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Study the Phyllotaxis with a New Molecular Depiction of Nuclear ITS Region of Locally Isolated Avicennia Marina Plant

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Abstract - Five leaf samples were collected from grey mangrove plants found along the coastline of Khor Al-Zubair oil port in the Khor Al-Zubair region, located southwest of Basra Governorate, Iraq. DNA was extracted from dried leaf material, and then the nuclear internal transcribed spacer (ITS) region, involving ITS1, the 5.8S rRNA region, and ITS2, ranging in size from 700 to 720 bp, was amplified using specific primers. Among the samples collected, three originated from plants exhibiting two leaves at each node (opposite leaves), while the remaining two samples were obtained from plants with three leaves at each node (whorled leaves). The current study primarily focuses on leaf arrangements termed as whorled leaves, which were observed in two of the collected samples, this pattern is unfamiliar in the Avicennia marina. Hence, there may be a correlation between the ITS region and the phyllotaxis pattern of these plants. Alignment analysis of a high-quality sequence of 692 base pairs revealed significant similarity with the internal transcribed spacer (ITS) of Avicennia marina. This sequence has been deposited in GenBank under the new accession number (PP237061), it was then, aligned with those from 16 other documented ITS regions of Avicennia marina specimens in NCBI to construct a comparative phylogenetic tree consisting of 32 nodes and 17 tips.

دراسة النمط الورقي مع تسجيل جديد لمنطقة ITS النووية لنبات القرم الرمادي (*Avicennia marina*) المعزول محليا

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المستخلص - تم جمع خمس عينات من أوراق نباتات المانغروف الرمادية المتواجدة في ساحل ميناء خور الزبير النفطي في منطقة خور الزبير، والذي يقع جنوب غرب محافظة البصرة في العراق. تم استخلاص الحمض النووي DNA من الأوراق وذلك بعد تجفيفها، ضُخمت المنطقة النووية ITS التي تتضمن ITS1، ومنطقة 5.8 rRNA، و ITS2والتي يتراوح حجمها من 700 إلى 720 زوج قاعدي باستخدام بوادئ متخصصة. من بين العينات التي تم جمعها، ثلاثة قد جُمعت من نباتات تظهر لديها ورقتان في كل عقدة (أوراق متقابلة)، بينما تم الحصول على العينتين المتبقيتين من نباتات تحتوي على ثلاثة أوراق في كل عقدة (أوراق متراصة). تُركز الدراسة الحالية بشكل أساسي على ترتيب الأوراق التي يطلق عليها مصطلح أوراق متراصة والتي لوحظت في اثنين من العينات التي تم جمعها، هذا النمط غير مألوف في Avicennia marina وبالتالي، قد تكون هناك علاقة بين منطقة ITS ونمط توزيع الأوراق(phyllotaxis) لهذه النباتات، أظهر تحليل Alignment للتسلسل عالي الجودة والذي يبلغ طوله 692 زوج قاعدي تشابهًا كبيرًا مع تتابعات ITS الخاصة بنبات المانغروف حيث تم تسجيل هذا التسلسل في بنك الجينات تحت الرقم الجديد P237061، وتم مقارنة هذا التسجيل الجديد مع منطقة ITS تابعة لـ 16 نبات مانغروف مسجل في موقع NCBI لبناء شجرة تطورية مقارنة تتألف من 32 عقدة و 17 نصلاً.

الكلمات المفتاحية: Avicenna marina، منطقة ITS النووية، المانغروف، النمط الورقي.

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Introduction

Mangroves thrive in the intertidal areas of tropical to sub-tropical latitudes, where they flourish as woody shrubs and tropical vegetation (Faridah *et al.*, 2019). Globally, mangroves span approximately 200,000 Km2 in terms of area (Himes *et al.*, 2018; Worthington *et al.*, 2020) , part of the family of Acanthaceae, it is a polyphyletic group of trees, consisting of more than 400 species (Wu *et al.*, 2018). These trees exhibit distinctive physiological and morphological variations, as well as resilience to hypersaline environments, tidal cycles, and variations in soil chemistry(Das *et al.*, 2015) (Sannigrahi *et al.*, 2020) . Significantly, mangroves function as a central source of abundant ecological resources, offering a protective haven for a variety of marine life to breed, seek shelter, and thrive. They also serve as a carbon sink to absorb carbon emissions. Nevertheless, excessive exploitation for wood and pollution of environment have significantly diminished the mangrove population and led to a decline in genetic diversity(Moreira *et al.*, 2017). Significantly, mangroves function as a central source of abundant ecological resources, offering a protective haven for a variety of marine life to breed, seek shelter, and thrive. They also serve as a carbon sink to absorb carbon emissions. Nevertheless, excessive exploitation for wood and pollution of environment have significantly diminished the mangrove population and led to a decline in genetic diversity(Asaf *et al.*, 2021).

The leaves of gray mangroves reach a length of up to 8 cm and a width of 5 cm, exhibiting an oval shape with pointed tips. Arranged opposite each other on the stems, these leaves are glossy green on the upper side, while the underside is characterized by a distinct pale and slightly hairy grey appearance. Stomata (pores) and salt glands are distributed across the entire leaf surface, with a higher concentration on the underside (Faridah *et al.*, 2019). Avicennia marina, commonly known as the grey or white mangrove, stands as a keystone species with remarkable ecological importance. However, exact identifying Avicennia marina and distinguishing it from related mangrove species stills a challenging mission, especially when relying solely on morphological features (Asaf *et al.*, 2021). Phyllotaxis indicates the arrangement of plant leaves on a stem. The essential arrangements are alternate and opposite leaf positioning. Sometimes it can also be whorled if many leaves appear to rise from the same node. In the opposite arrangement, two leaves face opposite sides of the plant stem (Godin *et al.*, 2020) The mangrove species Avicennia marina is intensely found in Australia and is identified by its opposite leaves (Fuenzalida *et al.*, 2023). Avicennia marina characterized by its leaves arranged in opposite pairs (Farooqui *et al.*, 2018; Mariano *et al.*, 2019).

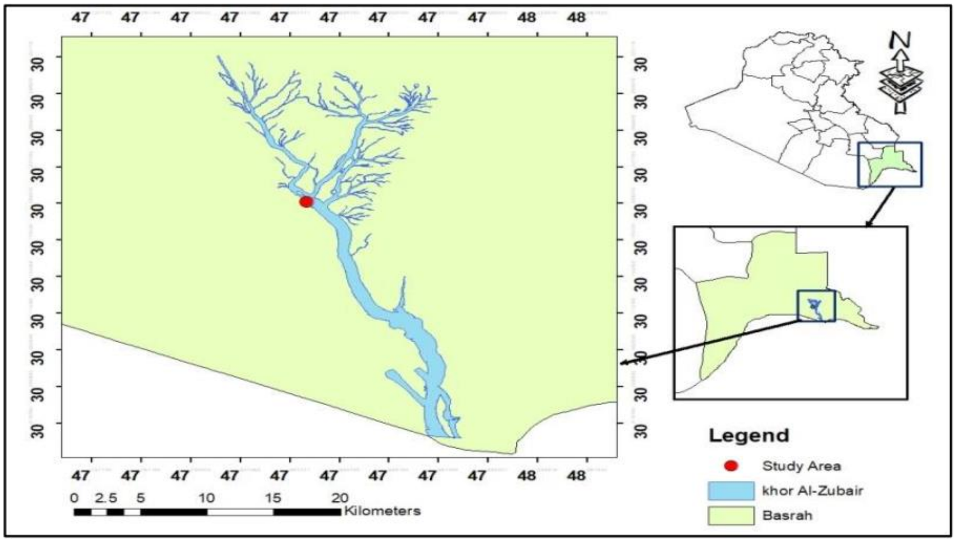
Recent achievement in molecular biology have exhibited a significant solution to this taxonomic challenge. The nuclear Internal Transcribed Spacer (ITS) region, encompassing three distinct segments-ITS1, the 5.8S rRNA region, and ITS2 has emerged as a good genetic marker for species identification and phylogenetic studies. With a size range of approximately 700 to 720 base pairs , the nuclear ITS region provide high resolution and discriminatory power, making it a valuable tool in identifying Avicennia marina and resolving taxonomic doubts within the mangrove genus(Glasenapp *et al.*, 2019).

The molecular technique applications in taxonomic research have altered our capacity to discover the genetic makeup of plant species. This advancement encourages researchers to make decisions aimed at preserving the characteristic ecological functions of mangrove habitats. By focusing on the role of the nuclear ITS region in identifying Avicennia marina, this study aims to explore the link between the examination of Avicennia marina phyllotaxis and the nuclear ITS region.

Materials and Methods

Sample collection

Five leaf specimens were gathered from gray mangrove plants situated along the coast of Khor Al-Zubair oil port in the Khor Al-Zubair region, located southwest of Basrah Governorate, Iraq, (latitude: 30°12'33.09"N, longitude: 47°52'24.22"E), (Figure 1).



(Figure 1) Sampling station

ITS Region Analysis; DNA Extraction and PCR

DNA was obtained from dried leaf material at room temperature using the Nucleospin Plant Kit II (Macherey & Nagel, Düren, Germany) (Lucas *et al.*, 2012). The efficiency of DNA extraction was assessed by observing bands on 1% agarose gels that were stained with ethidium bromide dye. The nuclear ITS region including ITS1, the 5.8S rRNA region and ITS2, with a size of 700 to 720 bp, was amplified by PCR. The primers P674 5′-CCTTATCATTTAGAGGAAGGAG-3′ (ITS5a) and P675 5′-TCCTCCGCTTATTGATATGC-3′ (ITS4) (Glasenapp *et al.*, 2019) were used, the expected PCR product was 700 to 720 bp . A *thermocycler (*Eppendorf, Germany) with lid heating was used for the PCR reactions. An initial denaturation step at 95℃ for 4 min was followed by 30 cycles with the following steps: denaturation at 95℃ for 25 s, primer annealing at 52℃ for 30 s and primer extension at 72℃ for 35 s. The reaction was terminated with a final hold at 72℃ for 10 min. Sequencing of the PCR products was done by Macrogen (Korea) using the primers P674 5′-CCTTATCATTTAGAGGAAGGAG-3′ and P675 5′-TCCTCCGCTTATTGATATGC-3′, the predicted amplified product size for the nuclear ITS region, covering ITS1, the 5.8S rRNA region, and ITS2, was within the range of 700 to 720 base pairs.

Construction of a Phylogenetic Tree

Geneious Prime 2019 software version 1.1(Geneious-Prime, 2024) was used to detect the sequences similarity. Multiple Sequence Alignment and the evolutionary relationship was determined by comparing the DNA sequence obtained from the sequencing step with the BLAST results obtained from NCBI. These relationships were constructed(Kapli *et al.*, 2020), and the tree was drawn using (Neighbour-Joining method) applied in Geneious Prime 2019 software version 1.1 which was downloaded from the official web site.

Results and Discussion

Avicennia Marina Phyllotaxis Description

Among the collected samples, three were obtained from plants featuring two leaves at each node (opposite leaves) (Figure 3), while the remaining two samples were derived from plants with three leaves at each node (whorled leaves) as shown in Figure 4. *Avicennia marina* is identified by its leaves arranged in opposite pairs pattern (Fuenzalida *et al*., 2023) .

(Figure 2) Plant samples of *Avicennia marina* with normal phyllotaxis characterized by the presence of two leaves at each node, arranged in an opposite manner.

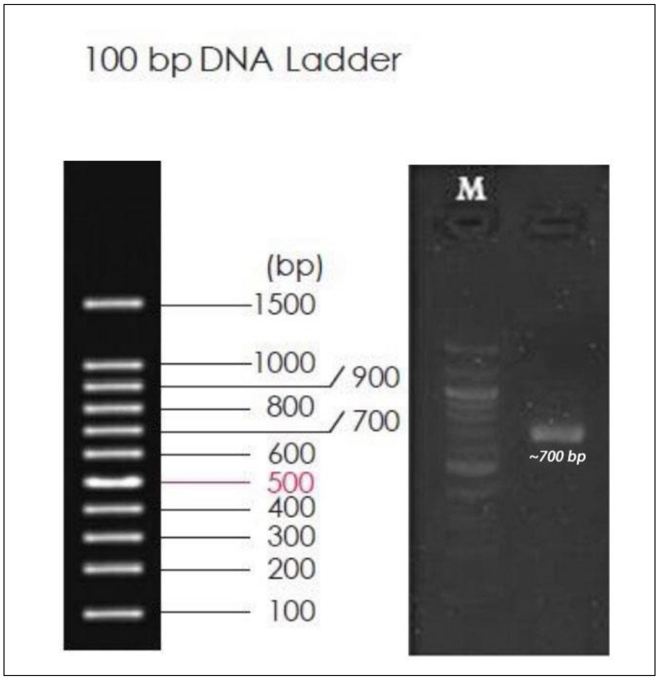
(Figure 3) Samples of *Avicennia marina* plant exhibiting abnormal phyllotaxis, marked by the occurrence of three leaves at each node, organized in a whorled leaf pattern.

Mangroves frequently develop various salt adaptive mechanisms (Nizam *et al*., 2022) ,in addition to alter the configuration of their leaves to enhance the balance of energy within the leaves, this includes features such as smaller, thicker leaves positioned vertically to reduce direct sunlight exposure, thereby reducing the rate of water loss per amount of carbon absorbed and maximizing heat dissipation (Khan *et al.*, 2020). current study is focused on the unique leaf arrangement known as whorled leaves, which we observed in two collected samples.

DNA Extraction and PCR

DNA was exclusively extracted from the whorled leaves of the *Avicennia marina* plant, followed by amplification using specific primers for the ITS region. Figure 5 illustrates a band of approximately 700 base pairs resulting from this process.

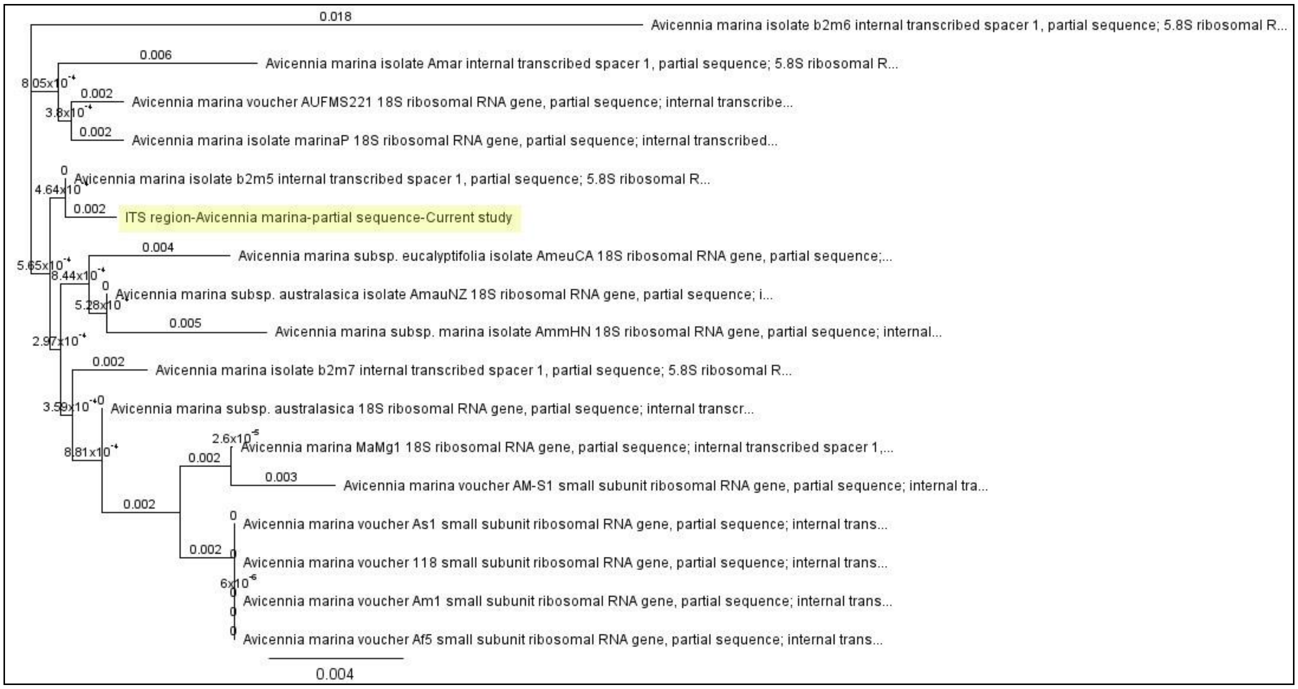
The PCR products of the specific gene submitted sequencing at MACROGEN/Korea's DNA sequencing service, available at "http://dna.macrogen.com." Subsequently, Geneious Prime 2019 software version 2.1 (Geneious-Prime, 2024) was employed to revise the sequence results. The analysis released a high-quality sequence of 692 base pairs. Further analysis involved comparing these sequences with the National Center for Biotechnology Information Database (NCBI) through the Basic Local Alignment Search Tool (BLAST) (BLAST, 2024), the alignment findings indicated a significant similarity with internal transcribed spacer (ITS) of *Avicennia marina*, the sequence was submitted in GenBank under new accession number (PP237061) (Alkanany *et al.*, 2024).



(Figure 4) An image of agarose gel electrophoresis displays band of the amplified ITS region, approximately 700 base pairs amplified ITS region

Phylogenetic Analysis

Conducting a comparative phylogenetic analysis with 32 nodes and 17 tips of the obtained ITS sequences highlighted notable sequence conservation within the studied region. Figure 5 demonstrates the proximity between the ITS region of *Avicennia marina*, showcasing the opposite leaves pattern in our study, and the internal transcribed spacer sequences of 16 other *Avicennia marina* specimens with whorled leaves arrangement archived in NCBI.



(Figure 5) Phylogenetic analysis of partial sequence of ITS region associated with *Avicenna marina*, considering various phyllotaxis pattern

Conclusion:

In conclusion, the study investigated leaf samples collected from grey mangrove plants in the Khor Al-Zubair region of Iraq. We identified a notable divergence in leaf arrangement patterns, particularly focusing on the uncommon occurrence of whorled leaves in *Avicennia marina*. The findings suggest a correlation between the ITS region and phyllotaxis patterns in these plants. Alignment analysis of the ITS sequence exhibited significant similarity with known *Avicennia marina* specimens, contributing to the phylogenetic understanding of this species. This study provides valuable insights into the genetic basis of leaf morphology in mangrove ecosystems.

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