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# Andrographis Echioides Molecular Characterization and Phylogenetic Study Using DNA Barcoding Technique

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.*

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## ABSTRACT

With the advancement in various molecular diagnostic tools, DNA Barcoding has emerged as a gold standard molecular diagnostic tool across the globe. Since ancient times, medicinal plants have been widely used in Indian Ayurvedic medicine for treating a variety of ailments. Plants of the genus *Andrographis* have been extensively used for treating different types of ailments. In this study, rarely studied medicinal plant species were isolated, sequenced at the genetic level and studied for their evolutionary characteristics using phylogenetic analysis. In the present study, the identity of *A. echioides* was confirmed by targeting different barcoding genes such as ribulose-bisphosphate carboxylase, internal transcribed spacer, RNA polymerase-beta subunit, maturase K, and photosystem II protein D1 genes using a phylogenetic approach. After successful isolation and amplification of genomic DNA, specific primers were utilised for sequencing of each barcoding gene, followed by nucleotide BLAST analysis to determine the sequence percent identity of each gene with that from other plant species. The best homologs were then utilised for conducting phylogenetic analysis which confirmed the identity of the plant as *Andrographis echioides.*

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*Keywords: Andrographis echioides; DNA barcoding; phylogeny.*

## ABBREVIATIONS

| | |
|---|---|
| *ITS* | *: Internal Transcribed Spacers* |
| *rbcL* | *: Ribulose-Bisphosphate Carboxylase* |
| *matK* | *: Maturase K* |
| *psbA* | *: Photosystem II Protein D1* |
| *rpoB* | *: RNA Polymerase-Beta Subunit* |
| *CTAB* | *: Cetyltrimethylammonium Bromide* |
| *PCR* | *: Polymerase Chain Reaction* |
| *MUSCLE* | *: Multiple Sequence Comparison by Log-Expectation* |
| *ML* | *: Maximum Likelihood* |

## 1. INTRODUCTION

Recent advancements in the field of molecular biology have made plant species identification possible by targeting selected gene sets which are conserved for the given plant species. One of the techniques of plant identification by molecular sequencing is known as DNA barcoding. DNA barcoding is an advanced technique which requires only a small part of the plant by which it maintains intact diversity since one does not need to sample the whole plant. This technique employs the principle of selection of DNA region targeted for amplification which remains conserved within a species. The success of DNA barcoding is now available to plant species ever since it was first proposed to detect animals, especially since the locus is known as cytochrome oxidase unit I, a mitochondrial gene was determined as a plant barcode [1].

The technique of DNA barcoding is now extended to identify fungal species through internal transcribed spacers (ITS) of nuclear ribosomal DNA [2]. In a recent study, molecular identification of plants by the number of DNA markers is proposed which are utilized either individually or in combinations [3,4]. Few previous investigations reported targeting by primers for the two-locus-based DNA barcoding and became prominently accepted especially for plastid genes such as ribulose-bisphosphate carboxylase (rbcL) and maturase K (matK) [5]. In many studies for plant identification at the species level, the use of the internal transcribed spacer (ITS) region of nuclear ribosomal cistron is advised as a DNA marker [6]. Additionally, plasmid DNA containing trnHsbA spacer region is also one of the choices for plant DNA barcoding. However, targeting this region is tough since it represents extensive length variations, also the presence of intraspecific micro inversions associated with palindromes and sequencing problems related to mononucleotide repeats.

Even though these markers are termed standard DNA barcodes, in some cases, they cannot be used directly. For example, animal feeding on plants when screened for their gut plant species sampled is difficult to target by these barcode markers since sample plant DNA register extensive damage. It is also noted that matK marker did not cover a broad spectrum of plant taxonomic units and hence responded to limited plant species identification. As an alternative now, the involvement of variable region targeting primers are used together and able to detect plants in a concluding analysis. In recent times, the use of a plastid intron located in the tRNA Leu UAA gene has been used prominently to study diet analysis. The method also has some drawbacks like that of trnH-psbA such as length variation, and hence the extent of utility for the marker in plant identification is questionable. Still, it is prominently prescribed for plant barcoding in insects mainly for beetles. The trnL intron has also been reported successful in the identification of below-ground plant richness (from roots) [7,8].

Since ancient times, medicinal plants have been extensively used in Indian Ayurvedic medicines for treating a variety of ailments. Out of the numerous plants well known for their medicinal use, approximately 20 out of 40 plants of the genus *Andrographis* occurring in India, have been used on a large scale in traditional Indian medicine for the treatment of dyspepsia, influenza, malaria, respiratory tract infections and as astringent and antidote for poisonous insect stings [9]. Understanding the importance of DNA barcoding of individual plant species, the current study aims to conduct DNA barcode profiling of *Andrographis echioides*, which have been previously known to exhibit anti-oxidant and anti-microbial properties against a few pathogenic organisms [9,10].

## 2. METHODS

### 2.1 Collection of Plant Specimen

The plant sample was collected from the Maharaja Sayajirao University of Baroda, Gujarat, India. The collected plant specimen was immediately flash frozen in liquid nitrogen and then stored at -80℃.

### 2.2 Genomic DNA Isolation and Visual Quantification

For extraction of genomic DNA, preserved plant samples were washed twice using distilled water and small fragments of nearly 0.5mm in length were cut. These fragments were treated with liquid nitrogen and crushed using mortar and pestle to obtain a fine powder of the plant fragments. The genomic DNA was isolated using the modified Cetyltrimethylammonium bromide (CTAB) protocol. The extracted genomic DNA was resuspended in 30 µL of TE buffer until further use. The extracted genomic DNA of the plant was run on 0.8% agarose gel for visual confirmation of the successful isolation of genomic DNA. A NanoDrop spectrophotometer was used to quantify the isolated genomic DNA and to adjust the DNA concentration of the sample before initiating the Polymerase Chain Reaction (PCR) process in PCR Thermocycler.

### 2.3 PCR amplification of Barcoding Genes from Genomic DNA

The PCR technique multiplies defined regions of template genomic DNA by involving marker primers. This analysis was conducted successfully by using the PCR Thermocycler. In the present study, a total of five genes were targeted by involving the genomic DNA of plant *A. echioides.* The target genes were rbcL, ITS, psbA, rpoB and matK. The details of primers utilized in DNA amplification are represented in Table 1. The amplified products obtained from the PCR process were run on 1.5 % agarose gel for visual confirmation of amplified gene sequences.

### 2.4 Phylogenetic Analysis of *Andrographis echioides*

FASTA sequences of closely related *Andrographis* plants were retrieved and aligned along with the resultant amplified barcode genes in MEGA 11 using Multiple Sequence Comparison by Log-Expectation (MUSCLE), a multiple sequence alignment method. Phylogenetic analysis of the *A. echioides* barcoding genes sequences was carried out using the distance-based method such Maximum Likelihood (ML) method along with the Bootstrap

**Table 1. Details of Primers utilized for DNA amplification**

| Primer Name | | Primer sequence 5’ to 3’ | Amplicon length in base pairs |
|---|---|---|---|
| rbcL | Forward primer | ATGTCACCACAAACAGAGACTAAAGC | |
| | Reverse primer | GTAAAATCAAGTCCACCRCG | 613 |
| ITS | ITS-1 Forward primer | TCCGTAGGTGAACCTGCGG | |
| | ITS-4 Reverse primer | TCCTCCGCTTATTGATATGC | 700 |
| matk | matk 320 Forward primer | CGATCTATTCATTCAATATTTC | |
| | matk 1326 Reverse primer | TCTAGCACACGAAAGTCGAAGT | 980 |
| psbA-trnHF | psbA- Forward primer | GTTATGCATGAACGTAATGCTC | |
| | trnHF-Reverse primer | CGCGCATGGTGGATTCACAATCC | 681 |
| rpoB - trnCGAR | rpoB - Forward primer | CKACAAAAYCCYTCRAATTG | 1083 |
| | trnCGAR Reverse | CACCCRGATTYGAACTGGGG | |

Phylogeny test with the number of bootstrap replications set at 1000 along with the Tamura-Nei model. *Rhinacanthus nasutus* (EU725798.1) and *Thunbergia erecta* (MZ555773.1) from relatively evolutionarily close taxa were set as an outgroup [11].

## 3. RESULTS

### 3.1 Genomic DNA isolation and Purification

The plant *A. echioides* was investigated for its genomic DNA by the given protocol and later was analyzed with the help of gel electrophoresis on 0.8% agarose gel. The result obtained showed the presence of genomic DNA above 10 kb. The visualization under gel documentation showcased high-quality genomic DNA separation run along with molecular marker as given in Fig. 1.

### 3.2 Amplification of Barcoding Genes

The targeting of rbcL, ITS, rpoB, matK, and psbA gene with the genome of *A. echioides* resulted in successful amplification of 200, 400, 900, 700 and 600 base pairs as recorded with 1.5% agarose gel documentation shown in Fig. 2.

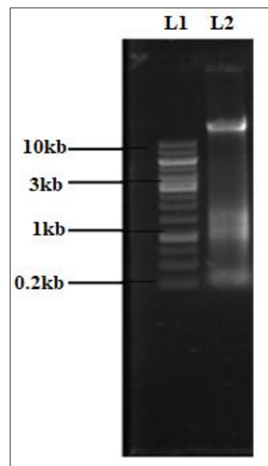


**Fig. 1. Results for genomic DNA extraction. The genomic DNA extracted from *Andrographis echioides* was analysed on 1.0% Agarose gel stained with Ethidium bromide (1mg/mL). L1: DNA ladder of 1kb. L2: gDNA of *Andrographis echioides***

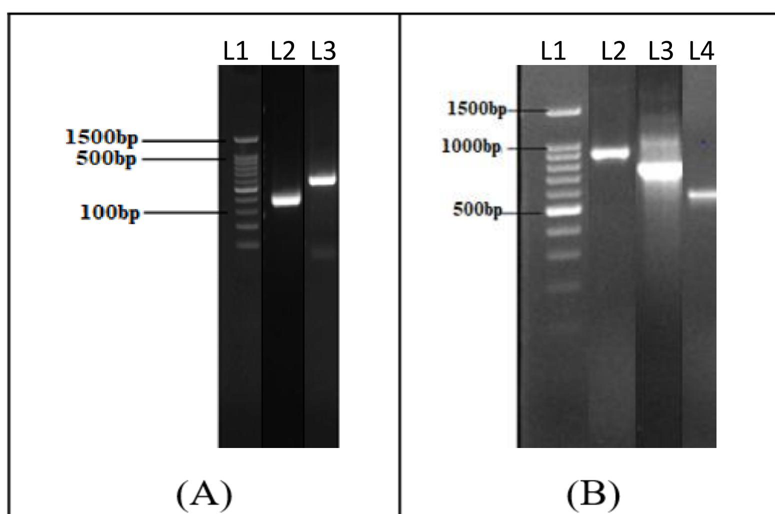


**Fig. 2. Result for PCR amplified genes from genomic DNA. (A) L1: DNA ladder of 1.5 kb, L2: amplified rbcL gene, L3: amplified ITS gene (B) L1: DNA ladder of 1.5 kb, L2: amplified rpoB gene, L3: amplified matK gene, L4: amplified psbA gene from *Andrographis echioides***

| | |
|---|---|
| 1 | GAACGTAATGCTCATAACTTCCCTTTAGATCTAGCTGCTATGGAAGCTCCAACAAATGGATAAGACTTGCTCTT<br>AGTGTATAGGAGTTTTTGAACATAGAATCCCATAAGGAGAAATAAACTTTCTTGATAGAACAAGAAAGTTTAT<br>TGCTCCTTGGTTTTCTTTTCATTTCATTTAATATTGTTTATTGTTTTTATTAATATTCTGCTTACCTAAACTTTTCT<br>TCTTTTCATTTTATAAAAATAAGTGGAAGACCTTCTAGTCTTAGGGATTGATTAATGATTGAGTATTATTCTCA<br>ATGAATTTTCTATCTAATATTGAATTCTAGGGGCGGATTAGCCAAGACGTTCG |
| 2 | AGTTCCGCCTGAAGAAGCAGGGGCAGCGGTAGCTGCCGAATCTTCCACTGGTACATGGACAACCGTGTGGACT<br>GATGGACTTACCAGCCTTGATCGTTACAAAGGGCGATGCTACAACATCGAGCCCGTTCTTGGAGAAACAGATC<br>AATATATTTGTTATGTAGCTTACCCTTTAGACCTTTTTGAAGAAGGTTCTGTTACCAACATGTTTACTTCCATTG<br>TAGGAAATGTATTTGGATTCAAAGCCCTGCGTGCTCTACGCCTGGAAGATCTGCGAATCCCTACTGCTTATATT<br>AAAACTTTCCAAGGTCCGCCTCATGGGATCCAAGTTGAGAGAGATAAATTGAACAAATATGGTCG |
| 3 | GATGCCTCTTCTATTTATTTATTACGATTCTTTCTCAACGAGTATTGTAATTGGAATACTCTTGTTAGTAGTCGG<br>TCAAAGAAAGACGATTCCTCTTTTTCAAAAAGAAATCAAAGATTCTTTTTATTCTTATATAATTCTCATGTATG<br>GGAATATGAATCCATTTTCTTCTATTTACGTAACCAATCTTCTAATTTCCGATCGACATCTTCTGGAGTTTTTTT<br>TGAACGAATCTATTTCTATGGAACAATAGAACGTCTTGGGAACGTTTTAGTTAAGGTTAAGGATTTTCAGGCG<br>AACCTATGGTTGGTCAAGGAACCTTGCATGCATTACATTAGGTATCAAAGAAGATTCATCTTGGCTTCAAAAG<br>GGACGTCAGTTTTAATGAATAAATGGAACTGCTACCTTGTCACTTTTTGGCAATGGCATTTTTCGCTGTGGTTT<br>CATCCAAGAAGGGTTTATAGAAACCAATTATCGAATCATTTCTTTGAATTTTTGGGCTATCTTTCAAGTGTCAG<br>GATCAAACCTTCAGTGGTACGGAGCCAAATTCTTCAAAATGCATTTCCAATCAATAATGCTATTAAGCAGTTT<br>GATACCCGTATTCAAATTAGGCCTTTAATTGCGTCATTGGCTAAAGCGAAATTTTGTAACGTATTAGGGCATCC<br>TATCAGTAAGCCGGTTTGGGCTAATTTATCAGATTCGAATATTATTGACCAATTTGGGCGTATA |
| 4 | TGCAGAAGATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCACTAGGCCAAGGGCACGC<br>CTGCCTGGGTGTCACCAATCGCCGCCCCAACCCCTGTGCCTCCGGCCACGGAGCGGGGCGAATGCTGGCCTCC<br>CGTGAGCACCGCCTCGCGGCTGGCTGAAAATCGGGTTCGTGGTGGATGCAGCGCCATGACAGACGGTGGTTG<br>AGCGTGACGTTCTCGAGGCCAGTCATGAGGGCGGCCTCCACCAGACGACTCCGTACCCAGCGACCCGCGAGC<br>GATGTCGATCGCCCACGACGCGACCTCAGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATATCAATAAGCGG<br>AGGAAAAGAAACTAACGAGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAGAGCCCACCATGAGAATCGG<br>TCGCCAGTGGCGTCC |
| 5 | CAGTTATTTGCAGGCGTTGCATTTATATAGTACAGCATCTTCAAATTGTAACCCTCCCATGGCATATAAGCTAC<br>TAATACGTTTTTCCCCAAAGAAAGTTCGCCACCAACTGTAGCAGCACCATCTGCTAAAATTTGTCCCCTTTTAA<br>TGCATTTACCCCGCTGAACCTGGGGTTTTTGATGCATACAAGTATTTTTGTTGGAACGTTGATACATAAGTAAT<br>GGAACGCTTAGAGTATCTCCATTGCCCGAGAAAAGGATCTTGTCAGTATCTATCGAAATAATCTTTCCCGCAC<br>GTTCGGCTATAGCAAGAGCCCCTGAATCTAGAGCTGTTTGTCGTTCCAACCCAGTTCCAACAATGCATTTCTCG<br>GACCGAGCAAGCGGAACTGCTTGACGTTGCTAAAAAA |

**Fig. 3. (1) Partial gene sequence of psbA from *Andrographis echioides* (2) Partial gene sequence of rbcL from *Andrographis echioides* (3) Partial gene sequence of matK from *Andrographis echioides* (4) Partial gene sequence of ITS from *Andrographis echioides* (5) Partial gene sequence of rpoB from *Andrographis echioides***

### 3.3 Sequencing of Marker Genes

Once the amplicon was obtained from the PCR for the gene-targeted *A. echioides* plant, it was successfully sequenced by Sanger Sequencing. The result showed, psbA gene was partially sequenced for 351 nucleotides. Similarly, genes such as rbcL, matK, ITS and rpoB were successfully sequenced for partial 360, 729, 510, and 455 base pairs respectively as shown in Fig. 3.

### 3.4 Phylogenetic Analysis

The evolutionary history was inferred using the Maximum Likelihood method [12]. The evolutionary distances were computed using the Tamura-Nei method [13,14] and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA 11.

### 3.5 Phylogenetic Analysis of psbA Gene

The analysis indicated that the psbA amplicon had 72% percent similarity with psbA gene of *Andrographis glandulosa* (Fig. 4). There was a total of 410 positions in the final dataset.

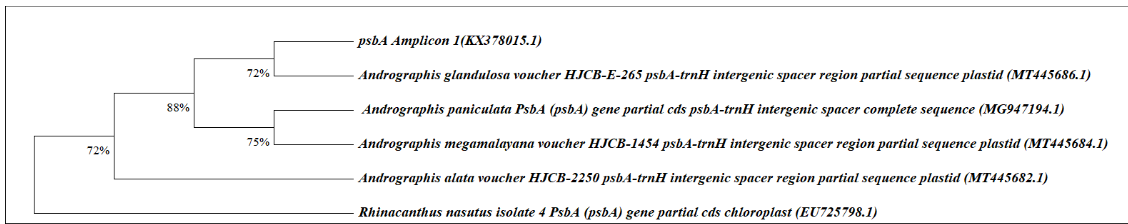


**Fig. 4. Phylogenetic tree of *Andrographis echioides* psbA gene sequence obtained by Maximum Likelihood (ML) method using MEGA 11 software. The accession numbers of the organisms are included in parentheses**

### 3.6 Phylogenetic Analysis of rbcL Gene

The analysis indicated that the rbcL amplicon had 51% percent similarity with rbcL gene of *Andrographis serpyllifolia* (Fig. 5). There was a total of 703 positions in the final dataset.

### 3.7 Phylogenetic Analysis of matK Gene

The analysis indicated that the matK amplicon had 79% percent similarity with matK gene of *Andrographis nallamalayana* (Fig. 6). There was a total of 915 positions in the final dataset.

### 3.8 Phylogenetic Analysis of ITS Gene

The analysis indicated that the ITS amplicon had 69% percent similarity with ITS gene of *Andrographis paniculate* (Fig. 7). There were a total of 738 positions in the final dataset.

### 3.9 Phylogenetic Analysis of rpoB Gene

The analysis indicated that the rpoB amplicon had 94% percent similarity with rpoB gene of *Andrographis paniculate* (Fig. 8). There were a total of 672 positions in the final dataset.

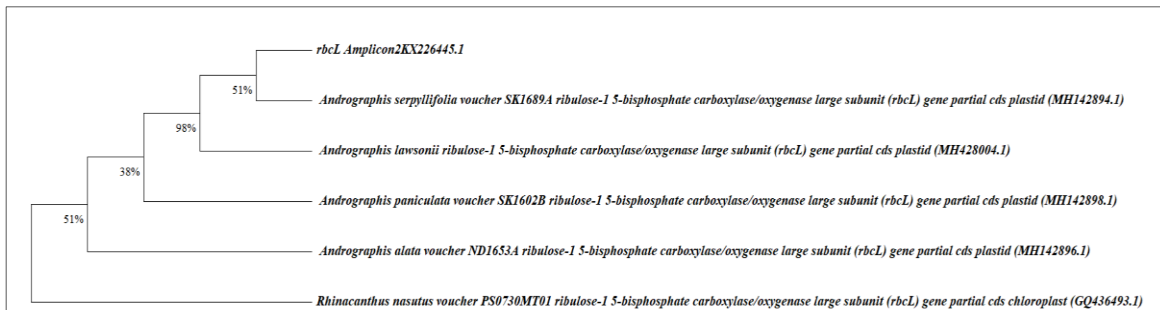


**Fig. 5. Phylogenetic tree of *Andrographis echioides* rbcL gene sequence obtained by Maximum Likelihood (ML) method using MEGA 11 software. The accession numbers of the organisms are included in parentheses**

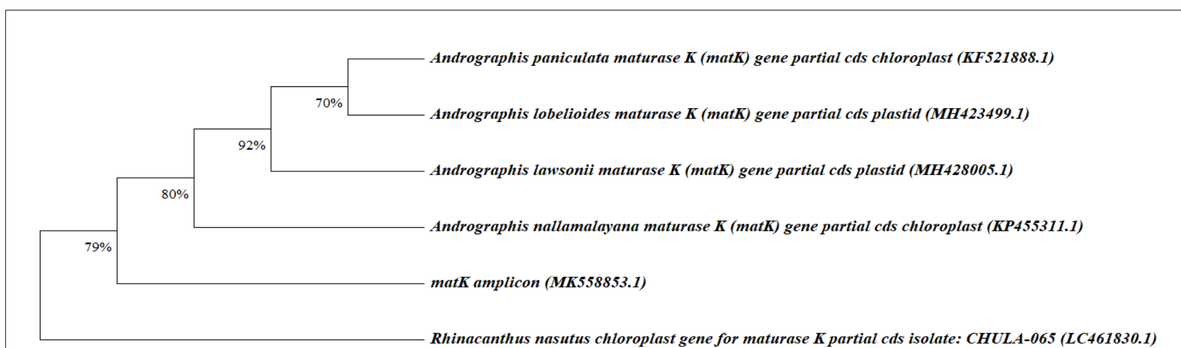


**Fig. 6. Phylogenetic tree of *Andrographis echioides* matK gene sequence obtained by Maximum Likelihood (ML) method using MEGA 11 software. The accession numbers of the organisms are included in parentheses**

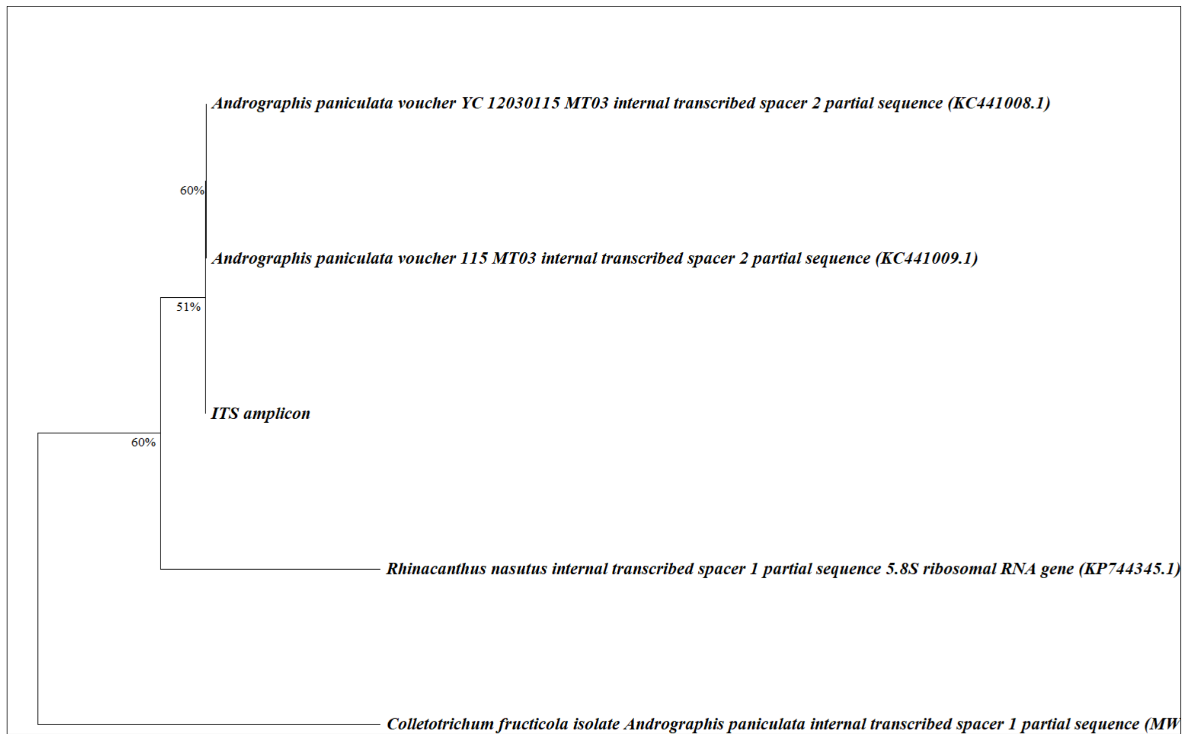


**Fig. 7. Phylogenetic tree of *Andrographis echioides* ITS gene sequence obtained by Maximum Likelihood (ML) method using MEGA 11 software. The accession numbers of the organisms are included in parentheses**

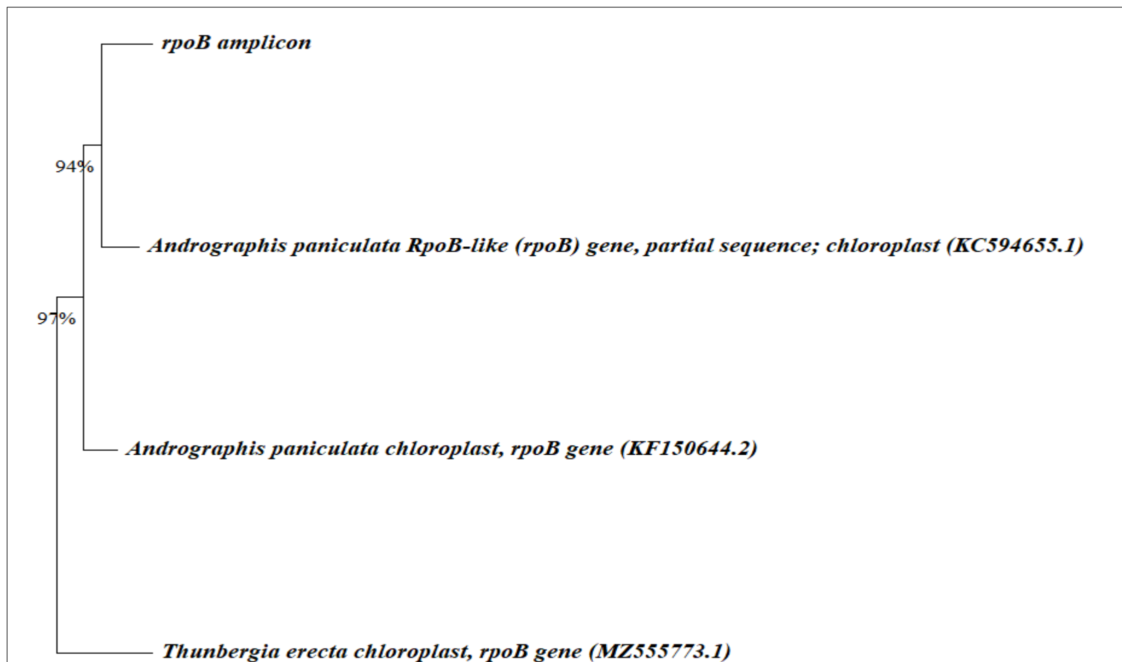


**Fig. 8. Phylogenetic tree of *Andrographis echioides* rpoB gene sequence obtained by Maximum Likelihood (ML) method using MEGA 11 software. The accession numbers of the organisms are included in parentheses**

## 4. DISCUSSION

In the present study, the identity of *A. echioides* was confirmed by targeting the ITS region. In a similar approach, a potential DNA barcode nuclear ribosomal ITS region was used previously to extensively classify plants on a phylogenetic basis [15,16,3]. According to a few authors, the ITS region is localized in the nuclear region represented some drawbacks [17,18]. Taking concern of it, the use of highly conserved chloroplast-based DNA barcodes is preferred along with nuclear ribosomal ITS [15,3]. As we also know the non-coding region is more vulnerable to genetic variation once compared with coding regions since they are responsible for protein-based functional constraints [19,20]. Taking into consideration the above points, the present study investigated the plastid-encoded psbA, matK and rpoB genes, which give more precise identification of *A. echioides*. Similar attempt earlier reported by Kress et al. [3] indicated that the use of plastid trnH-psbA spacer is a suitable locus for species identification.

## 5. CONCLUSION

The use of DNA barcoding for plant species identification by involving several nuclear and plastid-encoded genes able to identify *A. echioides* plant under investigation up to species level. The study showcased the more than 50% homology with every sequenced data obtained from psbA, rbcL, matk, ITS and rpoB gene which confirmed the experimental plant as *A. echioides*.

## DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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