FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 10 No. 16, June, 2026, pp 741 – 744
DOI: https://doi.org/10.33003/fjs-2026-1016-5851

DNA Barcoding of *Amaranthus Hybridus* Using Rpoc1 Chloroplast DNA Region

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Received: 31 Aug. 2026
Accepted: 01 Sep. 2026
Published: 14 Sep. 2026

ABSTRACT

Amaranthus hybridus L. (green amaranth) is an important leafy vegetable widely consumed in Nigeria and valued for its nutritional and medicinal purposes. However, accurate identification of the species can be difficult because of its morphological similarity to other *Amaranthus* species. This study employed DNA barcoding to identify and characterize *A. hybridus* using the chloroplast rpoC1 gene region. Young leaf samples were collected from *A. hydridus* grown at the department of Plant Biology, University of Ilorin, from seeds obtained from NACGRAB, Ibadan. Genomic DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit and the rpoC1 region was amplified using universal rpoC1F and rpoC1R primers. The amplified products were sequenced using the Sanger sequencing method. The resulting sequences were edited and assembled using SeqTrace while BLAST analysis was performed against sequences in the NCBI GenBank database. Phylogenetic analysis was conducted using the Maximum Likelihood method in MEGA 12. A consensus sequence of 531 bp was obtained. BLAST analysis showed100% sequence identity with a reference *A. hybridus* sequence (GQ248881.1) confirming the identity of the sample. The phylogenetic analysis also placed the study sample with the reference *A. hybridus* sequence with a strongly supported *Amaranthus* clade (96% bootstrap support). The study confirms the usefulness of the rpoC1 gene region for identifying *A. hydridus* and provides additional molecular data for species. The findings may support plant identification and biodiversity documentation of *Amaranthus* based products. Further studies using multiple barcode regions are recommended to improve discrimination among closely related species.

Keywords: *Amaranthus hybridus*; Molecular identification; Phylogeny; DNA barcoding; rpoC1

INTRODUCTION

The genus *Amaranthus* belonging to the family *Amaranthaceae* comprises about 70–75 species widely distributed across tropical, subtropical and temperate regions of the world (Xu *et al*., 2024, Stetter & Schmid, 2017). Among these, *Amaranthus hybridus* L. is one of the most common and widely utilized species popularly known as smooth amaranth, green amaranth or slim amaranth. In Nigeria and much of sub-Saharan Africa, the plant is valued as a leafy vegetable and forms an important part of the daily diet of rural and urban communities commonly referred to as "Efo Tete" among the Yoruba, "Inine" in Igbo and "Aleho" in Hausa (Achigan-Dako et al., 2014). Beyond its nutritional value, *A. hybridus* is traditionally used in the management of ailments such as anaemia, diabetes, hypertension and inflammatory conditions (Aderibigbe et al., 2021, Jimoh & Oladiji, 2022) and it is rich in essential minerals, vitamins, amino acids and phytochemicals including phenolics, alkaloids and flavonoids (Bharali et al., 2021,Achigan-Dako et al., 2014).

Despite its wide distribution and importance, accurate identification of *A. hybridus* remains difficult. Species within the genus *Amaranthus* exhibit considerable morphological variation which is influenced by environmental conditions such as growth stage, habitat and frequent hybridization among congeners further complicates its identification based on physical characteristics alone (Xu et al., 2024, Stetter & Schmid, 2017). Consequently, *A. hybridus* is often mistaken for closely related species such as *Amaranthus cruentus, Amaranthus retroflexus, and Amaranthus dubius*.

DNA barcoding offers a reliable molecular alternative to morphological identification which includes the use of short and standardized regions of DNA to identify organisms and analogous to how barcodes identify commercial products (Hebert et al., 2003). Commonly used plant barcode regions include matK,trnH-psbA, rbcL and ITS (Jimoh & Oladiji, 2022,COBOL, 2009). Among these, the chloroplast rpoC1 gene has attracted attention for ease of amplification, its relatively high interspecific variation and availability of universal primers suitable for a wide range of plant taxa (Dong et al., 2012). The marker has demonstrated effective species discrimination across diverse plant families which includes*Solanaceae, Fabaceae, Poaceae, and Amaranthaceae* (Fazekas et al., 2012, Ali et al., 2014, Linh et al., 2022). The rbcL marker has similarly proven effective for species level identification of economically important vegetable crops within Nigeria such as eggplant (*Solanum melongena*) (Abdulkareem et al., 2025A). A recent multi-loci study on *Adansonia digitata* in Nigeria similarly evaluated rpoC1 alongside ITS, rbcL and psbA-trnH thereby highlighting the value of testing this marker directly within local flora (Abdulkareem et al., 2025B). This variability appears elsewhere too where Abdulkareem *et al*. (2023) found that rpoC1 and ITS gave differing results when used to barcode *Vernonia amygdalina* in Nigeria thereby reinforcing that rpoC1's performance should be used to validate species by species rather than assumed (Kress & Erickson, 2007).

In Nigeria, molecular studies on indigenous plant species remain limited despite the country's rich plant biodiversity. Applying DNA barcoding to economically and medicinally important plants such as *A. hybridus* can improve accurate species identification, support development of a molecular reference database for Nigerian plants and aid the authentication of herbal products. This study therefore aimed to identify using molecular and phylogenetical character an accession of *A. hybridus* from Nigeria using the rpoC1 chloroplast gene region.

MATERIALS AND METHODS

Sample Preparation and DNA Extraction

Amaranthus hybridus seeds were obtained from the National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria, and germinated at the Department of Plant Biology, University of Ilorin, for four weeks. Fresh young leaf samples were harvested and used for genomic DNA extraction using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, USA), following the manufacturers protocol. DNA concentration and purity were assessed using a NanoDrop spectrophotometer, with A260/A280 ratios between 1.8 and 2.0 considered acceptable for downstream PCR amplification.

Amplification Primers

The rpoC1 gene region was amplified using the universal primers rpoC1F and rpoC1R, as described by Dong et al. (2012) (Table 1).

Table 1: Amplification Primers used for rpoC1 Barcoding of *Amaranthus Hybridus*

Primer	Sequence
rpoC1F	5′-GTTATGCATGAACGTAATGCTC-3′
rpoC1R	5′-CGCGCATGGTGGATTCACAATCC-3′

PCR Amplification and Sequencing

PCR amplification was carried out in a total reaction volume of 25 µl containing template DNA (~50 ng), 1× Taq buffer, 1.5 mM MgCl2, 200 µM dNTPs, 0.4 µM of each primerand 1 U Taq DNA polymerase. Thermal cycling conditions comprised an initial denaturation at 95°C for 5 minutes; 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; followed by a final extension at 72°C for 7 minutes. Amplification success was confirmed by agarose gel electrophoresis. Positive PCR products were purified and subjected to bidirectional Sanger sequencing using both rpoC1F and rpoC1R primers.

Data Analysis

Forward and reverse chromatograms were assembled into a consensus sequence using SeqTrace software. The consensus sequence was compared against the NCBI GenBank nucleotide database using BLAST to confirm species identity. Homologous rpoC1 sequences of related *Amaranthus* species and outgroup taxa were retrieved from GenBank and aligned using the MUSCLE algorithm implemented in MEGA version 12 (Linh et al., 2022). A Maximum Likelihood phylogenetic tree was constructed using the Tamura-Nei substitution model (Tamura & Nei, 1993), with node support assessed by 1,000 bootstrap replicates. Bootstrap values ≥70% were considered statistically significant. The Maximum Likelihood method has similarly been used to resolve the phylogenetic placement of other economically important Nigerian plant species for instance, ML analysis using ITS and psbA-trnH markers clarified the subgeneric classification of Curcuma longa (turmeric) with strong bootstrap support (Tiamiyu et al., 2024).

RESULTS AND DISCUSSION

Results

Genomic DNA was successfully extracted from *Amaranthus hybridus* leaf tissue and agarose gel electrophoresis confirmed intact DNA with high molecular weight and minimal degradation. Bidirectional Sanger sequencing and assembly produced a high-quality consensus sequence of 531 base pairs.

BLAST analysis of the consensus sequence against the NCBI GenBank database confirmed the molecular identity of the sample as *Amaranthus hybridus*, with 100% sequence identity to a verified herbarium voucher (GQ248881.1). Additional top hits included *A. retroflexus* (HQ593890.1), *A. arenicola* (KP318842.1), *A. albus* (KP318841.1), A. *blitum* (KP318843.1), *A. viridis* (KP318849.1), *Celosia cristata* (KP318851.1), and *Hemicroa pentandra* (ON756470.1) (Table 2).

Table 2: BLAST Hits for the rpoC1 Sequence of the *Amaranthus Hybridus* Sample Retrieved from NCBI GenBank

Species (BLAST hit)	GenBank Accession	Query Coverage (%)	Identity (%)	E-value
Amaranthus hybridus voucher NMNH Killip 40186	GQ248881.1	92	100.00	0.0
Amaranthus retroflexus voucher AP383	HQ593890.1	95	99.61	0.0
Amaranthus arenicola	KP318842.1	100	99.24	0.0
Amaranthus albus	KP318841.1	100	98.87	0.0
Amaranthus blitum	KP318843.1	100	98.87	0.0
Amaranthus viridis	KP318849.1	100	99.24	0.0
Celosiacristata	KP318851.1	100	96.22	0.0
Hemicroa pentandra	ON756470.1	99	96.77	0.0

The Maximum Likelihood phylogenetic tree (Figure 1) placed the consensus sequence in a well-supported monophyletic sister relationship with the *A. hybridus* reference voucher GQ248881.1 with a bootstrap value 40%, confirming species identity. The consensus sequence also clustered within a broader and strongly supported *Amaranthus* clade with bootstrap value 95% alongside *A. viridis, A. retroflexus, A. arenicola, A. albus, and A. blitum*, though relationships among these congeners were only resolved at the genus level, with several internal nodes showing weaker bootstrap support between 15–52%. *Celosia cristata* occupied a near-outgroup position outside the

Amaranthus clade, while *Hemicroa pentandra* formed the most basal, outgroup lineage, validating the rooting of the phylogeny.

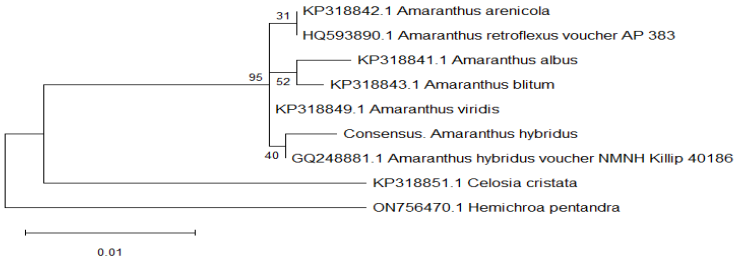

Figure 1: Maximum Likelihood phylogenetic tree of *Amaranthus hybridus* based on the rpoC1 chloroplast gene sequence

Discussion

The BLAST results obtained in this study confirm the effectiveness of DNA barcoding for resolving taxonomic uncertainty among morphologically similar plant species consistent with the foundational work of Hebert et al. (2003) and its extension to land plants by the CBOL Plant Working Group (9). The 100% sequence identity between the consensus sequence and the *A. hybridus* voucher GQ248881.1 supports the reliability of rpoC1 as a chloroplast marker for species level identification thereby corroborating the findings of Dong et al. (2012), who demonstrated that rpoC1 and related chloroplast loci exhibit sufficient variability for barcoding at low taxonomic levels.

The monophyletic clustering of the consensus sequence with *A. hybridus* confirms the molecular identity of the sample, while its broader genus level grouping with other *Amaranthus* species reflects known close evolutionary relationships within the genus. However, the generally low bootstrap support at several internal nodes and the paraphyletic relationships observed among congeneric species mirror challenges reported elsewhere in *Amaranthus* systematics. Stetter and Schmid (Moghaddam et al., 2023) similarly reported poorly resolved species boundaries within the genus using genotyping bysequencing data thereby attributing this to recent and rapid diversification among *Amaranthus* species. This suggests that while rpoC1 is adequate for confirming species identity against a reference database it may offer limited resolving power for reconstructing fine scale relationships among closely related, recently diverged congeners and a limitation also noted for plant DNA barcoding markers generally (Dong et al., 2012).

The basal placement of *Celosia cristata* and the outgroup position of *Hemicroa pentandra* are consistent with their expected phylogenetic distance from *Amaranthus* within the family *Amaranthaceae*, as reflected in broader family level phylogenomic work (Tiamiyu et al., 2024) which validate the rooting strategy used in this analysis.

These findings support the practical value of rpoC1 based barcoding for authenticating *A. hybridus* in contexts such as herbal product verification and given the plant's recognized nutritional and medicinal relevance (Xu *et al*., 2024, Achigan-Dako et al., 2014).

CONCLUSION

This study successfully identified and phylogenetically characterized an accession of *Amaranthus hybridus* using the chloroplast rpoC1 gene region. BLAST and phylogenetic analyses confirmed the molecular identity of the sample and its evolutionary relationships with other closely related *Amaranthus* species and outgroup taxa. The findings affirm rpoC1 as a reliable marker for species level confirmation, although single locus barcoding shows limited resolution for distinguishing closely related congeners which is an issue future study may address using multi locus approaches. This work contributes a molecular reference for *A. hybridus* to the global DNA barcode database with implications for plant breeding, biodiversity documentation and conservation.

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