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Biochemical Identification of Some True Halophytes Using DNA Barcoding in the Egyptian Red Sea Coast

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Abstract

Halophytes are a group of plants that thrive in high salinity. These plants are overused and endangered due to a combination of environmental factors and human behaviors. One of the ways to preserve the biological diversity of local plants is through their clear documentation to preserve the intellectual property rights of countries. Biochemical characterization using DNA barcoding is a relatively new species identification and authentication method using a short section of DNA from a specific gene or genes. In this study, ten halophytic plant species were collected from their different growth habitats along the Red Sea coast of Egypt. Then two standard chloroplast DNA loci (matK & rbcL) were used as DNA barcoding markers to document the collected species. They were successfully amplified and sequenced; the resulting barcode sequences have been deposited to the GenBank database. The sequence similarity (BLAST analysis) and the phylogenetic-based methods showed that the rbcL barcode has a high universality among the different tested taxa, whereas matK offers slightly low universality. In this context, we found that using the previously proposed barcode of rbcL alone may be difficult to differentiate between the closely related species (*Tetraena alba* and *Tetraena coccinea*). Two partial Coding Sequences (CDs) of the matK gene from *Atriplex farinosa* and *Tetraena alba* were deposited for the first time into the GenBank database (accession numbers OM164029 and OM16403, respectively). This finding is noteworthy as it represents a previously unreported addition to the available genetic information for these two plant species.

Keywords: DNA barcoding, Biodiversity, Halophytes, Red Sea, matK, rbcL

1. Introduction

The Red Sea is a narrow, elongated sea that runs between Africa and Asia. It is bordered by Egypt, Sudan, Saudi Arabia, Yemen, Eritrea, and Djibouti. The Red Sea is a tropical sea with a warm, clear climate with many endemic and native coastal plants and marine species [1]. Its plant ecosystem is diverse and unique, with a variety of species adapted to the region's arid and saline conditions. The coastal regions of the Red Sea in Egypt harbor diverse plant species, such as mangroves, sea grasses, and salt-tolerant shrubs, which form the blue carbon ecosystems. These ecosystems effectively mitigate climate change by sequestering carbon dioxide, a greenhouse gas that contributes to global warming [2, 3, 4]. They also provide crucial habitats for various marine and terrestrial species, including fish, crabs, and birds. As such, the blue carbon ecosystems play a vital role in maintaining the region's biodiversity [5, 6, 7].

Halophytes are a group of plant species that can thrive in high-salinity soils. They are typically found in seashores, mangrove swamps, and saline semi-deserts. The proportion of plant species that are classified as halophytes is relatively low, estimated to be only around 2% of the total plant species. They are widely distributed worldwide, particularly in arid and semi-arid areas. Along the Red Sea coast, halophytes are an important component of the plant ecosystem scattering

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in various communities along the coastline [6, 8]. These plants possess various mechanisms to cope with the high levels of salt in their environment, such as specialized root systems, excretion of salt through their leaves, and water storage in their leaves and stems [9]. They have recently taken an interest in benefiting from them in various economic fields, such as using them in livestock feeding, desalinizing and fertilizing soils, bioenergy crops, and as a source of some industrial and pharmaceutical chemicals [10, 11, 12]. Halophytes are promising models for studying salt tolerance mechanisms due to their complex defense mechanisms against salt stress. These mechanisms include the production of osmolytes, alterations in ion balance, intracellular compartmentalization of toxic ions, and scavenging systems for Reactive Oxygen Species (ROS) [13]. Similarly, Halophytes provide a valuable resource for the identification of genes and promoters that respond to salt stress. These can be utilized in genetic engineering to create crop plants with enhanced tolerance to salinity. This can promote sustainable agriculture in saline environments and enhance crop productivity in areas affected by salt stress [14].

However, like the marine ecosystem, the Red Sea's plant ecosystem also faces threats from climate change, pollution, and coastal development. Egypt has taken various measures to safeguard the plant and marine ecosystems of the Red Sea in response to the challenges they face. These conservation initiatives include the creation of marine protected areas, conservation efforts conducted both in their natural habitats (*in-situ* conservation) and outside their natural habitats (*ex-situ* conservation), enforcement of fishing regulations, and the implementation of ecological restoration and plantation projects [15].

In recent years, molecular tools such as DNA barcoding have been increasingly utilized to document and characterize plant genetic resources for the conservation of biodiversity, as well as to combat biopiracy and protect the intellectual property rights of countries [16, 17]. DNA barcoding is a reliable and efficient tool for accurately identifying and authenticating plant species. This technique uses short DNA sequences from specific genes to distinguish between closely related plant species that may be difficult to differentiate based on their physical characteristics alone, and discover new cryptic species. This can be particularly important for identifying and protecting rare or endangered plant species [18, 19]. By accurately identifying plant species and documenting their genetic profiles, DNA barcoding can also aid significantly in the conservation and protection of plant genetic diversity, as well as the development of conservation strategies, such as the establishment of protected areas and implementation of *in-situ* and *ex-situ* conservation measures [20, 21].

DNA barcodes for plants involve various plastid regions such as maturase K (matK), ribulose 1,5 bisphosphate carboxylase (rbcL), intergenic spacer region (trnH-psbA), RNA polymerase B (rpoB) and RNA polymerase C1 (rpoCl) with Internal Transcribed spacer (ITS) of the nuclear ribosomal DNA (nrDNA) region. Despite these options, the standard DNA barcode for plant identification, as suggested by the Consortium for the Barcode of Life (CBOL) Plant Working Group, is a combination of two genetic loci: rbcL and matK, which shows the most satisfactory species discrimination rate and universality results [22].

In this study, the matK and rbcL markers were used as a DNA barcode to investigate the true halophytic plant species collected from the coastal area of the Red Sea in Egypt. Consequently, the obtained sequences were compared to established reference sequences in the publicly available GenBank database to confirm the plant species' identity and test the feasibility of DNA barcoding in discriminating between them. Furthermore, the successful DNA barcoding sequences were deposited in the GenBank database for molecular documentation and authentication of the investigated plants.

2. Materials and methods:

2.1. Plant materials

Fresh, healthy plant tissues were collected from their habitats **(Supplementary Table S1)** and immersed in a liquid nitrogen tank immediately till used for DNA extraction. In addition, their sites of collection and Global Positioning System (GPS) coordinates (Latitudes and Longitudes) were recorded. All plant species were identified by Dr. Omran Ghaly, Plant Ecology and Range Management Department, Desert Research Center (DRC), Cairo, Egypt, and the voucher specimens were deposited in the Herbarium of DRC. The freeze cuttings from the plants were preserved in -80°C refrigerators for long-term *ex-situ* conservation at the National Research Centre (NRC), Egypt.

2.2. DNA extraction, barcode amplification & sequencing

The total genomic DNA was isolated from dried leaves and tissues using Qiagen DNeasy Plant mini kit (Qiagen, Germany) according to the manufacturer's protocol. In some cases, when the Qiagen extraction kit was unsuccessful, Cetyl Trimethyl Ammonium Bromide (CTAB) was also used, as the modified method by Sahu et al. [23]. Two plastids' regions matK and rbcL, widely used as standard plant barcodes, were amplified separately with a Biometra Tone 96G thermal Cycler (Analytik Jena GmbH, Germany). The PCR reactions were carried out in a total volume of 50 µl with the following components: 1x Green GoTaq® Flexi Buffer (Promega, USA), 2.5 mM MgCl2, 0.2 mM dNTPs (Promega, USA), 10 pmol of each primer (Macrogen Inc., South Korea), 1.25 units of GoTaq® G2 Flexi DNA Polymerase (Promega, USA), 50-80 ng template DNA and Nuclease-Free Water to the final volume. The thermocycling conditions for the PCR amplification of matK and rbcL barcode regions are summarized in **(Table 1)**, and details regarding the primers can be found in **(Table 2)**.

Table 1. The thermocycling conditions for PCR amplification of the matK and rbcL barcode regions

PCR step	Temperature	Time	No. of cycles
Initial denaturing	95°C	5 min	
Denaturing	95°C	30 sec	35 cycles
Annealing	50°C *(*matK) / 55°C (rbcL*)*	1 min	
Extension	72°C	1 min	
Final extension	72°C	7 min	

The PCR products were examined using 1.5% agarose gels with ethidium bromide (0.5 µg/ml) (Thermo Scientific Inc., USA) dissolved in 1x Sodium Borate (SB) buffer (10 mM NaOH + 40 mM Boric acid, pH 8.5). The gels were visualized and photographed using InGenius LHR Gel Documentation System (Syngene, UK), and the size of PCR products was determined by comparing them with 100 bp DNA Ladder (Genedirex, Taiwan) **(Figs. 1 and 2)**. The amplified products were purified using the Promega Wizard SV Gel & PCR cleanup system (Promega, USA) according to the manufacturer's protocol.

The purified PCR products were sequenced in both directions at Macrogen Inc. (Macrogen Inc., South Korea). The same PCR primers have been used for sequencing. After sequencing, the low-quality ends of the forward and reverse trace files were trimmed and the solid sequences without stop codons or gaps were assembled using the CodonCode Aligner v9.0.2 (CodonCode Co., USA). The final nucleotide sequences have then been deposited in GenBank and the provided accession numbers are mentioned in **(Supplementary Table S1)**.

Table 2. The primers used for PCR and sequencing

Loci	Primer	Sequence (5'->3')	Average expected product length	Reference
matK	matK-390F	CGATCTATTCATTCAATATTTC	936 bp	[24]
	matK-1326R	TCTAGCACACGAAAGTCGAAGT		[24]
rbcL	rbcL-1F	ATGTCACCACAAACAGAAAC	743 bp	[25]
	rbcL-724R	TCGCATGTACCTGCAGTAGC		[25]

2.3. DNA barcoding analysis

2.3.1. Plant identification

To evaluate DNA barcoding efficiency in identifying the examined specimens at the family, genus, and species levels, each plant barcode sequence (matK and rbcL) underwent a test using the Basic Local Alignment Tool (BLAST) against the GenBank database. The top-5 alignment hits with the lowest E-values and highest bit-scores were selected for the subsequent phylogenetic analysis **(Supplementary Tables S2 and S3)**. The plant sequence with mismatched alignment (i.e., failed to be identified as a correct genus or species) was omitted from the phylogenetic analysis.

2.3.2. Estimation of the best-fitting model for nucleotide substitution patterns description

The phylogenetic analysis between analyzed species was estimated in MEGA11 software using the Maximum Likelihood (ML) method [26]. Sequences were aligned using the MUSCLE (Multiple Sequence Comparison by Log Expectation) algorithm implemented in MEGA11 and analyzed to find the best ML model to describe the nucleotide substitution patterns. ML fits of 24 different nucleotide substitution models were tested **(Figs. 3, 4, and 5)** and the model with the lowest Bayesian Information Criterion scores (BIC) is considered the best to describe the substitution pattern [27].

2.3.3. Phylogenetic analysis by Maximum Likelihood (ML) method

Using the best-fitting ML model, the phylogenetic relationship was inferred in MEGA11, and the phylogenetic trees were drawn for the two analyzed DNA barcodes (matK and rbcL) and the combined matK+rbcL sequences [26]. The best bootstrap (BS) consensus tree inferred from 500 replicates represents the phylogenetic relationship of the taxa analyzed. The numbers displayed on the branches of the tree represent bootstrap values (BS value), which indicate the level of support for the tree's structure at each point of divergence. Higher bootstrap values indicate stronger support for the tree's topology [28]. To generate the initial tree, the Neighbor-Join (NJ) and BioNJ algorithms were applied to a matrix of pairwise distances calculated using the Tamura 3-parameter model. The tree with the highest log likelihood value was selected. In these phylogenetic trees, species that are close neighbors are considered to be closely related. The trees were then analyzed to determine if the species formed monophyletic groups. Monophyletic species were considered to be correctly identified, while non-monophyletic species were considered to be incorrectly identified. Non-uniformity of evolutionary rates among sites was modeled using a discrete Gamma distribution (G) with 5 rate categories. All nucleotide positions in the alignments with less than 95% site coverage were eliminated; thus, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). This analysis involved 48, 57, and 105 nucleotide sequences with a total of 425, 489, and 481 positions for matK, rbcL, and the combined matK+rbcL sequences, respectively, in the final datasets **(Figs. 6, 7, and 8)**.

2.4. Evolutionary divergence analysis

Each analyzed sequence was grouped with its top-5 matched hits, and the genetic divergences between and within groups, based on matK, rbcL, and the combined matK+rbcL nucleotide sequences, were calculated in MEGA11. The analysis was performed using the Tamura 3-parameter model [26], and the variation in rates among sites was modeled using a gamma distribution. The shape parameter for the gamma distribution was 0.95 for matK, 1.58 for rbcL, and 2.72 for the combined matK+rbcL sequences. The study also took into account the differences in composition bias among the sequences during evolutionary comparisons. Alignment gaps, missing data, and ambiguous bases were treated by the partial deletion option, as mentioned above. The codon positions analyzed in this study included the first, second, and third positions of the coding sequences, as well as noncoding regions. The number of base substitutions per site from averaging over all sequence pairs between and within groups is shown in **(Supplementary Tables S4, S5, and S6)** for matK, rbcL, and the combined matK+rbcL sequences, respectively.

Also, DNA barcoding gap, which represents the maximum intraspecific distance (distances within groups) compared to the minimum distance to the nearest neighbor (distances between groups), was calculated for all analyzed groups to assess the extent of overlap between the lowest interspecific and highest intraspecific genetic distances. The calculated barcoding gaps were compared with the previous phylogenetic trees to emphasize the delimitation and correct identification of species.

3. Results

3.1. Species identification

The PCR amplification of matK and rbcL barcodes for the ten plant species was successful, resulting in sharp single bands suitable for accurate DNA sequencing. The amplified sequence lengths ranged between 900-924 bp for the matK barcode and 705-726 bp for the rbcL barcode **(Figs. 1 & 2)**. The assembled and aligned sequence lengths ranged between 297-774 bp for matK and 489-540 bp for rbcL. After BLAST analysis, the successful

alignment rate of rbcL to assign the tested plants to their correct genus and species recorded in GenBank were 98% and 54%, respectively for rbcL, and 74% and 30%, respectively, for matK.

BLAST analysis for the matK sequence of *Tetraena alba* (Zygophyllaceae) showed mismatched assign with the top-5 hits to individuals belonging to Chenopodiaceae. In the same way, *Tetraena coccinea* (Zygophyllaceae) was assigned incorrectly to individuals belonging to Acanthaceae. The average similarity percentage of the matK barcode between the tested plant species and the others retrieved from GenBank was more than 98%, and more than 96% in the rbcL barcode for almost all species. The average bit score of the matK barcode for each plant species ranged between 510-1430, and that of the rbcL barcode ranged between 760-998. The highest average bit score of the matK BLAST analysis was for *Suaeda monoica* (=1416) with a 99.74% average similarity percentage and the lowest one for *Tetraena coccinea* (=512) with an average similarity of 88.21%. For the rbcL BLAST analysis, the highest average bit score (=998) was for *Tamarix aphylla*, *Limonium axillare*, and *Tetraena alba* with a 100% average similarity percentage, while the lowest value (=760) was for *Rhizophora mucronata* with an average similarity of 94.68% **(Supplementary Tables S2 and S3).**

Up to the date of November 1, 2023, the most striking result to emerge from the data is the first-time submission of two partial coding sequences of the matK gene from *Atriplex farinosa* and *Tetraena alba* into the GenBank database, with accession numbers OM164029 and OM16403, respectively. This finding is remarkable because it adds new genetic information for these particular plant species, which was previously unavailable in the public database.

Fig. 1. Agarose gel electrophoresis for amplified samples by using the primer matK for the samples (1) *Atriplex farinosa*, (2) *Avicennia marina*, (3) *Limonium axillare*, (4) *Nitraria retusa*, (5) *Rhizophora mucronata*, (6) *Salicornia fruticosa*, (7) *Suaeda monoica*, (8) *Tamarix aphylla*, (9) *Tetraena alba* and (10) *Tetraena coccinea*. (**M**) Genedirex 100 bp DNA Ladder.

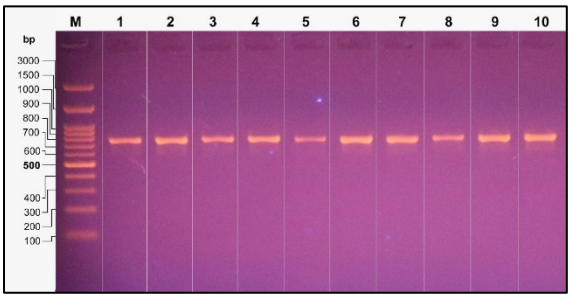
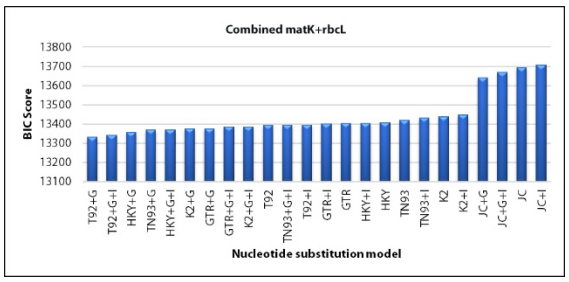


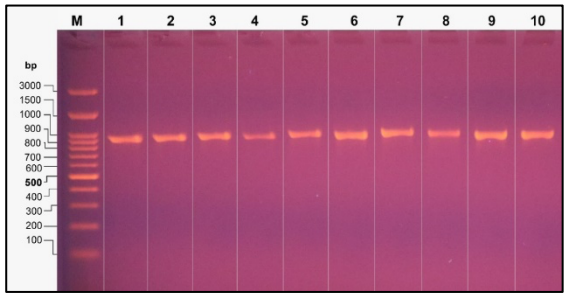
Fig. 2. Agarose gel electrophoresis for amplified samples by using the primer rbcL for the samples (1) *Atriplex farinosa*, (2) *Avicennia marina*, (3) *Limonium axillare*, (4) *Nitraria retusa*, (5) *Rhizophora mucronata*, (6) *Salicornia fruticosa*, (7) *Suaeda monoica*, (8) *Tamarix aphylla*, (9) *Tetraena alba* and (10) *Tetraena coccinea*. (**M**) Genedirex 100 bp DNA Ladder.

3.2. Phylogenetic relationship

The best-fitting ML model for matK, rbcL, and the combined matK+rbcL sequences, as indicated by the BIC score, was the Tamura 3-parameter (T92+G) model which is used as a monophyly-based species delimitation method **(Figs. 3, 4, and 5)**.



Phylogenetic trees obtained for all analyzed sequences (matK, rbcL, and the combined tree) exhibited almost a similar topology, except for the matK tree in which the mismatched *Tetraena alba* and *Tetraena coccinea* groups were omitted. The ML tree-building method with (T92+G) model showed



nine clustered monophyletic groups (*Atriplex* group, *Avicennia* group, *Limonium* group, *Nitraria* group, *Rhizophora* group, *Salicornia* group, *Suaeda* group, *Tamarix* group, and *Tetraena* group) that were

notably well-separated from one another within the tree **(Figs. 6, 7, and 8)**.

However, in the rbcL tree and the combined tree, *Tetraena alba* and *Tetraena coccinea* individuals did not show a clear separation into two separate groups, but their individuals overlapped in one cluster at a genus level. Similarly, the two GenBank-retrieved species (JQ218439.1 *Avicennia marina* and MH878940.1 *Rhizophora x annamalayana*) failed to be grouped with their correct groups. Comparing the three trees, regardless of the previous disappointing resolution, it can be seen that the other species were successfully identified because their individuals were clustered into a monophyletic group, separating them from their closest species in the tree. This phylogenetic analysis supported the monophyly of the clustered clades (BS value >95).

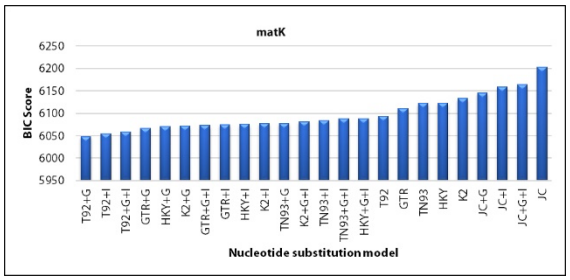


Fig. 3. Maximum Likelihood fits of 24 different nucleotide substitution models for matK sequences according to BIC scoring.

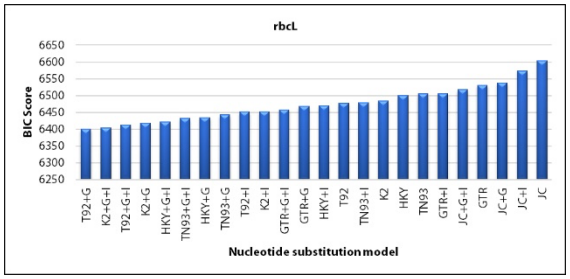


Fig. 4. Maximum Likelihood fits of 24 different nucleotide substitution models for rbcL sequences according to BIC scoring.

Fig. 5. Maximum Likelihood fits of 24 different nucleotide substitution models for the combined matK+rbcL sequences according to BIC scoring.

3.3. Estimation of sequence divergence

The average pairwise distances between matK, rbcL, and the combined (matK+rbcL) nucleotide sequences within groups were estimated at 0.0120, 0.0990, and 0.5380, respectively. On the other hand, the average distance between different groups was 0.2800, 0.1830, and 0.6580 for matK, rbcL, and the combined matK+rbcL nucleotide sequences, respectively **(Supplementary Tables S4, S5, and S6)**. For matK nucleotide sequences, the lowest distance between the groups was 0.1002 (found between the *Suaeda* group and *Salicornia* group), which was nearly four times higher than the highest distance within groups (0.0235) estimated for the *Salicornia* group, which indicates the presence of a barcoding gap. Despite that, the average genetic distances between species (interspecific) and within species (intraspecific) overlapped in the case of the rbcL and the combined matK+rbcL nucleotide sequences.

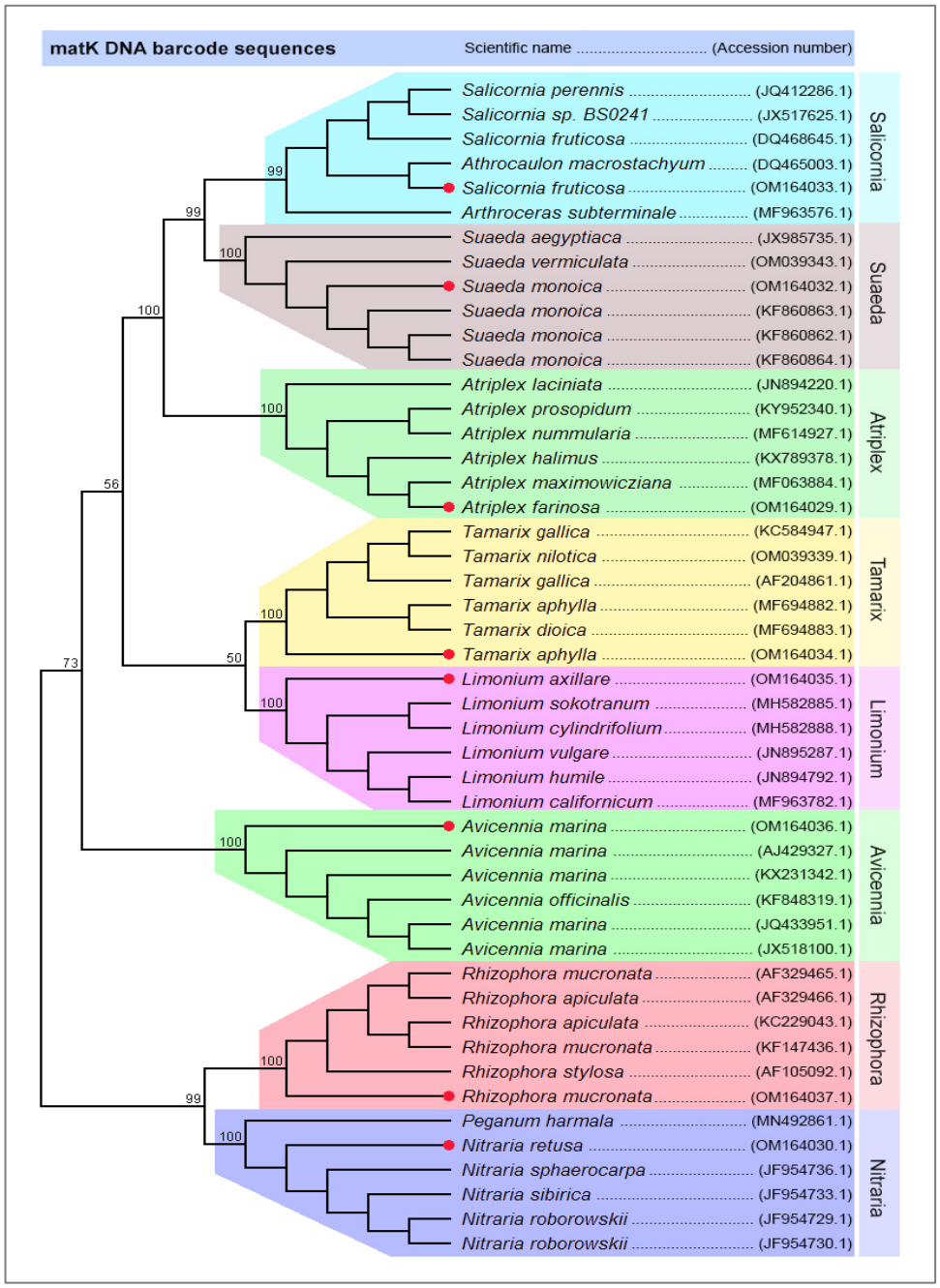


Fig. 6. Phylogenetic tree obtained from ML analysis based on matK DNA barcode sequences. Bootstrap values (BS) are indicated on the nodes, and group names are displayed on the right side of the tree. The red dots represent the taxa being examined in the current study, and they were aligned against the top-5 sequences retrieved from GenBank. The accession numbers are written next to the scientific names.

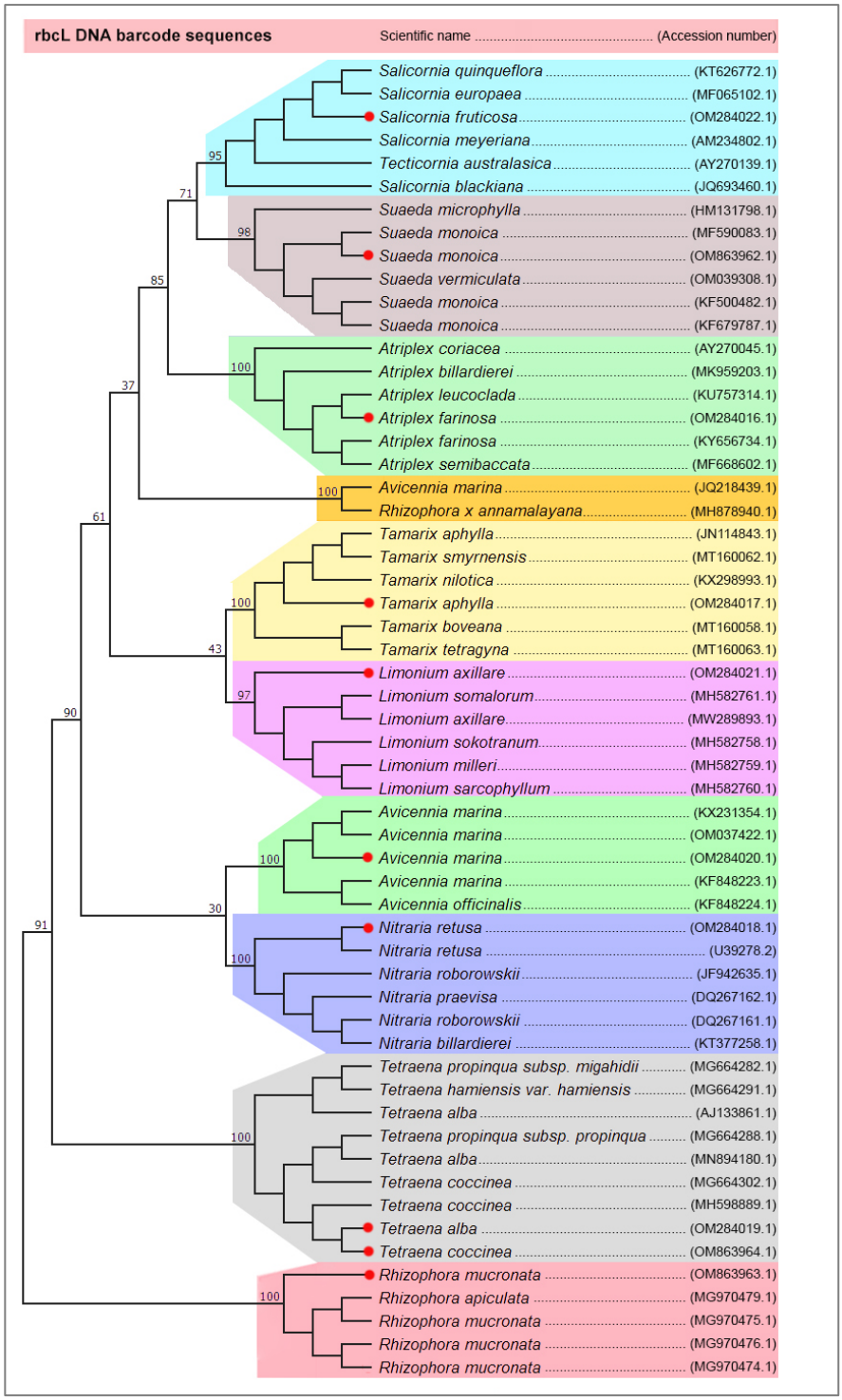


Fig. 7. Phylogenetic tree obtained from ML analysis based on rbcL DNA barcode sequences. Bootstrap values (BS) are indicated on the nodes, and group names are displayed on the right side of the tree. The red dots represent the taxa being examined in the current study, and they were aligned against the top-5 sequences retrieved from GenBank. The accession numbers are written next to the scientific names.

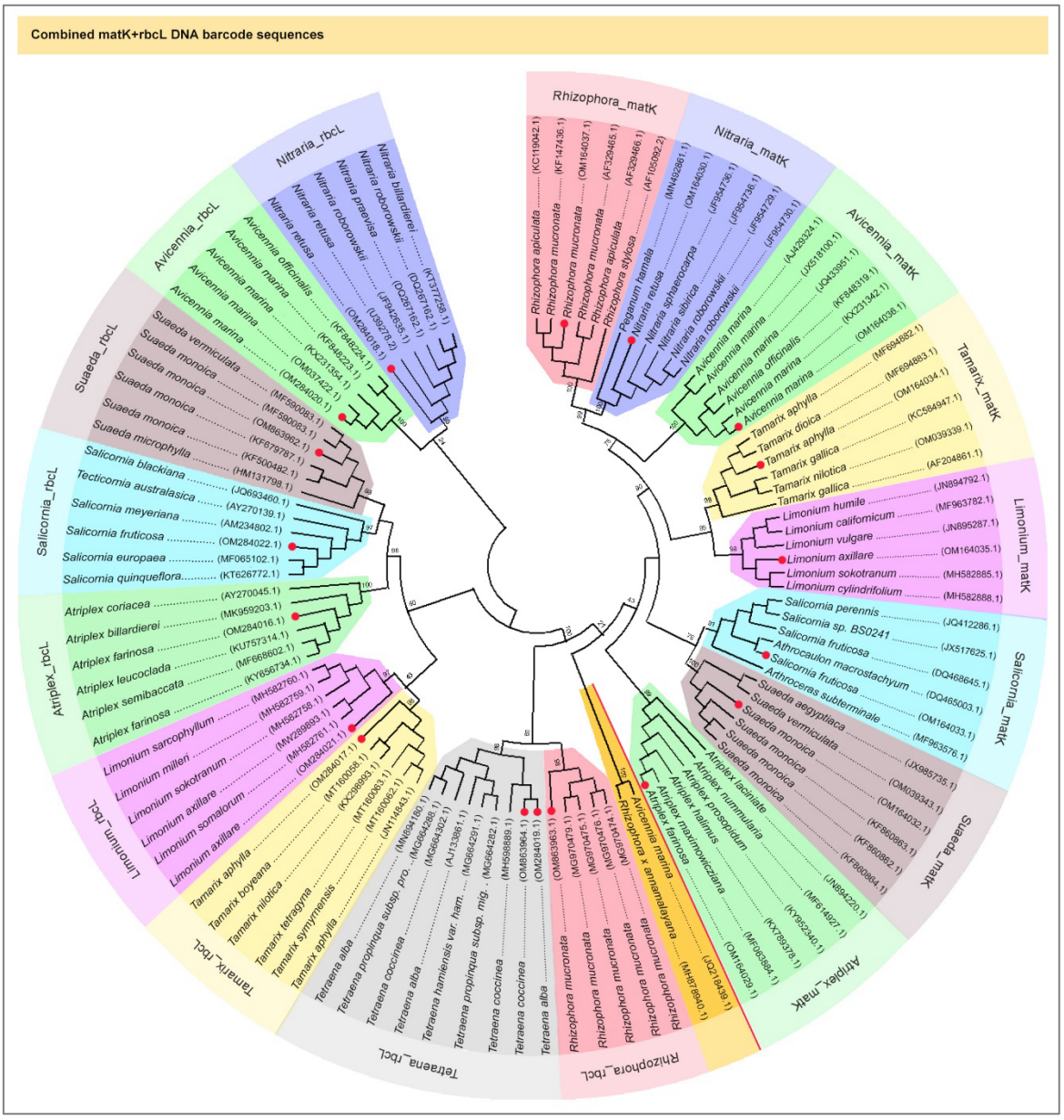


Fig. 8. Phylogenetic tree obtained from ML analysis based on combined matK+rbcL DNA barcode sequences. Bootstrap values (BS) are indicated on the nodes, and group names are displayed on the tree's outer layer. The red dots represent the taxa being examined in the current study, and they were aligned against the top-5 sequences retrieved from GenBank. The accession numbers are written next to the scientific names.

4. Discussion

A desirable DNA barcode should possess universal applicability, reliability, cost-effectiveness, and high discriminatory power. The commonly used DNA regions for plant DNA barcoding are the two plastid genes of rbcL and matK, as recommended by the CBOL Plant Working Group [22]. To assess the barcoding resolution in the present study, the sequence similarity method (BLAST) was applied, and the phylogenetic-based method showed that the rbcL barcode has a high universality among the different tested taxa, whereas matK offers slightly low universality. In this context, it has been found that using the previously proposed barcode of rbcL alone may be difficult to differentiate between the closely related species (*Tetraena alba* and *Tetraena coccinea*). These findings agree with the previous studies showing that the rbcL gene was highly conserved across a wide range of plant taxa, making it a useful marker for phylogenetic analyses at higher taxonomic levels (Family or genus level). However, its low variability limits its utility for resolving relationships among closely related taxa. In contrast, matK has been shown to have higher variability than rbcL, making it a useful marker for resolving relationships among closely related taxa. Nevertheless, the effectiveness of using it as a universal plant barcode is restricted due to its limited success in amplification within certain plant groups [29, 30]. Using multiple DNA regions in combination with rbcL can increase the accuracy of plant species identification in several ways. Firstly, different DNA regions evolve at different rates, and some may be more informative than others for certain plant groups. For example, the trnH-psbA intergenic spacer region is highly variable in some plant groups and can provide additional resolution when combined with rbcL [31]. Secondly, using multiple DNA regions can reduce the impact of homoplasy, where different species have the same or similar DNA sequence, by providing additional genetic data that can distinguish between closely related species. Finally, using multiple DNA regions can also increase confidence in species identification by providing concordant results across different markers. Therefore, combining rbcL and other DNA regions can provide a more comprehensive and accurate approach to plant species identification, which is particularly important for biodiversity assessment and conservation applications. Li et al. [29] suggested that the incorporation of the Internal Transcribed Spacer (ITS) as a complementary marker to the previously recommended core plant barcodes (matK + rbcL) for seed plants increases their universality and discriminatory power of them. However, its lower amplification success and sequencing difficulties remain a drawback, particularly in gymnosperms. This finding is in line with previous studies indicating that this nrDNA region evolves rapidly, leading to genetic variations that can differentiate closely related species [32, 33]. This finding is consistent with that of Li et al. [34], who found that the Internal Transcribed Spacer (ITS) performed the highest rate of species discrimination of some flowering plants (>95%) among the three markers (matK, rbcL, and ITS) with only a slight improvement in species resolution when combining the three DNA barcodes.

5. Conclusion

Our research has demonstrated that plant DNA barcodes can be used to quickly differentiate between species, which is helpful in biodiversity assessment. The combination of matK and rbcL used as a DNA barcode for plant species identification has been shown to work well for a wide range of plant taxa. However, it has some limitations, particularly when distinguishing between closely related species. One reason is that the variation in the matK and rbcL regions may not be sufficient to differentiate between very similar species. Additionally, some plant groups may have experienced recent or ongoing hybridization or incomplete lineage sorting, which can result in shared or incomplete variation in the DNA barcode regions. To overcome these limitations, we suggest using additional DNA loci or developing other molecular markers to more accurately distinguish between closely related species. Our deposition of new barcoding sequences to the GenBank is a step toward expanding the availability of authentic plant DNA barcodes in public databases. The significance of this addition lies in the potential for these sequences to serve as valuable tools for future studies involving these species, particularly in the areas of taxonomic classification and phylogenetic analysis.

Conflicts of Interest
There are no conflicts to declare.

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