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

<table>
<thead>
<tr>
<th>S. No</th>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>1</td>
<td>ITS1</td>
<td>GCATCGAT</td>
<td>TCTCCGC</td>
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<tr>
<td></td>
<td></td>
<td>GAAGAAC</td>
<td>TTATTGAT</td>
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<tr>
<td></td>
<td></td>
<td>GCAGC</td>
<td>ATGC</td>
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<tr>
<td>2</td>
<td>ITS2</td>
<td>ATGCGATA</td>
<td>GACGCTTC</td>
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<td></td>
<td>CTTGGTGT</td>
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<td>ACAAT</td>
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<td>3</td>
<td>rbcL</td>
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<td>TCGCATGT</td>
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<td></td>
<td>AAAC</td>
<td>TAGC</td>
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<td>4</td>
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<td></td>
<td></td>
<td>TCCCTCTA</td>
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<tr>
<td></td>
<td></td>
<td>TCCC</td>
<td>CGAG</td>
</tr>
<tr>
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<td>trnK</td>
<td>TACTCTAC</td>
<td>AAAGGGKC</td>
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<td>CRRTGAGT</td>
<td>TCAACCTA</td>
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<td></td>
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<td>TAGCAAC</td>
<td>CARGAAC</td>
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Table 2: PCR Reaction mixture

<table>
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<tr>
<th>Components</th>
<th>Volume (μL)</th>
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<tr>
<td>Emerald Amp® GTPCR master mix</td>
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<tr>
<td>Forward primer (5 pmol/μL)</td>
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</tr>
<tr>
<td>Reverse primer (5 pmol/μL)</td>
<td>1</td>
</tr>
<tr>
<td>Plant DNA (100 ng)</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>7</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3: Annealing temperature and amplicon size

<table>
<thead>
<tr>
<th>S. No</th>
<th>Gene Name</th>
<th>Annealing Temperature (°C)</th>
<th>Amplicon Size (bp)</th>
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<tbody>
<tr>
<td>1</td>
<td>ITS1</td>
<td>55</td>
<td>411</td>
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<tr>
<td>2</td>
<td>ITS2</td>
<td>55</td>
<td>515</td>
</tr>
<tr>
<td>3</td>
<td>rbcL</td>
<td>60</td>
<td>731</td>
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<td>trnL</td>
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<td>472</td>
</tr>
<tr>
<td>5</td>
<td>trnK</td>
<td>60</td>
<td>758</td>
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</tbody>
</table>

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.
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 MgCl₂) 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 Fisher 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
TGTTGAAATTTGCGAGAATCCCGTGAACCATCG
AGTCTTTTGACGACGCTTGGCCGCCGCCGGAGAC
CATACGCAGCCGAGGCACTCGTCCGCTGGGTC
GTCACGCATCGTTGCCGCCCCACACAA
CCCCCGGGGGGAAAGTTGTCGCAGGCGCG
CGGAGACTGCGTTCGCTGGGCTCTGGCCGC
TGCCGCTTGGCCAAAATCGATCTTTTCC
GGACGCCGAGCAGGAGCAGTGGTTGGGAG
AACAGCCTCTCGAGCTCCAGTCGCGCGCC
CCCGGCTCTCCGTGCAGGACTCGCGGGAC
CCTTTGACGCGCCTCTGGGCGATGCTCGC
TTCCGACCCCAAGCTCAGGGGGACTACC
CGCTGACTTTAAGCATATCAAATAACGGGAG
GGAA

>ITS2
TTGCGCCCCAAAGCCCATCAGGCGAGGCA
CGTCTGCTCGGGTGTACGATCGTCCGCC
CCCGCACAACAAACCCCCCGGGGAAAGG
TGGCGCGCGGGCGAGGACTGCGCTGCC
GTGCTGCTCCGCTCGCTGGTGGCCAAA
TCCAGTCTTTGCGACGCGAGCCGCAAG
GATCGGTTGGTGAAGCAGCCTCTCGAGC
TCGACTGCGCGCCGCGCTCCGCTGCA
GGGACTCGCGGACCTTTGACGCGCTCC
GGGATGGTCCGCTCGGCGGCCACGAGT
AGGGACAGTACCGCTGTAGTTAAGCAC
ATAACATAGCGAGGAAAGGAAAACGTACC
Azadirachta indica A. Juss

AGGATTCCTCAGTAAACGGCAGCGGAACC
GGGAGAGGCCAGCTTGAAGATCGGGCGT
CCCGAGCTCGGAAATGCTCTTGA

>rbcl
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GAAGGATCAAGATGCTGAGAAATTTAATA
CACGCTTAACTCCTTACAGATCCT
CGCCGAGGAAGCAGGGGCTGCGTAGCT
GCGGAATCTTCTACTGGATACATGGGAC
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GAGGCCGTTTGGAGAAGAAATCAATA
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TACTAATGATAATCAGGCAACAAAA
AACGATATATGCTTTACCTTACTCATAT
TAGTAAACATTCTTTGTGTACTTCAAA
GAAATGGTGATCTGATTATTTTGTCTCA
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GAACGTGTGCTTAAGTGGGGTTTTTGGAGT
GTTTCAATTTATTGGAAAGGGTGCTGG
GCGGAATCCCTTTTTGACTCTGACCTTGA
GATTTCACATTATAGTAAACATATAG
gAACAAAC
DNA Barcode of Azadirachta indica A. Juss