Bangladesh J. Plant Taxon. **32**(1): 65-75, 2025 (June) DOI: https://doi.org/10.3329/bjpt.v32i1.82393

MOLECULAR CHARACTERIZATION AND MULTILOCUS DNA BARCODE-BASED DELIMITATION OF *DURANTA ERECTA* L. MORPHOTYPES FROM NIGERIA

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Keywords: *Duranta*; Phylogenetic relationship; Plastid marker; Species identification; Phenotypic plasticity.

Abstract

This study assessed whether the observed morphological variation among eight distinct forms of *Duranta erecta* in Nigeria reflects true genetic divergence or represents phenotypic plasticity within a single species. The forms are distributed across geo-political zones in Nigeria and were characterized based on leaf coloration, margin types, and branching architecture. These forms exhibit variations in their chemical compositions, suggesting potential differences in their DNA profiles. DNA was extracted from leaf samples of all eight morphological forms, and conventional PCR was employed to amplify three marker regions: *ITS*, matK, and rbcL. The amplified fragments were visualized on 1% agarose gel electrophoresis, sequenced, and analyzed phylogenetically using MEGA-11. The matK marker exhibited 100% sequence identity, indicating minimal variation among the forms. In contrast, rbcL and *ITS* displayed 99% sequence identity, with *ITS* revealing greater polymorphic variation. Phylogenetic tree analysis showed the highest support values for rbcL, followed by *ITS* and matK. The combined topologies generated from the three markers revealed no significant differences in the evolutionary history of the eight *Duranta erecta* forms. This result suggests a gene flow among the forms, confirming their classification as a single species.

Introduction

The genus *Duranta* L., (Verbenaceae) comprises shrubs, often exhibiting a climbing growth habit. The branches are typically spiny, particularly on older stems, and young branches are pubescent. Of the approximately 17 species in the genus (Munir, 1995), *Duranta erecta* is the most widespread and extensively cultivated for ornamental purposes. In Nigeria, it is widely grown as an ornamental hedge plant, for beautification, boundary demarcation, and urban landscaping due to its hardiness and aesthetic appeal.

Beyond its ornamental value, *D. erecta* has a long history of ethnomedicinal use. It is traditionally employed in the treatment of tumors, malaria with spleen inflammation, scorpion stings, insect bites, dysentery, and diarrhea. In countries such as Burkina Faso, Ghana, Nigeria, and Tanzania, it is also used for treating infections, parasitic and digestive system disorders, and diabetes (Maregesi *et al*., 2008; Ghaisas *et al*., 2009; Awah *et al*., 2010).

D. erecta is distinguished by its axillary racemose inflorescences, membranous sparsely puberulent leaves, relatively long calyx teeth apicules, and a short corolla tube measuring 7–9 mm, which distinguish it from other related species (Sanders, 2001).

Despite its well-defined morphology, *D. erecta* exhibits significant phenotypic variation in Nigeria, particularly in leaf shape, flower color, thorn, and reproductive traits. These inconsistencies have led to taxonomic debates, with some researchers suggesting the existence of

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multiple forms or subspecies (Liu *et al*., 2012; Moroni *et al*., 2019). While morphometric and chemometric studies (Sagaya and AbdulRahaman, 2023a, b) have attempted to address these complexities, a molecular characterization remains crucial for resolving ambiguities, as morphological traits alone can be influenced by environmental factors and phenotypic plasticity.

DNA barcoding has emerged as a powerful tool for species delimitation, particularly in cases where morphological distinctions are unreliable (Heinrichs *et al*., 2011). This technique relies on short, standardized DNA sequences called "barcodes" to distinguish between species. Although highly effective in animals, especially through the use of the cytochrome c oxidase subunit I (COI) gene (Chen *et al*., 2010). Barcoding in plants presents unique challenges due to slower mutation rates, widespread hybridization, and polyploidy (Fazekas *et al*., 2009). To address this, plant DNA barcoding primarily relies on chloroplast (rbcL, matK) and nuclear (*ITS*) regions (Besse *et al*., 2021). The rbcL gene is widely used for its high amplification success across plant taxa (Kress and Erickson, 2007), while matK provides higher evolutionary resolution (Lahaye *et al*., 2008). The *ITS* region, due to its high variability, offers superior discriminatory power (Sass *et al*., 2007) and has been recommended by the Consortium for the Barcode of Life (CBOL, 2009) for plant identification alone or in combination with other barcode.

This study employs *ITS*, matK, and rbcL markers to molecularly characterize the eight morphologically distinct forms of *D. erecta* in Nigeria. Resolving the taxonomic confusion surrounding this species is essential for clarifying its molecular relationships and improving the understanding of the various forms cultivated in Nigeria.

Materials and Methods

Sample Collection and DNA Extraction

Fresh leaf samples from eight distinct morphological forms of *Duranta erecta* were collected from four states across three geopolitical zones: Kwara (North Central), Kebbi and Sokoto (North West), and Borno (North East) in Nigeria (Table 1). The leaves were cleaned, and genomic DNA was extracted using the Qiagen DNeasy Plant Mini Kit, following the protocol outlined by Lee *et al*. (2016). The extracted DNA was stored at –20°C in the DNA Bank of the Molecular Plant Systematics Research Group (MPSRG), University of Ilorin, Nigeria until further use**.**

Polymerase Chain Reaction (PCR) Amplification and Agarose Gel Electrophoresis

Three target DNA region were amplified: two plastid regions (*matK* and *rbcL*) and one nuclear ribosomal region (ITS) (Table 2). These regions were selected due to their frequent use in plant species identification, high interspecific variability and amplification efficiency (Kress *et al*., 2009; Kress *et al*., 2007; Lahaye *et al*. 2008). PCR reactions were carried out in a 25 µl reaction mixture containing 12.5 µl of Taq 2X PCR master mix (New England Biolabs), 1 µl each of forward and reverse primers (10 µM), 9.5 µl of double-sterilized distilled water (ddH_2O), and 1 µl of DNA template. A control reaction was prepared by substituting ddH_2O for the DNA template.

To prepare the DNA samples for electrophoresis, 5 µl of the extracted DNA was mixed with 1 µl of 6X gel-loading buffer containing 0.25% bromophenol blue and 30% sucrose in TE buffer (pH 8.0). A 0.8% agarose gel was prepared using 0.5 µg/ml SYBR Green in 0.5X Tris-Borate-EDTA (TBE) buffer. The DNA mixture was loaded onto the gel, and TBE buffer was used as the running buffer. Electrophoresis was performed for one hour at 75 V. The gels were visualized using a Genei UV transilluminator, and photographs were captured under a UV lamp using a Nikon digital camera (AKZ-S9 model) (Fig. 1).

Table 1. Brief descriptions and coordinates of the *Duranta erecta* forms employed in this study.

Sl. No.	Forms of *D. Erecta*	Sample sources (states)	Geopolitical zones	Gps coordinate	Brief morphological description of samples at their location
1	Green bush (GB)	Kwara	North Central	8º28'48.30672N 4º40'34.9824E	Erect stem with serrate to entire green leaves, branches long rarely with a single node with fascicle leaves poorly developed.
2	Yellow bush (YB)	Kwara	North Central	8º28'48.30672N 4º40'34.9824E	Branches composed of several nodes and internodes with fascicle serrated to entire yellow leaves well develop.
3	Variegated yellow (VY)	Kwara	North Central	8º28'48.30672N 4º40'34.9824E	Erect stem with serrate to dentate variegated yellow leaves, branches are a bit longer with decussate opposite thorn and leaves.
4	Variegated white (VW)	Kwara	North Central	8º28'48.30672N 4º40'34.9824E	Erect stem with serrate to dentate variegated white leaves, branches are a bit longer with decussate opposite leaves.
5	Thorny green (TG)	Kebbi	North West	12º27'16.22N 4º12'2.14E	Erect stem with fully serrated green leaves, branches are upright, armed with thorn on opposite sides.
6	Variegated yellow double (VYD)	Kebbi	North West	12º27'16.22N 4º12'2.14E	Erect stem with serrate to dentate plane with variegated yellow leaves, branches are a bit longer with decussate opposite leaves.
7	Plain yellow (PY)	Sokoto	North West	13º1'37.77N 5º14'20.998E	Erect stem with serrate to dentate plain yellow leaves, branches are straight with decussate opposite thorn and leaves.
8	Broad green (BG)	Borno	North East	11º47'24N 13º10'12E	Widely spread branches with half serrated to entire glabrous leaves.

Note: GPS coordinates were converted to standard degree–minute–second (DMS) notation for consistency. Morphotype codes (e.g., GB, YB, VY) are used consistently in the text and figures to aid cross-reference.

Table 2. Gene regions and their respective sequences.

Primer name	Sequence	Reference
*ITS*1	TCCGTAGGTGAACCTGCGG	White *et al*. (1990)
*ITS*4	TCCTCCGCTTATTGATATGC	White *et al*. (1990)
rbcL_F	ATGTCACCACAAACAGAGACTAAAGC	Levin, (2003)
rbcL_R	GTAAAATCAAGTCCACCRCG	Kress and Erickson, (2007)
matK_390f	CGATCTATTCATTCAATATTTC	Cuenoud *et al*. (2002)
matK_132r	TCTAGCACACGAAAGTCGAAGT	Cuenoud *et al*. (2002)

Sequence Quality, Alignment, and Phylogenetic Analyses

Raw sequence data were analyzed using a combination of software tools. SeqTrace 0.9.0 (Singh and Kumar, 2012) to view raw sequence data and generate consensus sequences. Alignment of DNA sequences was carried out using AliView version 1.17-beta1 (Larsson, 2014). Finalized sequences were submitted to the NCBI GenBank database, and accession numbers were obtained. Phylogenetic tree construction was performed using MEGA 11 (Tamura *et al*., 2021).

The DNA sequences of *D. erecta* samples were subjected to BLAST analysis in the NCBI database (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) for species verification. Nucleotide composition (A, T, G, C content) and sequence lengths were determined using the online GC

Content Calculator (https://www.sciencebuddies.org/science-fair-projects/references/genomics-g-c-content-calculator).

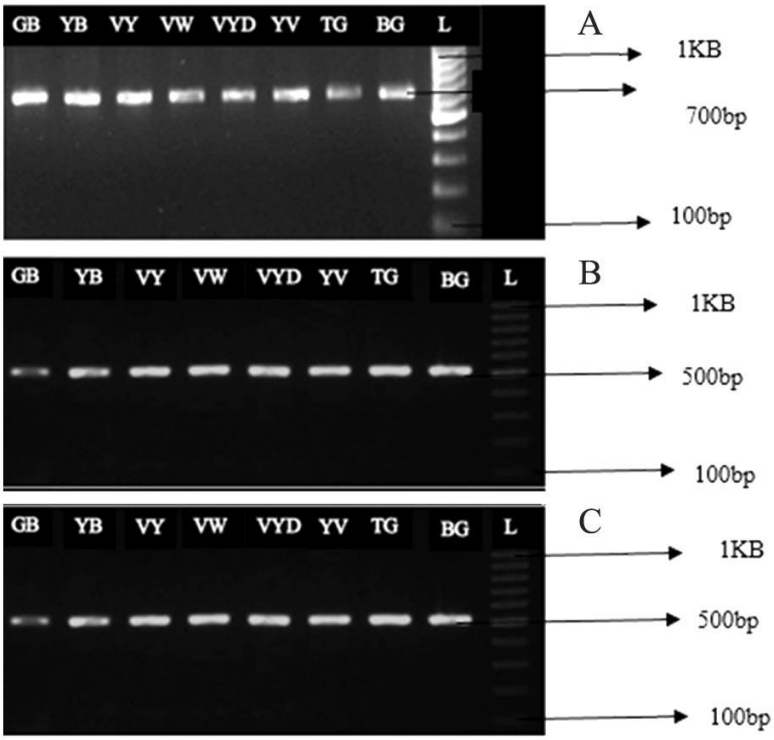

Fig. 1. Image of the Gel electrophoresis for PCR quality check (A= rbcL; B= *ITS* and C= matK

Note: L: Ladder; BG: Broad green; TG: Thorny green; YV: Plain yellow; VYD: Variegated yellow double; VW: Variegated white; VY: Variegated white; YB: yellow bush and GB: Green bush.

For phylogenetic reconstruction, *Parodianthus ilicifolius* (GenBank Accession: DQ463786) was selected as the outgroup based on BLAST similarity and previous taxonomic placement. The sequence variation and similarity percentages were also assessed. Phylogenetic trees were constructed using both the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods implemented in MEGA v11 (Tamura *et al.,* 2021). The optimal nucleotide substitution model was determined using Akaike Information Criterion (AIC), which selected the Tamura 3-parameter (T92) model. Node support was assessed with 1000 bootstrap replicates for both individual and concatenated barcode sequences. Gaps and ambiguous positions were excluded from the analyses to ensure accuracy.

Results and Discussion

DNA Extraction and Amplification

High-quality genomic DNA could initially not be obtained from *Duranta erecta* forms due to their high content of secondary metabolites, which form complexes with proteins and nucleic acids (Agawane *et al*., 2019; Inglis, 2018). This issue was effectively mitigated by incubating leaf samples at 65°C for 35–45 min, which disrupted problematic polyphenolic compounds and

reduced viscosity caused by co-precipitated polysaccharides (Sablok *et al*., 2009; Schenk *et al*., 2023). Schenk *et al*. (2023) emphasized that adjustments of extraction protocols improved DNA purity and subsequent PCR success.

All three DNA-barcode regions (matK, rbcL, and nuclear ITS) were successfully amplified from the eight *D. erecta* forms. The observed amplicon sizes (875 bp for matK, 570 bp for rbcL, and 671 bp for ITS) fell within recommended ranges (CBOL, 2009; Li *et al*., 2011; Kumar *et al*., 2015). However, incomplete amplification was noted for ITS in the broad green form and rbcL in the variegated yellow form, likely due to sequence variation or incomplete concerted evolution (Mirarab *et al*., 2016).

BLAST Identification and Sequence Variation

BLAST analysis identified all barcode sequences as *D. erecta* with 92–100% sequence identity and E-value of zero (0), confirming species-level identification. High identity scores and low E-values are indicative of accurate taxonomic placement (Wahyuni *et al*., 2023). Among the barcodes, matK displayed 100% similarity, *ITS* 98–99%, and rbcL 92–99%. These results affirm the reliability of DNA barcodes in taxonomic identification.

Sequence nucleotide composition and G–C content are summarized in Tables 3–5 and illustrated in Fig. 2. The *ITS* region exhibited the highest G–C content (64.8–66.5%), followed by rbcL (44.7–46.4%) and matK (34.6–35.1%). This ordering aligns with earlier findings (Castro *et al*., 2015; Tang *et al*., 2016; Song *et al*., 2021), and supporting the distinct genomic characteristics of each barcode region.

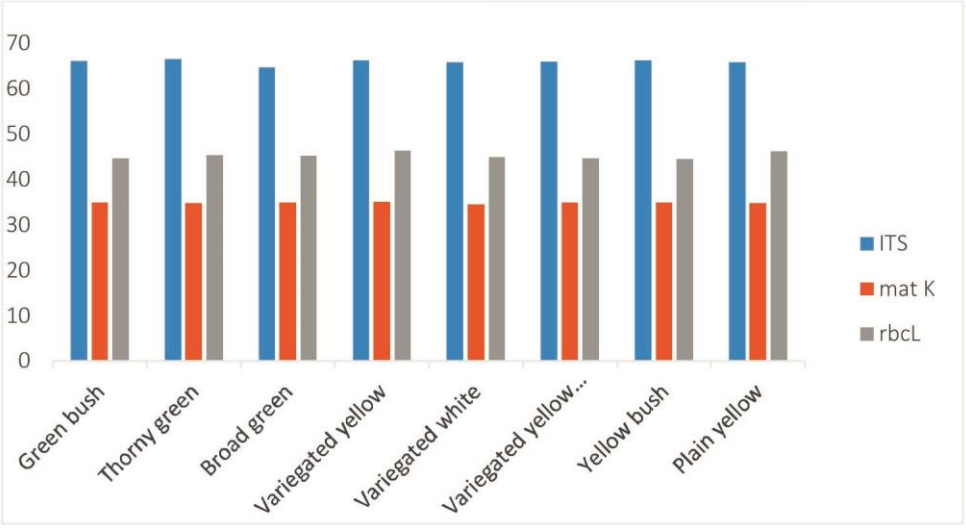

Fig. 2. Percentage of Guanine -Cytosine (G-C) content for *ITS*, matK and rbcL for the eight *Duranta erecta* forms.

Multiple sequence alignment (Table 6) revealed 77 variable sites (11.48%) in *ITS*, 62 (10.88%) in rbcL, and 18 (2.06%) in matK. The ITS region exhibited 66 single-nucleotide polymorphisms (SNPs), with the broad green form showing the highest levels of polymorphism and indel frequency. The high variability in *ITS* supports its potential as a discriminating marker in DNA barcoding studies (Pang *et al*., 2011; Wang *et al*., 2011; Su *et al*., 2015). Comparative studies (Fu *et al*., 2011; Castro *et al*., 2015) further affirm *ITS* is superior in resolution over chloroplast loci.

Table 3. The size and nucleotide content of the *ITS* genes of *Duranta erecta* forms.

Plant samples	Size (bp)	A (bp)	T (bp)	G (bp)	C (bp)	G-C content (%)
Green bush	652	125	96	199	232	66.1
Thorny green	657	126	94	201	236	66.5
Broad green	236	41	42	63	90	64.8
Variegated yellow	656	126	95	201	234	66.3
Variegated white	637	125	92	193	227	65.9
Variegated yellow double	658	128	96	199	235	66.0
Yellow bush	664	127	97	203	237	66.3
Plain yellow	662	130	96	201	235	65.9

Table 4. The size and nucleotide content of the matK genes of *Duranta erecta* forms.

Plant Samples	Size (bp)	A (bp)	T (bp)	G (bp)	C (bp)	G-C content (%)
Green bush	850	246	307	137	160	34.9
Thorny green	860	247	314	137	162	34.8
Broad green	848	246	306	136	160	34.9
Variegated yellow	845	244	304	137	160	35.1
Variegated white	871	252	318	139	162	34.6
Variegated yellow double	871	251	316	139	165	34.9
Yellow bush	852	246	308	138	160	35
Plain yellow	856	247	311	137	161	34.8

Table 5. The size and nucleotide content of the rbcl genes of *Duranta erecta* forms.

Plant Sample	Size (bp)	A (bp)	T (bp)	G (bp)	C (bp)	G-C content (%)
Green bush	548	152	151	125	120	44.7
Thorny green	544	150	147	127	120	45.4
Broad green	570	158	154	134	124	45.3
Variegated yellow	332	87	91	76	78	46.4
Variegated white	538	150	146	125	117	45
Variegated yellow double	546	151	151	125	119	44.7
Yellow bush	554	156	151	127	120	44.6
Plain yellow	457	121	125	99	112	46.2

Table 6. Variation of the DNA barcodes of individual locus and their combinations.

Parameters	*ITS*	rbcL	matK
Conserved	589	492	854
Variable site	77	62	18
Parsimony information site (PI)	11	18	5
Singleton	66	41	6
Percentage of variable point	11.48	10.88	2.06
Average pairwise distance	0.0112	0.0084	0.0015

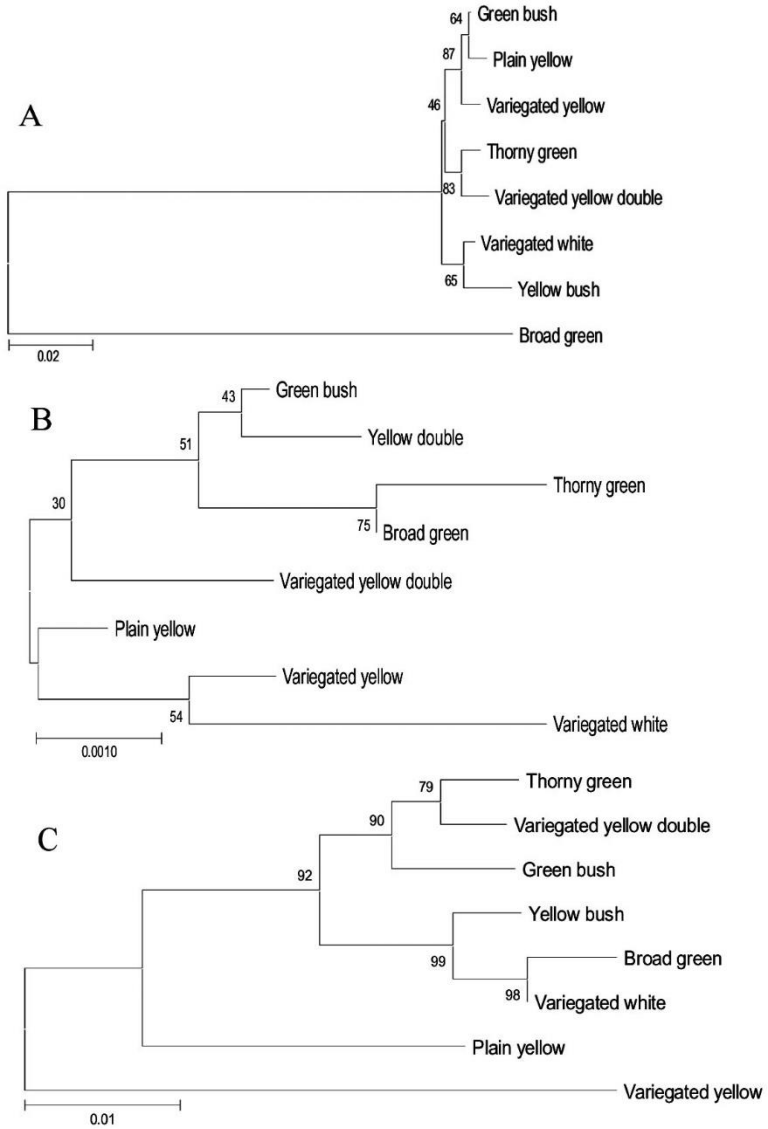

Fig. 3. Neighbor joining tree constructed based on the A: *ITS;* B: matK and C: rbcL sequences with a bootstrap of 1000 replicates.

Phylogenetic Analysis

Neighbor-Joining (NJ) analysis produced bootstrap supports of ≥79%, 46%, and 27% for rbcL, ITS, and matK, respectively (Fig. 3). In contrast, Maximum Likelihood (ML) analysis produced stronger support values of 95, 81 and 50%, respectively (Fig. 4). These results suggest rbcL offers the highest phylogenetic resolution, outperforming ITS and matK. This trend corroborating previous reports (CBOL, 2009; Kress *et al*., 2009; Oyebanji *et al*., 2020). The lower resolution of matK supports the report of Parks *et al*. (2009), highlighting its limited effectiveness in recently diverged taxa.

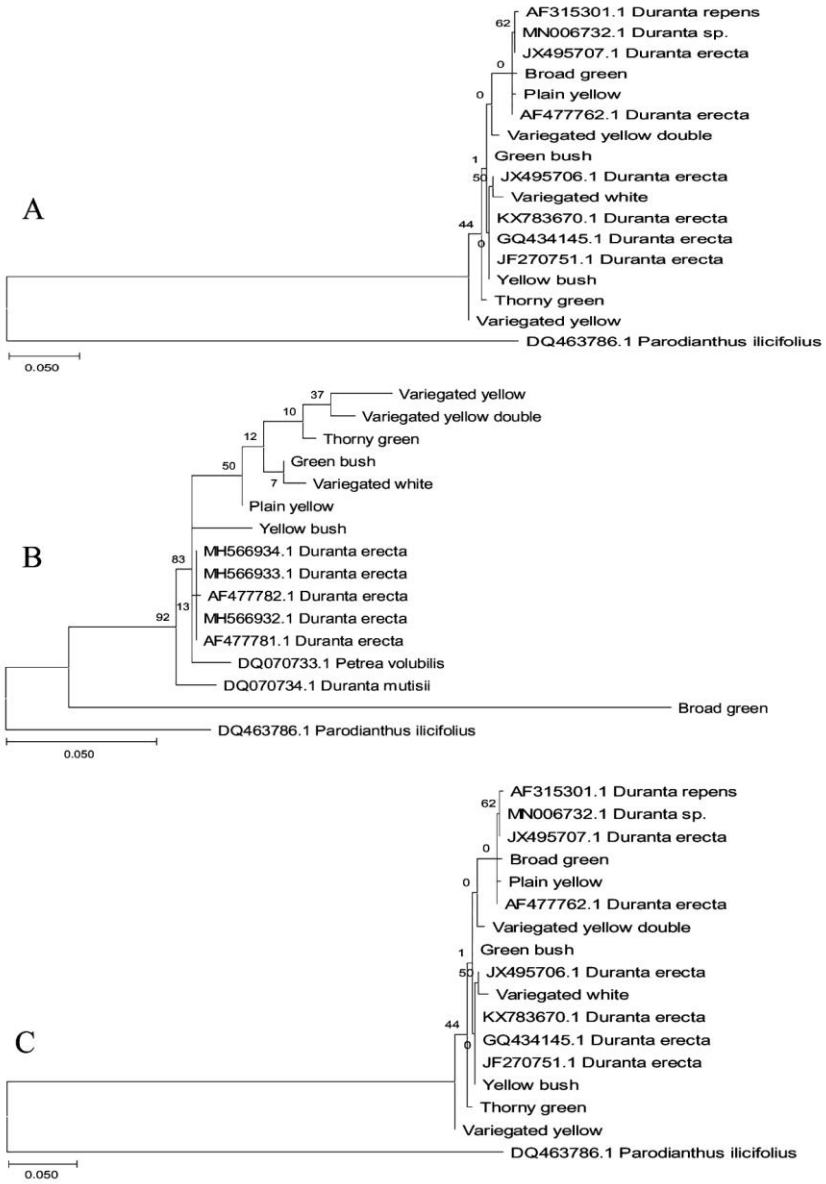

Fig. 4. Maximum Likelihood phylogram for A: rbcL; B: *ITS* and C: matK sequences in *Duranta erecta* and the sampled accessions.

NJ trees distinctly separated the plain yellow form from other *D. erecta* forms, while ML results clustered most forms with reference sequences. Concatenated analyses of two-locus and three-locus combinations yielded modest gains in intraspecific structure; notably, the variegated yellow double and thorny green forms clustered together across rbcL+matK, matK+ITS, and rbcL+ITS analyses, though this association did not hold in the three-locus tree (Fig. 5). This indicates potential gene flow among the forms and suggests genetic cohesion within the species.

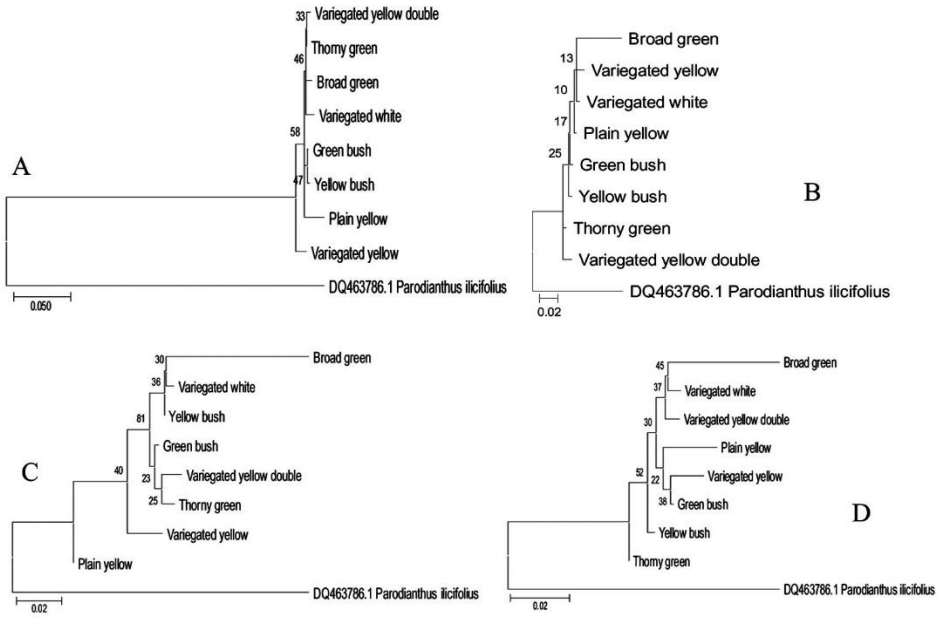

Fig. 5. Maximum likelihood phylogram for the combined A: rbcL+matK*;* B: matK+*ITS;* C: rbcL+*ITS;* D: rbcL+matK+*ITS* sequences in *Duranta erecta*.

This study confirms that rbcL is the most effective barcode for discriminating *D. erecta* forms, offering high amplification success, high BLAST identity, and strong phylogenetic resolution. The *ITS* region, despite lower amplification stability, exhibits highest sequence variability and potential for distinguishing closely related forms. The matK region, while useful for standardization, demonstrated limited resolution in this taxonomic context.

These findings align with CBOL (2009) recommendations for multi-locus barcoding and support the inclusion *ITS* as a potent combination for intraspecific discrimination. Given the potential hybrid origin of *D. erecta* and its capacity for gene flow, caution should be taken when interpreting sensitive morphological forms. Future work could further improve resolution using whole plastome sequencing or genomic SNP analysis to robustly address intraspecific variation.

Acknowledgements

The authors wish to express their appreciation goes to the curator of Department of Plant Biology, University of Ilorin Herbarium in person of Mr Bolu Ajayi for his support on the provision and identification of the plant specimen used for this study. Also, to the laboratory technologist for the facilities provided and time given during the research work.

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(Manuscript received on 15 December 2024; revised on 25 May 2025)