Genomic DNA Extraction and Quantification:

Reference Solution

Homogenize the pulverized *Azadirachta indica A. Juss* plant material in a clean and pre-chilled mortar and pestle by grinding it using liquid nitrogen. Perform DNA isolation according to manufacturer's protocol (NucleoSpin® Plant II, Macherey-Nagel, Germany). Briefly, to 100 mg of homogenized plant material, add 400 µL of lysis buffer and vortex in a 1.5 ml microcentrifuge tube. Incubate the content at 65°C for 30 minutes after adding 10 µL RNAse A to the mix. Further, load the lysate onto the filter and place it in a fresh collection tube (2 mL) and centrifuge at 11,000 x g for 2 min. Collect the clear flow-through and add 450 µL of binding Buffer and mix carefully. Centrifuge the mixture in second column along with a fresh collection tube (2 mL) at 11,000 x g for 1 min and discard the flow-through. Repeat this step, if the volume still exceeds 700 µL, wash the column first with 400 µL wash Buffer 1 and then with 700 µL wash Buffer 2 through centrifugation at 11,000 x g for 1 min and finally discard the flow-through. Dry the column by performing a brief spin at 11,000 x g for 1 min. Perform DNA elution by incubating the column into a fresh 1.5 mL microcentrifuge tube with 50µL Elution Buffer at 65°C followed by centrifugation for 1 min at 11,000 x g. Repeat this step with another 50 µL elution buffer (65°C) to elute more DNA into the same tube. Store the DNA at 4°C for further analysis. Quantify the DNA obtained using the Nanodrop 2000 spectrophotometer (Thermo Scientific™) using the Elution Buffer as the reference. Check the quality of DNA by gel electrophoresis on a 1% agarose gel containing Ethidium bromide. Load the extracted plant DNA sample (100ng/ µL) in the wells along with 2 µL 6X DNA loading dye (2.5% Ficoll®, 11mM EDTA, 3.3 mM Tris-HCl, 0.017% SDS, 0.015% bromophenol blue). Carry out the electrophoresis at 90V and 300 mA for 40 minutes. Visualize the DNA in the gel under UV and confirm its integrity.

Test Solution

PCR amplification of genes for DNA barcoding

Perform Polymerase Chain Reaction (PCR) amplification for different DNA barcode genes (Table 1) from the isolated genomics DNA of *Azadirachta indica A. Juss*.to generate the DNA barcode using the gene specific universal primers (Table 1) and defined conditions. Get the forward and reverse primers as mentioned in Table 1 synthesized and perform PCR amplification with the reaction mixture as shown in Table 2.

Perform PCR using the following program, having annealing temperature as mentioned in Table 3.

Initial denaturation at 94°C for 3 minutes, PCR amplification for 40 cycles at 94°C for 1 minute, Annealing temperature (Table 3) for 1 minute, & 72°C for 1 minute and final extension at 72°C for 10 minutes.

Visualization of PCR product:

Resolve the amplified PCR products by 1.5% agarose gel electrophoresis in Tris-acetate

EDTA buffer along with 100bp DNA ladder as size standard. Carry out electrophoresis at 100V for 40 minutes and visualize under the UV lamp to detect the gene amplification (Figure 1).

Table 1: List of Universal Primers

S. No	Gene Name	Forward Primer	Reverse Primer
1	*ITS1*	GCATCGATGAAGAACGCAGC	TCCTCCGCTTATTGATATGC
2	*ITS2*	ATGCGATACTTGGTGTGAAT	GACGCTTCTCCAGACTACAAT
3	*rbcL*	ATGTCACCACAAACAGAAAC	TCGCATGTACCTGCAGTAGC
4	*trnL*	GGTTCAAGTCCCTCTATCCC	ATTTGAACTGGTGACACGAG
5	*trnK*	TACTCTACCRTTGAGTTAGCAAC	AAAGGKGCTCAACCTACARGAAC

Table 2: PCR Reaction mixture

Components	Volume (µL)
Emerald Amp® GTPCR master mix	10
Forward primer (5 pmol/ µL)	1
Reverse primer (5 pmol/ µL)	1
Plant DNA (100 ng)	1
Nuclease free water	7
Total reaction volume	20

Table 3: Annealing temperature and amplicon size

S. No	Gene Name	Annealing Temperature (°C)	Amplicon Size (bp)
1	*ITS1*	55	411
2	*ITS2*	55	515
3	*rbcL*	60	731
4	*trnL*	60	472
5	*trnK*	60	758

Gel Elution and Purification:

Excise the gel piece (while visualizing under UV) having the amplified gene fragments using sterile scalpel blade for elution and purification using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Add 500 µL of binding buffer NT1 to each excised fragment in a fresh 1.5 ml microcentrifuge tube. Incubate the sample for 10 minutes at 50°C and then load the dissolved gel mixture on to the NucleoSpin® Gel and PCR Clean-up column fitted into a collection tube (2 mL), followed by centrifugation at 11,000 x g for 1 minute. Discard the flow through and wash the column with 700 µL wash Buffer NT3 and centrifuge at 11,000 x g for 1 minute. Carry out the elution by placing the column into a fresh 1.5 mL microcentrifuge tube, by adding 50 µL Elution Buffer NE (at room temperature) to the column. Centrifuge the column for 1 min at 11,000 x g to elute the PCR amplicon and repeat the step with 50 µL Buffer NE again to elute the remaining PCR amplicon into the same tube. Concentrate the eluted amplicon to a final concentration of 40 ng/ µL.

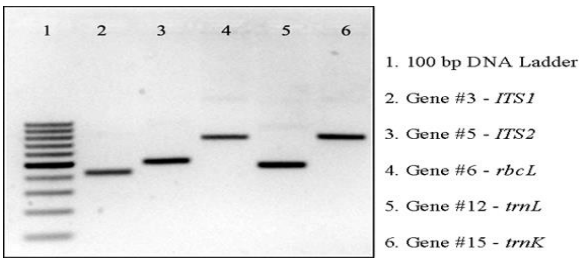

Figure 1: Amplicon of *Azadirachta indica A. Juss* empaneled genes.

DNA Sequencing:

To 40 ng of PCR amplicon in 2µL volume, add 1µL of appropriate forward or reverse primers, 2µL of Bigdye ready reaction termination mix (2.25 µL of Bigdye having 6FAM ddC, VIC ddA, NED ddG, TED ddT), 1µL of sequencing buffer (containing 200 mM Tris-HCl pH 9.0 and 5 mM $MgCl_2$) and 4µL Milli Q water, mix well and centrifuge briefly. Perform Sequencing amplification using the following cycling program: denaturation at 94°C for 5 minutes; amplification for 25 cycles at 96°C for 10 seconds, 55°C for 5 seconds & extension at 60°C for 4 minutes and finally store at 4°C for 60 minutes. Post amplification, mix the amplicon with 10 µL sterile Milli Q water, 2 µL of 3M sodium acetate (pH 4.6) and 50µL ethanol and incubate at room temperature for 15 minutes. Centrifuge the mixture for 30 minutes at 3500 x g. Remove and discard the supernatant, wash the DNA pellet with 70% ethanol and again centrifuge again and finally dissolve in 10 µL Formamide. Execute the sequencing of the amplicon in 3730XL sequence analyzer (Thermo Fischer Scientific™) and analyze the read out sequences using Sequencing analysis 5.2 Software (USA).

Evaluation and interpretation of results: Convert the obtained sequence chromatogram to FASTA format and screen for the identification of closest matching species using NCBI- BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Analyze the sequences further for barcode generation using CCMB DNA barcode generator.

Sequence of *Azadirachta indica A. Juss*

>ITS1

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TGTGAATTGCAGAATCCCGTGAACCATCG
AGTCTTTGAACGCAAGTTGCGCCCCAAGC
CATCAGGCCGAGGGCACGTCTGCCTGGGT
GTCACGCATCGTTGCCCCCCCCACACAAA
CCCCCCCGGGGGAAGGTGTCGGGGCCGGG
CGGAGACTGGCCTCCCGTGCGCTGCCCGC
TCGCGGTTGGCCCAAATCCGAGTCTTTCG
GCGACCGAGCCGCGACGATCGGTGGTGAG
AACAAGCCTCTCGAGCTCCAGTCGCGCGC
CCGCGTCCCCGTGTCAGGGACTCGCGGAC
CCTTTGCACGCCCTCTGGGCGATGCTCGC
TTCGCGACCCCAGGTCAGGCGGGACTACC
CGCTGAGTTTAAGCATATCAATAAGCGGA
GGAA
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>ITS2

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TTGCGCCCCAAGCCATCAGGCCGAGGGCA
CGTCTGCCTGGGTGTCACGCATCGTTGCC
CCCCCCACACAAACCCCCCCGGGGGAAGG
TGTCGGGGCCGGGCGGAGACTGGCCTCCC
GTGCGCTGCCCGCTCGCGGTTGGCCCAAA
TCCGAGTCTTTCGGCGACCGAGCCGCGAC
GATCGGTGGTGAGAACAAGCCTCTCGAGC
TCCAGTCGCGCGCCCGCGTCCCCGTGTCA
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>*rbcl*
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CGCCCGAGGAAGCAGGGGCTGCGGTAGCT
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TGTGTGGACCGATGGGCTTACTAGCCTTG
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AGGATCTACGAATCCCTCCCGCGTATTCT
AAAACTTTCCAAGGACCACCTCATGGGAT
CCAAGTTGAGAGAGATAAATTGAACAAGT
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GCGGTGGACTTGACTTTACCAAAGATGAT
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>*trnL*
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TTTGAATGATTGACAATCCATATAATTGC
TCATACTGAAACTTACAAAGTCTTCTTTT
TGAAGATTCAAGAAATGAAATTCTCCGTG
CAAGACTTTGCATACTTTAGTTTTGTCTT
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GTAAAATAAGGATGGTGTGTCGGAAATGG
TCGGGATAGCTCAGTTGGTAGAGCAGAGG
AC

>*trnK*
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GCGGAATCCCTTTTTGACTCTGCACCTGT
GATTCCACTATTATTAGTAAACAATAATG
gAACAAC

DNA Barcode of Azadirachta indica A. Juss