

Identification of a Herbal Powder by Deoxyribonucleic Acid Barcoding and Structural Analyses

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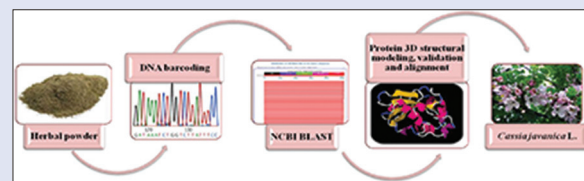
ABSTRACT

Background: Authentic identification of plants is essential for exploiting their medicinal properties as well as to stop the adulteration and malpractices with the trade of the same. **Objective:** To identify a herbal powder obtained from a herbalist in the local vicinity of Rajkot, Gujarat, using deoxyribonucleic acid (DNA) barcoding and molecular tools. **Materials and Methods:** The DNA was extracted from a herbal powder and selected *Cassia* species, followed by the polymerase chain reaction (PCR) and sequencing of the *rbcL* barcode locus. Thereafter the sequences were subjected to National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) analysis, followed by the protein three-dimension structure determination of the *rbcL* protein from the herbal powder and *Cassia* species namely *Cassia fistula*, *Cassia tora* and *Cassia javanica* (sequences obtained in the present study), *Cassia Roxburghii*, and *Cassia abbreviata* (sequences retrieved from Genbank). Further, the multiple and pairwise structural alignment were carried out in order to identify the herbal powder. **Results:** The nucleotide sequences obtained from the selected species of *Cassia* were submitted to Genbank (Accession No. JX141397, JX141405, JX141420). The NCBI BLAST analysis of the *rbcL* protein from the herbal powder showed an equal sequence similarity (with reference to different parameters like E value, maximum identity, total score, query coverage) to *C. javanica* and *C. roxburghii*. In order to solve the ambiguities of the BLAST result, a protein structural approach was implemented. The protein homology models obtained in the present study were submitted to the protein model database (PM0079748-PM0079753). The pairwise structural alignment of the herbal powder (as template) and *C. javanica* and *C. roxburghii* (as targets individually) revealed a close similarity of the herbal powder with *C. javanica*. **Conclusion:** A strategy as used here, incorporating the integrated use of DNA barcoding and protein structural analyses could be adopted, as a novel rapid and economic procedure, especially in cases when protein coding loci are considered.

Key words: Deoxyribonucleic acid barcoding, herbal powder, identification, protein three-dimension structure, *rbcL*

SUMMARY

- Authentic identification of plants is essential for exploiting their medicinal properties as well as to stop the adulteration and malpractices with the trade of the same. A herbal powder was obtained from a herbalist in the local vicinity of Rajkot, Gujarat. An integrated approach using DNA barcoding and structural analyses was carried out to identify the herbal powder. The herbal powder was identified as *Cassia javanica* L.



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INTRODUCTION

Medicinal plants have been used by mankind since ages to cure common as well as serious health ailments. Indian flora comprises of an affluent floristic diversity of medicinal plants. Medicinal values of these plants are exploited in major modes of therapy such as homeopathy, aroma therapy, traditional ayurvedic medicine, Unani, herbal therapy, etc. The process of herbal drug development often experiences (a) deliberate or indeliberate adulteration, and (b) unintentional substitution due to lack of authentic information.^[1] Hence, the identification of the plant products are of prime importance in preventing the adulteration and malpractices with the trade of the same.^[2] Unambiguous identification and authentication of the plants used for the production is therefore an elementary and critical step at the beginning of an extensive quality assurance process. Many different methods have been used since long for the identification of medicinal plants such as traditional pharmacognostic analysis,^[3,4] chromatographic and spectroscopic,^[5-7] physical techniques,^[8,9] and molecular markers.^[10] The latter are more suitable for identification as deoxyribonucleic acid (DNA), in contrast to ribonucleic acid, is a stable macromolecule that is found in all tissues, including the dead ones.

Among these, molecular marker techniques provide valuable data on diversity through their ability to detect variation at the DNA level, and have been routinely employed as they are easy, rapid and reliable to perform. Many DNA based methods have been used previously for detecting and differentiating plant materials from their contaminants viz. Random Amplified Polymorphic DNA,^[11,12] polymerase chain reaction (PCR)-Restriction Fragment Length Polymorphism,^[13,14] high-resolution melting analysis,^[15,16] DNA barcoding,^[17-21] etc. Among

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these, DNA barcoding is a comparatively recent technique, which uses DNA sequences from a small fragment of the genome to identify organisms.^[22] Furthermore, bioinformatics approaches have been of immense help for the in-depth understanding of the plant systems in various ways.^[23,24]

The *rbcl* gene codes for the large subunit of Rubisco enzyme in plants, is exclusively chloroplastic in origin and is a very useful molecule in phylogenetic analysis^[25] as well as designated as a core barcode in plants.^[26] It has been used in identification of medicinal materials earlier.^[18,27,28] In this study, a herbal powder was obtained from a herbalist in the local vicinity of Rajkot, Gujarat. Thereafter, the DNA was extracted, followed by the PCR and sequencing of the *rbcl* barcode. The basic local alignment search tool (BLAST) results, showed an equal sequence similarity to *Cassia roxburghii* and *Cassia javanica*. Hence, the comparative protein structural analysis was carried out to solve the ambiguity observed in the BLAST results.

MATERIALS AND METHODS

In this study, an easy, inexpensive, and rapid approach was used for the identification of a herbal powder, using the molecular and bioinformatics tools.

Deoxyribonucleic acid isolation from the medicinal extract and *Cassia* species

The DNA was isolated from the unknown medicinal extract using the cetyltrimethylammonium bromide method.^[29] The same was done for the *Cassia* sp. available in the local vicinity viz. *Cassia tora*, *Cassia fistula* and *C. javanica*.

Polymerase chain reaction amplification, nucleotide sequencing of the *rbcl* gene and basic local alignment search tool analysis

The *rbcl* gene was amplified from the herbal powder as well as the selected species of *Cassia* (mentioned earlier) with the primer pair: *rbcl*_F (5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3')^[30] and *rbcl*_R (5'-GTA AAA TCA AGT CCA CCR CG-3').^[31] The PCR amplification was conducted using 25 µL of reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 25 mM MgCl₂, 10 mM of each dNTPs, 10 pmol each of forward and reverse primers, 2 µL of template DNA and 1 units of Taq DNA polymerase (Genie, India) (without proof-reading activity) on a Veriti (96 Well Fast Thermal Cycler), Applied Biosystems, USA. 5 µL of each PCR product was electrophoresed through 2% agarose gel to determine the size of fragment. Positive products were then purified using the Exo-Sap-IT reagent (USB, Cleveland OH, USA) using the manufacturer's recommended protocol (2 µL reagent per 5 µL amplified DNA product). Thereafter, the purified samples were subjected to nucleotide sequencing using Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, NY, USA) as per manufacturer's protocol. After purification, the reaction products were analyzed on an ABI PRISM Genetic Analyzer 3130 (Applied Biosystems, NY, USA). Sequence editing was performed using Bioedit.^[32] The nucleotide sequences from the samples of: *C. tora*, *C. fistula*, *C. javanica* were submitted to the National Center for Biotechnology Information (NCBI) Genbank database using the standalone submission tool Sequin. The obtained *rbcl* sequence of the herbal powder was compared with sequences in the NCBI-Genbank using the BLAST. In order to increase the taxonomic coverage for the further bioinformatics analyses, the *rbcl* sequences of *Cassia abbreviata* and *C. roxburghii* were retrieved from the GenBank and used in addition to those plants for which the wetlab studies were carried out. All the nucleotide sequences were translated computationally using Bioedit^[32] in order to obtain the protein sequences, which were further used for the three-dimension (3D) structural analyses.

Homology modeling of the *rbcl* proteins

The protein 3D structures of *rbcl* proteins of different *Cassia* species along with that from the herbal powder were elucidated using the homology modeling approach. The 3D models were checked for their validity using the Ramchandran plot analysis. The models were also stereochemically checked with the help of Verify 3D server.^[33] Furthermore, the protein 3D models were submitted to the protein model database (PMDB)^[34] with the PMDBIDs: PM0079748-PM0079753 [Table 1].

Structural analyses using bioinformatics' tools

The multiple structural alignment was carried out using PROMALS3D server.^[35] Further in order to clear ambiguity of the previously obtained BLAST results, the pair-wise structural alignment analyses was also carried using herbal *rbcl* protein as template and that of *C. javanica* and *C. roxburghii* as targets.

RESULTS AND DISCUSSION

In the present investigation, a herbal powder, procured from a local herbalist, was tested with the plant DNA barcoding approach. *rbcl* is a core barcode locus^[26] and otherwise has been used in the phylogenetic analysis of plants since decades.^[36-41] The DNA was successfully isolated from the herbal powder evidenced in the form of sharp bands on the 1% agarose gel [Figure 1]. The *rbcl* barcode locus was amplified (~550 bp) [Figure 2] and sequenced on the Applied Biosystems 3130 Genetic Analyzer. The sequences obtained in this study were submitted to NCBI (Accession No. JX141397, JX141405, JX141420). The sequence was then compared with other sequences in the database using the NCBI-BLAST tool [Figure 3]. The query sequence showed an identity of 89% and query coverage of

Table 1: Ramchandran plot analyses of the homology models (using Rampage)

Plant name	PMDBID	Number of residues in the favored region (%)	Number of residues in the allowed regions (%)	Number of residues in the outlier regions (%)
<i>Cassia abbreviata</i>	PM0079748	97.1	1.7	1.2
<i>Cassia fistula</i>	PM0079749	94.7	4.3	1.1
<i>Cassia javanica</i>	PM0079750	97.2	2.8	0.0
<i>Cassia roxburghii</i>	PM0079751	97.5	2.5	0.0
<i>Cassia tora</i>	PM0079752	93.2	6.8	0.0
Herbal powder	PM0079753	94.0	4.0	2.0

PMDB: Protein model database

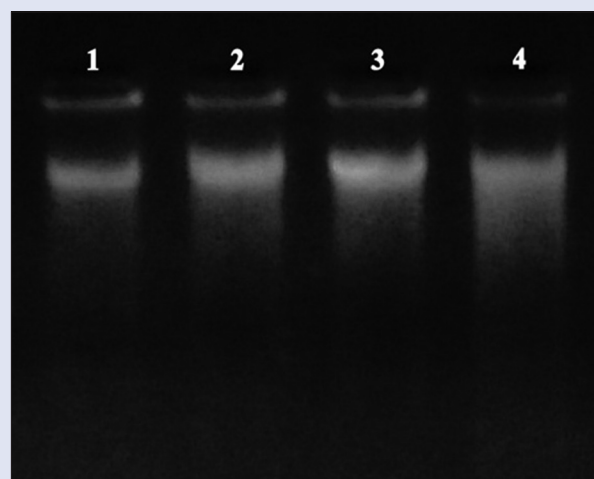


Figure 1: Agarose gel electrophoresis of the genomic deoxyribonucleic acid from the herbal powder and other *Cassia* species (Lane 1: Herbal powder; Lane 2: *Cassia fistula*; Lane 3: *Cassia javanica*; Lane 4: *Cassia tora*)

96% (with the same maximum score, total score and e-value) to two species of the *Cassia* genus: *C. javanica* and *Cassia roxburghii*. In addition, the nucleotide sequences of *Cassia roxburghii* (with accession no. JQ301861) and *C. abbreviata* (with accession no. JF265329.1) were retrieved from Genbank and used for the further bioinformatics' analyses.

The nucleotide sequences were computationally translated to amino acid sequences and the homology modeling of the protein sequences was carried out for the herbal powder as well as the selected *Cassia* species [Figure 4]. The hypothetical protein 3D structures were validated using bioinformatics tools. To assess stereochemical quality and structural integrity of the model, RAMPAGE^[42] and verify 3D^[33] servers were used.

The homology models obtained so were checked for the stereochemical viability using RAMPAGE server^[42] (<http://mordred.bioc.cam.ac.uk/rapper/rampage.php>) [Table 1]. The Rampage server assesses the accuracy of the generated hypothetical models based on the Ramchandran plot analyses of the same. The Ramchandran diagram plots phi versus psi dihedral angles for each residue in the protein. The predicted models were found to be acceptable since all had more than 90% of the residues in the favored region. Verify 3D analyzes the compatibility of an atomic model (3D) with its own amino acid sequence (1D) and hence tests the accuracy of the model. The models were also checked

for the 3D-1D profiles using Verify 3D server^[33] [Table 2]. Residues with a score over 0.2 are considered reliable. In the present study, the profiles for all the predicted models were >0.2 indicating the correctness of the predicted models. This indicates the correctness of the models predicted in this study. Hence, the predicted models were used for the further bioinformatics analysis. The multiple structural alignment of the predicted homology models was carried out using PROMALS3D server^[35] using the default parameters in order to find the structural distance between the *rbcl* proteins of the herbal powder with that of the *Cassia* species. The promals 3D server analyses the sequence-structure relationships in form of multiple structural alignment and generates a phylogenetic tree. In this study, the phylogenetic tree [Figure 5] shows, the structural similarities between the *rbcl* protein of the medicinal extract with that of *C. javanica*, *C. roxburghii* and *C. abbreviata*. Furthermore, in order to clear ambiguity of the NCBI-BLAST results, the pairwise structural alignment analyses was carried out using the RCSB Protein Databank Protein Comparison Tool.^[43] In this study, the medicinal extract *rbcl* protein was used as a template and the *rbcl* proteins of *C. javanica* and *C. roxburghii* were used as targets. A lower

Table 2: Three-dimension - one-dimension profile of the homology models (using verify three-dimension)

Plant name	Three-dimension - one-dimension score
<i>Cassia abbreviata</i>	0.61
<i>Cassia fistula</i>	0.46
<i>Cassia javanica</i>	0.35
<i>Cassia roxburghii</i>	0.62
<i>Cassia tora</i>	0.35
Herbal powder	0.38

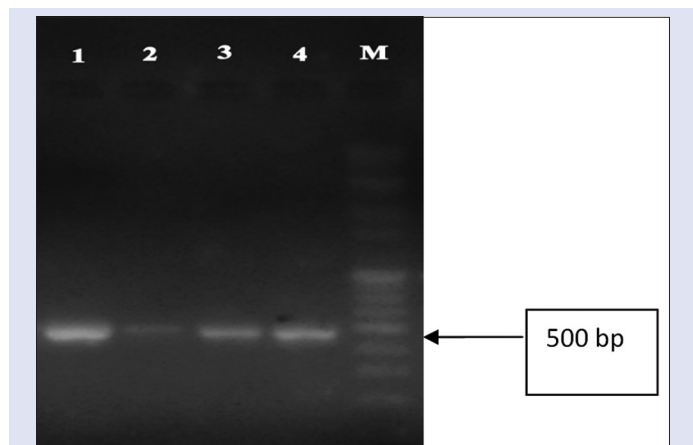


Figure 2: Agarose gel electrophoresis of the amplified *rbcl* gene from the selected samples (Lane 1: Herbal powder; Lane 2: *Cassia fistula*; Lane 3: *Cassia javanica*; Lane 4: *Cassia tora*)

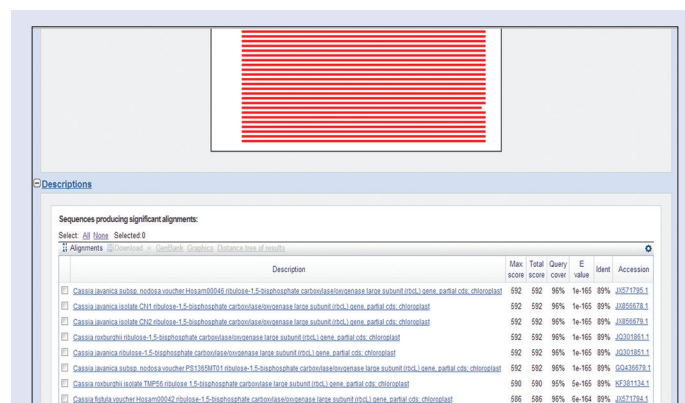


Figure 3: Ambiguity observed in the National Center for Biotechnology Information basic local alignment search tool output

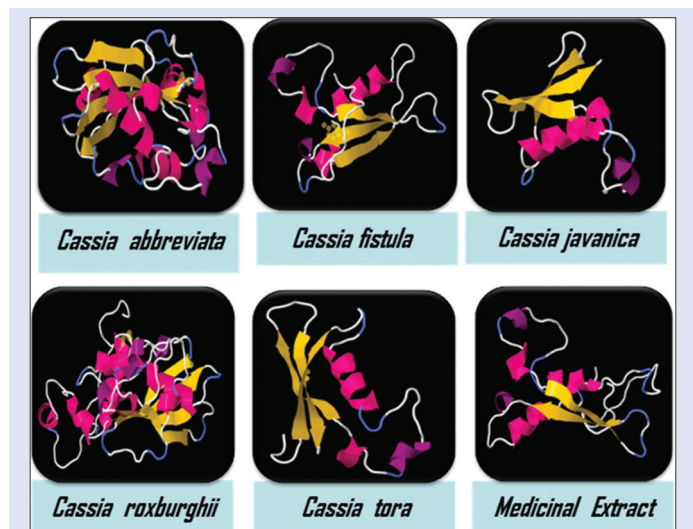


Figure 4: Homology modelling of the *rbcl* protein from selected samples

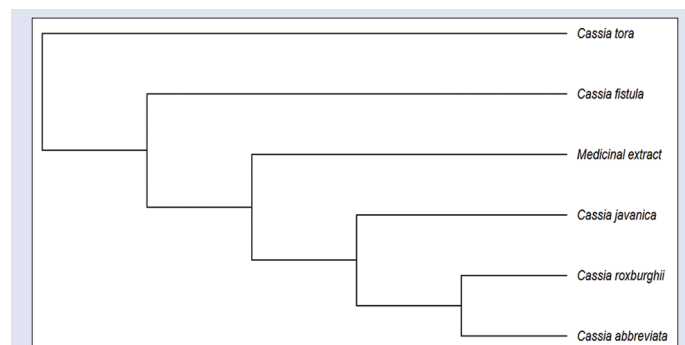


Figure 5: Phylogenetic tree based on the multiple structural alignment between herbal powder (medicinal extract) and *Cassia* species

Table 3: Pairwise structural alignment of *rbcl* protein from the medicinal extract with that of *Cassia* spp

Template	Target	RMSD
Herbal powder	<i>Cassia javanica</i>	0.81
	<i>Cassia roxburghii</i>	1.42

RMSD: Root mean square deviation

root mean square deviation (RMSD) value was observed in the pairwise *rbcl* protein structural alignment of the herbal powder with *C. javanica*, than that with *C. roxburghii* [Table 3]. A lower RMSD value implies, a closer relationship between the molecules under study. Hence, the herbal powder had a closer relationship with the *C. javanica*, which is also evidenced in the phylogenetic tree obtained from the multiple structural alignment. Hence, the herbal powder was identified as *C. javanica*.

There has been quite a discussion about the use of the sequence databases and similarity searching in the literature.^[44-47] Little and Stevenson^[48] found that BLAST can give accurate identification at the genus, but not the species, when DNA barcode sequences are considered. Similarly, there have been identification related problems, in doing similarity searching with BLAST.^[49] Hence, a strategy to solve such cases, is imperative, especially when protein coding loci are considered. The sequence similarity results, can be further confirmed by the protein structure data, as in this study; particularly when ambiguous BLAST results are obtained. Thus, the combined use of DNA barcoding and protein 3D structural analyses can help the identification of samples. The present study can therefore be used as an example of this new approach in conjunction with routine approaches of species' identification.

CONCLUSION

Hence this strategy, incorporating the integrated use of DNA barcoding and protein structural analyses could be adopted, as a novel, rapid and economic procedure for identification of plant species, especially in cases when protein coding loci are considered.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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