

GENETIC CHARACTERIZATION AND BARCODE CONSTRUCTION OF INDIGENOUS RICE VARIETIES AND ELITE LINES FOR INTELLECTUAL PROPERTY RIGHTS PROTECTION

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ABSTRACT

For the protection of farmers and consumer interests, rice varietal identification is a crucial part from a breeding point of view as well as seed production, and marketing. In this study, a total of 120 SSR primers extensively covering the whole rice genome were used to develop the varietal-specific DNA fingerprints of six indigenous rice varieties and nine elite rice lines. Among them, sixty-seven SSR primers were found polymorphic that amplified a total of 158 alleles. Diversity analysis of markers depicts the average number of amplified alleles (2.35), number of different alleles (Na: 0.534), effective alleles (Ne: 0.525), genetic diversity (0.375), observed heterozygosity (Ho: 0.120), expected heterozygosity (He: 0.062), fixation index (F: 0.936), Shannon's diversity index (I: 0.089) and Polymorphic Information Content (PIC: 0.314). The phylogenetic tree, principal coordinate analysis (PCoA), and structure analysis grouped all the studied rice genotypes into two major categories that were subdivided into four subgroups depicting the underlying genetic variations. The amplified allelic pattern and size of twenty-four SSR primers were used to construct the varietal-specific barcodes, a digitalized representation of DNA fingerprints, clearly and precisely showed genetic variation. The genotype-specific DNA fingerprints and the molecular marker information were used to generate the QR (quick response) barcode that can quickly scan through digital devices for rapid identification and management of varieties. These DNA fingerprints and barcodes of fifteen rice genotypes can ensure plant seed purity and can be used to protect intellectual property rights varieties.

Keywords: SSR, Rice, DNA fingerprint, Barcode, varietal identification, QR code.

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INTRODUCTION

Plant breeder's rights ensure the breeder's exclusive rights towards its new variety production, market, import, export, and offer for sale. This effectively forbids other parties from producing, marketing, or distributing the same variety without the breeder's consent. It also encouraged the breeders to develop new crop varieties while protecting the breeders' intellectual property rights that receive compensation for direct and indirect commercial exploitation of their varieties. These rights are granted to any approved variety based on their novelty, distinctness, uniformity, and stability (Abbas, 2020; Akshay *et al.*, 2022). Conventionally,

morphological descriptors are routinely used for maintaining the purity of varieties and for showing the identity of varieties including Distinctness, Uniformity, and Stability (DUS) but these morphological descriptors have many flaws including pleiotropic effects, epistatic interactions, and the environment's impact on trait expression (Vanisri Satturu *et al.*, 2018).

To overcome these the DNA profile is a prerequisite to grant breeders the right for a variety along with DUS as they allow breeders to accomplish indirect selection, in contrast to morphophysiological markers, as they can be utilized at any growth stage of crop-like seedling, active growth phase or seeds and they are not influenced by environment as well they have simple

inheritance pattern (Yu and Chung, 2021). Molecular marker technology offers immense help in the analysis of genetic variation among species. These are like a signpost on the genome positioned within or near the genetic locus which might be regulating a certain character of interest. They are co-inherited across generations having the same genetic locus in the segregating populace (Iqbal *et al.*, 2021).

Rice (*Oryza sativa* L.) is an extremely valuable food crop in the world that is consumed by more than half of the human population daily and is Pakistan's 2nd major staple grain. Rice has a diploid genome ($2n=24$) with a small genome size of 430 Mb, making it usable for the various genomic mapping resources (Kurata *et al.*, 1994). The loss of genetic diversity because of extended domestication and cultivation requires genetic insights studies for the development, improvement, and adaptation of climate-resilient fine rice varieties with enhanced yield and quality are prerequisites of this era to achieve food and nutritional security issues (Zheng *et al.*, 2023; Zainuddin *et al.*, 2024). Molecular markers facilitate fast and precise varietal identification, which is required for efficient crop management and characterization. Previously, RAPD, ISSR, and AFLP markers were used for the fingerprinting and characterization of agricultural varieties and germplasm but these days SSR and SNPs molecular markers widely used and recommended for DNA fingerprinting in rice by the International Union for the Protection of New Plant Varieties (UPOV) due to their good reproducibility, clear distribution in genome, and easy data standardization (Li *et al.*, 2023). The genetic diversity or relatedness information is more precisely depicted by SSR marker than SNP because SSR are more neutral, co-dominant and multi-allelic in nature (Singh *et al.*, 2013). SSR markers are thus a superior option for molecular screening of rice as they are co-dominant, bands produced are naturally orthologous, present in all gene pools and well distributed, highly reproducible which is crucial for genetic analysis and cost-effective (Sawant *et al.*, 2023). These markers are widely used for the assessment of seed purity, traceability, and adulteration in rice due to their widespread distribution on the whole rice genome (Vemireddy *et al.*, 2014). In rice, different DNA markers were used for DNA fingerprinting as ISSR (Tiwari *et al.*, 2013), RAPD (Arif *et al.*, 2005), SSR (Nader *et al.*, 2019; Li *et al.*, 2019) and SNPs (Choudhury *et al.*, 2023). The DNA barcode, based on DNA fingerprints, is a digital representation of genetic variation between the genotypes at the DNA level. Barcodes, QR codes, and PINs are widely used for easy access and secure things. Digital devices such as smartphones can scan the code through their camera to easily access the information anywhere. The objective of present study is to identify and authenticate rice variety using SSR-based DNA fingerprints and barcodes that will

uniquely differentiate variety on their marker profile and enhance the protection of breeder's intellectual property rights through easy varietal traceability and seed purity testing. This study uniquely established the genetic identity of each rice variety based on SSR marker profile by creating the molecular barcodes in Pakistan for the first time.

MATERIALS AND METHODS

Plant Material: Seeds of nine elite high-yielding rice lines along with six indigenous rice varieties including two basmati varieties, two non-basmati varieties, and two coarse rice varieties were collected from the National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agriculture Research Center (NARC), Islamabad and RRI-KSK (Rice Research Institute-Kala Shah Kaku, Punjab, Pakistan) (Table 1). Seeds were grown in pots filled with compost in the greenhouse. Fresh leaves were harvested and stored at -80°C for DNA extraction.

Table 1. List of fifteen rice genotypes used for developing DNA fingerprints.

Sr. #	Rice varieties/Lines	Code	Morphological description
1	NIGAB-48	G1	Green Super Rice
2	NIGAB-158	G2	Green Super Rice
3	NIGAB-176	G3	Green Super Rice
4	NIGAB-210	G4	Green Super Rice
5	NIGAB-252	G5	Green Super Rice
6	NIGAB-277	G6	Green Super Rice
7	NIGAB-305	G7	Green Super Rice
8	NIGAB-357	G8	Green Super Rice
9	NIGAB-366	G9	Green Super Rice
10	Super Basmati	B1	Basmati variety
11	Basmati-2000	B2	Basmati variety
12	PK-1121	NB1	Non-basmati variety
13	PK-386	NB2	Non-basmati variety
14	KSK-282	Cs1	Coarse Variety
15	KSK-434	Cs2	Coarse Variety

Genomic DNA extraction and DNA marker

Frozen plant leaves were fine ground in liquid nitrogen and the 100mg leaf powder was used for genomic DNA extraction using the GeneJET Plant Genomic DNA Purification Kit (Thermoscientific, USA, Cat. No. K0791). The eluted genomic DNA was quantified using Nanodrop and Qubit (serial no. A11645200561, Biospec-nano (230 V), Japan), and working dilution of concentration 30-35ng/μl was prepared with autoclaved Milli-Q water. In the present study, 120 SSR (Simple sequence repeats) primers, 10 from each rice chromosome were selected and

synthesized, intended to evaluate the genetic diversity and varietal identification between the fifteen rice lines/varieties. The SSR primers selected in the current study, covers the whole rice genome and these were retrieved notable published literature including the UK Food Standard Agency (FSA) (FSA, 2004); Eurofins (Nader *et al.*, 2019). The selected primers are very explicit and used for DNA fingerprinting of rice around the globe. The information including chromosome number, annealing temperature, primer sequence, and repeat motif of all SSR primers was determined from rice genome annotation databases (<http://www.gramene.org>) and given in Supplementary Table S1. The 10 μ M working dilutions of primers were prepared for PCR amplification.

Polymerase chain reaction (PCR): PCR amplification using SSR primers was carried out following the method of (McCouch *et al.*, 2002) with minor modifications using 3 mM MgCl₂, 1X of PCR (NH₄)₂SO₄ Buffer and 0.3 μ l of 5U/ μ l Taq DNA polymerase (ThermoFisher Scientific, Vilnius, Lithuania Cat. No. Ep0402), 2 μ l of 2 mM dNTPs (ThermoFisher Scientific, Vilnius, Lithuania Cat. No. R0181), 1 μ l of 10 μ M Primers in total reaction volume of 20 μ l, having 30 ng (2 μ l) template DNA, and 1 μ l of each of forward and reverse primers in 96 well plates (0.2 mL) (Thermo Scientific, USA, Cat. No. AB-0600). PCR profiling condition was optimized as 94°C for 5 min as initial denaturation step followed by 35 cycles of denaturation (95°C for 45 sec), annealing (55°C for 60 sec), extension (72°C for 90 sec) and final extension of 72°C for 10 min was carried out in PCR thermocycler (Applied Biosystems, Veriti 96-well thermal cycler, Model No. 9902, Singapore). The PCR products were resolved on 3% agarose gel (Thermoscientific TopVision Agarose, Cat. No. R0492) dissolved in 1X TAE buffer (Solarbio Life Science, Beijing, Cat No. T1060) and stained with 2.5 μ l/100ml ethidium bromide (1 mg/mL). The amplified band was visualized in a gel documentation system (Clever Scientific, UK) under Ultraviolet light.

Amplification band Scoring and Data analysis: The amplified product was scored as band presence (1) and absence (0) while comparing with the 50bp DNA ladder (ThermoFisher Scientific, Cat. No. SM0323). The genetic parameters of primer such as major allele frequency (MAF), genetic diversity, heterozygosity, and Polymorphic information content (PIC) were analyzed using PowerMarker V3.25 (Liu and Muse, 2005). The average No. of different alleles (Na), No. of effective alleles (Ne), Fixation index (F), Observed Heterozygosity (Ho), Expected heterozygosity (He), and Shannon's informative index (I) were calculated using GenAlEx 6.503 (Smouse and Peakall, 2012). The molecular variance (AMOVA) among and within the population and principal coordinate analysis (PCoA) was performed

using GenAlEx 6.503 software. To study the significance of the variance component linked to identifying the possible genetic structure of the population the nonparametric permutation procedure was applied with 9999 permutations and the F_{ST} pairwise correlation estimate between the populations was calculated using GenAlEx 6.503 (Smouse and Peakall, 2012). The genetic relationship based on Nei's genetic distance value was calculated by the Unweighted pair group of arithmetic means (UPGMA) and Neighbor Joining (NJ) clustering method using PowerMarker V3.25 (Liu and Muse, 2005). Cophenetic correlation coefficient (CCC) in value is used to identify the best clustering method, that depicts the NJ (0.9745) is better clustering to group the selected rice genotypes than UPGMA (0.9173), as higher CCC value near to 1 indicates better fit to original distance. NJ-based dendrogram to show the similarity and distance among the studied rice leaf samples was viewed using FigTree v1.4.4 software. Major genetic structure present underlining the selected genotypes was accessed using the Bayesian model-based clustering approach in structure v3.3.4 (Pritchard *et al.*, 2000). The actual subpopulation (K) was estimated to run the admixture and burn-in iteration 10,000 to simulate with burning period length up to 200,000 Monte Carlo Markov Chain (MCMC) replicates. The K value was run from 1 to 10 with ten independent runs for each run and the optimum K value for subpopulation was identified using ad-hoc quantity ΔK proposed by (Evanno *et al.*, 2005) estimated and visualized by StructureSelector (Li and Liu, 2018).

Barcode Construction based on Molecular Marker: Two SSR markers from each rice chromosome were selected for the construction of a barcode based on the method of (Li *et al.*, 2019). The amplification pattern of Super Basmati cultivated rice variety in Pakistan was used as the control to construct the digits barcodes for each rice line/variety used in this study whereas, the association of allelic variation and the allele size data arranged in chronological order was used to construct the bar-based barcode (Vanisri Satturu *et al.*, 2018). The QR codes for all fifteen rice varieties were generated through an online tool (<https://www.qr-code-generator.com/>). The rice variety name and its DNA fingerprint digits-based barcode along with the primer name were used in the description.

RESULTS

SSR markers efficiency analysis: A total of 120 SSR markers, ten from each 12 rice homologous pair of chromosomes were employed for fingerprinting of studied material (Supplementary Table S1). Out of 120 SSR markers, 53 primers were monomorphic (44.16%) while 67 (55.83%) were polymorphic among the studied material. The information on polymorphic SSR primer

sequence, chromosomal position, repeat motif, genetic diversity, and PIC value is given in Supplementary Table S2. The 67 SSR primers amplifies a total of 158 alleles ranging from 2 to 5 per primer with an average of 1.85. The PIC value ranged from 0.062 (RM 125) to 0.63 (RM 11300) with an average value of 0.314, while the average genetic diversity found while studying fifteen rice lines/varieties is 0.23 that ranged from 0.06 to 0.70 depicting the narrow genetic diversity among the studied material.

Genetic relationship through clustering: The genetic relationship was performed, and four groups were identified based on molecular data of fifteen rice lines/varieties (Figure 1). The group-I consists of one elite rice line NIGAB-366 (G9), which has maximum variation with indigenous basmati varieties B1 and B2. The group-II consists of 8 rice lines/varieties including all the elite rice lines submitted for varietal registration in Pakistan. Group-III consists of two indigenous cultivated rice varieties, the coarse rice, the group-IV consists of four fine rice indigenous varieties including the basmati (aromatic) and non-basmati (non-aromatic). The maximum similarity and differences based on Nei's frequency were given in Supplementary Table S3. The maximum similarity was seen among elite lines G2 and G3, G5 and G7 while from indigenous rice lines, the basmati varieties B1 and B2 showed maximum similarity with each other (Figure 1).

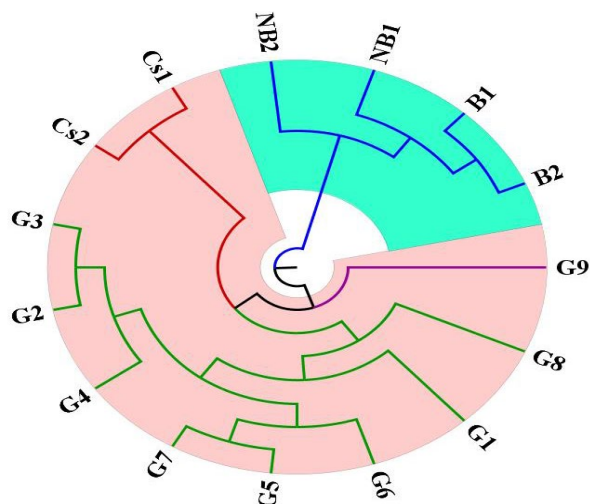


Figure 1. Cluster analysis of fifteen rice genotypes using NJ-based method.

The four groups are represented with assorted colors Purple (Group-I); Green (Group-II); Red (Group-III); Blue (Group-IV). The two colors represent two clusters, as the first big cluster (Peach) consists of GSR

lines and coarse varieties whereas the second cluster (Green) consists of the Basmati and Non-basmati fine varieties.

Genotype diversity analysis among the rice genotypes:

There was a significant allelic difference among the four population groups (G, B, NB, and Cs) (Table 1). The values of Na, Ne, I, Ho, He, and uHe were highest in population group G which includes all NIGAB rice elite lines followed by population group Cs which consists of all the coarse rice varieties. The fixation index was less than 0 in all populations revealing that more heterozygote is present than the homozygous (Figure 2; Supplementary Table S4). PCoA analysis characterized all the studied rice genotypes into the two-dimensional plane where the first two PCA components account for 25.13% and 12.71% of genetic variations. The accessions of NIGAB were mostly present in the left quadrants while the cultivated Pakistan varieties were scattered into the right quadrant (Figure 3A). Molecular variance analysis revealed 66% molecular variance among the populations and molecular variance within the population was 34% (Figure 3B) showing the highly significant variations among the populations, the Wright's F statistic F_{ST} pairwise estimate among the populations also divulges significant differentiation among them (Table 2). The P-value=0.002 suggests the observed variance is highly significance. The Bayesian clustering approach was used to analyze the population structure of 15 rice genotypes. The evanno's ΔK method clustered the genotypes into two major subpopulations as K=2 (Figure 3C). The populations with >80% probability is assigned to respective groups where the other was categorized as admixture. Subpopulation one consists of 8 individuals whereas, subpopulation two has 4 and 3 individuals fall in admixture. The subpopulations are shown with assorted colors and the length colors in the respective bar are the estimated sample's contribution to the subpopulation (Figure 3D).

Genotype Specific DNA fingerprint and Barcodes:

The 67 polymorphic primers were repeatedly repeated and evaluated to select the 24 primers covering all the twenty-four chromosomes of rice (two from each chromosome) to develop genotype-specific DNA fingerprints and to design the novel barcodes for varietal identification and authentication (Table 3). The alleles amplified by polymorphic primers were assigned different codes as A, B, and C, based on allele size in ascending order (Figure 4). The difference in the pattern of allele code distinguishes the genotypes from each other. The schematic process of fingerprinting and barcoding is discussed in Figure 5.

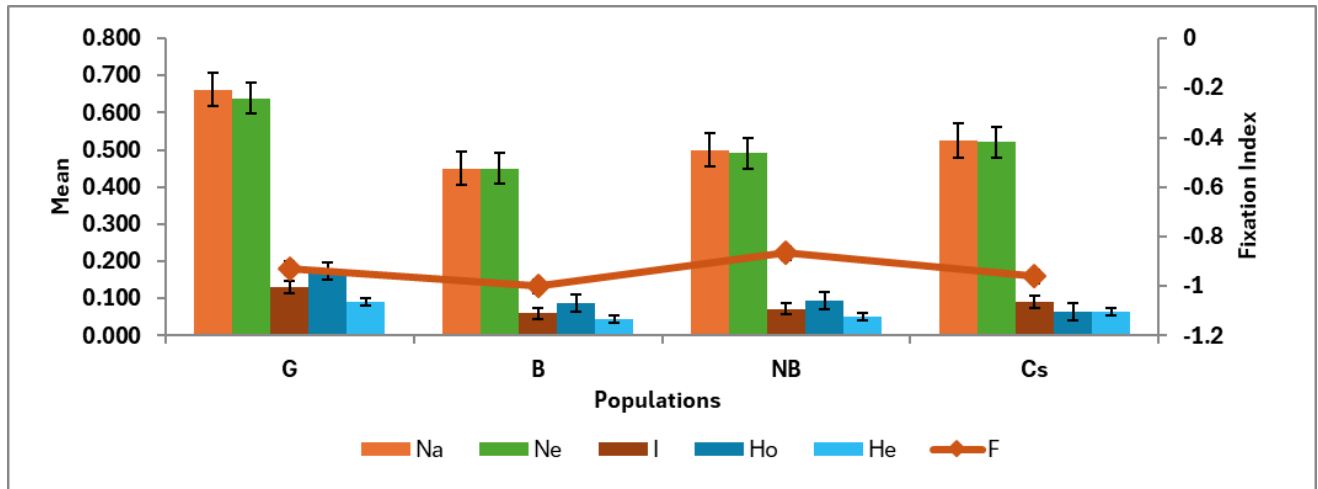


Figure 2. Allelic pattern across the fifteen rice genotypes assigned to four population groups (G, B, NB, Cs). The average No. of different alleles (Na), No. of effective alleles (Ne), Shannon’s informative index (I), Observed Heterozygosity (Ho), Expected heterozygosity (He), and Fixation index (F) of Green Super Rice lines (G), Basmati variety (B), Non-Basmati Variety (NB) and Coarse Variety (Cs). Bar represents the standard Error.

Table 2. AMOVA based on SSR marker between the rice genotypes and pairwise F_{ST} estimate.

Source	Degree of freedom	Sum of Square	Mean sum of Square	Estimated Variance	Percentage %
Among Pops	3	101.878	33.959	7.020	34%
Within Pops	11	147.056	13.369	13.369	66%
Total	14	248.933		20.388	100%

$P(\text{rand} \geq \text{data}) = 0.002$; Significance $P < 0.05$

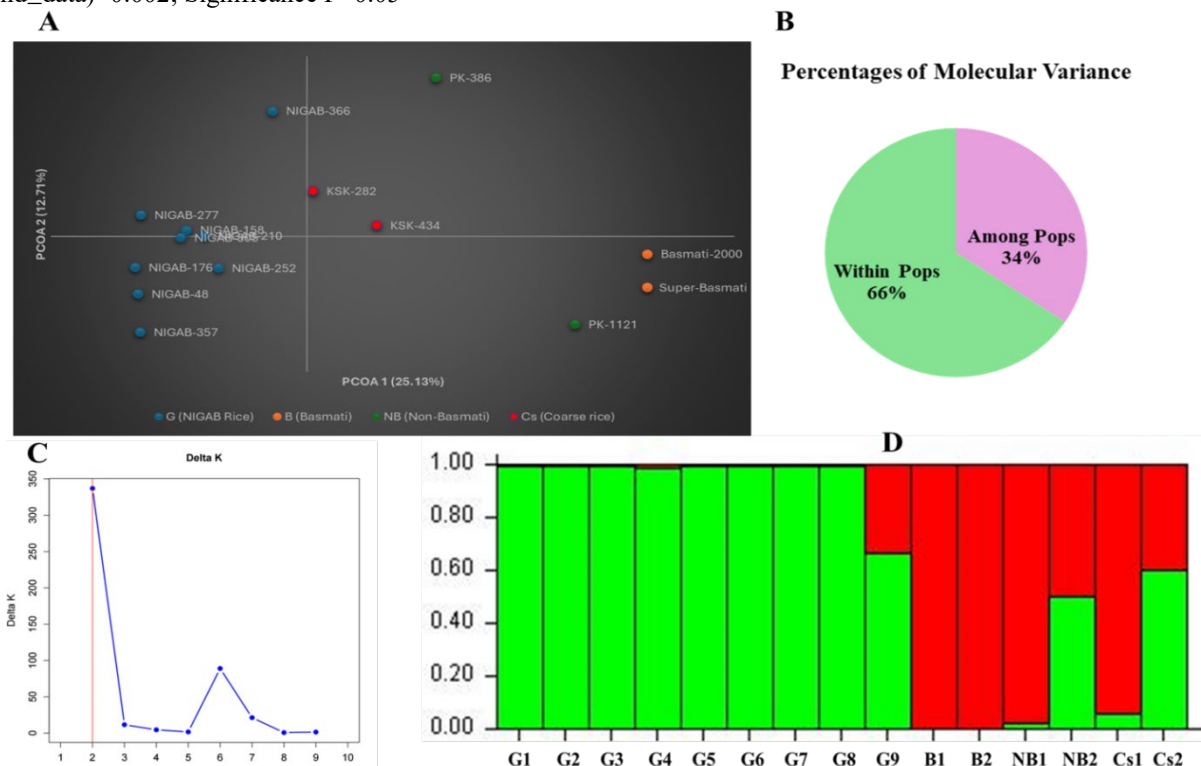


Figure 3. Structure variation among the fifteen rice genotypes. (A) Principal Coordinate Analysis (PCoA); (B) Analysis of molecular variance (AMOVA); (C) Distribution of Delta K value that based on rate of change of $L(K)$ value; (D) Histogram of structure population estimated with $K=2$

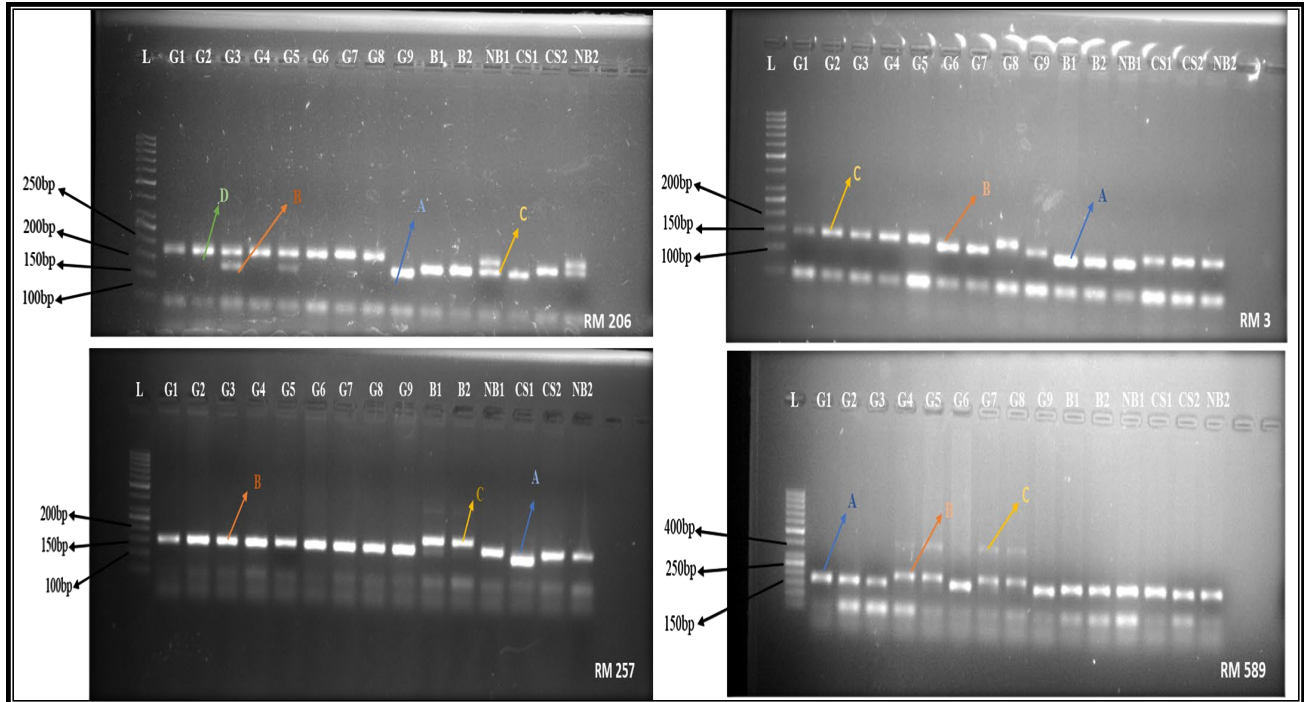


Figure. 4 Amplification pattern of fifteen rice genotypes obtained using primer RM206; RM3; RM257 and RM589.

L: 50bp DNA Ladder, G1=NIGAB-48; G2=NIGAB-158; G3=NIGAB-176; G4=NIGAB-210; G5=NIGAB-252; G6=NIGAB-277; G7=NIGAB-305; G8=NIGAB-357; G9=NIGAB=366; B1=Super Basmati; B2=Basmati-2000; NB1=PK-1121; CS1= KSK-282; CS2=KSK-434; NB2= PK-386.

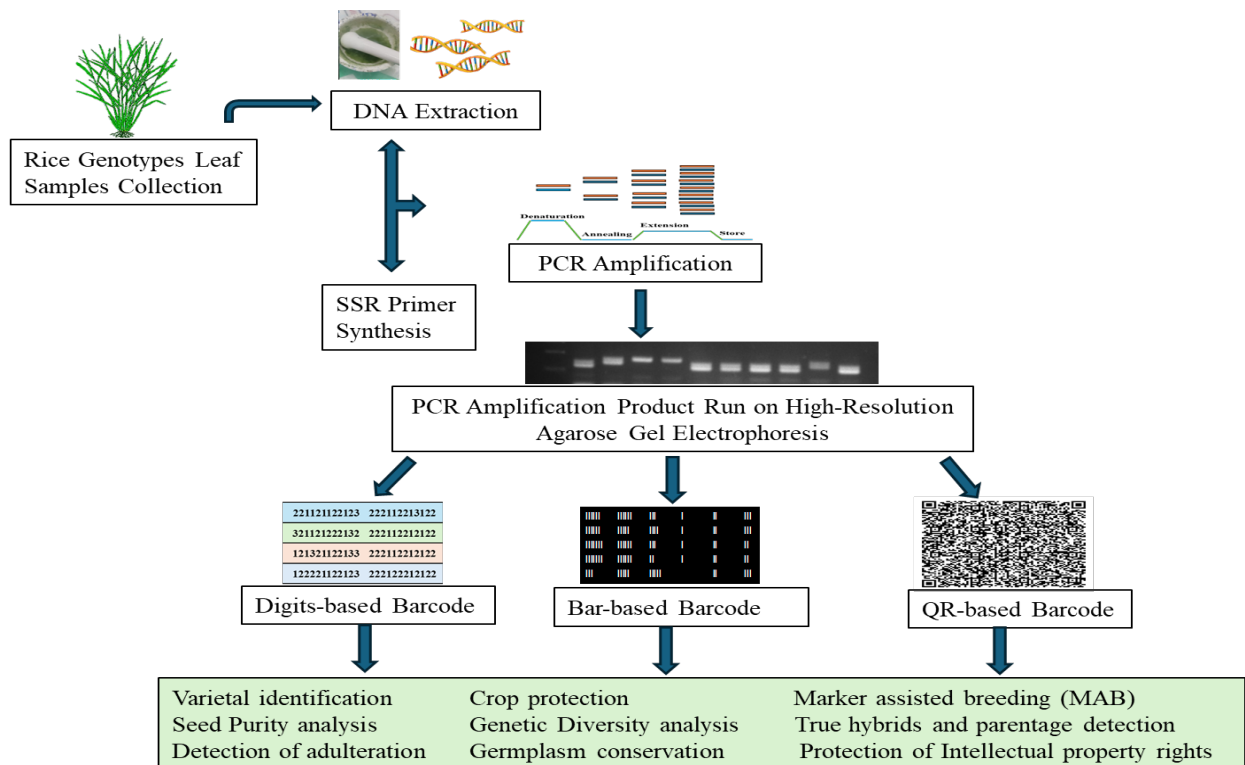


Figure 5. A schematic representation of DNA fingerprinting, Barcode and QR code development process in rice genotypes for varietal identification and authentication

Table 3. Information of 24 SSR primers used for DNA fingerprinting and barcode development of fifteen rice genotypes.

Sr. No.	SSR	Chr. No.	Fwd. Sequence	Rev. Sequence	Amplicon Size	Repeat Motif	MAF	Allele No.	Gene Diversity	PIC
M1	RM11300	1	GGTGAGGGAGGTACCGAAGTACTAGG	AAGTACCGAAGTACTAGG	492	(TA)48	0.4	4	0.6911	0.6345
M2	RM1287	1	GTGAAGAAAGCATGGTAAATG	CTCAGCTTGCTTGTGGTTAG	162	(AG)17	0.6	2	0.48	0.3648
M3	RM110	2	TCGAAGCCATCCACCAACGAAG	TCCGTACGCCGACGAGGTCGAG	156	(GA)15	0.467	4	0.5889	0.5026
M4	RM154	2	ACCTCTCCGCCTCGCCTCCTC	CTCCTCCTCCTGCGACCGCTCC	183	(GA)21	0.633	2	0.4644	0.3566
M5	RM16	3	CGCTAGGGCAGCATCTAAA	AACACAGCAGGTACGCGC	181	TCG)5(GA)16	0.8	2	0.32	0.2688
M6	RM55	3	CCGTCGCCGTAGTAGAGAAG	TCCCGGTTATTTTAAAGGCG	226	(GA)17	0.933	2	0.1244	0.1167
M7	RM10772	4	GCACACCATGCAAATCAATGC	CAGAAACCTCATCTCCACCTTCC	138	(GA)16	0.633	5	0.5578	0.5238
M8	RM241	4	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG	138	(CT)31	0.867	2	0.231	0.2044
M9	RM430	5	AAACAACGACGTCCCTGATC	GTGCCTCCGTGGTTATGAAC	173	(GA)25	0.867	2	0.231	0.2044
M10	RM153	5	GCCTCGAGCATCATCATCAG	ATCAACCTGCACTTGCCTGG	201	(GAA)9	0.867	2	0.231	0.2044
M11	RM3	6	ACACTGTAGCGGCCACTG	CCTCCACTGCTCCACATCTT	145	(GA)2GG(GA)25	0.4	3	0.64	0.5632
M12	RM589	6	ATCATGGTCGGTGGCTTAAC	CAGGTTCCAACCAGACACTG	186	(GT)24	0.667	2	0.444	0.3457
M13	RM47	7	ACTCCACTCCACTCCCCAC	GTCAGCAGGTCGGACGTC	229	(AG)7-(AG)11	0.933	2	0.124	0.1167
M14	RM11	7	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG	140	(GA)17	0.8	2	0.32	0.2688
M15	RM201	8	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	158	(CT)17	0.733	2	0.391	0.3146
M16	RM210	8	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG	140	(CT)23	0.733	2	0.391	0.3146
M17	RM219	9	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG	202	(CT)17	0.8667	2	0.231	0.2044
M18	RM257	9	CAGTTCCGAGCAAGAGTACTC	GGATCGGACGTGGCATATG	147	(CT)24	0.8	3	0.3378	0.3092
M19	RM222	10	CTTAAATGGGCCACATGCG	CAAAGCTTCCGGCCAAAAG	213	(CT)18	0.733	2	0.391	0.3146
M20	RM216	10	GCATGGCCGATGGTAAAG	TGTATAAAACCACACGGCCA	146	(CT)18	0.667	3	0.4978	0.445
M21	RM206	11	CCCATGCGTTAACTATTCT	CGTTCCATCGATCCGTATGG	147	(CT)21	0.6	3	0.524	0.4403
M22	RM167	11	GATCCAGCGTGAGGAACACGT	AGTCCGACCACAAGGTGCGTTGTC	128	(GA)16	0.933	2	0.124	0.1167
M23	RM19	12	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	226	(ATC)10	0.733	2	0.391	0.3146
M24	RM247	12	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG	133	(CT)16	0.8	2	0.32	0.2688

PIC: Polymorphic Information Content

MAF: Major allele frequency

Chr. No: Chromosome Number

RM: Rice Microsatellite

Bar-based barcodes: The allelic variation of 24 selected SSR primers was used for barcode development by first scoring the alleles based on allelic size and then sorting the allelic size data in chronological order from lowest to highest (Figure 4). The barcode was developed first by scoring the identity code (of each studied material and

then finding the code key (Table 4A, 4B). The bars based on allele size were then drawn in linear scale for all the studied material (Figure 6). These bar-based DNA barcodes are unique in terms of each variety depicting the genotype differences and can be used for identification and authentication for varietal protection.

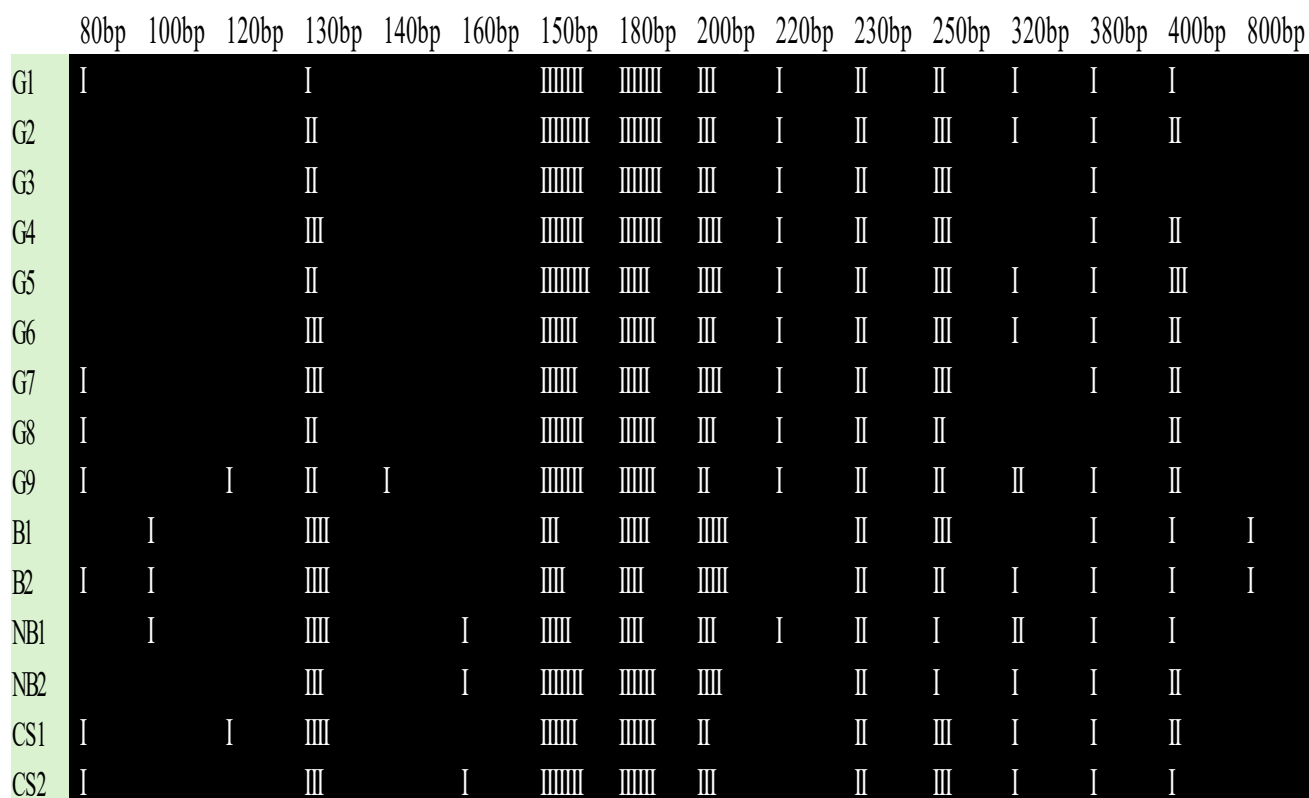


Figure 6. Bar-based barcodes of fifteen rice genotypes generated with size-based allelic variation with 24 polymorphic SSR primers

G1=NIGAB-48; G2=NIGAB-158; G3=NIGAB-176; G4=NIGAB-210; G5=NIGAB-252; G6=NIGAB-277; G7=NIGAB-305; G8=NIGAB-357; G9=NIGAB=366; B1=Super Basmati; B2=Basmati-2000; NB1=PK-1121; CS1= KSK-282; CS2=KSK-434; NB2=PK-386.

Digits-based barcodes: Twenty-four SSR primers based on their reproducibility and clear banding pattern were selected as core primers for the construction of barcodes. The fingerprint pattern of each primer for fifteen rice lines/varieties was obtained. The Super Basmati (B1) was taken as control, and the fingerprint pattern of this variety is marked as 1. The fingerprint codes of all other studied materials were recorded and marked as 1, 2, 3, 4, and so on (Figure 7). These codes were arranged sequentially as the chromosome numbers as ordered from chromosome 1

to chromosome 12 resulting in a codes combination of 24 SSR (Table 3) as two from each chromosome. The 24-digit codes formed from 24 SSR primers in rice were a unique identity pattern for each variety of rice (Table 5).

Quick Response (QR) codes: The online tool was used to generate the QR code for all studied rice varieties. The information related to variety and its fingerprint can be retrieved by scanning the QR code easily. The QR codes of all varieties are given in Figure 8.

Table 4A. Code key of fifteen rice genotypes generated with 24 polymorphic SSR primer allelic variations

Geno- types*	RM** 11300	RM 1287	RM 110	RM 154	RM 16	RM 55	RM 10772	RM 241	RM 430	RM 153	RM 3	RM 589	RM 47	RM 11	RM 201	RM 210	RM 219	RM 257	RM 222	RM 216	RM 206	RM 167	RM 19	RM 247
G1	ABC***	B	BC	AB	B	A	D	A	A	B	C	A	A	B	B	B	B	B	B	B	D	A	B	A
G2	AC	B	BC	BC	B	A	ABC	A	A	B	C	A	A	B	B	AB	B	B	B	B	D	A	B	A
G3	A	B	BC	BC	B	A	D	A	A	B	C	A	A	B	B	AB	B	B	B	E	BD	A	B	A
G4	A	B	BC	BC	B	A	AC	A	A	B	C	BC	A	B	B	AB	A	B	B	A	D	A	B	A
G5	ABC	A	BC	BC	B	A	C	A	A	B	C	BC	A	B	B	B	B	B	B	A	BD	A	B	A
G6	AC	A	BC	BC	B	A	BC	A	A	B	B	A	A	B	B	B	B	B	B	A	D	A	B	A
G7	A	A	BC	ABC	B	A	C	A	A	B	B	BC	A	B	B	B	B	B	B	A	D	A	B	A
G8	A	A	B	AB	B	A	C	A	A	B	C	BC	A	B	B	B	A	B	B	A	D	A	B	A
G9	ABC	B	ABC	AB	B	E	ABC	A	A	B	B	A	A	B	B	B	B	B	B	A	A	B	B	A
B1	A	B	BC	BC	A	A	C	B	B	B	A	A	AB	A	A	B	B	C	A	A	B	A	A	B
B2	B	A	BC	ABC	A	A	C	B	B	B	A	A	AB	A	A	B	B	C	A	A	B	A	A	B
NB1	B	A	BC	BC	A	A	BC	A	A	B	A	A	A	A	A	B	B	B	B	A	BC	A	A	B
NB2	ABC	A	BC	B	B	A	C	A	A	A	B	A	A	B	B	B	B	B	A	A	BC	A	A	A
CS1	ABC	A	AC	ABC	B	A	C	A	A	A	B	A	A	B	A	B	B	A	A	A	B	A	B	A
CS2	AB	A	BC	ABC	B	A	C	A	A	A	B	A	A	B	B	AB	B	B	A	B	C	A	B	A

*G1=NIGAB-48; G2=NIGAB-158; G3=NIGAB-176; G4=NIGAB-210; G5=NIGAB-252; G6=NIGAB-277; G7=NIGAB-305; G8=NIGAB-357; G9=NIGAB-366; B1=Super Basmati; B2=Basmati-2000; NB1=PK-1121; CS1= KSK-282; CS2=KSK-434; NB2= PK-386.

**RM: Rice Microsatellite/Simple sequence repeat.

***ABCD: Alleles based on allelic size in chronological order from lowest to highest (Detailed of allele size is provided in Table 4B)

Table 4B. Identity Code of fifteen rice genotypes generated with 24 polymorphic SSR primer amplified allelic size.

Alleles*	RM** 11300	RM 1287	RM 110	RM 154	RM 16	RM 55	RM 10772	RM 241	RM 430	RM 153	RM 3	RM 589	RM 47	RM 11	RM 201	RM 210	RM 219	RM 257	RM 222	RM 216	RM 206	RM 167	RM 19	RM 247
A	250	150	120	80	150	230	150	180	150	180	100	180	230	130	130	180	180	150	200	130	140	130	200	150
B	320	180	150	180	180		320	200	180	200	130	200	800	150	150	130	200	200	220	150	150	150	250	180
C	400		380	250			400				150	400						250			160			
D																					180			
E																								

*ABCD: Alleles based on allelic size amplified from lowest to highest and scored from agarose gel electrophoresis

**RM: Rice Microsatellite/Simple sequence repeats.

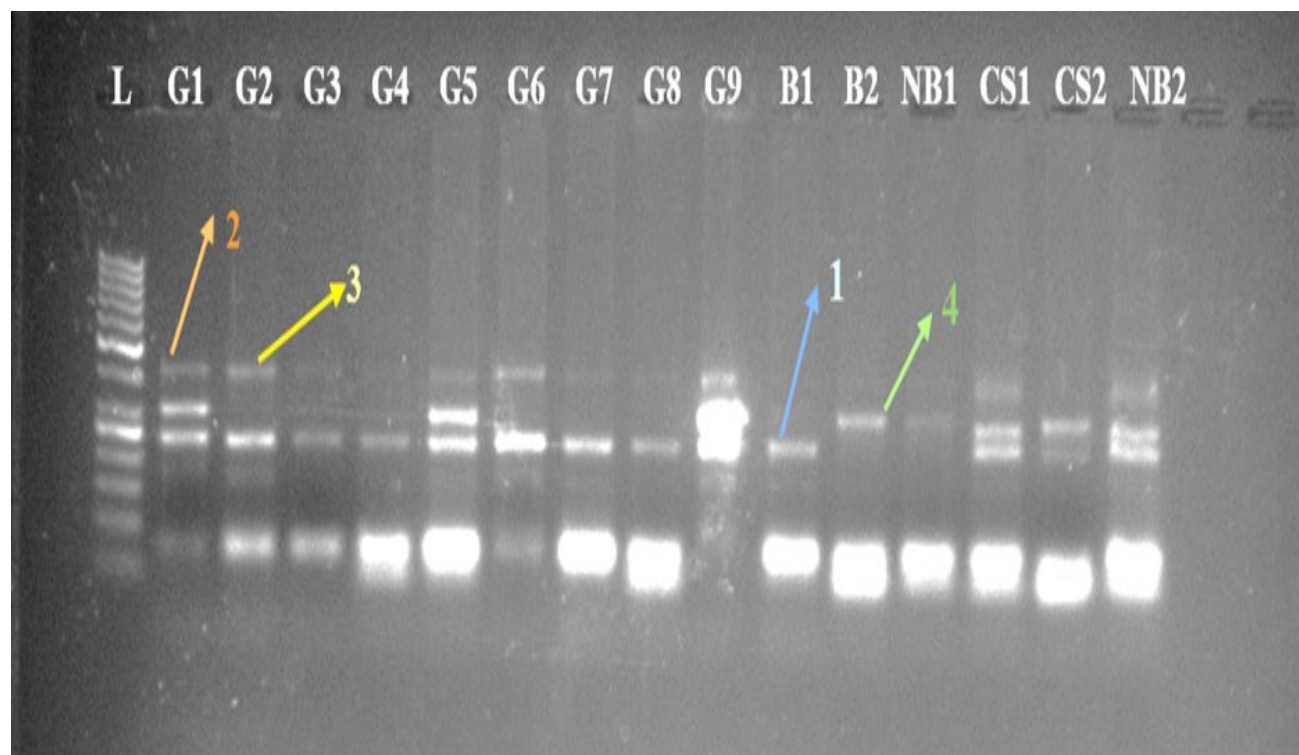


Figure 7. Amplification pattern of fifteen rice genotype with primer RM11300

L: 50bp DNA Ladder, G1=NIGAB-48; G2=NIGAB-158; G3=NIGAB-176; G4=NIGAB-210; G5=NIGAB-252; G6=NIGAB-277; G7=NIGAB-305; G8=NIGAB-357; G9=NIGAB-366; B1=Super Basmati; B2=Basmati-2000; NB1=PK-1121; CS1= KSK-282; CS2=KSK-434; NB2= PK-386

Table 5. DNA barcode of fifteen rice genotypes developed with an allelic pattern of amplification with 24 SSR primers.

Rice Lines/Varieties	Codes	Primer (M1-M12)	Primer (M13-M24)
Super Basmati	B1	111111111111	111111111111
NIGAB-48	G1	211221022122	222112222122
NIGAB-158	G2	311121222121	222212222122
NIGAB-176	G3	111121022121	222212203122
NIGAB-210	G4	111121322123	222222212122
NIGAB-252	G5	221121122123	222112213122
NIGAB-277	G6	321121222132	222112212122
NIGAB-305	G7	121321122133	222112212122
NIGAB-357	G8	122221122123	222122212122
NIGAB-366	G9	21122222232	222112211222
Basmati-2000	B2	421311111111	111111131111
PK-1121	CS1	223321122332	221113131122
PK-386	CS2	222321322331	222212221122
KSK-282	NB2	222121122332	222112214112
KSK-434	NB1	421111222111	211112234111

Barcodes were developed with 24 SSR primers (M1-M24) and organized according to sequential order of chromosomes number from 1 to 12 as RM11300 (Chr 1), RM1287 (Chr 1), RM110 (Chr 2), RM154 (Chr 2), RM16 (Chr 3), RM55 (Chr 3), RM10772 (Chr 4), RM241 (Chr 4), RM430 (Chr 5), RM153 (Chr 5), RM3 (Chr 6), RM589 (Chr 6), RM47 (Chr 7), RM11 (Chr 7), RM201 (Chr 8), RM210 (Chr 8), RM219 (Chr 9), RM257 (Chr 9), RM222 (Chr 10), RM216 (Chr 10), RM206 (Chr 11), RM167 (Chr 11), RM19 (Chr 12), RM247 (Chr 12)

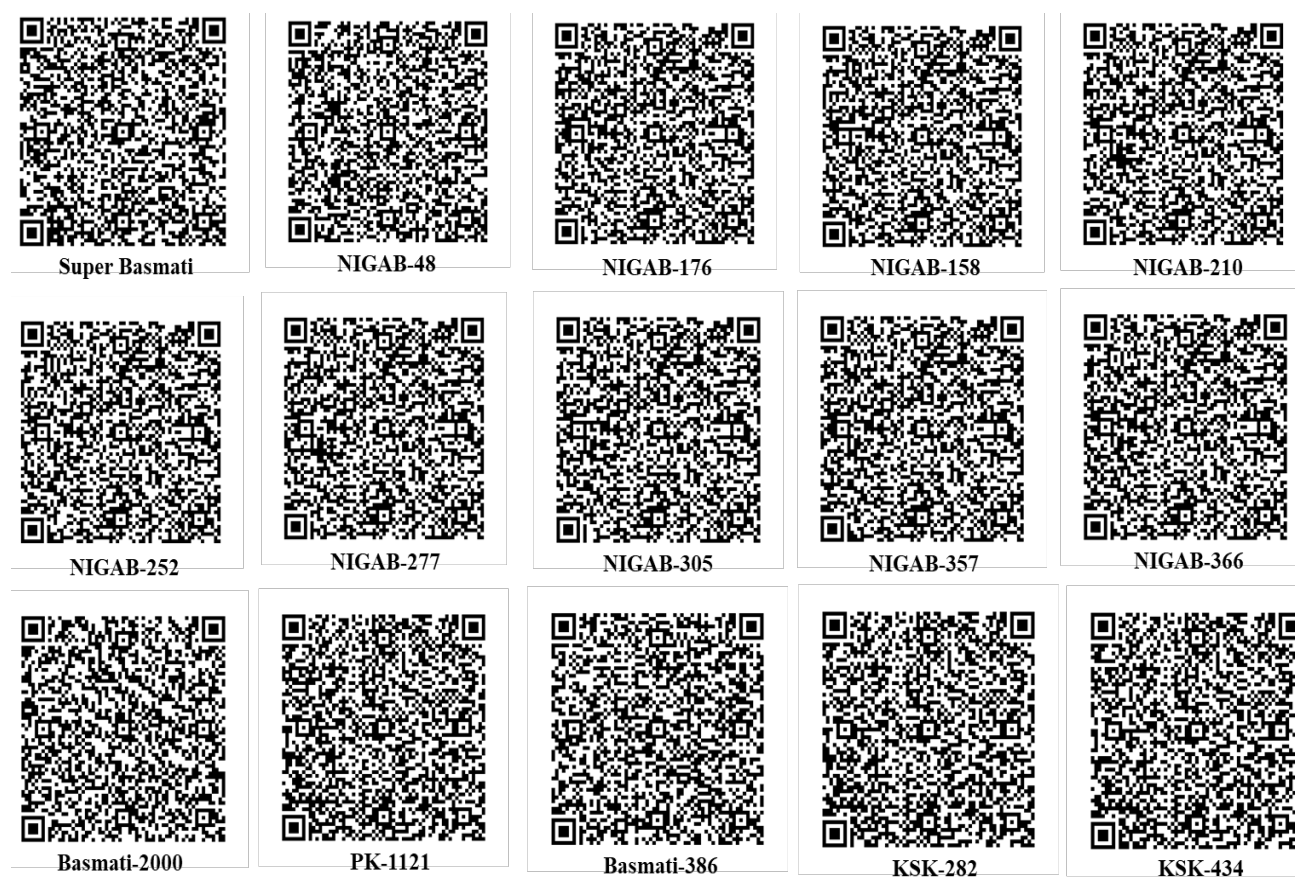


Figure 8. QR code for all fifteen rice genotypes

DISCUSSION

DNA fingerprinting based on molecular markers at the DNA level allows the identification of plant varieties with high precision. The allelic amplification data allows the comparison among the genotypes for genetic variation studies. The polyallelic SSR discriminates between the varieties more precisely without the influence of the environment. Genotyping using molecular markers such as SSR is economical and the combination of these 24 SSRs (used in the current study) is easy to apply for the fingerprint development for new varieties in comparison to indigenous varieties. The average number of alleles amplified in current study is 1.85 where previously reported average 3 alleles per locus amplified 158.

The 67 SSR primers amplified to a total 158 alleles ranging from 2 to 5 per primer with an average of 1.85. The PIC value of a marker is its discriminatory potential and is based on the amplified number of alleles and their frequency distribution. It ranged from 0 (monomorphic) to 1 (highly polymorphic). The AMOVA analysis revealed the high significance of data that the genetic difference among population is not random and it most probably occur due to actual genetic structure rather

than by chance. In two-dimensional plane of PCoA, the first two PCA components explains less variance percentage that is 25.13% and 12.71% revealing less genetic variations and not properly separate the clustering that is further confirmed through the genetic structure that's clearly distribute the populations in K=2 groups. The mean PIC value estimated in the current study using 67 polymorphic SSR primers is 0.314, ranging from 0.062 to 0.63. Previous studies reported the allele number ranged from two to three with a 2.05 average and the PIC value ranged from 0.312 to 0.368 with an average PIC value of 0.212 and using 19 polymorphic primers on 62 Indian rice *indica* genotypes (Bhargavi *et al.*, 2021). The PIC value of Indian rice using SSR markers were ranged from 0.04 to 0.5 with an average of 0.25 (Singh *et al.*, 2013). The higher PIC value, genetic diversity, and heterozygosity reveal the broader geographical distance among the studied plant genotypes (Hameed *et al.*, 2023). The AMOVA analysis revealed the high significance of data that the genetic difference among population is not random and it most probably occur due to actual genetic structure rather than by chance. In two-dimensional plane of PCoA, the first two PCA components explains less variance percentage that is 25.13% and 12.71% revealing less genetic variations and not properly separate the clustering that is further confirmed through the genetic

structure that's clearly distribute the populations in K=2 groups. In the present study the two sets of studied material as the cultivated rice varieties were separated from the newly developed varieties based on the NJ-based cluster analysis. The Indigenous varieties were grouped while the elite rice lines from NIGAB were grouped separately making them differentiated from each other at the DNA level. A similar difference was also perceived through principal coordinate analysis where the indigenous rice varieties were grouped on the right side of the two-dimensional plane while the elite rice lines were grouped on the left side. The population structure analysis also differentiated the populations into two major groups depicting the genetic distance among the studied genotypes.

The SNPs marker are single-nucleotide mutations, they are more robust and evolutionarily conserved than SSRs, which have a higher mutation rate and make it more difficult to identify true genetic differences due to allele variants within the same segment but SSR markers are co-dominant in nature, highly reproducible, highly polymorphic, higher mutation rate, evenly distributed over the genome and cost effective, making them excellent choice of molecular screening (Ellegren, 2004; Mammadov *et al.*, 2012). DNA barcodes based on DNA profiling of cultivars using SSR primers are digital representations of genetic variation among the genotypes. Barcodes are unique representations of varietal fingerprints, these were early developed for multiple crops using microsatellite markers such as *Solanum melongena* L. (Chinnappareddy *et al.*, 2012), *Psidium guajava* (Naga Chaithanya *et al.*, 2017), and *Vitis vinifera* L. (Galbacs *et al.*, 2015). In rice, 32 SSR primers were used for the barcode development of medium slender and 35 SSR for long slender rice grain varieties (Vanisri Satturu *et al.*, 2018). 62 rice varieties were amplified with 19 SSR primers for fingerprint and barcode development (Bhargavi *et al.*, 2021). Allele size barcode was developed in eighteen rice varieties using 53 SSR primers (Harisha *et al.*, 2021). In the current study, the different band positions amplified with 24 polymorphic SSR primers, the G9 and CS1 amplified a unique band at position 120 bp, whereas the Basmati varieties (B1 and B2) amplified the unique band at 800 bp position. The fine rice varieties (B1, B2, and NB1) amplified a unique band of 100bp. The G3 and G4 amplified a unique band at positions 220bp and 380bp. It is sometimes tough to read all text to retrieve a single piece of information, so the particulate information related to varietal-specific fingerprints was transformed into quickly accessible codes, the QR codes that can easily scan through your digital devices and can be printed into baggage of variety for easy identification for both the authorizer and purchaser. The breeder can also retrieve information about the alleles and banding patterns of specific genes or a variety. The DNA

fingerprint-based QR code was previously used for melon (Gao *et al.*, 2012), Clerodendrum species (Gogoi *et al.*, 2020), Tree Peony (Yang *et al.*, 2021), broccoli (Xie *et al.*, 2022), and rice (Bhargavi *et al.*, 2021).

The genotyping data, barcodes and QR code developed in the current study covering all the twelve rice chromosomes inferred that each studied material has its unique identity and that commercial varieties were not the same as the elite varieties. The present study significantly fulfills the requirement of the varietal registration process of Pakistan's Federal Seed Certification and Registration Department (FSC&RD) along with finding the purity and authenticity of rice cultivars from future exploitation. To our knowledge, this is the first attempt at DNA barcode development for commercial rice varieties and elite varieties using SSR primers in Pakistan.

Conclusions: The genetic diversity of fifteen rice elite lines and varieties was determined using 120 SSR primers distributed across all rice chromosomes, showing distinct groups present among them, that represents the distinctive source of genetic variation. In the current study, 24 polymorphic SSR primers were utilized to create a unique fingerprinting profile of rice varieties, which were represented visually in a unique barcode way based on the presence of an allele and its size. The genetic diversity and varietal identification established in this work have molecular and breeding implications and can be used for the preservation of intellectual property rights of newly developed rice varieties for sustainable rice production in Pakistan.

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Data Availability Statement: All the data are presented in the main text and supplementary file.

Ethics Approval: It is not applicable.

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