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Genetic Diversity and Polymorphism of *Eucalyptus* L'Hėr Species by ITS Sequence

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Abstract

Genus *Eucalyptus* belongs to the family Myrtaceae that consists of more than 700 species, various hybrids and varieties. The majorly distributed species that are grown in Iraq are *Eucalyptus alba, E. macarthurii, E. siderophloia* and *E. camaldulensis, E. tereticornis, E. vicina*. Most *Eucalyptus* species are highly dependent on rainfall, and this is challenged by climatic changes owing to global warming making it difficult to effectively match the availability of mature trees and the market demand, especially for use as power transmission poles. With the widespread availability of other naturally occurring *Eucalyptus* species, it has become important to determine the genetic diversity and to analyze the phenotypic traits of these species for suitability as power transmission poles. However, the absence of complete genomic data for this plant greatly limits its ability to progress genetically. The goal of this study was to examine the ribosomal RNA gene in *Eucalyptus* plants and control the sequencing system on a large scale in this species. Samples from plants were used to extract genomic DNA. Detection analysis was performed using PCR methods. Sequence analysis of ribosomal RNA gene was conducted with data analysis. Gel electrophoresis revealed bands of extracted nucleic acid from the ribosomal RNA gene of plants. The ribosomal RNA gene had several transitions and transversion mutations when compared to other globally known ones. Our findings demonstrated that the ribosomal gene developed in this study can be used for detecting the genus of *Eucalyptus* plants and obtaining 14 species belonging to the genus of the *Eucalyptus* was registered at the Gene Bank as new species in Iraq.

Keywords: Sequencing, Ribosomal RNA gene, *Eucalyptus* plants, Iraq.

التنوع الوراثي وتعدد الأشكال لأنواع الأوكالبتوس من خلال تسلسل ITS

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الخلاصة

ينتمي جنس *Eucalyptus* إلى عائلة Myrtaceae ويتألف من أكثر من 700 نوع، ومختلف الهجائن والضروب. الأنواع الأكثر انتشارا التي تزرع في العراق هي *Eucalyptus alba*، *E. macArthurii*، *E.*

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sideropholia، *E. camadulensis*، *E.tereticornis* و *E. vincia*. تعتمد معظم أنواع الأوكالبتوس بشكل كبير على هطول الأمطار، ,الامر الذي يمثل تحديا بسبب التغيرات المناخية التي تعود الى الاحتباس الحراري ممايجعل من الصعب أن تتوفر بيئة مناسبة تدعم وفرة من الأشجار الناضجة وتلبي حاجة السوق. مع توافر واسع النطاق لأنواع الأوكالبتوس التي تنمو بشكل طبيعي، يصبح من المهم تحديد التنوع الجيني وتحليل السمات المظهرية لهذه الأنواع. ومع ذلك، فإن عدم وجود بيانات جينية كاملة لهذا النبات يحد بشكل كبير من قدرته على التقدم وراثيا. كان الهدف من هذه الدراسة هو فحص جين الحمض النووي الريبي الريبوزومي في نباتات الأوكالبتوس والتحكم في نظام التسلسل على نطاق واسع في هذا النوع. واستخدمت عينات من النباتات لاستخراج الحمض النووي الجينومي. تم إجراء تحليل الكشف باستخدام طرق PCR، تحليل تسلسل الجين الحمض النووي الريبي الريبوزومي مع تحليل البيانات. كشف الترحيل الكهربائي عن اشرطة من الحمض النووي المستخرج من الجين الحمض النووي الريبوزي الريبوسومي من النباتات. كان لجين الحمض النووي الريبي الريبوزومي عدة طفرات انتقالية واستبدالية بالمقارنة مع غيرها من الطفرات المعروفة عالميا. وفقًا لنتائجنا، أثبت أن الجين الريبوزومي الذي تم تطويره في هذه الدراسة يمكن استخدامه لاكتشاف جنس نباتات الأوكالبتوس والحصول على 14 نوعًا ينتمي إلى جنس الأوكالبتوس تم تسجيله في بنك الجينات كانواع جديدة في العراق.

Introduction

The first discoverer of *Eucalyptus* plant was a French botanist Louis Hèritier in 1788. *Eucalyptu*s is a tall plant, of Australian origin and includes nearly 700 species [1]. It returns to one of the genera into the Myrtaceae family and is an aromatic evergreen flowering tree grown everywhere, like Iraq [2]. The genus has roughly 37 species in the Iraqi flora, especially in Baghdad, which is unclassified species [3]. There is a general belief that all-natural products and herbal remedies of this plant are safe to use [4]. Because of its economic importance and good properties, it has been cultivated primarily to produce high-value solid wood products [5].

Pharmacological studies have revealed that *Eucalyptus* possesses gastrointestinal, anti-inflammatory, analgesic, antidiabetic, antioxidant, anticancer, antimicrobial, antiparasitic, insecticidal, repellent, oral and dental, dermatological, nasal and many other effects (6). It has also been observed that high doses of *Eucalyptus* oil could damage lung, liver, kidney and heart, with lung being the organ most affected by the oil, followed by the liver, kidney, and then the heart which was less affected by eucalyptus oil, indicating that eucalyptus oil, when administered orally in high doses, causes clear toxicity effects. However, eucalyptus oil is a safe medicine for external use (7).

Early research revealed that open-pollinated progenies' breast-high diameters varied significantly by age among them [8]. A population breeding of *Eucalyptus* showed high genetic variability. Its populations from other distribution zones have also showed considerable genetic variations [9]. Due to its exceptional performance and high breeding value, *Eucalyptus* has been recognized as a valuable wood tree in China [8].

Genome survey sequencing is a crucial and economical method for learning detailed genetic and genomic details about the various phenotypes of organisms [10] and creating accurate molecular markers for plants breeding [11]. Genomic research has accelerated in size, scope, and speed with the advent of sequencing. The full genetic structure of *Eucalyptus*, however, is mostly unknown. The majority of *Eucalyptus* genome studies have mostly centered on a small number of commercially significant species, such as *E. grandis* and *E. camaldulensis* [12].

Numerous publications about the emergence of 18S ribosomal markers for *Eucalyptus* have been published globally during the past ten years [13]. Comparatively, substantial research on the EST 18S ribosomal or genomic development of *Eucalyptus* has been reported infrequently. In a recent phylogenetic reconstruction in *Eucalyptus* [14], despite the whole-genome analysis on *E. grandis* having been reported, it was found that *Eucalyptus* belongs to an independent subgenus that is distinct from other *Eucalypts* [9].

The development of *Eucalyptus* in molecular biology is significantly limited by the nonappearance of the mentioned genome for the plant. It is challenging to address the research demands of genetic diversity analysis and the building of a *Eucalyptus* genetic map since there are still few 18S ribosomal primers appropriate for genetic analysis. In the current study, we used sequencing technologies, along with genomic analysis, to assess the *Eucalyptus* genome. This research may help to increase the exploitation of *Eucalyptus* gene resources and advance genetic advancement.

This study aimed to investigate and determine the sequencing genome of ITS of *Eucalyptus* using 18s ribosomal RNA gene.

Materials and Methods

1- Samples Collection: Two hundred and thirteen samples were gathered from various Baghdad regions between November 2020 and April 2021. From this number, only 20 samples appeared with variations in morphological characters.

2- DNA Extraction and PCR Program: The cell wall must be broken in order to release the cellular constituents. This is usually done by grinding the leaves in liquid nitrogen with a mortar and food grinder (15) and then using the Favor Prep TM Plant Genomic DNA Extraction Mini Kit, genomic DNA is extracted from plants. DNA was extracted using a "Genomic DNA extraction kit" and kept at -20°C until use. PCR was performed on the PCR system with a reaction volume of 25 µL, including 5 µL of Master Mix, 1 µL of each primer pair ITS as in (Table 1) 16.5 µL of nuclease-free water, and 1.5 µL of DNA. The amplification program was as follows: initial denaturation at 95°C for 5 min, denaturation at 95℃ for 45 sec., annealing at 52°C for 1 min., extension 1 at 72°C for 1 min and extra extension at 72°C for 5 min for 35 cycles. The PCR products were subjected to 2% agarose gel electrophoresis at 70 volts for 1 h. UV- trans-illuminator was used to visualize DNA bands.

Table 1: Primers for amplifying *18S ribosomal R*NA gene.

Primer	Sequence	Primer Sequence	Tm (°C)	GC%	Size of Product (bp)
ITS	F	**5′- TCCGTAGGTGAACCTGCGG -3′**	60.3	50 %	**550-650**
	R	**5′ TCCTCCGCTTATTGATATGC-3′**	57.8	% 41	

3- Sequencing of *18S ribosomal RNA* Gene: Following company's instructions, the resolved PCR amplicons were commercially sequenced in both forward and reverse directions (Macrogen Inc. Geumchen, Seoul, South Korea). The clear chromatographs that were acquired from the ABI (Applied Biosystem) sequence files were then further examined, with the annotation and variances being shown utilizing bioinformatics analysis. Additionally, the virtual locations and other information of the obtained PCR fragments were detected by comparing the observed sequences of local samples with the retrieved sequences.

Results and Discussion

1- Results of PCR for Detection of the Amplicon of Ribosomal RNA Gene: Gel electrophoresis revealed bands of extracted nucleic acid from the 18S ribosomal RNA gene (Figure 1).

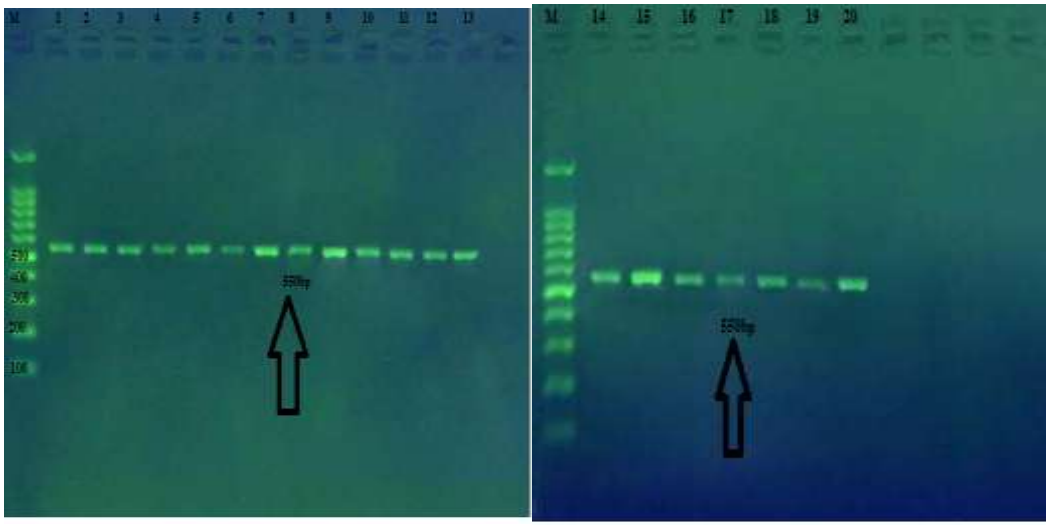

Figure 1: Gel electrophoresis *of ITS* gene in *Eucalyptus* species

2- Sequencing Analysis of 18S ribosomal RNA Gene: Mega 6 software was used for the sequencing study which revealed differences between the small subunit ribosomal RNA gene's nucleotide sequence and those of other internationally recognized genes. Transversion and transition appeared in *Eucalyptus* species. These alterations are detailed in Table 2.

Table 2: Genetic variation small subunit ribosomal RNA gene

18S ribosomal RNA Gene							
No.	**Type of Substitution**	**Location**	**Nucleotide**	**Sequence ID with Compare**	**Sequence ID with Submissions**	**Source**	**Identities**
1	**Transversion**	**251**	**A\T**	**ID: AF390524.1**	**ID: OP696596.1**	**Eucalyptus curtisii**	**99.63%**
	Transversion	**270**	**T\A**				
2	**Transversion**	**445**	**T\G**	**ID: KM064861.1**	**ID: OP696597.1**	**Eucalyptus globoidea**	**99.58%**
	Transversion	**446**	**T\G**				
3	**Transition**	**291**	**A\G**	**ID: KP142202.1**	**ID: OP696598.1**	**Eucalyptus leucoxylon**	**99.63%**
	Transition	**420**	**T\C**				
4	**Transversion**	**489**	**T\G**	**ID: AF190365.1**	**ID: OP696596.1**	**Eucalyptus erythrocorys**	**99.81%**
5	**Transition**	**334**	**C\T**	**ID: KM064884.1**	**ID: OP696600.1**	**Eucalyptus macarthurii**	**99.81%**
6	**Transversion**	**356**	**A\C**	**ID: KM065011.1**	**ID: OP696601.1**	**Eucalyptus botryoides**	**99.63%**
	Transition	**375**	**A\G**				
7	**Transversion**	**490**	**A\T**	**ID: KM064783.1**	**ID: OP696602.1**	**Eucalyptus nicholii**	**99.81%**
8	**Transition**	**322**	**T\C**	**ID: KM065009.1**	**ID: OP696603.1**	**Eucalyptus pauciflora**	**99.81%**

9	**Transition**	**546**	**A\G**	**ID: KM064957.1**	**ID: OP696604.1**	**Eucalyptus delegatensis**	**99.82%**
10	**-------**	**------**	**------**	**ID: HM116970.1**	**ID: OP696605.1**	**Eucalyptus siderophloia**	**100%**
11	**-----**	**------**	**-----**	**ID: MH628295.1**	**ID: OP696606.1**	**Eucalyptus alba**	**100%**
12	**Transversion**	**240**	**G\C**	**ID: ON090297.1**	**ID: OP696607.1**	**Eucalyptus camaldulensis**	**99.26%**
	Transversion	**397**	**T\G**				
	Transversion	**398**	**T\G**				
	Transversion	**414**	**T\G**				
13	**Transition**	**468**	**A\G**	**ID: HM116971.1**	**ID: OP696608.1**	**Eucalyptus vicina**	**99.81%**
14	**Transversion**	**553**	**C\A**	**ID: ON090297.1**	**ID: OP696609.1**	**Eucalyptus camaldulensis**	**99.81%**
15	**Transition**	**258**	**T\C**	**ID: KM064884.1**	**ID: OP696610.1**	**Eucalyptus macarthurii**	**99.82%**
16	**------**	**------**	**-------**	**ID: AY864901.1**	**ID: OP696611.1**	**Eucalyptus tereticornis**	**100%**
17	**Transition**	**650**	**C\T**	**ID: HM116970.1**	**ID: OP696612.1**	**Eucalyptus siderophloia**	**99.82%**
18	**Transversion**	**502**	**T\G**	**ID: HM116971.1**	**ID: OP696613.1**	**Eucalyptus vicina**	**99.63%**
	Transversion	**607**	**G\T**				
19	**-----**	**-----**	**------**	**ID: MH628295.1**	**ID: OP696614.1**	**Eucalyptus alba**	**100%**
20	**Transversion**	**52**	**T\A**	**ID: AY864901.1**	**ID: OP696615.1**	**Eucalyptus tereticornis**	**99.63%**
	Transition	**93**	**T\C**				

In this study, substitutions in the 18S ribosomal RNA gene were discovered in the ITS of *Eucalyptus.* Table 2 shows that some of the *Eucalyptus* ribosomal RNA genes have no substitutions, while others have several transition and transversion mutations at many locations with multiple identities ranging from 99% to 100%. A/G, T/C and C/T are examples of transition mutations, whereas A/T, T/A, T/G, G/T, A/C and G/C are examples of transversion mutations. Generally speaking, these ribosomal RNA gene alterations could lead to a specific species of *Eucalyptus* plant.

Sequences of the 18S ribosomal RNA or microsatellite DNA are crucial for adaptive evolution in rapidly changing settings [16]. Numerous tree species' ITS in plant genomes have been examined, and the counts varied greatly amongst them. The identities of the ITS in *E. alba* (100%) were similar to those found in *E. siderophloria* and *E. tereticornis* [13], and the similarities between *E. delegatensis* and *E. macarthurii* in *E. delegatensis* (99.82%) may be based on the *Eucalyptus* genomes from Gen Bank that Rabello et al. (2009) reported [11].

On the other hand, 99.63% of the ITS of *E. curtisii* were identical to those found in *E. leucoxylon*, *E. botryoides*, *E. vicina*, and *E. tereticornis*. *E. globoidea's* identities were also (99.58%) not closely related to any *Eucalyptus* species. *E. camaldulensis'* identities were also (99.26%) not closely connected to any *Eucalyptus* species. However, mononucleotide was among the ITS search criteria in the latter. Even using the same technique, the resultant distribution frequency for 18S ribosomal analysis would change due to the inter-species

variations, depth of sequencing data, quality of sequence splicing data, various 18SR searching software and searching criteria [17]. The *E. erythrocorgs* genome's identities were 99.81% closely similar to those of *E. nicholii* and *E. pauciflora*, and this trinucleotide may be the most abundant one ever discovered [18]. The genomic 18SR primers that were chosen for the screening test, showed informational behavior in *E*. species.

Conclusion

Our findings demonstrated that the ribosomal gene developed in this study can be used for detecting the genus of *Eucalyptus* plants and that 18S ribosomal primers were appropriate for genetic analysis. Sequencing technologies, along with genomic analysis, were used to assess the *Eucalyptus* genome. This research may help to increase the exploitation of *Eucalyptus* gene resources and advance genetic advancement.

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