Molecular Depiction of Thirteen Indian Toxic Plants with ITS Markers

Kiran Kumari, Saurabh Bhargava, Rajvinder Singh*

Department of Genetics, Maharshi Dayanand University, Rohtak (124001), Haryana, India.

Received 05 Dec. 2019; Accepted 31 May. 2020; Available Online 07 Sep. 2020

* Corresponding Author: Rajvinder Singh
Email: ajayrajaryan@rediffmail.com
doi:10.26735/YGUY5295

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

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.

Production and hosting by NAUSS

Arab Journal of Forensic Sciences & Forensic Medicine 2020; Volume 2 Issue (2), 159-169

Technical Note
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 incompatible 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).

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’GGAAGTAAAGTCCGTAACAAGG3’) and ITS 4 (reverse primer 5’TCCCTCGGTTATTGATATGC3’) [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 1- Master mixture for PCR amplification. |
|-----------------------------------------------|
| **PCR Reaction Mixture**                       |
| 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 enzyme                 |
| 0.1 µL                                       |
| Distilled water                              |
| to make up the volume 50 µL                  |

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

| 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
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 phylogenetic 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

---

**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%             |
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              | ![Phylogenic tree](image) |
| Thevetia neriifolia; HQ386699.1            |                        |
| Thevetia peruviana; KJ436390.1             |                        |
| Thevetia peruviana; KJ436389.1             |                        |
| Thevetia neriifolia; MT106637.1            |                        |
| Calotropis procera; MK446934.1             | ![Phylogenic tree](image) |
| Calotropis procera; AM396900.1             |                        |
| Calotropis procera; KR215629.1             |                        |
| Calotropis procera; KR149556.1             |                        |
| Calotropis procera; KR149555.1             |                        |
| Lantana camara; KU877464.1                 | ![Phylogenic tree](image) |
| Lantana camara; MH558635.1                 |                        |
| Lantana camara; MH768341.1                 |                        |
| Lantana camara; MH558637.1                 |                        |
| Lantana camara; MH558636.1                 |                        |
| Solanum villosum; KC540791.1               | ![Phylogenic tree](image) |
| Solanum villosum; KC540789.1               |                        |
| Solanum villosum; KC540788.1               |                        |
| Solanum villosum; KC540795.1               |                        |
| Solanum villosum; GU323359.1               |                        |
| Plant Name                  | Accession Number         |
|----------------------------|--------------------------|
| 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               |

*Graphs and additional information not shown in this representation.*
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.

Acknowledgment

The authors pay their hearty gratitude to Dr. S.S. Yadav, Assistant Professor in the Department of Botany, Maharshi Dayanand University, Rohtak (India) for suggesting literature and contributing in morphological identification of study plants.

Author Contribution Statement

Equal contribution.

Conflict of Interest

None

Funding information

No funding was received for this study

References

1. Yuan H, Ma Q, Ye L, Piao G. The traditional medicine and modern medicine from natural products. Molecules. 2016; 21(5):559-77.doi: 10.3390/molecules21050559
2. Bhatia H, Sharma YP, ManhasRK, Kumar K. Ethnomedicinal plants used by the villagers of district Udhampur, J&K, India. J Ethno pharmacol. 2014; 151(2):1005-18.doi: 10.1016/j.jep.2013.12.017
3. Bateman J, Chapman RD, Simpson D. Possible toxicity of herbal remedies. Scottish Medical Journal. 1998;43(1):7-15.https://doi.org/10.1177/003693309804300104
4. Joharchi MR, AmiriMS. Taxonomic evaluation of misidentification of crude herbal drugs marketed in Iran. Avicenna journal of phytomedicine. 2012;2(2):105-12.https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4075662/
5. Calahan J, Howard D, AlmalkiAJ, Gupta MP, Calderon AI. Chemical adulterants in herbal medicinal products: a review. Plantamedica. 2016; 82(6):505-15.doi: 10.1055/s-0042-103495
6. Chan TY. Worldwide occurrence and investigations of contamination of herbal medicines by tropane alkaloids. Toxins. 2017;9(9):284-94.https://doi.org/10.3390/toxins9090284
7. Ghorbani A, Saeedi Y, de Boer HJ. Unidentifiable by morphology: DNA barcoding of plant material in local markets in Iran. PloS one. 2017; 12(4):e0175722. https://doi.org/10.1371/journal.pone.0175722
8. Budowle B, Van Daal A. Forensically relevant SNP classes. Biotechniques. 2008; 44(5):603-10. doi: 10.2144/000112806
9. Magalhaes T, Dinis-Oliveira RJ, Silva B, Corte-Real F, Nuno Vieira D. Biological evidence management for DNA analysis in cases of sexual assault. Sci World J. 2015; 2015: 1-11. doi: 10.1155/2015/365674
10. Hebert PD, Cywinska A, Ball SL. Biological identifications through DNA barcodes. Proc Biol Sci. 2003; 270:313-21. doi: 10.1098/rspb.2002.2218
11. Kress WJ, Erickson DL. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. PLOS One. 2007; 2: e508. https://doi.org/10.1371/journal.pone.0000508
12. Ford CS, Ayres KL, Toomey N, Haider N, Van Alphen Stahl J, Kelly LJ, Wikstrom N, Hollingsworth PM, Duff RJ, Hoot SB, Cowan RS. Selection of candidate coding DNA barcoding regions for use on land plants. Bot J Linn. 2009; 159(1):1. doi: 10.1111/j.1095-8339.2008.00938
13. Bruni, I., De Mattia, F., Galimberti, A., Galasso, G., Banfi, E., Casiraghi, M., & Labra, M. Identification of poisonous plants by DNA barcoding approach. Int J Legal Med 2010; 124(6):595-603. doi: 10.1007/s00414-010-0447-3.
14. CBOL Plant Working Group. A DNA barcode for land plants. Proc Natl Acad Sci. 2009; 106:12794-7. doi: 10.1073/pnas.0905845106
15. Hollingsworth PM, Graham SW, Little DP. Choosing and using a plant DNA barcode. PLoS One. 2011; 6(5):e19254.https://doi.org/10.1371/journal.pone.0019254
16. Li M, Cao H, But PPH, Shaw PC. Identification of herbal medicinal materials using DNA barcodes. J Syst Evol. 2011; 49(3):271-83.https://doi.org/10.1111/j.1759-6833.2011.01032
17. Kress WJ. Plant DNA barcodes: Applications today and in the future. J systevol. 2017; 55(4):291-307.https://doi.org/10.1111/jse.12254
18. Yao H, Song J, Liu C, Luo K, Han J, Li Y, Pang X, Xu H, Zhu Y, Xiao P, Chen S. Use of ITS2 region as the universal DNA barcode for plants and animals. PloS one. 2010; 5(10):e13102.doi: 10.1371/journal.pone.0013102
19. Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri botanical garden. 1995; 82(2):247-77. doi: 10.2144/000112806
20. White TJ, Bruns TD, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Acad press. 1990:315-22. https://www.researchgate.net/publication/223397588
21. Kim WJ, Ji Y, Choi G, Kang YM, Yang S, Moon BC. Molecular identification and phylogenetic analysis of important medicinal plant species in genus Paonia based on rDNA-ITS, matK, and rbcL DNA barcode sequences. Genet Mol Res. 2016; 15(3):1-12. doi: 10.4238/gmr.15038472
22. DasGupta B, Konwar KM, Muandoiu II, Shvartsman AA. DNA-BAR: distinguisher selection for DNA barcoding. Bioinformatics. 2005; 21:3424-6. doi:10.1093/bioinformatics/bti547
23. Munch K, Boomsma W, Willerslev E, Nielsen R. Fast phylogenetic DNA barcoding. Philos TR Soc B. 2008; 363(1512):3997-4002. doi: 10.1098/rspb.2008.0169
24. Rach J, DeSalle R, Sarkar IN, Schierwater B, Hadrys H. Character-based DNA barcoding allows discrimination of genera, species and populations in Odonata. Proc R Soc B. 2008; 275:237-247. doi: 10.1098/rspb.2007.1290
25. Velzen R, Weitschek E, Felici G, Bakker FT. DNA barcoding of recently diverged species: relative performance of matching methods. PloS one. 2012; 7(1):e30490. https://doi.org/10.1371/journal.pone.0030490
26. Maheshwari J K. Illustrations to the Flora of Delhi. 1966.
27. Kumar S. Flora of Haryana. Bishen Singh Mahendra Pal Singh. 2001.
28. Frohne D, PfanderHJ. Poisonous Plants: A Handbook for Doctors, Pharmacists, Toxicologist, Biologists and Veterinaries. Timber Press. 2005.
29. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. Ribosomal DNA spacer-length polymorphism in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci. 1984; 81(24):8014-8. doi: 10.1073/pnas.81.24.8014
30. Wagner DB. Nuclear, chloroplast, and mitochondrial DNA polymorphisms as biochemical markers in population genetic analyses of forest trees. New Forests. 1992;6(1-4):373-90. doi 10.1007/BF00120653
31. Wallinger C, Juen A, Staudacher K, Schallhart N,
Mitterrutzner E, Steiner EM, Thalinger B, Traugott M. Rapid plant identification using species-and group-specific primers targeting chloroplast DNA. PLoS One. 2012; 7(1):e29473. doi: 10.1371/journal.pone.0029473

32. Hershkovitz MA, Zimmer EA and Hahn WJ. Ribosomal DNA sequences and angiosperm systematics. In: P.M. Hollingsworth, R.M. Bateman and R.J. Gornall eds. Molecular systematics and plant evolution. Taylor & Francis, London. 1999: 268-326.

33. Manokar J, Balasubramani SP, Venkatasubramanian P. Nuclear ribosomal DNA–ITS region based molecular marker to distinguish Gmelina arborea Roxb. Ex Sm. from its substitutes and adulterants. J Ayurveda Integr Med. 2018; 9(4):290-3. https://doi.org/10.1016/j.jaim.2017.10.001

34. Poczai P, Hyvönen J. Nuclear ribosomal spacer regions in plant phylogenetics: problems and prospects. MolBiol Rep. 2010; 37(4):1897-912. doi: 10.1007/s11033-009-9630-3

35. Kelchner SA. The evolution of non-coding chloroplast DNA and its application in plant systematics. Ann Missouri Bot. 2000: 482-98. https://doi.org/10.2307/2666142

36. Zhang D, Jiang B, Duan L, Zhou N. Internal Transcribed Spacer (ITS), an ideal DNA barcode for species discrimination in Crawfurdia wall (gentianaceae). Afr J Tradit Complement. 2016; 13(6):101-6. doi: 10.21010/ajtcam.v13i6.15

37. Cai J, Ma PF, Li HT, Li DZ. Complete plastid genome sequencing of four Tilia species (Malvaceae): a comparative analysis and phylogenetic implications. PLoS One. 2015; 10(11): e0142705. https://doi.org/10.1371/journal.pone.0142705

38. Zaya DN, Ashley MV. Plant genetics for forensic applications. In Plant DNA Fingerprinting and Barcoding. Humana Press. 2012:35-52. doi: 10.1007/978-1-61779-609-8_4.

39. Han J, Zhu Y, Chen X, Liao B, Yao H, Song J, Chen S, Meng F. The short ITS2 sequence serves as an efficient taxonomic sequence tag in comparison with the full-length ITS. Bio Med research international. 2013:1-7. doi: 10.1155/2013/741476

40. Wattoo JI, Saleem MZ, Shahzad MS, Arif A, Hameed A, Saleem MA. DNA Barcoding: Amplification and sequence analysis of rbcl and matK genome regions in three divergent plant species. Adv life sci. 2016; 4(1):3-7. http://www.als-journal.com/articles/vol4issue1/412.16/298