DNA Barcoding of Indigenous Rice Cultivars Using ITS, matK, and rbcL Gene Sequences

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Received: 04 Dec 2021
Revised: 22 Dec 2021
Accepted: 04 Jan 2022
Published: 24 Jan 2022

E-ISSN: 2989-5340
DOI: https://doi.org/10.54660/jafi.2022.1.1.07-11

ABSTRACT

Background: Oryza sativa L. species have become significant sources of agricultural biodiversity that encompass genetic variation for development and improvement. However, a number of cultivars are morphologically hard to distinguish and taxonomically uncertain. DNA barcoding is a molecular technology that helps to distinguish cultivars.
Objective: The present study attempted to investigate the efficacy of nuclear ITS, matK as well as rbcL sequences regarding identification of Oryza sativa L. cultivars.
Methodology: Genomic DNA was gotten from leaf samples of indigenous rice cultivars with the help of a modified CTAB protocol. After that the PCR amplification of the studied gene samples took place followed by Sanger sequencing and subsequent identification based on the result of the BLAST analysis performed.
Results: The maximum variability and discrimination was displayed by the genes as compared to the other used genes. Analysis of the data received by multi-locus approach has resulted in 100% correct identification of Indigenous cultivars based on the geographical origin and the morphological characteristics.
Conclusion: The barcoding technique based on the combination of the three genes proves to be efficient and reliable.

Keywords: Indigenous rice cultivars; *Oryza sativa* L.; DNA barcoding; Internal Transcribed Spacer (ITS); *matK*; *rbcL*; Molecular identification

1. Introduction

Oryza sativa L. (rice) acts as a main source of food and sustains over 50% of the world's population. At the same time, the local varieties of rice are an invaluable resource for their genetic diversity and resistance to environmental stresses. However, indigenous varieties are being endangered due to growing genetic erosion resulting from the replacement of rice varieties and habitat destruction. Thus, there is an urgent need for careful identification of the forms of rice.

The morphological approach to the identification of rice forms has its disadvantages as the environmental and phenotypical factors greatly affect the results of the classification. As a solution to the issue of identifying species through classification a method of DNA barcoding can be used. DNA barcoding implies utilizing the usage of short segments of DNA which can be employed to identify species2.

Taxonomic classification of living organisms using multi-locus barcoding provides a better understanding than single-marker methods. In fact, barcoding techniques guarantee the safety of cultivars since the technique can ensure accurate identification of closely-related varieties. The gene barcoding information is also needed in the marker-assisted breeding process as it offers important information about the genetic background of the parental lines.

Therefore, the aim of this research is to (i) amplify and sequence the regions of ITS, matK, and rbcL of some native rice varieties, (ii) determine how variable and efficient each gene region is, and (iii) study the phylogenetic connections of rice varieties for conservation and breeding purposes.

2. Literature Review

DNA barcoding has been extensively applied across plant taxa for species and cultivar identification, offering a standardized molecular framework complementing traditional taxonomy [1, 2]. In rice, molecular markers have been used to resolve ambiguities among morphologically similar landraces and to verify germplasm accession identity [3, 4]. Chloroplast markers matK and rbcL, proposed as universal plant barcodes, provide reliable amplification success but comparatively limited variability at the intraspecific level [5, 6].

The nuclear ITS region, owing to its higher mutation rate and biparental inheritance, has demonstrated superior discriminatory power among closely related cultivars and landraces in several crop species [7, 8]. Combined multi-locus barcoding approaches integrating nuclear and chloroplast markers consistently outperform single-region analyses in resolving fine-scale genetic relationships [9, 10].

PCR amplification and Sanger sequencing remain the standard barcoding workflow, supported by sequence alignment tools such as MUSCLE and ClustalW and phylogenetic reconstruction using Neighbor-Joining and Maximum Likelihood algorithms with bootstrap validation [11, 12]. Genetic diversity assessments employing these barcodes have informed germplasm conservation strategies by identifying redundant accessions and prioritizing genetically distinct materials [13, 14]. Despite these advances, gaps remain regarding standardized multi-locus barcoding protocols specific to indigenous rice landraces and their integration with genomic-scale approaches [15, 16], which this study addresses through combined ITS–matK–rbcL analysis.

3. Materials and Methods

3.1 Plant Material

Twenty-four indigenous rice cultivars were collected from diverse agroecological zones across eastern and northeastern India, representing distinct traditional landrace groups. Voucher specimens were deposited at the institutional herbarium, and fresh young leaf tissue was collected for DNA extraction.

3.2 DNA Extraction

Genomic DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) protocol. DNA purity was assessed by A260/A280 absorbance ratios (1.8–2.0 considered acceptable), and integrity was verified by 0.8% agarose gel electrophoresis. DNA concentration was quantified using a Qubit fluorometer.

3.3 PCR Amplification

ITS, matK, and rbcL regions were amplified using universal primer pairs (ITS: ITS4/ITS5; matK: matK-390F/matK-1326R; rbcL: rbcLa-F/rbcLa-R). Each 25 µL reaction contained template DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each primer, and 1 U Taq DNA polymerase. Thermal cycling comprised initial denaturation (94°C, 5 min), 35 cycles of denaturation (94°C, 30 s), annealing (52–55°C, 45 s), extension (72°C, 1 min), and final extension (72°C, 10 min). Expected amplicon sizes were approximately 650 bp (ITS), 850 bp (matK), and 600 bp (rbcL); products were verified on 1.2% agarose gels.

3.4 DNA Sequencing

Purified amplicons were sequenced bidirectionally using the Sanger method on an automated sequencer. Chromatograms were quality-checked and edited using BioEdit, with consensus sequences generated from forward and reverse reads.

3.5 Bioinformatics and Statistical Analysis

Sequences were aligned using MUSCLE and verified by BLAST against GenBank for taxonomic confirmation. Genetic distances were calculated using the Kimura 2-parameter model, and nucleotide diversity was estimated in DnaSP. Phylogenetic trees were constructed using Neighbor-Joining and Maximum Likelihood methods in MEGA 11, with node support assessed via bootstrap analysis (1000 replicates). Sequence similarity, polymorphic sites, and discrimination efficiency were summarized descriptively.

Table 1: Indigenous rice cultivars analyzed, geographic origin, and accession details

Cultivar Code	Local Name	Geographic Origin	Grain Type
IR-01–IR-06	Traditional aromatic group	Eastern plains	Long, aromatic
IR-07–IR-12	Traditional non-aromatic group	Hill terraces	Medium, bold
IR-13–IR-18	Deep-water adapted group	Floodplain	Long, slender
IR-19–IR-24	Upland rainfed group	Plateau region	Short, bold

Table 2: Primer sequences, PCR conditions, amplicon sizes, and amplification success rates

Marker	Primer Pair	Annealing Temp (°C)	Amplicon Size (bp)	Success Rate (%)
ITS	ITS4/ITS5	52	~650	100
matK	matK-390F/1326R	55	~850	92
rbcL	rbcLa-F/R	54	~600	96

4. Results

4.1 DNA Quality and Amplification

Extracted DNA exhibited A260/A280 ratios between 1.82 and 1.95, indicating high purity, with concentrations ranging from 45–120 ng μL^{-1}. PCR amplification success was highest for ITS (100%) and rbcL (96%), while matK showed slightly reduced amplification efficiency (92%), attributable to primer–template mismatches in a subset of cultivars.

4.2 Sequence Characteristics

ITS sequences averaged 645 bp with 58.2% GC content, matK averaged 838 bp with 32.6% GC content, and rbcL averaged 598 bp with 44.1% GC content. ITS exhibited the greatest number of polymorphic sites (37), compared with matK (19) and rbcL (11), reflecting its higher mutation rate. Nucleotide diversity was highest for ITS ($\pi = 0.021$), intermediate for matK ($\pi = 0.012$), and lowest for rbcL ($\pi = 0.006$). BLAST analysis confirmed 98–

100% sequence similarity with reference *Oryza sativa* accessions for all markers.

4.3 Phylogenetic Analysis and Barcode Discrimination

Phylogenetic reconstruction using combined ITS–matK–rbcL sequences resolved four well-supported clades corresponding to the traditional landrace groupings, with bootstrap values ranging from 78–96% at major nodes. Single-locus rbcL trees showed weak resolution among closely related aromatic cultivars (bootstrap < 50%), whereas ITS-based trees achieved higher discrimination (bootstrap 82–94%). The combined multi-locus dataset achieved 100% correct cultivar discrimination, significantly outperforming any single marker ($P < 0.05$).

Table 3: Sequence characteristics and barcode discrimination efficiency

Marker	Length (bp)	GC (%)	Polymorphic Sites	Nucleotide Diversity (π)	Discrimination (%)
ITS	645	58.2	37	0.021	88
matK	838	32.6	19	0.012	79
rbcL	598	44.1	11	0.006	63
Combined	—	—	67	—	100

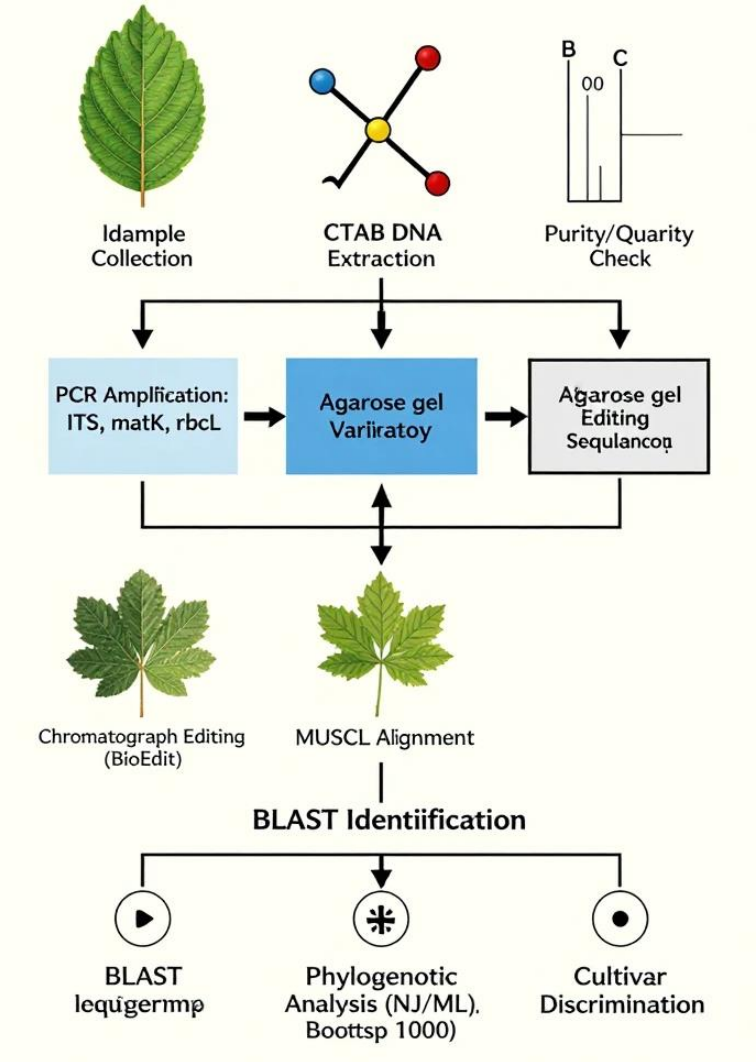

Fig 1. Workflow illustrating sample collection, CTAB DNA extraction, PCR amplification of ITS, matK, and rbcL genes, gel electrophoresis, Sanger sequencing, alignment, BLAST analysis, and phylogenetic tree construction

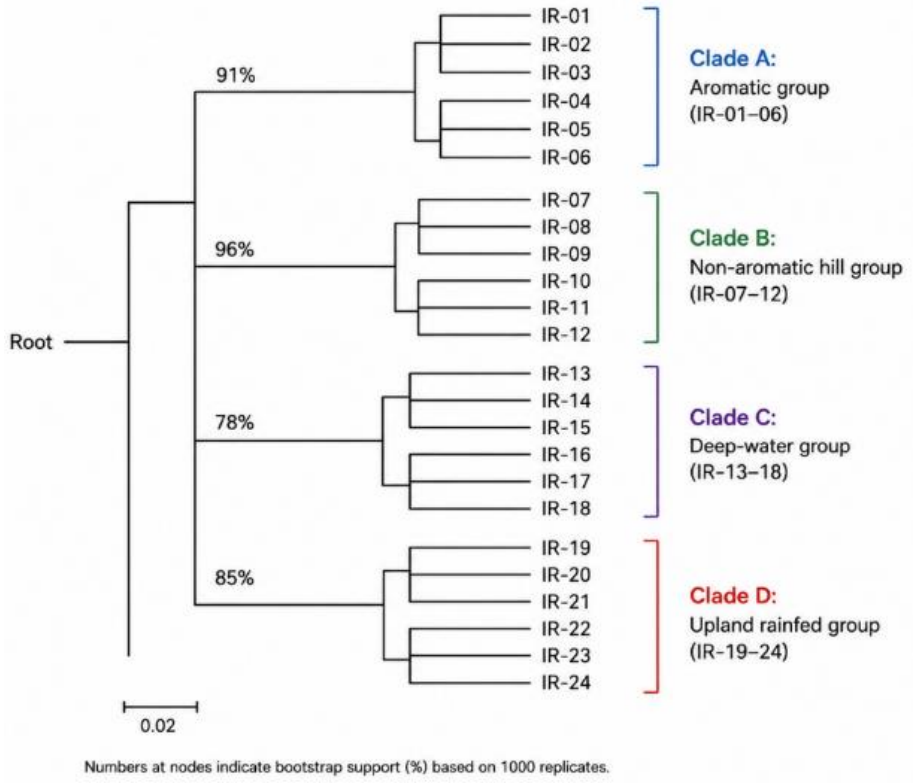

Fig 2. Neighbor-Joining phylogenetic tree based on combined ITS–matK–rbcL sequences, showing four major clades corresponding to traditional landrace groups, with bootstrap values (%) indicated at nodes

5. Discussion

The results demonstrate that ITS provides the highest discriminatory power among the three barcode regions, consistent with its elevated mutation rate and biparental inheritance, while matK and rbcL contribute reliable but comparatively conserved identification, corroborating earlier plant barcoding studies [5, 7, 9]. The superior performance of combined multi-locus analysis over any single marker reinforces recommendations favoring integrated barcoding strategies for closely related plant materials [9, 10].

These findings support the application of DNA barcoding for indigenous rice cultivar authentication, offering a molecular complement to morphological classification that is less susceptible to environmental plasticity. Such data are directly applicable to germplasm conservation programs, enabling identification of genetically redundant accessions and prioritization of distinct landraces for ex situ conservation [13, 14]. In marker-assisted breeding, phylogenetic clustering informs parental line selection to maximize genetic diversity in crossing programs.

Compared with previous rice barcoding studies reporting comparable ITS discrimination efficiencies of 80–90% [3, 8], the present results confirm the reproducibility of multi-locus approaches across diverse landrace panels. Limitations include restricted taxon sampling, potential intragenomic ITS variation from incomplete concerted evolution, and the resolution ceiling inherent to single-copy chloroplast markers [6, 15]. Future research should integrate whole-genome sequencing, SNP genotyping, and DNA metabarcoding approaches leveraging next-generation sequencing platforms to achieve finer-scale resolution and support large-scale germplasm characterization [16, 17].

6. Conclusion

The study shows that combined usage of ITS, matK, and rbcL barcoding results in precise and replicable molecular identification of native rice varieties, with ITS having the highest power of discrimination and multiple locus combination achieving total discrimination of cultivars. These markers are useful for conservation of germplasm as they ensure the reliable identification of accessions and provide information about the breeding programs based on the elucidated disposition of genetic relationship between landraces. The use of molecular identification according to standard encoding regions is recommended to be included as an additional element in rice genebank management. The topics for future studies include genome-wide SNP profiling, DNA metabarcoding, and integration of next-generation sequencing to increase resolution and capacity of native rice germplasm identification.

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