RESEARCH ARTICLE

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UTILIZATION OF 384 SNP GENOTYPING TECHNOLOGY FOR SEED PURITY TESTING OF NEW INDONESIAN RICE VARIETIES INPARI BLAS AND INPARI HDB

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SUMMARY

Purity of a variety is critical for producing good quality seeds of commercial varieties. Inpari Blas and Inpari HDB are 2 new Indonesian rice varieties released in 2014 which are resistant to main rice diseases such as blast, bacterial leaf blight and tungro. The objectives of this research were to identify genotype profiles of new rice varieties by using 384 SNP markers and to establish the basis for assessing and monitoring seed purity for these varieties. The study revealed 41 polymorphic markers between Inpari Blas and Inpari HDB which were mapped in chromosomes 2-5, 7-9, 11 and 12. The total of 41 SNPs was detected by intra-varietal genotyping method as the unique markers for Inpari Blas and Inpari HDB varieties. Those unique markers could be used for variety identification to assess and monitoring of seed purity for these varieties. Three pure homozygous lines from both Inpari Blas (i.e. line number 16, 21 and 22) and Inpari HDB (i.e. line number 10, 15 and 18) were detected by inter-varietal genotyping. The seed from those selected lines can be used further as breeder seed for Inpari Blas and Inpari HDB, respectively. Significant results from inter-varietal polymorphisms indicated that the inter-varietal genotyping method could be used for selecting homozygous lines in order to detect genotype purity of Inpari Blas and Inpari HDB varieties. In general, the 384 SNP-chip platform could be used further for other varietal seed purity detection.

Key words: Inpari HDB, Inpari Blas, genotyping, single nucleotide polymorphism (SNP), seed purity

Key findings: SNP marker platforms provide a powerful tool for the molecular-genetic analysis of rice. The distribution of specific SNP markers across chromosomes is important to define genotype profiles. Intra and intervarietal polymorphisms are useful in breeding programs for checking of genotype purity during commercial seed development of Inpari Blas and Inpari HDB varieties.

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INTRODUCTION

The purity of rice seeds for commercial the seed production is critical for seed production and prone to contamination causing the purity of seeds qualified being inferior. Impurity may be caused by out-crossing or mixing of seed during harvest. The use of seeds with lower purity will

produce off type or segregating plants to some characters and cause a decline in production levels and superior genetic characters. The seed purity test in conventional characterization done by morphological and agronomical trait characterization. This often hampered by a long time and also influenced by environmental factors. To address this problems, the use of molecular marker has been much used for various types of crops (Kumar *et al.*, 2012; Hipi *et al.*, 2013; Shavrukov *et al*., 2014). New SNP (single nucleotide polymorphism) marker technology platforms offer opportunities to detect the intra-varietal variation for checking of genotype purity during development of commercial cultivars.

The development of superior varieties that are resistant to diseases is one of the main targets in breeding rice. Inpari Blast and Inpari HDB are the 2 varieties which are resistant to main rice diseases including blast (caused by fungus pathogen *Pyricularia grisea*) and bacterial leaf blight (caused by bacteria pathogen *Xanthomonas oryzae*) released by 2013. In addition both varieties are also resistant to tungro. The yield production of Inpari HDB reach 6,14 ton/ha, while Inpari Blas reach 6.28 ton/ha (dried grain milled, the average). (Somantri *et al.*, 2013).

Every new commercial rice variety should be tested for 'DUS (distinct, uniform and stable)' screening platform. This is accordance with the rules of the international for the protection UPOV guideline of new varieties (UPOV, 2002; Choi, 2012). Distinct means varieties must be clear that a different character with one or more varieties of which already exist; *Uniform* means varieties have characters in one of the population must be uniform; and Stable means varieties have characters observed in a variety has to be unchanged after repeated planted on the next generation. The DUS screening results of the new varieties as the basis of ownership defined by Plant Breeder Rights (PBR) (Ma, 1999).

The seed multiplication of Inpari Blas and Inpari HDB varieties were facing problems in the seed pure or breeder seed providing which are the main requirement of the commercial seed. During the production of pure seed, there is a high risk of contamination with the seeds of other lines. This can happen due to poor maintenance or operations causing the physical mixing of seed or also caused by the effects of pollination from other sources. Testing for the seed purity of released varieties takes a long time and there is the influence of the environment. To address this issue, molecular markers techniques for testing the purity of varieties may be used.

Genotyping of cultivars and breeding materials using molecular markers is very important tool for modern plant breeders. Molecular markers facilitate unmistakable cultivar identification the similarity within germplasm collection (Gupta *et al*., 1999). SNPs markers are one of the most important type of molecular markers and there have been significant recent development in breeding program for rice (Jehan *et al.*, 2006; Liao 2010). The new SNP marker platforms offer the opportunity to investigate the relationships between rice cultivars from different regions (Shavrukov *et al*., 2014). Utilization of SNP markers in the form of chips would be applied in order to determine the graphical genotypes of the genomes of Inpari Blas and Inpari HDB. The objectives of this research were to identify genotype profiles of the new released rice varieties, Inpari HDB and Inpari Blas by using the SNP-chip maker platforms and to establish the basis for assessing and monitoring seed purity for these varieties.

MATERIALS AND METHODS

Inpari HDB is the doublé haploid variety derived from crossing between IR64 and wild rice species *Oryza rufipogon* (Acc.IRGC105491). In the population development, the anther culture was done using F1 to derive the first doublé haploid lines (DH0). During the seed multiplication, good performing lines were selected. This process were repeated until obtained the advance DH4 lines. Continued selecting were done based on phenotype and genotype performance which finally was selected the lines BIO5 as an original breeding ID line for Inpari HDB variety. This line was resistant to Brown Planthopper, Bacterial Leaf Blight, dan Tungro virus.

Inpari Blas variety is selected from the advanced backcross (BC5) population, progenies from IR64 and *O. rufipogon* (Acc. IRGC.105491) crossing. Selection based on phenotype and genotype were repeated during the population development. The lines BIO111 was selected as a promising lines for the original breeding ID for Inpari Blas variety. This line was resistant to blast due to the *Pir7* blast resistance gene (Utami *et al*., 2008).

Fifty individual lines of Inpari Blas and Inpari HDB varieties were grown-out in Cianjur and Sukabumi field trials. The agronomic measurement and plant protection were adopted as recommendation practices for raising a healthy crop. Visual evaluation of genetic purity was conducted based on the important morphological characters throughout the growth period. The individual lines were selected as for purity test purposes. Young leaves of each lines were collected for genomic DNA extraction. Genomic DNA was isolated and purified according to Thermo Scientific King Fisher Plant DNA kits. The DNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, 2013). A minimum of 5 µl of genomic DNA (50 ng/µl) was required for the Golden Gate assay. DNA was stored in TE buffer (10 mM Tris, pH 7.5: 1 mM EDTA), and at least 10% of the samples were duplicated within the samples for quality control as recommended. The DNA concentration was standardized into 50 ng/µl for the final concentration. The purity standard was determined according to the ratio of A260/A280 in range 1,8-2,0 (Sambrook and Russell, 2001).

The genotypes profiling were performed using the custom OPA 384 SNP-chip with using Illumina's GoldenGate assay kit. The chip was designed based on 1536 and 384 SNP sets which were optimized based on previous research (Utami *et al.*, 2013; Utami *et al.*, 2014). The selected SNPs were polymorphic could differentiate across rice subspecies, i.e, *indica, japonica* and *tropical japonica*. The selected SNP markers were distributed on all twelve rice chromosomes.

Genotyping was performed using Illumina's Bead Array GoldenGate (GG) assay (Fan *et al*., 2003). The core of this technology is a collection of 3-micron silica beads that are assembled in wells, which are etched on the miniaturized matrix surface and evenly spaced at ~5.7 microns distance. Each bead is covered with hundred thousand copies of a specific oligonucleotide that act as the capture sequences in GoldenGate assays. A high-resolution confocal scanner (iScan) reads the arrays and generates intensity data. These data are converted into genotypic data by the built-in genotype-calling software called 'Genome Studio'. The resulting SNP calls were then re-formatted into subsequent data analysis. Flapjack 1.15.03.02 software was used for visualizing graphical genotypes (Milne *et al*., 2010). The genotype profile data obtained were then used for screening the impure lines which has some heterozygous alleles in each SNP loci detected. The SNP markers which detected the most heterozygous alleles were indicated having the ability to differentiate each tested line.

RESULTS AND DISCUSSION

Distribution of SNP markers on chromosomes

The SNPs that used for this study were designed based on a previously study that showed highly polymorphic performance among the different subspecies of rice, *indica, tropical japonica* and *japonica* (Utami *et al.*, 2013). Figure 1 showed the distribution of the SNP markers which were distributed on the twelve chromosomes of rice genomes.

Chromosome 1 is the longest chromosome which was covered by selected 38 SNP markers (44,4 Mb in length). The mean length covered by 1 SNP marker of chromosome 1 is 1,2 Mb. While chromosomes 10 is the shortest chromosomes in length (19,8 Mb) which was covered by 13 selected SNP markers. The mean length of chromosome 10 covered by 1 SNP marker is 1,5 Mb.

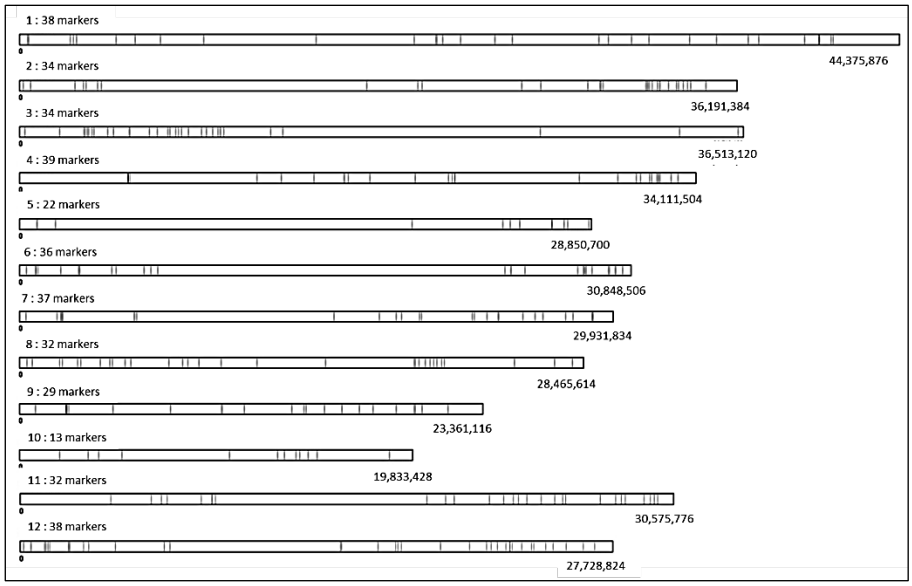

Figure 1. Distribution of 384 SNP markers were used to detect the genotype profile of the selected lines of Inpari Blas and Inpari HDB. This figure was produced by Flapjack software.

The purity of Inpari Blas and Inpari HDB were considered based on the homozygous alleles (AA, BB) and heterozygous (AB) alleles. The proportion of homozygous and heterozygous genotype profile of Inpari Blas dan Inpari HDB in each chromosome were shown in Table 1.

Table 1 showed that heterozygous (AB) alleles proportion comparing with homozygous allele (AA and BB) on Inpari Blas variety was 9.2%. The homozygous alleles, AA and BB were 71.9% and 85.3%, respectively. For Inpari HDB, the proportion of heterozygous (AB) alleles comparing with homozygous alleles (AA dan BB) were 8.14%. Therefore the purity of Inpari HDB variety was slightly higher than Inpari Blas variety due to having the proportion of lower heterozygous alleles. Homozygous allele AA and BB proportion Inpari HDB variety were reach 75.5% and 79.2% respectively.

Genotype profiles were obtained further analyzed using Flapjack program to interpret the genotype profiles inter and intra varietal (Figures 2 and 3). Based on this analysis, individual plants could be selected that are homozygous.

Genotyping intra varietal

Genotyping for intra-varietal analysis (Figure 2) was conducted to select SNP markers which could differentiate (polymorphism) the genotype profile of Inpari Blas and Inpari HDB. The selected SNP were used as the unique markers for both varieties. In addition, selection was also done to select the SNPs that have the highest homozygous alleles, so the markers could be used to detect the purity of Inpari Blas and Inpari HDB. Thus, these markers have the capacity to detect the specific characters to identify Inpari Blas and Inpari HDB varieties. The SNP IDs could be used for assessing and monitoring of seed purity for these varieties.

Based on intra-varietal analysis, 41 SNP markers were selected as the polymorphic markers to differentiate the genotype profiles of Inpari Blas and Inpari HDB. These SNP markers were mapped on chromosomes 2-5, 7-9, 11 and 12. These SNPs identify the specific polymorphisms between Inpari Blas and Inpari HDB (Table 2). The specific single nucleotide could be used for variety identification and monitoring of seed purity for Inpari Blas and Inpari HDB varieties.

Table 1. The homozygous and heterozygous allele proportion of Inpari Blas and Inpari HDB.

Genotypes		Inpari Blas								Inpari HDB							
		AA		BB		AB		NC		AA		BB		AB		NC	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Chromosome – Total Markers	1-38	813	11.6	916	11,9	133	9,41	38	2.0	764	40.2	947	49.8	112	5.9	77	4.1
	2-34	810	11.6	775	10,1	106	7,50	9	0.5	776	45.6	773	45.5	109	6.4	42	2.5
	3-34	95.4	1.4	590	7,7	106	7,50	50	2.9	902	53.1	620	36.5	110	6.5	68	4.0
	4-39	92.8	1.3	89.0	1,2	32	2,26	100	5.1	941	48.3	821	42.1	125	6.4	63	3.2
	5-22	409	5.9	526	6,8	106	7,50	59	5.4	449	40.8	504	45.8	79	7.2	68	6.2
	6-36	943	13.5	671	8,7	152	10,76	34	1.9	926	51.4	670	37.2	148	8.2	56	3.1
	7-37	848	12.1	785	10,2	146	10,33	71	3.8	708	38.3	886	47.9	128	6.9	228	6.9
	8-32	705	10.1	759	9,9	123	8,70	13	0.8	708	44.2	737	46.1	114	7.1	41	2.6
	9-29	800	11.4	437	5,7	143	10,12	70	4.8	736	50.8	490	33.8	144	9.9	80	5.5
	10-13	104	1.5	457	5,9	73	5,17	16	2.5	63	9.7	483	74.3	77	11.8	27	4.2
	11-32	573	8.2	764	9,9	179	12,67	84	5.2	665	41.6	600	37.5	180	11.2	155	9.7
	12-38	796	11.4	920	12,0	114	8,07	70	3.7	667	35.1	999	52.6	127	6.7	107	5.6
Total		6989	100	7689	100	1413	100	614	100	8305	100	8530	100	1453	100	1012	100
Proportion			71.9		85.3		9.63				75.5		79.2		8.63		

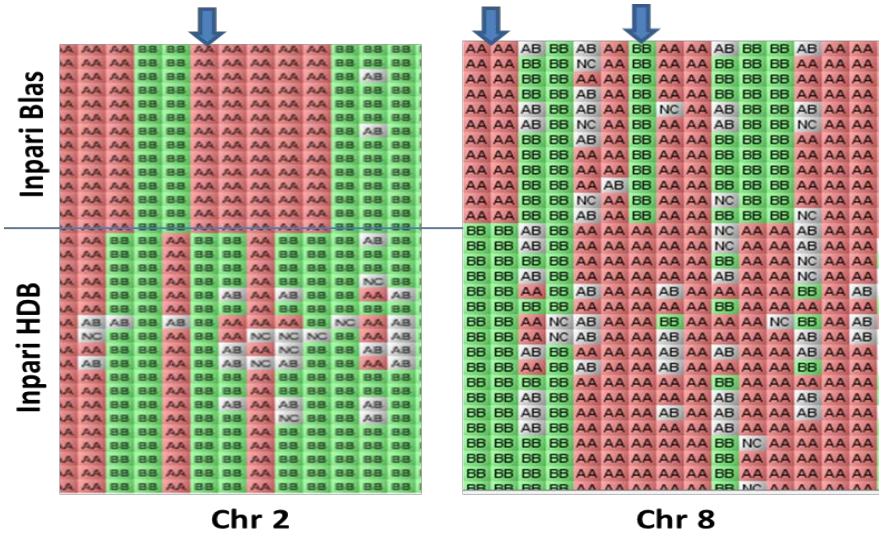

Figure 2. Genotyping intra-varietal analysis to select polymorphism and homozygous SNP markers for detecting the genotype identity of Inpari Blas and Inpari HDB varieties. The arrows marked the SNP loci that were polymorphic between Inpari Blas and Inpari HDB and contained homozygous alleles.

Inter-varietal genotyping

Genotyping inter-varietal analysis (Figure 3) were conducted to select the homozygous profile genotypes on each line of Inpari Blas and Inpari HDB which were grown and characterized at the different location, Cianjur and Sukabumi, West Java. The lines which have homozygous genotype profiles both at Cianjur and Sukabumi field trials were chosen as the source of breeder's seed of Inpari Blas and Inpari HDB varieties.

Based on this analysis, the 3 pure homozygous lines for Inpari Blas, line numbers 16, 21 and 22 were selected as the sources of seed production. While for Inpari HDB, the line numbers 10, 15 and 18 were selected. The seed from these selected lines could be used further as breeder's seed for Inpari Blas and Inpari HDB. Agronomic performance of Inpari Blas and Inpari HDB in the one of field trials at Cianjur, West Java are shown in Figure 4.

SNP markers are a powerful tool for the study of genetic polymorphism (Mammadov *et al.*, 2012). Fifty individual lines of Inpari Blas and Inpari HDB varieties were evaluated in the field trials at Cianjur and Sukabumi, West Java. They were analyzed with 384 SNP markers using Golden Gate technology platform from Illumina (Illumina, 2009), for Intra- and inter-varietal genetic polymorphic and the results were used for further molecular-genetic study.

Table 2. The identity of 41 polymorphic SNP markers.

No.	Chr	SNP markers	Genomic position (bp)	Specific ID	
				Inpari HDB	Inpari HDB
1	2	id2002229	4,143,587	A	C
2	2	id2010357	25,352,506	T	C
3	2	TBGI112898	29,316,294	C	T
4	2	id2012408	29,430,824	A	G
5	2	TBGI118359	33,351,136	G	C
6	2	TBGI118464	33,362,836	C	A
7	2	TBGI118590	33,475,496	T	A
8	2	TBGI119254	33,843,912	A	G
9	2	TBGI120561	34,637,604	G	C
1	3	id3006493	12,674,348	T	C
2	3	id3006808	13,276,088	T	C
3	3	id3015619	33,290,594	A	G
1	4	TBGI187378	5,407,451	C	T
2	4	id4004294	14,864,656	A	G
3	4	id4007024	21,641,804	A	G
4	4	TBGI204000	21,966,144	C	T
5	4	TBGI204006	21,966,410	T	A
1	5	TBGI264404	28,119,310	T	C
1	7	TBGI315077	2,087,279	C	T
2	7	TBGI315139	2,136,556	C	T
3	7	TBGI315142	2,137,503	T	C
4	7	TBGI315255	2,196,880	A	G
5	7	TBGI315185	2,159,646	T	A
1	8	id8000131	338,278	A	C
2	8	id8000244	658,673	T	C
3	8	id8000984	3,108,387	A	G
1	9	TBGI367885	2,361,688	T	C
2	9	TBGI367899	2,401,112	A	G
3	9	id9006377	19,020,266	A	C
1	11	id11007625	8,468,863	T	C
2	11	TBGI427500	8,984,345	G	A
3	11	TBGI427505	9,007,794	A	C
4	11	TBGI427506	9,007,834		
5	11	TBGI446461	23,718,964	C	T
6	11	TBGI446468	23,719,236	T	C
7	11	TBGI446469	23,719,250	C	T
8	11	TBGI46470	23,719,334	G	A
9	11	TBGI446479	23,719,916	A	G
10	11	TBGI446483	23,720,096	C	T
11	11	TBGI446447	13,718,510	C	G
1	12	id12001224	2,963,765	T	C

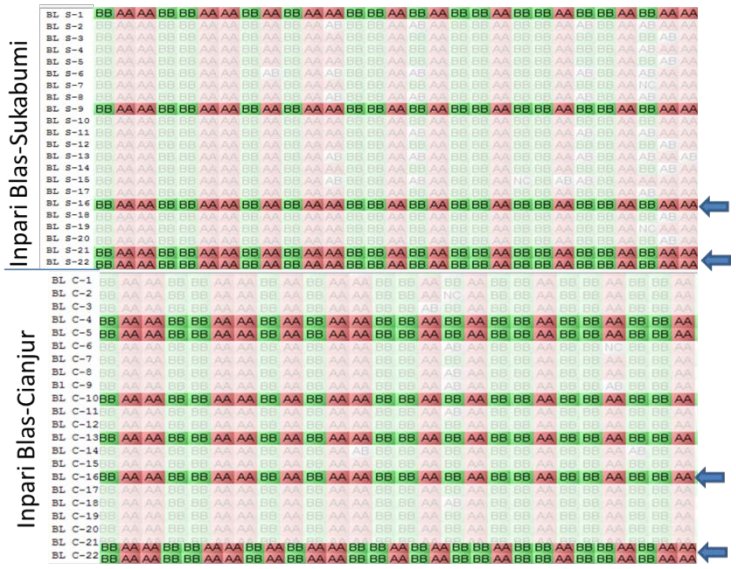

Figure 3. Inter-varietal genotyping analysis to select homozygous genotypes on each lines of Inpari Blas and Inpari HDB, which were grown and characterized at Cianjur and Sukabumi. The arrow marked the lines which have same homozygous profile both in Cianjur and Sukabumi, the lines number were: 16, 21 and 22. These fixed homozygous lines were choosed as source of seed purity.

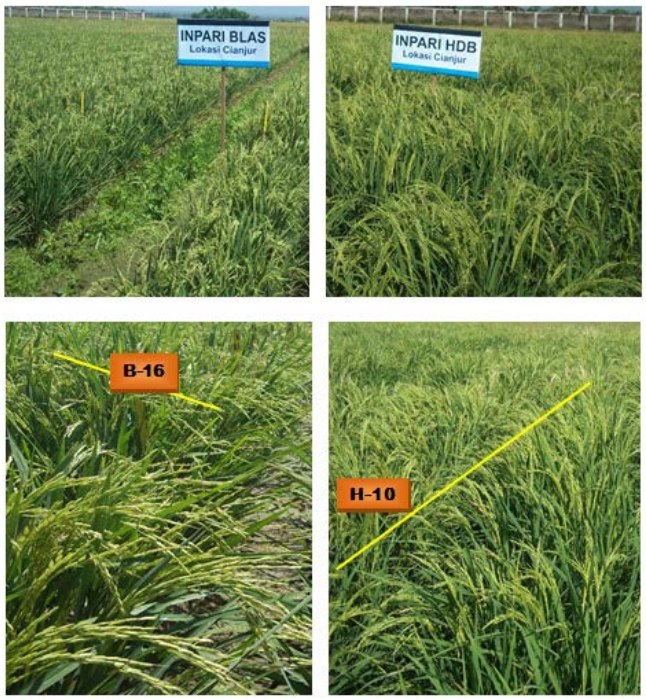

Figure 4. Field performance of Inpari Blast and Inpari HDB in Cianjur, West Java.

This study was similar to Shavrukov *et al.* (2014) which investigate the relationships between wheat cultivars. Using SNP-chip is one method to distinguish between homozygous alleles and heterozygous are needed to test the purity of seeds or seedlings (Thomson *et al*., 2010). These results also show that the 384-SNP chip can be used to test the genetic purity of rice not only on the population results of crosses but also on out crosses, the seeds are mixed or confused.

One of the targets of this study was to identify specific SNP markers for Inpari Blas and Inpari HDB that could be used for assessing and monitoring of seed purity; 41 SNP markers were identified it was means around 10,7% of the total SNP markers. This percentage of the polymorphic SNP markers is likely dependent on the differences in the sets of SNP markers and germplasm used in different studies (Trick *et al.*, 2012; Wurschum *et al*., 2013). The specific SNP markers were clustered tended to be distributed on the distal regions of the edge start point map for each chromosome. For example, on chromosomes 2, 4, 5 and 1, the specific SNP markers were more distributed on 25,4-34,6 Mb rather than in around 4 Mb (Table 2). Nevertheless, in chromosome 7, all the specific SNP were distributed in 2,0 – 2,2 Mb.

Field performance of Inpari Blas and Inpari HDB lines

In the field trials, purity evaluation was conducted based on morphological traits including plant height, days to maturity, panicle exertion, panicle length and days to harvest. The characters of individual's line shown deviation from the standard characters were identified as off-type and they were supported by the homogenous genotype profiles comparing in the 2 different field trials.

CONCLUSION

The SNP marker platforms provide a powerful tool for the molecular-genetic analysis of rice. The distribution of specific SNP markers across chromosomes is an additional defining characteristic of genotype profiles. The results of this study reporting that based on intra varietal genotyping found 41 polymorphic SNP markers between Inpari Blas and Inpari HDB. Based on theinter varietal genotyping, 3 pure homozygous lines of Inpari Blas and Inpari HDB that cauld be used as a breeder's seed source. Intra - and inter - varietal polymorphism have important applications in breeding program for checking of genotype purity during commercial seed development of Inpari Blas and Inpari HDB varieties.

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