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ARTICLE

Physicochemical and Genetic Analysis of an Endemic Rice Variety, Njavara (*Oryza sativa* L.), in Comparison to Two Popular South Indian Cultivars, Jyothi (PTB 39) and IR 64

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Njavara is a medicinal rice strain, endemic to Kerala, South India, bestowed with medicinal qualities. Genetic variations and some of the physicochemical properties were studied using standard molecular protocols and compared with those of nonmedicinal rice varieties: Jyothi and IR 64. Njavara showed 11 unique positive and 36 unique negative markers to differentiate it from Jyothi and IR 64. Genetic similarity coefficient studies showed two well-defined clusters separating Njavara from Jyothi and IR 64. All the three varieties had waxy gene Wx^a allele. Njavara had $(CT)_n$ repeats at $(CT)_{10}$, while Jyothi and IR 64 had repeats at $(CT)_{11}$ in the 5′-untranslated region of waxy gene. Njavara showed a CGTG sequence, while Jyothi and IR 64 had a CGCG sequence at the 14th exon of Sbe 1 gene. Njavara, Jyothi, and IR 64 have similar amylose equivalent (AE), which was confirmed by microsatellite markers. The SSR primers for protein content and setback viscosity primer (RM 4608) were observed to be polymorphic in case of Njavara. Njavara rice, with a distinct gene pool and medicinal properties, can be exploited as a nutraceutical rice.

KEYWORDS: Njavara; medicinal rice; waxy (*Wx*) gene; starch synthase (SS); starch branching enzyme (SBE)

INTRODUCTION

Rice, *Oryza sativa*, belongs to the family Graminae. Rice is a diploid ($2n = 24$) self-pollinating, monocot plant with a relatively small genome size of about 430 Mb. Rice is the staple food to one-third of the world's population. Nearly half of the world's rice cultivation occurs in Asia. There is a wide genetic variability among the wild and cultivated varieties of rice. Molecular markers have proven to be powerful tools to assess and elucidate the genetic variability between natural population and cultivars of rice. Random amplified polymorphic DNA (RAPD) is the most extensively used marker to study genetic diversity in rice, due to its ease and low cost (*1*).

Grain quality is an important parameter for preference of a rice variety. Physiochemical characters such as apparent amylose content, protein content, gelatinization temperature, and gel consistency affect the grain quality. Many QTL analyses for grain quality of rice have been reported (*2*).

Starch is a major component in rice (90−93%). Starch comprises of two glucose polymers, amylose and amylopectin. Amylose is a linear α-1,4-glucan with small amounts of α-1,6-branch linkage, and amylopectin is a α-1,4-glucan with ∼5% of α-1,6-linkages (*3*). Physicochemical properties of rice vary with variation in the starch granule size and shape, relative proportion of amylose and amylopectin, their chain length distribution, and the occurrence and spacing of branched points in the amylopectin molecule (*4*). The physicochemical properties of various starches in relation to their thermal, pasting, and textural properties have been reported (*5*).

Biosynthesis of starch in rice is catalyzed by four classes of enzymes, ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (SDBE), and these exist in various isoforms (*6*, *7*). Hirose and Terao (*6*) have reported five types of 10 SS isoforms, namely two granule-bound starch synthase (GBSS) isoforms (GBSSI and GBSSII), one SSI isoform in the SSI type, three SSII isoforms (SSIIa [SSII-3], SSIIb [SSII-2], and SSIIc [SSII-1]) in the SSII type, two SSIII isoforms (SSIIIa [SSIII-2] and SSIIIb [SSIII-1]) in the SSIII type, and two SSIV isoforms (SSIVa [SSIV-1] and SSIVb [SSIV-2]) in the SSIV type. Granule-bound starch synthase (GBSS) encodes Waxy gene (*Wx*), necessary for the synthesis of amylose (*8*). The combined action of SS, SBE, and SDBE is required for formation of amylopectin from amylose (**Figure 1**). The *Wx* is encoded in chromosome 6 and has the two allelic forms Wx^a and Wx^b in nonwaxy rice (*9*). The difference in expression of the *Wx* gene is believed to be due to post-transcription regulation (*10*). The alleles differ by a single base at the 5′-end of the splice site of intron 1, and the substitution in the Wx^b allele decreases the splicing efficiency at that site, resulting in less transcript, protein, and amylose.

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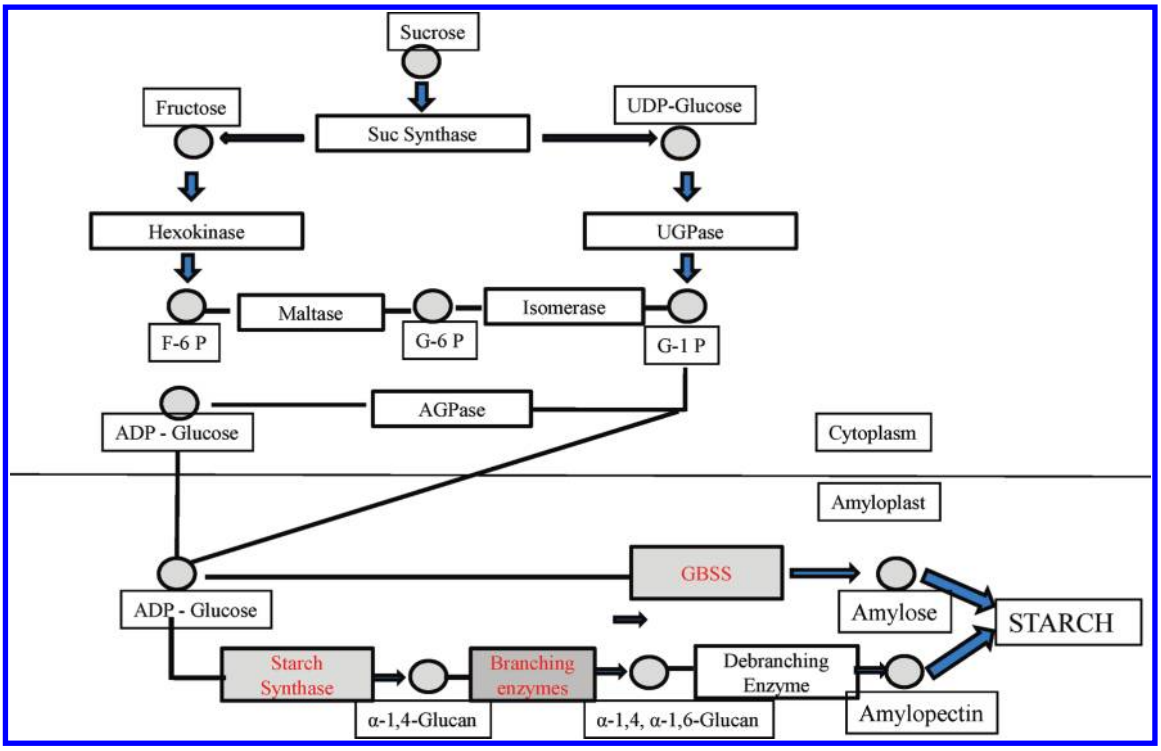

Figure 1. Starch biosynthesis pathway, giving a diagrammatic representation of the major metabolites and enzymes involved in conversion of sucrose to starch. Carbon enters the amyloplast as hexose phosphate or ADP glucose. ADP glucose is converted to amylose by GBSS (granule-bound starch synthase) and to amylopectin by starch synthase (SSI, SSII, SSIII, SSIV), starch branching enzyme (SBE1, SBE3, SBE4), and starch debranching enzyme (SDBE).

The starch biosynthesis pathway from sucrose is presented in **Figure 1**. Soluble starch synthase (SSS) is localized in the plastid stroma (*6*) and catalyzes the chain-elongation reaction of the α-1,4-glucosidic linkage by transferring a glucose moiety from ADP-glucose to the nonreducing end of the linkage. Starch branching enzyme (SBE) introduces a α-1,6-glucosidic linkage into a α-polyglucan. The starch debranching enzyme (SDE) hydrolyzes α-1,6-glucosidic linkage of α-polyglucan. In rice, numerous microsatellites have been identified and serve as an efficient tools to study allelic diversity (*11*).

The present work aims to study the genetic variability and microsatellite polymorphism in amylose content, protein content, waxy gene, SS, and SBE gene in the Indian rice varieties Njavara, Jyothi (PTB 39), and IR 64 (**Figure 2**). Njavara (*Oryza sativa* L.) is one of the medicinal red rice varieties endemic to parts of Kerala in South India. It is believed to be a progenitor of Asiatic rice with an unadulterated gene pool (*12*). Njavara is similar to ordinary rice, with husk color varying from golden yellow to brownish black, depending upon the soil conditions (*13*). Njavara is believed to be bestowed with many medicinal properties, finding immense use in treatments of arthritis, cervical spondylitis, peptic ulcer, muscle wasting, skin diseases, and certain neurological problems. Dehusked Njavara paddy is the main component of Njavara *kizhi*, a bolus prepared by cooking rice with milk and certain herbs such as *Sida rectusa* and *Alpinia galanga*, a specialized Ayurvedic therapy for treatment of paralysis, arthritis, and neurological problems. The preparation is used for massaging, leading to extensive perspiration of the body. The duration of the treatment varies from disease to disease and season to season. This makes the body supple, increases blood circulation, and removes stiffness from joints. Njavara paddy has low yield and found to be moderately resistant to pests. Regular consumption of Njavara is believed to increase body weight and is recommended for babies and lactating mothers. Recently an anticancer gene, Bowman-Brik trypsin inhibitor protein, has been identified

Figure 2. Dehusked paddy samples of (A) Njavara, (B) Jyothi (PTB 39), and (C) IR 64.

in Njavara (*14*). Njavara is at the brink of extinction, due to low yields and limited usage for Ayurvedic treatments only. Jyothi (PTB 39) and IR 64 are two nonmedicinal rice varieties. Jyothi, like Njavara, is a red rice variety and IR 64 is white rice; both rice varieties are consumed in South India. Jyothi is a hybrid variety (PTB 39) with a parentage of PTB 10 and IR 8. IR 64 is a high-yielding, hybrid, nonpigmented variety with a parentage of IR 5657 and IR 2061.

MATERIALS AND METHODS

Rice materials. Njavara paddy was brought from Padma Ayurveda, Mannar (Kerala), India, while Jyothi (PTB 39) and IR 64 paddy were procured from Agriculture Products Marketing Cooperative (AMPC) market in Bandipalya, Mysore, India. Paddy harvested in December 2003 was obtained and stored at room temperature for 1 year and 5 months and then shifted to cold (4−6 °C) until use.

Physicochemical Analysis. The paddy samples were dehusked using a rubber roller dehusker (Satake Corporation, Tokyo, Japan) and ground into flour (~60 mesh) using a rice mill (Surabhi, India). The brown rice flour was stored at 4 °C until use. The brown rice flour was used to determine apparent amylose content (ACC), protein content, swelling power, pasting (Brabender viscography, Type 801202 (Duisburg, Germany)), and thermal properties (differential scanning calorimetry

(DSC), Mettler Toledo, Columbus, OH). The data was used in this study for marker association analysis.

Estimation of Amylose Content/Equivalent. Amylose from defatted brown rice flour was estimated using the method of Sowbhagya and Bhattacharya (*15*). Total amylose equivalents were derived. Potato amylose was used as a standard.

Estimation of Protein. The micro-Kjeldahl method was employed to determine the total nitrogen and the crude protein ($N \times 5.95$) (*16*).

Estimation of swelling power (SP). The swelling powers of brown rice flour of Njavara, Jyothi, and IR 64 were determined at 50−98 °C (boiling point of water at Mysore, India, 750 m altitude), according to the modified method of Unnikrishnan and Bhattacharya (*17*). Samples of brown rice (500 mg, db) were heated in about 20 mL of distilled water at the aforementioned temperatures for 30 min with intermittent stirring. The weight of each sample was adjusted to 25 mg on cooling to room temperature and centrifuged at 3000 rpm for 45 min. The supernatant was decanted, and the residue was weighed for swelling power determination. The swelling power of the flour was determined using the formula

$$\text{swelling power (SP)} = (\text{wt of wet residue in mg})/(500 - (\text{wt of the dried sample in mg}))$$

Estimation of Viscography Parameters. A Brabender Type 801202 viscograph (Duisburg, Germany) fitted with a 700 cmg sensitivity cartridge was used for determining viscography of rice varieties as per the method of Halick and Kelly (*18*). Experiments were carried out in aqueous medium independently in triplicate.

Brown rice flour (10% dry weight basis) was stirred in water in a Braun mixer and made up to 500 mL. The slurry was poured into the Brabender biscograph bowl and heated at the rate of 1.5 °C/min at 75 rpm. The Brabender thermo regulator was set at 30 °C, at the beginning of the experiment. The slurry was heated to 95 °C, maintained at 95 °C for 20 min, and then cooled to 30 °C at the same rate, as mentioned above. The changes in viscosity (Brabender curves) occurring in the slurry were recorded on the viscograms attached to the instrument. The viscosity was measured in Brabender units (BU).

The Brabender amylograms were read to give the following parameters: peak viscosity (PV), hot paste viscosity (HPV), and cold paste viscosity (CPV). The following parameters were derived: break down (BD), the difference between peak viscosity and hot paste viscosity; setback, the difference between cold paste viscosity and peak viscosity. The values shown in **Table 2** are the average values of three original parameters (PV, HPV, and CPV) and the derived parameters (BD, SB).

Estimation of Thermal Parameters. DSC (Mettler Toledo, Columbus, OH) was employed to conduct the thermal analyses of brown rice flour. Samples with glass-distilled water (1:2) were hermetically sealed in DSC pans and equilibrated for 1 h at room temperature. The samples were heated at 10 °C/min over a temperature range of 25−110 °C, using an empty pan as reference. Equipment was calibrated using indium as the reference material.

Genomic DNA Extraction. DNA was extracted from leaf and seed samples of Njavara, Jyothi, and IR-64 according to the method of Doyle and Doyle with little modification (*19*). Leaf samples were grounded into a fine powder using liquid nitrogen. The samples were incubated in extraction buffer (0.1 M Tris−HCl pH 8.0, 0.5 M NaCl, 0.5 M EDTA, and 0.01 M β-mercaptoethanol) and 10% SDS at 65 °C for 30 min. The samples were then centrifuged at 4000 rpm at 4 °C for 10 min followed by filtration through mira cloth. DNA was precipitated with an equal volume of isopropyl alcohol, followed by centrifugation at 4000 rpm for 10 min at 4 °C. The DNA pellets were air-dried for 1 h. The DNA was resuspended in TE buffer (10 mM Tris−HCl (pH 8.0), 1 mM EDTA (pH 8.0)). The samples were then treated with RNase A (0.5 μg/mL) and proteinase K (50 μg/mL) at 37 °C for 30 min to remove RNA and protein contaminations, respectively. The reaction was terminated by adding equal volumes of isoamyl alcohol and chloroform (24/1, v/v) and centrifuged at 12 000 rpm for 5 min. The aqueous phase was pooled into a fresh tube and incubated with 95% ethanol for 15 min at room temperature. The DNA was pelleted by centrifugation at 13 000 rpm for 10 min and then rinsed with 70% ethanol, air-dried, and stored at 4 °C, until use, after dissolving in 100 μL of TE buffer.

DNA was extracted from the paddy grain (polished rice) samples by the modified CTAB method of Murray and Thompson (*20*). A brief account of the method used for the extraction of DNA is as follows: seed samples (polished rice) were ground into a fine powder using liquid nitrogen. The samples were incubated in prewarmed modified CTAB buffer (*20*), 10 mM Tris−HCl pH 8.0, 20 mM Na_2EDTA pH 8.0, 20 mM NaCl, 2% CTAB, and 0.2% (v/v) β-mercaptoethanol) at 65 °C for 1 h. DNA was extracted with an equal volume of chloroform and isoamyl alcohol (24/1), followed by centrifugation at 10 000 rpm for 10 min at 25 °C, to allow phase separation. The upper aqueous layer was collected and transferred into polypropylene tubes. DNA was precipitated using ice-cold isopropyl alcohol. DNA was pelleted down by centrifugation at 25 °C for 10 min at 11 000 rpm. The DNA pellets were washed with 70% ethanol and air-dried for 1 h. The DNA was resuspended in 200 μL of TE buffer (10 mM Tris−HCl (pH 8.0), 1 mM EDTA (pH 8.0)). The samples were then treated with RNase A (10 mg/mL) and proteinase K at 37 °C for 6 h to remove RNA and protein contamination. The reaction was terminated by adding equal volumes of phenol and chloroform (1/1, v/v). Phase separation was done by centrifugation at 8000 rpm for 5 min. The aqueous layer was retained and washed with chloroform. DNA was precipitated at −20 °C, overnight, using absolute ethanol containing 3 M sodium acetate (1/10 volume). The DNA was pelleted by centrifugation at 11 000 rpm for 10 min at 4 °C and then rinsed with 70% ethanol, air-dried, and stored at 4 °C, until use, after dissolving in 100 μL of TE buffer. The quantity and purity of DNA was confirmed by spectrophotometry and electrophoresis, using agarose (0.8%) gel.

Random Amplified Polymorphic DNA (RAPD) Analysis. A total of 23 decamer primers were used for RAPD analysis. The primers were procured from Bioserve, Bangalore, India. The PCR amplifications were performed in a Eppendorf Mastercycler Gradient (Germany) and carried out in a final volume of 20 μL in a reaction mixture containing 2 μL (8−21 ng) of template DNA, 30 pm of random primer, 2.5 mM dNTPs each, 1PCR assay buffer (containing 100 mM KCl, 20 mM Tris−HCl (pH 8), 1 mM DTT, 0.5% Igepal, and 0.5% Tween 20, 50% glycerol, 100 mM $MgCl_2$), 0.5 unit of DNA Taq polymerase (Chromous Biotech, Bangalore, India). The PCR program consisted of 1 cycle of 5 min at 94 °C, 29 cycles of 1 min at 94 °C, 1 min at 40 °C, 2 min at 72 °C, and a final extension of 6 min at 72 °C, the temperature was ultimately kept at 16 °C. The PCR products were separated on 1.8% agarose gel (Sigma, St. Louis, MO) and stained with ethidium bromide (0.001%), and the number of bands was recorded using a gel documentation system (Herolab, EASY 442K).

Microsatellite (SSR) Analysis. SSR markers linked closely to amylose content, protein content, and setback viscosity were identified according to the maps available at http://www.gramene.org, and works published by Aoki et al. (*21*) and Wada et al. (*22*) were procured from Bioserve (Bangalore, India). The PCR amplifications were performed in a Mastercycler Gradient (Eppendorf AG, Germany). The PCR program consisted of 1 cycle of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C, and a final extension of 5 min at 72 °C. The PCR products were analyzed by electrophoresis using a 4% metaphor agarose gel (QA-Agarose TM, Q-Biogene, Bangalore, India), stained with ethidium bromide, visualized using a UV transilluminator, and photographed with a gel documentation system (Herolab, EASY 442K).

Polymorphism in *Wx*, SBE1, and SS1 genes in the rice varieties were studied using known primer sequences (*23*−*25*). PCR conditions were maintained as 5 min at 94 °C, followed by 45 s at 94 °C, 60 s at 55 °C, and 60 s at 72 °C for 35 cycles and an extension of 7 min at 72 °C. The PCR program for waxy gene (GBSS, 484−485) was slightly modified for proper amplification. The PCR program consisted of 1 cycle of 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C, and a final extension of 8 min at 72 °C. The amplified products were mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA (pH 8), 0.1% bromophenol blue, and xylene cyanol), denatured at 90 °C for 3 min, and chilled immediately. The samples were analyzed by 7−8% polyacrylamide gel (based on separation of band) and photographed with a gel documentation system (Herolab, EASY 442K). The samples were run against 100 and 10 bp DNA ladders (Genei, Bangalore, India) for comparison. The amplified products of *Wx*1-*Wx*2 (*24*) and SS gene were electrophoresed on 4% metaphor agarose gel (QA-Agarose TM, Q-Biogene, Bangalore, India) for appropriate amplicon separation.

SNP and CAPS Analysis. The AGGTATA /AGTTATA polymorphism at the putative 5′-leader intron splice-junction of the *Wx* locus was detected by restriction endonuclease cleavage following *Acc*I (BRL) (Sigma, St. Louis, MO) digestion of PCR product (*26*). Sequences harboring G nucleotide showed digestion, while the undigested fragments indicated the presence of T nucleotide.

The ACTAGT/ACTACT polymorphism in the 3′-untranslated region of Sbe 3 was detected using the restriction endonuclease *Spe* I (Sigma, St. Louis, MO) (*26*). Samples with G nucleotide had the restriction site for Spe I endonuclease, giving two fragments of 215 and 295 bp. Polymorphism at the Sbe 1 site was detected using primers designed by Han et al. (*26*). The C/T nucleotide polymorphism at the Sbe 1 site was investigated by sequence analysis. The samples were electrophoresed on 1.2% agarose gels, stained with ethidium bromide, visualized with a UV transilluminator, and photographed with a gel documentation system (Herolab, EASY 442K).

Sequence Analysis. Amplified PCR fragments from all the three varieties using oligos *Wx* 5′ 484:W2R, Sbe 1 (SNP), and Sbe 3 492:493 were purified using a HiPura PCR Product Purification Kit (Himedia, Bangalore, India). The purified PCR products were cloned into pKRX-T plasmid (T/A Cloning Kit, SBS Genetech, India). Competent *E. coli* ($DH_5\alpha$ strain) was transformed using this plasmid. Transformed colonies were pooled and checked for the required DNA amplified fragment using PCR. The successful transformants were purified using a HiPura Plasmid Purification Kit (Himedia, Bangalore, India) and sequenced (MWG Biotech, Bangalore, India).

Resolving Power (Rp). The resolving power (Rp) for each primer was calculated following the method of Prevost and Wilkinson (*27*) for selecting primers that can distinguish a maximal number of accessions. The resolving power (Rp) of a primer is equal to $P \times$ Ib, where Ib (band informativeness) is defined as $\text{Ib} = 1 - [2 - |0.5 - p|]$, p being the proportion of the three rice varieties containing the bands.

Data Analysis. RAPD profiles of well-resolved and consistently reproducible fragments were scored as present (1) or absent (0) for each analysis. Bands with the same migration distance were considered homologous. A pairwise similarity matrix was computed and analyzed with NTSYS (*28*) version 2.02 using a simple matching coefficient (*29*). The similarity matrix was used to construct a dendrogram by the unweighed pair-group method with arithmetical averages (UPGMA). Analysis of variance was performed for physicochemical characters using the SPSS system for Windows version 7.5 (SPSS Inc., Chicago, IL). Duncan's multiple range tests was conducted for comparison of means at $P < 0.05$.

RESULTS

RAPD Analysis. The total amplified products of 23 RAPD primers resulted in a total number of 193 bands with an average of 8.39 bands per primer, of which 38.34% were polymorphic (**Table 1**). Thus, the average number of 8.39 bands per primer is considerably high, representing their robustness for use in fingerprinting applications. The bands were scored by eye in two independently prepared sets and were unaffected by the DNA extraction method and PCR replication. The resolving power (Rp) of the RAPD primers ranged from 0.78 (OPA 01 and OPBD 05) to 6.33 (OPAH 18) (**Figure 3**). OPC 07 and OPAE 09 (**Table 1**) showed the maximum number of polymorphic bands (66.67%). The RAPD analysis helped to identify unique positive and/or negative markers for Njavara. Njavara showed 11 unique positive and 36 unique negative markers for the 23 RAPD primers studied, to differentiate between Jyothi and IR 64. OPAH 18 was characterized by the maximum number of negative markers (7) for Njavara (**Figure 3**). These markers are of immense use in varietal protection/identification of Njavara from other rice varieties, especially when there is a possibility of adulteration of Njavara with other varieties.

The genetic similarity (GS) coefficients between Njavara−Jyothi and Njavara−IR 64 were 0.0676 and 0.1014, respectively. The RAPD-based dendrogram (**Figure 4**) obtained from genetic similarity (GS) coefficients show two well-defined clusters separating Njavara from Jyothi and IR 64 to form the first cluster, while Jyothi and IR 64 form the second cluster.

Table 1. Total Number of Bands (TNB), Number of Polymorphic (NPB) Bands, Percentage of Polymorphic Bands (% PB) and Resolving Power (Rp) Obtained per RAPD Primer

sample no.	primer	sequence (5′−3′)	TNB	NPB	% PB	Rp
1	OPA 01	5′ CAGGCCCTTC 3′	9	1	11.111	0.78
2	OPA 02	5′ TGCCGAGCTG 3′	6	1	16.667	0.89
3	OPA 08	5′ GTGACGTAGG 3′	11	4	36.364	4.11
4	OPA 11	5′ CAATCGCCGT 3′	9	2	22.222	4.33
5	OPA 16	5′ AGCCAGCGAA 3′	8	5	62.5	4.11
6	OPA 17	5′ GACCGCTTGT 3′	7	4	57.143	3.33
7	OPA-20	5′ GTTGCGATCC 3′	9	3	33.333	2.56
8	OPC 07	5′ GTCCCGACGA 3′	6	4	66.667	3.22
9	OPC 08	5′ TGGACCGGTG 3′	7	2	28.571	1.67
10	OPC 13	5′ AAGCCTCGTC 3′	7	2	28.571	1.67
11	OPC 14	5′ TGCGTGCTTG 3′	8	5	62.5	4.89
12	OPC 15	5′ GACGGATCAG 3′	8	5	62.5	4.00
13	OPAE 09	5′ TGCCACGAGG 3′	9	6	66.667	4.89
14	OPAE 14	5′ GAGAGGCTCC 3′	9	2	22.222	1.67
15	OPAF-02	5′ CAGCCGAGAA 3′	9	5	55.556	4.00
16	OPAF-14	5′ GGTGCGCACT 3′	10	2	20	1.67
17	OPAG 04	5′ GGAGCGTACT 3′	11	2	18.182	1.67
18	OPAG 15	5′ CCCACACGCA 3′	8	4	50	3.11
19	OPAH 18	5′ GGGCTAGTCA 3′	11	7	63.636	6.33
20	OPAS 19	5′ TGACAGCCCC 3′	8	1	12.5	0.89
21	OPBB 09	5′ AGGCCGGTCA 3′	6	3	50	2.44
22	OPBD 05	5′ GTGCGGAGAG 3′	11	2	18.182	0.78
23	OPBD 17	5′ GTTCGCTCCC 3′	6	2	33.333	1.56
total			193	74	38.342	

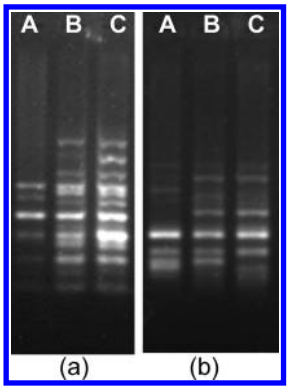

Figure 3. Randomly amplified polymorphic DNA (RAPD) analysis for the primer OPAH 18 (a) and OPAE 09 (b): (A) Njavara; (B) Jyothi (PTB 39); (C) IR 64.

Microsatellite Analysis. *Amylose Content*. Njavara, Jyothi, and IR 64 were observed to have intermediate amylose content (~23%) (**Table 2**). The microsatellite marker RM5005 (qAC7) showed a polymorphic band for Njavara compared to the two popular varieties (**Figure 5**, part 1); all the other markers were monomorphic for the trait.

Protein Content. Rice is a major source of protein in Asian countries and is rich in lysine. Njavara showed higher protein content (~10%) compared to Jyothi and IR 64 (~8%) (**Table 2**). SSR markers were selected from the QTLs identified by Wada et al. (*22*). They reported three QTLs governing protein content in rice, located at chromosomes 2 (qPC 2), 6 (qPC 6), and 9 (qPC 9) . The SSR primers RM 5470, RM 1369, and RM1896, located at qPC 2, qPC 6, and qPC 9 QTLs, respectively, were observed to be polymorphic in case of Njavara (**Figure 5**, part 2). These may account for the higher protein content in Njavara.

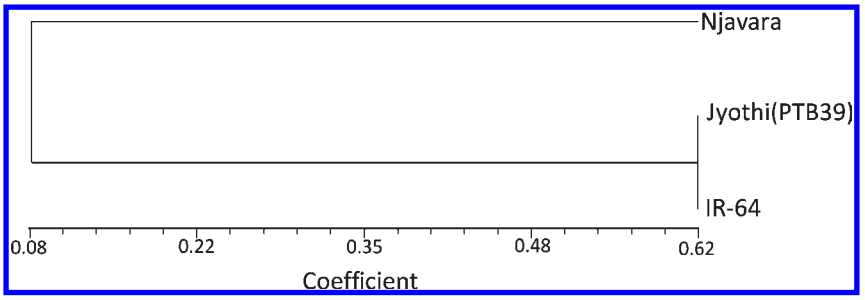

Figure 4. Dendrogram of genetic relationship among rice varieties obtained from cluster analysis of RAPD data.

Table 2. Comparison of the Mean Values of Physicochemical Properties of Brown Rice Flour[a]

				thermal properties					pasting properties				
variety	AAC (%)	protein (%)	SP (%)	T_0 (°C)	T_p (°C)	T_c (°C)	ΔH(J/g)	GT (°C)	PV (BU)	HPV (BU)	CPV (BU)	BD (BU)	SB
Njavara	22.70[a]	9.52[a]	11.76[a]	63.81[b]	69.58[b]	77.26[b]	4.28[b]	70.12[a]	840[a]	670[a]	1725[a]	170[a]	885[a]
Jyothi	22.83[a]	7.97[b]	13.03[a]	59.62[c]	65.72[c]	72.24[c]	3.99[c]	66.38[b]	500[c]	430[b]	1140[b]	70[b]	640[b]
IR 64	24.25[a]	7.97[b]	13.06[a]	69.38[a]	75.40[a]	80.66[a]	4.73[a]	71.25[a]	653[b]	443[b]	1270[b]	210[a]	616[b]

[a] Mean significantly different at $P < 0.05$. Legend: AAC, apparent amylose content; T_0, initiation temperature; T_p, peak temperature; T_c, conclusion temperature; ΔH, enthalpy; GT, gelatinization temperature; PV, peak viscosity; CPV, cold paste viscosity; BD, breakdown viscosity; SB, setback viscosity.

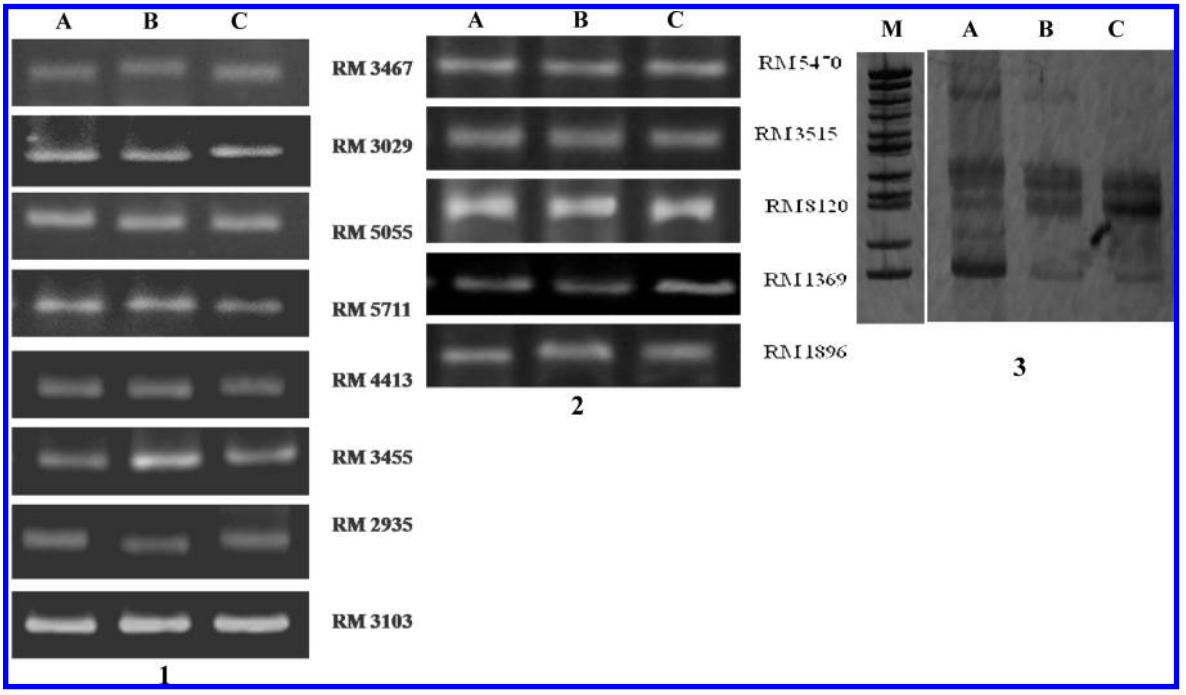

Figure 5. Microsatellite profiles of rice obtained with (1) amylose primers, (2) protein primers on 3% metaphor agarose gel, and (3) setback viscosity (RM 4608) on 7% silver-stained polyacrylamide gel: (A) Njavara; (B) Jyothi (PTB 39); (C) IR 64; (M) molecular marker (10 bp).

Setback Viscosity. Njavara was observed to have a higher setback viscosity (885 BU) compared to the other two varieties. Microsatellites (RM 4332 and RM 4608) identified by QTL studies (*21*) were used to study the polymorphism of these alleles. In our study we failed to get amplification for the microsatellite RM 4332 at the required base pair length (160 bp). However, microsatellite primer RM 4608 showed polymorphism at the required base pair (135 bp) (**Figure 5**, part 3).

Waxy Gene. In the present study, both the waxy (*Wx*) gene markers showed polymorphism (**Figure 6**, parts 1 and 2). The amplified product size ranged between 400 and 500 bp for *W1*−*W2* waxy primer (**Figure 6**, part 1) and between 120 and 125 bp for *Wx* primer 484−485 (**Figure 6**, part 2).The sequence analysis of *Wx* gene (484-W2R) gene indicated that the $(CT)_n$ repeat for Njavara was $(CT)_{10}$, while Jyothi and IR 64 had $(CT)_{11}$ (**Figure 7**, part 6). Though Jyothi and IR 64 had the same $(CT)_n$, repeat variation in gelatinization temperature and pasting properties was observed (**Table 2**).

Starch Synthase (*SS*) *and Starch Branching Enzyme* (*SBE*). The physicochemical characters of Njavara, Jyothi, and IR 64 made us consider that the IR 64 has longer chains of amylopectin, requiring higher temperatures to dissociate completely than are required for shorter chains, followed by Njavara and Jyothi (**Table 2**). Earlier reports have identified the occurrence of four sets of allele variations in SS gene, SS-A, SS-B, SS-C, and SS-D, with the SS-A class having the most recurrent occurrence among the nonwaxy rice varieties (*23*). The SS1 microsatellites showed polymorphism in case of the three varieties. Njavara and IR 64 were observed to have amplicon size less than 200 bp (monomorphic band), while Jyothi had a product size above

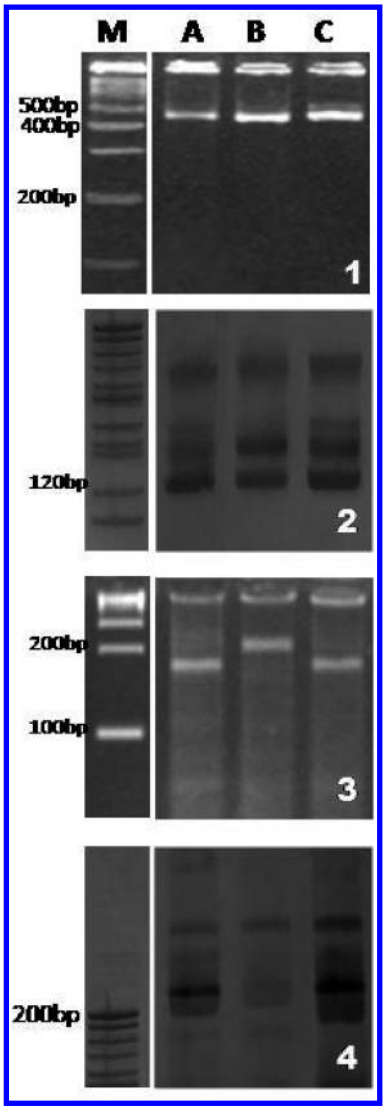

Figure 6. Microsatellites used to study polymorphism of alleles for (1) waxy gene (*Wx1*−*Wx2*), (2) waxy gene (484−485), (3) starch synthase gene, and (4) starch branching enzyme: (A) Njavara; (B) Jyothi; (C) IR 64; (M) marker.

200 bp (**Figure 6**, part 3). As per the reports of Bao et al. (*23*), IR 64 was observed to have SS-C allele (199 bp). This agrees with the present study. Thus, it can be concluded that Njavara too has the SS-C allele.

Starch Branching Enzyme (*SBE*). The SBE microsatellite $(CT)_n$, located on the sixth chromosome of intron 2 of SBE gene (*11*), showed amplification above 200 bp for all three varieties, with Jyothi and IR 64 having monomorphic bands while Njavara has an amplicon size of lower base pair length (**Figure 6**, part 4). Bao et al. (*23*) have reported four SBE alleles, SBE- A, SBE-B, and SBE-D with an insertion sequence of 11 bp (CTCTCGGGCGA) above 200 bp and SBE-C (without an insertion sequence). IR 64 has been reported to have SBE A allele (206 bp) (*23*). With IR 64 as reference it could be concluded that Jyothi has SBE-A allele and Njavara has SBE-B or SBE-D allele. The SBE-A allele is reported to be unique for *indica* rice varieties (*30*).

SNP and CAPS Analysis. All three rice varieties, Njavara, Jyothi, and IR 64, had the AGGTATAC sequence (G SNP) at the putative leader intron 5′-splice site of waxy gene (**Figure 7**, parts 1 and 2). The PCR amplicons had size > 200 bp and were cleaved by Acc I endonuclease. This was confirmed by sequence analysis of the cloned PCR product (**Figure 7**, part 6). Njavara, Jyothi, and IR 64 were observed to have amylose content ∼23%, and this agrees with the studies of Bligh et al., (*31*) that non waxy rice with AAC > 18 shows AGGTATAC polymorphism. The CAPS marker designed from the 14th exon of Sbe 1 gene (*21*) had amplicon size 400−500 bp for the three varieties (**Figure 7**, part 3). The sequence analysis of the amplicons showed that Njavara had the CGTG and Jyothi and IR 64 had the CGCG sequence (**Figure 7**, part 7). However, this C/T substitution does not change the amino acid profile (*26*).

The CAPS marker Sbe3, located at the 3′-untranslated region of the Sbe gene (*21*), showed amplicon size above 500 bp. All three varieties had a restriction site for Spe I endonuclease, indicating the presence of C nucleotide (**Figure 7**, parts 4 and 5). The sequence analysis further confirmed the sequence as ACTACT in all three varieties studied. The result agrees with the presence of C nucleotide reported in the case of IR 64 by Bao et al. (*23*).

DISCUSSION

DNA fingerprinting is an important tool for varietal protection to prove ownership or derivation of plant lines (*32*). DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationship studies. Molecular markers have been successfully used to study genetic diversity and distribution in rice (*1*). The present study showed that Njavara behaves as an "isolate". The genetic diversity detected by microsatellite and AFLP studies on Njavara concluded that it represented a distinct unadulterated gene pool or must have undergone independent divergence from the ancestral gene pool (*12*). Our study also supports and confirms the earlier report that Njavara constitutes a discrete genetic makeup.

Carbohydrate and protein are the two major components in rice. Physical and nutritional properties, cooking and eating quality, texture, and aroma play a major role in consumer preferences, which vary with geographical and cultural variations. On the basis of rice requirement either for ceremonial or medicinal purposes or food products, the choice of rice variety varies as well. The key constituents governing cooking and eating quality are: amylose content (AC), gelatinization temperature (GT), gel consistency (GC), grain appearance, cooked grain elongation, and fragrance of cooked rice (*5*, *33*). Brown rice consists of 75−85% carbohydrates. Thus, starch properties are important determinants of rice quality. Starch composition mainly differs in the proportion of amylose and amylopectin. Amylose contents (AC) for the three varieties in the present study are comparable, but still they differed in their physicochemical characters. Rice breeders are focusing on efforts to improve rice grain quality. The use of molecular markers has facilitated the understanding of quantitative trait loci (QTLs) and marker-assisted selection (MAS). Microsatellites related to physicochemical properties such as amylose, protein, and setback viscosity identified by QTL analysis have been reported (*21*, *22*). In the present study, amylose content was known to be associated with variation at the *waxy* (*Wx*) locus on chromosome 6, and several modifiers have also been reported in the same region by others (*34*). All the markers used in the present study were monomorphic between Njavara, Jyothi, and IR 64, except the QTL at qAC 7 with the nearest marker RM 5055. However, this QTL appears to be a minor factor controlling amylose synthesis.

Rice has a relatively favorable amino acid composition with a high amount of lysine and protein digestibility. This makes rice a reasonably good source of protein in diets with limited animal protein availability. However, one of the main nutritional

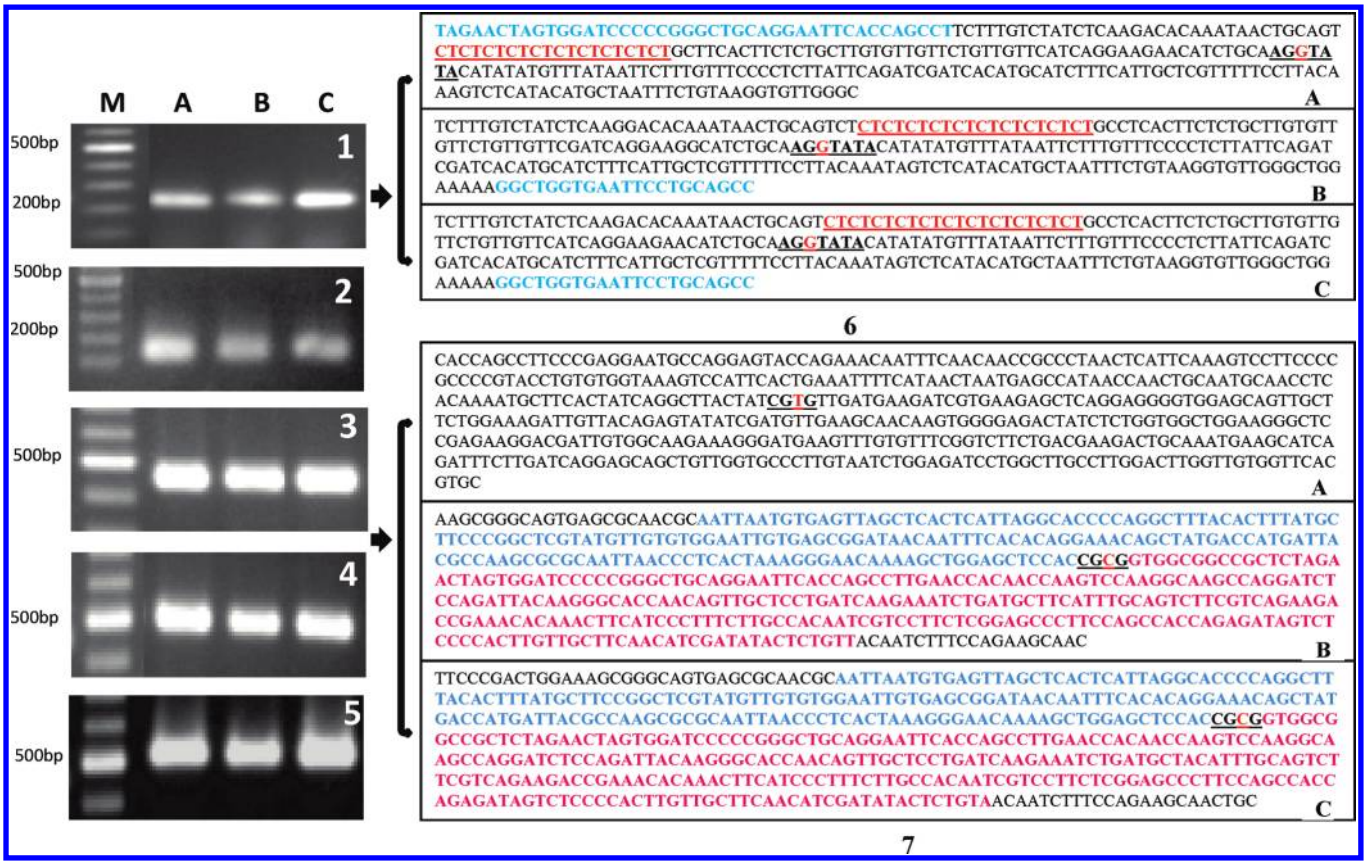

Figure 7. SNP/CAPS profile of (1) waxy (*Wx*) gene (484 and W2R) PCR product, (2) waxy (*Wx*) gene PCR product digested with Acc I, (3) SBE 1 gene (490 and 491) PCR product, (4) SBE 3 gene (492 and 493) PCR product, (5) SBE 3 PCR product digested with Spe I (alignment of amplified sequences with primer . (6) *Wx* 484-W2R, the polymorphic base $(CT)_n$ repeats and C/G SNP highlighted in red and the pKRX-T vector sequence highlighted in blue. (7) SBE 1 (SNP), the polymorphic bases C/T SNP highlighted in red. Sequences with C nucleotide are cleaved by Acc II restriction enzyme to give two fragments of size 182 bp (blue) and 274 bp (pink). Legend: (A) Njavara; (B) Jyothi; (C) IR 64; (M) marker (100 bp).

problems occurring worldwide, and especially in rice-consuming developing nations, is protein-energy malnutrition. These can be overcome by supplementation, fortification, and dietary diversification. Improvements in rice technology include a variety of approaches, namely enhancing nutritional quality through plant breeding, genetic modification, and improvements in rice fortification techniques. In the present study, all the markers RM 5470, RM 1369, and RM1869 nearest to QTL qPC2, qPC6, and qPC9, respectively, showed polymorphism. Wada et al. (*22*) showed that the Koshihikari allele (*japonica* cultivar with good eating quality) at qPC2 and qPC6 was responsible for the decrease in protein content, while the QTL at qPC9 increased protein content. Higher protein content in Njavara could be attributed to these QTLs.

The amount of long chains of amylopectin is one of the determinants of grain quality and is associated with setback viscosity (*21*). Setback viscosity indicates the precipitation of linear molecules or consistency of starch on cooling. When starch is cooked in water, the starch granules swell with an increase in temperature, leading to breakdown of the granules followed by leaching of amylose and long chains of amylopectin. The setback viscosity is believed to be correlated with the long chains of amylopectin molecule (*35*), encoded near the waxy (*Wx*) locus on the short arm of chromosome 6 (*21*). The *Wx* locus is reported to have a significant effect on pasting properties, including setback viscosity. Njavara showed relatively high setback viscosity compared to Jyothi and IR 64. QTL studies showed a polymorphic band for RM4608 for Njavara, possibly indicating that the expression of this allele for long-chain synthesis of amylopectin is different from that of Jyothi and IR 64. Polymorphisms within the *Wxa* alleles are associated with differences in amylose content or viscosity characteristics among *Wxa* types (*22*, *23*), although the association of these polymorphisms with long-chain content of amylopectin associated with viscosity has not been investigated.

Association of *Wx*, SS, and SBE microsatellites and SNPs/CAPS used in the present study are well-documented (*24*−*26*, *30*, *36*). Variations in $(CT)_n$ repeats at the 5′-untranslated region of *Wx* gene explained most of the variations in AAC content (*2*, *23*−*25*, *30*, *31*). Various studies have reported 14 $(CT)_n$ repeats, ranging from $(CT)_8$ to $(CT)_{22}$ in *Wx* gene (*25*, *26*, *31*). $(CT)_{11}$ allele is the most common in nonwaxy rice varieties. Ayres et al. (*25*) reported that $(CT)_{11}$ allele groups have an amylose content greater than 23%. In this study, Njavara showed repeats at $(CT)_{10}$ while Jyothi and IR 64 had repeats at $(CT)_{11}$. Our findings are in accordance with earlier reports (AAC 23−24%). The G/T polymorphism is also associated with amylose content in nonwaxy rice varieties. This single base pair change is responsible for altering the regulation of *Wx* gene expression at the posttranscriptional level (*36*). Sano (*9*) and Hirano et al. (*36*) reported that the AGGTATAC sequence coincides with the presence of W^a allele. It is not clearly understood whether the $(CT)_n$ repeat polymorphism at the *Wx* gene acts at the transcriptional and/or translational level or is linked to the G/T polymorphism which is responsible for variation in amylose content (*30*).

Jyothi and IR 64, having the same $(CT)_{11}$ repeats and G SNP at *Wx* loci, showed variations in pasting and thermal properties (**Table 2**). Our studies are in accordance with those of

Han et al. (*26*) and Ayres et al. (*25*). They reported that *Wx* $(CT)_n$ repeats and SNP could not account for all the variation in ACC and physicochemical properties.

In conclusion, the genetic analysis of Njavara rice showed that it has a distinct unadulterated gene pool. The molecular markers for physicochemical properties confirmed the higher protein and pasting and thermal properties of Njavara.

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LITERATURE CITED

(1) Ge, S.; Oliveira, G. C. X.; Schaal, B. A.; Gao, L. Z.; Hongm, D. Y. RAPD variation within and between natural populations of the wild rice *Oryza rufipogon* from China and Brazil. *Heredity* **1999**, *82*, 638–644.

(2) Bao, J. S.; Wu, Y. R.; Hu, B.; Wu, P.; Cui, H. R.; Shu, Q. Y. QTL for rice grain quality based on a DH population derived from parents with similar apparent amylose content. *Euphytica* **2002**, *128*, 317–324.

(3) Hizukuri, S.; Takeda, Y.; Maruta, N.; Juliano, B. O. Molecular structures of rice starch. *Carbohydr. Res.* **1989**, *189*, 227–235.

(4) Gunaratne, A.; Hoover, R. Effects of heat-moisture treatment on the structure and physiochemical properties of tuber and root starches. *Carbohydr. Polym.* **2002**, *49*, 425–437.

(5) Vandeputte, G. E.; Derycke, V.; Geeroms, J.; Delcour, J. A. Rice starches. II. Structural aspects provide insight into swelling and pasting properties. *J. Cereal Sci.* **2003**, *38*, 53–60.

(6) Hirose, T.; Terao, T. A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). *Plant* **2004**, *220*, 9–16.

(7) Nakamura, Y. Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. *Plant Cell Physiol.* **2002**, *43*, 718–725.

(8) Smith, A. M.; Denyer, K.; Martin, C. The synthesis of the starch granule. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1997**, *48*, 67–87.

(9) Sano, Y. Differential regulation of *waxy* gene expression of rice endosperm. *Theor. Appl. Genet.* **1984**, *68*, 467–473.

(10) Wang, Z. Y.; Zeng, F. Q.; Shen, G. Z.; Gao, J. P.; Snustad, D. P.; Li, M. G.; Zang, J. L.; Hong, M. M. The amylose content in rice endosperm is related to the post transcriptional regulation of waxy gene. *Plant J.* **1995**, *7*, 613–622.

(11) Akagi, H.; Yokozeki, Y.; Inagaki, A.; Fujimura, T. Microsatellite DNA markers for rice chromosomes. *Theor. Appl. Genet.* **1996**, *93*, 1071–1077.

(12) Nair, S. P.; Thomas, G. Njavara collection: A composite but distinct gene pool. *IRRN* **2001**, *30*, 1.

(13) Menon, M. V. Njavara the healing touch. *Sci. Rep.* **2004**, Feb. 28−30.

(14) http://www.newindpress.com/NewsItems. *Scientists identify anti-cancer gene in Njavara rice*. **July 2007**.

(15) Sowbhagya, C. M.; Bhattacharya, K. R. A simplified colorimetric method for determination of amylose content in rice. *Starch* **1979**, *23*, 53–56.

(16) AOAC. *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Washington, DC, 2000.

(17) Unnikrishnan, K. R.; Bhattacharya, K. R. Swelling and solubility behaviour of parboiled rice flour. *J. Food Technol.* **1981**, *16*, 403–408.

(18) Halick, J. V.; Kelly, V. J. Gelatinization and pasting characteristics of rice varieties as related to cooking behaviour. *Cereal Chem.* **1959**, *36*, 91–98.

(19) Doyle, J. J.; Doyle, J. L. Isolation of plant DNA from fresh tissue. *Focus* **1990**, *12*, 13–15.

(20) Murray, M. G.; Thompson, W. F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4325.

(21) Aoki, N.; Umemoto, T.; Yoshida, S.; Ishii, T.; Kamijima, O.; Matsukura, U.; Inouchi, N. Genetic analysis of long chain synthesis in rice amylopectin. *Euphytica* **2006**, *151*, 225–234.

(22) Wada, T.; Uchimura, Y.; Ogata, T.; Tsubone, M.; Matsue, Y. Mapping of QTLs for physicochemical properties in *japonica* rice. *Breeding Sci.* **2006**, *56*, 253–260.

(23) Ayres, N. M.; McClung, A. M.; Larkin, P. D.; Bligh, H. F. J.; Jones, C. A.; Park, W. D. Microsatellites and a single-nucleotide polymorphism differentiate apparent amylose classes in an extended pedigree of US rice germplasm. *Theor. Appl. Genet.* **1997**, *94*, 773–781.

(24) Larkin, P. D.; Park, W. D. Association of *waxy* gene single nucleotide polymorphisms with starch in rice (*Oryza sativa* L.). *Mol. Breed* **2003**, *12*, 335–339.

(25) Bao, J. S.; Corke, H.; Sun, M. Microsatellites, single nucleotide polymorphisms and a sequence tagged site in starch-synthesizing genes in relation to starch physicochemical properties in nonwaxy rice (*Oryza sativa* L.). *Theor. Appl. Genet.* **2006**, *113*, 1185–1196.

(26) Cai, X. L.; Liu, Q. Q.; Tang, S. Z.; Gu, M. H.; Wang, Z. Development of a molecular marker for screening the rice cultivars with intermediate amylose content in *Oryza sativa* subsp. *Indica*. *J. Plant Physiol. Mol. Biol.* (*China*) **2002**, *28*, 137–144.

(27) Han, Y. P.; Xu, M. L.; Liu, X. Y.; Yan, C. J.; Korban, S. S.; Chen, X. L.; Gu, M. H. Genes coding for starch branching enzymes are major contributors to starch viscosity characteristics in waxy rice (*Oryza sativa* L.). *Plant Sci.* **2004**, *166*, 357–364.

(28) Prevost, A.; Wilkinson, M. J. A new system of comparing PCR primers applied 651 to ISSR fingerprinting of potato cultivars. *Theor. Appl. Genet.* **1999**, *98*, 107–112.

(29) Rohlf, F. J. *NTSYS-pc: Numerical taxonomy and multi-variate analysis system, Version 2.02*; Exeter Software, State University of New York at Stony Brook, Stony Brook, NY, **1998**.

(30) Sokal, R. R.; Michener, C. D. A statistical method for evaluating systematic relationships. *Univ. Kansas Sci. Bull.* **1958**, *38*, 1409–1438.

(31) Bao, J. S.; Corke, H.; Sun, M. Microsatellites in starch-synthesizing genes in relation to starch physicochemical properties in waxy rice (*Oryza sativa* L.). *Theor. Appl. Genet.* **2002**, *105*, 898–905.

(32) Bligh, H. F. J.; Till, R. I.; Jones, C. A. A microsatellite sequence closely linked to the waxy gene of *Oryza sativa*. *Euphytica* **1995**, *86*, 83–85.

(33) Saker, M. M.; Youssef, S. S.; Abdallah, N. A.; Bashandy, H. S.; El Sharkawy, A. M. Genetic analysis of some Egyptian rice genotypes using RAPD, SSR and AFLP. *Afr. J. Biotech.* **2005**, *4* (9), 882–890.

(34) Vandeputte, G. E.; Vermeylen; Geeroms, J.; Delcour, J. A. Rice starches. III. Structural aspects provide insight in amylopectin retrogradation properties and gel texture. *J. Cereal Sci.* **2003**, *38*, 61–68.

(35) He, P.; Li, S. G.; Qian, Q.; Ma, Y. Q.; Li, J. Z.; Wang, W. M.; Chen, Y.; Zhu, L. H. Genetic analysis of rice grain quality. *Theor. Appl. Genet.* **1999**, *98*, 502–508.

(36) Hirano, H. Y.; Eiguchi, M.; Sano, Y. A single base change altered the regulation of the waxy gene at the posttranscriptional level during the domestication of rice. *Mol. Biol. Evolution* **1998**, *15*, 978–987.

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