

Using *matK* and *rbcl* as DNA barcodes for Identifying and Authenticating Selected Species of Euphorbiaceae in Nigeria

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Abstract

A molecular systematic study of seven species of the Euphorbiaceae family was conducted using two chloroplast DNA genetic barcode markers Ribulose - 1, 5 - biphosphate carboxylase (*rbcl*) and Maturase K (*matK*) to provide DNA barcodes for their unique identification from the Barcode of Life Database (BOLD) system. The seven species were of genus *Acalypha*: *Acalypha hispida* and *Acalypha wilkesiana*; genus *Euphorbia*: *Euphorbia heterophylla* and *Euphorbia hirta*; genus *Jatropha*: *Jatropha curcas* and *Jatropha gossypifolia* and genus *Manihot*: *Manihot esculenta*. For *rbcl*, only *A. wilkesiana*, *E. heterophylla*, *E. hirta*, *J. gossypifolia* and *M. esculenta* were amplified by PCR and barcoded even though they all showed high initial DNA quality before subjecting them to PCR for amplification and sequencing. For *matK*, only *E. heterophylla*, *J. gossypifolia* and *M. esculenta* were amplified and barcoded. Barcoding results showed that *rbcl* and *matK* barcode regions can be used to identify and distinguish between the various species of *Euphorbiaceae* studied. The DNA sequences of the species were used to determine phylogenetic relationship using *Mega 7* Software. The dendrogram showed that both *rbcl* and *matK* categorized the species into three groups validating existing systematic. Sequenced DNA submitted in 2020 to the BOLD system showed this was the first time these species were barcoded.

Keywords:

DNA sequencing, Phylogeny, DNA barcode, Dendrogram, *rbcl*, *matK*

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Introduction

The family Euphorbiaceae is the sixth largest plant family worldwide (Zahra *et al.* 2014) with cosmopolitan species because its distribution extends across most habitats in the world. They are economically important as sources of food, fibre, ornamentals, raw materials for industry and useful secondary metabolites used as medicines (Hohmann *et al.*, 2003; Jassbi *et al.*, 2004; Vasas *et al.*, 2004; Ahmad *et al.*, 2006; Bijekar and Gayatri, 2014). The seven species studied are from genus *Acalypha* - *Acalypha hispida* and *Acalypha wilkesiana*; genus *Euphorbia* - *Euphorbia*

heterophylla and *Euphorbia hirta*; genus *Jatropha* - *Jatropha curcas* and *Jatropha gossypifolia* and genus *Manihot* - *Manihot esculenta*. Although macro-morphological studies conducted indicate that the seven selected species are related to each other (James *et al.*, 2020) and anatomical characterization of their leaves and roots point to similar conclusions (James *et al.*, 2022a & b), research on their molecular systematics and barcoding is limited. Molecular characterization provides a quicker and more precise way of determining relationship among species that are closely related than

morphological and anatomical lines of evidence (Hollingsworth *et al.*, 2011; Awomukwu *et al.*, 2015). DNA barcoding is used to accurately identify species, study extent and distribution of variation in species gene-pools, provide correct information for taxonomic clarification and breeding purposes, for the reconstruction of phylogenetic trees, and uncover distinctiveness among closely related taxa (Peirson *et al.*, 2013; Swarna *et al.*, 2015; Tallei *et al.*, 2021; Ho *et al.*, 2021). Therefore in order to eliminate any discordance between morphological and anatomical phylogeny and obtain a more precise means of determining the relationships among these related species than those from morphological and anatomical investigation, DNA barcoding was conducted as a means for precise rapid authentication, identification and phylogenetic placement of species based on DNA sequences allowing researchers to efficiently and promptly recognize known species and discover new species hitherto unknown. It is also important for estimating and understanding molecular rate of evolution in plants and for conducting in-depth comparative studies of the extent of regional biodiversity among plants and other organisms (Khan *et al.*, 2016; Harnelly *et al.*, 2018; Pfeiler, 2018; Gao *et al.*, 2019). While DNA barcoding is no longer exactly novel given that its use was formalized since 2003 (DeSalle and Goldstein, 2019), it still offers good value for money and provides a means to categorize plants using specific genes and appropriate markers. Criteria considered in choosing DNA barcodes for plants include diversity of the taxon, comprehensive amplification of the PCR, the capacity to correctly differentiate the species, and the ability to analyze and apply required bioinformatics (Kress and Erickson, 2008). An ideal DNA barcode should also be routinely recoverable with a single pair of primers, easy to use and should lend itself to two-way sequencing without the need to edit manually the sequences (Kress *et al.* 2005; CBOL, 2009). In their review articles Vijayan and Tsou (2010) and Hollingsworth *et al.* (2011), each listed separately, fifteen DNA markers used as potential barcoding markers in plants although none has been guaranteed to provide a foolproof recognition of all plants. Two most used genetic markers are the chloroplast DNA Ribulose - 1, 5 - biphosphate carboxylase (*rbcl*) and Maturase K (*matK*). The Consortium for the Barcode of Life (CBOL)

recommended that *rbcl* and *matK* be used in combination for barcoding of plants and this has been largely accepted by scientists even though researchers are still exploring other promising options (CBOL, 2009; Pang *et al.*, 2010; Jing *et al.*, 2011; Aubriot *et al.*, 2013; Zhao *et al.*, 2015; Pahlevani, 2017; Wang *et al.*, 2017; Tallei *et al.*, 2021). After using Internal Transcribed Spacer (ITS), ITS2, *matK* and *rbcl* for species identification of some *Euphorbiaceae*, Pang *et al.* (2010), reported that ITS, and ITS2 were successful in identifying over 90 percent at the species level and 100 percent at genus level. Awomukwu *et al.* (2015) working in Nigeria, used *matK* and *rbcl* genetic markers to successfully categorize and authenticate the genus *Phyllanthus*. Oshingboye and Ogundipe (2018), also in Nigeria, conducted DNA barcoding of the sub family *Mimosoideae* using *trnL* intron, plastid *rbcl*, *matK* and ribosomal ITS. Moreover, Abdel-Hamid *et al.* (2021) conducted DNA barcoding of some taxa of genus *Acacia* using *rbcl* and *matK*. On the other hand, El-Banhawy *et al.* (2021), working in Egypt employed *ETS*, *matK*, *trnL* intron, and *trnL* intergenic spacer including the whole *ITS* section as possible DNA barcodes for *Euphorbia*. They found that subunits ITS, ITS1 and ITS2 in BLASTn showed high level discrimination among species, but the Maximum Likelihood analysis using *trnL* intron produced better resolution of phylogenetic tree than other sections. On rare occasions, genetic markers could give conflicting results as declared by Tallei *et al.* (2021) who reported that *matK* and ITS gave conflicting groupings in their study. In our study, *matK* and *rbcl* were used to investigate seven *Euphorbiaceae* species, to provide suitable barcodes from DNA for their unique identification from the Barcode of Life Database (BOLD) system.

Materials and Methods

Collecting and Identifying Plant Materials

Ten (10) fresh and healthy plant samples from each of the four genera in the family *Euphorbiaceae* (Fig 1) were collected in separate bags from five (5) locations in Rivers, Delta, Abia, Bayelsa and Imo States, Nigeria. Leaves, stems and roots were separated and preserved for study. Samples of the species were identified by comparison using proven specimens from the herbarium and affirmed using taxonomic tools and descriptions

available in documented floras (Ozimeke *et al.* 2019). Identified pressed samples were deposited at the University of Port Harcourt and Rivers State University Herbaria for

reference and further studies. The samples were preserved for 3 years at a temperature of about 20°C and 50% relative humidity.

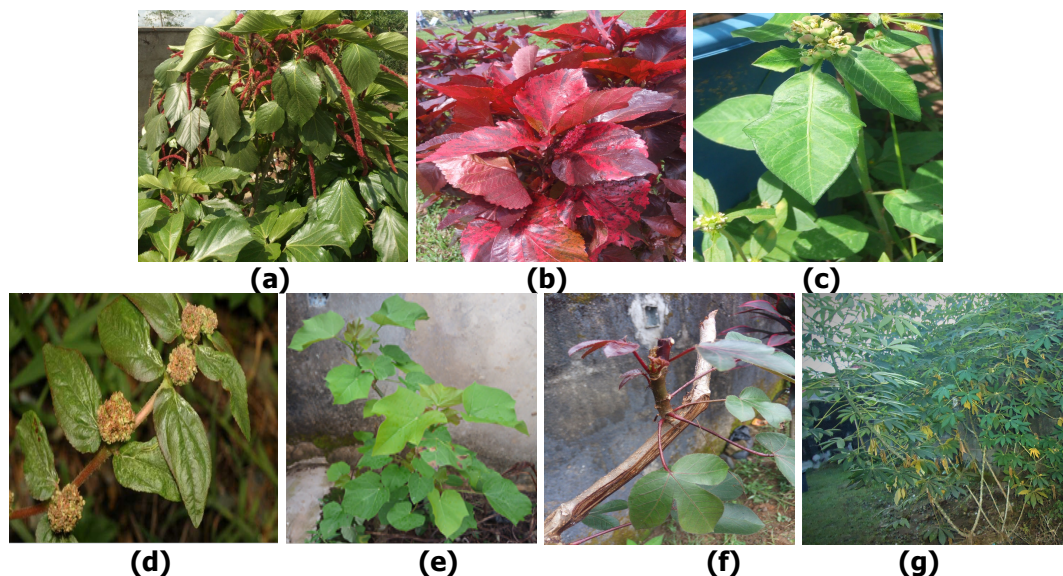


Fig 1. The seven species of Euphorbiaceae studied (a) *Acalypha hispida*; (b) *Acalypha wilkesiana*; (c) *Euphorbia heterophylla*; (d) *Euphorbia hirta*; (e) *Jatropha curcas*; (f) *Jatropha gossypifolia*; (g) *Manihot esculenta*

Extraction of DNA, Amplification and Sequencing

DNA Extraction

Briefly, DNA was obtained from 150mg finely cut fresh healthy leaf samples using the Zymo Research Quick-DNA Plant/Seed Miniprep Kit protocol provided by adding beta-mercaptoethanol for optimal performance to

bring the Genomic Lysis Buffer to dilution of 0.5% (v/v). All other protocols were carried out as described in Manen *et al.* (2005)

Amplification of DNA using *rbcl* marker

In Tables 1, 2 and 3 the methods used for PCR profiles and primers for the *rbcl* region are presented.

Table 1: Primers used for the *rbcl* PCR Amplification of the Gene Regions

Region	Primers Name	Primers Sequence 3'-5'	Primers Length
<i>rbcl</i>	<i>rbcl</i> F	5'-ATGTCACCACAAACAGAGACTAAAGC-3'	26
	<i>rbcl</i> R	5'-GTAAAATCAAGTCCACCRGC-3'	20

Table 2: Recipe for the Direct *rbcl* PCR Amplification Method

PCR Components	C ₁ = Stock Concentration	C ₂ = PCR Concentration	V ₁ = PCR Volume (μl)	Number of Samples	Cocktail (μl)
Master Mix (MX)	2x	1x	15	X8	120
Primer (F)	25μM	0.5μM	0.6		4.8
Primer (R)	25μM	0.5μM	0.6		4.8
Template	—	—	1		
H ₂ O	—	—	12.8		
	Final	Volume (V ₂)	30		102.4

Table 3: PCR Cycling Conditions for the *rbcl* Gene Regions Amplified

PCR Steps		9 cycles		35 cycles		
Initial denaturing	Denaturing	Annealing temperature	Extension	Extension	Final Extension	Hold temperature
95°C	95°C	60°C	72°C	72°C	72°C	10°C
5min.	30sec.	30sec.	30sec.	30sec	5min	∞

Amplification of DNA using matK marker

In Tables 4, 5 and 6 the methods used for PCR profiles and primers for the matK region are shown

Table 4: Primers used for the *matK* PCR Amplification of the Gene Regions

Region	Primers Name	Primers Sequence 3'-5'	Primers Length
<i>MatK</i>	<i>matK</i> -390F	3'-CGATCTATTCATTCAATATTTTC-5'	22
	<i>matK</i> R	3'-TCTAGCACACGAAAGTCGAAGT-5'	22

Table 5: Recipe for the Direct *matK* PCR Amplification Method

PCR Components	C ₁ = Stock Concentration	C ₂ = PCR Concentration	V ₁ = PCR Volume µl)	Number of Samples	Cocktail (µl)
Master Mix (MX)	2x	1x	15	X8	120
Primer (F)	25µM	0.5µM	0.6		4.8
Primer (R)	25µM	0.5µM	0.6		4.8
Template	—	—	1		
H ₂ O	—	—	12.8		
	Final	Volume (V ₂)	30		102.4

Table 6: PCR Cycling Conditions for the *matK* Gene Regions Amplified

PCR Steps		9 cycles		35 cycles		
Initial denaturing	Denaturing	Annealing temperature	Extension	Extension	Final Extension	Hold temperature
95°C	95°C	60°C	72°C	72°C	72°C	10°C
5min.	30sec.	30sec.	30sec.	30sec	5min	∞

Sequencing

The BigDye Terminator Kit on a 3510 ABI sequencer by Ingaba Biotechnological, Pretoria South Africa Sequencing was used for the sequencing. Each sample was sequenced in both sense and antisense direction in triplicate with sequencing carried out at a definitive volume of 10ul. The main components were 0.25 ul BigDye® Terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The cycling regime and sequencing conditions followed 32 cycles of 96°C for 10seconds, followed by 55°C for 5seconds and finally 60°C for 4min.

DNA Barcoding

The *rbcl* and *matK* gene regions were preferred for DNA barcoding in this study because they are the default gene markers universally used in identifying and validating plant species on Barcode of Life Database (BOLD) Systems. Each species of

Euphorbiaceae studied had a unique nucleotide barcoding pattern in BOLD and were identifiable from this unique pattern. Only barcode data / results for the successfully sequenced 5 species using *rbcl* and the 3 species using *matK* marker regions are presented.

BLAST Analysis of DNA sequence data.

The acquired sequences were edited with the aid of Bioinformatics Algorithm Trace Edit. From the National Centre for Biotechnology Information (NCBI) data base, similar sequences were downloaded by means of Basic Local Alignment Search Tool for Nucleotide (BLASTN) and aligned making use of Multiple Alignment with Fast Fourier (MAFFT). The Neighbor-Joining method in MEGA 7.0 was used in inferring the evolutionary history (Saitou and Nei, 1987). Felsenstein's (1985), bootstrap consensus tree inferred from 500 replicates is taken to

represent the evolutionary history of the taxa analyzed. The Jukes-Cantor method was used in computing the evolutionary distances (Jukes and Cantor, 1969).

Results and Discussions

Amplification of DNA Barcode Regions

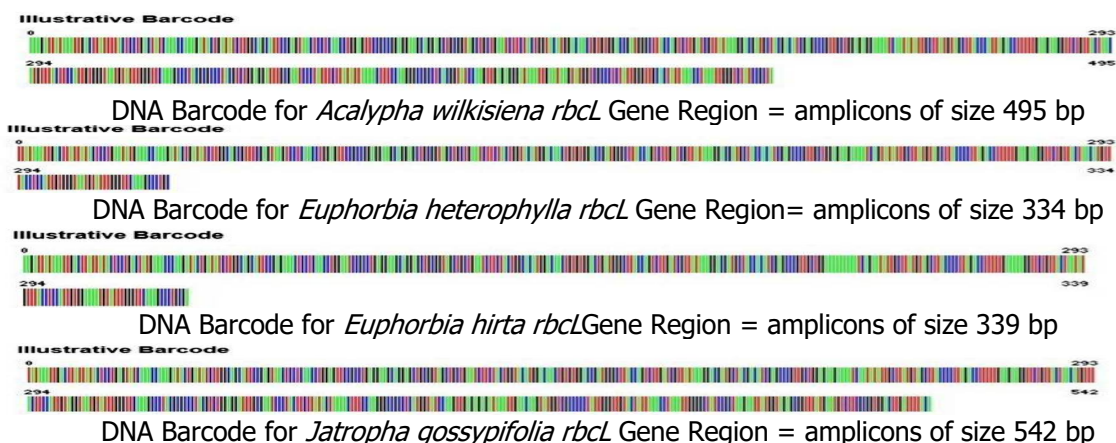
For *rbcl*, only *A. wilkesiana*, *E. heterophylla*, *E. hirta*, *J. gossypifolia* and *M. esculenta* DNA samples were amplified whereas those for *A. hispidia* and *J. curcas* did not amplify. For *matK*, only *E. heterophylla*, *J. gossypifolia* and *M. esculenta* DNA samples were amplified whereas *A. wilkesiana*, *A. hispidia*, *E. hirta*, and *J. curcas* did not amplify even though they all showed high initial DNA quality before PCR amplification and sequencing. The entire process was repeated thrice with the same results. Tharangamala *et al.* (2013) and Tallei *et al.* (2021) reported that sometimes, plant DNA can be problematic and not readily amplified. Other researchers had stated that certain compounds in plant tissues like polysaccharides and phenolic compounds sometimes inhibit PCR amplification (Jobes *et al.* 1995; Wilson, 1997). Ganie *et al.* (2015) declared that secondary metabolites such as alkaloids, tannins, polysaccharides, essential oils, and phenolics, among others even in very small quantities may inhibit PCR or even influence isolation of DNA. Since the number of secondary metabolites in the plant is elevated with aging, the age of materials used may further compound the problem, becoming more severe as the material gets older. Contaminations like these often inhibit the activities of critical enzymes such as restriction endonucleases, polymerases and ligases among others. We posit therefore that the

DNA amplification failure was not from the extraction of the DNA samples because the purity level and quality of the DNA samples tested after extraction were very high. In future, use of markers like ITS, and ITS2 that may not be as impacted by secondary metabolites could be considered (Li *et al.* 2011, El-Banhawy *et al.* 2021).

DNA Barcoding

Barcode results of the species that were successfully sequenced using *rbcl* and *matK* markers from the BOLD System are presented in Fig 2 and in Fig 3 respectively. The majority of *Euphorbiaceae* species may be easily and correctly identified based on morphological traits, but in the vegetative state, identification could be problematic, especially when some of the morphological characteristics overlap between species. An example of such a situation occurs in the genus *Acalypha* between *Acalypha hispidia* and *Acalypha wilkesiana* which have similar characteristics morphologically but can be differentiated from each other by the nature of their flowers as *A. wilkesiana* is monoecious whereas *A. hispidia* is dioecious. These two species can very easily be misidentified, at the seedling and vegetative stages when flowers are absent. Consequently, some researchers have pointed out that when closely related taxa are involved *rbcl* and *matK* show less species discriminating ability and that nuclear *ITS* should be included with the combination of *matK* + *rbcl* to have better discriminating ability in closely related species. (Li *et al.* 2010).

rbcl





DNA Barcode for *Manihot esculenta* *rbcL* Gene Region= amplicons of size 298 bp

Fig. 2. DNA barcode results from the BOLD System for five species of Euphorbiaceae amplified and sequenced in the *rbcL* Gene Region

matK



DNA Barcode for *Euphorbia hirta* *matK* Gene Region= amplicons of size 301 bp



DNA Barcode for *Jatropha gossypifolia* *matK* Gene Region = amplicons of size 577 bp



DNA Barcode for *Manihot esculenta* *matK* Gene Region = amplicons of size 233 bp

Fig. 3. DNA barcode results from the BOLD System for three species of Euphorbiaceae amplified and sequenced in the *matK* Gene Region

The barcoding results showed that both *rbcL* and *matK* chloroplast gene barcode regions identified these plant species. Awomukwu, *et al.* (2015) studying species of *Phyllanthus* using *matK* and *rbcL* markers reported successful barcodes of the studied species of the genus *Phyllanthus* of the plant family *Phyllanthaceae*. Wattoo *et al.* (2016) also declared that *matK+rbcL* as barcodes would provide an accurate way for species identification and discrimination of families of *Solanaceae*, *Euphorbiaceae* and *Fabaceae*. It is interesting to note that *Acalypha wilkesiana*; *Euphorbia heterophylla*; *Euphorbia hirta*; *Jatropha gossypifolia*; and *Manihot esculenta* had no previous submitted sequence records on BOLD Systems as at the time of their submission in 2020, indicating that this was the first time these five species were submitted and registered on the BOLD website (<http://www.boldsystems.org>).

Molecular Phylogenetic Analysis

For the successfully sequenced *Euphorbiaceae* species using *rbcL* and *matK* markers, MEGA

version 7 was used in conducting phylogenetic and molecular evolutionary analysis as described by Kumar *et al.* 2016. The substitution patterns were homogeneous among lineages and the compositional distance correlated with the number of differences between sequences. The dendrograms generated from MEGA 7 software are presented in Fig 4 and Fig 5 revealing the molecular diversity of the *Euphorbiaceae* species being analyzed using *rbcL* and *matK* gene regions respectively. The evolution record was deduced through Neighbor-Joining protocol (Saitou and Nei, 1987). The dendrogram generated from *rbcL* generated three groups or clusters. The first group is made up of *Acalypha wilkesiana* and *Manihot esculenta*, the second group contains only *Jatropha gossypifolia* while *Euphorbia heterophylla* and *Euphorbia hirta* occupied the third group (Fig. 4).

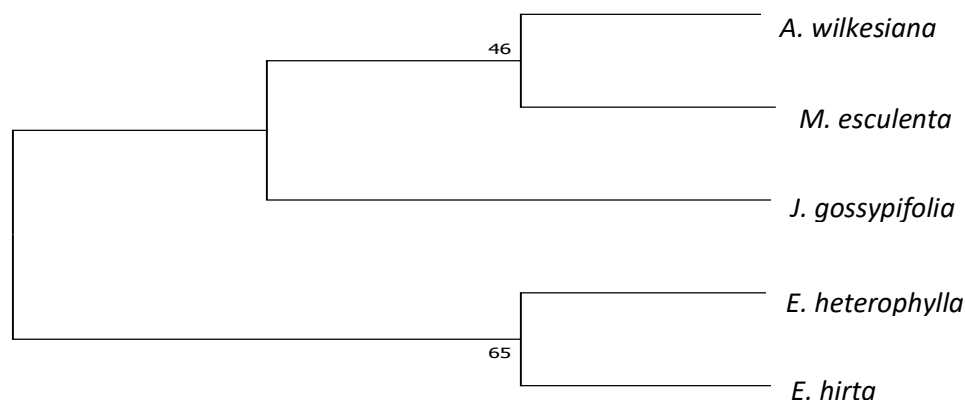


Fig. 4. Dendrogram of the Molecular Phylogeny of the five successfully sequenced Euphorbiaceae Species Studied using *rbcL* Marker

For the *matK*, three groups were obtained. The first, second and third groups consist of *Jatropha gossypifolia*, *Manihot esculenta* and *Euphorbia hirta* respectively (Fig 5). Ho *et al.*

(2021) used *rbcL* and *matK* to group jewel orchids in Vietnam into four different species with significant variations.

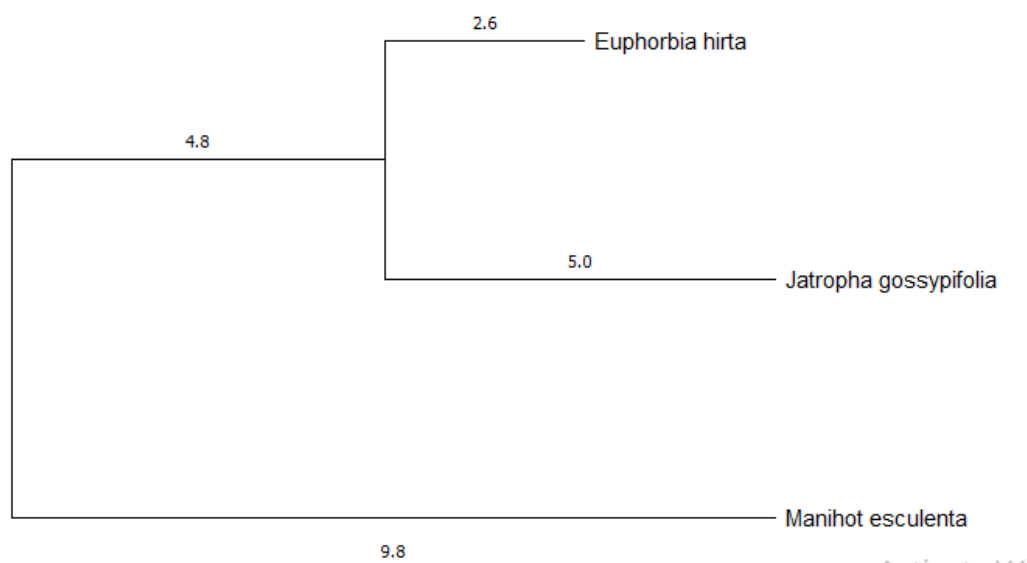


Fig. 5. Dendrogram of the Molecular Phylogeny of the three successfully sequenced Euphorbiaceae Species Studied using *matK* Marker

Conclusion

Using plastid Ribulose bisphosphate carboxylase large chain (*rbcL*) and Maturase K (*matK*) chloroplast genes as markers proved to be promising tools for authentication of the successfully sequenced Euphorbiaceae species and could in combination with other systematic lines of evidence provide better information regarding the classification of the plant group. Sequenced DNA submitted in 2020 to the BOLD system showed this was the

first time these species were barcoded. The dendrograms based on the DNA information of the *rbcL* and *matK* regions grouped these selected species into three groups for both markers.

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