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Identification of CITES-Listed Euphorbia royleana through DNA Barcoding Technology: A New Facet in Wildlife Forensics

التعرف على الفربيون الصباري "Euphorbia Royleana" المدرج ضمن اتفاقية الاتجار الدولي بأنواع النباتات والحيوانات البرية المهددة بالانقراض "CITES" من خلال استخدام تقنية الترميز الشريطي للحمض النووي: وجه جديد لعلوم الأدلة الجنائية للحياة البرية

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Abstract

Disorganized and chaotic collection of the Euphorbia plant species from the wild is one of the major reasons for its endangered status. According to CITES, the trade in *Euphorbia royleana* species is prohibited under Appendix II. However, the trade continues unabated as current identification methods do not discriminate between closely related species.

In the present study, a DNA barcoding method has been used to establish inter- and intra-specific divergences of both *matK* and *rbcL* regions by using pairwise genetic distance measurement methods for evaluating the maximum barcoding gap.

The *matk* and *rbcL* yielded a 100% amplification and sequencing success rate to distinguish closely related species of *Euphorbia royleana* unambiguously. The matk and rbcL showed average interspecific genetic distance divergence values of 0.031and 0.015,

Keywords: Forensic Science, Wildlife Forensics, Plant DNA Barcoding, Euphorbia Royleana, CITES.

المستخلص

يعد الجمع غير المنظم والعشوائي لأنواع نبات الفربيون التي تعيش في الحياة البرية أحد الأسباب الرئيسية لحالتها المعرضة للخطر. فوفقًا لاتفاقية الاتجار الدولي بأنواع النباتات والحيوانات البرية المهددة بالانقراض "CITE"، يعد الاتجار في أنواع نباتات الفربيون محظورًا بموجب الملحق الثاني من الاتفاقية. وعلى الرغم من ذلك، فما يزال الاتجار في هذه النباتات مستمرًا بكامل قوته؛ نظرًا لأن الطرق المستخدمة حاليًا للتعرف على هذه النباتات لا تميزها عن الأنواع ذات الصلة بها بشكل وثيق.

وقد تم في هذه الدراسة استخدام طريقة الترميز الشريطي للحمض النووي "DNA" لإيجاد تباعدات بين الأنواع وبين النوع الواحد لمنطقتي جيني matK وrbcL من خلال استخدام طرق قياس للمسافات الجينية لكل زوج من أجل تقييم أقصى فجوة للترميز الشريطي.

وحقق الجينان matK وrbcL تكبيرًا بنسبة 100% ومعدل نجاح تسلسل للتمييز بصورة لا يكتنفها أي غموض بين الأنواع المرتبطة بشكل وثيق بنبات الفربيون الصباري. وقد أظهر كل من الجينين matK وrbcL متوسط قيم تباعد للمسافات الجينية بين الأنواع المختلفة

الكلمات المفتاحية: علوم الأدلة الجنائية، علوم الأدلة الجنائية للحياة البرية، الترميز الحمض النووي للنباتات، الفربيون الصباري، اتفاقية الاتجار الدولي بأنواع النباتات والحيوانات البرية المهددة بالانقراض "CITES".

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respectively. The maximum number of species-specific SNPs was observed in *matK* sequences at seven consecutive sites, which could distinguish *Euphorbia royleana* from closely related species.

The best candidate barcoding region to identify *Euphorbia royleana* was found to be *matK* with a single-locus barcoding approach. Furthermore, the species discrimination method was developed with the help of species-specific SNPs derived from the *matK* barcoding region to accurately authenticate *Euphorbia royleana*, and it provided 100% species resolution.

مقدارها 0.031 و0.015 على التوالي. كما لوحظ وجود أقصى عدد من أشكال النوكليوتيد المفرد "SNPs" الخاصة بأنواع النباتات في تسلسلات matK على سبعة مواقع متعاقبة يمكن أن تميز الفربيون الصباري عن الأنواع الأخرى المرتبطة به بشكل وثيق.

علاوة على ذلك، تم التوصل إلى أفضل منطقة مرشحة للترميز الشريطي من أجل التعرف على الفربيون الصباري وهي استخدام جين matK مع أسلوب ترميز شريطي أحادي الموضع. كما تم إيجاد طريقة للتمييز بين أنواع النباتات بمساعدة SNPs الخاص بأنواع النباتات المشتق من منطقة الترميز الشريطي لجين matK وذلك للتحقق الدقيق من الفربيون الصباري، حيث اتسمت هذه الطريقة بدقة في التمييز بين الأنواع بنسبة 100%.

1. Introduction

Medicinal plants are wildly harvested for their medicinal properties. Many species of these plants are becoming endangered or vulnerable to extinction due to their over-trading. The United Nations Office on Drugs and Crime (UNODC) Worldwide database suggests that is 14.3% of seizures regulated by CITES (Convention of International Trade in Endangered Species) and other related organizations belong to the category of plants [1]. The reports from Trade Records Analysis of Flora and Fauna in Commerce (TRAFFIC) suggests illegal trade of plants extract from the wild species is around 7 billion USD [2].

Euphorbia royleana is a succulent cactus-like shrub, which belongs to the Euphorbiaceae family. The plant is extracted from the wild due to its broad spectral medicinal properties used to treat inflammation and arthritis [3], as well as asthma, cough, anemia and jaundice [4].The surplus extraction of this plant from the wild has led to its vulnerability. Therefore, Euphorbia royleana has been listed in CITES Appendix-II [5]. Because plant populations are exploited by pharmaceutical businesses, enormous volume exchange at national and international levels has been observed. This might be one of the major causes for their rapid depletion in various regions of the Indian subcontinent [6]. The illegal extraction of medicinal plants like Euphorbia royleana from the wild has been unabated for a long time. Either our labs lack identification methodologies and are unable to discriminate between closely related species, or there is no legislation available that can prevent this plant from being traded and extinction [7]. The taxonomic status of the Euphorbia species is dubious, as the plant displays similar morphtypes dispersed in Euphorbia genus: the similarity of morphological attributes has caused it to be characterized into sub-divisions or segments by certain taxonomists. Furthermore, proposals and studies to conclusively identify the plant only based on morphological and anatomical characteristics are still inconclusive [8]. Because the Euphorbia species exhibits an internal variation with similar morphotypes, morphological identification of species has become difficult as well as controversial. It has, therefore, become very difficult to distinguish it from closely related species. Such limitations need to be overcome by using advanced, validated scientific methods like DNA barcoding techniques.The ability of these techniques to discriminate species, as well as provide a delineation of closely related species, is quite promising. DNA barcoding technology has the potential to evolve as a new and effective facet of wildlife forensics [9-14]. CITES has listed the Euphorbia royleana species in appendices II; therefore

taxonomic delineations from closely related species is urgently required with inexpensive and validated methodologies[15]. DNA barcoding technology is well accepted and validated by various scientific groups and can be useful in the conservation of animal species as well [16]. Similar studies are urgently required in wildlife forensics, specifically with respect to the identification of other medicinal and aromatic plants that are traded illegally.

Recently, various scientific experts has used DNA barcoding technology for the identification of plants. They have suggested 13 regions for the identification of plants. The plant DNA barcoding regions used for the identification are nrITS, nrITS2, accD, ndhJ, ycf5, trnLrpoC1, rpoB, matK, trnH-psbA, rbcL, atpF-H, psbK-I, and UPA [17].

The agreement on a single-locus universal plant DNA barcoding region is still the major concern in plant DNA barcoding studies [17]. The technology has given suitable results with fresh as well as dried herbal products, but it has certain limitations. Firstly, the validation of candidate loci with respect to specific genus and secondly, the lack of accessible databases of DNA sequences in the NCBI National Center for Biotechnology Information (NCBI), GenBank [18,19]. No DNA sequences are available for BLAST search in GenBank, NCBI, and Bold Systems. Therefore, it was thought desirable to make a comprehensive first attempt to collect and analyse Euphorbia royleana species with the help of DNA barcoding technology to establish a barcode reference library by using both a single and a multi-locus approach. The present study has been designed to evaluate the applicability of recommended universal primers of either matK or rbcL or both for successful amplification of desired barcoding regions. The study utilizes the Consortium of Barcode of Life (CBOL) recommended matK and rbcL barcoding region to assess the candidature for more specific species resolution in discriminating closely related species. As the use of a DNA barcoding technique in forensic cases requires a validation of candidate markers with respect to amplification success rate and sequence success, the present study investigated the reliability and reproducibility of the results obtained according to the guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM).

2. Materials and Methods

Euphorbia royleana plants were collected from three different sites: the Medicinal Botanical Garden in Sarangpur, the Medicinal Botanical Garden in Dumreda, Shimla, and the Medicinal Botanical Garden, Mandi, in Himachal Pradesh state of India to check the intra-plant species variations, if any. The samples were preserved on marked FTA classic cards in zip lock bags containing silica beads. The Euphorbia royleana product samples (10) were also collected from the local market of Chandigarh, a region in India.

2. 1 DNA extraction and amplification

The FTA cards were punched and then homogenized with 400 µL of pL1, followed by RNase solution, which was further incubated for 10 minutes at 65oC. The DNA extraction was carried out using a NucleoSpin® Plant II Kit (Macherey-Nagel) in accordance with the manufacturer's protocol. The extracted results were checked with the help of 0.8% agarose gel, which was prepared by dissolving in 100 mL of 0.5X TBE buffer. The template DNA used in PCR amplification was 40-50 ng/µL. The primers utilized for PCR amplification were 390f and 1326r for matk and rbcLa_f and rbcL724_rev for rbcL [20,21]. Reaction conditions as per the recommended guidelines provided by the Consortium of Barcode of Life (CBOL) plant-working group are shown in Table-1.

Table 1- *Universal primers utilized and reaction conditions for matK, rbcL and ITS barcoding regions.*

Target	Primer References	Direction	Sequence (5' ⟶ 3')	Reaction Conditions		
matK	390f [17]	Forward	CGATCTATTCATTCAATATTTC	30 - sec	°C 98	} cycles 40
				5 - sec	°C 98	
	1326r [17]	Reverse	TCTAGCACACGAAAGTCGAAGT	10 - sec	°C 50	
				15 - sec	°C 72	
				60 - sec	°C 72	
				∞-	°C 4	
rbcL	rbcLa_f [17-18]	Forward	ATGTCACCACAAACAGAGACTAAAGC	30 - sec	°C 98	} cycles 40
				5 - sec	°C 98	
	rbcL724_ rev [17-18]	Reverse	GTAAAATCAAGTCCACCRCG	10 - sec	°C 58	
				15 - sec	°C 72	
				60 - sec	°C 72	
				∞-	°C 4	
ITS	ITS-5F [19]	Forward	GGAAGTAAAAGTCGTAACAAGG	30 - sec	°C 98	} cycles 40
				5 - sec	°C 98	
	ITS-4R [19]	Reverse	TCCTCCGCTTATTGATATGC	10 - sec	°C 58	
				15 - sec	°C 72	
				60 - sec	°C 72	
				∞-	°C 4	

The nuclear ITS (Internal Transcriber Spacer gene) was also tested with two different primer sets: ITS-5F and ITS-4R [22], with the above-mentioned reaction condition. However, the desired amplification could not be achieved, even after repeated trials, which led to its exclusion from the present study. The selected DNA regions (matK and rbcL) were amplified by using a reaction volume of 20-µL. The 20 µL reaction mixture includes 1x PCR buffer 1.5 mM MgCl2, 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 μL DNA, 0.2 μL DNA polymerase enzyme (Genei®), 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers. The sequencing reaction for both forward and reverse primers was performed by using the standard manufactured protocol of the Big Dye Terminator (v3.1) using Cycle Sequencing Kit (Applied Biosystems, USA).

2. 2 Sequence analysis

The sequences obtained for both forward and reverse sequences for each barcoding region, matK and rbcL, were assembled and edited by using Mega 7.0 software. A NCBI BLAST nucleotide tool was used to study the homology of obtained sequences with sequences available in the NCBI database. The edited sequences were then aligned with CLUSTAL W present in MEGA 7.0 software with 15 as a gap-opening penalty, 6.66 as gap extension

Table 2- *Accession number of DNA sequences published in NCBI GenBank and BOLD systems.*

Barcoding region	NCBI GenBank Accession Number	BOLD Process IDs
Maturase Kinase (matK)	MK002729 and MK0027271	ERR001-19, ERR002-19, ERR003-19
Ribulose biphosphate carboxylate large subunit (rbcL)	MH765673.1 and MH765674.1	ERR013-19, ERR007-19, ERR008-19

penalty, and with 5-transition weight. The sequences of matK and rbcL were analyzed individually and genetic distances were evaluated for single locus and multilocus barcodes. The guidelines given by CBOL were followed to study the variability of interspecific and intraspecific genetic distances using pairwise genetic distance [23]. The informative parameters of DNA sequences were generated with the help of MEGA 7.0 by using informative parsimony sites, degenerate, coverage and CpG, mean GC content, a variable number of nucleotides, and the average length of the sequences.

2. 3 Data analysis and sequence submission to NCBI

The sequence data obtained from the present study were examined with the help of ABI sequencing analysis. MEGA 7.0 and CLUSTAL W tools were also utilized to compare various parameters of the sequences obtained. The sequences of closely related species like Euphorbia maculate, Euphorbia Hita, and Euphorbia abyssinicia were obtained from the NCBI Gen Bank in FASTA format. The interspecies similarity was studied with the help of the MEGA 7.0 genetic software, and the discriminatory power of the matK, rbcL and ITS barcoding regions were assessed. The sequences obtained from the study were edited with the help of sequencing analysis and were published in NCBI.

The contribution is expected to enrich the plastid and nuclear genomic database of Indian plant species, as it will be highly useful in dealing with the problem of species identification through BLAST analysis. The aligned sequences for all the three tested barcodes were sequentially analyzed for p-distance (pair-wise distance) and are shown in Table-3 and Figure-2.

3. Results

The success rate for amplification and sequencing of both plastid barcoding regions, i.e. matK and rbcL,was 100% with regards to the universality of primers. The four sequences generated during the course of the present investigation were published in GenBank NCBI and BOLD systems, as shown in Table-2. No DNA barcoding studies had been undertaken before this study on the Euphorbia royleana species; therefore, no congeneric sequences corresponding to Euphorbia royleanaare found in a NCBI BLAST search. Among search results obtained through BLAST nucleotide hits, the DNA sequences of Euphorbia royleana showed most similarity with other genera of the family Apocynaceae. The PCR amplicons of the two barcoding regions, i.e. matK and rbcL, showed a consistent size:1kb for matK and 800kb for rbcL, which is in agreement with the mean size of the respective marker, as shown in Figure-1. The sequence characteristics of both plastid candidate barcoding regions (matK and rbcL) are shown in Table-3.

The matK sequence size varied from 474 bp to 534 bp with 52 variable sites and 867 conserved sites. It is interesting to note that the matK region showed maximum parsim informative sites (27) with respect to Euphorbia royleana and its closely related species. The alignment length of the matK barcoding region was 613 bp within the aligned region.

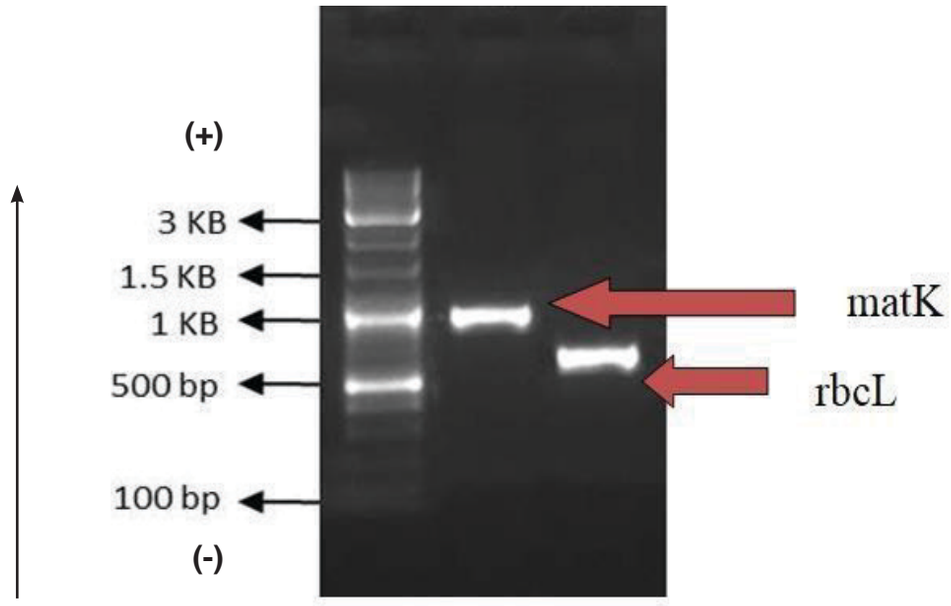

Figure 1- *PCR amplification gel images of matK and rbcL barcoding region showing 1kb amplified product in case of matK and 700bp product in case of rbcL.*

Table 3- *Nucleotide sequence parameters of Euphorbia royleana for matK and rbcL barcoding regions.*

S. No	Parameter Studied	matK	rbcL
1.	Mean Inter-specific distances	0.031	0.015
2.	Mean Intra-specific distances	0.0	0.00
3.	Number of Conserved Sites	867	1321
4.	Number of Variable Sites	52	38
5.	Number of Parsim Info Sites	27	10
6.	Number of Singleton sites	25	13
7.	0-Fold	883	942
8.	2-Fold	328	294
9.	4-Fold	146	143
10.	Coverage	653	532
11.	CpG	148	150

The plastid barcoding region, rbcL, showed alignment length of 525 bp and was observed to be extremely conserved within the four tested species of Euphorbia genus, resulting in 1321 conserved sites with 38 variable regions and 10 parsim informative sites. From the overall sequences generated and observed, matK showed more variations with maximum number of informative sites.

3.1 Distance analysis and barcoding regions for species identification

The analysis of barcoding gaps assists in es-

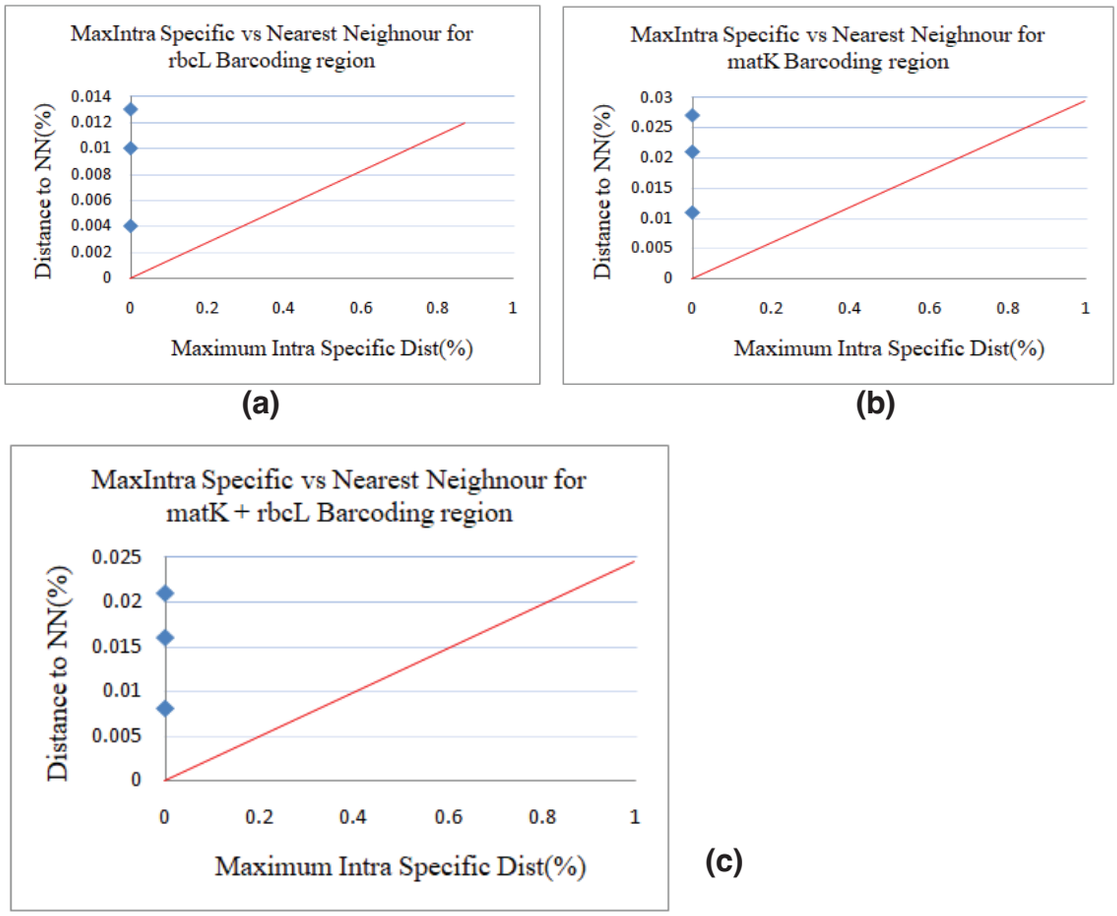

Figure 2- *Graph showing barcoding gap plotted against the distances to the nearest neighbor (NN) vs. the maximum intra-specific distances (%) for three barcoding region i.e. for (a) rbcL (b) matK (c) matK+rbcL. The pair wise genetic distance revealed discrimination ability for Euphorbia species. Individuals with same genetic distance are represented with single dot. Linear intersecting line indicated the presence of barcoding gap with respect to three barcoding loci tested.*

tablishing the distances within conspecific and congeneric species. The scatter graphs plotted for barcoding gap analysis within the nearest neighbor (NN) of Euphorbia royleana against the genetic distance of conspecific species revealed mean interspecific genetic distance greater than the mean intraspecific genetic distance in cases of both matK and rbcL barcoding regions (Figure-2). Among the closely related individuals concerning the matK barcoding region, the highest distance of 0.027 was recorded for *Euphorbia hirta*. Euphorbia maculate and Euphorbia abyssinicia showed a barcoding gap of 0.021 and 0.011, respectively, in terms of their nearest neighbor to *Euphorbia royleana*. The matK sequences demonstrated a barcode gap, i.e.031 pairwise genetic distance, which makes it the best candidate barcode in comparison to rbcL to identify *Euphorbia royleana* and its closely related species (Figure-2). In terms of matK barcoding loci, Euphorbia maculate and Euphorbia abyssinicia recorded the lowest NN distance of 0.011 and 0.021 (matK) amongst the four species; whereas Euphorbia hirta showed maximum genetic distance with a 0.027 barcoding gap, which makes matK a potent candidate barcode in terms of barcoding gap analysis and the best marker for the discrimination of Euphorbia

royleana from its closely related species available on the market.

Among the closely related individuals of *Euphorbia royleana*, the highest distance of 0.013 was recorded for Euphorbia hirta for the rbcL barcoding region. Euphorbia maculate and Euphorbia abyssinicia showed the maximum genetic barcoding gap in terms of their nearest neighbor with Euphorbia, which was observed to be 0.010 and 0.004, respectively. The interspecific genetic distance study can successfully discriminate between two closely related species, as shown in Figure-1. Meanwhile, rbcL exhibited a maximum inter-specific divergence of only 0.015, making it a less suitable choice in comparison to matK for species identification.

The maximum inter-specific genetic distance in a multilocus region (matK+rbcL) was observed to be 0.024, revealing it a potent choice as a candidate loci, beside the matK barcoding region. In terms of matK+rbcL barcoding loci, Euphorbia maculate and Euphorbia abyssinicia recorded the NN distance of 0.016 and 0.008, respectively, whereas Euphorbia hirta showed maximum genetic distance with a 0.021 barcoding gap.

The maximum genetic distance to the nearest region (NN) in a multilocus barcoding region (matK+rbcL) when compared to single-locus matK revealed lesser genetic distance. Thus, matK as single locus barcode can be considered the best candidate marker for the discrimination of closely related species of Euphorbia royleana a vailable in the market.

The scatter graphs have been plotted for maximum intra-specific distances versus the Nearest Neighbor distances to validate and authenticate the subsistence and enormity of the barcode gap with three tested candidate barcodes. From the graph, it can be clearly observed that no maximum intra-specific divergence existed between conspecific species of Euphorbia royleana. Based on the utility of the single-locus barcode approach, matKcan be considered as the prospective candidate barcode for the recognition of Euphorbia royleana.

Similarly, with respect to the multilocus barcode approach, barcoding gap was observed compared with matK loci, which establishes the competence of a multilocus approach in plant DNA barcoding technology. The core barcode (matK+rbcL), as suggested by CBOL guidelines, suggests a barcoding gap between 0.008–0.021 among the individuals of the species with their nearest neighbor, thus suggesting suitability for the identification of Euphorbia royleana as shown in Figure-2.

3. 2 Species specific SNPs with respect to matK and rbcL barcoding regions

Regarding rbcL barcode, not even a single nucleotide polymorphism was observed for Euphorbia royleana, which could discriminate it from other species. However, in the case of other species like Euphorbia abyssinicia, two SNPs were located at 127 and 535 in which Adenine (A) and Thymine (T) were replaced with guanine and cytosine(C), respectively, in other reaming species. Euphorbia hirta showed only one SNP where cytosine was replaced by Thymine at position 193 and Euphorbia maculate showed one SNP at position 430, where thymine was replaced with guanine.

The best single locus, matK, presented opportunities to differentiate species-specific sequences at different positions between 219 bp to 844 bp region as shown in Figure-3.

The 7 valuable, specific SNPs of Euphorbia royleana were located as follows:

- Position 299 cytosine(C) was replaced by guanine.
- Position 412 adenine (A) was replaced by thymine (T).
- Position 460 cytosine (C) was replaced by

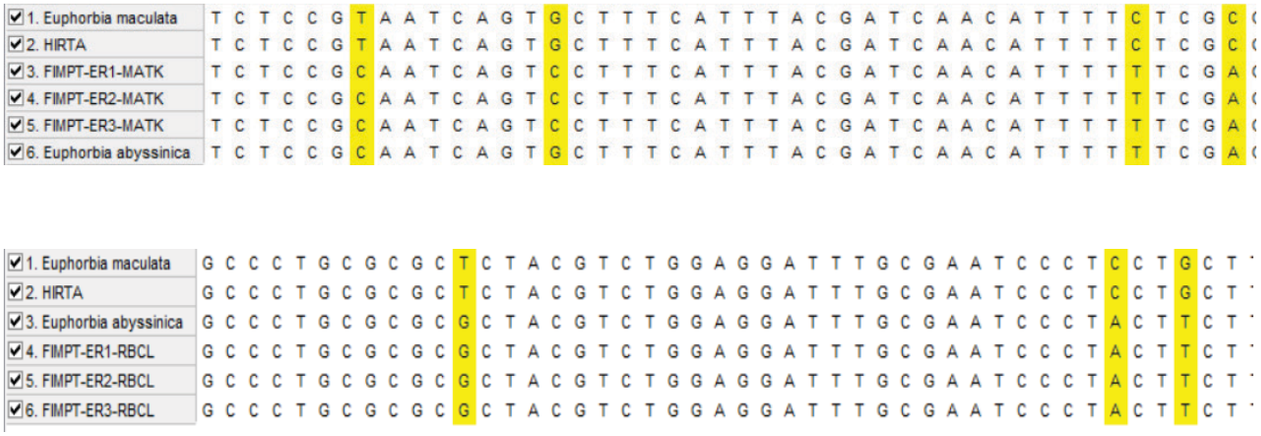

Figure 3- *Single nucleotide polymorphism (SNP) alignment of matK and rbcL DNA Barcoding region variability with respect to closely related species.*

adenine(A).

- Position 597 thymine (T) was replaced by guanine(G).
- Position 719 cytosine (C) was replaced by thymine(T).
- Position 820 cytosine (C) was replaced by adenine(A).
- Position 822 thymine (T) was replaced by cytosine (C)

Similarly, in Euphorbia hirta the valuable SNP was located at position 319 in which cytosine is replaced by guanine. Regarding Euphorbia abyssinicia, two valuable SNPs, 241 and 279, were identified in which adenine and thymine (T) are replaced by cytosine (C). Only one valuable SNP was observed with respect to Euphorbia maculate at position 251 in which cytosine (C) was exchanged with thymine (T) in all the remaining closely related species.

3. 3 Validation of DNA barcoding

DNA sequences of matK and rbcL barcoding regions were observed to be highly distinctive for all Euphorbia species. DNA extracted from herbal samples collected from the market yielded more degraded DNA compared to the DNA obtained from fresh plant samples. PCR amplification and DNA sequencing was still success ful regarding matK and rbcL, whereas the ITS barcoding region was not successfully amplified and, therefore, was not included in the present study. In the present study, PCR amplifications with 2.0 mM to 7.0 mM MgCl2 concentrations showed stable amplification results. The annealing temperature of 500C with ± 3°C for matK was observed to produce good amplification results. The annealing temperature for rbcL was standardized to be 580C. Even when changing the cycle number and different DNA Taq polymerases, obtained from different manufacturers, results remained the same. All PCR products obtained in the validation study were sequenced and aligned with positive controls of Euphorbia royleana and were 100% similar in the DNA sequence alignments.

4. Discussion

DNA barcoding technology is able to authenticate and discriminate between the molecular identity of Euphorbia royleana species and its closely related species of E. royleana.

The present study was the first published attempt to represent the molecular phylogeny of the threatened and endangered Euphorbia royleana species listed in CITES Appendix-II. The bioinformatics analysis of the markers suggests that the

barcoding regions can precisely discriminate between the closely related species.

The molecular study with plastid loci can be helpful in providing resolution among both conspecific species and congeneric species (Figure-1). From the tested plastid loci, the matK region as a single locus showed the maximum competence for the identification of species in Euphorbia royleana.

The plant molecular systematic study requires a candidate barcode, which could deliver relevant information on all taxonomic levels. The second most important aspect in choosing a plant DNA barcode was maximum discriminatory ability and higher evolutionary rate of the marker. The matK barcoding region showed potential variable coding regions, specifically in cases of angiosperms, and has also been recommended by CBOL to be a barcode for land plants [22,23]. However, matK exhibits low amplification in certain plant species due to less universality of certain primers. In the present study, the matK primer was tested for its applicability with respect to the Euphorbia royleana species. The results obtained suggest that matK satisfies the two criteria to be chosen as a candidate barcode to distinguish Euphorbia royleana from other conspecific and congeneric species. The matK region provided the most variable sites and with maximum parism informative sites with 100% successful amplification and sequencing results. The universality of this primer pair was tested using 10 (plants) of euphorbia royleana, and the primers showed strong amplification (100%) and sequencing (100%) success.

In comparison to the rbcL plastid region, the matK barcode provides superior phylogenetic evolutionary rate, specifically in the case of euphorbia royleana. The results obtained are in agreement with the results of other genus-level studies [24,25]. The lowest genetic distance was asingle-locus rbcL region, which limits its utility in Euphorbia genus. The large size of the rbcL,i.e. ~1430 bp length, and more conserved region in the markers show contentious limits for accurate identification at the species level. The whole barcoding region getting successfully amplified and sequenced for clear species discrimination is necessary and a primary requirement for successful identification.

The conditions which make a barcode strong for identification of species, are because of its small size and ability to pairing with universal primers easily [11,22]. The high success rate in terms of amplification and sequencing of the chloroplast matK coding region offers a better qualification as a candidate barcode, either as a single locus or in combination with rbcL. The matK barcoding region contains a larger number of nucleotide substitutions with a maximum number of variable sites and maximum parsim informative sites than rbcL loci from the plastid genome. Moreover, the main aspect of the matK barcoding region is the barcoding gap, which is demonstrated through higher inter-specific divergence values versus intraspecific divergence among matK sequences.

The clear distinction of closely related species is an important aspect from a legal point of view (CITES) to distinguish between legal and illegal trade. In the present study, the molecular identification method, especially DNA barcoding, successfully provided a potential tool to delineate closely related species. Similar observations were also made by earlier studies [22,23,26,27].

The investigation of single universal DNA barcode is suitable for the identification of all types of land plants. The scientific community/CBOL has also recommend matK+rbcL two-locus barcode for land plants, which is in line with the conclusions made in the present study. Moreover, we have performed a comprehensive validation as well as evaluation of

closely related species that were creating problems in tackling illegal trades due to inaccurate identification. The present study suggested two barcoding approaches to accurately authenticate the species as well as delineate the congeneric species, first with the combination of matK+rbcL and secondly with a single-locus matK barcoding region. The choice of barcoding region varies with respect to genus and species under question. Therefore, these types of validation studies are urgently required to affirm the choice of candidate barcoding loci so that standardized procedures can be adopted to help the forensic science community to successfully resolve such types of cases.

SNPs based on chloroplast DNA with respect to matK and rbcL regions are shown in Figure-3 and are well suited for specific molecular marker development. The SNP specific targeted study can be a valuable addition in establishing marker based kit and direct usage to identify specific plant species.

Species richness in NCBI

The present study was successful in developing a reference DNA barcode database for Euphorbia royleana, CITES listed important medicinal plants. DNA sequence data generated from the current study have been deposited and accepted at the NCBI nucleotide bank. The sequences published in NCBI GenBank were assigned accession numbers as follows:

- MK002729 and MK002727.1 format K region with 534bp and 474bp sequence length, respectively.
- MH765673.1 and MH765674.1 for rbcL region with 449bp and 658bp sequence length, respectively.

5. Conclusion

The unique sequence of the candidate barcode matK obtained in the present study provided precise leads in expedition of the molecular individuality of Euphorbia and its closely related species. The best candidate barcoding region for the identification of Euphorbia royleana species turned out to be matK with a single locus barcoding approach and have maximum interspecific genetic variations i.e. the matK showcase maximum genetic distances within different species of same genus. The results obtained (the novel sequences) in the present study have successfully contributed to NCBI GenBank for the identification of the Euphorbia royleana plant and will ultimately help in the delineation of closely related species. Additionally, the species-specific SNPs derived from the matK barcoding region established its importance in providing accurate species discrimination. The insertion of diverse conspecific populations and congeneric species helped in gaining imminent impending approach toward conservation of Euphorbia species.

It is expected that the sequences obtained in the present study will be useful in regulating the illegal wildlife trade in medicinal plants, by identifying natural herbal and aromatic plant species with the DNA barcoding technique for regulatory purposes and ensuring the quality and safety of natural medicines by validating the authenticity of natural herbal materials.

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Ethics approval and consent to participate

Ethical approval was not required.

Conflict of Interest

The authors declare that they have no conflict of interest.

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