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SSR marker-based DNA fingerprinting of *Sub1* introgressed lines in the background of traditional rice varieties of Assam India

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Rice varieties are usually characterized by agro-morphological descriptors used for seed certification and seed characterization by following distinctiveness, uniformity, and stability (DUS) test. But in fact, these primary distinguishing morphological descriptors among rice varieties are very limited and hence face problems to distinguish germplasm accessions. Germplasm certification in NBPGR requires a DNA fingerprinting profile to explain germplasm uniqueness compared to existing varieties. Varietal identification has gained a key role worldwide, particularly in plant variety protection. Sixty-two morphological descriptors studies have shown the *Sub1* introgressed advanced lines E-6, C-210, C-196, 1189-1 and 1160-1 are distinct from the other varieties for more than 15morphological traits, based on these variations the lines were selected for DNA fingerprinting. About68 SSRs markers were used for DNA fingerprinting in seven genotypes, two of which were parents (Ranjit, Bahadur) and three *Sub1* introgressed advanced lines (E6, C210, C196) in Ranjit background, and two *Sub1* introgressed advanced lines (1189-1, 1160-1) in Bahadur background. DNA fingerprinting was done on these genotypes of rice using SSR markers. Among the 68 SSR markers, total 65 markers were amplified and three were found not amplified. Out of 65 markersfour of them *viz*. RM 152, RM 172, RM 251, and RM 346 showed better polymorphism with amplicon size ranges from 155-163 bp, 150-159 bp, 137-147 bp, and 166-175 bp, respectively, and remaining 61 showed monomorphic amplification. Therefore, SSR (Simple-sequence repeats) based DNA fingerprinting helped to differentiate Ranjit, Bahadur, E-6, C-210, C-196, 1189-1, and 1160-1. Hence, the research reveals that newly developed high-yielding *Sub1* introgressed advanced lines in the background of traditional Assam rice varieties (Ranjit and Bahadur) are unique in their identity.

Keywords: DNA Fingerprinting, Rice, SSR, Varietal identification and protection

Rice (*Oryza sativa* L.) is the major staple food representing roughly half of the world's population (Sellamuthu *et al.* 2011). It is cultivated in wide range of climatic conditions and throughout a huge geographical area (Rahman *et al.* 2009). The major variations ofrice genotypes are reported in Asia, specifically in China and the Indian subcontinent on morphological, biochemical, and molecular aspects (Nadir *et al.* 2017; Qiu *et al.* 2017). The rice origin was reported near the north-eastern part of India (Assam) (Zhao *et al.* 2010), as well as the south-western part of China (Yunan), both of which are strongly recommended to the Himalayan subtropical upland (Choi *et al.* 2017). *Oryza rufipogon* and *Oryza nivara* are the two types of *Javanica* rice which are known to be the immediate descendants of *Oryza sativa* L (Chang *et al.* 1976). North-East India, including Assam, is regarded to be one of the main rice origin centers representing a precious rice gene pool reservoir (Chetia *et al.* 2018; Gautam *et al.* 2018). Assam is a major rice-growing state, with 2.54 million (M) ha of cropped area out of a total of 3.3 million (M) ha, and is considered to be a hot spot of rice genetic resourcearound the world (Sahoo *et al.* 2019 and Sharma *et al.* 2017). The Assam Center (near Bangladesh) is also known as the Javanica rice's center of origin (Cheng *et al.* 1976).

Elite cultivars that have been improved have the potential to increase yields while also ensuring a consistent supply of rice. On the practical management of seed distribution, it is essential to select and clearly define cultivated varieties. Newly developed varieties should be distinct from other varieties as per protection of plant varieties and farmers' right authority (PPV and

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Suppl. Data available on respective page of NOPR

FRA) National guidelines (2007), for DUS (Distinctiveness, Uniformity, and Stability) test. In agricultural system, plant variety and cultivar identification are one of the most key factors. A large number of elite cultivars and landraces of cereal crops have made it challenging to classify and differentiate varieties based on morphological traits, as these get environmentally influenced, and thus phenotypic stability is the product of adaptation to these evolving climatic conditions. To overcome this, scientists have designed and used DNA markers, statistical testing, and software to characterize and identify suitable cultivars or varieties for cultivation in breeding programmes. The key purpose of using molecular markers is to identify genetic variation in DNA sequence within and among plant species.

Molecular markers often generate new sources of variation by introducing useful traits from related species. The International Union for the Protection of New Plant Varieties group evaluates various DNA markers parameters before their routine use in determining the DUS characterization of varieties and existing genotypes (Bredemeijer *et al.* 2002). The advancement in genomics technologies and the availability of various markers make the job easy to resolve the issue of identity (Kirst *et al.* 2005). Also, the genetic tagging and marker-assisted selection utility using microsatellites were demonstrated and found informative within rice varieties which act as an alternative approach to improve the varieties (Chen *et al.* 1997; McCouch *et al.* 1997; Akagi *et al.* 1996; Panaud *et al.* 1996; Olufowote *et al.* 1997). Microsatellite markers give a high degree of polymorphism and also provide greater informative existence. Hence these SSR markers are useful in developing specific rice genotype DNA profiles. Such profiles will be useful if rice varietiesare to be differentiated unequivocally to obtain plant variety protection (Rahman *et al.* 2009). This has therefore been widely used in identification, plant variety registration, seed purity control, high accuracy, and low-cost reliability.

PCR-based SSR markers are widely used in rice, and they are scattered throughout the plant's genome (McCouch *et al.* 1997). Average genome-wide coverage of rice SSR marker is one marker for every 6 centimorgans, which is adequate to assess the genotype identification (Olufowote *et al.* 1997). SSR markers are generally multi-allelic and co-dominant with expected frequency of heterozygosity greater than 0.7 which are convenient and allowing for precise discrimination among closely related individuals (Du *et al.* 2013). Microsatellite markers specificity and high information content enable the identification of individuals based on allele frequencies inter-laboratory. As SSR markers information can easily be exchanged by labs based on primer sequence, data comparison attempts to standardize fingerprinting data are straightforward which enable SSR markers as a choice for DNA fingerprinting (Singh *et al.* 2016). Previous studies of rice reported the excellent potential of SSR markers in genetic characterization and individual identification (Tamilkumar *et al.* 2009). Characterization and documentation methods have recently been used in many varieties/landraces/cultivars/wild species of *Oryza Sativa* L, *Triticum aestivum*, *Zea mays*, *Brassica napus*, *Glycine max*, and other crop s pecies (Rahman *et al.* 2010, Rahman *et al.* 2020, and Yadav *et al.* 2020). In the current study, research was done to assess the unique fingerprinting profiles of the selected *Sub1* introgressed advanced lines and their parental lines by SSR markers. Each variety has a unique fingerprinting profile that can be used as an identification mark for varietal protection.

Materials and Methods

Plant material

The plant material used in DNA fingerprinting analysis for the present investigations consisted of 7 genotypes out of which two were parents (Ranjit, Bahadur) and three *Sub1* introgressed advanced lines (E6, C210, C196) in Ranjit background and two *Sub1* introgressed advanced lines (1189-1, 1160-1) in Bahadur background. Seeds of Ranjit and Bahadur were collected from RARS, AAU, Titabar. Assam's most popular traditional semi-tall varieties are Ranjit and Bahadur. These *Sub1* introgressed advanced lines were selected based on DUS characters for maximum resemblance to their recurrent parents. The rice genotypes along with their parents were evaluated for various agro-morphological traits based on 62 DUS descriptors provided by Protection of Plant Varieties and Farmers Right Act guidelines (2007). These were evaluated on the basis of their trait difference from their parent. A collection of 68 informative SSR markers have been used to identify a specific fingerprint (Suppl. Table 1). These SSR markers were picked based on their maximum genome coverage from 12 chromosomes of rice.

DNA extraction and SSR marker based screening

Genomic DNA was isolated from 3 weeks old leaf tissues of these lines along with their parental checks

with slight modifications to the miniprep protocol. DNA quality was tested with 0.8% agarose gel electrophoresis. PCR amplification was done as per Panaud *et al.* (1996) using 68 SSR primers synthesized at Metabion International, USA. The sixty-eight SSR primers were scattered throughout all the 12 rice chromosomes. PCR amplification assay was performed in 10 µL reactions comprising 10 ng/µL of DNA template, 1 µL of TBE buffer (containing 200 mM Tris-HCl with pH 8. 3, 500 mM KCl, 15 mM MgCl2), 0.25 µL of 1 mM dNTP, 0.5 µL of 5 µM forward and reverse primers and 0.25 µL of Taq DNA polymerase (3 U/µL) (initial denaturation for 5 min at 94°C, followed by 35 cycles for 1-minute denaturation at 94°C, 1 min annealing at 55 °C and 2 min extension at 72°C with a final extension for 10 min at 72°C). The PCR products have been mixed using bromophenol blue gel loading dye and checked on 3% agarose gel to get amplification. The bands were visualized after 0.5 mg/mL ethidium bromide gel staining and were documented using Gel imager (UVP, UK). In order to calculate the amplified band size for each microsatellite marker, we used molecular weight size markers (100 bp DNA ladder) using software DNA frag Ver. 3.03 (Nash *et al.* 1991).

Results

Agro-morphological descriptors

The three *Sub1* introgressed advanced lines *viz.* C196, E6, and C210 along with Ranjit were evaluated to know the difference among them by using various agro-morphological traits. The content presented in (Tables 1 and 2) can be used to identify the distinctive characteristics of different rice genotypes with ease. The genotype C-196 showed difference in morpho-physiological traits from Ranjit, E-6 and C-210 for leaf anthocyanin colouration, leaf distribution of anthocyanin colouration, leaf sheath anthocyanin colouration, leaf pubescence of blade surface, leaf auricles, leaf anthocyanin colouration of collar, leaf length of blade, leaf width of blade, stem length, stem intensity of anthocyanin coloration of nodes, panicle number per plant, lemma and palea colour, grain weight of 1000 fully developed grains, grain length, grain width and decorticated grain width (Table 1). The genotype E-6 showed difference in morpho-physiological traits from Ranjit, C-196 and C-210 for basal leaf sheath colour, leaf length of blade, leaf width of blade, lemma anthocyanin colouration of apex, stem length, stem anthocyanin colouration, stem intensity of anthocyanin coloration of nodes, panicle number per plant, lemma and palea colour, panicle exertion, grain weight of 1000 fully developed grains, grain length, grain width, decorticated grain length and decorticated grain width (Table 1). The genotype C-210 showed difference in morpho-physiological traits from Ranjit, C-196 and E-6 for basal leaf sheath colour, leaf auricles, leaf length of blade, leaf width of blade, stem length, stem intensity of anthocyanin coloration of nodes, grain weight of 1000 fully

Table1 — List of observation of morpho-physiological descriptors for which the Sub1 introgressed advanced lines showed variations with Ranjit (Parental check)

Characteristics	Ranjit	C-196	E-6	C-210
Basal leaf: Sheath colour	Green	Green	Absent	Green
Leaf: Anthocyanin colouration	Absent	Present	Absent	Absent
Leaf: Distribution of anthocyanin colouration	--	In blotches	--	--
Leaf Sheath: anthocyanin colouration	Absent	Present	Absent	Absent
Leaf: Pubescence of blade surface	Medium	Weak	Medium	Medium
Leaf: Auricles	Present	Absent	Present	Absent
Leaf: Anthocyanin colouration of collar	Absent	Present	Absent	Absent
Leaf: Length of blade in (cm)	49. 7cm	37	61. 5	42. 8
Leaf: Width of blade (cm)	1	1. 5	1. 5	2
Lemma: Anthocyanin colouration of apex	Medium	Medium	Strong	Strong
Stem: Length (excluding panicle; excluding floating rice) (cm)	100	105	99. 8	116
Stem: Anthocyanin colouration	Absent	Absent	Present	Present
Stem: Intensity of anthocyanin coloration of nodes	Absent	--	Medium	Weak
Panicle: Number per plant	10	8	11	10
Lemma and Palea: Colour	Straw	Brown spot	Brown spots on straw	Straw
Panicle: Exertion	Well exerted	Well exerted	-	Well exerted
Grain: Weight of 1000 fully developed grains	19. 8	17. 8	17. 6	17. 6
Grain: Length	8. 12	7. 8	7. 98	7. 78
Grain: Width	1. 94	1. 54	1. 64	1. 5
Decorticated grain: Length	5. 82	5. 58	5. 6	5. 44
Decorticated grain: Width	1. 66	1. 3	1. 44	1. 28

Table2 — List of observation of morpho-physiological descriptors for which the *Sub1* introgressed advanced lines showed variations with Bahadur (Parental check)

Characteristics	Bahadur	1189-1	1160-1
Basal leaf: Sheath colour	Green	Green	Dark
Leaf: Intensity of green colour	Medium	Medium	Dark
Leaf: Anthocyanin colouration	Absent	Absent	Present
Leaf: Distribution of anthocyanin colouration	--	--	In blotches
Leaf Sheath: anthocyanin colouration	Absent	Absent	Present
Leaf sheath: Intensity of anthocyanin colouration	--	--	Strong
Leaf: Anthocyanin colouration of collar	Present	Absent	Present
Leaf: Length of blade in (cm)	39	34. 5	33
Leaf: Width of blade (cm)	2	1. 5	1. 8
Lemma: Anthocyanin colouration of keel	Medium	Weak	Medium
Lemma: Anthocyanin colouration of area below apex	Medium	Weak	Medium
Lemma: Anthocyanin colouration of apex	Strong	Weak	Strong
Stem: Length (excluding panicle; excluding floating rice) (cm)	100	131	100
Stem: Anthocyanin colouration of nodes	Absent	Present	Absent
Stem: Intensity of anthocyanin coloration of nodes	Absent	Medium	Absent
Stem: Anthocyanin colouration of internodes	Absent	Medium	Absent
Panicle: Curvature of main axis	Deflexed	Drooping	Deflexed
Panicle: Number per plant	10	7	11
Spikelet: Colour of tip of lemma	Brown	Yellow	Brown
Leaf: Senescence	Late	Early	Late
Grain: Weight of 1000 fully developed grains	23	20	20
Grain: Length	8. 28	7. 98	7. 78
Grain: Width	2. 04	1. 54	1. 84
Decorticated grain: Width	1. 94	1. 46	1. 66

developed grains, grain length, grain width, decorticated grain length and decorticated grain width (Table 1).

Similarly, the two *Sub1* introgressed advanced lines *viz*. 1189-1and 1160-1 along with Bahadur were evaluated to know the difference among them by using various agro-morphological traits. The genotype 1189-1 showed difference in morpho-physiological traits from Bahadur and 1160-1 for leaf anthocyanin colouration of collar, leaf length of blade, leaf width of blade, lemma anthocyanin colouration of keel, lemma anthocyanin colouration of area below apex, lemma anthocyanin colouration of apex, stem length, stem anthocyanin colouration of nodes, stem intensity of anthocyanin coloration of nodes, stem anthocyanin colouration of internodes, panicle curvature of main axis, panicle number per plant, spikelet colour of tip of lemma, leaf senescence, grain weight of 1000 fully developed grains, grain length, grain width and decorticated grain width (Table 2). The genotype 1160-1 showed difference in morpho-physiological traits from Bahadur and 1189-1 for basal leaf sheath colour, leaf intensity of green colour, leaf anthocyanin colouration, leaf distribution of anthocyanin colouration, leaf sheath anthocyanin colouration, leaf sheath intensity of anthocyanin colouration, leaf anthocyanin colouration of collar, leaf length of blade, leaf width of blade, panicle number per plant, grain weight of 1000 fully developed grains, grain length, grain width and decorticated grain width (Table 2). These lines *viz*. E-6, C-210, C-196, 1189-1, and 1160-1 could be easily distinguished during early growth stages and later from parental varieties based on 62 morphological descriptors from which variability was reported.

Fingerprinting of Sub1 introgressed lines using SSR Markers

The majority of SSR markers were derived from the rice genome's genomic and EST regions. These are produced from conserved regions of the genome, making it easy to distinguish between varieties and hybrids. In the current study, 68 SSR primer pairs covering various chromosomes were used to check a set of five genotypes along with their parents as a check. Among them, total 65 primers got amplified and three were not amplified. Out of 65 markers four showed distinguish and polymorphic amplification

Table 3 — Comparisons between SSR band size and selected breeder's traits of rice genotypes

S. No	Genotype Name	Band positions of Primers (bp)			
		RM 152	RM 172	RM 251	RM 346
1	Ranjit	156	150	137	175
2	Bahadur	163	159	147	173
3	E-6	155	152	144	166
4	C-210	160	156	140	170
5	C-196	158	154	137	168
6	1189-1	160	155	144	169
7	1160-1	157	157	139	172

while remaining 61 was found amplified monomorphic. These four primers were good enough to identify unique allelic patterns for specific *Sub1* introgressed advanced lines (Suppl. Fig. 1). The SSR markers such as RM152 produced unique alleles in Ranjit (156 bp), Bahadur (163 bp), E-6 (155 bp), C-210 (160 bp), C-196 (158 bp), 1189-1 (160 bp) and 1160-1 (157 bp). RM172 produced unique alleles in Ranjit (150 bp), Bahadur (159 bp), E-6 (152), C-210 (156 bp), C-196 (154 bp), 1189-1 (155 bp) and 1160-1 (157 bp). RM 251 produced unique alleles in Ranjit (137 bp), Bahadur (147 bp), E-6 (144), C-210 (140 bp), C-196 (137 bp), 1189-1(144 bp) and 1160-1 (140 bp). RM 346 produced unique alleles in Ranjit (175 bp), Bahadur (173), E-6 (166 bp), C-210 (170 bp), C-196 (168 bp), 1189-1 (169 bp), and 1160-1 (172 bp) and hence these markers could be used for differentiation of these cultivars of rice genotypes (Table 3). These SSR markers RM 152, RM 172, RM 251, and RM 346 produced unique alleles in E-6, C-210, C-196, 1189-1, and 1160-1 and hence could be used for differentiation of these five *Sub1*introgressed advanced lines from their parental checks (Suppl. Fig. 1). Hence these markers can be designated as genotype-specific SSR markers for identifying these advanced lines in present varietal testing programme.

Discussion

Morphological markers have been frequently used in the genus *Oryza* for descriptive purposes and are used in plant variety protection for distinguishing the individual varieties based on their distinctness, uniformity, and stability test. Characterization and assessment of accessions are the pre-requisites for utilizing the variety available for breeding. Hence, the rice genotypes are characterized to identify cultivar-specific traits and cultivar-specific SSR fingerprint which could be used for variety identification. In the current investigation the 62 agro-morphological DUS studies shown that E-6, C-210, C-196, 1198-1 and 1160-1 are distinct from the other traditional varieties (Ranjit and Bahadur) for more than 15 morphological traits, based on these variations the lines were selected for DNA fingerprinting. The similar studies were earlier reported in foxtail millet, sorghum and pearl millet (Natesan *et al.* 2020; Santhiya *et al.* 2020; Natesan *et al.* 2021).

Agro-morphological variation among the lines gives an opportunity for better selection of plant varieties but they are affected by environment. So, the DNA markers are unlimited in number apart from morphological and biochemical markers and are unaffected by environmental and/or developmental stages (Ovesna *et al.* 2002; Saker *et al*. 2005). Genetic markers are formed as a result of a variety of different forms of DNA mutations, including substitutions, rearrangements, and errors in tandem replication of repetitive DNA (Tamilkumar *et al.* 2009). Molecular fingerprinting is of utmost importance in protecting the novelty of a newly evolved plant variety. DNA fingerprinting reveals that RM 152, RM 172, RM 251 and RM 346showed better polymorphism out of 65 amplified primers. The amplicon size for the four polymorphic markers ranges from 155-163 bp, 150-159 bp, 137-147 bp, and 166-175 bp, respectively. The remaining three primers were not amplified. It is assumed that the absence of bands from the three primers is due to the incompatibility of such primers with the rice cultivars under investigation (Natesan *et al.* 2020). It is possible that the failure to amplify well using primers is due to an improper PCR programme (Santhiya *et al.* 2020 and Natesan *et al.* 2021). Furthermore, primers that are incompatible with the analyzed rice DNA sequences may result in the result not being amplified because there is no complementary match between the rice DNA and the primary sequences used. Hence, the research reveals that newly developed high-yielding *Sub1* introgressed advanced lines are unique in their identity. Similar,

findings were also reported in foxtail millet, sorghum and pearl millet (Natesan *et al.* 2020; Santhiya *et al.* 2020 and Natesan *et al.* 2021). According to a previous study, 29 rice varieties were identified using three microsatellite markers and 93 rice types were identified using five microsatellite markers (Rahman *et al.* 2006). Similarly, the DNA fingerprinting of 110 rice cultivars in Bangladesh was done using five polymorphic SSR markers, which included RM153, RM251, RM333, RM335, and RM475 (Rahman *et al.* 2010). The benefit of using microsatellite markers includes easy sharing and comparing the results among laboratories. These findings can be helpful in distinguishing rice varieties. Microsatellites are considered as an option for varietal characterization due to their multiallelic and codominant nature (Smith *et al.* 1996). Poljuha *et al*. (2008) reported a minimum of 3 SSR markers was adequate for fast, unambiguous discrimination of olive varieties. Microsatellites were reported to be more effective in identifying in-cultivar variation. Ravi *et al.* (2003) reported SSR markers can identify very small variations than RAPD between closely related breeding lines. Another rice study recorded six, adequate SSLPs to differentiate among 71 related lines (Olufowote *et al.* 1997). When 12 rice parental lines were subjected to molecular fingerprinting and genetic distance analyses, 62 out of 100 SSR markers examined were found to be polymorphic. The majority of the primers have identified rice lines with different band sizes which will act as a fingerprint profile and useful for the identification of hybrids. The results revealed that the hybrid rice parental lines have little genetic variation, which indicates that the genetic base needs to be broaden to minimize heterosis in rice breeding programme (Rajendran *et al.* 2012).

Morphological characteristics have been used for characterizing and distinguishing plants (cultivars), both quantitative and qualitative because the new plant species need to be differentiated from the other identified varieties as these are distinct from other varieties, as well as uniform in their characteristics and are genetically stable. DUS regulations specify the procedure for performing tests for the distinctiveness, uniformity, and stability of new plant varieties to grant the breeder's right and the time for providing the material needed for review in cases where the right of priority has been asserted and the first request has been refused or withdrawn. Hence, DNA-based markers have opened up new horizons for detecting variation at a molecular level. Of all DNA markers, SSR markers provide a rapid approach to analyze and compare DNA. The DNA fingerprinting data will be used for varietal identification.

Conclusion

These *Sub1* introgressed advanced lines which showed variation for more than 15 characters out of 62 DUS descriptors and unique banding profiles by SSR DNA fingerprinting imply that they are novel and have a unique banding profile. The set of microsatellite markers utilized in the experiment allows for a thorough evaluation of rice genotypes and the detection of distinct DNA profiles. The data collected can be utilized for varietal evaluation and the establishment of a database of all rice cultivars/landraces grown in Assam, as well as for the provision of further genetic information on agronomic and quality aspects. As a variety, these lines are predicted to benefit One million flood-affected growers in Assam (India) and aid in enhancing rice productivity in the state's flood-prone ecosystem.

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Conflicts of interest

All authors declare no conflict of interest.

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