Evaluation of single and multilocus DNA barcodes towards species delineation in complex tree genus Terminalia,

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Abstract

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Protocol

DNA extraction and Quantification

Step 1.

Isolation of Genomic DNA

One - two gram of deveined leaves was washed with sterile distilled water & blotted with tissue paper to remove water completely. The dried leaf samples were homogenized with liquid nitrogen in prechilled mortar and pestle. The homogenate powder was immediately added in 5-7 ml / sample freshly prepared pre-warmed (50 ^{0}C - 55 ^{0}C) extraction buffer and mixed gently. The centrifuge tube was incubated at 65 ^{0}C in water bath for 1-2 hr with occasional gentle swirling to ensure complete lysis. The homogenate was mixed with equal volume of chloroform-isoamyl alcohol (24:1) and mixed vigorously. The mixture was centrifuged at 10500 rpm, for 12 minutes at RT. The aqueous supernatant was transferred with a wide bore pipette to a new centrifuge tube. An additional chloroform-isoamyl alcohol (24:1) purification step was performed to remove proteins and potentially interfering secondary metabolites. 0.6 volumes of cold isopropanol were added to the supernatant, mixed gently and stored at 4 ^{0}C for 1-2 hr. The mixture was centrifuged at 12000 rpm, for 15 minutes at RT to precipitate DNA. Discarded the supernatant and the pellet was washed with 70% ethanol. The pellet was dried in vacuum for 10-15 minutes and resuspended in 500 µl of TE buffer. After complete dissolution, the nucleic acids were incubated with RNase-A (final concentration 10µg/ml) for 30 minutes at 37 ^{0}C. The mixture was extracted with equal volume of chloroform-isoamyl alcohol (24:1) and mixed vigorously and was centrifuged at 11500 rpm, for 12 minutes at RT. The aqueous supernatant was transferred to fresh microcentifuge tube and added 2 volumes of cold ethanol. The microcentifuge tube was mixed vigorously and incubated at -20 ^{0}C for overnight precipitation. DNA was pelleted by spinning at 14000 rpm 15 minutes at 4 ^{0}C. The pellet was washed with 70% ethanol and dried in vacuum for 10-15 minutes. The precipitated DNA was finally dissolved in 100µl of sterile distilled water and stored at -20 ^{0}C.

Quality check and quantification of isolated DNA

Isolated DNA was checked for its quality and quantity by electrophoresis on a 0.8% agarose gel and spectrophotometric analysis (NanoDrop, ND-1000, USA). A double digested λ DNA (EcoRI and HindIII) and 2-Log DNA ladder were used as standard to assess the size of DNA bands. The DNA was diluted to a final concentration of 25-50 ng/µl for PCR amplifications.

PCR amplification

Step 2.

Two plastid barcodes (the coding genes *matK* and *rbcL*) and a nuclear internal transcribed spacer (*ITS*) were amplified according to PCR reaction conditions following guidelines from the Consortium of Barcode of Life (CBOL) plant-working group and sequenced using universal primers.

The selected DNA regions were amplified by using a standard PCR, whereby amplification for each primer set was carried out in a 50-µl volume solution containing 1X Taq DNA polymerase buffer, 200 µM each dNTP (dATP:dTTP:dCTP:dGTP in 1:1:1:1 parts), 10 pmol of each primer (forward and reverse), 1 unit of Taq DNA polymerase and 25 ng of template DNA.

Region	Primer	Sequence (5´-3´)	Thermocycling conditions
ITS	ITS5a	5'-CCTTATCATTTAGAGGAAGGAG-3'	94°C 5 min; [30 cycles: 94°C 1 min; 50°C 1 min; 72°C 1.5 min]; 72°C 7 min
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	
rbcL	rbcL1F	5'-ATGTCACCACAAACAGAAAC-3‘	95°C 2 min; [35 cycles: 94°C 1 min; 55°C 30 s; 72°C 1 min]; 72°C 7 min
	rbcL724R	5'-TCGCATGTACCTGCAGTAGC-3‘	
matK	matK 390F	5'-CGATCTATTCATTCAATATTTC-3’	95°C 2 min; [30 cycles: 94°C 1 min; 48°C 30 s; 72°C 1 min]; 72°C 7 min
	matK 1326R	5'-TCTAGCACACGAAAGTCGAAGT-3'	
psbA-trnH	fwdPA	5'-GTTATGCATGAACGTAATGCTC-3	94°C 5 min; [35 cycles: 94°C 1 min; 55°C 30 s; 72°C 1.5 min]; 72°C 7 min
	revTH	5'-CGCGCATGGTGGATTCACAATCC-3'	
	psbA	5'-GTTATGCATGAACGTAATGCT-3'	94°C 5 min; [35 cycles: 94°C 1 min; 55°C 30 s; 72°C 1.5 min]; 72°C 7 min
	trnH	5'-CGCGCATGGTGGATTCACAATCC-3'	

PCR purification

Step 3.

PCR purification of the amplified product was done using the nucleo spin purification kit by following the manufacturer’s protocol as follows:

1. Mixed 1 volume of sample with 2 volumes of Buffer SET (e.g., mix 100 µl PCR reaction and 200

µl Buffer SET). For sample less than 100 µl adjusted the volume to 100 µl using Buffer SET or water.

2. Placed a SureExtract Spin PCR/Gel Extraction Kit Column into a Collection Tube (2 ml) and loaded the sample. Centrifuged for 1 min at 11,000 × g. Discarded flowthrough and placed the column back into the collection tube.
3. Added 700 µl Buffer SET3 to the SureExtract Spin PCR/Gel Extraction Kit Column. Centrifuged at 11,000 × g for 1 min. Discarded flow through and placed the column back into the collection tube.
4. Repeated step 3. Discarded flowthrough and placed the column back into the collection tube.
5. Centrifuged at 11,000 × g for 2 min for complete removal of Buffer SET3.
6. Placed the SureExtract Spin PCR/Gel Extraction Kit Column into a new 1.5 ml microcentrifuge tube. Added 15 – 50 µl Buffer SEB (pre warmed at 70˚C) allowed it to stand at room temperature (18 - 25˚C) for 1 min. Centrifuged for 1 min at 11,000 × g.

Sequencing of amplicons

Step 4.

Sequencing PCR Mix:

Buffer: 1.75 µl

Reaction Mixture: 0.5 µl

Primer F/R: 1 µl

Template: 60-100 ng

Water: Adjusted to final volume of 10 µl

Sequencing PCR Steps: 30 cycles

Initial denaturation	96°C for 1 min.
Denaturation	96°C for 10 sec.
Annealing	50°C for 5 sec.
Extension	60°C for 4 min.
Hold	4°C forever

Sequencing Purificaton:

Solution A: 10 µl M.Q. Water + 2 µl 125 mM Edta

Solution B: 50 µl Ethanol + 2 µl 3 M CH_3COONa (pH 4.6)

- Added Soltion A and B to the sequencing pcr product.
- Incubated for 15 minutes at RT.
- Centrifuged at 12000/RT/30 min.
- Washed with 70% ethanol (500 µl)
- Centrifuged at 12000/RT/10 min.
- Pellet vaccum dried.
- Dissolved in HDF = 12 µl.

Sequencing Run:

Denatured for 2 min at 96°C.

Sequencing run were performed on an ABI 3130 XL automated sequencer (Applied Biosystems).

Sequence analysis

Step 5.

- The electropherograms obtained for each region were base-called using PHRED.
- Raw sequences obtained for each region were assembled and edited using CodonCode Aligner v.3.0.1 (CodonCode Corporation).
- The sequences were blasted on NCBI BLAST under the programme BLASTN 2.2.1+ and on to BOLD using Identification Request for checking their homology with other available sequences.
- The edited sequences were then aligned with Muscle 3.8.31 on the EMBLEBI website (http://www.ebi.ac.uk) under default parameters and adjusted manually in BioEdit v7.1.3.0.