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Genetic authentication of medically significant legumes of Fabaceae family using the standardized DNA barcoding method

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Growing global demand for herbal medicine and nutritional supplements has increased due to the high usage of traditional herbs in recent decades. Various plant materials are in high demand as a result of the growing popularity of herbal goods. Chemical approaches, macroscopic and microscopic investigation, and plant taxonomy are examples of traditional identification techniques. Global commercial interest in traditional herbs identification at genetic level has increased due to high risk of adulteration in the herbs. The main method used to identify herbs genetically is DNA barcoding. The ability to determine the material's purity is the main advantage of this method. In this study, four medically significant plants were purchased from the local herbal market in Karachi. Using polymerase chain reaction technology and sequencing of the universal internal transcribed spacer region (ITS_2) a nuclear barcode region, samples were accurately identified. The selected bean samples were identified as Plant A: *Vigna mungo*; Plant B: *Lens culinaris*; Plant C: *Clitoriaternateas*; and Plant D: *Vignaradiata*, appears to have a ribosomal nuclear ITS_2 region that exhibits more than 90% similarity with the reference barcode sequences indicating a high rate of species identification and discrimination. Therefore, amplification and sequencing a universal barcode region in the DNA offers a revolutionary tool for identifying species, preserving genetic diversity, and using plant species responsibly that have medical importance.

Keywords: DNA barcoding, internal transcribed spacer region, adulteration, Polymerase chain reaction, Amplification

INTRODUCTION

Traditional medicine in many cultures across the world places a high value on the use of medicinal herbs to treat a wide range of ailments. Through the years, traditional knowledge about medicinal plants has been transmitted. Any food or medicine commodity produced requires high-quality raw ingredients with sufficient nutritional content and palatable flavor. (Gorini, 2023). Any food product's authenticity can be determined by whether or not it has the necessary biological element (Pereira, 2013). When it comes to processed foods, it can be challenging or even impossible to identify the pure material from the adulterant phenotypically that is, just by visual inspection if the structure and particle size/shape of the raw materials are altered or damaged as a result of processing techniques (Bosmali, 2012). Therefore, developing a system for detecting adulteration that is trustworthy, accurate, and has a strong discriminating capacity is essential. Since ancient times, plants have been used as a source of medicine.

To authenticate the source elements in food, a variety of chemical analysis techniques have been developed, including mass spectrometry (MS), thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), NMR spectroscopy, and Fourier transform infrared (FT-NIR) spectroscopy

(Karppinen, 2022). In these cases, the analysis possible using these methods is limited. Thus, whenever possible, techniques aimed at molecular biology-level identification such as DNA-based approaches are crucial. DNA barcoding has developed into a powerful tool for categorizing plant species despite the absence of physical features (Chen et al. 2023). It makes it possible to quickly and accurately identify the species.

A short, standardized section of the DNA sequence is used in DNA barcoding, a method for classifying and identifying species. The DNA barcoding approach is based on the use of the barcode region, a highly conserved section of the genome (de Boer et al. 2015). The sequence obtained after PCR amplification of this region is compared to a reference database of known species. The identification of organisms including fungus, animals, and plants has been done using this technique (Barcodes, 2006).

The internal transcribed spacer (ITS_2) barcode region of the plant genome is a helpful DNA barcode for identifying the plant species and has been utilized extensively in plant barcoding studies (Hassan, 2023; Michel et al. 2016). The ribosomal RNA gene's ITS_2 region, which lies between the small and large subunits, varies greatly between and within species. The ITS_2 region can likewise be amplified by PCR rather easily, and it can be sequenced using common sequencing methods. The ITS_2 region has been used to identify numerous plant species, including medicinal plants

It is difficult to accurately and quickly authenticate medicinal plants and their adulterants because of the magnitude of the global medicinal plant trade. Therefore, our objective is to provide a practical and reliable tool for identifying these medicinal plants and their adulterants in commerce and guaranteeing the security of their use.

The CTAB procedure was used to extract the whole DNA for this sample. Using ITS_2-S_2F and ITS_4 primers, the internal transcribed spacer (ITS_2) region of the plant genome was amplified, then sequenced and analyzed via bioinformatics tools.

The standardization of herbal products depends on the precise identification of medicinal plants because the chemical makeup and therapeutic qualities of herbs might vary depending on the species and growing conditions. DNA barcoding can be used to identify the correct species in herbal products and to check for impurities or adulterants.

Our study serves as a proof-of-concept for application of DNA barcoding in Pakistan. This method may be used to identify therapeutic plants in a reliable and effective manner, which could have significant ramifications for the herbal market and conventional medicine.

MATERIALS AND METHODS

In the present study, four different legume samples belonging to the family Fabaceae were collected from the local herbal market from Karachi in 2022 All corresponding voucher samples are deposited in the herbarium of the Hamdard laboratories Waqf Pakistan (HLWP) (Figure 1a).

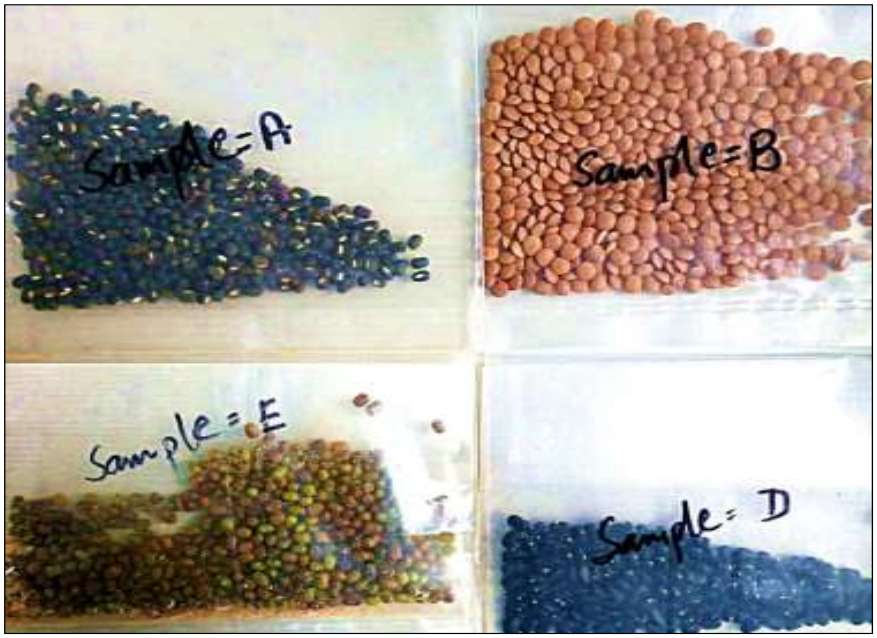

Figure 1a: Legume Samples morphological condition

DNA Extraction, Amplification and Sequencing

Prior to DNA extraction, the tested material was properly cleaned with 75% alcohol solution to prevent any microbial DNA contaminating in the sample. One gran of each sample was then ground into powder in liquid nitrogen using a mortar pestle. Using the CTAB method, genomic DNA was extracted according to the modified protocol (Doyle, 1991). In order to amplify the ITS_2 region,

ITS_2-S_2F ATGCGATACTTGGTGTGAAT and ITS_4 TCCTCCGCTTATTGATATGCprimers were used as recommended by the Consortium of Barcode of Life-Plant Group The PCR reaction mixture were 1µl (about 30 ng) of genomic DNA, 10 µl of 2x PCR master mix Dream taq (Thermo Fischer Scientific, USA), 1 µl of forward primer ,1 µl of reverse primer and 7 µl of nuclease free water PCR of 35 cycles with running conditions of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min.A final extension of PCR product at 72 °C for 7 min to complete the PCR reaction in a mini Amp thermal cycler (Applied Bio system, USA). The PCR product was assessed on 2% Agarose gel in the electrophoresis unit and a 100 bp DNA ladder (Thermo Fischer Scientific, USA) was use for size determination of the amplified product. The PCR product was stores at -20℃ for downstream process.

Sequence Analysis and Primer design

The PCR products were purified using a QIA rapid PCR purification kit (Tiangen Biotech, Beijing, China), and then immediately sequenced bi-directionally on an ABI 3730XL sequencer (Applied Bio system, USA) using the original amplification primer as the sequencing primer (figures 2-5). The initial forward and backward movements were put together using a 3.0 Codon Code Aligner. The entire ITS_2 sequences were subjected to carried out the nucleotide blast with the highest similarity score and lowest E value. The barcode gaps were manually edited in the pairwise alignment view using BLAST. Molecular Evolutionary Genetic Analysis' MEGA11 software (Molecular Evolutionary Genetic Analysis; (https://www.megasoftware.net/)was used to align multiple sequences using Clustal W v10.1.8(https://www.genome.jp/tools-bin/clustalw) (Gao et al. 2010).

RESULTS AND DISCUSSION

Amplification and Sequencing Results of ITS_2 barcoding gene

DNA barcode primer of the ITS_2 region of the selected samples were successfully amplified with determined size of the PCR product as shown in figure 1b. DNA bands obtained from the amplification reveals the amplicon length of ± 370 bp after size comparison with the reference gene ruler of 100 bp.

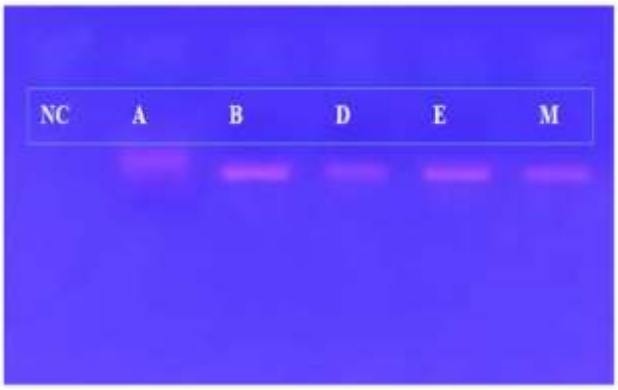

Figure 1b: **Amplified products of DNA barcode primer of the ITS2 region. NC:** negative control, Plant **A:** *Vigna mungo L. hepper*; **B:** *Lens culinaris Medik*; **D:** *Clitoris ternasea L.* **E:** Vignaradiate R. *wilczek*, and **M**: f100bp DNA ladder.

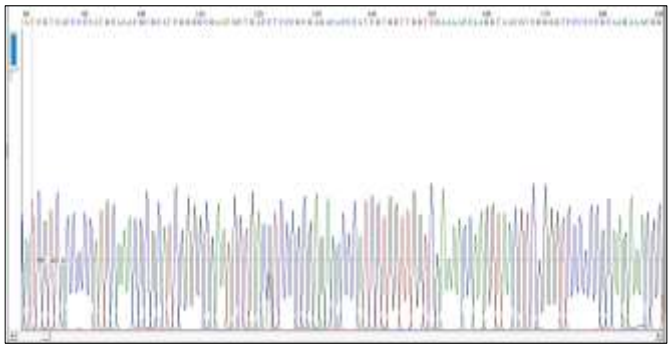

Figure 2: Trace/ABI file of Sample (A) Vigna mungo L. Hepper forward primer (ITS_2 S_2F)

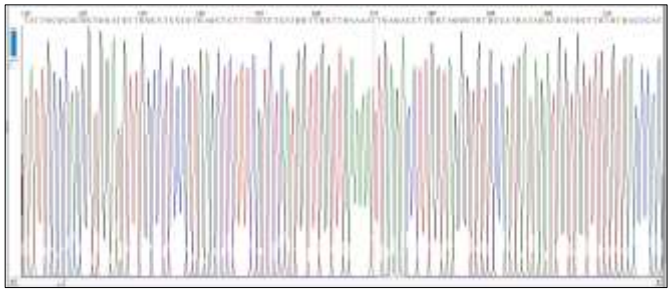

Figure 3: Trace /ABI file of sample (B) Lens culinaris Medik forward primer (ITS_2 S_2F).

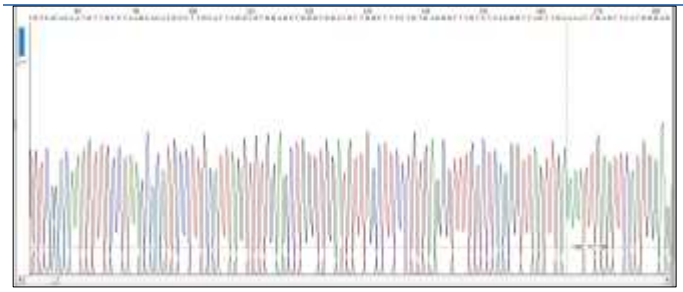

Figure 4: Trace /ABI file of sample (D) Cilitoria ternacia L Forward primer (ITS_2 S_2F).

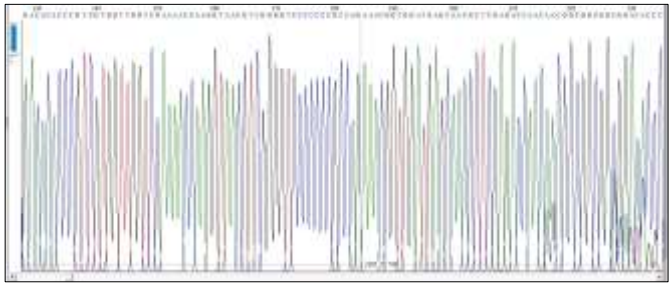

Figure 5: Trace / ABI file of sample (E) Vigna ridiata (L) R.Wilczek Forward primer (ITS_2 S_2F).

BLAST RESULTS

BLASTn analysis (NCBI gene bank) was conducted to identify the respective plants with the reference matched sequences in the NCBI database. Results shows that the inclusive samples were observed with more than 90 % identical match with the reference sequence in the NCBI database.

No gaps or indels were present in any of the sequence. The BLAST alignment scores of the genetically verified samples are described in Table 1.

Table 1: BLAST alignment scores with the reference barcoding IDs of the identified samples

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Sample	Herb Name	Percent Identity (%)	Accession ID
Specie A	Vigna mungo	99.66%	**MF467915.1**
Specie B	Lens culinaris	100%	**KJ787202.1**
Specie D	Clitoria ternatea	100%	**MG561852.1**
Specie E	Vigra radiata	100%	**KM210321.1**

When comparing the resulting DNA sequences of the tested samples with the reference samples in NCBI database, the alignment details of the sequenced barcoding regions i.e. the queried regions were compared with respect to the entire sequence length to the subject/reference sequence**.** As figures 6-9 indicates the detailed alignment report:

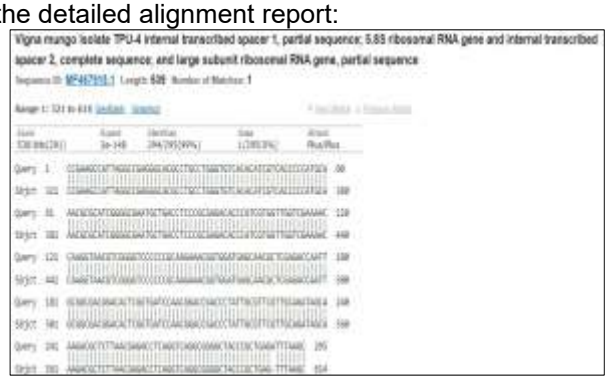

Figure 6:ITS_2 barcoding gene alignment with reference sequence of Vigna mungo

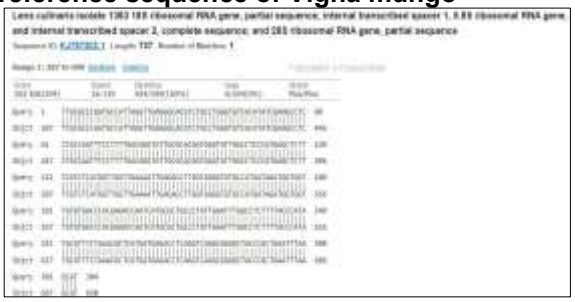

Figure 7: ITS_2barcoding gene alignment with reference sequence of Lens culinaris

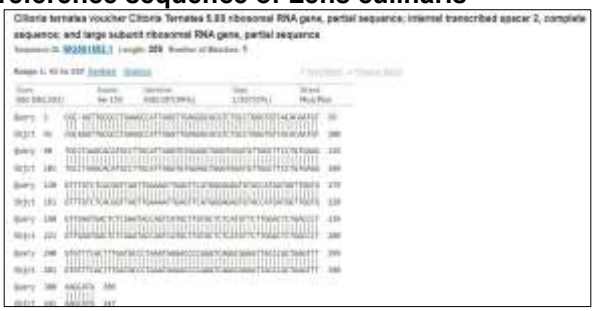

Figure8: ITS_2 barcoding gene alignment with reference sequence of Clitoris ternatea.

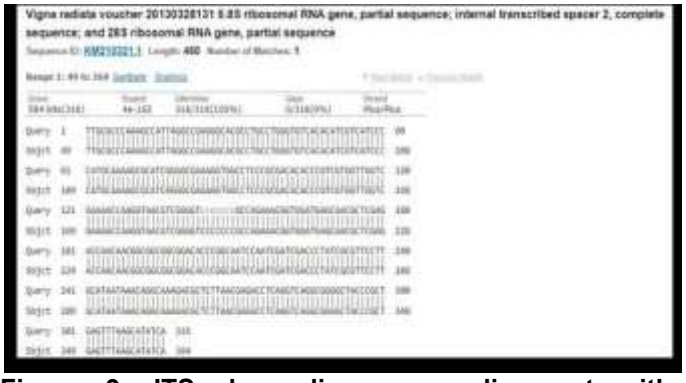

Figure 9: ITS_2 barcoding gene alignment with reference sequence of Vigna radiata.

The intra and inter sequence conserved regions were evaluated using the Molecular evolutionary genetic

analyzer tool. All the resulting identified sequences were subjected to multiple sequence alignment using the Clustal W tool to analyze the single overlapping sequence similarities within the aligned sequences. Figure *10* illustrates the multiple sequence alignment of all the samples which shows the variations of single nucleotide base at multiple points in the sequence. These sequence variations illustrate the genetic discrimination of various species within different plant families and these variations have also implement impact on the physical appearance and other cellular and subcellular functionalities of the plants. Figure 10 illustrates the multiple sequence alignment of the identified samples.

Figure 10: Clustal W based alignment of the ITS_2 barcoding region in MEGA X

Phylogenetic analysis

Tree topology of the ITS barcoding region was obtained using the neighbor-joining analysis.

Results reveals that ITS gene can be used to elaborate the taxonomic positions of the species. Phylogenetics is based on sequence variations that are examined by specified algorithms. Mostly the sequence variations occur due to insertions, deletions or substitutions in gene sequences that shows the evolutionary processes. Phylogenetic analysis using kimura 2-P model with a bootstrap1000 showed a highest similarity in BLAST search with the reference sequences in NCBI. In this analysis, 4 identified sample sequence and 7 reference sequence retrieved from NCBI database and subjected to phylogenetic tree construction using the MEGA X tool and the results are illustrated in figure 11.

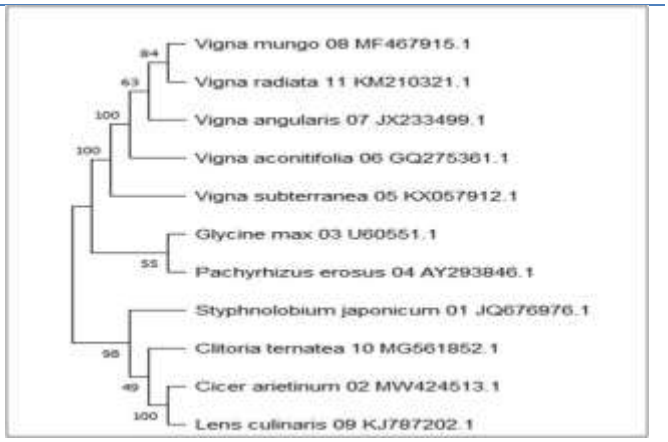

Figure 11: Phylogenetic tree by Neighbor joining analysis of evolutionary relationship between identified bean samples and the closest species as per ITS region sequence

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown in figure 11. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 274 positions in the final dataset. Evolutionary analyses were conducted in MEGA11. Results of the tree indicates that the highest evolutionary similarities were observed in *Vigna* Genus species *Vignasubterranea*(KX057912.1), *Vignaaconitifolia* (GQ275361.1),*Vignaangularis* (JX233499.1). Therefore, the analysis reveals the evolutionary similarities and divergence b/w the identified species.

Correct identification is necessary for traditional herbal plants to be utilized safely in treating human illnesses (Rydberg, 2010). In contrast to the traditional phenotyping-based taxonomy, DNA barcoding, whether at the chloroplast-plastid, nuclear, or both regions, is a more modern approach that is gaining acceptance. The conserved barcode area's molecular fingerprints highlight the genetic relevance and crop development potential.

Our findings show that the nuclear barcoding region of the plants with high similarity scores had large intragenic variability. The ITS_2 barcode region has a substantial level of discrimination efficiency, according to the results. Findings from the study would be helpful in building a reference database for accurate species identification. The primary objective of DNA barcoding, which involves the extraction, amplification, and

sequencing of genomic DNA, is to quickly build a reference library.

Molecular techniques appear to be the best advancement for classifying indistinguishable plant species in general. For the selected plant genotypes' barcode references, reliable species identification is accessible as an addition to the DNA sequencing database and gene bank for prospective informatics applications in future medical research. The DNA database can be used to target the underutilized conservational and ethno biological traditional potential of this medicinal crop.

CONCLUSIONS

Differentiating between species is done using DNA barcoding at the nuclear region. DNA barcoding analysis of intra- and inter-specific divergence may be successfully conducted on the ribosomal nuclear ITS_2 region. The results of this study reveal the molecular insights of species genetic authentication, genetic preservation, and secure use of species of medically significant plants. The use of DNA barcode technology for species identification and discrimination in commercially and medicinally important plants, as well as for the detection of adulteration in the food and pharmaceutical industries, may be feasible. The findings back up more study into genetic relationships for upcoming crop development plans for food, nutrition, and medicine.

Supplementary materials

Not applicable.

Author contributions

All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable.

Data Availability Statement

The materials are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest

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