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Genetic identification and authentication of members of *zingiberaceae* family using ITS_2 amplification

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Global commercial interest in the identification of therapeutic plants has increased due to the significant risk of adulteration in the herbs. The main methods for genetically identifying herbs include DNA barcoding. The main advantage of this method is that it can determine the material's purity. This study focuses on the precise identification of three significant species of the *Zingiberaceae*using amplification and sequencing of the nuclear internal transcribed spacer region (a barcode region). The ribosomal nuclear ITS_2 region of the chosen plant species, according to the results, exhibits 100% identity with the reference genome, indicating a high rate of species identification. According to the study's findings, the ITS_2 standard barcode offers a revolutionary tool for identifying species, preserving genetic diversity, and safely using plant species with medical importance.

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

INTRODUCTION

The use of medicinal herbs to cure a variety of ailments is highly valued in traditional medicine in many cultures all over the world. Traditional knowledge about medicinal plants has been passed down over the years(Bordoloi et al. 2023). Plants have been utilized as a source of medicine for ages. However, due to the need for knowledge of plant taxonomy, it may be difficult to identify medicinal herbs (Shneyer, 2009). Despite the lack of morphological characteristics, DNA bar coding has become a potent technique for classifying plant species (Rydberg, 2010). It allows for the swift and precise identification of the species.

DNA bar coding is a technique for classifying and identifying species that uses a condensed, standardized segment of the DNA sequence(Chen et al. 2023). The barcode region, a highly conserved portion of the genome, is used in the DNA bar coding method(SHI et al. 2011). A reference database of known species is used to compare the sequence acquired after PCR amplification of this area. This method has been used to identify several creatures, including fungi, mammals, and plants (Saha et al. 2020).

In many plant bar coding research, the internal transcribed spacer (ITS2) barcode region of the plant genome has been used to identify the plant species (Yao et al. 2010). The ITS2 region, which is located between the small and large subunits of the ribosomal RNA gene, differs significantly between and within species (Nasarodin et al. 2023). Similar to how quickly the ITS2 region can be amplified by PCR, it can also be sequenced using standard sequencing techniques. Numerous types of plants, including medicinal plants, have been identified using the ITS2 region(Jiang et al. 2023).

It is difficult to accurately and quickly authenticate medicinal plants and their adulterants because of the magnitude of the global medicinal plant trade(Kumar et al. 2023). 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.

Cardamom (*Amomum subulatum*)

Amomum subulatum (Large Cardamom) is a perennial herbaceous crop, planted in swampy places throughout hills near water streams(Parveen et al. 2018). Approximately half of the world's cardamom is produced in the Sikkim State of India, making it the world's greatest producer. Phytochemical analysis has revealed the presence of presence of carbohydrates, flavonoids, amino acids, steroids, triterpenoids, glycosides, tanninsetc.(Bisht et al. 2011).*A subulatum* is used as mouth freshener, foul-breath and to treat palpitation. Due to its medicinal properties like Analgesic , Anti-inflammatory, Anti-bacterial activities, it is stated to be an official medication in the Ayurveda Pharmacopoeia (Shukla et al. 2010).

Siamese ginger(*Alpinia galanga*)

Alpiniagalanga is a ginger-like perennial herb is native to Indonesia, India, Sri Lanka, China,

Vietnam, Java, and Sumatra (Chudiwal et al. 2010). Phytochemical analysis has revealed the presence of tannins, flavonoids, sterols, and glycosides. Leaf and flower essential oils mainly contain 1,8-cineole, α-terpineol terpinen-4-ol, camphor, and a- and b-pinenes(Khairullah et al. 2020). Alpinia galangal is used to treat various diseases like diabetes mellitus, cardiovascular diseases and bronchitis (Eram et al. 2019).

Ginger (Zingiber officinalis)

Zingiberofficinale, a member of the Zingiberacae family, is a well-known spice worldwide. It is a long-leaving perennial creeper with thick tuberous rhizome, yellow-green blooms, and long leaves. This kind of storage root has a strong flavor. Phytochemical analysis has revealed the presence of gingerol and paradol, shogaols, etc(Yousfi et al. 2021). Pharmacological uses included the gastric griping, low appetite, helminthes, antitussive, detoxicant, and anti-inflammatory(Kumar et al. 2011).

Besides the medicinal significance of these herbs the major problem with respect to its commercialization is the high rate of adulteration which could be health risky for human consumption. Thus this problem derives the need of accurate identification prior to use in the formulations. Therefore, DNA barcoding is the best method for standardization of the medically significant herbs.

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(Basavaraju et al. 2023).

Our study serves as a proof-of-concept for application of DNA bar coding 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

Plant Material

Three medicinal herbs belonging to the family *Zingiberaceae* were selected for the actual genetic authentication purpose. These samples were selected for the genetic discrimination at the specie and sub specie level. Thus providing aid in the pure selection of herbs from the adulterated mixing. All corresponding voucher samples are deposited in the herbarium of the Hamdard laboratories Waqf Pakistan (HLWP).

Genomic DNA isolation and PCR amplification

Prior to DNA extraction, the surfaces of the herbal plant material were properly cleaned with 75% alcohol solution to prevent any microbial DNA contaminating in the sample. One piece of ~1-2 gm 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 TCCTCCGCTTATTGATATGC primers 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.

DNA Sequencing and Analysis

Using the original amplification primer as the sequencing primer, the PCR products were immediately sequenced directionally on an ABI 3730XL sequencer (Applied Biosystem, USA) after being purified using a QIA quick PCR purification kit (Tiangen Biotech, Beijing, China).

Bioinformatics analysis

The initial forward and backward movements were put together using a 3.0 Codon Code Aligner. The entire ITS_2 sequences were subjected to carry out the nucleotide blast with the highest similarity score and lowest E value. The barcode gaps were manually edited

in the pair wise 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)

RESULTS AND DISCUSSION

Amplification and Sequencing Results of ITS2 barcoding gene

DNA barcode primer of the ITS2 region of the selected plants were successfully amplified with determined size of the PCR product as shown in fig 1**.** DNA bands obtained from the amplification reveals the amplicon length of ± 370 bp after size comparison with the reference gene ruler of 100 bp.

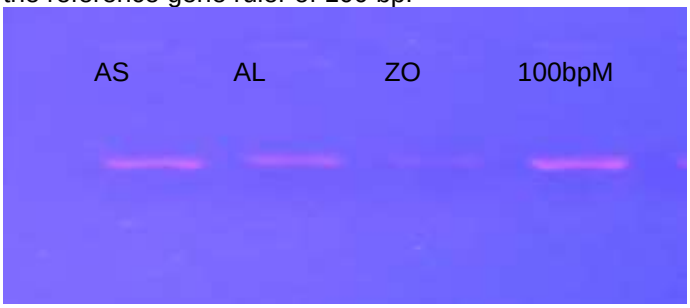

Figure 1: AS (*Ammomans subulatum*) ,AL(*Alpinia galganal),*ZO(*zingiber officinalis*) as the 100p reference gene ruler.

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 100 % identical to the reference sequence of *Ammomumsubulatum, Alpinia galangal and Zingiberofficinalis.*

No gaps or indels were present in any of the sequence. As illustrated in Fig 2, 3 and 4.

After the BLAST identification, the identified sequences were subjected to the multiple sequence alignment which indicates the similarity ratio in the targeted regions of all the identified herbs

Correct identification is necessary for traditional herbal plants to be utilized safely in treating

Human illnesses. In contrast to the traditional phenotyping-based taxonomy, DNA bar coding, 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.

Table1: BLAST of tested herbs with reference genome sequence

S.No	Sample	Herb Name	Percent Identity (%)	Reference Barcoding gene ID
1	Specie 01	*Amomum subulatum*	100%	OK104763.1
2	Specie 02	*Alpiniagalanga*	100%	MN545635.1
3	Specie 03	*Zingiberofficinalis*	100%	OQ220932.1

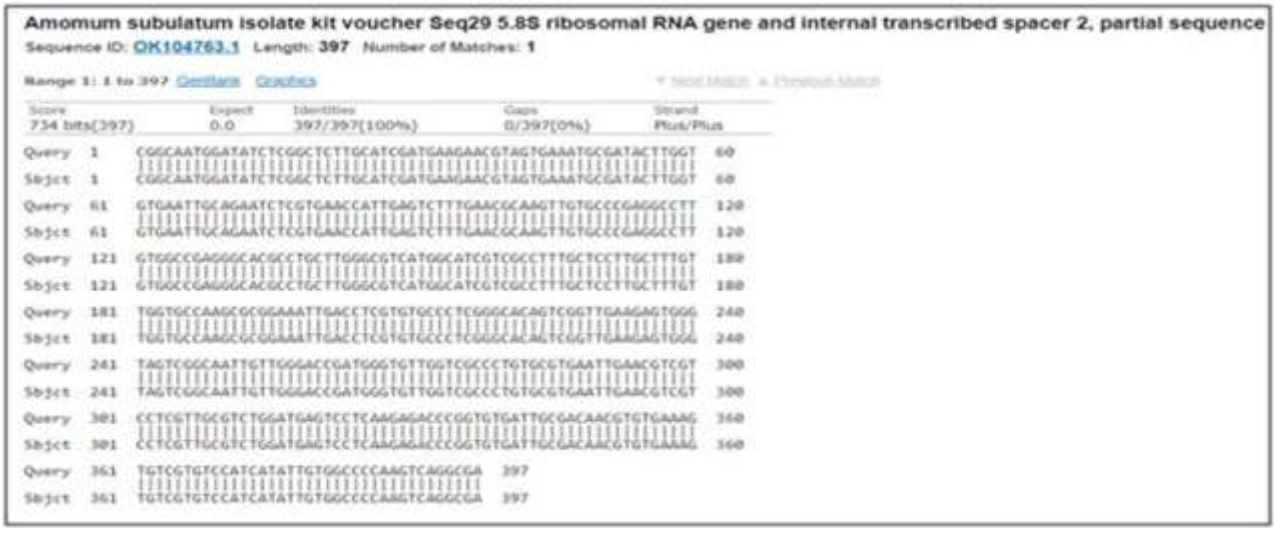

Figure2: Reference ITS2 barcoding gene alignment match with Ammomum subulatum gene sequence

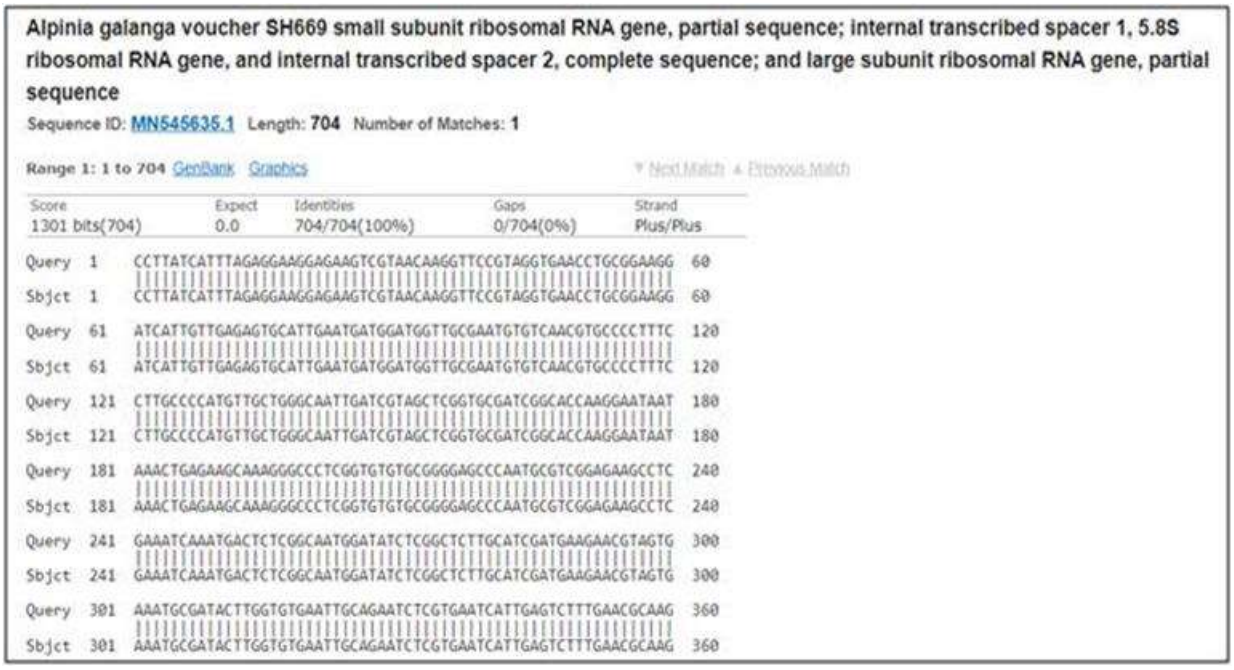

Figure 3: Reference ITS2 barcoding gene alignment match with Alpinia galanga gene sequence

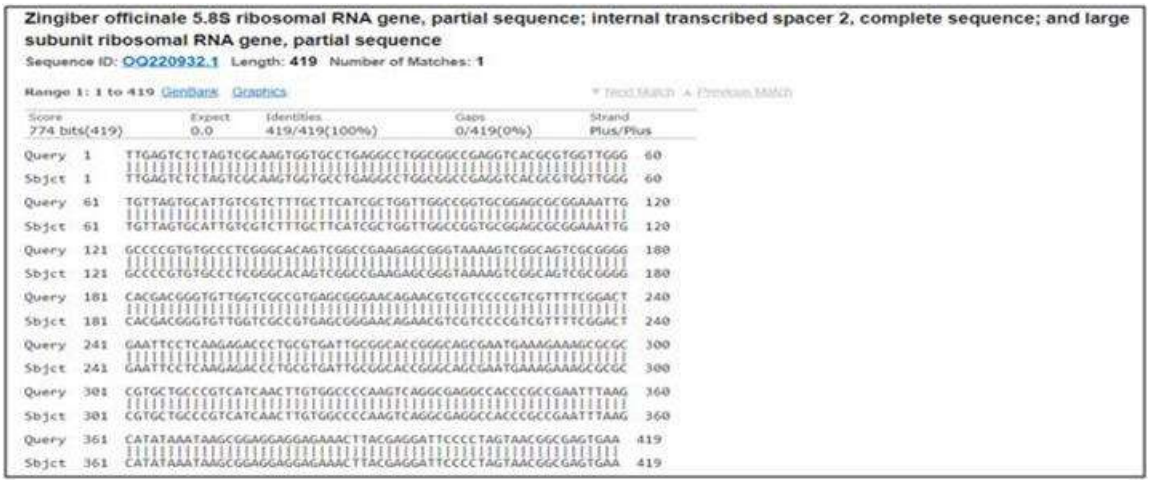

Figure 4: References ITS2 barcoding gene alignment match with Zingiber officinalis gene sequence

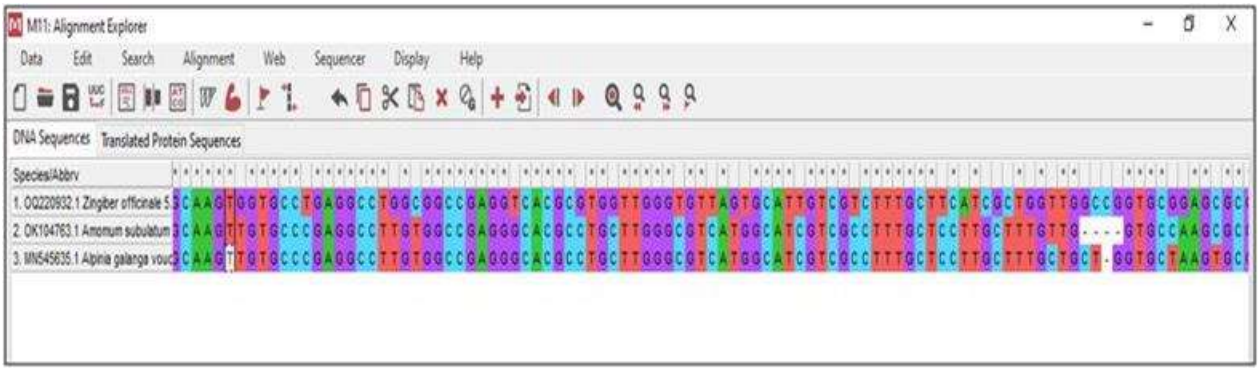

Figure 5: Clustal W based sequence alignment of the identified herbs

Our findings show that the nuclear bar coding region of the plants with high similarity scores had large iatrogenic 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 bar coding, 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 bar coding at the nuclear region. DNA bar coding 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.

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Supplementary materials

The supplementary material / supporting for this article can be found online and downloaded at: https://www.isisn.org/article/

Author contributions

Conceptualization, SR and MJ; data curation, MJ, formal analysis; MJ, methodology design; SR and MJ, resources; SR and HAB, writing –Original Draft; MJ, writing, reviewing and editing; SR, SJ, AS; Project ideology All authors have read and agreed to the published version of the manuscript. 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

All of the data is included in the article/Supplementary Material.

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Conflict of interest

The authors declare no conflict of interest.

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