J. Appl. Res Plant Sci. Vol. 5(1), 58-62, 2024, https://doi.org/10.38211/joarps.2024.05.219

Research Article

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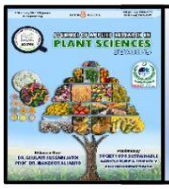

Available on https://www.joarps.org
Journal of Applied Research in Plant Sciences (JOARPS)
ISSN: 2708-3004 (Online), 2708-2997 (Print)

DNA Barcoding Based Identification of *Rosa x Damascene* and *Prunus dulcis* Herbs Using ITS_2 Barcoding Gene Amplification.

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Article Received 10-09-2023, Article Revised 28-10-2023, Article Accepted 08-11-2023.

Abstract

The considerable risk of adulteration in the herbs has raised commercial interest in the identification of medicinal herbs globally. DNA barcoding is the primary techniques for identifying the herbs at genetic level. This technique's key benefit is that it can identify the material's purity. This study focuses on the accurate identification of species utilizing Polymerase chain reaction-based nuclear universal internal transcribed spacer region (a barcode region) amplification and sequencing in 2 medically significant plants (*Rosa x damascene* and *Prunus dulcis*) procured from the local herbal market of Karachi in the year 2022. Results suggest that the ribosomal nuclear ITS_2 region of the selected plant species shows the 100 % identity with the reference genome, therefore it has shown a good rate of identification at the species level. The findings of this concludes that ITS_2 is the novel standard barcode that involve in species identification, genomic conservation, and secure utilization of the medically significant plant species.

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

Introduction

Traditional medicine in many cultures across the world places a high value on the intake of medicinal herbs to treat a wide series of ailments (Stoeckle *et al.*, 2011). Through the years, traditional knowledge about medicinal plants has been transmitted. Since ancient times, plants have been used as a source of medicine. Despite this, it might be challenging to identify medicinal herbs because it requires expertise in plant taxonomy (Ahmed, 2022). 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, Ichim, & Newmaster, 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, Meyer, Taveras, & Molina, 2016). The ribosomal RNA gene's ITS2 region, which lies between the small and large subunits, varies greatly between and within species. The ITS2 region has been used to identify numerous plant species, including medicinal plants.

Almond (*Prunus dulcis*): Almond, scientifically known as Prunus dulcis, is a flowering plant in the Rosaceae family. Almond kernel extract contains fatty acids, phenolic acids, phenolic compounds, flavonoids, phytosterols, and vitamins in addition to phytochemicals (Sayed, Mostafa, Haggag, & Hassan, 2023). The majority of the fatty acids were oleic acid (76.23%) and linoleic acid (15.43%) (Prgomet, Gonçalves, Domínguez-Perles, Pascual-Seva, & Barros, 2017). According to these findings, the extraction of almonds is a valuable source of fatty acids, soluble lipid vitamins, phytosterols, flavonoids, and phenolic compounds (Singh et al., 2022). Due to population growth, almond kernels are now a popular food choice because of their excellent nutritious content. According to studies on the micro- and macronutrient composition of almonds, nuts include a

variety of nutritional constituents, including lipids, amino acids, carbohydrates, minerals, and vitamins (Keser, Demir, & Yılmaz, 2014).

Rose (*Rosa x damascene*): Rosa x damascene belongs to the Rosaceae family as well. It is a vital plant with therapeutic applications in contemporary medicine that are both affordable and successful (Gavra *et al.,* 2022). Rosa x damascene has a wide spectrum of bio pharmacological properties, including those of depressants, hypoglycemic, analgesic, anti-inflammatory, and antioxidant. The fundamental elements of essential oils, geraniol and citronellol, are what give them their pharmacological effects for the treatment of numerous human illnesses in a variety of dosage forms (Aleksidze *et al.*, 2021)

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. Using ITS_2-S_2F and ITS_4 primers, the internal transcribed spacer (ITS_2) region of the plant genome was amplified, then subjected to DNA sequencing 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 (Han *et al.*, 2016). 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.

Material and Methods

Plant Materials: Two medicinal herbs samples (*Rosa x damascene* and *Prunus dulcis* belonging to the family Rosaceae 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).

Genomic DNA isolation and PCR amplification: Prior to DNA extraction, the surfaces of the herbal plant material were washed with 75% ethanol to prevent any microbial DNA contaminating in the sample. One piece (stem) of ~1-2 gm of each sample was then crushed into powder in liquid nitrogen (Sahu, Thangaraj, & Kathiresan, 2012). 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 Conglomerate 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 run on 2% Agarose gel and then run in the electrophoresis unit along with a reference 100 bp DNA ladder (Thermo Fischer Scientific, USA) used for size determination of the amplified product. The PCR product was stored at -20℃ for downstream process.

DNA Sequencing and Analysis: 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.

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 carried out the nucleotide detonation with the highest parallel score and lowest E-value. The barcode slits were manually edited in the pairwise placement view using BLAST. Molecular Evolutionary Genetic Analysis' MEGA11 software; (https://www.megasoftware.net/) was used to align multiple sequences.

Results and Discussion:

Amplification and Sequencing Results of ITS_2 barcoding gene: DNA barcode primer of the ITS_2 region of the selected plants were successfully amplified with determined size of the PCR product as shown in fig 3.1**.** DNA bands obtained from the amplification reveals the amplicon length of ± 370 bp after size comparison with the reference gene ruler of 100 bp.

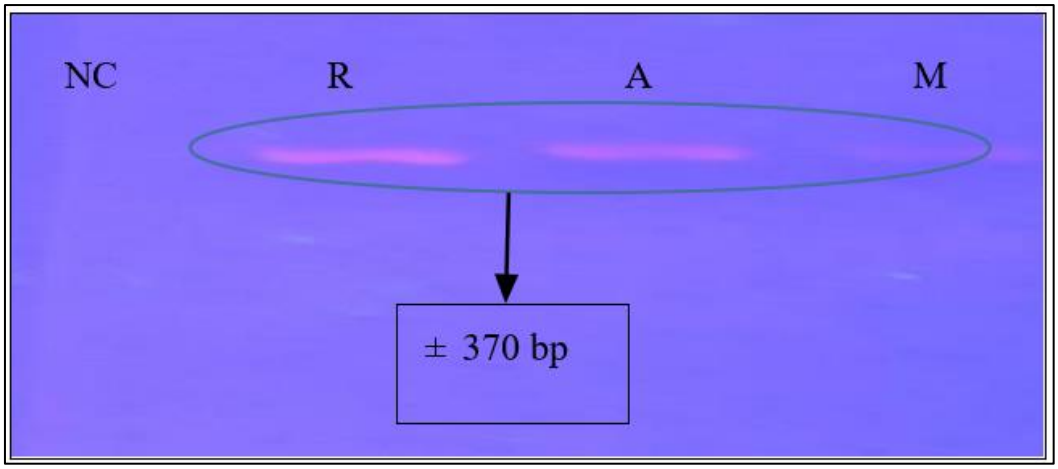

Figure. 1. NC negative control lane, R (*Rosa x damascene*), A (*Prunus dulcis)* are ITS_2 barcode amplified bands and M is the 100bp DNA ladder

DNA Sequencing Chromatogram interpretation: The chromatogram shows the different colored peaks. Eah peak denotes a different base which is detected under emitted fluorescence based detection in the ABI sequencer (Matsuo, 2021). On the top of the graph the position no of each base is indicated. The corresponding peak color of the denoted base call is compare with the base mentioned on the top to ensure the validity of our results. *Fig 2* and *3* illustrated the sanger sequencing results of both selected samples .

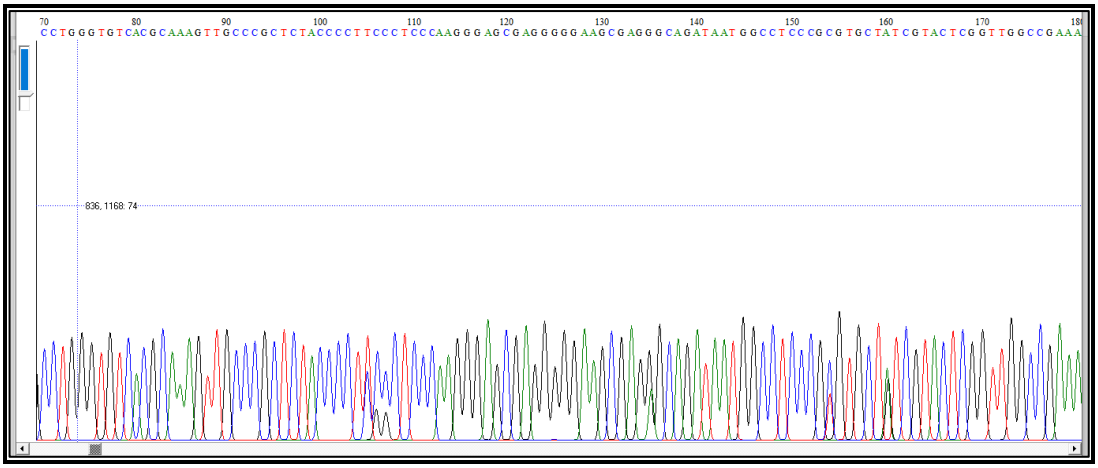

Figure. 2. Trace AB1file Rosa x damascenes forward primer $ITS_2 S_2 F$

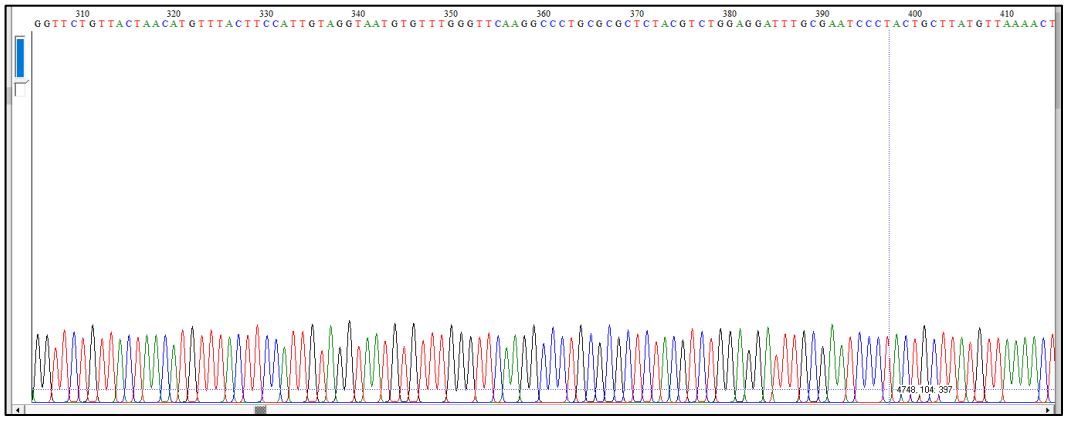

Figure. 3 Trace/ABI file of Prunus dulcis forward primer ITS_2S_2F

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 100 % identical to the reference sequence of *Rosa x damascene and Prunus dulcis*. The reference sequence ID matched with our tested samples are also indicated in the database. No gaps or

indels were present in any of the sequence. As illustrated in Fig 4 and 5.

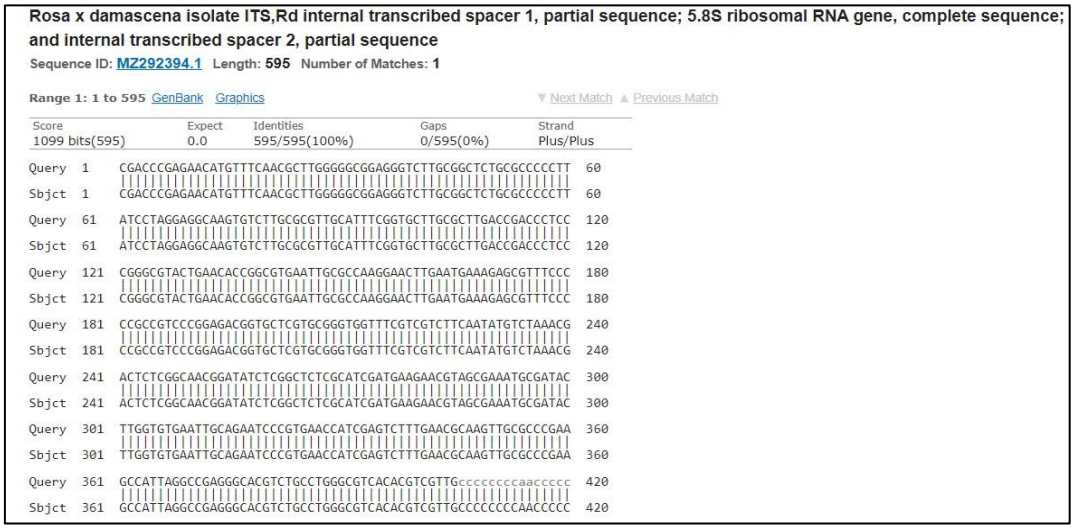

Figure. 4. ITS_2 barcode alignment with the reference sequence of Rosa x damascene

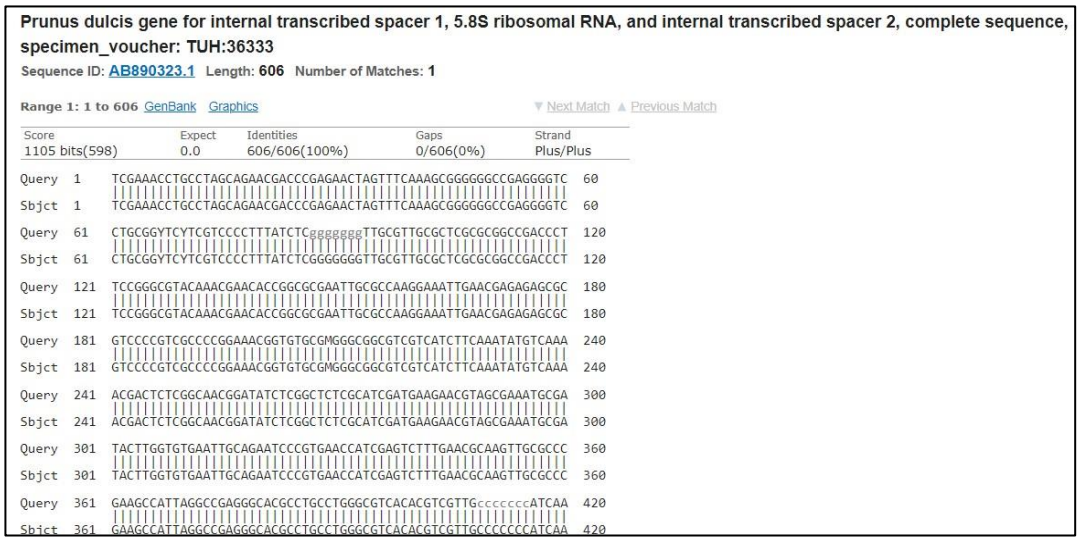

Figure. 5. ITS_2 barcode alignment with the reference sequence of Prunus dulcis

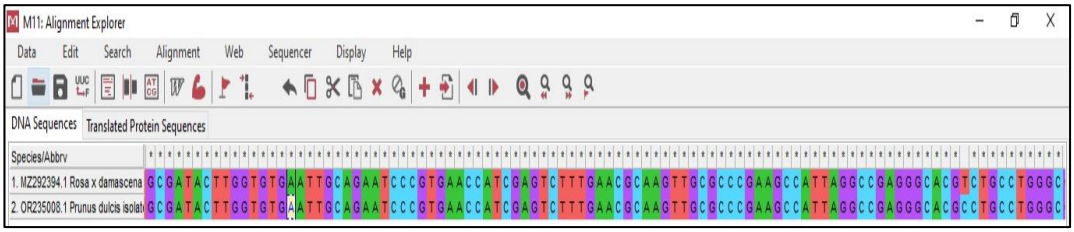

Figure. 6. Clustal W based ITS_2 barcode alignment of Rosa x damascene and Prunus dulcis in MEGA X

MEGA Alignment: The MEGA alignment of the genetically identified samples was done using the Clustal W tool through which the conserved regions b/w the aligned sequences were observed and the single nucleotide differences were also observed b/w these sequences (Dev, Sijimol, Prathibha, Sreekumar, & Muralidharan, 2020).

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.

Conclusion:

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 reveals 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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