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PCR STANDERDISATION & DNA BARCODING TO ASSESS THE VARIATION IN DNA AMONG THE SPECIES WITH *HILDEGARDIA POPULIFOLIA*

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ABSTRACT

The present study deals with; the isolation of DNA was done by four different methods, and among these CTAB was found good. Hind III was used to digest the DNA. PCR procedure is followed in order to increase the copies of selected part of DNA, chloroplast matK specific primer was used, to analyze the genetic variation.

KEYWORDS

DNA barcoding, Hildegardia polulifolia.

INTRODUCTION

1.1 *HILDEGARDIA POPULIFOLIA* IN GENERAL:

Hildegardia populifolia (Roxb.) Schott. & Endl. a deciduous tree species is known to represent by sole surviving population comprising about 20 trees in Kalrayan hills of Tamilnadu (Nayar & Sastry 1990). World Conservation Monitoring centre (1998) assessed the conservation status of species as critically endangered following IUCN version 2.3 (IUCN, 2008). It was reported earlier endangered in 1997(Walter & Gillette, 1998). Rao *et al.* (1998) recognized five subpopulations for endangered species in Rayalaseema districts of Andhra Pradesh. FRLHT, a Bangalore based NGO conducted a camp workshop in 2001 and assessed 50 threatened taxa of Andhra Pradesh, following IUCN version 3.1 and recorded 4 taxa under critically endangered category including *Hildegardia populifolia* (Jadhav *et al.,* 2001). Sarcar and Sarcar (2002) recommended its revision to `critically endangered` status. The species is also listed endangered in the red list of threatened vascular plant species in India (Rao, Kameswara *et al*., 2003). Nayar (2007) reported ten endemic species of Eastern Ghats facing population crash including *Hildegardia populifolia*.

1.1.1. Systematic position of Hildegardia *populifolia*:

Malvales (Sterculiaceae, Tiliaceae, Bombacaceae and Malvaceae) should be considered an enlarged family; in Malvaceae nine sub families were recognized (Bayer *et al.,* 1999; Alverson *et al.,* 1999). One of these sub families, the sterculioideae comprises the members of the family Sterculiaceae, and this merging has been strongly supported by Angiosperm Phylogeny Group (2003) and Hinsley (2008). The present study adopts the same concept and hence the current position of the taxon as follows:

Kingdom	**: Plantae**
Subkingdom	**: Tracheobionta**
Super division	**: Spermatophyta**
Division	**: Angiosperms**
Class	**: Eudicots**
Subclass	**: Rosidae**
Order	**: Malvales**
Family	**: Malvaceae**
Sub-family	**: Sterculioideae**
Tribe	**: Sterculieae**
Genus	**: *Hildegardia***
Species	**: *Hildegardia populifolia***

The sub family Sterculioideae is well defined group, characterized by apetalous (lacking petals), exinvolucellate (lacking an epicalyx) flowers with a fleshy, usually petaloid, fused calyx, an absence of staminoids, a monodelphous stamina column, an androgynophore (a stalk separating the calyx from the stamens and styles), and apocarpous ovaries and fruits. The flowers are typically monoceous, i.e., have separate male and female flowers borne on the same plant. The subfamily comprises 12-14 genera (Hinsley, 2008) including *Hildegardia.*

1.1.2. Habitat

On the slopes between 300- 700m in dry deciduous forests, especially among boulders; saplings found among rocky crevices.

1.1.3. Vernacular names:

Telugu: daalibuda, Gali buddaga, Pichipoliki, Buddapoliki; Tamil: Malaipuvarasu; English: poplar leaved ardor (Lushington).

1.2. DNA BARCODING:

In 2003, Paul Hebert, researcher at the University of Guelph in Ontario, Canada, introduced '**DNA barcoding'** as a way to identify species. Barcoading uses a very short genetic sequence from a standard part of a genome products using, the way a super market scanner distinguishes products using the black stripes of the Universal Product Code (UPC). The two processes may look very similar to untrained eye, but in both cases the bar codes are different. DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species. It differs from molecular phylogeny in that the main goal is not to determine classification but to identify an unknown sample.

Until now, biological specimens were identified using morphological features like the shape, size and color of the body parts. In some cases trained technicians could make identification using morphological keys but in most of cases experienced professional taxonomist is needed. If the specimen is damaged or it is in immature stage of development even specialist may be unable to make identification. This problem could overcome by Barcoding; even non-specialists can obtain barcodes from tiny amounts of tissue.

The gene region that is being used as a standard bar code for animals groups is 648 base pair region in mitochondrial cytochrome oxidase 1 gene (CO1). CO1 is proving highly effective in identifying birds, butterflies, fishes, flies and many other animal groups. CO1 is not effective barcode in plants because it evolves too slowly, but two gene regions in the chloroplast, matK or rbcl, have been approved as barcode regions for plants.

In plant chloroplasts, the tRNA^{lys} (UUU) gene (trnK) contains a group II intron (tanKII), which encodes the matK open reading frame. The trnK intron of the plant encodes the matK open reading frame (ORF), which has been used extensively as a Phylogenetic marker for classification of plants. The matK ORFs belongs to the ML (mitochondrial like) subclass of group II intron ORFs, indicating that they were derived from a mobile group II intron of the class. Group II intron are self-splicing RNAs and mobile elements found in Eubacteria, archea and some organelles of fungi, plants and algae (Lambowitz and Zimmerly, 2004). The *mat*K gene, formerly known as *orf*K, is emerging as yet another gene with potential contributions to plant molecular systematics and evolution (Johnson and Soltis, 1994, 1995; Steele and Vilgalys, 1994; Liang and Hilu, 1996; Gadek, Wilson, and Quinn, in press). The gene, ≈1500 base pairs (bp), is located within the intron of the chloroplast gene *trn*K, on the large single-copy section adjacent to the inverted repeat. A homology search indicates that the 102 amino acid positions at the carboxyl terminus are structurally related to portions of maturase-like polypeptide and might be involved in splicing Group II introns (Sugita, Shinozaki, and Sugiura, 1985; Neuhaus and Link, 1987; Ems et al., 1995).

MATERIALS AND METHODS

DNA purification kit (PureFast® Plant Genomic DNA purification kit), PCR Master

Mix, Agarose gel electrophoresis consumables and Primers purchased from HELINI Biomolecules, Chennai, India.

IJPBS |Volume 2| Issue 3 |JULY-SEPT |2012|272-277

2X Master Mix:

It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2mM MgCl2, 1µl of 10mM dNTPs mix and PCR additives.

Agarose gel electrophoresis:

Agarose, 50X TAE buffer, 6X gel loading buffer and Ethidium bromide are purchased from HELINI Biomolecules, Chennai.

Procedure:

Genomic DNA extraction from plant leaves:

1. 10mg of leaf is taken into fresh 1.5ml centrifuge tube.
2. Added 400µl of lysis buffer and 40µl of Proteinase K [10mg/ml] and grinded with micro pestle, briefly vortexed.
3. Incubated in water bath at 65°C for 15 min.
4. Cool to room temperature and Added 130µl of Solution Lysis buffer-2.
5. Inverted gently for 5 times and incubate for 5 minutes in room temperature.
6. Centrifuge it for 10 min at 10000rpm.
7. Transferred the supernatant into fresh tube.
8. Added 500µl of Binding Buffer and gently mixed.
9. Transferred cleared lysate into Pure Fast column and centrifuged at 10000rpm for 1minute.
10. Discard flow through and added 500µl of Wash Buffer and Centrifuge at 10000 rpm for 1 minute.
11. Discard flow through and added 500µl of Wash Buffer-2 and centrifuged at 10000rpm for 1min. Repeated Wash Buffer-2 wash one more time.
12. Discarded flow through and Centrifuged column for additional 2minutes to remove any residual ethanol.
13. Eluted DNA by adding 100µl of Elution Buffer and Centrifuged for 1min.
14. Quality and Quantity of extracted DNA is checked by loading in 1% agarose gel and 1µl of extracted DNA is used for PCR amplification.

PCR Procedure:

[25µl of Master Mix contains: 10X Taq buffer, 2mM Mgcl2, 0.4mM dNTPs mix, and 2U Proofreading Taq DNA polymerase]

1. Reactions set up as follows;

Components Quantity
In PCR vial
Master Mix 25µl
Chloroplast matK specific Primer - forward (10pmoles/µl) 1µl
F: 5'- GGG TTG CTA ACT CAA TGG TAG AG-3'
Chloroplast matK specific Primer- reverse (10pmoles/µl) 1µl
R: 5'- TGG GTT GCC CGG GGC TCG AAC-3'
Genomic DNA 1µl
Water, nuclease free 22µl
Total volume 50µl

2. Mixed gently and spin down briefly.
3. Place into PCR machine and program it as follows;

Initial Denaturation: 94ºC for 3 min
Denaturation: 94ºC for 1 min
Annealing: 58ºC for 1min 30 cycles
Extension: 72ºC for 1min
Final extension: 72º C for 5 min

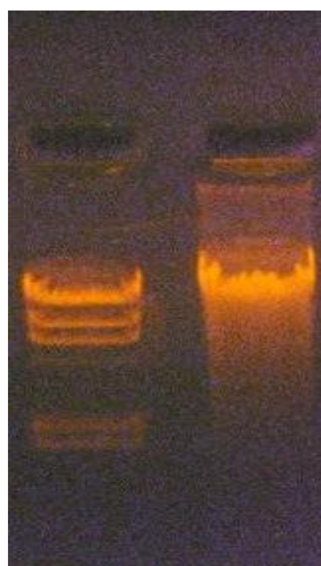
Loading:

1. Prepare 2% agarose gel. [2gm of agarose in 100ml of 1x TAE buffer]
2. Mix 8µl 6X Gel loading dye to each PCR vial and loaded 5µl of PCR sample.
3. Run electrophoresis at 50V till the dye reaches three fourth distances and observe the bands in UV Trans-illuminator.

Agarose gel electrophoresis:

1. Prepared 2% agarose. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven).
2. When the agarose gel temperature was around 60ºC, added 5µl of Ethidium bromide.
3. Poured warm agarose solution slowly into the gel platform.
4. Kept the gel set undisturbed till the agarose solidifies.
5. Poured 1X TAE buffer into submarine gel tank.
6. Carefully placed the gel platform into tank. Maintained the tank buffer level 0.5cm above than the gel.
7. PCR Samples are loaded after mixed with gel loading dye along with 10µl HELINI QuickRef 250bp DNA Ladder.
8. Run electrophoresis at 50V till the dye reaches three fourth distance of the gel.
9. Gel viewed in UV Trans-illuminator and observed the bands pattern

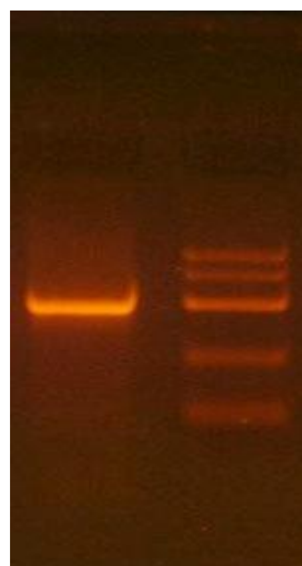
RESULTS



Lane 1 lane 2

Fig.1: Plant DNA isolation

Lane 1: Lambda DNA/ Hind III digest
Lane 2: Plant DNA isolated



Sample Ladder

Fig. 2: amplification of matK
Sample: ~500bp PCR product

Sequence of matK of *H. populifolia*

>GCTTCTTCTTTGCATTTATTACGTTTCTCTCTCTACGAGTATTGTAATTTGAAGAGTTTTCTTACTCCAAAGAAA
TCTATTTTGATTTTTAATGCAAGATTATTCTTGTTCCTATATAATTCTCATGTATGTGAATACGAATCCATTTTCC
TTTTTCTCCGTAACCAATCTTCTCATTTACGAGCAACATATTCTGGAGTCTTTCTTGAACGCATTTATTTCTATGG
AAAAATAGAGTATCTTGTAGAAGTCTTTTATAATGATTTTCAGAACAACCTATGGTTGTTCAAAGACCCTTTCAT
ACATTTTATTAGGTATCAAGGAAAGGCAATTCTGGCCTCAAAAGATACGTCTCTTCTGATGAATAAGTGGAAA
TATTACTTTGTCGATTTATGGCAATATCATTTTTACATGTGGTCTCAATCAGGAAGAGTCCGTAGAAATCAATC
ATCTAAATATTCTCTCGACTTTCTTGTGCTAGAA

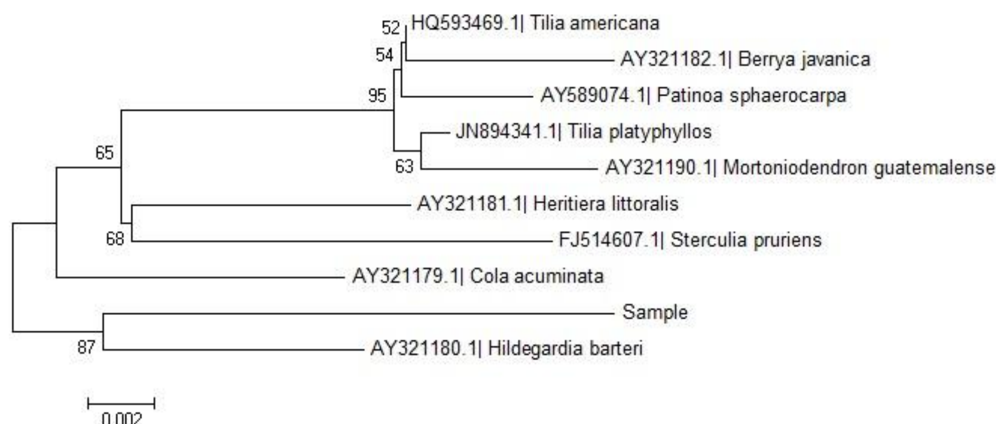


Fig.3: Phylogenic tree of *H. populifolia*

DISCUSSION

The incorporation of DNA sequence data in order to infer relationships among green plants has revolutionized systematic botany considerably. Although sequence data from all three genomes are available now for innumerable plant species, thus facilitating primer design, numerous plastid gene regions

that serve as prominent markers in angiosperm Phylogenetics are rarely used among early land plants. Reasons are gene-rally, two fold, and either genomic reorganizations or mutations in the priming site hamper amplification of the target region with known primers (Wicke & Quandt, 2009).

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