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DEOXYRIBONUCLEIC ACID BARCODING OF *MORINDA CITRIFOLIA* (RUBIACEAE) USING RIBULOSE BISPHOSPHATE CARBOXYLASE GENE MARKER

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ABSTRACT

Aim: The current research aims to establish the DNA barcode of *M. citrifolia* using rbcL gene sequences, contributing to the development of a molecular database for medicinal plants.

Methods: The procedure involved genomic DNA extraction from *M. citrifolia* leaves using plant genomic DNA extraction kit from Bioneer. Molecular identification was performed using ribulose-1,5-biphosphate carboxylase large subunit (rbcL) gene polymerase chain reaction (PCR) amplification, gel electrophoresis, then Sangersequencing. The obtained sequence was compared in National Centre for Biotechnology Information-Basic Alignment Search Tool (NCBI-BLAST) with similar sequences and the novel sequence was deposited in the Gene bank. Phylogenetic analysis on the rbcL region was established using NCBI blastn 2.16.0+ online database with the neighbor-joining (NJ) procedure.

Results: The PCR revealed a single band on the gel electrophoresis with an approximate size of the rbcL-PCR amplicon, 630bp in comparison with the 1000bp plus DNA ladder, successful amplification and sequencing of a 526-base pair DNA fragment, which was subsequently aligned and analyzed through BLAST, revealing a high sequence similarity (94–95%) to other *Morinda* species in the GenBank database. Phylogenetic analysis confirmed the close relationship of *M. citrifolia* with related taxa where an accession number of OR730434 was retrieved from the GenBank.

Conclusion: This study highlights the importance of DNA barcoding using the *rbcL* gene for accurate identification and authentication of *Morinda citrifolia*. High sequence similarity with related species and phylogenetic analysis confirm its utility for species identification and evolutionary studies. The sequence, deposited in GenBank, contributes to the molecular database, supporting future research and conservation efforts in herbal medicine.

Keywords: DNA barcoding, *Morinda citrifolia*, rbcL gene, medicinal plants, species authentication

INTRODUCTION

Traditional medicine has relied heavily on plants for centuries, with numerous species being recognized for their therapeutic

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properties. One such plant is *Morinda citrifolia* L. (commonly known as Noni), belonging to the Rubiaceae family, which has gained attention due to its wide-ranging medicinal applications. *M. citrifolia* has been traditionally used across different cultures to treat a variety of ailments, including arthritis, infections, hypertension, and diabetes [1]. The popularity of this plant in traditional medicine underscores the need for accurate species identification, as morphological similarities with other species in the *Morinda* genus can lead to frequent misidentification [2]. This misidentification poses significant risks for consumers, who may unwittingly consume adulterated or substituted products that could lead to adverse effects or reduced therapeutic efficacy [3].

Morphological identification, the conventional method for classifying plants, relies on observable traits such as leaf shape, flower structure, and overall plant size. However, these characteristics can vary within a species due to environmental factors, age, or genetic variability, making it difficult to accurately distinguish between closely related species [4]. Additionally, the reliance on expert taxonomists for accurate identification can introduce subjectivity, as such methods require extensive training and experience. This variability highlights a critical limitation in traditional taxonomic approaches, especially when applied to medicinal plants where precise identification is paramount for safety and efficacy [5].

In recent years, DNA barcoding has emerged as a reliable molecular tool for species identification, offering a standardized approach to overcome the limitations of traditional taxonomy. DNA barcoding uses short, standardized genetic sequences that are unique to each species, effectively providing a "barcode" that allows for rapid and accurate species identification [5]. This technique has become especially relevant in the field of ethnomedicine and phytotherapy, where the misidentification of medicinal plants can lead to the use of incorrect species, potentially compromising patient safety and treatment outcomes [3]. The concept of DNA barcoding was pioneered by Paul Hebert and his team, who demonstrated its utility in differentiating species using mitochondrial DNA in animals. Since then, the approach has been adapted for plants, with particular markers proving effective in plant species discrimination [3].

Among the various genetic markers used in plant DNA barcoding, the ribulose bisphosphate carboxylase large subunit (rbcL) gene, located within the chloroplast genome, has gained prominence. The rbcL gene is known for its high amplification success rate, universality across plant taxa, and ability to resolve phylogenetic relationships within diverse plant groups [6]. As an essential component of the photosynthetic enzyme RUBISCO, rbcL encodes a large subunit that is well-conserved across species, making it an ideal candidate for DNA barcoding in plants [4]. Studies have shown that rbcL is particularly useful for differentiating plant species within the same genus, allowing researchers to reliably identify closely related species

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that are often indistinguishable through morphological characteristics alone [2; 3]. Furthermore, DNA barcoding offers a valuable tool for regulatory authorities and practitioners in traditional medicine, enabling the authentication of medicinal plant materials to ensure quality control and safety. Establishing a molecular reference for *M. citrifolia* will support the sustainable use of herbal resources in Nigeria and help maintain consumer trust in traditional medicine practices. As DNA barcoding continues to gain traction in plant taxonomy, its application in the authentication of medicinal plants like *M. citrifolia* could greatly enhance the safety and efficacy of phytotherapeutic products [4; 6].

Fig.1: ***M. citrifolia*** **from the Pharmacognosy Garden in the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria.**

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

Fresh leaves of *Morinda citrifolia* were collected from the Pharmacognosy Garden, Ahmadu Bello University (ABU) Zaria. The plant material was verified and authenticated by the Department of Botany, ABU Zaria (Voucher No: *Morinda citrifolia ABU01862*).

2.1.2 Extraction Kit

The DNA was extracted using the Accuprep Plant Genomic DNA Extraction Kit (Bioneer), which is a commercial kit specifically designed for efficient DNA isolation from plant tissues.

2.1.3 Polymerase Chain Reaction

The nucleotide sequences of rbcL primer below were used for the PCR amplification:
Forward:
5’ATGTCACCACAAACAGAGACTAAAGC-3’
Reverse: 5’-GTAAAATCAAGTCCACCRCG-3’
These primers were designed to amplify a portion of the rbcL gene, commonly used for plant DNA barcoding. Accu Power Hot Start PCR Premix (Bioneer), which contains thermostable DNA polymerase, dNTPs, and $MgCl_2$.

2.2 Methodology

2.2.1 DNA Extraction

Genomic DNA was extracted from approximately 100 mg of fresh, finely ground *M. citrifolia* leaf tissue using the Accuprep Plant Genomic DNA Extraction Kit (Bioneer). The extraction protocol was based on standard procedures outlined by the kit manufacturer with modifications to improve yield and purity. The leaf tissue

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was homogenized in the provided Plant Tissue Lysis Buffer (PL buffer) in the presence of Proteinase K and RNase A to ensure complete cell lysis and the removal of RNA contaminants. The process of DNA purification included sequential centrifugation steps to remove cellular debris. The final DNA was eluted in a 50 µL volume of elution buffer as per the manufacturer's instructions. Isolated plant genomic DNA was stored at −20ºC for further use [3].

2.2.2 Polymerase Chain Reaction Amplification.

The PCR amplification was carried out in a total reaction volume of 20 µL. The reaction mixture consisted of:16 µL of nuclease-free water, 1 µL of each primer (forward and reverse), and 2 µL of DNA template (extracted genomic DNA)
The following optimized PCR conditions were applied to amplify the rbcL gene region:

Table 1: PCR Conditions for Amplification of rbcL Primer

Steps	Temp	Time
Initial Denaturation	94°C	5 minutes
35 Cycles of Amplification		
Denaturation:	94°C	30 seconds
Annealing:	54°C	30 seconds
Extension:	72°C	60 seconds
Final Extension	72°C	5 minutes

This amplification protocol was based on the method described [7], as the rbcL gene is known to produce reliable and reproducible amplification products for species identification.

2.2.3 Gel Electrophoresis

To confirm the successful amplification of the rbcL gene, the PCR product was analyzed by gel electrophoresis. A 1.2% agarose gel was prepared, and the PCR product was loaded along with a 100 bp DNA ladder as a size standard. The gel was stained with ethidium bromide, which fluoresces under ultraviolet (UV) light allowing for observation of distinct band representing different size of molecules. The electrophoresis was run at 110–120V for 30 minutes. The presence of a distinct band at approximately 630 bp was used to verify the success of the amplification [8].

2.2.4 DNA Sequencing and Analysis

Following gel electrophoresis, the purified PCR product was sent for Sanger sequencing. The obtained sequence was analyzed using Finch TV software. The sequence was compared with reference sequences in the NCBI BLAST database to identify the closest matches to *Morinda citrifolia* and other *Morinda* species. Phylogenetic analysis was performed using MEGA X software to construct a phylogenetic tree based on the sequences, as described by Tamura *et al*., [8]. Additional analyses were conducted to assess base composition, GC content, and Open Reading Frames (ORFs). Codon usage and translation were also analyzed using online bioinformatics tools such as BioPython[9], GoogleColab [10]and Expasy [11].

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Table 2: PCR Reaction Setup for rbcL Gene Amplification

Reagent	Quantity
Nuclease-free water	16 µL
Forward Primer (5 µM)	1 µL
Reverse Primer (5 µM)	1 µL
DNA Template	2 µL
AccuPowerHotStart PCR Premix	2 µL

2.2.5 DNA Sequencing Submission for Accession Number

The sequence data in FASTA format was prepared and logged in to the NCBI Bank it submission tool. Details about the sequence, organism, and relevant metadata was filled into the submission form and submitted. This was processed by NCBI and a unique accession number was assigned.

3.0 RESULTS

3.1 DNA EXTRACTION

Genomic DNA was successfully extracted from *M. citrifolia* leaves, yielding high purity DNA. There were no visible signs of degradation, indicating successful isolation of high-quality genomic DNA suitable for further analysis.

3.2 PCR Amplification

Electrophoresis analysis revealed a single, bright band at approximately 630 bp, consistent with the expected size for the rbcL amplicon in *M. citrifolia* [12].

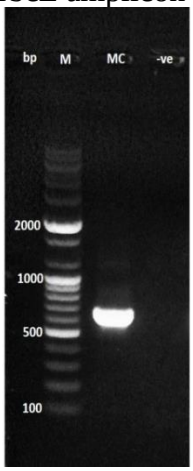

Figure 2: PCR Product Visualized on an Agarose Gel

3.3 Sequencing and BLAST Analysis

The chromatogram and nucleotide sequence obtained from the *M. citrifolia* PCR product was analyzed using Finch TV software (Fig. 3 and 4). The sequence was subjected to BLAST analysis for comparison with known sequences in the GenBank database. The BLAST results indicated high similarity to *Morindapubescens* and other species within the Rubiaceae family.

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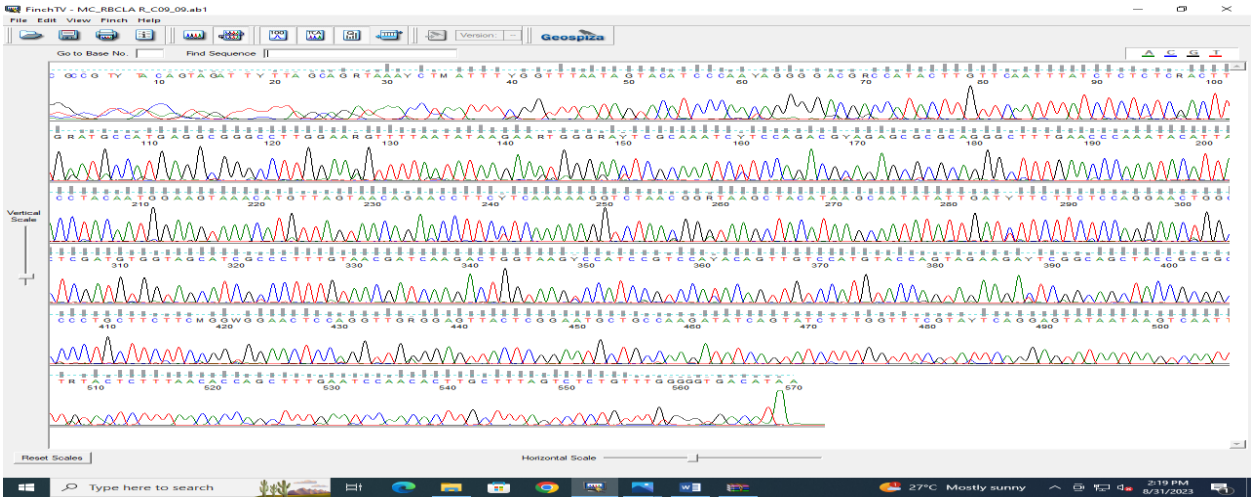

Figure 3: Chromatogram of Nucleotide Sequence Analysis from Finch TV

TTAGCAGTAAACTATTTGGTTTAATA
GTACATCCCAAAGGGGACGCCATACT
TGTTCAATTTATCTCTCTCACTTGATG
CCATGAGGCGGGCCTTGGAAGTTTTA
ATATAAGAATGGGATCGCAAATCTCC
AGACGAGAGCGCGCAGGGCTTTGAA
CCCAAATACATTACCTACAATGGAAG
TAAACATGTTAGTAACAGAACCTTCT
CAAAAAGGTCTAACGGTAAGCTACAT
AAGCAATATATTGATTTCTTCTCCAG
GAACTGGCTCGATGTGGTAGCATCGC
CCTTTGTAACGATCAAGACTGGTAAG
CCATCCGTCCAACAGTTGTCCATGTA
CCAGTAGAAGATCGGCAGCTACCGC
GGCCCCTGCTTCTTCGGGGAACTCCA
GGTTGGGAGTTACTCGGAATGCTGCC
AAGATATCAGTATCTTTGGTTTCGTAT
CAGGAGTATAATAAGTCAATTTTACT
CTTTAACACCAGCTTTGAATCCAACA
CTTGCTTTAGTCTCTGTTTGGGGGTGA
CATAA

Nuclueotide base sequence from *M. citrifolia*

3.4 Characteristics of the Sequence

The nucleotide sequence was 526 base pairs in length. The base composition analysis revealed the following base counts: A = 148, T = 149, C = 115, G = 114. The GC content of the sequence was 43.54%, consistent with typical rbcL gene sequences from related species.

3.5 Open Reading Frame (ORF) Detection

ORF detection revealed multiple sequences of interest. Below is a summary of the detected ORFs:

ORF 1: *Seq('LAVNYLV...')*
ORF 2: *Seq('QTIWFN...')*
ORF 3: *Seq('SSKLFGLIV...')*

This analysis confirmed the presence of valid ORFs, which are important for functional predictions in further comparative studies.

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Figure 4: Open Reading Frame (ORF)

3.6 Codon Usage Analysis and Translation

Codon usage was analyzed, and the following codon frequency was observed: TTT: 14, TTC: 7, TTA: 10, TAC: 10, GGT: 8, GGG: 7. The codon usage pattern reflects a typical plant gene, particularly in the rbcL gene, where variation in codon frequency can provide insights into the evolutionary relationships of plant species.

select all *10 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Morinda citrifolia chloroplast, complete genome	Morinda...	845	845	100%	0.0	95.10%	153113	NC_047302.1
Morinda citrifolia voucher Bremer 3302 (UPS...	Morinda...	845	845	100%	0.0	95.10%	127492	KY378694.1
Morinda citrifolia Chiang Mai University, Facu...	Morinda...	845	845	100%	0.0	95.10%	751	LC633824.1
Morinda pubescens isolate TMP94 ribulose ...	Morinda...	832	832	97%	0.0	95.35%	578	KF432025.1
Morinda pubescens voucher VCW 26 ribulos...	Morinda...	830	830	97%	0.0	95.18%	592	MT083904.1
Morinda officinalis chloroplast, complete gen...	Gynocht...	828	828	100%	0.0	94.56%	153398	NC_028009.1
Gynochthodes officinalis isolate QB02 ribulo...	Gynocht...	828	828	100%	0.0	94.56%	738	ON926572.1
Damnacanthus indicus chloroplast, complete...	Damnac...	828	828	100%	0.0	94.56%	153997	NC_060411.1
Gynochthodes parvifolia chloroplast, complet...	Gynocht...	828	828	100%	0.0	94.56%	153069	NC_054151.1
Gynochthodes cochinchinensis voucher SZG...	Gynocht...	828	828	100%	0.0	94.56%	153022	NC_053818.1

Figure 5: A Tabular Display of the Nucleotide BLAST results

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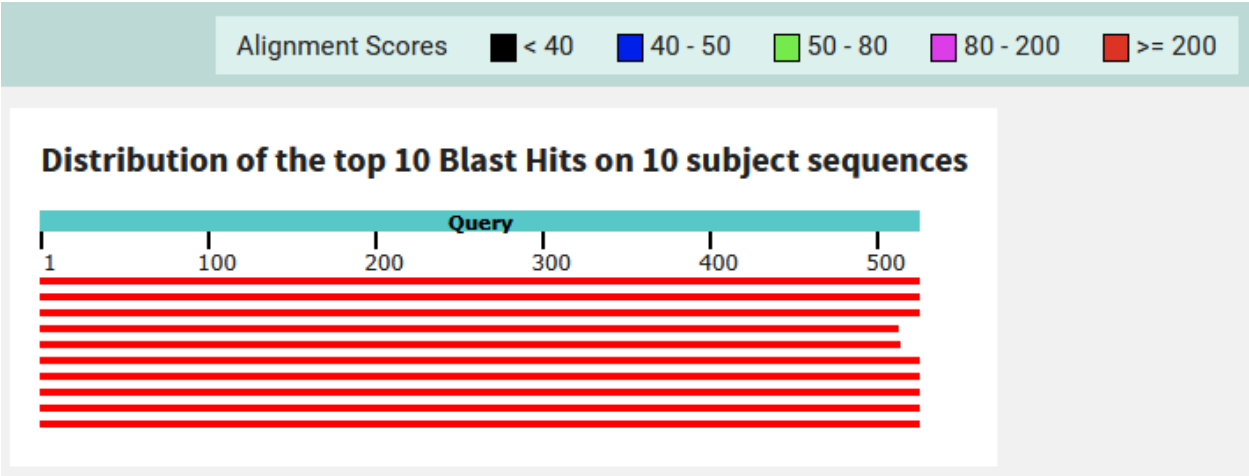

Figure 6: BLAST alignment between the query sequence and the top 10 most similar sequences in the GenBank database

3.7 Phylogenetic Analysis

The phylogenetic tree constructed from the sequence alignment showed that *M. citrifolia* clustered closely with *Morindapubescens* and other species within the Rubiaceae family. This supports the close evolutionary relationship between these species.

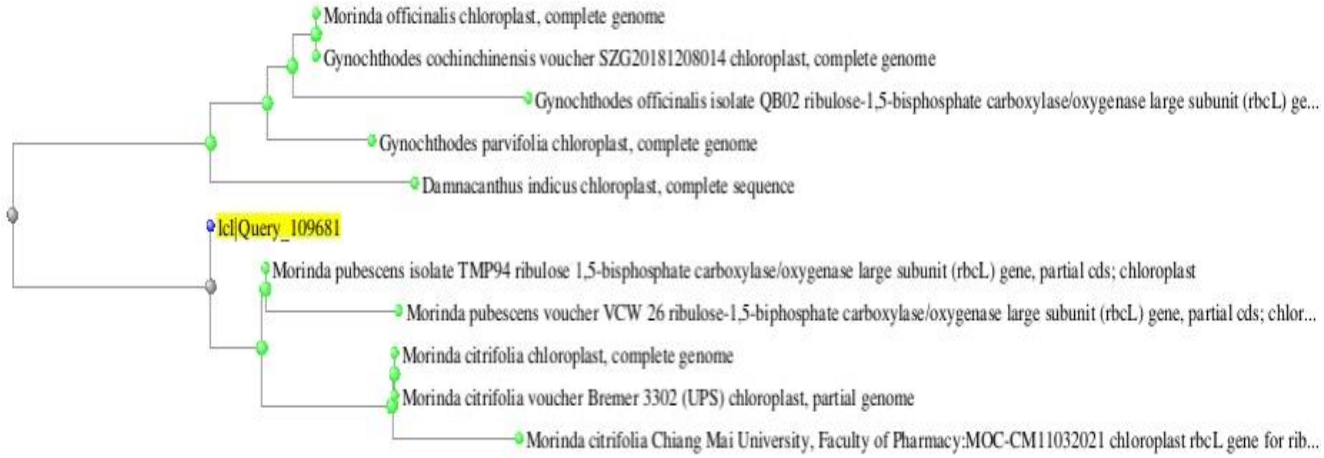

Figure 3.7: Phylogenetic tree showing the relationship between the query nucleotide sequence and sequences from the GenBank

3.8 Accession Number

The sequence was submitted to GenBank and assigned the accession number OR730434.

4.0 DISCUSSION

The quality of the genomic DNA extracted from *M. citrifolia* was critical for the successful PCR amplification of the rbcL

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gene. The extraction of high-quality genomic DNA from *Morinda citrifolia* leaves yielded DNA of sufficient purity for subsequent molecular analyses, without any visible degradation. This step is crucial in ensuring that the DNA is free from contaminants that could interfere with downstream processes such as PCR amplification and sequencing [13]. The successful amplification of a 630 bp fragment using the rbcL primers, observed as a bright band in electrophoresis, confirms that the genomic DNA was intact and the PCR reaction was efficient. This confirmed the successful amplification of a region suitable for DNA barcoding, as rbcL is widely used due to its relatively conserved nature and ability to differentiate plant species at the genus and species levels [14]. The clear amplification and clean band indicated minimal contamination and the efficient PCR process, key factors for obtaining high-quality sequence data, making it a reliable marker for species identification [14].

Following PCR amplification, the nucleotide sequence of the rbcL gene was successfully generated and analyzed. The obtained sequence, with a length of 526 base pairs, displayed high congruence with *Morinda pubescens* and other species within the Rubiaceae family [12; 15]. This is typical for the *rbcL* region used in plant DNA barcoding [16]. The BLAST analysis revealed a sequence similarity of 94-95%, an E-value of 0.0, and query coverage of 97% for *M. pubescens* and 100% for *M. citrifolia*, indicating a very high degree of match reliability and a high degree of homology with related species in the GenBank database. Such a high similarity reinforces the evolutionary closeness between *M. citrifolia* and *M. pubescens*, which share similar morphological traits, medicinal uses, and ecological niches [17].

The results of this study, particularly the nucleotide composition and GC content of the *rbcL* gene in *Morinda citrifolia*, align with findings from previous research on plant DNA barcoding [12, 18]. The GC content of 43.54% is consistent with typical *rbcL* sequences in plants, which generally range between 40-45% [12, 18]. This moderate GC content is characteristic of the *rbcL* gene, a chloroplast marker widely used for its universality and reliability in species identification and phylogenetic studies [16]. The base composition (A = 148, T = 149, C = 115, G = 114) further supports the conserved nature of *rbcL*, as similar patterns have been reported in other members of the Rubiaceae family [17, 19].

The detection of multiple open reading frames (ORFs) in the *M. citrifolia* sequence is significant for understanding the functional potential of the gene. Although the rbcL gene is primarily involved in encoding the large subunit of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) which is essential for photosynthesis [19], the presence of multiple ORFs suggests the possibility of other functional elements within the sequence that could be explored in future studies. The identification of valid ORFs suggests that the gene is functionally complete [20].

Codon usage can vary across different plant species, and this variation provides insights into the evolutionary history and adaptation

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mechanisms of plant species [21]. The codon frequencies for rbcL in *M. citrifolia* align with expected patterns seen in other plant species, suggesting that there are no unusual codon preferences in this sequence [22, 23]. These findings support the hypothesis that codon usage patterns can offer valuable evolutionary insights, particularly when comparing closely related species within a family. Codon usage analysis revealed a typical pattern for a plant species, with certain codons appearing more frequently, such as TTT, TTA, and TGC [23]. Such analysis is important as it provides insights into the evolutionary pressures acting on the *M. citrifolia* genome. The predominance of certain codons could be a result of selective pressures to optimize the efficiency of protein synthesis under specific environmental conditions [21, 22]. The codon usage pattern observed in this study is consistent with that of other Rubiaceae species, supporting the idea that plant species within this family share similar genetic signatures at the molecular level [21, 24, 25]. The codon usage analysis revealed that TTT (Phenylalanine) is the most frequent codon, occurring 14 times, followed by TTA (Leucine) and TAC (Tyrosine), each occurring 10 times**.** Additionally, GGT (Glycine) and GGG (Glycine) appeared 8 and 7 times, respectively. The dominance of TTT suggests a strong preference for Phenylalanine in the rbcL gene of *Morinda citrifolia*, which may be due to species-specific tRNA adaptation and translational efficiency [22]. The moderate presence of Leucine (TTA) and Tyrosine (TAC) further suggests their functional importance in the RuBisCO enzyme, possibly contributing to its structural stability and enzymatic activity [24, 25]. The usage of Glycine codons (GGT and GGG) may reflects the role of Glycine in maintaining protein flexibility and function [23].

The phylogenetic tree constructed from the sequence alignment confirmed that *M. citrifolia* clusters closely with *M. pubescens* and other species within the Rubiaceae family, corroborating the results of the BLAST analysis [26]. This clustering is consistent with previous studies that have used molecular markers to confirm the phylogenetic relationships within the Rubiaceae family [17]. This phylogenetic relationship is also consistent with previous studies that have placed these species within the same taxonomic group due to shared morphological traits, such as leaf structure and flower morphology [17]. The tree further validates the application of DNA barcoding as an effective tool for elucidating the evolutionary history of medicinal plants. The close clustering of these species indicates a relatively recent common ancestor, highlighting the utility of molecular markers like rbcL in resolving phylogenetic relationships at various taxonomic levels [20].

The sequence obtained in this study was submitted to GenBank and assigned the accession number OR730434, ensuring that it can be accessed and used for future comparative analyses. Depositing sequences in public databases like GenBank contributes to the global effort of cataloging plant genetic resources, supporting biodiversity conservation and facilitating future research.

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While the rbcL gene has proven useful for species-level identification, it may not always provide sufficient resolution in cases of closely related species. The use of a single genetic marker can sometimes lead to misidentifications, particularly when species exhibit very similar genetic sequences [27]. Therefore, to improve the accuracy of plant identification, a multi-locus approach that includes other markers, such as matK or ITS, is recommended. This strategy has shown to increase the resolution of DNA barcoding in plant species that are difficult to differentiate based on a single gene [28]. Additionally, future studies could benefit from examining a wider range of accessions from different geographical regions to capture the genetic diversity within *M. citrifolia* and its close relatives.

The use of DNA barcoding for the authentication of *M. citrifolia* highlights its potential role in ensuring the integrity of herbal products, particularly in light of the growing demand for natural health products worldwide [29]. As adulteration of medicinal plants becomes a pressing issue in the herbal medicine industry, DNA barcoding can provide a quick and accurate method for species identification, ensuring that consumers receive the correct products [15]. Additionally, understanding the genetic diversity of medicinal plants is essential for conservation efforts. The results of this study contribute to a better understanding of the evolutionary relationships within the Rubiaceae family, which can inform conservation strategies, particularly for species that are at risk due to overharvesting or habitat loss [30].

5.0 CONCLUSION AND RECOMMENDATION

This study successfully demonstrated the application of the rbcL gene for DNA barcoding and phylogenetic analysis of *Morinda citrifolia*. The molecular identification of this species provides a foundation for future studies on the authentication and conservation of medicinal plants. Given the close genetic relationship between *M. citrifolia* and *M. pubescens*, further studies that incorporate additional genetic markers and broader geographical sampling are necessary to enhance the resolution of species identification and gain a more comprehensive understanding of the genetic diversity within these plants. Moreover, integrating DNA barcoding with other molecular techniques could yield deeper insights into the pharmacological potential of *M. citrifolia* and other closely related species.

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Competing Interests

The authors declare that they have no competing interest related to this research.This study was conducted solely for academic and scientific purposes,with no

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commercial or financial influence from external entities.All findings and conclusion are presented transparently without bias or conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them

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