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DNA Barcoding of *Vernonia amygdalina* using *ITS* and *RPOC 1* Multi Loci Gene Regions

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ABSTRACT

Vernonia amygdalina is one of the most well-known plants found in Africa and Asia and the most cultivated species of the genus *Vernonia*. The knowledge of how living and extinct species are related to one another supports much of evolutionary Biology. Therefore, this study was aimed at carrying out the molecular identification of *Vernonia amygdalina* using *ITS* and *RPOC1* regions. In this study, the identification and phylogenetic analysis of *Vernonia amygdalina* was done through amplification of the DNA using *ITS* and *RPOC1* primers. The ITS and RPOC1 sequences were compared with the existing sequences in the NCBI GenBank using BLAST searches then the sequences were aligned and maximum likelihood and parsimony trees were constructed using MEGA11. The result showed that ITS sequence length is 627bp while the RPOC1 sequence is 422bp. The blast result for ITS primer sequence showed 93% - 96% similarity against 6 accessions, 70% - 74% against 8 accessions, 64% - 69% against 6 accessions, and 82% similarity against 1 accession. Whereas the RPOC1 primer sequence gave 96% - 99% similarity against all accessions in the BLAST results. *Vernonia amygdalina, Hirpicium diffusum, Cyanthillium cinereum, Sonchus ustulatus, Olearia odorata, and Olearia laxiflora* were observed as out groups for their respective trees with the RPOC1 primer producing the highest percentage with all BLAST matches ranging from 96% to 99%. This indicates that the RPOC1 primer produced higher identification to species level.

Keywords: DNA Barcoding; *Vernonia amygdalina*; ITS; RPOC 1; Multi loci gene.

INTRODUCTION

Vernonia is one of the most well-known plants found in Africa and Asia. It is a genus of approximately 1000 species of trees, shrubs, woody climbers, or herbs in the family Asteraceae. *Vernonia amygdalina* (commonly known as bitter leaf due to its bitter taste) is the most cultivated species of the genus *Vernonia* and it has been the most noticeable species in the family of Asteraceae that had been studied in Africa (Martucci *et al.*, 2014). Aside from its numerous uses, *V. amygdalina* is also used locally in traditional medicine in South-Eastern Nigeria where it is referred to as "Onugbu" among the Igbo -speaking individuals of Eastern Nigeria. It is known as "Ewuro" among the Yoruba-speaking individuals of South-Western Nigeria and "Shuwaka" among the Hausa-speaking individuals of Northern Nigeria. Also, the different local names of *V. amygdalina* include; English-Bitter leaf, Tanzania-Omjunso, Nigeria-Onugbo, Ewuro, Etidot, Ityuna, Oriw, Chusa-doki, Shiwaka, Malaysia-South Africa leaf, Rwanda-Umubilizi, Cameron-Suwaaka, Uganda-Labwori, Omubirizi, Ekibirizi, Ghana-Awonoo, Awonwene, Jankpantire, Congo-Mpasinyioso, Zimbabwe-Musikavakadzi, Gabon-Ndoki, China-Ikaruga, Kenya-Olulusia, Ethiopia-Grawa, Graw, etc.(Nwakanma *et al.*, 2011; Hamzah *et al.*, 2013; Egharevba *et al.*, 2014; Martucci *et al.*, 2014; Francis, 2015).

Understanding phylogenetic relationships among species is fundamental for many studies in Biology, the knowledge of how living and extinct species are related to one another supports much of evolutionary Biology. Therefore, knowing the relationships among species is a significant goal in its own right and underlies our system of phylogenetic classification (Telford *et al.*, 2015). Phylogenetic analyses typically use a network of numerous steps, beginning from sequencing, to contamination removal, homology and orthology detection, multiple sequence alignment, gene tree inference, and finally species tree reconstruction. Since DNA sequencing was developed and sequence data were first used for phylogenetics, our understanding of the tree of life has transformed profoundly. For nearly two decades, molecular phylogenies were determined by data from one or a few genes, typically generated using PCR amplification and Sanger sequencing. Therefore, the development of new sequencing technologies brought about huge datasets comprising numbers of genes that have increased by orders of magnitude (Streicher *et al.*, 2016; Emms and Kelly, 2019). The molecular identification of *Vernonia amygdalina* was carried out by extracting the DNA to determine the sequence using ITS and RPOC1 primers, comparing the ITS and RPOC1 sequences with the existing sequences in the NCBI GenBank using BLAST searches, aligning the sequences and constructing maximum likelihood and maximum parsimony trees using MEGA11.

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MATERIALS AND METHODS

DNA Extraction

Young leaf samples of *V. amygdalina* were used. The specimens were cleaned and then ground with a sterile mortar and pestle. A DNeasy Plant Mini Kit and an automated DNA extraction instrument (QIAshredder Spin column, DNeasy mini spin column) were employed for DNA isolation; gel electrophoresis and an electro photometer were also employed for extracted DNA quality and concentration determination.
The primers pairs used for sequencing.

PRIMERS	SEQUENCES
ITS-1	5' TCCGTAGGTGAACCTGCGG 3'
ITS-4	5' TCCTCCGCTTATTGATATGC 3'
RPOC1 – F	5' GCAAAGAGGGAAGATTTCG 3'
RPOC1 – R	5' CCATAAGCATATCTTGAGTTGG 3'

Amplification and Sequencing of *ITS* and *RPOC1* regions

PCR amplification was performed with the barcode markers as shown above. Primers used were synthesized by Inqaba Biotec. South Africa. The PCR was carried out with a total reaction volume of 30µl in a thermo cycler (Eppendorf, Germany) (Table 1). The reaction mixture consisted of 20-50 ng of genomic DNA, 10X PCR buffer, 2.5µM dNTPs, 5pmol primers and 1µ of Taq DNA polymerase (Genet.Bio. Korea). The thermo cycler PCR condition for amplification took an initial denaturation of 95ºC for 5 min followed by 35 cycles of denaturation at 95ºC for 30secs. Annealing was at 55 ^{0}C for 30s and extension was at 72^{0}C for 1 min. Final extension was at 72^{0}C for 5mins**.** The amplicon verification was carried out on a 1% agarose gel and was purified with EZ- 10 Spin Column PCR product purification Kit (Bio Basic Inc. Ontario, Canada). Template preparation and optimum concentration for cycle sequencing reactions needed for the assay is shown in Table 2. The PCR setup for cycle sequencing reaction was done in a total reaction volume of 10µl and the thermocycler PCR condition for cycle sequencing reaction are an initial denaturation 96^{0}C for 1 min in 1 cycle. This was followed by 30 cycles of denaturation at 96^{0}C for 10s and annealing at 50^{0}C for 5s. Extension for the reaction was at 60^{0}C for 4 min and final extension was done at 60^{0}C for 7 min. The product was then purified. The various alleles were sequenced using 3130xl Genetic analyser (Applied Biosystems, CA, USA). The editing of the sequences obtained were 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 CodonCode Aligner version 4.24 (Codon Code Corporation).

The DNA sample to be sequenced was combined in a tube with RPOC1 primer and ITS primer, DNA polymerase, and DNA nucleotides (dATP, dTTP, dGTP and dCTP). The four dye-labelled, chain terminating dideoxynucleotides were added as well, but in much smaller amounts than the ordinary nucleotides. The mixture was first heated to denature the template DNA (separate the strands), then cooled so that the primers can bind to the single-stranded template. When the primer bound, the temperature was raised again, allowing DNA polymerase to synthesize new DNA starting from the primer. No further nucleotides can be added, the strand ended with the dideoxy. This process was repeated in several cycles. The dideoxynucleotide was incorporated at every single position of the target DNA in at least one reaction.

Table 1: Preparation of PCR mixture

S. No.	PCR Components	Volume (µl)
1	Milli Q water	23.0
2	Template	1.0
3	10X Taq Buffer with MgCl2	3.0
4	5 pmol/µlrbcL	1.0
5	2.5 mMdNTPs	1.0
6	1 U/µlTaq DNA Polymerase	1.0
	Total Reaction Volume	30.0

Table 2: Thermo-cycler PCR condition for barcode amplification

Step	Temperature (˚C)	Time	No. of cycles
Initial denaturation	95	5 min.	1
Denaturation	95	30 sec.	35
Annealing	55	30 sec.	
Extension	72	1 min.	
Final extension	72s	5 min.	1

Taxa Assignment

Basic Local Alignment Search Tools (BLAST) searches were applied to both ITS and RPOC1 sequences using the National Center for Biotechnology Information (NCBI) database. The generated sequences of ITS and RPOC1 of A. amygdalina were used as query sequences using E value a $<1x10^{-5}$ and maximum hits (99 or 100%) with species in the reference database of NCBI.

The sequences were aligned using Aliview fast and lightweight alignment viewer and editor of sequences and Phylogenetic analyses were conducted in MEGA11 (Tamura *et al.*, 2021) and the phylogenetic trees were inferred with the maximum likelihood and maximum parsimony method based on the Tamura-Nei model (Tamura and Nei, 1993). The topologies of the phylogenetic trees were evaluated using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

RESULTS

The results on molecular identification of *V. amygdalina* using DNA barcodes of ITS and RPOC1 fragments are presented in this chapter (Tables 3-6 and Figures 1-6).

Table 3: Blast result for ITS primer sequence.

SN	Blast match	Blast similarity	Phylogenetic affinity
1.	*Vernonia humblotii*	94%	*V. amygdalina*
2.	*Decaneuropsis eberhardtii*	96%	*V. amygdalina*
3.	*Vernonia cumingiana*	94%	*V. amygdalina*
4.	*Decaneuropsis cumingiana*	96%	*V. amygdalina*
5.	*Hesperomannia swezeyi*	96%	*V. amygdalina*
6.	*Hesperomannia arborescens*	93%	*V. amygdalina*
7.	*Pleurocarpae adenticulata*	82%	*V. amygdalina*
8.	*Hesperomannia arbuscula*	93%	*V. amygdalina*
9.	*Baccharoides adoensis*	69%	*V. amygdalina*
10.	*Gorteria diffusa*	70%	*V. amygdalina*
11.	*Echinops jesdianus*	64%	*V. amygdalina*
12.	*Monosis volkameriifolia*	69%	*V. amygdalina*
13.	*Acilepis sutepensis*	71%	*V. amygdalina*
14.	*Acilepis attenuate*	71%	*V. amygdalina*
15.	*Echinops ceratophorus*	64%	*V. amygdalina*
16.	*Dubyaea chimiliensis*	74%	*V. amygdalina*
17.	*Hirpicium diffusum*	70%	*V. amygdalina*
18.	*Lapsana communis*	68%	*V. amygdalina*
19.	*Vernonia garnieriana*	66%	*V. amygdalina*
20.	*Rhagadiolus angulosus*	74%	*V. amygdalina*
21.	*Hirpicium echinus*	70%	*V. amygdalina*
22.	*Dubyaea tsarongensis*	74%	*V. amygdalina*

BLAST = Basic local alignment search tool.

Phylogenetic affinities of ITS primers of *Vernonia amygdalina* with the identification numbers then scientific name of the accessions written. Maximum Likelihood method and Tamura and Nei (1993) were used to infer the evolutionary history. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and nodes labeled with Bootstrap values itemized as percentages and based on 1000 replications.

In the phylogenetic tree, the sample *Vernonia amygdalina* ITS sequence forms a clade with

paraphyletic association with *EF155745.1:129-563 Baccharoides adoensis* and a node with bootstrap value 69.6% containing amongst other members 3 species of *Vernonia* namely *JN715905.1:148-560 Vernonia garnieriana, EF155819.1:1-598 Vernonia humblotii and HG004807.1:81-699 Vernonia cumingiana.*
With respect to the clade with bootstrap value 97.3% containing a monophyletic group with 2 members each of *Vernonia and Decaneuropsis* viz *EF155819.1:1-598 Vernonia humblotii, HQ158396.1:22-639 Decaneuropsis cumingiana, HQ158397.1:22-641 Decaneuropsis eberhardtii and HG004807.1:81-699 Vernonia cumingiana* forms a polyphyletic association with *Vernonia amygdalina* ITS sequence. *Vernonia amygdalina* ITS sequence forms a polyphyletic association with a clade of bootstrap value 75.2% consisting of *JN715905.1:148-560 Vernonia garnieriana* and other species of *Dubyaea, Hirpicium and Echinops.*

Table 4: Blast result for RPOC1 primer sequence.

SN	Blast match	Blast similarity	Phylogenetic affinity
1.	*Centratherum punctatum*	98%	*V. amygdalina*
2.	*Cyanthillium cinereum*	99%	*V. amygdalina*
3.	*Ixeris polycephala*	98%	*V. amygdalina*
4.	*Echinacea purpurea*	98%	*V. amygdalina*
5.	*Sphagneticola calendulacea*	98%	*V. amygdalina*
6.	*Echinacea speciosa*	98%	*V. amygdalina*
7.	*Gundelia tournefortii*	99%	*V. amygdalina*
8.	*Rudbeckia hirta*	99%	*V. amygdalina*
9.	*Ratibida columnifera*	99%	*V. amygdalina*
10.	*Centipedae latinoides*	98%	*V. amygdalina*
11.	*Mikania fasciculata*	98%	*V. amygdalina*
12.	*Mikania salviifolia*	98%	*V. amygdalina*
13.	*Mikania obtusata*	98%	*V. amygdalina*
14.	*Lactuca raddeana*	99%	*V. amygdalina*
15.	*Dimerostemma asperatum*	99%	*V. amygdalina*
16.	*Sonchus pinnatus*	98%	*V. amygdalina*
17.	*Sonchus ustulatus*	99%	*V. amygdalina*
18.	*Olearia odorata*	98%	*V. amygdalina*
19.	*Olearia laxiflora*	98%	*V. amygdalina*
20.	*Ixeris repens*	98%	*V. amygdalina*
21.	*Petasites japonicus*	98%	*V. amygdalina*

BLAST = Basic local alignment search tool.

Phylogenetic Analysis by Maximum Likelihood Method with ITS primer

Phylogenetic Analysis by Maximum Parsimony Method with ITS primer

Phylogenetic affinities of ITS primers of *Vernonia amygdalina* with the identification numbers then scientific name of the accessions written. Maximum parsimony method and Tamura and Nei (1993) were used to infer the evolutionary history. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and nodes labeled with Bootstrap values itemized as percentages and based on 1000 replications.

In the phylogeny tree, the Sample *Vernonia amygdalina* ITS sequence is clustered with *EF155745.1:129-563 Baccharoides adoensis* both forming a clade with bootstrap value of 84.1%. With respect to the node with 48.1% bootstrap value, the Sample *Vernonia amygdalina* ITS sequence forms a paraphyletic association with 2 other species of *Vernonia* namely *V. humblotii* and *V. cumingiana.*
The sample *Vernonia amygdalina* ITS sequence is also in a paraphyletic relationship with *JN715905.1:148-560 Vernonia garnieriana* with respect to the node with 58.3% bootstrap value while the Sample *Vernonia amygdalina* ITS sequence is in polyphyletic association with monophyletic clusters of *Echinops, Dubyaea species.* With respect to the node 100% boostrap value. *MG833043.1:172-614 Hirpicium diffusum* was observed to be an outgroup.

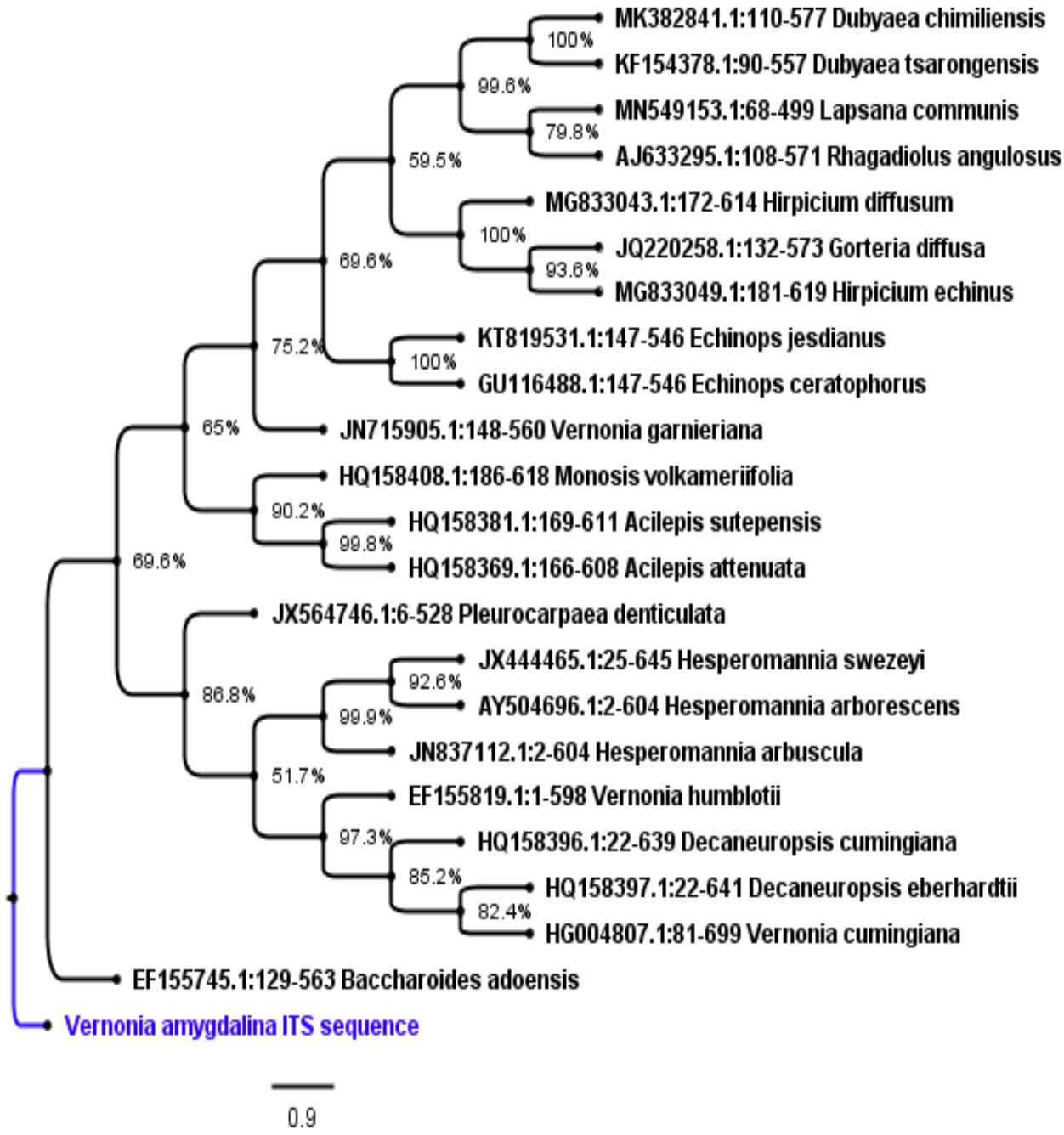


Figure 1: Phylogeny tree by Maximum parsimony method of ITS primers of *Vernonia amygdalina*

Phylogenetic Analysis by Maximum Parsimony Method with RPOC1 primer

Phylogenetic affinities of RPOC1 primers of *Vernonia amygdalina* with the identification numbers then scientific name of the accessions written. Maximum parsimony method and Tamura and Nei (1993) were used to infer the evolutionary history. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and nodes labeled with Bootstrap values itemized as percentages and based on 1000 replications.

In the phylogenetic tree, the sample *Vernonia amygdalina* rpoC1 sequence formed a clade with *KT345133.1:723-1167 Centratherum punctatum* with a bootstrap value of 49.6%. Also, with respect to the node with bootstrap value of 13.4% percent, the sample *Vernonia amygdalina* formed a paraphyletic relationship with a clade with bootstrap value 68%

containing several species viz; *Echinacea purpurea, Rudbeckla hirta var pulcherrima, Ratibida columnifera, echinacea speciosa, Mikania species as well as centipeda elatinoides.*

The sample was also in a polyphyletic group with 3 major clades with respect to the node with bootstrap value of 99.3% consisting of several species such as *Ixeris spps., Lactuca raddeana, Sonchus pinnatus, Gundelia tournefortii, Cyanthilium cinereum, Sonchus ustulatus and Petasites japonicus*. While 2 species of *Olearia* viz MW229257.1:17894-18338 *Olearia odorata* and MW229254 .1:17875-18319 *Olearia laxiflora* were observed to be the outgroup.

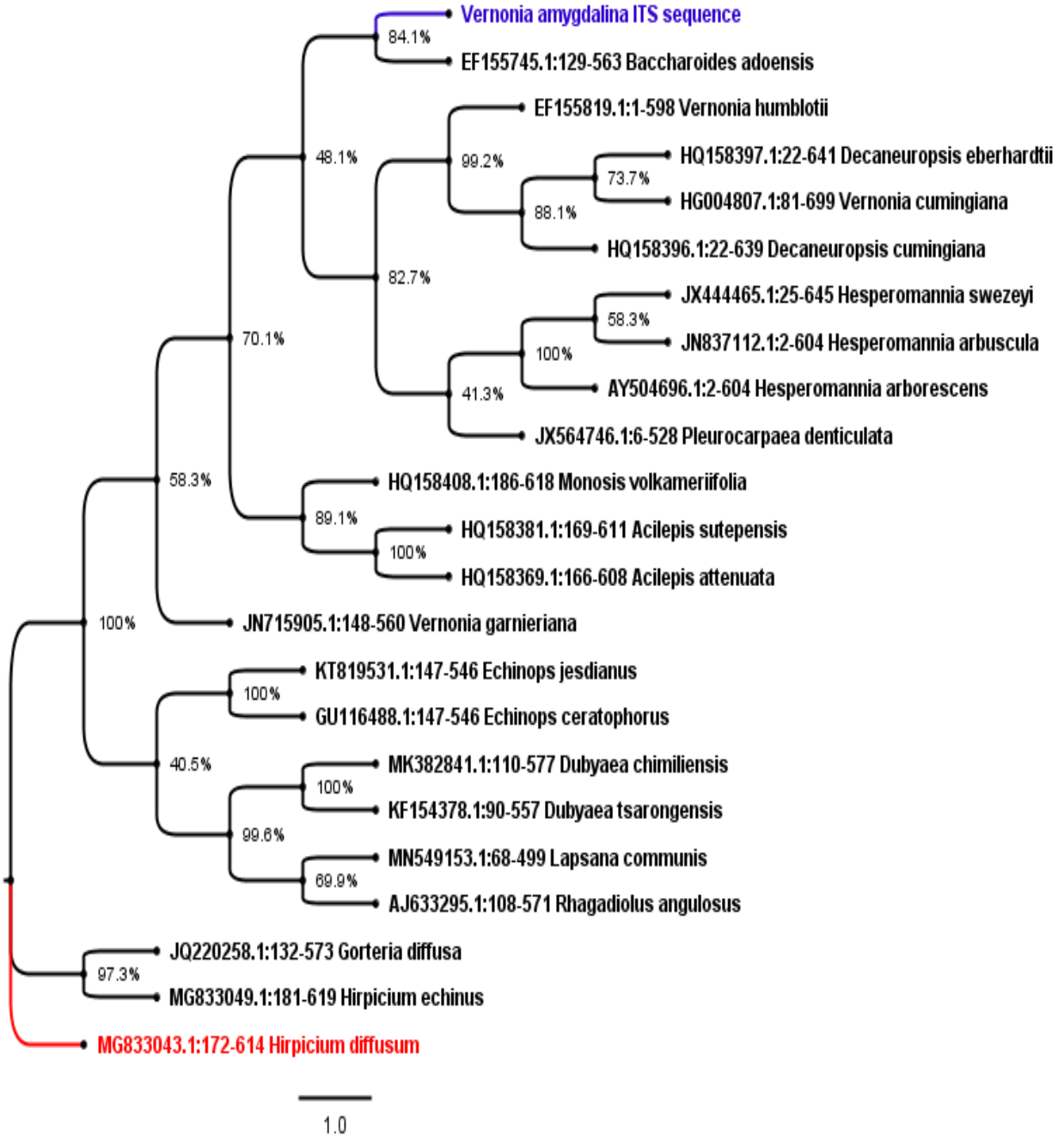


Figure 2: Phylogeny tree by Maximum parsimony method of ITS primers of *Vernonia amygdalina*

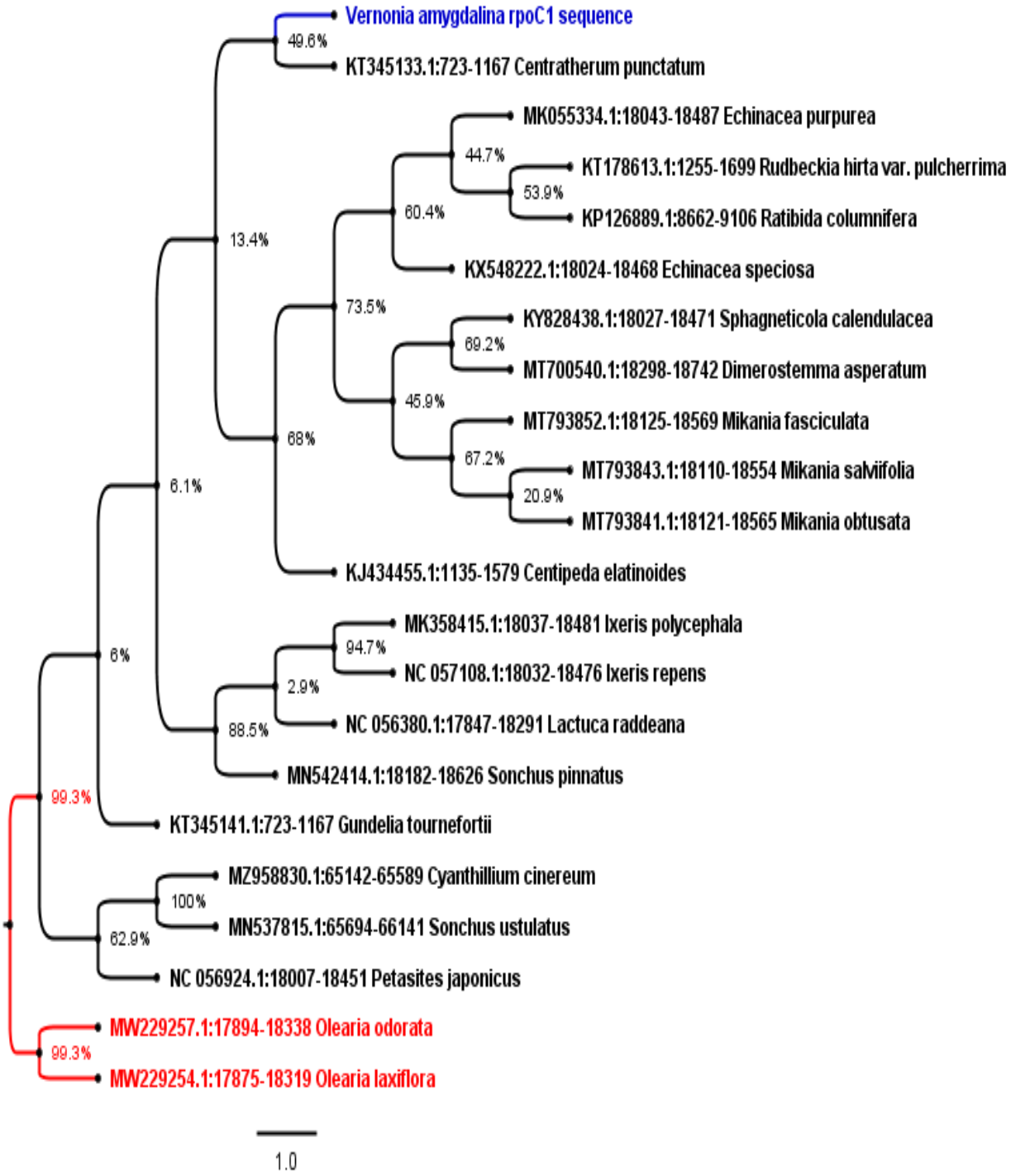


Figure 3: Phylogenetic affinities of RPOC1 primers of *Vernonia amygdalina* by Maximum parsimony method.

Phylogenetic Analysis by Maximum Likelihood Method with RPOC1 primer

Phylogenetic affinities of RPOC1 primers of *Vernonia amygdalina* with the identification numbers then scientific name of the accessions written. Maximum Likelihood method and Tamura and Nei (1993) were used to infer the evolutionary history. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and nodes labeled with Bootstrap values itemized as percentages and based on 1000 replications.

In the phylogeny tree, the sample *Vernonia amygdalina rpoC1 sequence* clustered with *KT345133.1:723-1167 Centratherum punctatum* with a bootstrap value of 68.4% forming a clade. Also, with respect to the node with 13.2% bootstrap value, the sample *Vernonia amygdalina rpoC1 sequence* formed a paraphyletic relationship with a monophyletic group consisting of 2 species of *Ixeris* namely *MK358415.1:18037-18481 Ixeris polycephala and NC 057108.1:18032-18476 Ixeris repens a*s well as other species such as *KT345141.1:723-1167 Gundelia tournefortii, NC 056380.1:17847-18291 Lactuna raddeana* and *MN542414.1:18182-18481 Sonchus pinnatus*

The sample *Vernonia amygdalina rpoC1 sequence* formed a polyphyletic relationship with a monophyletic clade containing 2 species of *Olearia* namely *Olearia odorata* and *Olearia laxiflora* with accession numbers *MW229257.1:17894-18338 and MW229254.1:17875-18319* respectively. *MZ958830.1:65142-65589 Cyanthilium cinereum* and *MN537815.1:65694-66141 Sonchus ustulatus* were observed as the outgroup.

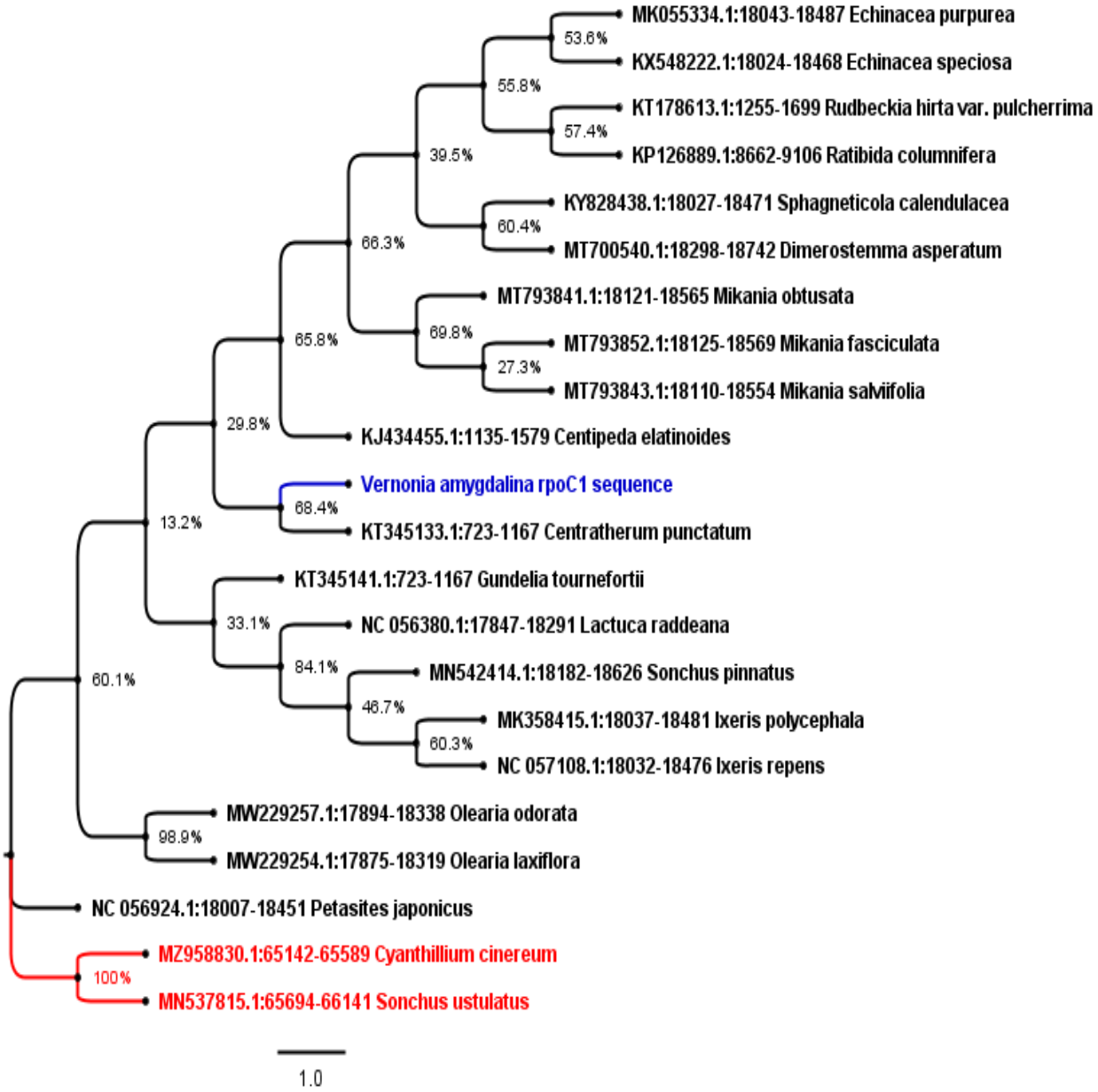


Figure 4: phylogenetic affinities of RPOC1 primers of *Vernonia amygdalina* with Maximum Likelihood method.

Phylogenetic Analysis by Maximum Parsimony Method with Concatenated sequences

Phylogenetic affinities of concatenated sequences of *Vernonia amygdalina* with the identification numbers then scientific name of the accessions written. Maximum parsimony method and Tamura and Nei (1993) were used to infer the evolutionary history. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and nodes labeled with Bootstrap values itemized as percentages and based on 1000 replications.

In the phylogeny tree of concatenated sequences of *Vernonia amygdalina* ITS sequence and *Vernonia amygdalina* rpoC1 sequence formed a paraphyletic relationship relative to each other and conversely in a polyphyletic relationship with 2 other species of *Vernonia* viz *V. cumingiana* and *V. humblotii* with accession numbers EF155819.1:1-598 and HG004807.1:81-699 respectively with respect to the node with bootstrap value 68.2%.

With respect to the major clade held by a node with bootstrap value of 20.1% the concatenated sequences formed a paraphyletic relationship with several species viz *Ratibida columnifera, Echinacea purpurea, Dimerostemma asperatum* and *Mikania fascuculata* while the 2 concatenated sequences formed a polyphyletic relationship with other species including *Ixeris polycephala, Sonchus pinnatus, Lactuca raddeana, Gundeelia tournefortii and Petasites japonicus while Olearia odorata with* accession number *MW229257.1:17894* was observed as an outgroup.

Phylogenetic Analysis by Maximum Likelihood Method with Concatenated sequences

Phylogenetic affinities of concatenated sequences *Vernonia amygdalina* with the identification numbers then scientific name of the accessions written. Maximum Likelihood method and Tamura and Nei (1993) were used to infer the evolutionary history. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and nodes labeled with Bootstrap values itemized as percentages and based on 1000 replications.

Vernonia amygdalina ITS sequence and *Vernonia amygdalina* rpoC1 sequence formed a paraphyletic association relative to each other in the phylogeny tree. The two *V. amydalina* ITS and rpoC1 sequences respectively are in a polyphyletic association with other species of Vernonia viz *EF155819.1:1-598 Vernonia amygdalina* and *HG004807.1:81-699 Vernonia cumingiana* which formed a clade scoring a bootstrap value of 98%. This polyphyletic relationship was extended to other species in the major clades held by nodes scoring 97%, 69% and 83% respectively with members including *Gorteria diffusa, Hirpicium diffusum, Rhagadiolus angulosus, Centipeda elatinoides, Mikania fasciculata, Dimerostemma asperatum, Raibida columnifera, Echinicea purpurea, Lactuca raddeana, Ixeris polycephala and Sonchus pinnatus* amongst others while *MW229257.1:17894-18338 Olearia odorata and NC 056924.1:18007-18451 Petasites japonicus* were observed as outgroup.

Table 5: Out group for ITS and RPOC1 primers.

PRIMER	PHYLOGENETIC TREE	OUT GROUP
ITS	Maximum likelihood	*Vernonia amygdalina*
	Maximum parsimony	*Hirpicium diffusum*
RPOC1	Maximum likelihood	*Cyanthillium cinereum*
		Sonchus ustulatus
	Maximum parsimony	*Olearia odorata*
		Olearia laxiflora

Table 6: Blast similarity for ITS and RPOC1 primers.

PRIMER	BLAST SIMILIARITY	NUMBER OF ACCESSIONS
ITS	93%-96%	6
	82%	1
	70%-74%	8
	64%-69%	6
RPOC1	96%-99%	21

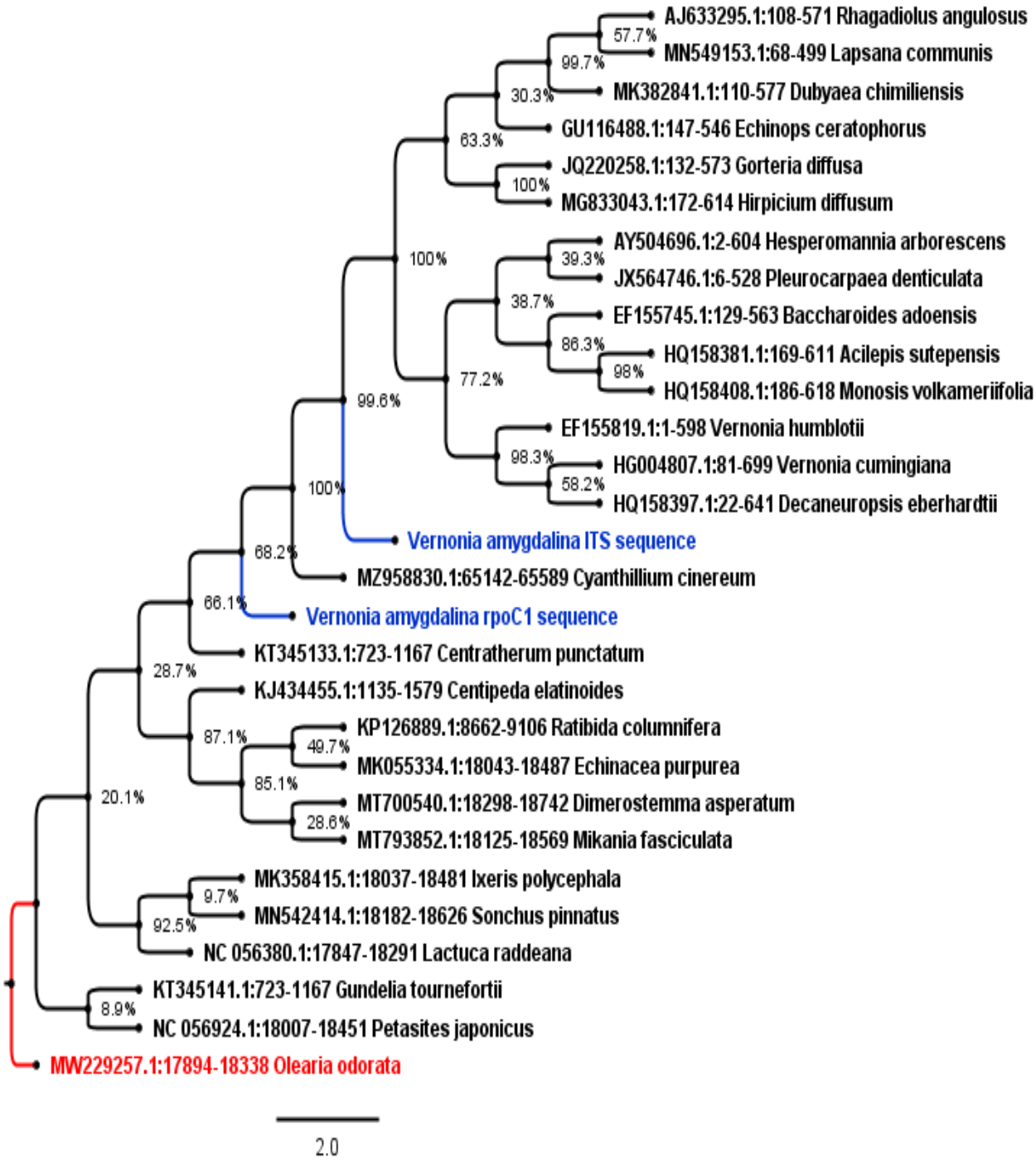


Figure 5: Phylogenetic affinities of concatenated sequences of *Vernonia amygdalina* Maximum parsimony method.

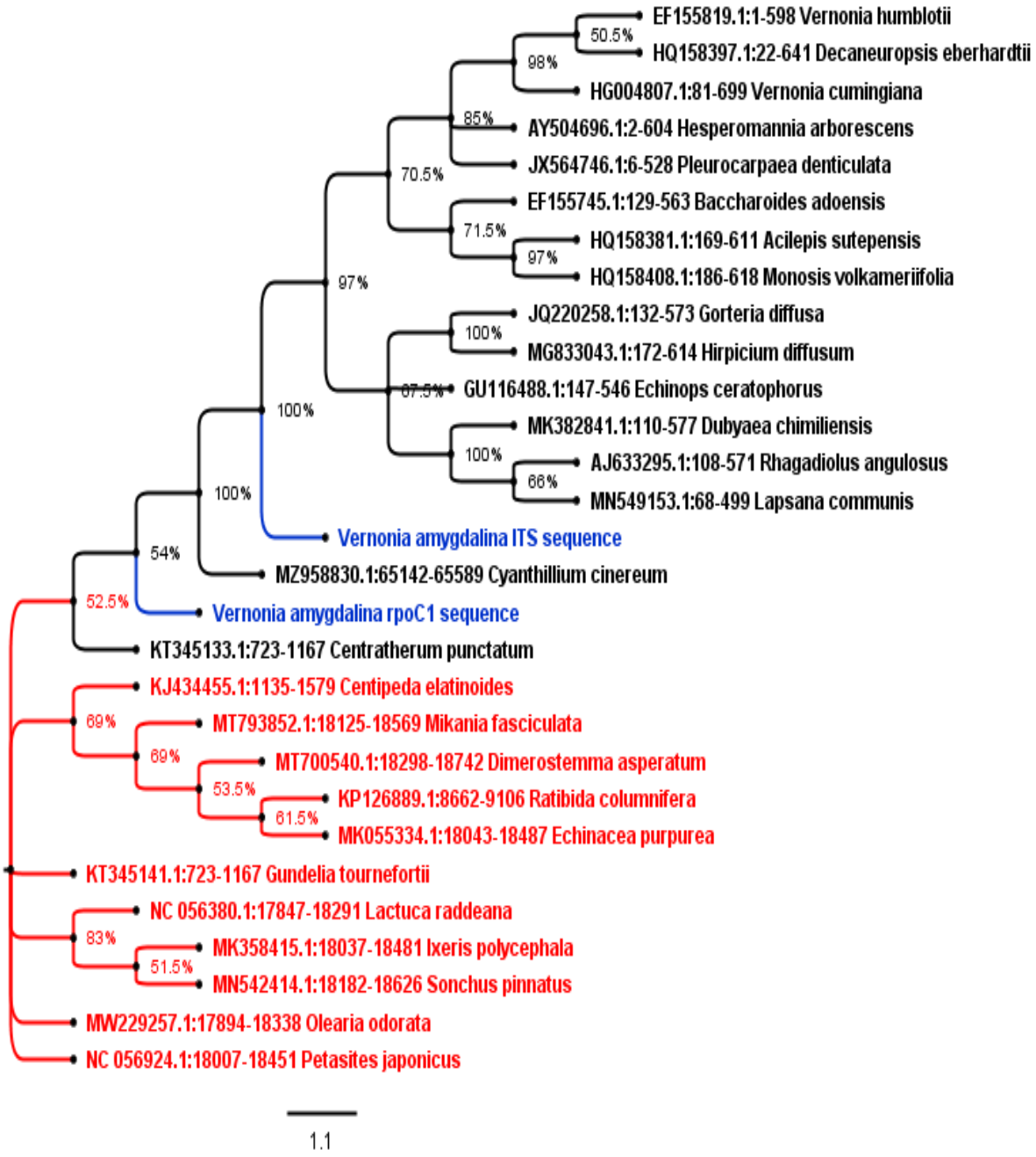


Figure 6: Phylogenetic affinities of concatenated sequences *Vernonia amygdalina* by Maximum Likelihood method.

DISCUSSION

The proper identification and evaluation of biologically important plant families play a key role in understanding evolutionary history of many important plant's species, these methods enable researchers to find similarity and differences among different plant families. Among this methods DNA barcoding is a standard and rapid method that can be used by researchers to identify and classify different organisms and Phylogenic study of necessary and important plant species is useful to identify new species (Khan *et al.*, 2015; Onstein *et al.*, 2015). Several genes are applied or used for DNA barcode studies which include rbcL, matK, trnH-psbA ,ndhf , trnL-trnF 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 and this agrees with the work of Kress *et al.* (2005).

Two primers namely ITS and RPOC1 were used for this study, the RPOC1 primer produced the highest e-value percentage with all BLAST matches ranging from 96% to 99%. Therefore, the RPOC1 primer is the most appropriate for this study. This study is the first to use ITS or RPOC1 primers for the molecular characterization of *Vernonia amygdalina*. Other studies have used primers such as OPB, OPH and OPT primers (Aikpokpodion *et al.*, 2018), OPA, OPC, OPD, and OPE primers (Nwakanma *et al.*, 2018) and rbcL primer (Eraga *et al.*, 2020).

A phylogenetic tree displays taxonomic groups in hierarchical order. In such trees, as we have in this study, species or taxonomic groups whose evolution from a common ancestor is relatively recent, share a familiar branch point called Node and they are clustered together as a monophyletic group. Several statistical methods are available for constructing phylogenic trees based on molecular data which include the distances method, parsimony method, and maximum likelihood method, amongst others. The maximum parsimony and maximum likelihood methods were used in this study unlike in the study of Aikpokpodion *et al.* (2018) whereby a cluster analysis using an unweighted pair group method with arithmetic averages (UPGMA) was used. The maximum parsimony method searches for the topology that involves the smallest number of evolutionary steps and relies on fewer assumptions compared to the likelihood method. Whereas the maximum likelihood method aims at finding a tree that maximizes the probability of observing the data for a specific substitution model.

Indicative from the phylogenetic analyses adopting Maximum Likelihood method, the sample *Vernonia amygdalina* ITS sequence shows an evident evolutionary relationship with several members of Asteraceae family which *V. amygdalina* belongs, indicative from Figure 1, the sample forms a clade with paraphyletic association with *EF155745.1:129-563 Baccharoides adoensis* proving that the ITS primers used was able to successfully identify or characterize molecularly to the family level. Also, the sample showed a great association (paraphyletic) with 3 members of the genus *Vernonia* viz *JN715905.1:148-560 Vernonia garnieriana, EF155819.1:1-598 Vernonia humblotii and HG004807.1:81-699 Vernonia cumingiana* indicating a further specificity of ITS for-DNA barcoding of Plants in the genus.

Inference from the BLAST (Basic Local Alignment Search Tool) analysis conducted (Table 3), the sample *Vernonia amygdalina* ITS sequence showed significant similarity to members of *Vernonia genus scoring* significantly high BLAST similarity percentages such as 94% by both *Vernonia humblotii* and *Vernonia cumingiana* with *Vernonia garnieriana* scoring a lower BLAST similarity percentage of 66%. It is worth noting that *Decaneuropsis eberhardii* and *Decaneuropsis cumingiana* both with 96% similarity score *belongs* to the tribe *vernonieae*, sub family *vernonieae* and family *Asteraceae* (Robinson and Kkvarla, 2007) in which the sample under study also belongs thus further supporting the efficiency of ITS for-DNA barcoding of Plants in the genus.

Inferences from phylogenetic analysis using the Maximum parsimony method (Figure 2) showed similar affinity with the Sample *Vernonia amygdalina* ITS sequence as obtained from the Maximum likelihood method as regards the phylogenetic relationship with species such as *Baccharoides adoensis, Vernonia garnieriana, Vernonia humblotii, Vernonia cumingiana* as mentioned and discussed above, thus indicating a great deal of reliability on the two methods (Maximum Likelihood and Parsimony). However, the maximum parsimony method revealed more distant species in polyphyletic association with the sample Belonging to the same Family-*Asteraceae* such members include *Echinops jesdianus, Echinops ceratophorus, Dubyaea chimiliensis, Dubyaea tsarongensis, Lapsana communis and Rhagadiolus angulosus.* While *Hirpicium diffusum* was observed as outgroup.

Phylogenetic analysis by Maximum Parsimony with rpoC1 primer revealed that sample *Vernonia amygdalina* rpoC1 sequence (Figure 3) showed great affinity with *Centratherum punctatum* both forming a monophyletic group consisting of two members of the same family (*Asteraceae*) but different genus, indicating a lesser specificity of rpoC1 primers compared to ITS primers in DNA barcoding and characterization of plants to the (*Vernonia*) genus level. The above statement is further corroborated with observance of species in paraphyletic relationship with sample *Vernonia amygdalina* rpoC1 sequence relate not at the genus level but only at the family level such species includes *Echinacea purpurea, Rudbeckla hirta var pulcherrima, Ratibida columnifera, echinacea speciosa, Mikania species as well as centipeda elatinoides. Olearia odorata and Olearia laxiflora* were observed as the outgroup.

The results shown from the Phylogenetic Analysis by Maximum Likelihood Method with RPOC1 primer (Figure 4) were consistent with that of the above i.e. Maximum parsimony method where the sample *Vernonia amygdalina* rpoC1 sequence showed great

affinity with *Centratherum punctatum* as they both clustered together forming a clade. It is also evident that rpoC1 could not reveal the genetic affinities beyond the family level as all species sample *Vernonia amygdalina* rpoC1 sequence had affinities with whether para or polyphyletic as evident in Figure 4 belonged to *Asteraceae* and no single specie was identified in the genus level. Also, *Cyanthilium cinereum* and *Sonchus ustulatus* were observed as the outgroup.

The BLAST result for rpoC1 primer as shown in Table 4, revealed 7 species having a very high BLAST similarity percentage value of 99% and 14 species scoring 98% with sample *Vernonia amygdalina* rpoC1 sequence, this implies a very high phylogenetic relationship between the species and the sample, furthermore this result corroborate the finding that rpoC1 primer identified only to the family level as all the 21 members observed belonged to the same family *Asteraceae* as sample *Vernonia amygdalina* rpoC1 sequence.

The Maximum parsimony and maximum likelihood method gave different bootstrap values for the sequences of the ITS and RPOC1 primers as shown in Table 5, as well as the concatenated sequences. The maximum likelihood tree for the ITS primer sequence presented *Vernonia amygdalina* as an out group (Figure 1) whereas the maximum likelihood tree for the RPOC1 primer presented the sister species *Cyanthillium cinereum* and *Sonchus ustulatus* as the out-group while it grouped *Vernonia amygdalina* in a clade of its own having *Centratherum punctatum* as its sister species (Figure 4). However, the maximum likelihood tree of the concatenated sequences showed that the ITS primer sequence is an evolved form of the RPOC1 primer sequence with bootstrap support of 100% (Figure 6). On the other hand, the maximum parsimony tree for the ITS primer sequence showed *Hirpicium diffusum* as the out group and *Vernonia amygdalina* in a clade and *Baccharoides adoensis* as its sister species with an 84% bootstrap support value (Figure 2). Whereas the maximum parsimony tree for the RPOC1 primer sequence showed *Olearia odorata* and *Olearia laxiflora* as the out group and presented them as sister species with a 99.3% bootstrap support value while the *Vernonia amygdalina* has *Centratherum punctatum* as its sister species with a 49.6% bootstrap support value (Figure 3). However, the maximum parsimony tree of the concatenated sequences showed the ITS primer sequence and RPOC1 primer sequence to be in different clades with the ITS primer sequence as an evolved form of the RPOC1 primer sequence with bootstrap support of 100% for ITS primer sequence and 68.2% for the RPOC1 primer sequence (Figure 5).

The sequences of the ITS and RPOC1 primers gave different blast similarity species from the NCBI database, it also gave different blast similarity values against different accessions of *Vernonia amygdalina* employed from the NCBI database as shown in Table 6. The ITS primer sequence gave 93% - 96% similarity against 6 accessions, 70% - 74% similarity against 8 accessions, 64% - 69% similarity against 6 accessions, and 82% similarity against 1 accession it was blasted against. Whereas the RPOC1 primer sequence gave 96% - 99% BLAST similarity.

CONCLUSION

This study delivers primary data for assessment which will be beneficial for the extensive use in research on DNA barcoding. Nevertheless, additional evolution on procedures to improve clean species level identification, PCR amplification processes, as well as the discovery of novel primers and local authenticated databases can offer significant roles in the effective application of plant barcoding. The presence of genetic variation in the ITS and RPOC1
primer sequences of *Vernonia amygdalina* has been established.

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