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Molecular Depiction of Thirteen Indian Toxic Plants with ITS Markers

التوصيف الجزيئي لثلاثة عشر نباتًا هنديًا سامًا باستخدام معلمًات ITS

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Abstract

Plant identification is an overwhelming task due to different biological attributes and great diversity in plant species. In the absence of physical markers, molecular techniques have become useful for the identification of species of origin of medicinal plant seeds, pastes, and formulations of suspected plants.

The Internal Transcribed Spacer (ITS) region of nuclear rRNA was amplified from thirteen different toxic plant species by using universal primer ITS 1 & 4. Nucleotide sequences of all selected plants were submitted in NCBI and accession numbers were acquired.

The results of this study give accurate identification of thirteen plant species and proved the ITS region of 18s-26s nuclear ribosome to be an important tool for phylogenetic analysis and species identification of plants. The sequence was aligned with top matched reference sequence and presented in Clustal Omega software for making a phylogenetic neighbour tree.

The significance of these findings is paramount in forensic toxicology scenarios especially when fragmentary plant material is found in the stomach/intestine and its morphological identification becomes impossible. In these circumstances, the PCR based molecular technique surely plays a significant role in solving complicated forensic cases.

Keywords: *Forensic Science, Forensic Botany, ITS Region, Universal Primers.*

المستخلص

غالبًا ما يكون تحديد النباتات مهمة شاقة بسبب السمات البيولوجية المختلفة والتنوع الكبير للأنواع النباتية. وفي ظل غياب العلامات الفيزيائية تصبح التقنيات الجزيئية مفيدة في الاستخدام للتعرف على أنواع منشأ البذور الطبية والمعاجين والمستحضرات النباتية المشتبه بها. وفي هذه الدراسة تم تضخيم منطقة ITS من rRNA لثلاثة عشر نوعًا مختلفًا من النباتات السامة باستخدام البادئ الشامل ITS 1 & 4. وتم تقديم تسلسل النيوكليوتيدات لجميع النباتات المختارة في NCBI وتم الحصول على أرقام القيد.

وتعطي نتائج هذه الدراسة تحديدًا دقيقًا لثلاثة عشر نوعًا نباتيًا وأثبتت أن منطقة ITS من الريبوسوم النووي 18s-26s لتكون أداة مهمة للتحليل الوراثي للنباتات، والتحقق من التنوع الجيني وتحديد الأنواع النباتية. وتمت مواءمة التسلسل مع أعلى تسلسل مرجعي متطابق وتم تقديمه في برنامج Clustal Omega لإنشاء شجرة النشوء والتطور التجاوري.

وتعتبر هذه النتائج ذات أهمية قصوى في سيناريوهات علم السموم الجنائي خاصة عندما يتم العثور على مادة نباتية مجزأة في المعدة/الأمعاء، ويصبح تحديدها المورفولوجي مستحيلاً. في هذه الظروف من المؤكد أن التقنية الجزيئية القائمة على تفاعل البوليميراز المتسلسل تؤدي دورًا مهمًا في حل قضايا الجنائية المعقدة.

الكلمات المفتاحية: علوم الأدلة الجنائية، علم النبات الجنائي، منطقة ITS ، البوادئ الشاملة.

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1. Introduction

Since time immemorial, medical practitioners have used a wide variety of plants for the treatment of various diseases. Despite their medicinal properties, plants produce some harmful products like alkaloids, tannins, glycosides, toxalbumins, etc. that cause adverse toxic effects in humans and livestock [1]. A plant is regarded as poisonous and toxic if it causes gastrointestinal and dermatological disorders upon intentional or unintentional exposure [2]. Literature reports include cases where substitution or adulterations of incompatiblle plant ingredients in herbal products can cause health and safety problems [3-7]. The forensic expert generally analyzes toxic plants and their metabolites from the biological materials (stomach content and viscera) of victims. Plants material is identified by conventional morphological methods and molecular biological techniques. The metabolites in the forensic sample are identified by chemical methods. Trace amounts of scraped plant material (crushed seeds/leaves) recovered from the food, drinks, and body (stomach/vagina/rectum) need forensic identification and confirmation of plant species. Lack of morphological features and insufficient quantities make the forensic analyses more challenging.

The advancement in molecular biology that has the capability to enhance the individualization of small botanical evidence in routine forensic science, is exploited for such analyses [8-10]. Literature proposed the DNA barcoding and Cytochrome C oxidase 1 (CO1) gene of the mitochondrial genome for taxonomical identifications. CO1 is often used for animal species differentiation and has a comparatively lesser use in plant species differentiation. DNA barcoding can be used to differentiate species of the ingredients of herbal preparations during all stages of product processing. DNA can be extracted from small amounts of material such as those found as leaf litter, dung, pollen, seeds, dried or degraded herbarium voucher specimen, fecal sample, permafrost preserved sub-fossils, and ancient sediment cores [7, 11-13]. The literature contains numerous examples where various DNA profiling methods including Randomly Amplified Polymorphic DNA (RAPD), Short Tandem Repeats (STRs), and microsatellites were used to link suspect-victim-crime scenes. The success rate of accurate identification of species from pieces of botanical evidence depends on the taxonomic coverage of the reference database. DNA barcodes using mitochondrial, plastid, and nuclear genomes as well as four primary gene regions i.e. rbcL, matK, trnH-psbA, and internal transcribed spacer (ITS) have been proposed for forensic analyses of suspected toxic plants [14-17]. ITS is considered as a local barcode in a plant when plastid DNA recovered in a low amount is used to identify genera, species as well as evolutionary studies [1,11,18]. The use of nuclear ribosomal RNA cistron as a unit of RNA polymerase I that consist of 18S, 5.8S, and 26S rRNA genes [19] is shown in Figure-1. The Internal transcribed spacer (ITS) consists of two non-coding regions, which are variable for molecular identification of plants, animals, and fungus, that can easily identify species and genera and have the ability to distinguish closely related plant species [17,19-21]. The literature contains various proposed methods to match DNA barcodes to a reference library for identification purpose i.e. similarity-based, phylogenetic tree based, character-based and statistical methods [22-25].

The present study evaluated the ability of the ITS marker to identify thirteen different toxic plant species. Identification is based on BLAST sequence similarity by taking the top five hit sequences to produce an optimized result.

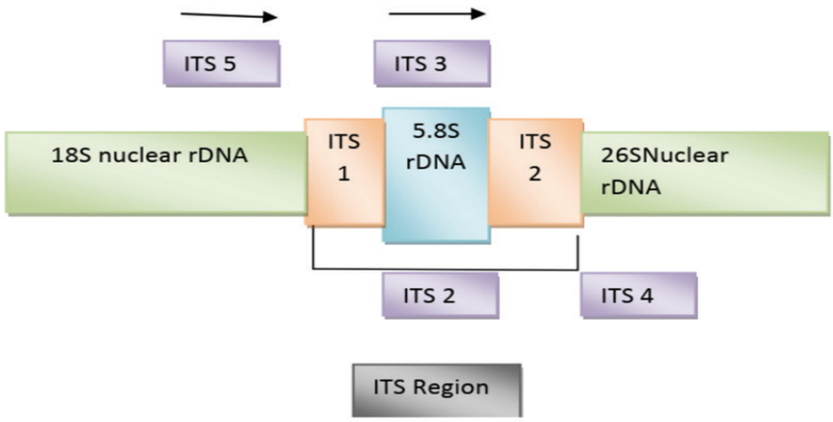


Figure 1- *ITS Region of nuclear rRNA [19].*

2. Materials and Methods

2.1 Study sites, collection & identification of samples

Thirteen potentially toxic plants of forensic interest were collected from Rohtak district of Haryana situated in the northern part of India. Samples were collected from the study sites during 2016-2019. Collected samples were morphologically identified using available monographs and flora of Haryana [26-28]. All plant specimens were deposited in the Forensic Chemical Sciences Laboratory of the Department of Genetics, Maharshi Dayanand University Rohtak, Haryana (India).

Table 1- *Master mixture for PCR amplification.*

PCR Reaction Mixture	Volume (50 µL)
Genomic DNA	~20 ng
dNTPs mix (2.5mM each)	1.0 μL
Forward Primer	0.5 μL
Reverse Primer	0.5 μL
Taq Buffer A (10X)	1X
Taq Polymerase enzyme	3U
Bovine Serum Albumin	0.1 μL
Distilled water	to make up the volume 50 μL

**ng=nanogram * µL= microliter *U=unit*

2.2 Isolation of DNA

All the collected fresh leaf samples of these toxic plants were washed individually with running water and left at 27 °C for 10 to 15 days. Dried leaves were ground into powder form with a sterilized mortar and pestle in liquid nitrogen. Approximately 100 mg of dried leaf powder of each selected plant was used for isolation of total genomic DNA following the Cetyltrimethyl Ammonium Bromide (CTAB) method [29]. The extracted DNA samples were electrophoretically analyzed in 0.8% Agarose gel and evaluated by the Gel documentation system (DNR Bio-Imaging Systems). The isolated DNA samples were stored at -20°C temperature for PCR amplification. Purity and quantity of DNA were checked using a UV spectrophotometer by calculating absorbance ratio A260/A280.

2.3 Polymerase chain reaction amplification of ITS region

Isolated nuclear DNA was amplified using a universal primer of ITS 1(forward primer 5'GGAAGTAAAAGTCGTAACAAGG3') and ITS 4 (reverse primer 5'TCCTCCGCTTATTGATATGC3') [20]. PCR amplification reactions were carried out in 50µl volumes. Table-1 indicates each PCR reaction tube contained the reaction mixture. Table-2 reveals the PCR experimental conditions for ITS gene amplification. PCR amplified products of respective bands size for ITS (~700bp) were checked in 2% agarose gel using StepUp™ 100 bp DNA ladder. Gels were photographed under a gel documentation system. Table-3 shows top matched homology with a coverage percentage and accession numbers for each species.

Sequencing was outsourced to Eurofins Scientific India Pvt. Ltd, Bangalore (India). Nucleotide sequence results (forward sequences) by Sanger dideoxy method, were aligned on NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify similarity/homology from the nucleotide database. The query fasta files were submitted to sequence submission tools for ribosomal RNA (rRNA) NCBI to obtained the accession number from https://www.ncbi.nlm.nih.gov/WebSub/. Phylogenetic analysis of closely related plant species were retrieved from the gene bank. Sequence alignment of thirteen identified plant species were presented in Clustal Omega software to make a phylogenetic neighboring tree.

Table 2-*Experimental conditions for PCR thermal cycling.*

Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Final Hold
94°C	94°C	55°C	72 °C	72 °C	4 °C
5 min	30 sec	30 sec	1:30 min	10 min	∞
1 cycle		35 cycles		1 cycle	

Table 3-*Gene Bank accession numbers of selected toxic plants along with query coverage and percent identity.*

Gene bank Accession No.	Scientific name	Query cover %	Percent identity %
VSN- FS001 AN-MH777018.1	Thevetia neriifolia	99%	99.00%
VSN-FS002 AN-MH734617	Calotropis procera	100	97.33%
VSN-FS003 AN-MH915658	Lantana camara	91%	99.11%
VSN-FS004 AN-MH734908	Solanum villosum	98%	97.44%
VSN-FS009 AN-MH923185	Jatropha curcas	99%	99.26%
VSN-FS012 AN-MH734909	Argemone mexicana	100%	97.47%
VSN-FS014 AN-MH734808	Citrullus colocynthis	96%	99.73%
VSN-FS016 AN-MH735183	Datura metel	97%	98.52%
VSN-FS021 AN-MH915657	Senna alata	98%	99.29%
VSN-FS022 AN-MH756619	Ipomoea carnea	90%	99.31%
VSN-FS026 AN-MH756191	Parthenium hysterophorus	97%	95.78%
VSN-FS040 AN-MH762134	Datura inoxia	91%	99.39%
VSN-FS060 AN-MH756599	Abrus precatorius	97%	97.18%

3. Results

Figure-2 shows the High-quality DNA was isolated from dried leaves of the thirteen selected toxic plants using standard protocol [29]. Quantity of DNA (approx. 1250 ng) was checked by using a UV spectrophotometer with an absorbance ratio (A260/A280=1.5-1.8). A dilution of 20 ng/µl was generated from 1250 ng/100 µl by adding a TE pH 8.0 buffer. Amplification of the extracted DNA for ITS regions was visualized through a gel documentation system (DNR Bio-Imaging Systems) and is shown in Figure-3.

The amplified sequence of ITS was a blast in Gene Bank utilizing BLASTn analysis and implemented in the GenBank web interface (http://blast.ncbi.nlm.nih.gov). The previously published sequence of the respective selected plant genus in GenBank was also used as a reference in this study. After nucleotide BLAST analysis for all sequences it was observed that selected samples have been correctly identified up to genera and species level, an NCBI accession number was obtained with its query coverage & identity percent as presented in Table-3. Gene Bank accession numbers were obtained after processing the data through ribosomal RNA (rRNA) NCBI tool as shown in Table-3. The closely related top matched sequences retrieved from GenBank were used for phylogenetic analysis. The phylogenetic analysis of the thirteen plant species was computed using blastn x software the neighbor-joining method as shown in Figure-4. All referenced sequences obtained from nucleotide BLAST analysis were then aligned in Clustal Omega software and their phylogenic tree with a neighbor-joining distance of the studies species are shown in Table-4. Some of the variations of names used for the same plants throughout different races, ethnics, and geographic range are likely the most important reasons for the mismatch in species identification by nucleotide BLAST analysis.

4. Discussion

Authentication of botanical pieces of evidence (such as tree barks, roots, leaves, seeds, etc.) is helpful in distinguishing closely related plant species and in identifying adulteration with substitute or adulterant plant species from dried raw drug specimens [31]. Earlier studies reported different markers for molecular identification of plant species. ITS as a DNA marker for molecular identification of some closely related plants, fungi as well as animals have proved successful [20,32]. The literature has reports on ITS region of nuclear ribosomal DNA used to identify and distinguish two plant species i.e. Gmelina asiatica and Mallotus nudiflorus to authenticate plant species [33]. Literature reports have emphasized genetic markers on nuclear rRNA, which is a more conserved DNA sequence and which has a low mutation rate. The ITS region of nuclear rRNA i.e. 500-700bp in angiosperms and 1500-3500bp in gymnosperm, with the help of their universal primer are commonly used for species

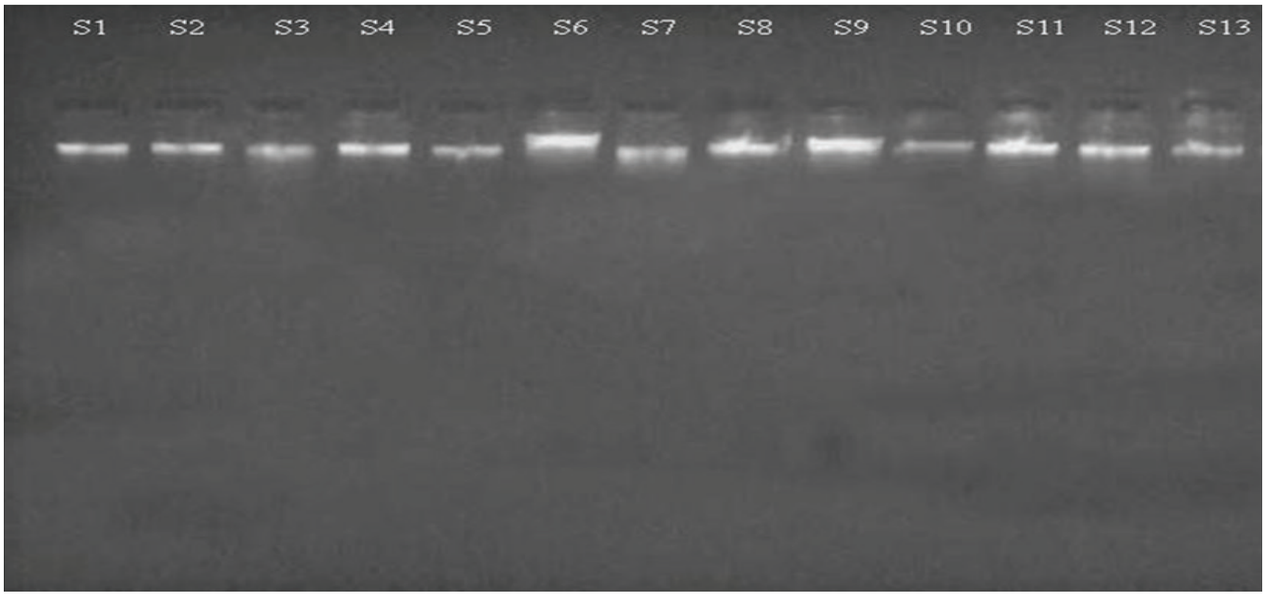


Figure 2- *Electropherogram of genomic DNA band of selected plants-S1: Thevetia neriifolia; S2: Calotropis procera; S3: Citrullus colocynthis; S4: Solanum villosum; S5: Argemone mexicana; S6: Datura metel; S7: Senna alata; S8: Parthenium hysterophorus;S9: Abrus precatorius; S10: Ipomoea carnea; S11: Datura inoxia; S12: Lantana camara; S13:Jatropha curcus.*

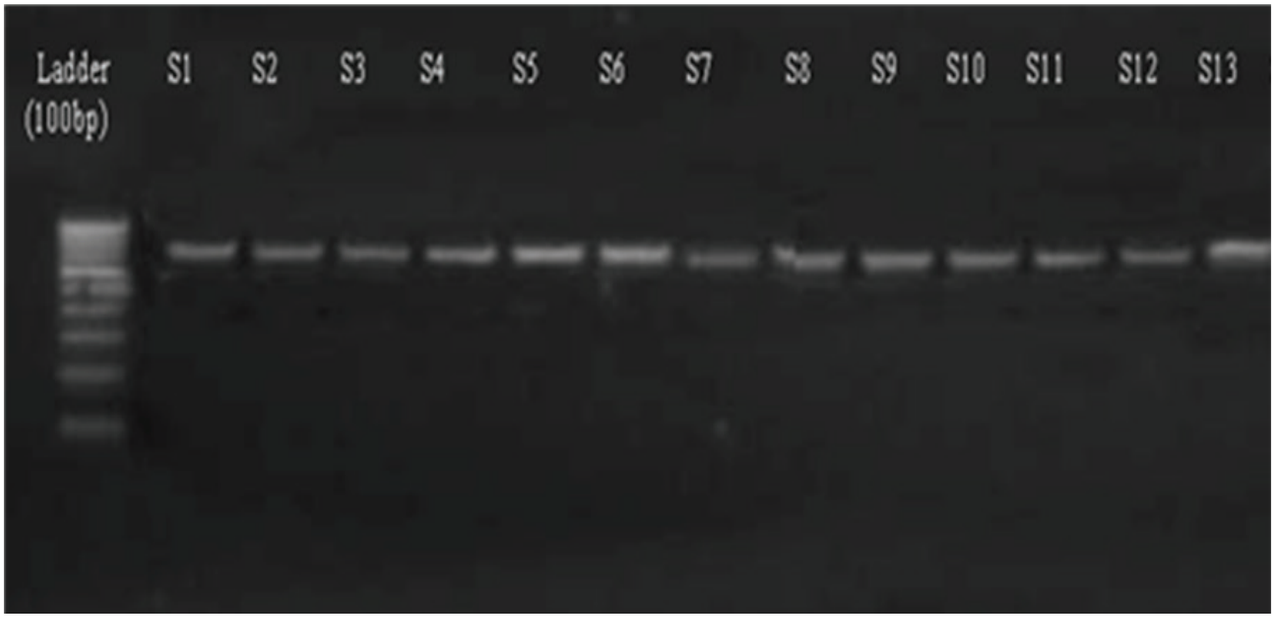


Figure 3- *Agarose gel electropherogram showing PCR amplified product of ITS 1 & 4 primer (~700 bp) of 13 plant species (S1 to S13) with 1000bp ladder; S1: Thevetia neriifolia; S2: Calotropis procera; S3: Citrullus colocynthis; S4: Solanum villosum; S5: Argemone mexicana; S6: Datura metel; S7: Senna alata; S8: Parthenium hysterophorus; S9: Abrus precatorius; S10: Ipomoea carnea; S11: Datura inoxia; S12: Lantana camara; S13: Jatropha curcus.*

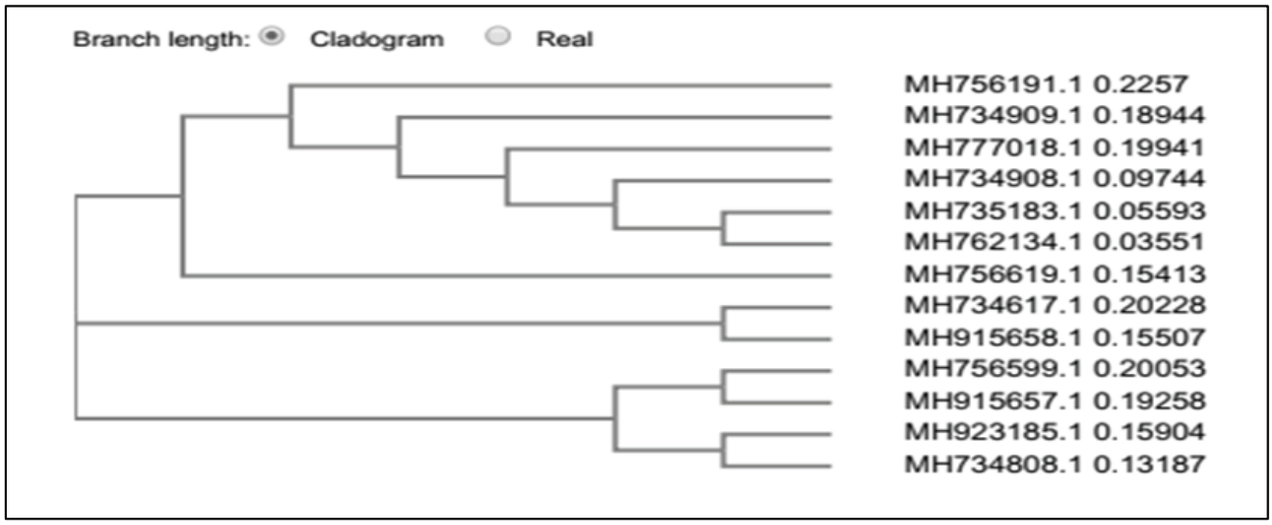
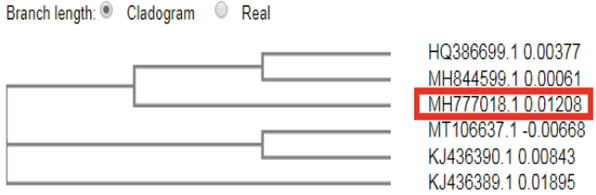
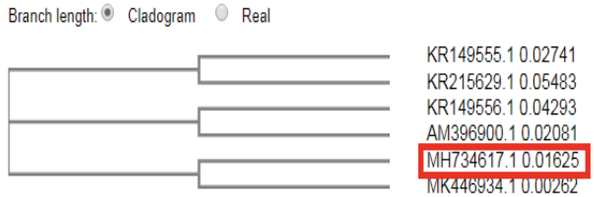
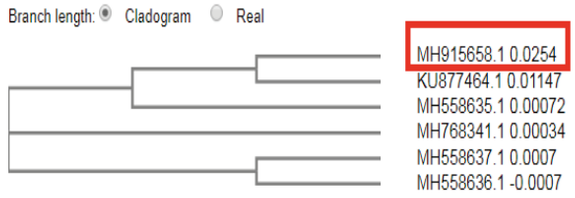
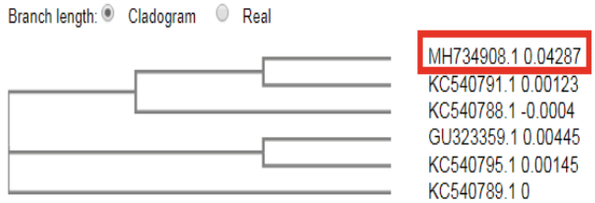
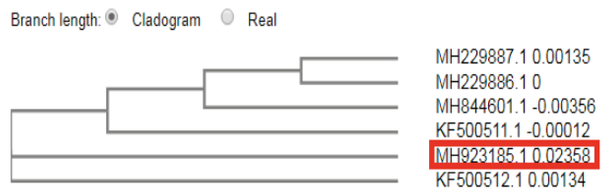
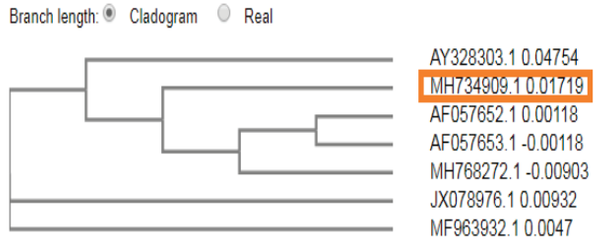
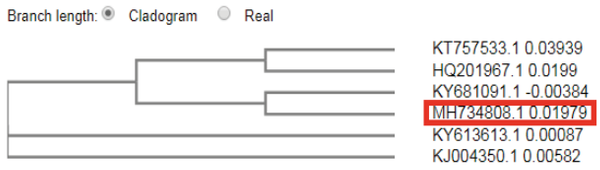
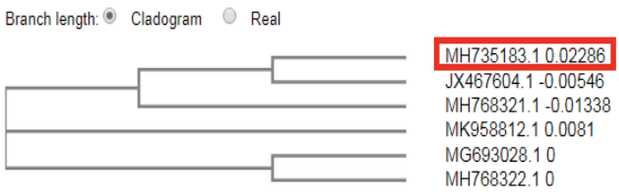
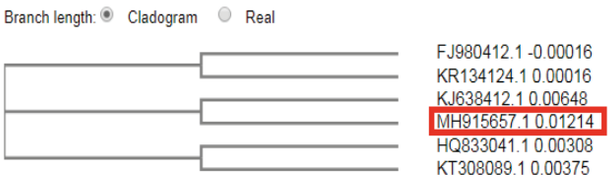
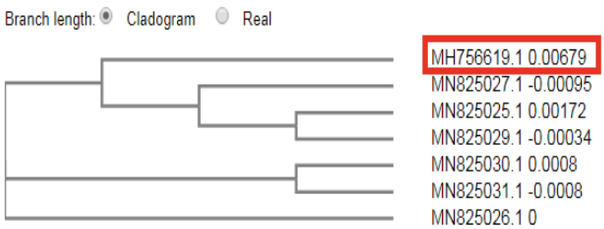
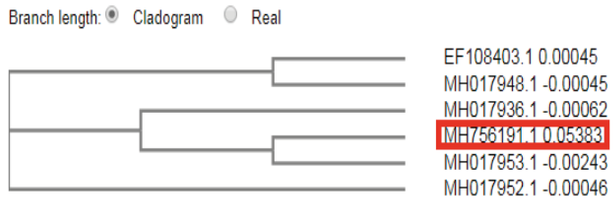
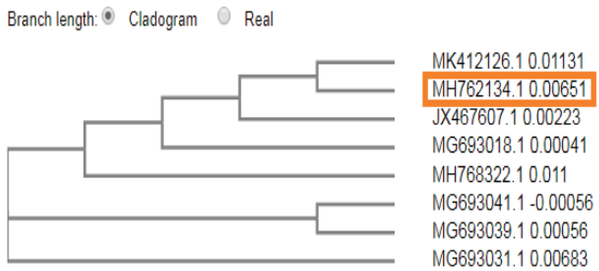
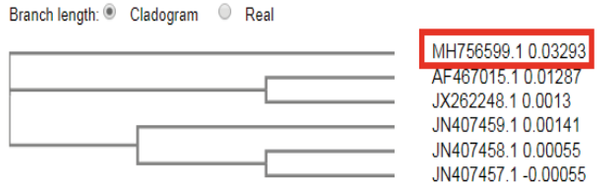
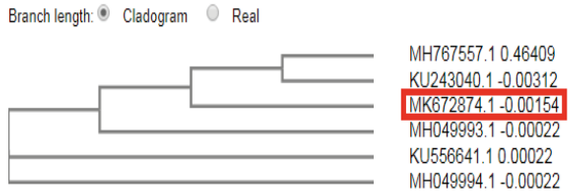
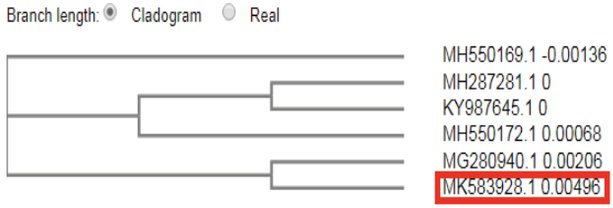
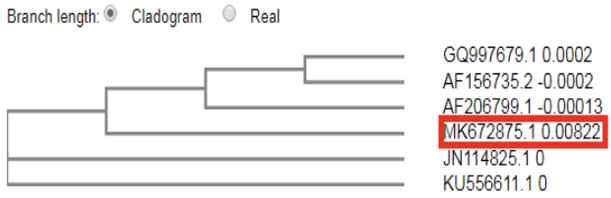


Figure 4- *Neighbour-joining tree of thirteen plant species of current study using ITS sequence.*

Table 4-*Blast match sequence with Neighbour-joining tree with studied species.*

Blast match sequence / Reference accession no	Phylogenic tree
Thevetia peruviana; MH844599.1 Thevetia neriifolia; HQ386699.1 Thevetia peruviana; KJ436390.1 Thevetia peruviana; KJ436389.1 Thevetia neriifolia; MT106637.1	
Calotropis procera; MK446934.1 Calotropis procera; AM396900.1 Calotropis procera; KR215629.1 Calotropis procera; KR149556.1 Calotropis procera; KR149555.1	
Lantana camara; KU877464.1 Lantana camara; MH558635.1 Lantana camara; MH768341.1 Lantana camara; MH558637.1 Lantana camara; MH558636.1	
Solanum villosum; KC540791.1 Solanum villosum; KC540789.1 Solanum villosum; KC540788.1 Solanum villosum; KC540795.1 Solanum villosum; GU323359.1	

Jatropha curcas; KF500511.1 Jatropha curcas; KF500512.1 Jatropha curcas; MH229886.1 Jatropha curcas; MH229887.1 Jatropha curcas; MH844601.1	
Argemone mexicana; MH768272.1 Argemone munita; MF963932.1 Argemone albiflora; JX078976.1 Argemone mexicana; AY328303.1 Argemone mexicana ; AF057653.1 Argemone mexicana; AF057652.1	
Citrullus colocynthis; KY681091.1 Citrullus colocynthis; KJ004350.1 Citrullus colocynthis; KY613613.1 Citrullus naudinianus; KT757533.1 Citrullus ecirrhosus; HQ201967.1	
Datura metel; MK958812.1 Datura metel; MH768322.1 Datura metel; MG693028.1 Datura metel; JX467604.1 Datura metel; MH768321.1	
Senna alata; KR134124.1 Senna alata; HQ833041.1 Senna alata; KJ638412.1 Senna alata; KT308089.1 Senna alata; KR134124.1	
Ipomoea carnea; MN825026.1 Ipomoea carnea ; MN825027.1 Ipomoea carnea; MN825025.1 Ipomoea carnea ; MN825031.1 Ipomoea carnea ; MN825029.1 Ipomoea carnea; MN825030.1	

Parthenium hysterophorus; MH017953.1 Parthenium hysterophorus; MH017952.1 Parthenium hysterophorus; MH017936.1 Parthenium hysterophorus; EF108403.1 Parthenium hysterophorus; MH017948.1	
Datura inoxia; MG693018.1 Datura inoxia; MG693031.1 Datura reburra; MH768322.1 Datura metel; MG693041.1 Datura wrightii; MG693039.1 Datura inoxia ; JX467607.1 Datura inoxia; MK412126.1	
Abrus mollis; JX262248.1 Abrus precatorius; JN407459.1 Abrus precatorius; JN407458.1 Abrus precatorius; JN407457.1 Abrus precatorius; AF467015.1	
Ricinus communis; MH767557.1 Ricinus communis; MH049994.1 Ricinus communis; MH049993.1 Ricinus communis; KU243040.1 Ricinus communis; KU556641.1	
Cassia roxburghii; MH550172.1 Cassia fistula; MH550169.1 Cassia fistula;MG280940.1 Cassia fistula; MH287281.1 Cassia fistula; KY987645.1	
Nerium oleander; KU556611.1 Nerium oleander; AF206799.1 Nerium oleander; JN114825.1 Nerium oleander; AF156735.2 Nerium oleander; GQ997679.1	

identification of plants or in phylogenetics [34]. However, no particular specific loci or combination of multi-loci has been reported in all plant species. The Consortium for the Barcode of Life (CBOL) Plant Working Group (2009) recommended core marker of plastid rbcL and matK along with supplemented marker such as plastid trnH-psbA and nuclear ribosomal internal transcribed spacer (ITS) use as a barcode primer to differentiate angiosperm plants after sequencing [13,35-36]. Several studies emphasized on three genomes in flowering plants i.e. plastid genome (e.g. rbcL, matK, rpoB, rpoC1, ycf1, and trnH-psbA), mitochondrial genome (ccmFN, matR, and rrn26) and nuclear genome (ITS) for authentication of plant species [37]. Literature studies advocated the ITS region be used as a barcode for phylogenetic studies of plants, animals, fungi, and protista. There has been a debate on the issue of which region should be used for species identification [38-40]. PCR based approaches are becoming imperative when conventional taxonomic identification fails to identify plant evidence in forensic investigation. Sometimes small fragments of plant species are found as evidence material and in such instances; identification of the species with conventional morphological methods becomes difficult. This experimental work might be interesting in the forensic field where cases related to adulterated or mixed plant materials are encountered. This present work focused on the suitability and specificity of ITS 1 and 4 primers on thirteen different plant specimens. So, further research has been ongoing on visceral matrices proving the suitability of these primers.

5. Conclusion

Plants are an integral part of human and animal lives but ignorance about their phytochemistry can be life threatening. Therefore, scientific awareness of toxic plants is mandatory for the public including laymen, farmers and the forensic community. Doctors and medical professionals can help themselves by thoroughly studying the morphological identification markers described here. It is also recommended for Indian forensic science laboratories to compile a proper database of Indian toxic plants encountered in poisoning cases for routine work. Future research and development work can be taken up in this direction. The use of molecular tools in the characterization of plants is a mainstay practice. In the present study, we developed a DNA-based protocol to identify and distinguish thirteen Indian toxic plant species.

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Author Contribution Statement

Equal contribution.

Conflict of Interest

None

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