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Genetic diversity assessment of four local rice (*Oryza sativa* L.) genotypes using RAPD markers

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ABSTRACT

Genetic diversity underpins the long-term security of any breeding programme, yet many locally adapted rice genotypes are still characterised largely by appearance rather than at the DNA level. This study used Random Amplified Polymorphic DNA (RAPD) markers to assess the genetic relationships among four rice (*Oryza sativa* L.) genotypes — MTU 1229, MTU 1166, MCU 103 and BPT 2595 — currently under trial in Andhra Pradesh, India. Genomic DNA was extracted from 10–15-day-old seedling leaves using a CTAB-based method and screened against ten decamer primers (OPF1–OPF10). Nine primers amplified reproducible products, together yielding 40 scorable bands of 300–900 bp, of which 24 (60%) were polymorphic and 16 (40%) monomorphic. Primer OPF2 was the most discriminating, producing eight bands with 87.5% polymorphism. A polymorphic amplicon shared by MCU 103 and BPT 2595 was excised, purified and sequenced; BLAST comparison placed the fragment at approximately 88% identity with a region of *Arabidopsis thaliana* chromosome 1, tentatively corresponding to a trithorax-like protein-coding sequence. The results confirm that even a modest RAPD primer panel can resolve meaningful genetic variation among closely related rice genotypes, supporting its continued use as a low-cost, first-pass tool for germplasm characterisation, parental selection, and the conservation of local rice diversity.

Keywords: *Oryza sativa*, RAPD markers, Genetic diversity, Polymorphism, Molecular characterisation, Rice germplasm

1. Introduction

Rice (*Oryza sativa* L.) is grown in at least 95 countries and remains the single most important source of dietary calories for a large share of the world's population. India alone accounts for roughly 43% of the global rice-growing area, and within India, Andhra Pradesh is one of the leading producing states, with close to 45 million hectares under the crop yielding well over a million tonnes annually. Given this scale, even modest gains in variety performance or resilience translate into substantial benefits for farmers and for food security more broadly.

Much of that improvement depends on genetic diversity — the raw material breeders draw upon to combine desirable traits such as yield, pest resistance and stress tolerance. Historically, diversity has been assessed through morphological and biochemical traits, but these markers are far from ideal: many agronomically important characters show low heritability, are polygenic, and are strongly influenced by growth conditions and environment. Two genotypes can therefore look alike in the field while being genetically quite distinct, or the reverse, making purely phenotypic selection an unreliable guide for breeding decisions.

Compounding the problem, local rice germplasm is disappearing at a worrying rate. Farmers increasingly favour high-yielding modern varieties, whose output can be almost three times that of traditional landraces, while local germplasm — often more tolerant of pests, disease and abiotic stress — is progressively abandoned. Reports suggest that dozens of local rice types have already been lost within the last two decades, with several more at risk. Before this material disappears entirely, there is a clear case for systematically screening and documenting the genetic variation that still exists, both to conserve it and to identify genotypes worth incorporating into active breeding lines.

Molecular markers offer a way to do this that sidesteps the shortcomings of morphological scoring. A molecular marker is simply a DNA sequence variant whose inheritance can be tracked directly, independent of environmental noise or developmental stage. Since the first such marker — restriction fragment length polymorphism (RFLP) — was described by Botstein *et al.* (1980)^[1], a large family of DNA-based marker systems has emerged, including RAPD, AFLP, SSR and SNP, most of which are detected using PCR-based chemistry.

Among these, Random Amplified Polymorphic DNA (RAPD) analysis remains attractive for laboratories without access to genome sequence information or expensive reagents. The technique uses a single short (9–10 bp) primer of arbitrary sequence to amplify anonymous fragments scattered across the genome (Williams

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et al., 1990) [2]. Because no prior sequence knowledge is required, the same primer set can, in principle, be applied to any species. RAPD needs only a small amount of template DNA (5–20 ng), is comparatively quick to run, and can reveal a useful degree of polymorphism in a single PCR step (Rafalski *et al.*, 1991) [3]. Its main drawback is that it behaves as a dominant marker — heterozygotes and homozygotes cannot be distinguished — but for a first-pass survey of diversity among genotypes, this limitation is rarely disqualifying.

The present study applies RAPD analysis to four rice genotypes currently under evaluation in Andhra Pradesh — MTU 1229, MTU 1166, MCU 103 and BPT 2595 — with three specific objectives: (i) to determine the genetic diversity and relatedness among these genotypes; (ii) to reveal nuclear DNA-level variation within each genotype; and (iii) to test whether RAPD markers are sensitive enough to detect genetic polymorphism among genotypes that are otherwise difficult to separate on morphological grounds alone.

2. Materials and methods

2.1 Plant material

Four rice genotypes — MTU 1229 (parentage MTU7029 × NLR3042), MTU 1166 (BPT5204 × MTU1071), MCU 103 and BPT 2595 (both with confidential parentage) — were obtained as seed from the Research Station, Anaparthi. Seeds were germinated and grown individually in test tubes under controlled conditions for 10–15 days to provide young, actively growing leaf tissue for DNA extraction (Figure 1). Fresh leaves were excised with sterile scissors, rinsed in distilled water followed by ethanol to remove surface microorganisms and contaminating DNA, blotted dry on tissue paper, and stored in labelled polythene bags at –20 °C until extraction.

2.2 Genomic DNA extraction

Total genomic DNA was isolated using a CTAB (cetyl trimethylammonium bromide)-based protocol, chosen because CTAB and its accompanying additive, polyvinylpyrrolidone, are particularly effective at removing the polysaccharides and polyphenols that commonly co-purify with DNA from plant tissue and interfere with downstream enzymatic reactions. Briefly, 100 mg of leaf tissue was homogenised and incubated in 500 µL CTAB extraction buffer (0.1 M Tris-HCl, 0.02 M Na₂EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol, 2% CTAB and polyvinylpyrrolidone, pH 8.0) at 60 °C for 30 minutes. Following centrifugation, the supernatant was extracted with an equal volume of

chloroform:isoamyl alcohol (24:1), repeated until the aqueous phase was clear. DNA was precipitated from the aqueous phase with 0.7 volumes of ice-cold isopropanol at –20 °C, pelleted by centrifugation, washed with 70% ethanol, air-dried, and finally resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA).

2.3 Assessment of DNA quality

The integrity of the extracted DNA was checked by electrophoresis on a 0.8% agarose gel prepared in 1X TBE buffer containing ethidium bromide, run at 70 V for 30–60 minutes alongside a 1 kb DNA ladder, and visualised under UV *trans*-illumination.

2.4 Primer selection

Ten decamer primers of the OPF series (OPF1–OPF10; Table 1) were used for RAPD amplification, selected on the basis of their reported ability to generate reproducible, informative banding patterns in cereal genomes.

Table 1. RAPD (OPF-series) primers used in the study.

Primer	Sequence (5'→3')
OPF-01	ACG GAT CCT G
OPF-02	GAG GAT CCC T
OPF-03	CCT GAT CAC C
OPF-04	GGT GAT CAG G
OPF-05	CCG AAT TCC C
OPF-06	GGG AAT TCG G
OPF-07	CCG ATA TCC C
OPF-08	GGG ATA TCG G
OPF-09	CCA AGC TTC C
OPF-10	GGA AGC TTG G

2.5 PCR amplification

Each 40 µL PCR reaction contained 150–200 ng genomic DNA (3 µL), 5 µL of 10X reaction buffer, 1 µL dNTPs (10 mM), 1 µL Taq DNA polymerase (2 U), 2 µL each of forward and reverse primer (10 pmol), and 26 µL sterile distilled water (Table 2). Amplification was carried out on a gradient thermal cycler with an initial denaturation at 95 °C for 5 minutes, followed by 34 cycles of denaturation (95 °C, 45 s), annealing (52 °C, 45 s) and extension (72 °C, 45 s), and a final extension at 72 °C for 5 minutes (Table 3).

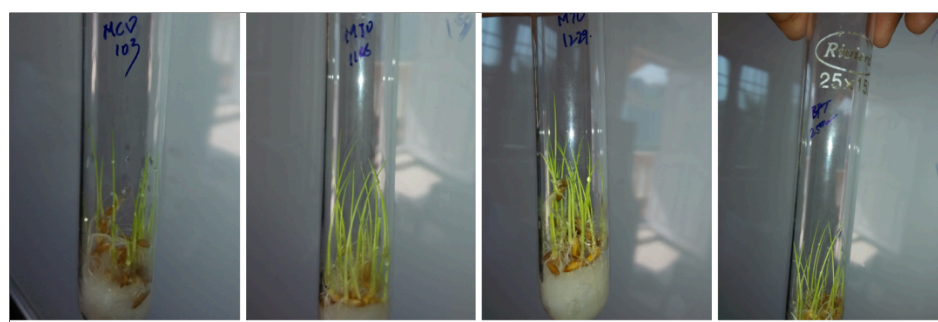


Figure 1. Ten- to fifteen-day-old seedlings of the four rice genotypes raised in test tubes for leaf sampling (left to right: MCU 103, MTU 1166, MTU 1229, BPT 2595).

Component	Volume	Primer 1 (10 pmol)	2 µL
Genomic DNA (150–200 ng)	3 µL	Primer 2 (10 pmol)	2 µL
10X reaction buffer	5 µL	Sterile distilled water	26 µL
dNTPs (10 mM)	1 µL	Final volume	40 µL
Taq DNA polymerase (2 U)	1 µL		

Table 2. Composition of the PCR reaction mixture.

Step	Temperature	Duration	Cycles
(continued on next page)			

Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	45 s	34
Annealing	52 °C	45 s	34
Extension	72 °C	45 s	34
Final extension	72 °C	5 min	1

Table 3. Thermal cycling profile used for RAPD-PCR.

2.6 Electrophoresis of amplified products and band scoring

Amplicons were resolved on 1.2–2.0% agarose gels in 1X TBE buffer at 70–80 V for 1–1.5 hours, using a 1 kb DNA ladder as a size reference, and documented on a gel imaging system. Bands were scored as present or absent across genotypes for each primer; a band was treated as polymorphic if it was not shared by all four genotypes.

2.7 Gel elution, sequencing and sequence analysis

To investigate the identity of a distinctive amplicon shared by two of the genotypes, PCR was repeated for MCU 103 and BPT 2595 under the same conditions, and the target band was excised from the gel with a sterile scalpel. DNA was recovered using a QIAquick Gel Extraction Kit following the manufacturer's protocol — gel solubilisation in Buffer QG at 60 °C, isopropanol addition, column binding, washing with Buffer PE, and elution in 22 µL elution buffer. The purified fragment was sequenced, the resulting chromatogram was examined using Chromas trace-viewing software, and the consensus sequence was compared against the NCBI nucleotide database using BLAST to identify the closest matching sequences.

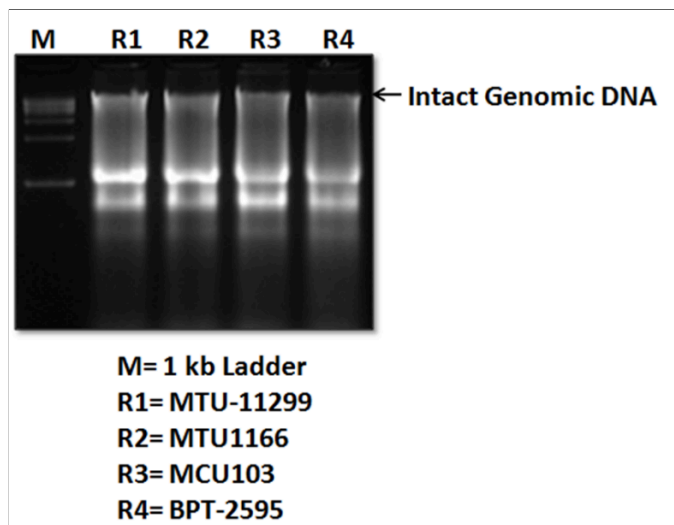
3. Results and discussion

3.1 DNA quality

Electrophoresis of the extracted genomic DNA produced a single, high-molecular-weight band for each of the four genotypes with minimal shearing or RNA contamination (Figure 2), confirming that the CTAB-based protocol yielded DNA of sufficient quality and quantity for reliable PCR amplification.

3.2 RAPD profiling and polymorphism

Of the ten OPF primers screened, nine amplified genomic DNA to give reproducible, scorable products (Figure 3); primer OPF5 failed to amplify under the conditions tested and was excluded from further analysis. Collectively, the nine informative primers generated 40 bands ranging from approximately 300 to 900 bp, of which 24 bands (60%) were polymorphic across the four genotypes and 16 (40%) were monomorphic (Table 4).

**Figure 2.** Genomic DNA of the four rice genotypes resolved on a 0.8% agarose gel. M: 1 kb ladder; R1: MTU 1229; R2: MTU 1166; R3: MCU 103; R4: BPT 2595.

The amount of polymorphism varied considerably between primers. OPF2 was the most productive and most discriminating primer, generating eight bands of which seven (87.5%) were polymorphic — the highest information content of any primer tested. OPF6 was similarly informative, with five of six bands (83.3%) polymorphic. By contrast, OPF1 produced only two bands with 50% polymorphism, and OPF9 generated five bands but just 40% polymorphism. OPF10, though it produced only two bands, showed complete polymorphism between genotypes. This spread is consistent with the general observation in RAPD studies that informativeness is primer-dependent rather than a fixed property of the technique itself — a pattern also reported by Rahman *et al.* (2007) [4] and Gholaki *et al.* (2003) [5] in their respective surveys of rice genetic diversity, where only a subset of the primers screened proved genuinely useful for distinguishing genotypes.

Table 4. RAPD primers, bands scored, size range and polymorphism observed among the four rice genotypes.

Primer	Sequence (5'→3')	Bands scored	Size range (bp)	Polymorphic bands	% Polymorphism
OPF-01	ACG GAT CCT G	2	300+	1	50.00
OPF-02	GAG GAT CCC T	8	300–900	7	87.50
OPF-03	CCT GAT CAC C	7	300–900	3	42.85
OPF-04	GGT GAT CAG G	7	400–800	3	42.85
OPF-05	CCG AAT TCC C	—	—	—	—
OPF-06	GGG AAT TCG G	6	300–800	5	83.33
OPF-07	CCG ATA TCC C	1	600–700	—	—
OPF-08	GGG ATA TCG G	2	600–700	1	50.00
OPF-09	CCA AGC TTC C	5	400–800	2	40.00
OPF-10	GGA AGC TTG G	2	300+	2	100.00
Total		40		24	
Average		20		12	

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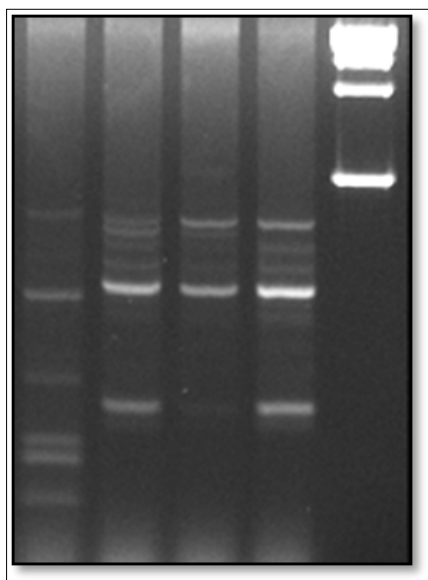


Figure 3. Representative RAPD banding pattern of the four rice genotypes generated with an OPF-series primer, alongside a 1 kb DNA ladder.

The overall level of polymorphism recorded here (60%) sits within, though towards the lower end of, the range reported in comparable RAPD studies of rice. Arif *et al.* (2005) [6], for instance, found roughly half of their primers polymorphic across 19 Pakistani rice genotypes, while Sing *et al.* (2003) [7] and Ravi *et al.* (2003) [8] reported much higher polymorphism (90–95%) when working with more genetically diverse panels that included wild relatives alongside cultivated varieties. This contrast is informative in its own right: the four genotypes examined here are closely related, elite or near-elite breeding lines under trial in the same region, so a comparatively modest — though still clearly detectable — level of polymorphism is exactly what would be expected, and is itself a useful finding for breeders trying to judge how genetically distinct these lines really are.

Weak or faint bands were occasionally observed alongside strong, clearly scorable ones. Such weak amplification is generally attributed to partial sequence mismatch between the primer and its annealing site rather than to a true absence of that locus (Thormann *et al.*, 1994; Clark *et al.*, 1993) [10, 9], and only unambiguous bands were included in the polymorphism scoring reported here.

3.3 Sequence identity of a shared polymorphic amplicon

Because MCU 103 and BPT 2595 shared a distinctive amplicon of particular interest, this fragment was re-amplified, gel-purified (Figure 4) and sequenced. The chromatogram obtained showed clean,

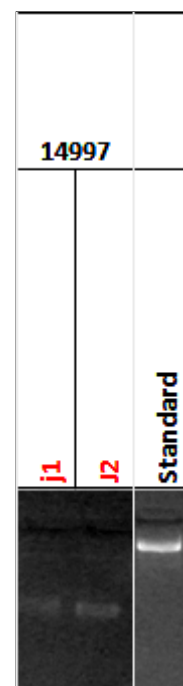


Figure 4. Gel elution of the target amplicon from MCU 103 (J1) and BPT 2595 (J2) prior to sequencing, alongside a standard.

An 88% identity to a dicot model species is, on its own, too low to claim direct orthology, and some caution is warranted given that RAPD amplicons are anonymous by design and were not intended to target coding sequence specifically. Nonetheless, the match is well above what would be expected by chance for a 334 bp window and suggests that this particular amplicon lies within, or close to, a conserved genic region rather than purely repetitive or non-coding DNA. Confirming the identity and function of this fragment in rice itself would require designing gene-specific primers from the rice genome sequence and repeating amplification and sequencing directly — a natural next step beyond the scope of the present RAPD-based survey.

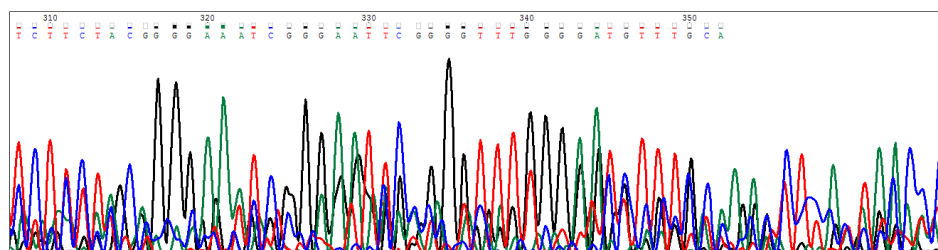


Figure 5. Representative chromatogram from sequencing of the BPT 2595 (J2) amplicon.

3.4 General discussion

Taken together, these results support the three objectives set out for this study. Clear genetic differentiation was detected among the four rice genotypes despite them being closely related breeding lines, confirming that meaningful diversity exists even within a small, elite panel. The RAPD banding patterns also revealed variation at the level of individual amplified loci, consistent with genuine nuclear DNA-level polymorphism rather than artefactual noise. Finally, the fact that

reproducible, scorable polymorphism was obtained from a modest ten-primer screen, without any prior sequence information for these particular genotypes, demonstrates that RAPD remains an effective and low-cost tool for detecting genetic polymorphism in rice varieties under active development. These findings are broadly consistent with earlier RAPD-based surveys of rice diversity (Kubo *et al.*, 2000; Ferdowsi, 2004; Chung *et al.*, 2005) [12, 13, 11], which likewise found that even a limited primer panel could resolve genotype-level differences not obvious from morphology alone.



Figure 6. BLAST alignment showing 88% identity between the sequenced amplicon and Arabidopsis thaliana chromosome 1 (trithorax-like protein region).

From a practical standpoint, the results suggest that pairs such as MTU 1229/MTU 1166 and MCU 103/BPT 2595 could be prioritised for closer comparison if breeders are looking to combine genetically distinct parental material, though a formal cluster or similarity analysis across a larger primer panel would be needed before making firm recommendations. The unexpected sequence similarity to an *Arabidopsis* trithorax-like gene region also flags the shared MCU 103/BPT 2595 amplicon as a candidate worth following up with targeted, sequence-based markers in future work.

4. Conclusion

This study demonstrates that RAPD markers, despite their technical simplicity, can resolve genetic differences among four closely related rice genotypes — MTU 1229, MTU 1166, MCU 103 and BPT 2595 — that are difficult to separate using morphology alone. Ten OPF-series primers generated 40 scorable bands, 60% of which were polymorphic, with primer OPF2 proving the most informative. Sequencing of a shared amplicon from MCU 103 and BPT 2595 revealed roughly 88% identity to an *Arabidopsis thaliana* genomic region, pointing to a putative gene-associated fragment worth further characterisation. These results reinforce the case for using RAPD analysis as an accessible first step in characterising local rice

germplasm, informing parental selection in breeding programmes, and supporting the conservation of genetically distinct rice lines before they are lost to the spread of a narrower set of high-yielding varieties.

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