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Potential use of DNA barcoding and phylogenetic analysis for the identification of medicinal plants of girnar forest

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Abstract

DNA barcoding is an efficient method for species level identification and also contribute in taxonomy and biodiversity. Bioinformatics plays very important role in analysis of barcode data. The cytochrome oxidase 1 (CO1) mitochondrial gene was successfully utilised for animal DNA barcoding but for plant it is difficult to standardise plant DNA barcode. Girnar Forest Sanctuary of Junagadh, Gujarat is very rich in medicinally important plants. Many researchers have identified many plant species of greater medicinal value by traditional method but in the present study the aspect was to study these plant species using molecular methods specially DNA barcoding. In the present study 6 different species which are very important in herbal medicine were identified using ribulose- 1, 5-bisphosphate carboxylase- (*rbcLa*) barcode. The species were identified efficiently by evolutionary relations with closely related species by constructing phylogenetic tress using neighbour joining method. Our study indicate that *rbcLa* was powerful barcode for species identification and also helpful in prevention of adulteration of medicinal plants.

Keywords: Adulteration, Bioinformatics, DNA barcoding, Medicinal Plants, *rbcLa*

1. Introduction

For the resolution of many biological issues such as diversity discovery, bio-security assurance, species conservation and pandemics preservation, species identification and correct classification are very crucial process [1, 2]. Many molecular methods have been increasingly investigated for the identification of medicinal plants [3, 4]. But the major disadvantage is that these techniques are not standardised for identification purpose [3]. Among different techniques, DNA barcoding has been recognised as simple, rapid, robust, cost effective and highly applicable approach for species identification with the advance in DNA sequencing [5, 6]. DNA barcoding employs sequence variation within a short standardised region of the genome "a barcode" to provide accurate identification at the species level [7, 8]. A 658 bp fragment of mitochondrial cytochrome C oxidase I (COI) has been designated as DNA barcode for animals [8]. For Plants, a two-locus chloroplast barcode using rbcLa-ribulose- 1, 5-bisphosphate carboxylase- and mat K-maturase K genes has recently gained support [9].

The adulteration of medicinal plants is difficult to achieve at international level. The traditional system of medicine utilizes medicinal plants to cure various ailments but the herbal industry suffers from substitution and adulteration of medicinal herbs with closely related species. The efficacy of the drug decreases if it is adulterated and in some cases can be lethal if it is substituted with toxic adulterants. The traditional methods of medicinal plant identification include organoleptic methods, macroscopic and microscopic methods and chemical profiling [10]. However neither method can identify the related species easily in processed products because the microscopic and macroscopic methods are depend too much on the availability of experts and are time consuming and laborious [11]. In the latter method, chemical profiles or markers may be affected by physiological and storage conditions. Authentication at the DNA level provides more reliability because, in contrast to RNA, DNA is more stable molecule and is found in all tissues. Therefore there is a need for development of DNA based method for authentication of medicinal plants [10]. So to ensure the safety in the extensive use of medicinal plants, molecular identification through DNA barcoding is necessary.

The aim of this study is to identify the very important medicinal plants of Girnar forest through DNA barcoding and phylogenetic analysis in order to prevent adulteration of medicinal plants.

2. Materials & Methods

2.1 Plant material

Samples used in this study were collected from Girnar forest, Junagadh, Gujarat and identified based on their morphological characters. Twig with good flowers was collected for the specimen with clear phyllotaxy and the branching system. Sufficient leaves were collected and put it into plastic bags containing silica beads. Plant materials were also pressed into blotting paper for herbarium preparation. The local name, botanical name, collection code, collection date, location, description of the floral parts and photos of the specimen were also recoded. Details on specimen ID, local name and botanical name based on morphological characters were summarised in Table 1.

Table: 1 Sampling ID, Local Name and Botanical Name

S. No.	Specimen ID	Vernacular Name	Botanical name	Family
1	S1	Popti	*Physalis angulata* L.	Solanaceae
2	S2	Ananthamul	*Hemidesmus indicus* (L.) R. Br. ex Schult.	Apocynaceae
3	S3	Sahdevi	*Vernonia cinerea* (L.) Less.	Asteraceae
4	S4	Indra dhanus	*Lantana camara* L.	Verbenaceae
5	S5	Rati dudheli	*Euphorbia hirta* L.	Euphorbiaceae
6	S6	Puntranjiva	*Putranjiva rouxburghii* Wall.	Putranjivaceae

2.2 DNA extraction, amplification and sequencing

Genomic DNA was extracted using fresh leaves and seeds of plants in an initial weight of approximately 60 mg for leaves and 30 mg for seeds. Extraction was carried out using Macherey NAGEL kit (Ref. No. 740663.24).

PCR was carried out using 1X final concentration of ReadyMix™ Taq PCR Reaction Mix (Sigma) and, template DNA (50 ng/µl) and was performed in Thermal cycler (Applied Biosystems Veriti®). Each PCR reaction contained 1X ReadyMix™ Taq PCR Reaction Mix (10 µl), 10 pmole of each forward and reverse Primers and 50-100 ng Template DNA. Two specific primers were used, rbcLaF: ATGTCACCACAAACAGAGACTAAAGC and rbcLaR: GTAAAATCAAGTCCACCRCG (Levin et al. 2003; Kress et al. 2007). PCR product was visualised on 2 % Agarose gel. The gel images were recorded in JPEG or TIF formats using gel documentation system (Biorad, USA). The gels were analyzed by using the software Image lab version 3.0 (Biorad, USA). The amplified products were purified using Gen Elute™ PCR Clean-up kit (Cat # NA 1020-1kt).

Successful PCR products were sequenced using BigDye® Terminator v 3.1 Cycle sequencing kit. The cycle sequencing was performed in 10 µl system in Thermal cycler (Applied Biosystems Veriti®). Each reaction mixture contained 4 µl Terminator Ready Reaction mix v 3.1, 1 µl Bigdye Sequencing buffer, 150 - 300 ng template, 10 pmole of each primer and deionized water to make the volume up to 10 µl. In order to achieve optimal results, complete removal of unincorporated dye terminators before performing capillary electrophoresis is necessary. So the sequencing products were purified using BigDye XTerminator® Purification Kit. Kit contains SAM™ Solution and BigDye® XTerminator™ Solution. Capillary electrophoresis of cycle sequenced products was Performed on 3500 XL platform (Applied biosystems).

2.3 Sequence alignment and data analysis

Sequence data from cycle sequencing were evaluated using sequencing analysis version 5.4 (Apllied Biosystems) and BioEdit, biological sequence alignment editor (Ibis Biosciences). Consensus sequences were generated after aligning gene sequences from forward and reverse primers. Short sequences (< 50 bp) and sequences with gaps were removed. Remaining sequences were exported into FASTA format and used for identification. Two methods of species identification including Maximum likelihood method of phylogenetic tree and BLAST based methods were performed. The multiple and pairwise alignment was performed using Clustal W multiple alignment tool. The phylogenetic trees were constructed with the neighbour joining (NJ) method based on p-distance model using MEGA 6.06 [12]. These sequences were subjected to sequence match analysis using Basic Local Alignment Search Tool (BLAST) on NCBI. Identification was done by 99% base-pair match of the sequence obtained to the closest available reference sequences of the corresponding species. After the preliminary analysis the sequence was submitted to BOLD SYSTEMS according to the guidelines provided onto BOLD website (http://www.boldsystems.org/).

3. Results and Discussion

Girnar is rich in plants of medicinal value and in general floral biodiversity and is the highest one in Saurashtra of Gujarat and due to difficulty to explored whole Girnar Forest Sanctuary, very important medicinal plants of the region were selected. Species identification by morphology faces several difficulties like phenotypic plasticity, genotypic variability, life stage and gender [8, 13, 14]. Other molecular techniques have standardization problem [3]. So DNA barcoding is the new approach with the greater advance in biotechnology to overcome these difficulties [5].

The medicinal important of selected plant species were shown in Table 2. The vouchers of all specimens were submitted to Biodiversity Gene Bank, Gujarat State Biotechnology Mission. The voucher ID given by the institution was shown in Table 3. The high quality DNA was successfully isolated from all 6 specimens and used for the amplification of rbcLa. The rbcLa fragment was successfully amplified from all 6 specimens. High quality sequences were obtained from all specimens. The sequences length was observed in between 550 to 570 bp. The obtained sequences were used to identify the specimens by using BLAST. All species showed 100 % identity and 0.00 E-value. The results based on the BLAST method indicated that rbcL have highest identification efficiency at species level. BLAST search in GeneBank produced the similar result as morphological one which was showed in Table 3. The results of BLAST match was also verified by neighbour-joining analysis. Levels of intra and inter specific divergence was calculated using pairwise distance model [12].

Table 2: Importance of Plant in Medicine

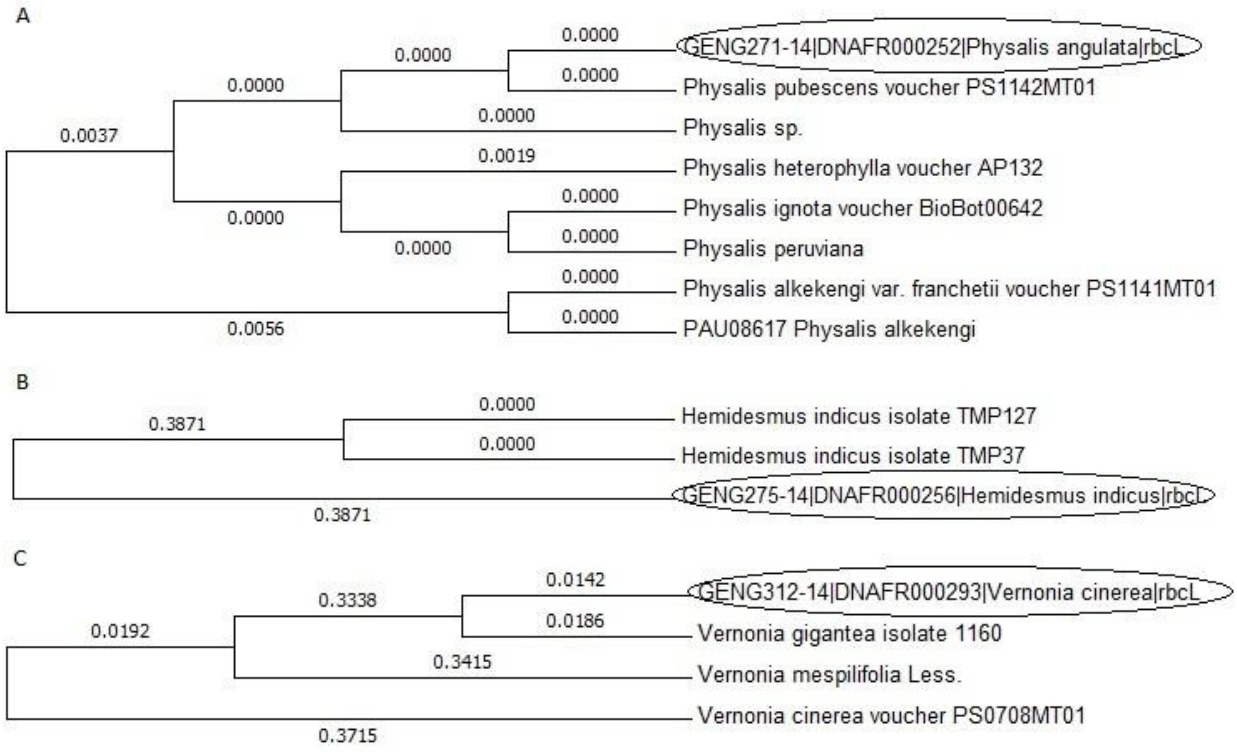
S. No.	Plant	Medicinal Value
1	*Physalis angulate*	Kills bacteria, viruses, cancer cells, germs, relieves pain, reduce inflammation, prevents ulcers, reduce fever etc.
2	*Hemidesmus indicus*	Diaphoretic, depurative, diuretic, immunosuppressant, aphrodisiac, antisyphilitic, antileucorrhoeic, galactogenic, anti-diarrhoel, antirheumatic, febrifuge, helps in indigestion, dysentery, cough, bronchitis, uterine hemorrhage and blood diseases, also useful in skin disease, fever, thirst, vomiting, poisoning,

		anemia etc.
3	*Vernonia cinerea*	Used as specific herb for leucorrhoea, dysuria, spams of bladder and for haematological disorder. Also used in asthma.
4	*Lantana camara*	Anticancer, antiproliferative, antibacterial, haemolytic, antioxidant, anti fungal, anti mutagenic, anti-inflammatory, wound healing, antifilarial activity
5	*Euphorbia hirta*	Increasing milk flow in women, increase urine output, antidiarrheal, antispasmodic, anti-inflammatory etc.
6	*Putranjiva rouxburghii*	Treatment of catarrh, skin disease, fever and sterility and also in rheumatism

Table 3: Sequencing Results

S. No.	Specimen ID	Voucher ID	Identification			Sequence Length(bp)
			Botanical name	Gene Bank		
				Identity	E-value	
1	S1	BIOMTFR-HER-000833	*Physalis angulata*	100 %	0.00	570
2	S2	BIOMTFR-HER-000838	*Hemidesmus indicus*	100 %	0.00	558
3	S3	BIOMTFR-HER-000840	*Vernonia cinerea*	100 %	0.00	579
4	S4	BIOMTFR-HER-000841	*Lantana camara*	100 %	0.00	558
5	S5	BIOMTFR-HER-000878	*Euphorbia hirta*	100 %	0.00	567
6	S6	BIOMTFR-HER-000880	*Putranjiva rouxburghii*	100 %	0.00	570

Phylogenetic analysis by neighbour joining method is another effective approach for species identification. According to Will and Rubinoff, morphology could not play a role for identification and classification through DNA barcoding [15]. A combination of DNA sequences with existing morphological characters is further suggested to accelerate species identification and classification [16]. The accuracy of DNA barcoding for identification of the species is depends on the extent of barcoding gap [17]. The barcoding becomes less reliable if the more overlap exists between intraspecific and interspecific divergence [18]. The barcoding gap is the difference between the maximum intraspecific and the minimum interspecific distances. For Physalis sp., Hemidesmus sp., Vernonia sp., Lantana sp. Euphorbia sp. and Putranjiva sp. net average interspecific variation were found to be 0.00, 0.77, 0.36, 0.77, 0.02, 0.00 respectively and intraspecific variation were found to be 0.00, 0.77, 0.74, 0.72, 0.02, 0.01 respectively. According to the neighbour-joining tree, each genus represented a monophyletic unit (Fig 1). Each number on the tree represents the branch length. Phylogentic tree was created for closely related species. The tree clearly indicated that our identification is very closely related to the same genus according to evolutionary relationship. Correctly identified specimens were submitted to Barcode of life Datasystem. The results of barcoding were shown in Table 4.



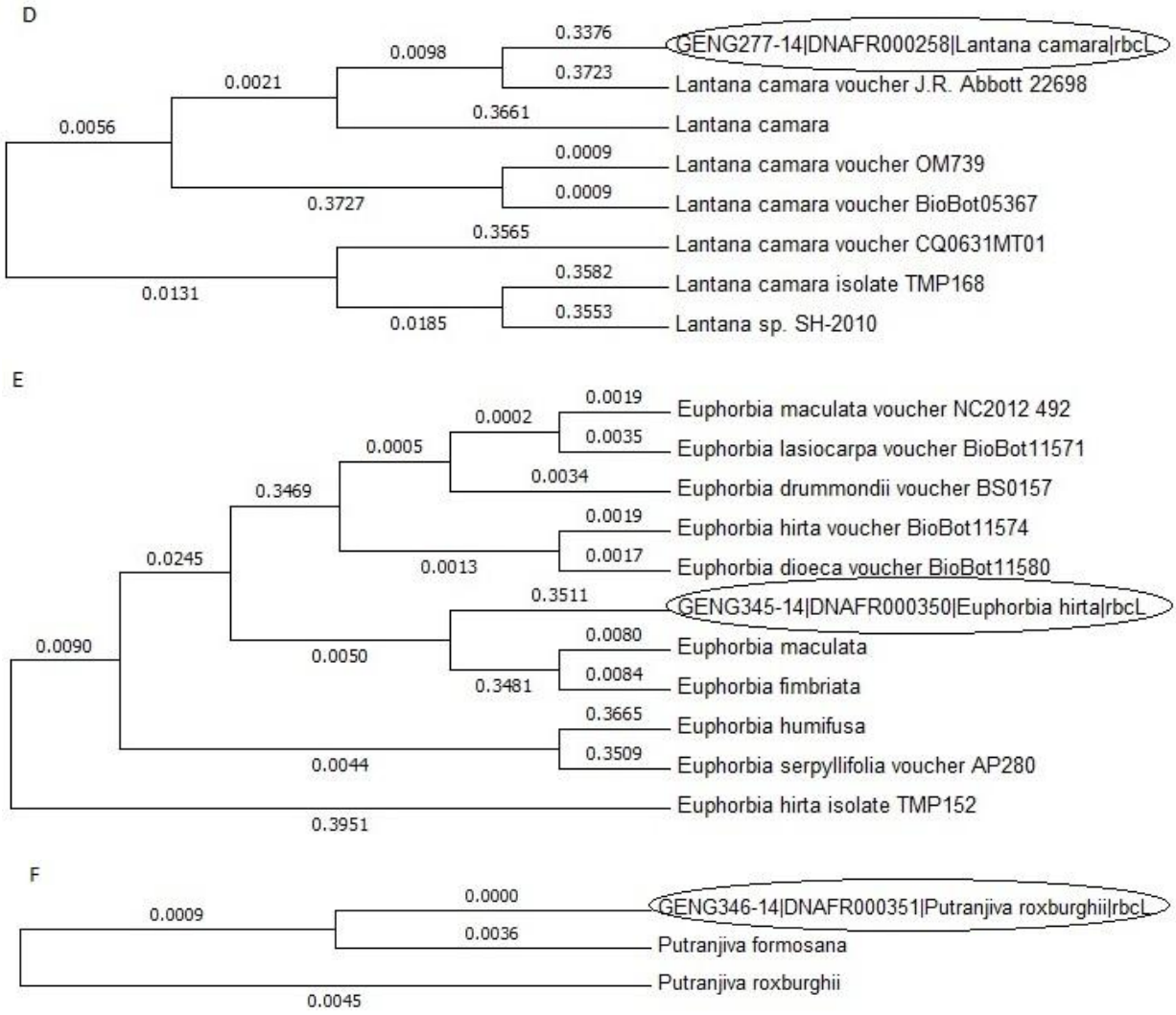


Fig 1: Phylogenetic tree constructed using neighbour-joining method. A. *Physalis angulata* B. *Hemidesmus indicus* C. *Vernonia cinerea* D. *Lantana camara* E. *Euphorbia hirta* F. *Putranjiva roxburghii*

Table 4: Barcoding Results

S. No.	Specimen ID	Identification		Sample ID	Process ID
		Botanical Name	Barcoding Identification		
1	S1	*Physalis angulata*	*Physalis angulata*	DNAFR000252	GENG271-14
2	S2	*Hemidesmus indicus*	*Hemidesmus indicus*	DNAFR000256	GENG275-14
3	S3	*Vernonia cinerea*	*Vernonia cinerea*	DNAFR000293	GENG312-14
4	S4	*Lantana camara*	*Lantana camara*	DNAFR000258	GENG277-14
5	S5	*Euphorbia hirta*	*Euphorbia hirta*	DNAFR000350	GENG345-14
6	S6	*Putranjiva rouxburghii*	*Putranjiva rouxburghii*	DNAFR000351	GENG346-14

The research findings have various applications: One is to provide insights into species-level taxonomy and contribute towards the taxonomic process of defining and delimiting species. The second, and major application, is to assist in the process of identifying unknown specimens to known species. DNA barcoding approaches are providing useful insights into cryptic species diversity. DNA barcodes could be a useful tool for plant conservation. Of particular importance is the ability to identify unknown plant material, such as from customs seizures of illegally collected specimens. It had potentials in biodiversity, bio security, discovery of new species and the prevention or detection of illegal trade.

4. Conclusion

The study was concluded as that rbcLa is helpful in identifying the plants at molecular level because it shows the higher amplification with 500-600 bp in length. This study demonstrates the efficacy of DNA barcoding in identification of medicinal plant. The study will be helpful to ensure the safety in their extensive use.

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