(RESEARCH ARTICLE)

Genomic analysis of chloroplast *mat*K and *rbc*L gene from *Flacourtia inermis* Roxb for plant DNA barcoding

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Abstract

Identification of medicinally significant plant species is helpful for their utilization as medicine. *Flacourtia inermis* Roxb are important medicinal plant. Aim: To Genotypic characterization of *Flacourtia inermis* through DNA sequence of *mat*K and *rbc*L gene. *Flacourtia inermis* Roxb DNA was isolated using NucleoSpin® Plant II Kit (Macherey-Nagel gene extraction) Kit. Isolated DNA was amplified by using PCR (*mat*K and *rbc*L gene primers). Amplified *mat*K and *rbc*L gene was sequenced by ABI 3500 DNA analyzer and the sequence quality was checked using sequence scanner software v1. The results exposed basic variability of the sequences. The *mat*K and *rcb*L gene phylogenic tree was constructed using MEGA 6 software. *Flacourtia inermis* Roxb DNA was successfully isolated using NucleoSpin® Plant II Kit (Macherey-Nagel gene extraction) Kit and confirmed by agarose gel electrophoresis. Isolated *mat*K and *rbc*L genomic DNA was successfully amplified by using PCR. From the *mat*K sequence, BLASTn analysis result *Flacourtia inermis* species showed 99.20% relationship with Flacourtia jangomas and *rbc*L BLASTn analysis result of *Flacourtia inermis* species showed100% relationship with Flacourtia jangomas at NCBI database. From this study, the result concluded the *mat*K and *rcb*L genomes can be used for DNA identification of *Flacourtia inermis*. The information obtained from the study would be helpful for further research involving genotypic identification of *Flacourtia* sp.

Keywords: *Flacourtia inermis*; *mat*K and *rbc*L gene; DNA sequence; Phylogenetic analysis

1. Introduction

Flacourtia inermis plant belonging to family Flacourtiaceae, is a autotrophic plant found in South India, and also found in Malaysia, Indonesia, the Philippines etc. [1] The most important task for a botanist is the identification of correct plant species.[2] DNA bar-coding is the standard method for species identification and differentiation. DNA bar-coding technique growing rapidly, and functional tool for species diversity examination and monitoring of the genomic evolution.[3] *Flacourtia inermis* Roxb is a medium size tree that grow up to 15m height. Genomic evaluation, a short genome sequence is used as a molecular marker for identifying the diversity that present among the plants. Plant genomic analysis sequence locus of nuclear ribosomal cistron internal transcribed spacer (ITS) regions is most commonly used. Several genes of mitochondria, chloroplast and nucleus genes have been used for evaluation sequence differences at genomic level. [4]

Phylogenetic evaluation is emerging as a capable tool for exploring correlations between the phylogenetic diversity and useful attributes of medicinal species. [5] Among all the genes *mat*K and *rbc*L gene sequence are used for the study of plant genomic evaluation. [6, 7] The development of DNA barcoding method to recognize these herbs is critically needed for the safety of patients. [8] Maine objective of work genotypic characterization of *Flacourtia inermis* through DNA sequence of *mat*K and *rbc*L gene.

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2. Material and methods

2.1. DNA isolation using NucleoSpin® Plant II Kit (Macherey-Nagel)

About 100 mg of the tissue is homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube. Four hundred microlitres of buffer PL1 is added and vortexed for 1 minute. Ten microlitres of RNase A solution is added and inverted to mix. The homogenate is incubated at 65°C for 10 minutes. The lysate is transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid is collected and the filter is discarded. Four hundred and fifty microlitres of buffer PC is added and mixed well. The solution is transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid is discarded. Four hundred microlitre buffer PW1 is added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid is discarded. Then 700 µl PW2 is added, centrifuged at 11000 x g and flow through liquid is discarded. Finally 200 µl of PW2 is added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column is transferred to a new 1.7 ml tube and 50 µl of buffer PE is added and incubated at 65°C for 5 minutes. The column is then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4°C.

2.2. Agarose Gel Electrophoresis for DNA Quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.3. PCR amplification

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM MgCl$_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers (Table 1). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

Table 1 Primers used for DNA amplification

Target	Primer Name	Direction	Sequence (5' → 3')
matK	390f	Forward	CGATCTATTCATTCAATATTTC
	1326r	Reverse	TCTAGCACACGAAAGTCGAAGT
*rbc*L	rbcLa_f	Forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcL724_r	Reverse	GTAAAATCAAGTCCACCRCG

2.4. PCR amplification reactions

matK

98 °C	-	30 sec
98 °C	-	5 sec
50 °C	-	10 sec
72 °C	-	15 sec
72 °C	-	60 sec
4 °C	-	∞

(98 °C 5 sec, 50 °C 10 sec) 40 cycles

rbcL

98 °C	-	30 sec
98 °C	-	5 sec
58 °C	-	10 sec
72 °C	-	15 sec
72 °C	-	60 sec
4 °C	-	∞

40 cycles (for 98 °C 5 sec, 58 °C 10 sec)

2.5. Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.6. ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37oC for 15 minutes followed by enzyme inactivation at 80oC for 15 minutes.

2.7. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufacture protocol.

2.8. Post Sequencing PCR Clean up

Make master mix-I of 10µl milli Q and 2 µl 125mM EDTA per reaction. Add 12µl of master mix I to each reaction containing 10µl of reaction contents and mix properly. Make master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction. Add 52 µl of master mix II to each reaction. Contents are mixed by inverting. Incubate at room temperature for 30 minutes. Spin at 14,000 rpm for 30 minutes and decant the supernatant and add 100 µl of 70% ethanol. Spin at 14,000 rpm for 20 minutes and decant the supernatant and repeat 70% ethanol wash. Finally decant the supernatant and air dry the pellet. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

2.9. Sequence Analysis

The sequence quality was checked using sequence scanner software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1.

2.10. Phylogenetic analysis

The basic sequence analysis including nucleotide frequencies, transition/ transversion ratio and variability in different regions of sequences were computed by Molecular Evolutionary Genetics Analysis by using MEGA. [9, 10].

3. Results

3.1. DNA isolation and Identification

Flacourtia inermis DNA was isolated using NucleoSpin® Plant II Kit. The Isolated DNA was checked using agarose gel electrophoresis. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). (Figure 1).
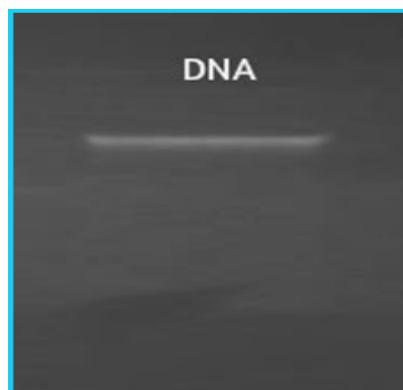

Figure 1 Isolated plant DNA conformed by agarose gel electrophoresis

3.2. *mat*K and *rbc*L gene

From the Isolated genomic DNA, *matK* and *rbcL* gene amplified by using primers 390f forward, 1326r reverse and rbcLa forward, rbcL724 reverse. Amplified *matK* and *rbcL* gene were confirmed by agarose gel electrophoresis. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). (Figure 2).
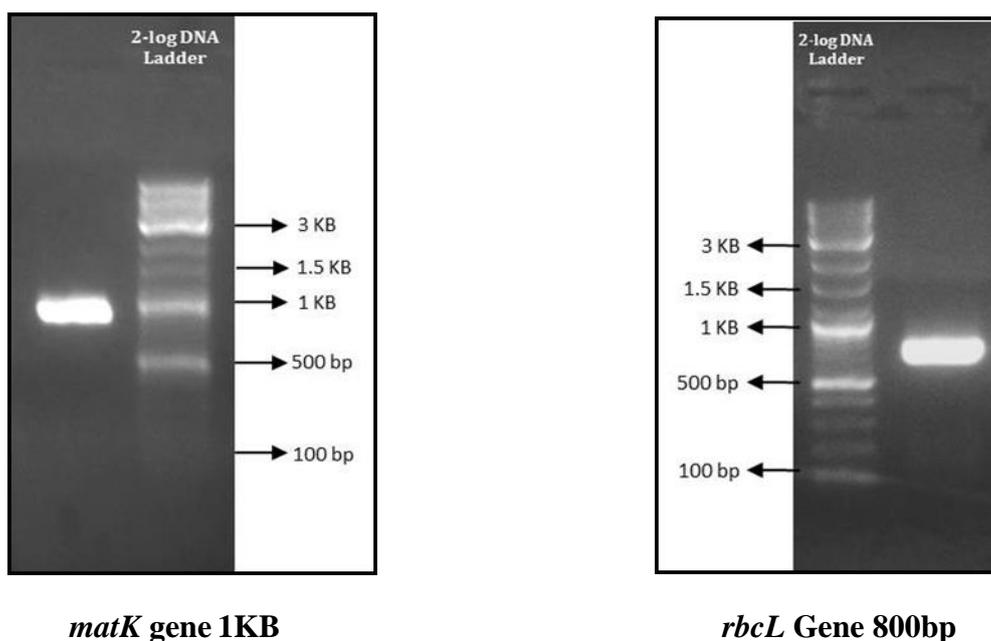

matK **gene 1KB**   *rbcL* **Gene 800bp**

Figure 2 Amplified *matK* and *rbcL* gene with ladder

3.3. Sequences

The amplified *matK* and *rbcL* gene sequence quality was checked using sequence scanner software v1 (Applied Biosystems) and sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1

3.3.1. matK Sequences SUB5886889

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>SR810-MATK
TTAAATTATGTCTCAGATGGACTAATACCTTACCCCATCCATCTAGAAAAATTGGTTCAAATCCTTCGCTCTTGGGTGAAAGATCCCTCCTCTTTGCATTTATTA
CGACTATTTCTTCACGAGTATTGGAATTGGAACAGTCTTATTATTCCAAAGAAATCTATTTCCATTTTTGCAAAAAATAATCCAAGATTTTTCTTGTTCCTATAT
AATTCTCATGTATATGAATACGAGTCCGTTTTCTTTTTTCTTCGTAATCAATCCTTTCATTTCCGATTAACATTTTCTCAGGTCTTTCTTGAGCGAATATATTTC
TATGGAAAAATAGAACATTTTGTAGAAGTCTTTACTAAGGATTTTCGGGACAGCTTATGCTTGCTCAAGGATCCTTTCATACATTATGTTAGATATCAAGGAAAA
TCCATTTTGGTCTCAAAGGATACGCCTCTTCTGATGAAAAATGGAAAGATTACCTTGTCAATTTATGTCAATGTCATTTTGATGTGTGCTTTCAACCAAAAAAG
ATCCATAAAAACCCATTATCATTATACAAGCATTCTTTCGACTTATTAGGCTATCTTTCAAGTGTACGACTAAACCTTTCAGTGGTACGGAGTCAAATGCTAGAA
AATGCATTTCTAATAGAGAATACTATGAATAAACTCGATACAACAGTTTCAATTATTCCTTTGATTGGATCATTAGCAAAAATGAAATTTTGTAACGCAGTAGGA
CATCCCATTAGTAAACCGACCTGGGCCGATTTCTCGGATTCTGATATTATCGACCGATTTGTCTGTATATGCAGAAATCTTTCTCATTATTATAGCGGATCCTCA
AGAAAAAAGAGTTTGTATCGAATAAAATATATA
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3.3.2. rbcL Sequences

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>SR810-RBCL
ATAAATTGACTTATTATACTCCTGACTATGAAACCAAAGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCCGCCCGAGGAAGCAGGGG
CCGCGGTAGCTGCTGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGATGGGCTTACCAGTCTTGATCGTTATAAAGGACGATGCTACGACATCGAGCCCG
TTGCTGGAGAAGAAAATCAATATATTGCTTATGTAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTAT
TTGGGTTCAAAGCCCTACGCGCTCTACGTCTGGAGGATTTGCGAATCCCCCCTGCTTATTCTAAAACTTTTCAAGGCCCACCTCACGGCATCCAAGTTGAGAGAG
ATAAATTGAACAAGTATGGTCGCCCCCTATTGGGCTGTACTATTAAACCTAAATTGGGGTTATCCGCTAAGAATTACGGTAGAGCAGTTTATGAATGTCTACGTG
GTGGACTTGATTTTACCAAAGATGATGAGAACGTGAACTCCCAACCATTTATGCGTTGGAGAGATCGTTTCTTATTTTGTGCCGAAGCACTTTATAAAGCACAGG
CTGAAACCG
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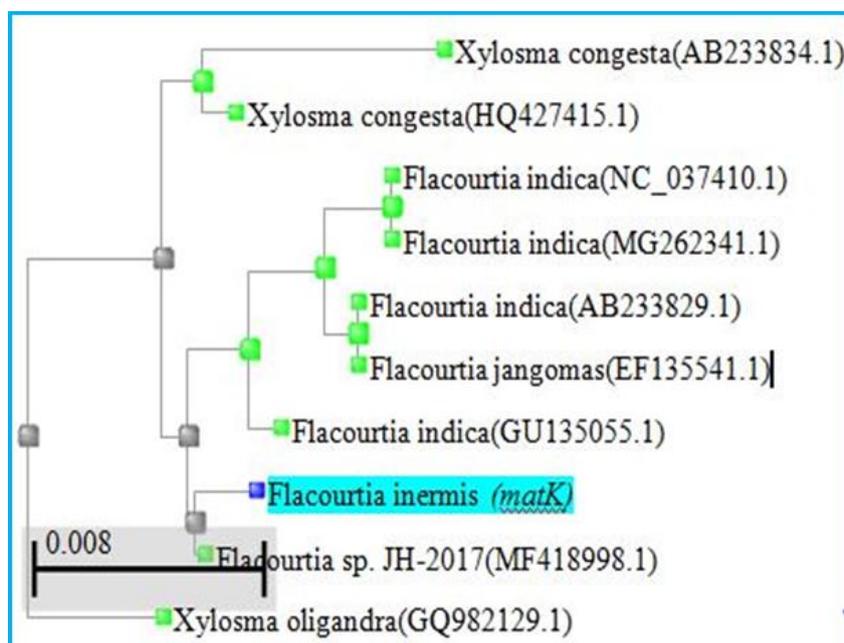
3.4. Phylogenetic analysis

Figure 3 The phylogentic tree of *Flacourtia inermis* using *matK* sequences

Note: Maximum Parsimony, maximum likelihood and tree generated from *matK* gene *Flacourtia inermis.* (Supporting material 1)
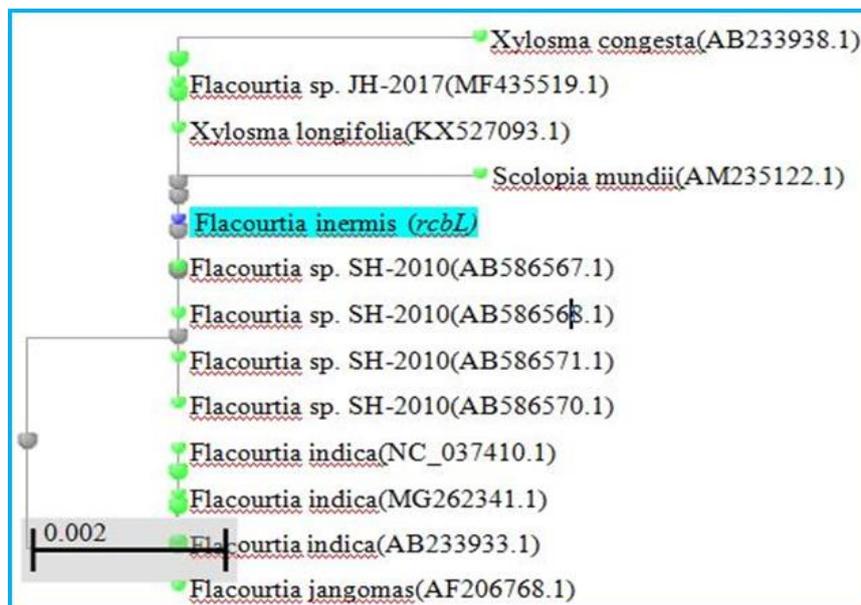
Figure 4 The phylogentic tree of *Flacourtia inermis* using *rcbL* sequences

Note: Maximum Parsimony, maximum likelihood and tree generated from *rcbL* gene *Flacourtia inermis.* (Supporting material 2).

4. Conclusion

In this work, genomes of *Flacourtia inermis* species were sequenced and evaluated. Two variant regions were screened and were formed to be potential DNA barcodes for the identification of *Flacourtia inermis*. The results exposed basic variability of the sequences. The *matK* and *rcbL* gene phylogenic tree was constructed using MEGA 6 software. *Flacourtia inermis* species show 99.20% relationship with *Flacourtia jangomas*. The phylogenic trees indicate that the *matK* and *rcbL* genomes can be used for the identification of *Flacourtia inermis*. The information obtained in this work would be a useful source for further research involving the identification and phylogenetic analysis of *Flacourtia sp* (Flacourtiaceae).

Compliance with ethical standards

Acknowledgments

Disclosure of conflict of interest

No conflicts of interest.

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