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MOLECULAR STUDY FOR TWO SPECIES OF ALOPECURUS L. (POACEAE) IN IRAQ

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Abstract
In this study was used the PCR technique to detect the two different species of Alopecurus L. Agrostideae tribe and this technique relied on amplifying the 18s ribosomal RNA gene belonging to the chloroplast in the leaves of plants. The results of this study showed the success of the specific primer for each species in amplifying the target gene used in this study, which in turn led to the success in conducting genetic analysis of the species under study based on the ribosomal RNA gene, as it showed 99% similarities between the samples used and their genetic sequence deposited in the Genebank and compared between them. By comparing the DNA sequences of the analyzed samples with the corresponding DNA sequences, the exact locations and other details of the PCR amplified fragments were determined. A comprehensive genetic tree was generated for Alopecurus texilis and Alopecurus tiflisensis, which was based on the DNA sequences of the genetic sequences traced in the examined samples from the Agrostideae. A. texilis and A. tiflisensis, are aligned with most of the corresponding sequences in linking proximal lineages. The results also showed the presence of one mutation in the nitrogenous bases in the samples examined for each species, Furthermore, the two species were also registered in the Genebank by the researcher under the serial numbers (MZ871594.1 for A. texilis and MZ871595.1 for A. tiflisensis).
Keywords: Poaceae, Alopecurus, DNA sequences, 18S ribosomal RNA gene.

Introduction

Poaceae (Gramineae) is one of the largest families between the angiosperms, and is represented in every phytogeographic region in the world. It comprises about 10,000 species and 651 genera. It is divided into six sub families (Clayton & Renvoize ,1986). This family encompasses tremendous ecological, morphological, physiological, and generic diversity and is divided into 651 to 765 genera (Kellogg , 1998). A large and comprehensive family that is widely distributed almost everywhere, and its genera of monocotyledonous flowering plants known as herbs, cereals, bamboos, natural grassland grasses, and species cultivated in meadows and pastures (Haston et al., 2009), consisting of about 780 genera and about 12,000 species (Christenhusz & Byng, 2016).

As for the number of species in Iraq, Al-Rawi (1964) mentioned 95 genera of the Poaceae family, comprising 250 species, while Ridda & Daood (1982) indicated that the number of genera is 101, comprising 265 species. Al-Musawi (1987) mentioned that it has 101 genera and 270 species, and the numbers are still increasing due to the development of recent taxonomic studies on the Poaceae family. Some molecular studies have been conducted on members of the Poaceae family, including the study of Soreng & Davis (2000), which is the first molecular study of some genera of the tribe

Aveneae, including Agrostis stolonifera. With the continuous progress and development in molecular studies, studies have increased to know the evolutionary origins of various plants and their close relationships, by identifying specific genes in specific regions within the nucleus, chloroplasts, or mitochondria, following their molecular and nucleotide sequences, and studying their genetic stability, and at the level of the Poaceae family, the molecular information provided the basis for the hypotheses of the evolution of weeds at the level of families and clans (Liang's, 1997).

Molecular analyzes have surprisingly improved the evidence for access to plant strains (Shinwari & Shinwari, 2010). However, many phylogenetic relationships within many groups of plants are still unclear and need to be further explored by analyzing the progeny of several ribosomal genes (Shinwari & Qaisar, 2011). The ribosomal rRNA genes are an integral part of cell metabolism and are often used to reconstruct the deep branches of plants throughout evolutionary history. Small (16S, 18S) rRNA subunits have been used in several attempts to infer plant life sequences throughout history (Woese, 1998).Its gene can be used as an internal control for phylogenetic scaling due to the high conservatism of the 3′ region, as it is highly conserved in many plant species and several recent studies indicate that rRNA was the best control (Jarosova & Kundu, 2010). Some researchers avoid using ribosomal RNA to normalize high abundance real-time PCR data compared to target gene expression although this ratio varies based on samples and treatments (Schmittgen & Zakrajsek, 2000). There are reports of S18 and S25 rRNA being used as endogenous genes due to the high gene expression of the amplicons used (Kim et al., 2003).The aim of this study is a molecular study of two species of the tribe Alopecurus L., using DNA sequencing technology, also this study is the first in Iraq and is also a few in the world, so it lacks sources that agree with the results obtained in this study.

Materials and Methods

Plant materials

Fresh plant samples collected from field trips were prepared. The leaves were dried, and then the leaves were ground. Initial tests were conducted at Kerbala University, College of Science.

primer was selected specifically for the genus Alopecurus, which is based on the S ribosomal RNA18 gene, and was designed in this study using the NCBI Genbank Sequence and Primer3 plus. The primer was provided by (Scientific Reseracher. Co. Ltd / Iraq) in (Table 1).

TABLE 1. Sequences of the primer used in the study

Primer		Sequence 5'-3'	PCR product size	Genbank design Code
Alopecurus spp.	F	TGGCCAAAGTCCTCGATAACC	501bp	KM523765.1

	R	TATAGGGTCCAAAGGCCATCA		

PCR amplification and DNA sequencing

The technique was used to detect the different genus Alopecurus , and this technique relied on amplifying the 18S ribosomal RNA gene belonging to the chloroplast. The plant DNA was extracted using the Genomic DNA Mini Kit, the extracted genomic DNA was examined using a nanophotometer (THERMO. USA), which verifies and quantifies the purity of the DNA by reading the absorbance at (260/280 nm). PCR reaction mix was prepared using (GoTaqTMGreen PCR Master Mix). According to the company's instructions as in (Table 2).

TABLE 2. PCR Master mix

PCR Master mix	Volume
DNA template 5-50ng	5µL
18S rRNA gene Forward primer (10pmol)	2µL
18S rRNA gene Reverse primer (10pmol)	2µL
Go taq Green Master mix	12.5µL
PCR water	3.5 µL
Total volume	25µL

Then, the PCR master mix tubes mentioned in the above table were transferred to Exispin vortex centrifuges at 3000 rpm for 3 min. Then they are placed in a PCR Thermocycler. Among the conditions of the PCR thermal cycler using the traditional PCR system, as in Table 3.

TABLE 3. PCR Heat Cycler Conditions

PCR step	Temp.	Time	repeat
Initial Denaturation	95ºC	5min.	1
Denaturation	95 ºC	30sec.	35 cycle
Annealing	60 ºC	30sec	
Extension	72 ºC	5min.	
Final extension	72 ºC	5min.	1
Hold	4 ºC	Forever	-

The PCR products were analyzed by agarose gel electrophoresis method. A DNA sequencing method was performed to identify the confirmed genotypes based on the 18S rRNA genes of the species under study and the PCR results were sent to Macrogen in Korea by DHL for DNA sequencing analysis by the AB DNA sequencing system. DNA sequence analysis was performed

using Molecular Phylogenetic Analysis (Mega 6.0) and multiple sequence alignment analysis for ClustalW alignment analysis based on the partial fimH gene. Genebank-based NCBI BLAST analysis. Finally, sequencing samples were submitted to the NCBI-Genbank database to obtain Genbank accession numbers.

Results and Discussion :

PCR amplification

Results of the molecular study showed the success of the specific primers for each species in amplifying the target gene used in this study, which in turn led to the success in conducting genetic analysis of the species under study based on the 18 ribosomal RNA gene and analysis involved this primer of the post-LDR region of (A1-A4) showed positive PCR amplification of the genus Alopecurus spp. with a size of bp 501 PCR (Fig. 1). The proportion of appearing 99% similarities between the species of this genus used and their genetic sequence deposited in the Genebank and compared between them. By comparing the DNA sequences of the analyzed samples with the corresponding DNA sequences, the exact locations and other details of the PCR amplified fragments were determined.

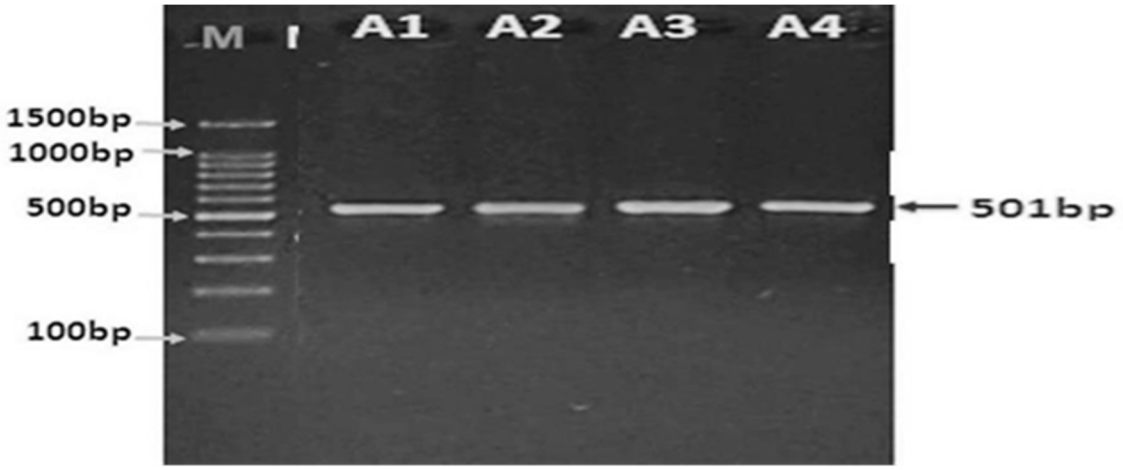

Fig. 1. Agarose gel electrophoresis PCR product of the 18s rRNA gene for samples of the genus Alopecurus spp. Where M: DNA ladder (100-1500 bp) iNtRON/ Korea. The post-LDR region of (A1-A4) showed positive PCR amplification of the genus Alopecurus spp. with a size of (bp 501 PCR).

DNA sequencing and species analysis Dendrogram

A comprehensive genetic tree was generated for Alopecurus texilis and Alopecurus tiflisensis, which was based on the DNA sequences of the genetic sequences traced in the examined samples from the Agrostideae. Alopecurus texilis and Alopecurus tiflisensis, are aligned with most of the corresponding sequences in linking proximal lineages .

This tree included the presence of 7 different species belonging to the same genus: Alopecurus glaciali, Alopecurus brevifol, Alopecurus ponticus, Alopecurus gerardii, Alopecurus lanatus, Alopecurus magellanicus, and Alopecurus glaucus (Fig. 2).
For Alopecurus texilis, it has been linked to the sequence number deposited in the Genebank KM523765.1, which belongs to a Canadian of the same genus. It was also found in the genetic tree near the Alopecurus texilis group, the presence of a closely related genus is Alopecurus glaucus with a percentage of about 99%, and thus a close sequence location can be identified for these two species. It also interacts significantly with the two species Alopecurus brevifol and Alopecurus ponticus with a percentage of approximately 99% as well.
It was also found that the genus Alopecurus tiflisensis with the serial number set by the researcher MZ871595 is locally isolated and did not match any serial number deposited in the gene bank, and showed less difference from other species of the genus Alopecurus, and the genetic distance of the total genetic changes between them was (0.0150-0.0050%) (Table 1).

Results also showed the presence of one mutation in the nitrogenous bases in the samples examined for the species Alopecurus texilis, (Fig. 3,4). As for the type Alopecurus tiflisensis, the presence of 6 base mutations was recorded, where two of the bases were replaced in different positions in the samples that were examined, documented and presented according to their positions in GenBank (Table 4). The two species were also registered in the Genebank by the researcher under the serial numbers (MZ871594.1 for Alopecurus texilis and MZ871595.1 for Alopecurus tiflisensis) Table 4. Results of the molecular study using the nucleotide sequencing method indicated that the confirmed identity of the plant samples used in the study was reached through the NCBI blast Genebank website. The obtained results showed high similarities in the sequences between the samples used and their genetic sequence in the gene bank.
There are very few studies on the clan Agrostideae, as it was studied morphologically only in the Iraqi Encyclopedia. Some molecular studies dealt with other races belonging to the same clan, but using different genes, such as the study of Mei (2007), which showed the presence of genetic affinity between the genera Calamagrostis and Polypogon of the Agrostideae, based on the molecular study that he conducted, and the study of Liang (1997) showed the existence of genetic affinity between the two genera, Phleum and Agrostis from the same clan, achieved genetic isolation at the level of the genera that support them by using a heterogeneous gene, the matK gene, on which most previous molecular studies, such as the study of Soreng & Davis (2000), Hilu et al., (1997) and Schneider et al., (2009) ; Al-Sumairi (2016) study also dealt with some species belonging to genera of the Agrostideae, as it used the matK gene. The Al-Ehdari study (2015) also dealt with species belonging to the two clans Aristideae and Stipeae of the Poaceae family, as it showed the nucleotide sequences of the nuclear region ITS1 and the chloroplast gene TRNL-F, providing support for previous taxonomic studies of these species.

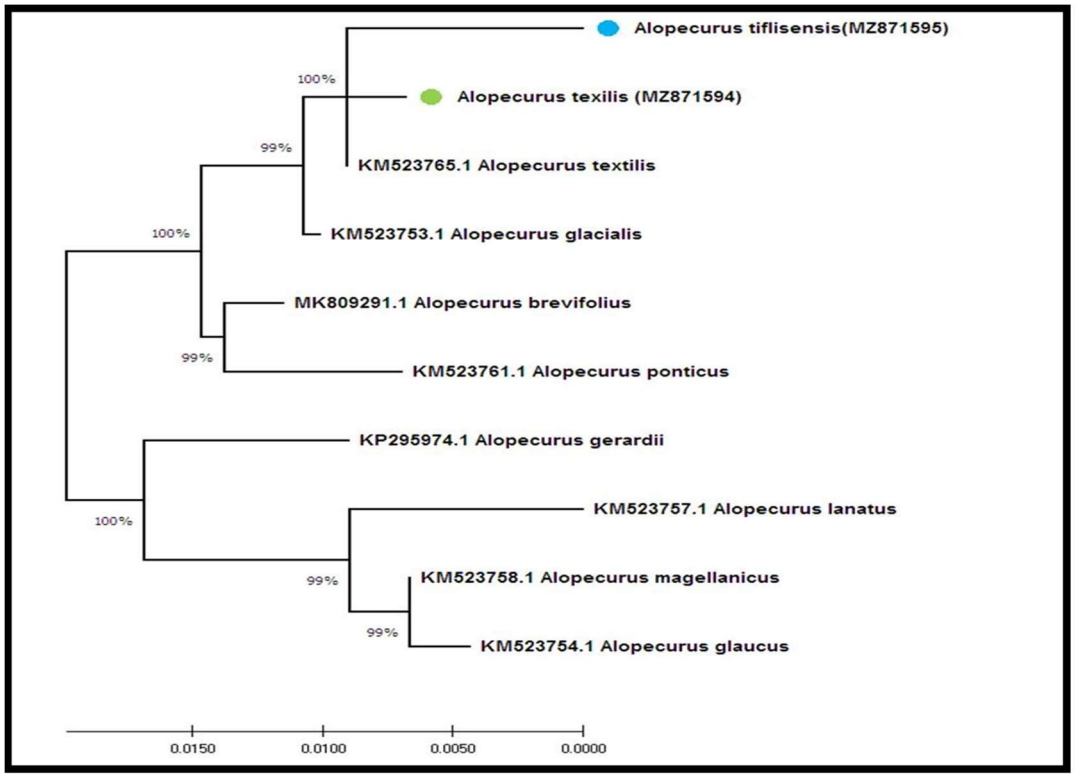

Fig. 2 . Phylogenetic tree of Alopecurus texilis and Alopecurus tiflisensis using MEGA X program.

TABLE 4: Sequence homology between Alopecurus texilis and Alopecurus tiflisensis based on NCBI-BLAST Genebank data from related Alopecurus species.

***Alopecurus* isolate No.**	**Genbank Accession number**	**NCBI-BLAST Homology Sequence identity (%)**			
		Number Mutation	**Type of Mutation**	**Polymorphism Percent (%)**	**Identity (%)**
Alopecurus texilis	MZ871594	1	G/T	0.22%	99.78%
Alopecurus tiflisensis	MZ871595	6	T/A, C/T, G/C, C/T,G/T, C/T	1.35%	98.65%

Alopecurus texilis (MZ871594)

Score	Expect	Identities	Gaps	Strand
821	0.0	446/447(99	0/447(0	Plus/Plu

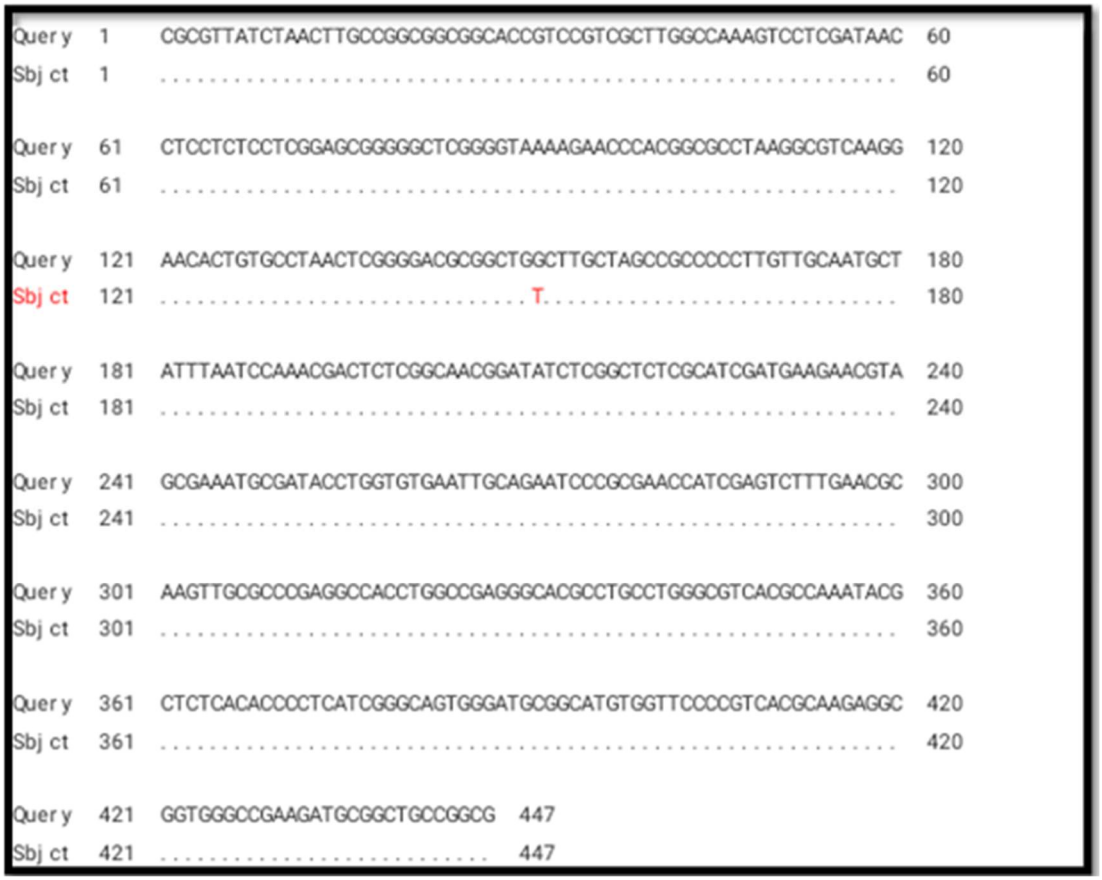

Fig. 3. Mutations recorded in Alopecurus texilis.

Alopecurus tiflisensis (MZ871595)

Score	Expect	Identities	Gaps	Strand
789	0.0	439/445(99	0/445(0	Plus/Plu

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Query  3    CGTTATCTAACTTGCCGGCGGCGGCACCGTCCGTCGCTTGGCCAAAGTCCTCGATAACCT  62
Sbjct  1    ............................A.....................T........  60

Query  63   CCTCTCCTCGGAGCGGGGGCTCGGGGTAAAAGAACCCACGGCGCCTAAGGCGTCAAGGAA  122
Sbjct  61   ..............................................................C...  120

Query  123  CACTGTGCCTAACTCGGGGACGCGGCTGGCTTGCTAGCCGCCCCCTTGTTGCAATGCTAT  182
Sbjct  121  ..........................................T.....................  180

Query  183  TTAATCCAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGC  242
Sbjct  181  ............................................................  240

Query  243  GAAATGCGATACCTGGTGTGAATTGCAGAATCCCGCGAACCATCGAGTCTTTGAACGCAA  302
Sbjct  241  ............................................................  300

Query  303  GTTGCGCCCGAGGCCACCTGGCCGAGGGCACGCCTGCCTGGGCGTCACGCCAAATACGCT  362
Sbjct  301  ..............................T.............................  360

Query  363  CTCACACCCCTCATCGGGCAGTGGGATGCGGCATGTGGTTCCCCGTCACGCAAGAGGCGG  422
Sbjct  361  ............................................................  420

Query  423  TGGGCCGAAGATGCGGCTGCCGGCG  447
Sbjct  421  ....................T.....  445
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Fig. 4. Mutations recorded in Alopecurus tiflisensis.

Conclusion :

Based on sequence analysis and blast result, it indicates that the DNA approach is a good application for the identification of these locally available species of Alopecurus L. This study showed the importance of molecular study as a tool for the separation of species of the genus Alopecurus L. Moreover, species identification in this study will help to develop further experiments with other species for this tribe Agrostideae. The data showed a significant difference at the level of molecular detection between species the collected of Alopecurus in Iraq.

Competing interests: The authors declare that they have no conflicts of interest

Authors' contributions: All authors have contributed equally. All authors read and approved the final manuscript.

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