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Article

Sequencing report of the rRNA amplicons of *Echinochloa crus-galli in Iraq*

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ABSTRACT
This study was conducted to identify the genetic polymorphisms of one wild grass sample species and assess the phylogenetic distribution these isolates occupy based on their internal transcribed (ITS) ribosomal sequences. This study amplified one genetic locus covering a portion of the ITS rRNA sequences. A direct sequencing strategy was performed for the observed PCR amplicons in the amplified genetic locus. Subsequently, a comprehensive phylogenetic tree was constructed in the observed variants for these sequences to reveal the accurate phylogenetic distances alongside other relative sequences. Our results indicated the identity of the investigated wild grass samples (assigned S1 and S2). Sequencing reactions indicated that our investigated samples belonged to *Echinochloa crus-galli* var. *crus-galli* (panicum grass), a common wild grass dominated in tropical Asia. Two ribosomal variations were identified in the investigated wild grass samples, 61C>T, observed in the S2 sample and 408C>A, observed in both the S1 and S2 samples. According to the identified results, the investigated samples were positioned within one distinct phylogenetic clade of these identified wild grass sequences in the currently generated comprehensive tree. These positionings were observed within the main clade of *Echinochloa crus-galli*. Based on the identified ribosomal sequences, it was found that the currently investigated samples may exhibit variable extents of diversities originating from several Asian and South American ancestors. Also, the currently constructed tree revealed that the investigated wild grass sequences belonged to one distinct clade of *Echinochloa crus-galli*, having many sequences of close phylogenetic connections. This clade occupied far away phylogenetic positions from the other comparable grasses within the same tree.
Keywords: rRNA amplicons, *Echinochloa crus-galli*, Rice

INTRODUCTION

Rice is a herbaceous plant belonging to the family Poaceae genus Oryza Oryza, which includes 25 species spread in the tropics of Asia, Africa, Central America and northern Australia. It is believed that rice originated from wild fathers O.spontanea and O.fatua[1,2]. The rice crop, Oryza sativa L. (Rise), is also one of the important strategic summer grain crops from an economic point of view, as it is considered a “basic” food for half the population of the globe or more, and its

production in East Asian countries constitutes 90% of global production[3]. Rice is very sensitive to the weed, especially in the early stages of growth, as research results indicated that losses in this crop sometimes reach 70% of the yield when not controlled, in addition to its poor quality[4] and [5].

METHODS OF SEQUENCING REACTIONS

DNA Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from both termini, forward and reverse directions, according to the instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations were not because of PCR or sequencing artifacts. By comparing the observed nucleic acid sequences of wild grass samples with the retrieved reference sequences of the wild grass database, the virtual positions and other details of the retrieved PCR fragments were identified[8].

Interpretation of sequencing data

The sequencing results of the PCR products were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed nucleic acids numbered in PCR amplicons and corresponding positions within the referring genome. Each detected variant within the targeted ribosomal sequences was annotated by SnapGene Viewer ver. 4.0.4 [9].

Comprehensive phylogenetic tree construction

A specific comprehensive tree was constructed in this study according to the cladogram construction described by [10]. The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server [11]. Based on the Clustal omega suit [12], multiple sequence alignments were made for the retrieved nucleic acid sequences. Subsequently, the neighbor-joining method built an inclusive tree and visualized it using the iTOL suit to generate a cladogram of clades construction [13]. The observed variants and their corresponding reference sequences were incorporated into the constructed comprehensive cladogram. The sequences of each classified phylogenetic species in the comprehensive tree were annotated accordingly.

RESULTS AND DISCUSSION

DNA Sequencing of PCR amplicons

Within this locus, two samples were included, which showed approximate lengths of the ribosomal fragment. The sequencing reactions indicated the confirmed identity of the amplified products by performing NCBI blasts. After positioning the amplified fragments within the rRNA sequences, the details of these sequences were shown within the amplified sequences. The NCBI BLASTn engine showed a high sequence similarity between the sequenced samples with Echinochloa crus-galli var. crus-galli sequences. The NCBI BLASTn engine indicated the presence of about 99% homology with the GenBank accession number KF163619.1 that belonged to Echinochloa crus-galli sequences. The accurate positions and other details of the retrieved PCR fragments were identified by comparing the observed nucleic acid sequences of these investigated samples with the retrieved nucleic acid sequences (GenBank acc. KF163619.1).

The exact positions and other details of the retrieved PCR fragment were identified by comparing the observed DNA sequences of the currently investigated samples with the retrieved DNA sequences (Fig. 1).

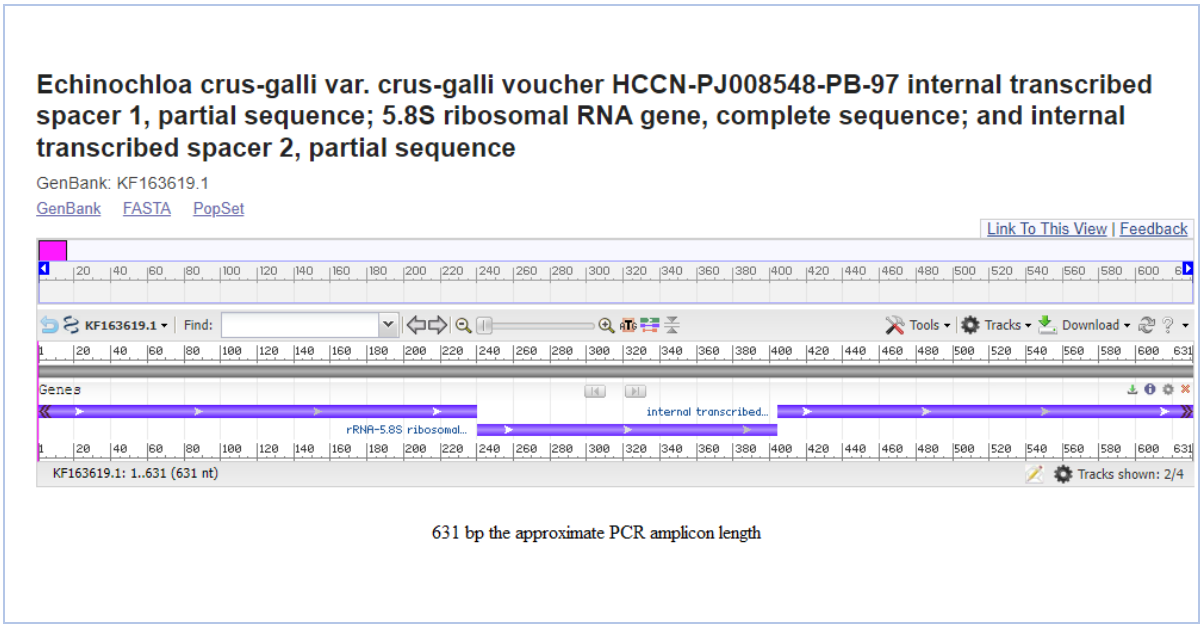


Figure 1. The exact position of the retrieved 631 bp amplicon partially covered a portion of the ITS-rRNA sequences within Echinochloa crus-galli genomic sequences (GenBank acc. no. KF163619.1).

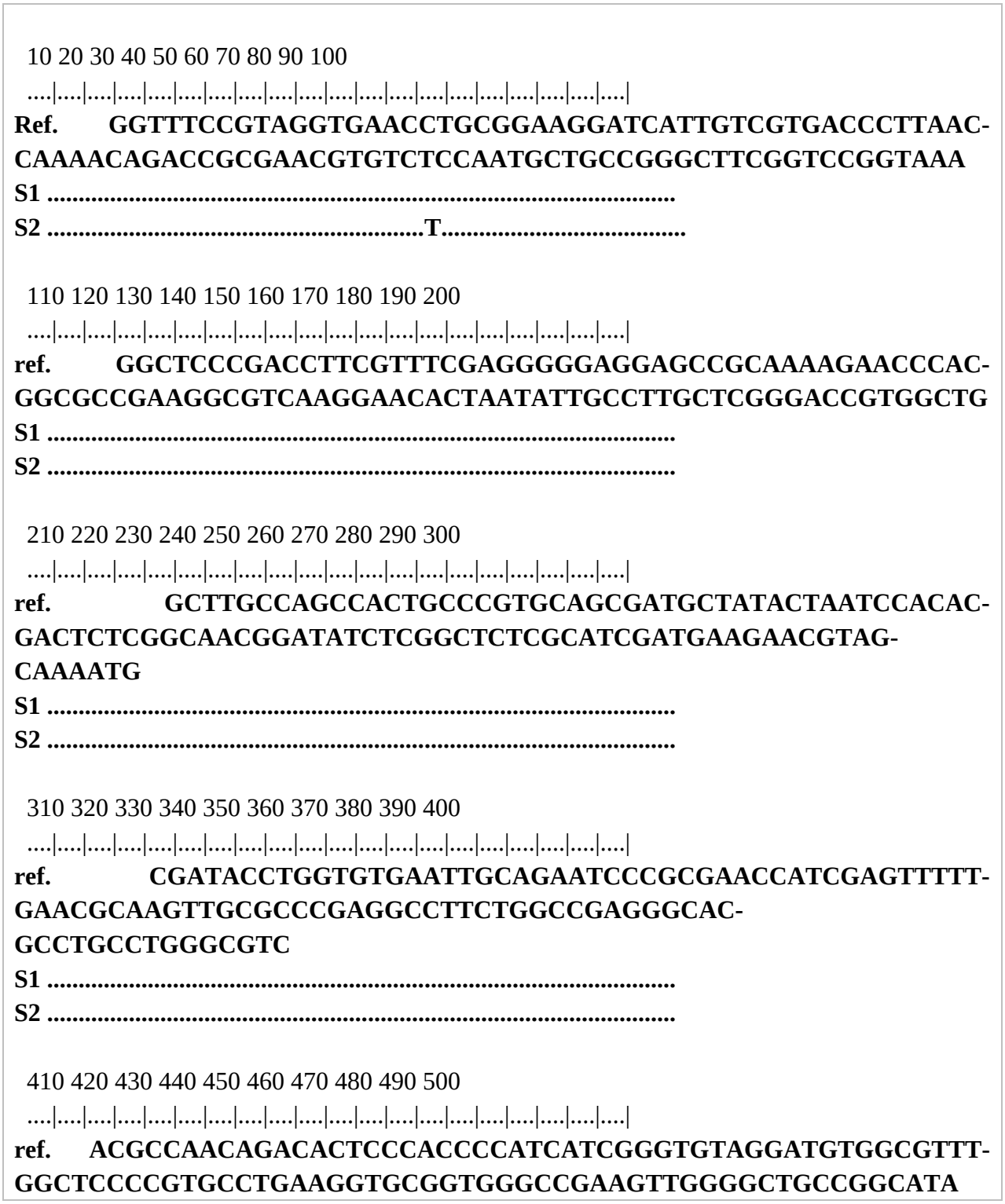
Interpretation of sequencing data

After positioning the 631 bp amplicons' sequences within the genomic sequences of the Echinochloa crus-galli, the details of its sequences were highlighted, and the total length of the amplified amplicons was also determined (Table 1).

Organism	Reference locus sequences (5′ - 3′)	length
Echinochloa crus-galli	GGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCGTGACCCTTAAC-CAAAACAGACCGCGAACGTGTCTCCAATGCTGCCGGGCTTCGGTCCGG-TAAAGGCTCCCGACCTTCGTTTCGAGGGGGAGGAGCCGCAAAAGAACCCAC-GGCGCCGAAGGCGTCAAGGAACACTAATATTGCCTTGCTCGGGAC-CGTGGCTGGCTTGCCAGCCACTGCCCGTGCAGCGATGCTATACT-AATCCACACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAA-GAACGTAGCAAAATGCGATACCTGGTGTGAATTGCAGAATCCCGCGAAC-CATCGAGTTTTTGAACGCAAGTTGCGCCCGAGGCCTTCTGGCCGAGGGCAC-GCCTGCCTGGGCGTCACGCCAACAGACACTCCCACCCCATCATCGGGTGTAG-GATGTGGCGTTTGGCTCCCCGTGCCTGAAGGTGCGGTGGGCCGAAGTT-GGGGCTGCCGGCATACCGTGTCGGGCACAGCACGTGGTGGGCGAC-TACAAGTTGTTCTCGGTGCAGCGTCCCGGCACGCAGCTAGCTT-GATGGCCCTAAGGACCCATGTACAACCGAAGCGCACTGTCGCTCGGAC-CGCGACCCCA	631 bp

Table 1. The positions and length of the amplified fragments were used to amplify a portion of the ITS rRNA gene within one type of wild grass genomic DNA sequence.

The retrieved sequences of ribosomal samples were aligned with their corresponding referring sequences. These sequences were prepared by positioning our investigated samples with the most relative sequences deposited in the NCBI database KF163619.1. Our results indicated the presence of two nucleic acid variants observed in two of the investigated samples, 61C>T, which was observed in the S2 sample and 408C>A, which was observed in both S1 and S2 samples. These differences in the analyzed samples' currently observed nucleic acid sequences were not found in the corresponding reference sequences (Fig. 2).



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S1 .....A.....................................................................................
S2 .....A.....................................................................................

  510 520 530 540 550 560 570 580 590 600
  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ref.      CCGTGTCGGGCACAGCACGTGGTGGGCGACTACAAGTTGTTCTCGGTG-
CAGCGTCCCGGCACGCAGCTAGCTTGATGGCCCTAAGGACCCATGTACAACC
S1 .....................................................................................
S2 .....................................................................................

  610 620 630
  ....|....|....|....|....|....|.
ref. GAAGCGCACTGTCGCTCGGACCGCGACCCCA
S1 ..............................
S2 ..............................
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Figure 2. Nucleic acid sequence alignment of two wild grass samples with the most relevant deposited genomic sequences of one type of wild grass species (*Echinochloa crus-galli*). The symbol "ref" refers to the NCBI reference sequences, while "S No.#" refers to sample numbers.

To confirm these variations, the sequencing chromatograms of the investigated samples, as well as their detailed annotations, were verified and documented, and the chromatograms of their sequences were shown according to their positions in the PCR amplicons (Fig. 3). The presence of these variants was confirmed in their original chromatograms. The absence of any possible technical error was also confirmed.

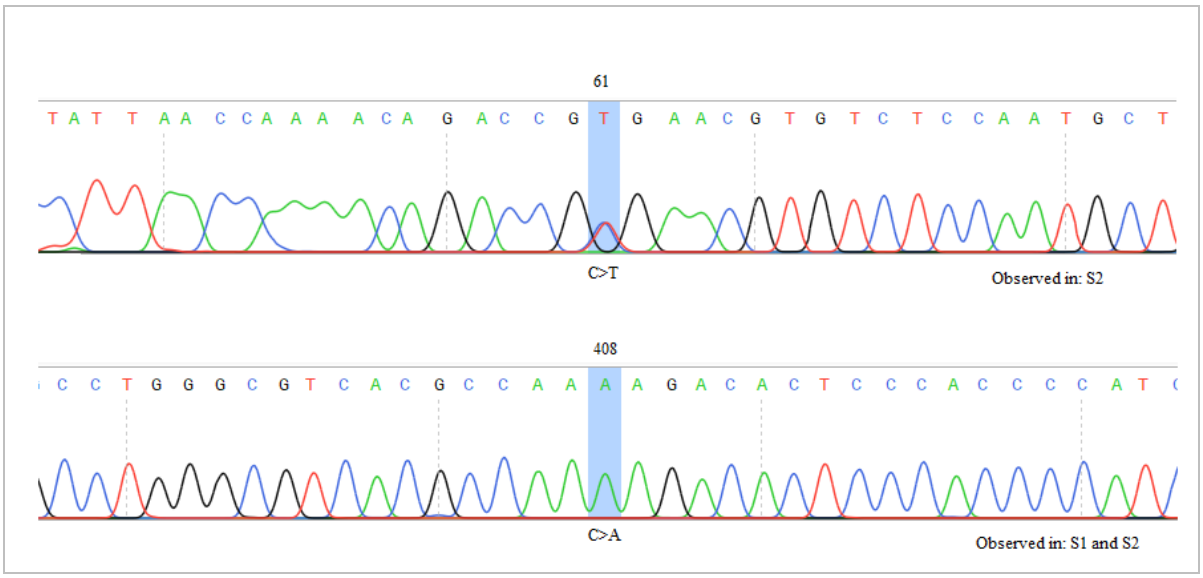

Figure 3. The chromatogram of the *Echinochloa crus-galli* DNA sequences. The clarity of the observed peaks refers to the strict contamination-free technical parameters followed to validate each variant in the present sample. The letter "S" refers to the code of the investigated samples having these variants in this study.

Comprehensive phylogenetic tree construction

A comprehensive phylogenetic tree was generated based on the investigated ITS ribosomal nucleic acid sequences in the analyzed wild grass sample. Along with the other deposited DNA sequences, this phylogenetic tree contained our screened wild grass samples aligned with their highly related sequences in a neighbor-joining mode. In the currently constructed tree, the total number of aligned nucleic acid sequences was 115, consisting of different sequences, four minor clades, and one major clade. This major clade represented the most crucial incorporated nucleic acid sequences within the currently constructed tree, namely Echinochloa crus-galli. Based on the analyzed genetic sequences, our ITS rRNA sequences were clustered into one major phylogenetic clade, which entailed a particular range of diversity of these wild grass sequences in terms of our analyzed rRNA sequences (Fig. 4). In this clade, the currently investigated S1 and S2 samples were incorporated. Concerning S1 samples, it was found that this sample was positioned in the immediate vicinity of the GenBank accession number KF010208.1, which was deposited from South Korea. Meanwhile, a slight tilt was exhibited by the S2 sample as it was observed in the vicinity of the GenBank accession number AM404346.1, which was deposited from Mexico. This slight tilt may be due to the observed 408C>T nucleic acid substitution that may induce a particular phylogenetic deviation from the original positions these grasses take. However, the variable international origins of both samples are also possible and could not be omitted from our consideration. This is due to the high similarity of S1 and S2 samples to Asian and South American strains of the same species. Irrespective of these differences, both investigated samples of Echinochloa crus-galli resided in closely related phylogenetic distances within this tree. Meanwhile, it was found that there are vast phylogenetic distances between Echinochloa crus-galli as this species was incorporated in a distinct place away from the other incorporated species within the tree. These distances indicated the presence of distinct phylogenetic differences among the ITS ribosomal sequences of Echinochloa crus-galli and other species of grasses (phylogenetic tree scale range 0.1). In addition to the described major clade of Echinochlct phylogenetic distance crus-galli, four types of grass were incorporated within the same tree as outgroup sequences. These outgroup grasses included Alloteropsis semialata (black seed grass), Coix lacryma-jobi (job tears), Saccharum hybrid (a tall perennial plant within the grass family), and Miscanthus sacchariflorus (Amur silver grass). These outgroup grasses were aligned to the Echinochloa crus-galli clade, in which our samples were incorporated to assess the actual phylogenetic distance between S1 and S2 samples and these clades. Our results found that the Echinochloa crus-galli clade occupied a distinct phylogenetic position away from these outgroup sequences. These data refer to the lower percentages of nucleic acid homology between the Echinochloa crus-galli clade and the other related clades of other grasses.

From the above-stated data, it is consequent to consider several potential ancestries of the investigated wild grass samples. These sorts of genetic distribution referred to the sensitivity of the utilized ITS rRNA-based ribosomal amplicons in the accurate discrimination among the investigated wild grass samples. Thus, the distinctive role of the generated phylogenetic tree in detecting the currently analyzed samples could not be excluded from the explanation. Accordingly, this notion further indicates these analyzed wild grass isolates and reveals accurate genotyping phylogenetic distributions alongside their relative wild grass sequences.

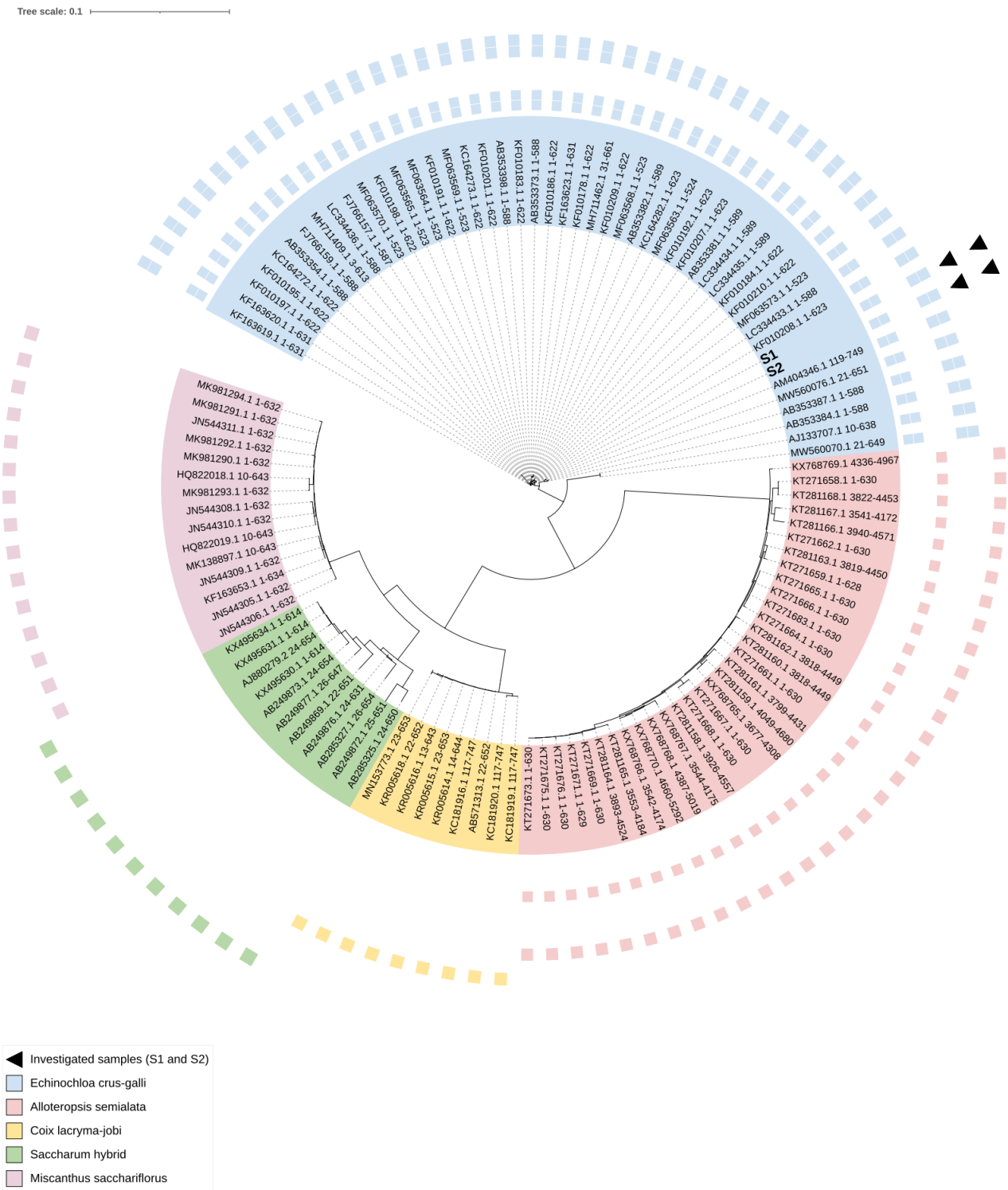

Figure 4. The comprehensive phylogenetic tree of the ITR rRNA sequences within the genomic sequences of one wild grass species, namely *Echinochloa crus-galli*. The variable colors refer to the variable grouping of the analyzed variants within their Genbank deposited sequences. The number "0.1" at the top left portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S" refers to the code of the investigated samples in this study.

DISCUSSION

The weed of Echinochloa crus-galli is considered one of the most important weeds spread with rice. It is an annual weed belonging to the Poaceae family, a smooth upright plant without desire, height 90 cm, the leaf is striped in shape, rough to the touch, the flower is a spike 15 cm long, and the seed is oval, pointed at both ends, smooth and glossy average The number of seeds produced by one plant is 5000 seeds. The plant is without ligaments and ears[6,7].

CONCLUSIONS

In conclusion, the present tree provided an inclusive tool for the guaranteed identity of the investigated wild grass samples. Therefore, using the PCR-sequencing strategy in the analyzed samples has confirmed the identity of these samples and showed the pattern of their phylogenetic distribution. This sort of observed diversity for this wild grass species may suggest the possibility of utilizing the current ITS rRNA sequences in accurately detecting and discriminating among a wide spectrum of other wild grass sequences. The same genus and species of *Echinochloa crus-galli* from rice weeds were present in Iraq.

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