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DNA BARCODING OF *Adansonia digitata* USING MULTI-LOCI GENE REGIONS (ITS, *rbcL*, *rpoC1* and *psbA-trnH*)

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ABSTRACT:

Adansonia digitata, also known as Baobab, is a tree species endemic to Africa. It belongs to the family Malvaceae. The species holds immense economic, cultural, and scientific value worldwide. As a result, it has been introduced to other parts of the world such as India, Sri Lanka, and Australia. Despite its immense value, information on its DNA barcodes for effective identification and conservation efforts of the species is inadequate in the literature. This study aimed to molecularly characterize *A. digitata* found in Nigerian flora using DNA barcodes from ITS, *rbcL*, *rpoC1,* and *psbA-trnH* primers. DNA was isolated from young leaves, and Sanger sequencing reactions were subsequently performed. Sequences obtained from each primer were subjected to Basic Local Alignment Search Tool (BLAST) analyses conducted on the National Center for Biotechnology Information (NCBI) website. A high percentage similarity range of 98-100% was recorded. Phylogeny was inferred using the Maximum likelihood method with a bootstrap test of 1000 replications. Results revealed a successful species-level identification of *A. digitata* by *rbcL*, ITS, and *PsbA-trnH* primers, as the consensus clustered with identical species with 39%, 88%, and 57% bootstrap support values, respectively. The DNA barcode of *A. digitata* obtained from the *rpoC1* primer submitted to the NCBI nucleotide database with accession number OR251003.1 is the first to be submitted to the database. The accession numbers for the *rbcL*, ITS, and *PsbA-trnH* primers are OQ694034, OP709538, and OR135362 respectively. This study provides DNA barcodes for the identification of *A. digitata* relevant for research, economic, and conservation endeavours.

KEYWORDS: Adansonia Digitata: BLAST: DNA Barcoding: Phylogeny: PSBA-TRNH: RBCL: ITS: NCBI: Maximum Likelihood: Sanger Sequencing.

1. INTRODUCTION

Adansonia digitata L. belongs to the Malvaceae family and is commonly known as the Baobab tree, native to Africa. The African baobab is a very long-lived tree with multipurpose uses. It is said that some species are over 1000 years old (Rahul *et al.,* 2015). *A. digitata* L. is iconic as it is emblematic, culturally significant, and essential in traditional medicine in Africa and India (Bellary *et al*., 2021). Several studies in different African countries, such as Benin, Burkina Faso, Malawi, Mali, Nigeria, Tanzania, and South Africa, have highlighted this deciduous stem-succulent taxon as a priority species for domestication and enhanced utilization (Gebauer *et al*., 2016).

The fruit of *A. digitata* is also used daily in the diet of rural communities in West Africa (Ibrahima *et al.,* 2013). In the Northern part of Nigeria, the leaves are relished for a local soup called "miyan kuka". The species contributes to rural incomes (Kamatou *et al.,* 2011) and has various important medicinal and food uses (Kaboré, 2011). Traditionally, the pulp is consumed in different forms. It is also used in the formulation and preparation of cereals and beverages. It is reported that baobab pulps have many nutrients, including Vitamin C, riboflavin, niacin, pectin and citric, malic, and succinic acids, while the oil also contains Vitamins A, D, and E (Donkor *et al.,* 2014). According to Silva *et al*. (2023), ethnopharmacological uses of various plant parts of *A. digitata* have been reported for hydration, antipyretic, antiparasitic, antitussive, and sudorific properties and in the treatment of diarrhoea and dysentery in many African countries. *A. digitata* has been reported to exert hypoglycemic, hypolipidemic, antimicrobial, analgesic, anti-inflammatory, antioxidant and antipyretic properties (Braca *et al*., 2018). Biochemical studies also showed that the pulp of *A. digitata* is rich in dietary fibres and carbohydrates (Eltahir & Elsayed, 2019).

Despite its wide distribution and importance, the taxonomy and conservation of *A. digitata* remain challenging due to high morphological variability, broad ecological adaptability, and potential hybridization with other *Adansonia* species (Jansen *et al*., 2020 ; Muthai *et al*., 2017;). Traditional morphological approaches often fall short in accurately distinguishing *A. digitata* from its congeners, especially in regions where multiple species may coexist or where phenotypic traits are influenced by

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environmental conditions. This taxonomic uncertainty hinders conservation planning and the sustainable management of genetic resources.

Molecular identification methods, particularly DNA barcoding, offer a robust alternative for resolving species boundaries and understanding genetic diversity within *A. digitata*. DNA barcoding is a species identification tool that utilizes a short segment of the genome, which provides a genetic signature or fingerprint of the species (Abdulkareem *et al*., 2024). The main advantage of this novel technique is that it requires a small sample of tissue (Trujillo-Argueta *et al*., 2022). Single-locus barcoding approaches, however, have shown limited resolution in species with complex evolutionary histories or low interspecific divergence (Karabanov *et al*., 2023). To overcome these limitations, a multi-loci DNA barcoding strategy is increasingly recommended. gene regions such as nuclear ITS and chloroplast markers *rbcL*, *rpoC1*, and *psbA-trnH* could be adopted to assess their utility in accurately identifying tax*a*. Each of these loci offers different levels of variability and phylogenetic resolution: while *rbcL* and *rpoC1* are highly conserved and functional for higher-level taxa, ITS and *psbA-trnH* provide greater discriminatory power at the species level due to their higher mutation rates and sequence variability (Letsiou *et al*., 2024).

The ITS region, located in the nuclear genome, evolves quickly and is highly variable, making it effective for distinguishing between closely related species. However, it can sometimes be difficult to amplify and may show variation within a single species. The chloroplast gene *rbcL* is widely used in plant barcoding due to its ease of amplification and alignment (Abdulkareem *et al*., 2024), though it is relatively conserved and may not separate closely related species well. The *rpoC1* region, another coding gene in the chloroplast genome, offers moderate variability and is also considered reliable for general plant identification (Abdulkareem *et al*., 2023). The *psbA-trnH* intergenic spacer, a non-coding region, tends to show higher levels of variation and has been useful in distinguishing species in several plant groups (Anakha & Hari, 2022). By combining these nuclear and chloroplast regions, a multi-locus barcoding approach increases the accuracy of species identification and helps overcome the limitations of using a single DNA marker.

The proper identification and evaluation of biologically relevant plant taxa play a crucial role in understanding phylogeny of many important plant's species; these methods enable researchers to find similarity and differences among different plant families. Several genes that are applied or used for DNA barcode studies include *rbcL, matK, trnH-psbA* and ITS separately or in combination. The availability of the sequences of barcoding genes in the databases is expected to rapidly increase thereby increasing their utilization in the identification of plant species subsequently. Therefore, establishing a local barcode database will be valuable for a broad range of potential ecological applications, including the building of community phylogenies, as opined by (Gostel & Kress, 2022).

Despite the potential of DNA barcoding, several barriers limit its application in the identification and conservation of *A. digitata*. These include inadequate representation in reference barcode databases, molecular infrastructure, and technical capacity in many countries. Addressing these challenges is essential for the development of integrative conservation strategies. Although baobabs are widely known, the readily available genetic information on the species, such as DNA barcodes of the species in Africa and particularly Nigeria, is inadequate. Thus, studies on DNA barcoding, especially using the multi-loci approach and investigating the phylogeny of *A. digitata*, are necessary.

2. MATERIALS AND METHODS

Collection of Plant Samples:

Fresh leaf samples of *Adansonia digitata* L. were collected in the early morning at approximately 6:40 AM from a mature tree located within the University of Ilorin campus, Ilorin, Kwara State, Nigeria (Latitude: 8.4799° N, Longitude: 4.5418° E). The sampling site was selected due to its accessibility, secure environment for repeated access if necessary, and the confirmed presence of morphologically typical *A. digitata* specimens, which represent the species within the region.

The plant species was identified based on morphological characteristics in accordance with the *Flora of West Tropical Africa* and verified by a plant taxonomist at the Department of Plant Biology, University of Ilorin. A voucher specimen (Voucher No: UIH/PLB/AD003) was prepared and deposited in the University of Ilorin Herbarium for future reference. The collected leaves were immediately placed in sterile zip-lock bags.

Genomic DNA Extraction:

One gram of fresh leaf tissue of *Adansonia digitata* was frozen with liquid nitrogen and ground into a fine powder. Total genomic DNA was isolated from the sample using the DNeasy Plant Mini Kit (Qiagen, USA). The DNA samples were stored at -40 °C prior to PCR analysis.

PCR Amplification and Sequencing Protocols:

PCR amplification was performed with the barcode markers as shown in Table 1. Primers used were synthesized by Inqaba Biotec. South Africa. The PCR was carried out with a total reaction volume of 30µl in a thermocycler (Eppendorf, Germany) containing the following components: 23.0 µL of Milli-Q water, 1.0 µL of template DNA, 3.0 µL of 10× Taq buffer containing $MgCl_2$, 1.0 µL of 5 pmol/µL forward and reverse primers, 1.0 µL of 2.5 mM dNTPs, and 1.0 µL of Taq DNA polymerase (1 U/µL).

The PCR setup for barcode amplification and cycle sequencing reaction PCR amplification was performed in a thermal cycler under the following conditions: an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 5 minutes to complete the reaction.

The cycle sequencing reactions were conducted using purified PCR products and performed in a 30-cycle protocol with the following thermal profile: an initial denaturation at 96°C for 1 minute, followed by 30 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes. A final extension step was carried out at 60°C for 7 minutes to ensure complete product formation. The product was then purified.

The various alleles were sequenced using the 3130xl Genetic Analyser (Applied Biosystems, CA, USA). The editing of the sequences obtained was manually carried out using Sequence Scanner software v1.0 Applied Biosystems, CA, USA), and the full-length sequences were assembled using a

local alignment algorithm, Codon Code Aligner version 4.24 (Codon Code Corporation).

Table 1: Primers used for sequencing

Primers	Sequences
ITS – 1	TCCGTAGGTGAACCTGCGG
ITS – 4	TCCTCCGCTTATTGATATGC
PsbA	GTTATGCATGAACGTAATGCTC
trnH	CGCGCATGGTGGATTCACAATCC
***rbcL*– F**	ATGTCACCACAAACAGAGACTAAAGC
***rbcL* – R**	GTAAAATCAAGTCCACCRCG
***rpoC1*-F**	GGCAAAGAGGGAAGATTTCG
***rpoC1*-R**	CCCATAAGCATATCTTGAGTTGG

Phylogenetic Analysis:

The phylogenetic analysis of *Adansonia digitata* was conducted using four DNA barcode regions: *rbcL*, *psbA-trnH*, *rpoC1*, and *ITS*. Forward and reverse sequences obtained from each region were manually checked and trimmed for quality using BioEdit v7.2 and subsequently assembled into consensus sequences using seqtrace 0.9.0. Consensus sequences for each marker were aligned using AliView v1.26 employing the MUSCLE algorithm with default gap opening and extension parameters.

The aligned sequences were subjected to nucleotide BLAST searches against the NCBI GenBank database to confirm species identity. The percentage identity of the best hits was recorded for each locus. Only high-quality alignments with clear, unambiguous base calls were used for further analyses.

Phylogenetic trees were constructed using the Maximum Likelihood (ML) method implemented in MEGA X (Tamura *et al.,* 1993). Prior to tree reconstruction, the best-fit nucleotide substitution model for each barcode region was determined using the Akaike Information Criterion (AIC) within MEGA X. The model used was the Tamura-nei model and the tree inference option was the Nearest-Neigbour-Interchange (NNI). Phylogenetic trees were generated for each locus independently, and the robustness of clades was assessed using 1,000 bootstrap replicates. Bootstrap values ≥70% were considered to provide strong support. *Theobroma cacao* (Malvaceae) was used as an outgroup in all phylogenetic reconstructions based on its close taxonomic relationship but clear distinction from the *Adansonia* genus. except for ITS sequences and *rpoC1* phylogenetic analyses, which had different outgroups.

3. RESULTS

Molecular Identification and Phylogenetic Analysis of the Extracted DNA:

Molecular identification was conducted to confirm the classification of the sample to the species level. The DNA sequence length of the ITS region, *psbA-trnH*, and rbcL was 739bp, 581bp, and 589bp, respectively. A BLAST search in the NCBI GenBank using these sequences showed a 98%-99% similarity score with multiple sequences of *Adansonia digitata* (Tables 2-5). The obtained sequences were submitted to the NCBI database, and their respective accession numbers are as follow: OQ694034, OP709538, OR135362 and OR251003 for the *rbcL*, ITS, *psbA-trnH* and *rpoC1* sequences respectively.

Phylogenetic Analysis by Maximum Likelihood Method with *RBCL* Primer:

In the phylogeny tree (Figure 1), the consensus; sample UIL *Adansonia digitata* 1B, with accession number OQ694034 clustered together with *Adansonia digitata* with accession number MN216530 and shows to be in a monophyletic group with another *Adansonia digitata* of accession number GU981721 in relation to *Adansonia rubrostipa*, thus, the four species form a clade. The node formed another branch with *Cola verticillata* of accession number KC628413, forming a paraphyletic group.

Table 2: BLAST Analysis Result showing Percentage Identity of sample UIL *Adansonia digitata* 1B using *rbcL* gene

Accession number	Species name	Percentage identity
GQ436701	*Scaphium lychnophorum*	98.95%
KM361014	*Tiliaxeuropaea* L.	98.95%
KX909588	*Magnoliophyta spp.*	98.29%
AJ233124	*Cola nitida*	98.95%
MN192725	*Tilia paucicostata*	98.97%
KC628413	*Cola verticillata*	99.29%
AJ233115	*Adansonia fony* var. *rubrostipa*	99.28%
GU981721	*Adansonia digitata* L.	99.31%
MN216530	*Adansonia digitata* L.	99.82%
AY864312	*Craigia* sp.	99.47%
MH658621	*Tilia chinensis*	99.12%
KF381124	*Pterospermum xylocarpum*	98.45%
KC627960	*Chlamydocola chlamydantha*	99.47%
MG833602	*Pseudobombax* sp.	98.95%

AY328183	*Heritiera parvifolia* Merr.	97.97%
JN114787	*Bombax ceiba* L.	99.11%
KY556637	*Bombax ceiba* L.	98.29%
KR530039	*Sterculia monosperma* Vent.	98.73%
AB925312	*Heritierajavanica*	99.06%
MH598894	*Abutilon pannosum*	98.46%
KX527079	*Firmiana simplex* (L.)	99.07%
HQ427249	*Reevesia pycnantha*	98.86%
AF022125	*Theobroma cacao* L.	98.58%
LC385908	*Theobroma cacao* L.	98.75%

Table 3: BLAST Analysis Result showing Percentage Identity of sample UIL *Adansonia digitata* 1B using ITS gene sequence

Accession Number	Species Name	Percentage Identity
KU145773	*Adansonia digitata L.*	96.46%
KU145771	*Adansonia digitata L.*	96.46%
KU145766	*Adansonia digitata L.*	96.46%
KU145751	*Adansonia digitata L.*	96.04%
HQ658372	*Adansonia digitata L.*	94.66%
KU145770	*Adansonia kilima*	96.57%
JX178939	*Adansonia kilima*	96.21%
JX178940	*Adansonia kilima*	96.20%
JN400326	*Adansonia kilima*	97.89%
AF028526	*Adansonia digitata L.*	97.89%
JN400317	*Adansonia digitata L.*	98.71%
KF760378	*Adansonia digitata L.*	98.72%
AF460193	*Adansonia digitata L.*	98.71%
KU145720	*Adansonia digitata L.*	97.11%
KU145696	*Adansonia digitata L.*	96.97%
KF760379	*Adansonia kilima*	98.15%
KX118407	*Adansonia digitata*	98.02%
KX118406	*Adansonia digitata*	98.01%
JN400327	*Adansonia kilima*	97.88%
KU145759	*Adansonia kilima*	96.18%
KU145731	*Adansonia kilima*	96.04%
JN400324	*Adansonia kilima*	97.75%
KF760383	*Adansonia kilima*	97.87%
KF760380	*Adansonia kilima*	97.87%

Table 4: BLAST Analysis Result showing Percentage Identity of sample UIL *Adansonia digitata* 1B using *PsbA-trnH* sequence

Accession Number	Species name	Percentage identity
JN400281	*Adansonia gregorii*	98.20%
JN400283	*Adansonia gregorii*	97.97%
JN400286	*Adansonia suarezensis*	96.93%
JN400278	*Adansonia perrieri*	96.24%
JN400280	*Adansonia madagascariensis*	93.78%
JN400287	*Adansonia digitata* L.	100.00%
JN400286	*Adansonia suarezensis*	96.93%
OK469474	*Pseudobombax ellipticum*	89.41%
KX248987	*Pachira dolichocalyx*	98.10%
OL312300	*Pachira insignis*	93.78%
GQ982172	*Cavanillesia platanifolia*	92.64%
KP095701	*Reevesia thyrsoidea*	84.86%
MF786076	*Catostemma commune*	89.44%
GQ982386	*Theobroma cacao* L.	86.35%
FJ038989	*Quararibea duckei*	91.51%

GQ982350	*Quararibea asterolepis*	90.69%
HG964052	*Magnoliophyta sp.*	84.88%
KR735343	*Hibiscus calyphyllus*	84.60%
GQ982301	*Ochroma pyramidale*	84.76%
JX997372	*Ochroma pyramidale*	93.33%
GQ982310	*Pachiraquinata*	93.35%
KX248382	*Eriotheca longitubulosa*	92.32%
MW048045	*Ochroma lagopus*	92.87%

Table 5: BLAST Analysis Result showing Percentage Identity of sample UIL *Adansonia digitata* 1B using *rpoC1* gene sequence

Accession Number	Species name	Percentage Identity
FJ038695	*Sterculia pruriens*	92.15%
OL701986	*Reevesia thyrsoidea*	92.15%
NC_063747	*Reevesia orbicularifolia*	92.15%
MN384259	*Thespesia populnea* (L.)	92.15%
NC_057077	*Ceibas peciosa*	91.93%
NC_037494	*Bombax ceiba* L.	91.93%
HQ901200	*Gossypium barbadense* L.	91.93%
KP221927	*Gossypium harknessii*	91.93%
NC_039570	G*ossypium* schwendimanii	91.93%
MK792868	*Gossypium hirsutum*L.	91.93%
NC_053355	*Abelmoschus moschatus* Medik.	91.93%
NC_060636	*Hibiscus trionum* L.	91.93%
KY635876	*Abelmoschus esculentus* (L.) Moench	91.93%
MW446504	*Hibiscus cannabinus* L.	91.93%
MW194065	*Plagianthus squamatus*	91.93%
NC_049129	*Malva wigandii*	91.93%
NC_063267	*Malva canariensis*	91.93%
NC_063266	*Navaea phoenicea* (Vent.)	91.93%
NC_053839	*Alcea rosea* L.	91.93%
MW194059	*Hoheria populnea*	91.93%
FJ395860	*Tilia* × *europaea* L.	92.37%
HQ594157	*Tilia americana* L.	92.37%
MT984473	*Tilia* × *europaea* L.	92.37%
MT984562	*Tilia cordata* Mill.	92.37%
MH169579	*Tilia amurensis*	92.37%
KT894774	*Tilia oliveri*	92.37%

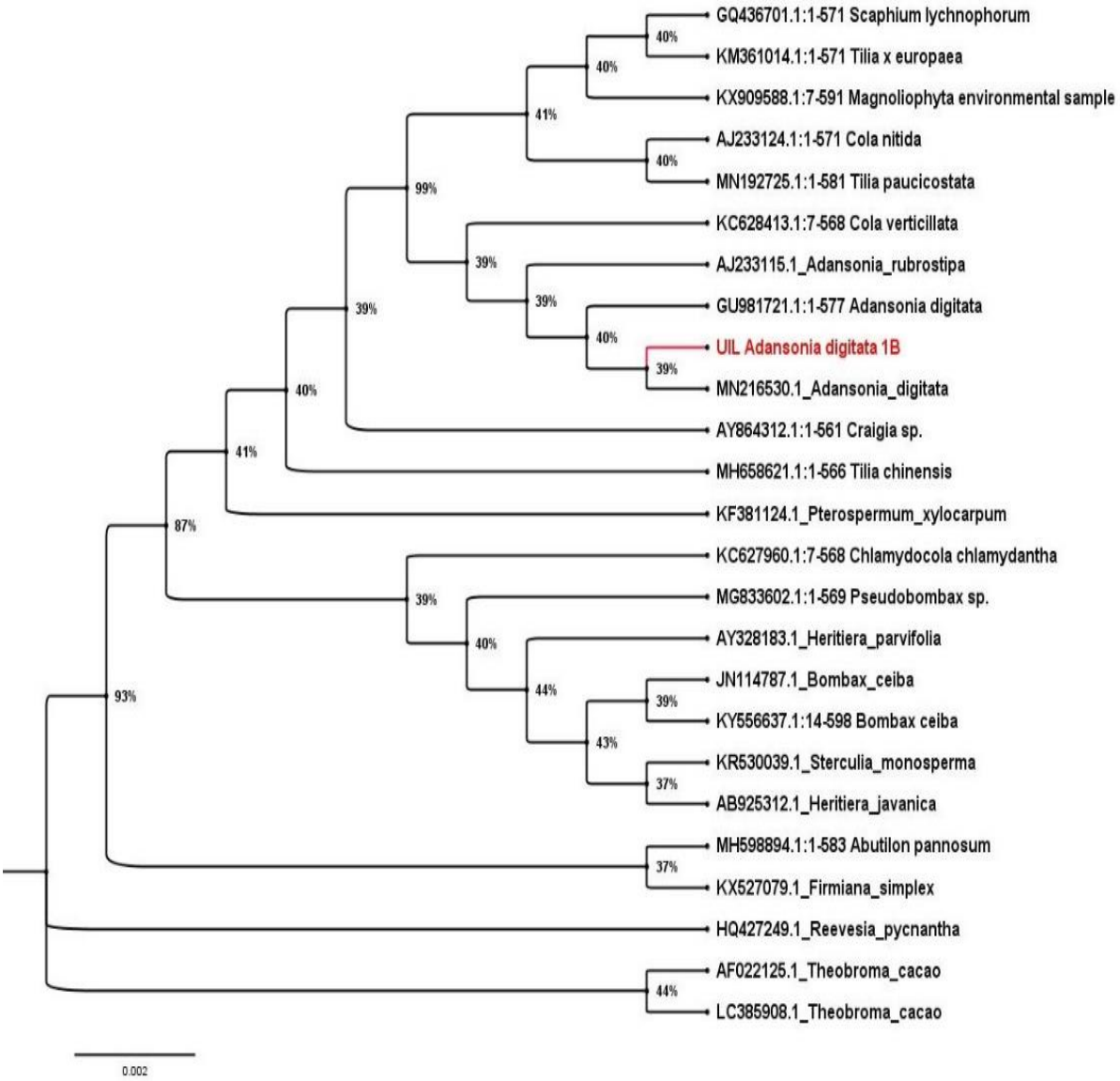

Figure 1: Maximum Likelihood phylogenetic analysis of *Adansonia digitata* based on *rbcL* chloroplast gene sequence.

The Phylogenetic tree of the *Adansonia digitata* sample UIL 1B showed complex evolutionary relationships using *rbcL* gene marker. The sample clustered with another *Adansonia digitata* (accession number MN216530) with a relatively low bootstrap value (39%), which shows moderate relationships between this two genetic information. The tree shows complex paraphyletic relationships with *Tilia* species, *Cola nitida* and *Scaphium lychnophiorum*, this grouping results shared but not identical relationships among these taxa. More complex evolutionary relationships are demonstrated by the *rbcL* tree forming polyphyletic relationships with *Bombax ceiba*, *Heritiera* species, and *Chlamydocola chlamydantha*, indicating possible genetic divergence and intricate evolutionary routes within the higher level of taxonomy. The overall tree structure is demonstrated with the high boot strap value with the two major clades (87%).

Phylogenetic Analysis by Maximum Likelihood Method with ITS Primer:

The Phylogenetic tree of the *Adansonia digitata* sample UIL 1B presented strong evolutionary relationships using ITS gene marker. The sample clustered with two *Adansonia digitata* (accession numbers KU145720 and KU145696) with a bootstrap value of 88% (Figure 2).

The tree shows strong paraphyletic relationships with *Adansonia digitata* and *Adansonia kilima*. This grouping results shared identical relationships among these taxa with bootstrap support value range (90-93%) indicating possible close genetic relationships.

The overall tree structure is demonstrated with the high bootstrap value with the two major clades (93%). In the tree, the clade associated with the sample revealed a monophyletic group containing 7 taxa: all (all *Adansonia digitata*) showing strong genetic relationship within the clade with a strong bootstrap support value (92%).

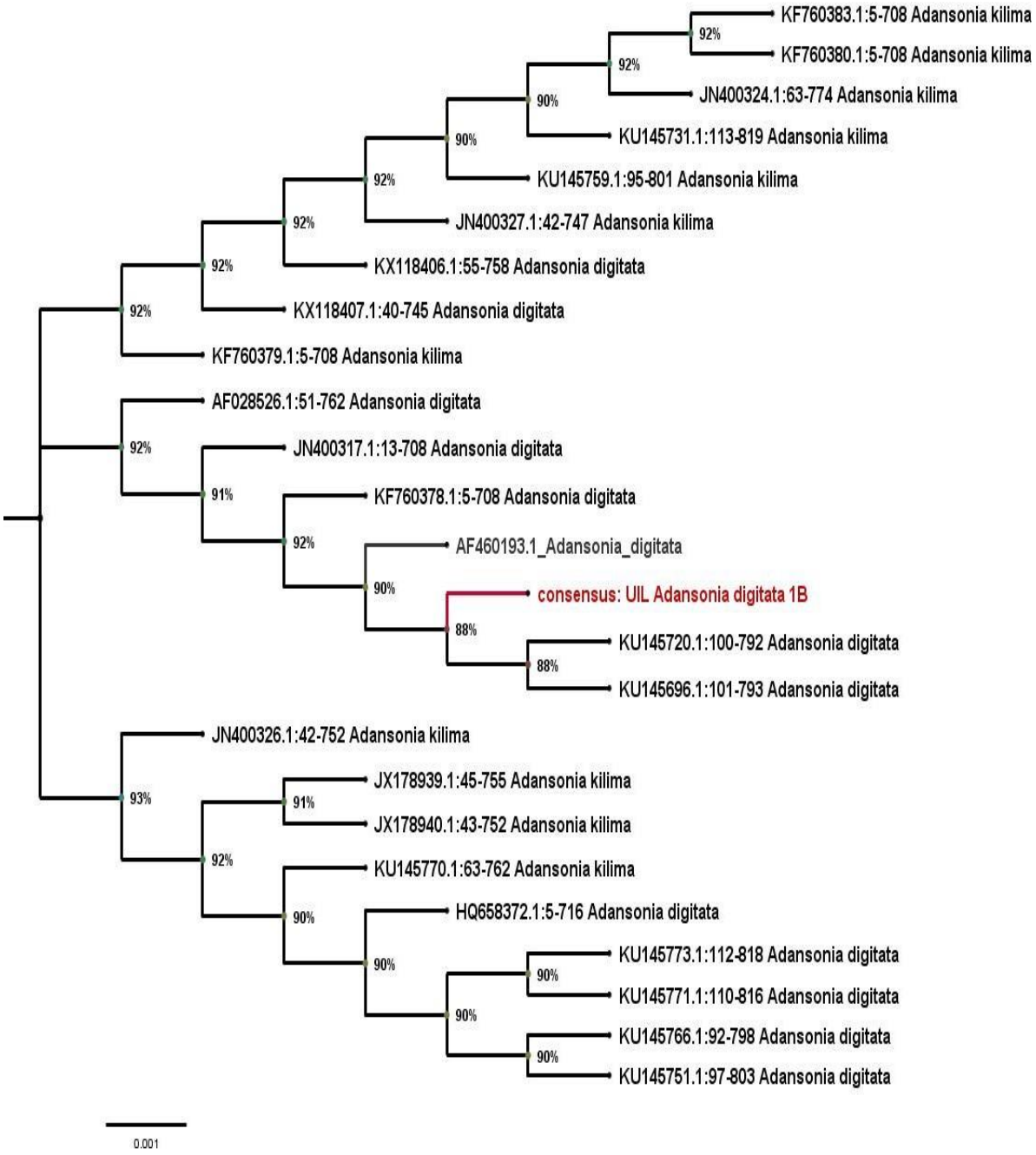

Figure 2: Maximum Likelihood phylogenetic analysis of *Adansonia digitata* based on ITS sequence

Phylogenetic Analysis by Maximum Likelihood Method with Psba–Trnh Primer:

The phylogenetic tree of the *Adansonia digitata* UIL 1B showed complex evolutionary relationships using *PsbA-trnH* gene marker. The sample clustered another *Adansonia digitata* (accession number JN400287) in the phylogenetic tree, with a bootstrap value of 57%, which was the only representative of the species in the tree, indicating a close relationship between between the duo (Figure 3). The tree showed monophyletic relationship between the Sample UIL *Adansonia digitata* 1B and other members of the Adansonia genus viz; *Adansonia gregorii*, *Adansonia suarezensis*, *Adansonia perrieri* and *Adansonia madagascariensis* indicating Close evolutionary relationship between members of the monoplyletic group

The tree reveals paraphyletic relationships with, *Adansonia suarezensis*, *Pseudobombax ellipticum, Pachira dolichocalix,* and *Pachira insignis* in relation to the sample demonstrated by a strong bootstrap value with the two clades (76%)

Complex evolutionary relationships were demonstrated by the phylogenetic tree forming polyphyletic relationships with species including *Catostemma commune*, *Reevesia thyrsoidea*, *Quararibea* species and *Hibiscus* calyphyllus with respect to the major clade with bootstrap value of 77%. Indicating possible genetic divergence and intricate evolutionary routes within the higher level of taxonomy. The outgroup turned out to be *Theobroma cacao* (accession number GQ982386).

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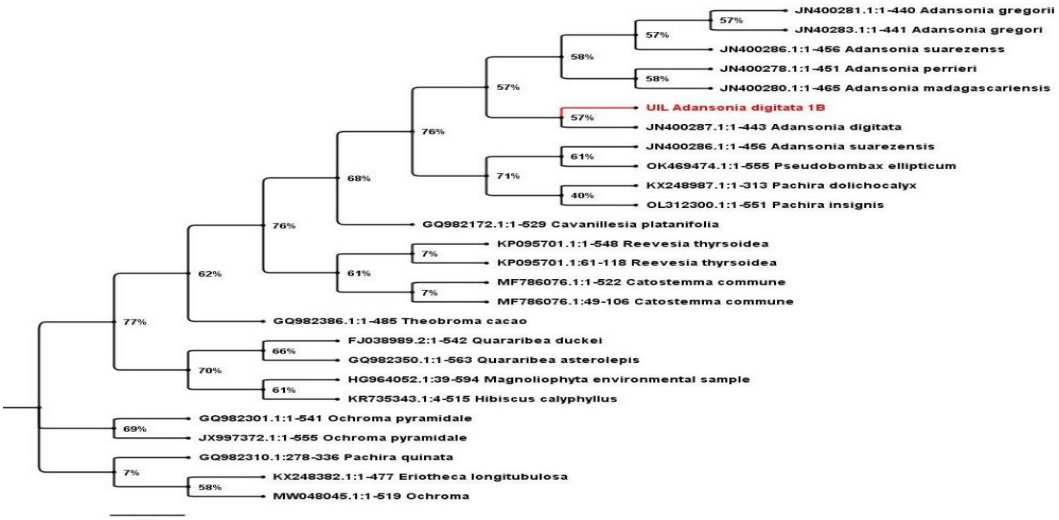

Figure 3: Maximum Likelihood phylogenetic analysis of *Adansonia digitata* based on *psbA-trnH* chloroplast spacer sequence.

Phylogenetic Analysis by Maximum Likelihood Method with *Rpoc1* Primer:

The Phylogenetic analysis using *rpoC1* gene marker did not show relationships based on the species and genus level. However,it revealed credible evolutionary relationships at the family level (Malvaceae). Also, the BLAST search from the NCBI database showed no affinity to *species* of *Adansonia digitata* (Table 5). The sample occurred in a monophyletic group demonstrated with a strong bootstrap value (100%) with taxa such as *Sterculia pruriens, Tilia cordata, T. europae, and T. americana* indicating close evolutionary relationships as they share the same most common recent ancestor (MCRA). The tree shows complex polyphyletic relationship with several notable members of Malvaceae viz; *Hibiscus cannabinus, Reevesia spp Abelmoschus esculentus, A. moschatus,* Gossypium species*, Bombax ceiba* and *Ceiba peciosa*. Thisgrouping results shared but not identical relationships among these taxa (Figure 4).

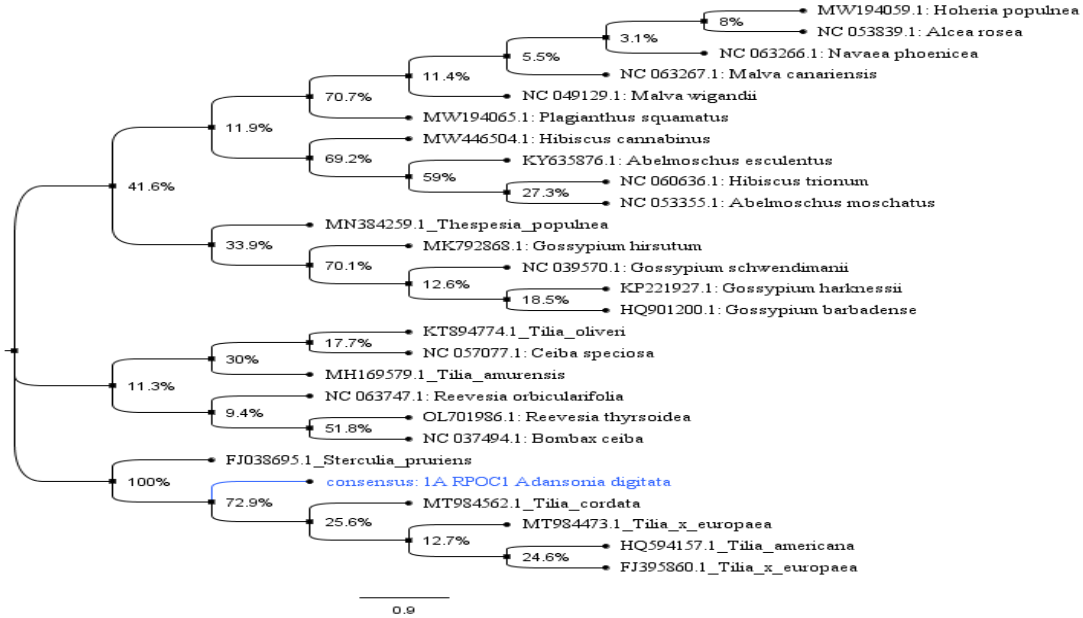

Figure 4: Maximum Likelihood phylogenetic analysis of *Adansonia digitata* based on *rpoC1* chloroplast gene sequence

Phylogenetic Analysis by Maximum Likelihood with Concatenated Sequences.

Phylogenetic tree was reconstructed based on a concatenated alignment of three barcode regions (*rbcL*, *psbA-trnH*, and ITS), excluding *rpoC1* due to the absence of corresponding sequence data (*Adansonia digitata*). The resulting tree revealed that the sample UIL *Adansonia digitata* 1B clustered with *Adansonia digitata* isolate 259, forming a clade supported by a moderate bootstrap value of 53%. The tree revealed a monophyletic consisting of four taxa including the sample, *Adansonia digitata* cp301, *Adansonia digitata* isolate 259 and *Adansonia kilima* supported with a bootstrap value (63%) suggesting a close phylogeny within members of the group. The tree revealed polyphyletic relationships with two members of the *Reevesia* genus namely *R. thysoidea* and *R. pycantha* both constituting a major clade. *Theobroma cacao*, turned out the outgroup (Figure 5).

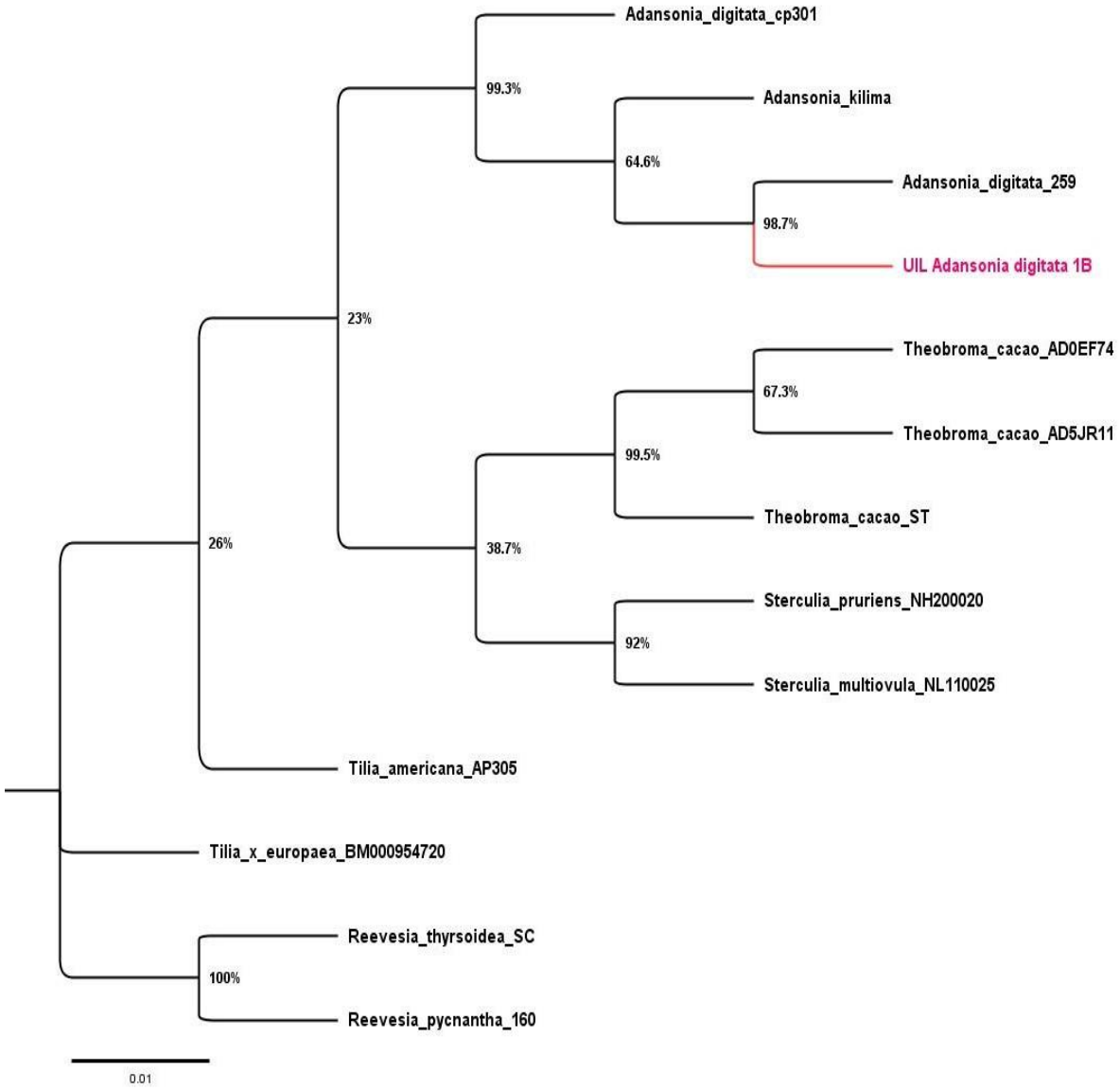

Figure 5: Maximum Likelihood phylogenetic analysis of *Adansonia digitata* based on concatenated chloroplast *rbcL*, *psbA-trnH* and nuclear ITS markers (excluding *rpoC1*).

Phylogenetic Analysis by Maximum Likelihood with Concatenated Sequence (Including *Rpoc1*):

A phylogenetic tree was reconstructed based on a concatenated alignment of three barcode regions (*rbcL*, *psbA-trnH*, and ITS), including *rpoC1.* The inclusion of the *rpoC1* region in the concatenated dataset resulted in a more robust and well-supported phylogenetic tree, as indicated by higher bootstrap values and clearer clade resolution. This is likely due to the informative nature of *rpoC1*, a moderately conserved chloroplast gene that contributes additional parsimony-informative sites, thereby enhancing the phylogenetic signal. In contrast, the dataset excluding *rpoC1* may have lacked sufficient informative characters, leading to weaker statistical support, underscoring the importance of incorporating complementary loci like *rpoC1* to improve the accuracy and reliability of multi-locus phylogenetic analyses.

In the phylogeny tree, the sample UIL *Adansonia digitata* 1B clustered with *Adansonia digitata* isolate 259 with a strong bootstrap value (98%) forming a clade, this underscores the great similarity between the sample and *Adansonia digitata*. The tree revealed a paraphyletic relationship between the consensus sample; UIL *Adansonia digitata* 1B and *Adansonia kilima* with a moderate bootstrap support value (65%) indicating some degree of phylogenetic relationship between the two as they belong to the same genus. The tree revealed a monophyletic group including *Adansonia kilima*, *Adansonia digitata* isolate cp 301 and the same cluster where all four taxa formed a major clade with strong bootstrap support value (99%). The phylogenetic tree also revealed polyphyletic association between the sample and *Sterculia* species viz; *S. puriens* and *S. multiovula* with a bootstrap value of 92%. Sample UIL *Adansonia digitata* 1B is in a complex polyphyletic association with *Tilia* species, *Reevesia* species. *Theobroma cacao* turned out as outgroups (Figure 6).

Figure 6: Maximum Likelihood phylogenetic analysis of *Adansonia digitata* based on concatenated chloroplast *rbcL*, *psbA-trnH* and *rpoC1* nuclear ITS markers.

4. DISCUSSION

DNA barcoding is a molecular technique for species identification is relatively simpler and more accurate than most conventional methods of species identification. DNA barcoding utilizes one or more standardized short DNA regions for taxon identification (Antil *et al*., 2023). Due to the limitations of conventional methods, molecular techniques are used to investigate the problems related to the identification and classification of species (Sarwar *et al.,* 2019). This study proved that the molecular method of species identification should be recommended as it gives more accurate results in species identification and even explaining the evolution of species.

The maximum likelihood phylogenetic analyses revealed a strong correlation between the consensus; sample UIL *Adansonia digitata* 1B and several *Adansonia* species including *Adansonia kilima, Adansonia rubrostipa, Adansonia gregorii*, *Adansonia madagascariensis* and a particularity stronger affinity to *Adansonia digitata* underscoring an effective species identification ability of the primers used. The maximum likelihood is the most used inference technique, especially for estimating evolutionary relatedness between isolates, possibly owing to its computational efficiency and robustness while theoretically relatively simple (Dhar & Minin, 2016).

The multi-loci approach of using more than a single primer gave more insight into species identification. In the work of Larrain *et al*. (2019), multi-locus strategy outperformed the mono-locus methods for the molecular identification of the *Mytilus* taxa. Also, in a study by Promputtha *et al.* (2005), it was suggested that some degree of inaccuracy was associated with using the ITS region alone for the identification of species and thus, recommends using multiple primers for effective species identification. adopting a multi-loci approach, the consensus; Sample UIL *Adansonia digitata* 1B clustered with *Adansonia digitata* in all but one of the four phylogenetic trees constructed using sequences obtained from ITS, *rbcL*, *psbA-trnH* and *rpoC1* markers, consistency of the clustering of consensus and *A. digitata* species in all the phylogeny except that of *rpoC1* cannot be coincidental.

Considering the BLAST (Basic Local Alignment Search tool) analyses conducted on the NCBI database, the consensus; sample UIL *Adansonia digitata* 1B scored strong similarity with *Adansonia digitata* in all primers used for the analyses except *rpoC1* primer this further corroborates the efficacy of the primers in species level identification of plants.

The consensus sample; UIL *Adansonia digitata* 1B obtained from the *psbA-trnH* primer, showed a tremendous 100% similarity with *Adansonia digitata* isolate 259 of accession number JN400287 obtained from the work of Pettigrew *et al*. (2012), who claimed the sample was obtained from west Africa. This study further narrows down the origin of the species as our results suggests that the sample might have been have obtained or similar to the species available in Nigeria. It also showed a 99.55% percentage identity with another isolate of *Adansonia digitata*. High percentage identity was also recorded with other species from the genus *Adansonia* notably *Adansonia rubrostipa* with 98.83% identity, *Adansonia gregorii* with 98.20% identity, and *Adansonia kilima* with 99.55% percentage identity. It is noteworthy that these species did not persist in other primers except *Adansonia kilima* which showed up with the ITS primer. Inferences from the DNA barcodes obtained from the Internal Transcribed Spacer (ITS) primer showed similarity with several samples of *Adansonia digitata* with an approximate percentage identity of 98% this result is indicative of the efficacy of the primer and this point to the fact that the DNA barcodes could be relevant for subsequent species identification. It also showed similarity with *Adansonia kilima* with approximately 97% identity. This is corroborated with the findings of Pettigrew *et al*. (2012) where it was reported that the ITS phylogeny demonstrated a genetic similarity between *A. digitata* and *A. Kilima*. Also, the variation in floral and pollen characters and

chromosome number was examined in specimens from Africa and identified a new diploid baobab species, it was found that *Adansonia kilima* sp., co-existed with *Adansonia digitata* in Africa. *Adansonia kilima* is superficially similar to *A. digitata* but can be differentiated on the basis of floral morphology, pollen, and chromosome number (Pettigrew *et al*., 2012). From the phylogeny tree, the bootstrap value of the clade containing the consensus; sample UIL *Adansonia digitata* 1B which clustered with *Adansonia digitata* has a value of 88%. Although, it has been pointed out that ITS primer only cannot be completely effective for species and genus identification, So, it can be said that from this study that ITS primer is the best primer for DNA barcoding of *Adansonia digitata* on the species level.

The BLAST analysis with the *A. digitata* DNA barcode from the *rpoC1* primer was rather noteworthy as there was no similar sequence of the species on the NCBI database, and our submission was the first on the database for the *rpoC1* region. This result can be attributed to the observation that no or not many DNA barcoding works have been done on *Adansonia digitata* using the *rpoC1* primer. However, *rpoC1* primers have been used for effective DNA barcoding of plant species such as *Medicago sativa* by El-Sherif and Ibrahim (2020) and Chen *et al*. (2022), who reported that *rpoC1* showed excellent power in identifying 18 of 21 *Fritillaria species* except three closely related species, including *Fritillaria cirrhosa*, *F. dajinensis*, and *F. omeiensis*. Abdulkareem *et al*. (2023) indicated that *rpoC1* produced higher identification to species level in identification of *Vernonia amygdalina*. However, in this study, *rpoC1* was able to identify members in the same family as *A. digitata* including *Reevesia* spp, *Theobroma* spp, *Ochroma* spp, *Ceiba spp*., *Tilia spp*.

The *psbA-trnH* primer showed to be a very good and efficient primer for the barcoding of *Adansonia digitata*. The DNA barcode BLAST gave a result of 100% species percentage identity with the sample. In the phylogenetic tree constructed with the *psbA-trnH* sequences, the consensus sample clustered with *Adansonia digitata* with a bootstrap value of 57%, a value that is higher than the bootstrap value from the phylogenetic tree of *rbcL* sequences of 39%. The relatively higher bootstrap support value however supports the results from other primers.

One notable outcome was the variation in bootstrap support across different gene regions. For instance, phylogenetic trees constructed using *rbcL* showed relatively low bootstrap values (e.g., 39%), reflecting limited phylogenetic resolution. Low bootstrap values indicate weak statistical support for particular clades and may result from several factors, including short sequence lengths, low variability within the marker, or high conservation among closely related species. While these values do not invalidate the clustering observed, they highlight the importance of combining multiple loci to improve confidence in species delineation.

Bolson *et al*. (2015) reported ITS and *trnH-psbA* as efficient DNA barcodes to identify threatened commercial woody angiosperms from Southern Brazilian Atlantic rainforests. Loera-Sánchez *et al*., (2020) opined *trnH-psbA* was efficient and promising for identification of forage legumes and grasses while Hassan (2023) reported DNA Barcode of *trnH-psbA* is a promising candidate gene for efficient identification of bitter and swe*et al*mond and related species.

Concatenation all common sequences in the 3 primers; ITS, *rbcL* and *psbA-trnH* that positively associated the consensus with *A. digitata* in separate phylogenetic analysis showed that the consensus; sample UIL *Adansonia digitata 1B* again clustered together with *Adansonia digitata* isolate 259. This gives a great level of confidence that the isolated DNA sequence can be identified as *Adansonia digitata*. Thereby successfully indicating that the sequences obtained in this study are representative DNA barcodes of *A. digitata.* It is noteworthy that the inclusion of the *rpoC1* markers in the concatenation analysis yielded divergent results. While *rpoC1* did not produce BLAST hits for *A. digitata* possibly due to the absence of *A. digitata rpoC1* sequences in public databases. its inclusion in the concatenated dataset significantly improved the robustness of the phylogenetic tree, with increased bootstrap support and clearer clade formation. This occurence could be explained by the conserved yet phylogenetically informative nature of the *rpoC1* gene. The lack of matching sequences in BLAST does not reflect poor marker performance but rather highlights the underrepresentation of *rpoC1* sequences for *A. digitata* in GenBank. In fact, this study contributes the first publicly available *rpoC1* sequence for *A. digitata*, representing a novel resource for future phylogenetic studies. Previous studies (El-Sherif & Ibrahim, 2020; Chen *et al*., 2022; Abdulkareem *et al*., 2023) have reported the efficacy of *rpoC1* in resolving plant taxa, suggesting its value in multi-locus barcoding approaches, especially when paired with more variable regions. The consistency of the results across independent markers and phylogenetic reconstructions indicates that the sequences generated in this study are reliable DNA barcodes for *Adansonia digitata*, contributing to its growing molecular resources and providing a foundation for future taxonomic and conservation studies.

CONCLUSION

Molecular identification is no doubt emphasized to be a more promising technique for species barcoding and identification. From the study, it can be concluded that ITS primer is a recommendable primer for the barcoding of *Adansonia digitata*., however *psbA-trnH* and *rbcL* are credible primers for *Adansonia digitata* identification. DNA barcoding of *Adansonia digitata* for easier identification and classification is necessary. This allows for insight into the related species, provides more information about the species in the gene bank database and ultimately curbs misidentification and interchangement of the species for another.

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Ethical Statement

Ethical approval was not necessary for this research. Because the authors didn't work on human and animal subjects.

Author Contributions

AK.A., contributed in writing the original draft; A.Y.A., performed data curation, and analysis. Y.R.A., contributed to the software. B.A., handled the resources and project administration. S.K.O., and L.A.A. assisted with the formal analysis and writing review and editing; O.B.U., contributed to the data curation; K.I. provided supervision and D.M.M., was responsible for overall

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