C. purpureus* and *Urochloa* spp the ITS1/ITS4 primers did not has polymorphic bands, which did not allow a clear determination of the genetic diversity between accessions of the same taxonomic entity. Furthermore, there were not differences in the *C. purpureus* x *C. americanus* hybrids that are in the germplasm under study. These results may be due to the need to include other primer pairs that complement those used or to genome sequencing, as indicated by Hsiao *et al.* (1998), who observed that the use of different primer combinations (ITS1/ITS4, ITS1/ITS2 and ITS3/ITS4) allowed determining the variability of nuclear ribosomal DNA among Poaceae species.

This contradicts what referred by Ahmadi *et al.* (2022), who stated that ITS markers can be used as a more appropriate assessment tool to analyze interspecific and intraspecific relations when distinguishing different genotypes, since nucleotide changes decrease as evolution progresses, so that only a few nucleotide changes occur. In this sense, the results obtained in this study show that the ITS are more effective in identifying variability between species of different genera and are not efficient in determining genetic differences between accessions of the same species.

The amplification products of *C. purpureus* revealed, in some cases, the presence of double, weak or diffuse bands. González (2002) and Nadeem *et al.* (2018) report that these characteristics in the amplification of the band patterns could be due to the composition of the gel and the buffer, as well as the voltage and current intensity conditions. In addition, some of the primers used may have more than one hybridization region in the genomic DNA of the studied samples, the presence of introns in the rDNA subunits, and the amplification technique or amplification of DNA from external contamination. So, it is necessary to check the influence of these factors on the amplification of ITS primers in subsequent assays.

Conclusions

It is concluded that the ITS1/ITS4 primers are effective in amplifying DNA from *C. purpureus* and *Urochloa* spp. accessions, demonstrating their usefulness as a tool for evaluating the quality of the extracted DNA and its amplification capacity by PCR. However, these primers do not have sufficient resolution to distinguish genetic differences between accessions of both species within the fragment of the ITS region analyzed.

Although ITS markers can be used to verify the amplification of *C. purpureus* samples and for general differentiation between species of the genus in the Poaceae family, it is recommended to complement these analyses with sequencing techniques to achieve a more precise genetic characterization.

The information generated in this study expands the genetic-molecular knowledge of *C. purpureus* and creates the initial conditions for future phylogenetic studies in the genus *Cenchrus,* as well as for the application of assisted selection strategies in improvement and conservation programs for its germplasm.

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