https://doi.org/10.33472/AFJBS.6.4.2024.751-760

African Journal of Biological Sciences

Forensic application of DNA barcoding to differentiate toxic plant from the common medicinal plants for consumer safety

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Abstract

Consumption of poisonous plants are unsafe for both human and animal health. Rapid identification of these plants is prerequisite for forensic investigation to provide reliable treatment. Lack of taxonomical features in fragmented, degraded or processed plants hindering the species identification process at poison control centres. Plant like *Argemone* contains toxic alkaloids Sanguinarine and Dihydro-sangunarineis potential threat to consumer safety. Accidental ingestion of any part of these plants or contamination with mustard oil are capable causing eye disease, epidemic dropsy and proteinuria.Therefore, the current study was employed DNA barcoding for identification from different parts of *Argemone* species. DNA was successfully extracted from the simulated cookedseeds, degraded leaf samples and fresh leaf samples of *Argemone mexicana*, *A. ochroleuca*, *A. pleiacantha*. A reference DNA barcode library of 27 sequences of *rbc*L barcode was assembled for six species of *Argemone*. Species identification was analysed by NCBI-BLAST, divergence analysis and maximum-likelihood phylogenetic tree. In all three methods, *rbcL* exhibited only at genus level identification. The phylogenetic tree constructed shows clear differentiation of *Argemone* form its closely related genera *Fumaria, Meconopsis Roemeria and Brassica*. When considering the rate of PCR amplification and sequencing success, rbcL could be used as a used as a primary marker and secondary makers like trnH-psbA or ITS2 could to be used for species identification. The current study provides the proof of concept that DNA barcoding could be useful in the forensic identification of toxic plants even from fragmented, deteriorated and cooked samples.

Keywords: DNA Barcode, toxicology, forensics, authentication, consumer safety

Introduction

Human life without plants is impossible as we rely on plants as a daily source of nourishment and medicine. However, some plants with toxic compounds pose a threat to human and animal welfare. When these plants come into touch with people or are consumed, they can cause severe allergies

or even death (1). Studies on forensic sciences revealed that more than 80% of poisoning cases were due to accidental ingestion of poisonous plants and approx. 20% are due to voluntary consumption (2-5). Besides, unintentional mixing of toxic plant material with medicinal herbs also resulted in emergency cases (6). For instance, poisoning induced by toxic plant like *Argemone* has been well documented in India. Chopra et al. (7) provided an overview on *Argemone* plant's distribution, botanical characters, chemical constituents, poisoning and its relation to epdemic dropsy. The medico legal aspects of *Argemone* poisoning were documented by Subrahmanyam (8). Das and Khanna (9) reviewed the Clinico-epidemiological, toxicological and safety evaluation studies on argemone oil.

Seeds of *Argemone* plant apparently have close resemblance with mustard seeds. Due to this reason, mustard seeds are often adulterated or misidentified with *Argemone mexicana* seeds either accidentally or intentionally. These seeds are mixed with mustard seeds during extraction of mustard oil extraction process. Toxic alkaloids Sanguinarine and Dihydro-sangunarine present in the *Argemone* oil may cause eye disease leading to blindness (10). Regular consumption of it may also causes disease called epidemic dropsy. Epidemic dropsy occurs due to the use of contaminated mustard oil (with which *Argemone* oil is completely miscible) for cooking and massage (11) and proteinuria (specifically loss of albumin) occurs, with a resultant edema as would occur in nephrotic syndrome. Ingestion of argemone oil causes hypertension, glaucoma, dropsy, diarrhea, vomiting and anemia. The toxicity is attributed to the presence of the alkaloid sanguinarine, which is also carcinogenic (12). Argemone oil toxicity poses a serious threat to human health and should be checked by appropriate regulatory measures. In several instances, the various parts of poisonous plants are obtained in their native, cooked, vomited, or degraded forms. In forensic toxicology, accurate identification of degraded plant components is extremely difficult, particularly when dealing with vomit sample remains. Samples may not have the morphological characteristics necessary for trustworthy species identification under these conditions. Plant species identification can be challenging in the absence of specific taxonomic traits, as noted by Xie Lie et al. (13), since a variable percentage of plant pieces are typically unidentified in forensic toxicology. Therefore, utilising DNA barcoding as an identification tool which does not rely on plant morphology and unaffected by the aforementioned issues could greatly overcome the challenges. Furthermore, live or dead tissue from any part of the plant at any point of its life cycle may be identified using DNA barcoding (1). The usefulness of DNA barcoding in forensic investigations was highlighted in various studies.

In order to identify toxic plants, Burniet al. (14) employed DNA barcoding techniquein a wide range of angiosperms that possess a variety of toxic compounds. The authors examined five DNA barcode regions (At103, sqd1trnHpsbA, rpoB, and matK) from nuclear and cpDNA.The results showed matK marker was the most effective in differentiation on species. Combination of matK with a nuclear marker like At103 was proposed for the identification of toxic hybrids from their parent species. In another study by Nithaniyal et al. (1) developed a trustworthy reference DNA barcode library for 100 poisonous plantsusingrbcL DNA barcodes to identify toxic from closely related species. Using the library, 100% and 89% of identification at the genus and species levels, respectively were reported. Further, when mapping of the toxic plant metabolites to the DNA-based phylogenetic tree revealed phylogenetically linked species also possessed similar hazardous chemicals. Jie Wang and colleagues (15) studied four DNA barcodes for identification of poisonous plants that are often found in China. The usefulness of DNA barcoding was evaluated by Tree-

Building (NJ), PWG-Distance, and Basic Local Alignment Search Tool (BLAST) methods. The authors noted that while the sequencing of trnH-psbA and ITS was less than ideal, the amplification success rate of the remaining three regions aside from the matK region was high. Meanwhile, the simulated stomach juice made matK more likely to be challenging to amplify and sequence. The results of the three methods indicated that the BLAST method had the lowest identification rates, while the PWG-Distance and Tree-Building methods had similar recognition rates. When primer versatility was taken into account, they discovered that the single barcode rbcL was the most effective and economical marker for clinical identification. The BLAST method was also found to be quicker and easier as a clinical diagnostic analysis method.

The current study particularly focused on the molecular differentiation of closely related species of *Argemone*. Degraded leaf, fresh leaf, cooked seeds, and fresh seeds, were selected for the DNA barcoding analyses. We aimed to (i) study the DNA isolation efficiency in fresh leaf, degraded leaf, fresh seeds and cooked or boiled seeds of *Argemone* species. (ii) Analyse the nucleotide variation of congeners in rapid differentiation of toxic plant and (iii) to apply DNA barcode as an identification tool in forensic toxicology to delineate toxic herb from commercial plants. This baseline study on *Argemone* congeners provides the proof of concept in DNA barcoding could be highly useful in forensic detection of the toxic plant during emergency cases and also complements consumer safety.

Material and method

Collection and preparation of plant sample

The noxious, erect, prickly, annual herb *A. Mexicana* grows in open fields, along roadsides and in cultivated lands. Fresh leaves and seeds were collected from the Patharia hill location near the Sagar district of Madhya Pradesh. Herbarium was deposited in the Department of Botany at Doctor Hari Singh Gour Central University (UGC-DSA/ASIST Sponsored Department) Sagar, Madhya Pradesh, India. In addition, closely related species like *A. ochroleuca* and *A. peliacanta* (? doubtful species) were also included to study species relationships. Herbarium specimen were authenticated by Dr. Pradeep Tiwari and deposited at Doctor Hari Singh Gour Central University Herbarium (BOT/H/01/10/02 and BOT/H/01/10/03). The collected fresh leaves and seeds were initially cleaned with running tap water followed by distilled water before DNA isolation. For the preparation of simulated degraded leaves sample, leaves were kept inside the moistened soil for 20 days. Simulated samples of cooked seeds were prepared by boiling them in water for 100^0C about 10-15 minutes. Further, the sample was labelled as S1 for fresh leaves of *A. mexicana*, S2 for degraded leaves *A. mexicana*, S3 for fresh seeds of *A. ochroleuca*, S4 for boiled seeds of *A. pleiacantha*, S5 for fresh leaves of *A. ochroleuca*, and S6 for degraded leaves of *A. pleiacantha*.

DNA Extraction, amplification and DNA Sequencing

The genomic DNA was extracted by following the standard protocol using (Xploregen discoveries plant kit). Around 100 mg of each sample was placed in a mortar and homogenized with 1 ml of extraction buffer and the homogenate was transferred to a 2 ml-microfuge tube. An equal volume of Phenol: Chloroform: Isoamlyalcohol (25:24:1) was added to the tubes and mixed well by gently shaking the tubes. The tubes were centrifuged at room temperature for 15 min at 14,000 rpm. The upper aqueous phase was collected in a new tube and an equal volume of Chloroform: Isoamly alcohol (24:1) was added and mixed. The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 14,000 rpm was transferred to a new tube. The DNA was precipitated

from the solution by adding 0.1 volumes of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol. After 15 min of incubation at room temperature the tubes were centrifuged at 4°C for 15 min at 14,000 rpm. The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and air dried. The DNA was dissolved in 100 µl of TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). To remove RNA 5 µl of DNAse free RNAseA (10 mg/ml) was added to the DNA.The quality of DNA was checked on 0.8% agarose using Agarose gel electrophoresis.

DNA barcodes were amplified by PCR using universal primers (rbcL-F: ATGTCACCACAAACAGAGACTAAAGC and rbcL-R: GTAAAATCAAGTCCACCRCG). PCR reaction mixture of contained 1X buffer with 1.5 mM MgCl2, 200 mM dNTPs, 5 pmol primers, and 1 unit Taq DNA polymerase. PCR was done in a thermal cycler (Eppendorf, Germany) using the following protocol: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, final extension at 72°C for 5 minutes, and hold at 16°C. The PCR products were checked by checked on 1% agarose, and purified using EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc. Ontario, Canada). The purified PCR products were sequenced bidirectional using the same PCR primers in 3130xl Genetic analyzer (Applied Biosystems, CA, USA) using Big Dye Terminator chemistry.

DNA sequence validation, assembly and phylogenetic analysis

The quality of each sequence was analysed using Sequence Scanner Software v.1.0 (Applied Biosystems, CA, USA). Full-length sequences were assembled, and consensus sequences were annotated using Codon Code Aligner version 4.2.4 (CodonCode Corporation, MA, USA). Sequence data were primarily validated by homology search using NCBI-BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Validated DNA sequences were submitted to NCBI database for open access. We assembled the reference DNA barcode library of *Argemone* by retrieving DNA barcodes form the GenBank database of NCBI (https://www.ncbi.nlm.nih.gov/). Total of 27 sequences were retrieved for the genus *Argemone* using stringent criteria proposed by Sivaraj et al. (16). In addition, we have retrieved 76 barcode sequences of *rbcL*, from closely related species to understand the genetic relationship among the species. Sequences from the genus *Brassica* was used as an out-group. Intra-specific genetic distances were calculated by assigning a query sequence to its closest match based on the genetic divergence using Taxon DNA v.1.6.2 **(17)**. Divergence was calculated as follows: per cent divergence = no. of mismatched nucleotides/ total no. of aligned nucleotides × 100. The sequence statistics were calculated using MEGA v.5.1 **(18)**. The phylogenetic tree was constructed using the combined dataset that comprised of our own DNA sequence and sequence data retrieved from the GenBank database. The maximum likelihood tree was constructed using Clustal W alignment tool in MEGA v.5.1 **(18)**. The total number of nucleotides in the aligned data set was 550 bp. All positions containing gaps and missing data were eliminated from the analysis. The K2P distance was set as an evolutionary model, and the bootstrap support was analysed with 1000 replications. The tree with a 70 % bootstrap value was generated and viewed in FigTree version 1.3.1 **(19)**.

Results and discussion

DNA isolation, PCR and sequence assembly

In the present study we have attempted to extract DNA from the simulated cooked and degraded samples along with fresh plant material.Isolation of DNA is successful for all the fresh leaf, degraded leaf and boiled seed samples of *Argenome* (Fig.1). According to Lei Xie et al. **(13)** the primary obstacle in the DNA barcoding technology is the laboratory protocols followed for DNA

extraction from the plant materials that are dried using silica gel, degraded samples, or even digested plant material. Nature of the sample will have a major impact on the quality and success of the DNA extraction of whole DNA. In this study, the yield of DNA was comparable in fresh leaf, degraded leaf and boiled seedsregardless to the nature of plant materials. The absorbance ratio at 260/280 nm was 1.8 for *A. mexicana* and *A. ochroleuca* (Table-1). Further, successful PCR amplification is crucial to access the utility of DNA barcodes which could be affected due to quality of the DNA extracted **(20,21)**. Although the seeds of *Argenome* contain high fatty acids, polyphenols and oil contents, PCR amplification was successfully in all the samples. The PCR positive amplicon were subjected to bidirectional DNA sequencing and a total of six DNA sequences from three species of *Argenome* were assembled for DNA barcode analysis. Sequence length variation was not found in the alignment and recovered the 600 bp of target sequence. Primary validation of sequence data were done by homology search using NCBI-BLAST algorithm. All the sequences were matched with similarity of 99.8% to 100% similarity with *Argenome* species. After validation, thesequences were submitted to the NCBI database under the accessions number OR480108, OR753280, OR640940, OR640941, OR906271, and OR906272.

Figures Legends

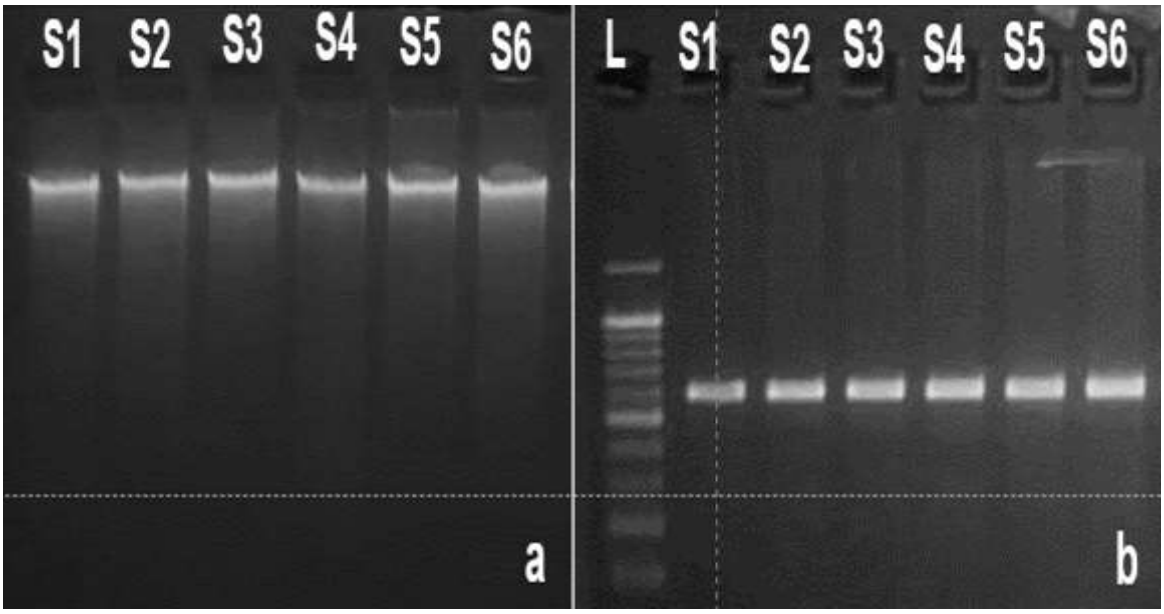

Figure 1. (a) DNA isolation and (b) PCR amplification of *rbcL* barcode.

S1-Fresh Leaves of *A. mexicana*; **S2**-Degraded leaves *A. mexicana*; **S3**-Fresh Seed *A. ochroleuca*; **S4**-Boiled seed *A. pleiacantha*; **S5**-Fresh Leaves *A. ochroleuca*; **S6**-Degraded leaves *A. pleiacantha*; L-100 kb marker.

Table Legends

Table -1 : Quantification of DNA isolated from different plant parts of *Argemone*

S. No.	Sample ID	NCBI Accession number	Botanical Name	Sample type	Spectrophotometer OD Value			ng/µl
					A260	A280	260/280	
1	S1	OR480108	*Argemone mexicana*	Fresh Leaves	0.596	0.33	1.8	28.7
2	S2	OR753280	*Argemone mexicana*	Degarded leaves	0.297	0.177	1.7	14.9
3	S3	OR640940	*Argemone ochroleuca*	Fresh Seed	0.397	0.232	1.7	19.9
4	S4	OR640941	*Argemone pleiacantha*	Boiled seed	0.349	0.22	1.6	17.5
5	S5	OR906271	*Argemone ochroleuca*	Fresh Leaves	0.412	0.232	1.8	20.6
6	S6	OR906272	*Argemone pleiacantha*	Degarded leaves	0.382	0.263	1.5	19.1

Divergence analysis of *Argemone* sequences

Genetic divergence was estimated by calculating pairwise combinations of 27*rbc*L sequences of the *Argemone* collected from this study along with retrieved sequences. The results showed a minimum of 0.0 % and a maximum of 0.2 % divergence among the six *Argemone* species. The average nucleotide composition of AT and GC contents were 54.6 % and 45.4 %, respectively. The number of conserved sites, variable sites, and parsimony informative sites were 566 bp, 4 bp, and 3 bp, respectively. Further, six species include *Argemone albiflora*, *A. mexicana*, *A. ochroleuca*, *A. pleiacantha*, *A. munita* and *A. subfusiformis* showed less variation at genetic level. Less genetic variation of *rbcL* in the congeners were also reported in *Acacia* **(22)**, *Hibiscus* **(23)** and *Terminalia* **(24,25)**. However, nuclear marker showed enough variation in congeners of angiosperms may be used as additional marker for this purpose **(1,26,27,28).**

Divergence analysis of *Argemone* and other closely related species

Genetic divergence was estimated by calculating pairwise combinations of 103*rbcL* sequences belonging to thirty-six species of five congeners in the family Papaveraceae. The results showed a minimum intra-specific divergence of 0.0 % and a maximum divergence of 0.2 %. Inter-species divergence ranged from 0.2 % to 1.0%. The average nucleotide composition of AT and GC contents were 54.7 % and 45.3 %, respectively and the number of conserved sites, variable sites, and parsimony informative sites were 485 bp, 85 bp, and 83 bp, respectively. Genetic divergence analysis showed that *rbcL* maker could able to differentiate at genus level than the species level. Earlier studies reported that that the rbcL marker is highly conserved and unlikely to show more variation in congeners **(29,30)**. Details of variable sites in *Argemone, Fumaria, Meconopsis Roemeria and Brassica* sequences are provided in the Supplementary Table.

Maximum likelihood tree analysis

The maximum likelihood tree constructed using *rbcL* maker contained 103 sequences representing thirty six species from *Argemone*, *Fumaria*, *Meconopsis, Roemeria* and *Brassica* (Out-group). Each genus formed a monophyletic clade without any overlapping between the species. All 27 sequences of *Argemone* formed a separate clade, which shows clear segregation from other species of Papaveraceae. This clade represented by two accessions of*Argemone albiflora*, fourteen accessions of *A. mexicana*, five accessions of *A. ochroleuca*, three accessions of *A. pleiacantha*, one accession of *A. munita* and two accessions of *A. subfusiformis* from the countries USA, China, Saudi Arabia, Canada and India. The identity of *A. Pleiacantha* was uncertain and the accessions were clustered with other species *Argemone* without any genetic differentiation. Interestingly, species of *Fumaria* formed two sub-cladesindicating the more genetic diversity in the genus (Fig. 1). One sub-clade consisted of *F. parviflora*, *F. microstachys*, *F. vaillantii*, *F. schleicheri*, *F. occidentalis*, *F. indica*, *F. densiflora*, and *F. bastardii*. Another sub-clade was formed by species such as, *F. muralis*, *F. purpurea*, *F. martini*, *F. agraria*, *F. capreolata*and *F. officinalis*. Nine species of *Meconopsis* formed a sister clade to *Argemone* and *Fumaria* (Fig. 2) **(31)**. The species *Roemeria argemone* formed a basal clade to *Argemone*, *Fumaria* and *Meconopsis* of Papaveraceae with *Brassica* as an out-group. The rbcL sequences analysed by maximum likelihood tree showed a clear segregation of *Argemone* only among closely related genus but not the species. Six species of *Argemone* does not show much differentiation due to low genetic divergence and the same was observed in ten *Meconopsis* species. Low genetic signal in *rbcL* marker of Papaveraceae reinforces the use of additional marker like *trn*H-*psb*A and ITS2 **(32), (33).**

Figure 2. Maximum-likelihood tree construction showing *Argemone* phylogenetic relationships

Conclusion

The common prickly herb *A. Mexicana* grows in open fields, cultivated lands and along roadsides across India. In agriculture, allopathic effects of *Argemone* were shown to affect germination and seedling vigour of wheat, mustard, fenugreek, sorghum, finger-millet, tomato, cucumber, and other crops **(34)**. Due to the environmental and other harmful impacts, *A. mexicana* have been listed as noxious weed most of the countries. They serve no economic purpose and possess characteristics that are harmful to humans, animals or the environment **(35).** Accidental consumption of these plants showed adverse consequence in both humans and animals **(36)**. Identification of *Argemone* in degraded form is much difficult since it lacks intact morphology when fragmented. In our study, we successfully extracted DNA from the simulated cooked and degraded samples of *Argemone*. A reference library of 103 sequences was assembled by including the closely related species from the genus *Argemone*, *Fumaria*, *Meconopsis* and *Roemeria*. Species identification was analysed by NCBI-BLAST, divergence analysis and maximum-likelihood phylogenetic tree. In all three methods, the possibility of using *rbcL* for the identification of the toxic plant *Argemone* exhibited only at genus level. However, by considering the rate of PCR

amplification and sequencing success, rbcL could be used as a primary validation purpose and secondary makers like trnH-psbA or ITS2 could to be used for species identification. The current study provides the foundation for DNA barcoding form the samples including fresh, deteriorated, and cooked vegetable samples which has wide application in plant forensics and poison control centers.

Acknowledgement: The authors P.Y., S.D.M and M.P.G. are thankful to facilities provided by the Shri Vaishnav Institute of Forensic Science, Shri Vaishanav Vidyapeeth Vishwavidyalaya, Indore, Madhya Pradesh, India. S.N. acknowledges the Director, Botanical Survey of India, Kolkata, India. Authors are thankful to Biokart Laboratory Pvt. Ltd. Bangalore, India for providing laboratory facilities for DNA Barcoding analysis.

Authors contributions: Conceptualization, data curation, P.Y.; writing-original draft, methodology, formal analysis, S.N.; formal analysis, writing-review and editing S.D.M and M.P.G. All authors have read and agreed to the published version of the manuscript.

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