Distinguishing Rice Genotypes using Morphological, Agronomical, and Molecular Markers

Pembedaan Varietas Padi Berdasarkan Karakter Morfologi, Agronomi, dan Marka Molekular

Untung Susanto, Nofi Anisatun Rohmah, and Made Jana Mejaya

Indonesian Center for Rice Research (ICRR)
Street 9th, Subang, West Java, Indonesia
Email: untungsus2011@gmail.com

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ABSTRAK. *Data lengkap karakteristik suatu varietas sangat bermanfaat untuk mengecek keautentikan suatu varietas yang benihnya diperdagangkan. Seringkali nama suatu varietas berubah dalam proses distribusi benih informal antar petani. Hal ini berpotensi merugikan pihak yang memiliki hak kekayaan intelektual varietas. Pencirian keautentikan suatu varietas berdasarkan karakter morfologi sering kali kurang memadai. Dewasa ini, telah tersedia teknologi marka molekuler, seperti SSR dan SNP yang lebih akurat dalam membedakan antarvarietas, relatif praktis, efektif, dan efisien. Penelitian ini bertujuan untuk membedakan sembilan varietas padi yang ditanam di lahan petani berdasarkan karakter morfologi (47 karakter), agronomi (9 karakter), marka SSR (12 marka terpilih terpaut karakter-karakter penting tanaman padi), dan marka SNP (384 marka) serta membandingkan akurasi masing-masing marka dalam membedakan varietas yang diuji. Kesembilan genotipe tersebut adalah empat varietas yang ditanam petani yang benihnya melalui distribusi informal, sehingga memiliki nama baru yang tidak terdapat dalam daftar varietas padi yang telah dilepas, serta lima varietas unggul yang telah resmi dilepas sebagai acuan. Penelitian untuk mendapatkan data morfologi dan agronomis tanaman dilakukan di Desa Ranca Jaya, Kecamatan Patok Beusi, Kabupaten Subang, Jawa Barat, pada MH 2011/2012, menggunakan rancangan acak kelompok dengan tiga ulangan. Ekstraksi DNA dari masing-masing varietas dilakukan dari contoh daun yang berasal dari materi yang sama dengan yang ditanam di lapang menggunakan metode CTAB yang dimodifikasi. Selanjutnya masing-masing contoh DNA diuji dengan 12 marka SSR dan 384 marka SNP. Hasil pengujian menunjukkan marka SSR terpaut karakter spesifik tanaman padi lebih akurat untuk membedakan varietas padi yang diuji dibandingkan dengan marka SNP (acak dan umumnya tidak terpaut suatu karakter tertentu) serta ciri-ciri agronomi dan morfologi. Karakter agronomi mampu membedakan beberapa varietas yang tidak dapat dibedakan berdasarkan karakter morfologinya. Secara keseluruhan, karakter morfologi, agronomi, dan molekuler dapat digunakan untuk menjamin perlindungan hak kekayaan intelektual atas suatu varietas.*

Kata kunci: Marka molekuler, morfologi, agronomi, padi.

ABSTRACT. Complete data on characteristics of a rice variety is very important to trace the authenticity of the variety at the field. Sometimes a name of a variety had changed, due to the informal seed distribution among farmers. This could become problem in the property right of the variety. Distinguishing among rice varieties using only morphological and agronomical traits are sometimes not sufficient. Currently, molecular markers such as SSR (Simple Sequence Repeats) and SNP (Single Nucleotide Polymorphism) markers have become available and are powerfull to distinguish rice genotypes. This research was aimed to distinguish nine rice varieties grown by farmers, using morphological characters (47 traits), agronomical characters (9 traits), SSR markers (12 primer pairs, related with important traits of rice plant), and 384 SNP markers, and to compare the effectiveness of each technique in distinguishing among genotypes. A field experiment was conducted in Ranca Jaya village, Patok Beusi, Subang, West Java during Wet Season (WS) of 2011/2012, using a Randomized Complete Block Design in three replications. A modified CTAB method was used to extract DNA for detection using 12 SSR markers and 384 SNP markers. The results revealed that the use of SSR markers that were linked to certain genes was more accurate than that of the SNP markers, agronomic, and morphological characters, in distinguishing differences among the 9 rice genotypes. The complete data of morphologic, agronomic, and molecular are useful to distinguish the authenticity of a variety in order to protect the intelectual property right attached on the variety.

Keywords: Morphological, agronomic, SSR, SNP, rice.

PENDAHULUAN

Complete characteristic data of a rice variety is very important to trace the authenticity of a variety in the field. Sometimes the name of a variety changed following its informal seed distribution among farmers, which may cause problem in relation with the property right of the variety. On the other hand, distinguishing rice varieties using only morphological traits would not be sufficient. Currently, genomic studies that had advanced and various molecular markers are available, such as RAPD (Random Amplified Polymorphism Devices) (Choudhury *et al*. 2001), AFLP (Amplification Fragment Length Polymorphism) (Fuentes *et al*. 1999, Virk *et al*. 2000), RFLP (Restricted Fragment Length Polymorphism) (Sun *et al*. 2001), SSR (Simple Sequence Repeats) (Hashimoto *et al*. 2004), and SNP (Single Nucleotide Polymorphism) (Takatsu *et al*. 2004, Raghavan *et al*. 2006, McNally *et al*.

2009), and even genomic sequence (Sasaki *et al*. 2005). Those markers are densely located accross the genome (McCouch *et al*. 2002), therefore they are more powerful to distinguish rice genotypes (Chuang *et al*. 2011, Jain *et al*. 2004) than the previously used markers, such as isozymes (Li *et al*. 2000, Fuentes *et al*. 1999, Virk *et al*. 2000) and morphological characteristics (Li *et al*. 2000).

Previous study revealed that RAPD could be used to fingerprint rice genotypes (Choudhury *et al*. 2001). For examples, 58 primers were enough to differentiate 1041 rice genotypes. AFLP is able to detect polymorphisms with higher efficiency than RAPD (+15%) and isozyme (+34%) (Fuentes *et al*. 1999). A total of 44 RFLP had been used to test 197 rice genotypes from various Asian countries, including wild relatives and identified that the genes diversities in rice varieties from South Asian countries was higher than those of South East Asian countries. Cultivated rice had the lowest gene diversity (Sun *et al*. 2001).

Furthermore, SSR gives more advantage due to more densely markers identified (McCouch *et al*. 2002), reliable results, possible for high throughput running (Coburn *et al*. 2002), and cost effective. McCouch *et al*. (2002) reported very dense markers had been validated and annotated SSR markers for rice, i.e. one SSR marker in every 157 kb, which is very useful for genetic study in rice. SSR markers has been widely used for genetic study and for breeding activity. Application of different markers, such as AFLP, isozyme, ISSR, and RAPD gave partial agreement among the markers. Particularly, AFLP and isoenzyme could be used more effectively for grouping the genotypes. On the other hand, SSR had been proven more powerfull than RAPD (Ravi *et al*. 2003).

SSR markers had been used to study genetic diversity, such as among local and improved rice varieties in Indonesia (Thomson *et al*. 2007); *Oryza rufipogon* (Song *et al*. 2003), and among popular varieties in Zhejiang Province of China (Zhu *et al*. 2012). SSR markers (164 primer pairs) had been used to measure the genetic diversity among 24 Philippines rice varieties carrying good quality traits (Lapitan *et al*. 2007). AFLP and SSR had been applied on 95 local and modern sake-brewing rice varieties together with the 76 popular rice varieties, and had been found that sake-brewing rice varieties had much smaller diversity than those of popular varieties (Hashimoto *et al*. 2004).

For breeding related purposes, SSR could be used to investigate genetic structures of breeding materials to identify the parent for crossing. SSR (101 markers) had been applied on 193 accessions internationally for rice breeding and showed that the materials were consisting of three groups, i.e. Group I, which corresponded with classical (the) indica sub species, whereas group II and III which belonged to the japonica subspecies. Genetic variability analysis revealed that selection for eco-geographical adaptation on multilocus associations was largely responsible for the maintenance of extensive variation in the primary gene pool of rice (Yu *et al*. 2003). In Cuba, application of 10 SSR markers on 39 traditional and 11 modern rice varieties revealed that a higher heterozygosity was found in traditional varieties (68% of the total microsatellite alleles). Majority of traditional varieties were distantly related to the improved varieties (Alvarez *et al*. 2007).

SSR had also been used to fingerprint, i.e., to distinguish the difference among rice genotypes (high yielding varieties, local varieties, and wild relatives) that was useful for plant variety protection (Rahman *et al*. 2009). SSR had been used to distinguish traditional and improved varieties to ensure purity and quality of rice to be exported from Thailand (Chuang *et al*. 2011). SSR could also distinguish among indian aromatic, indica and japonica varieties (Jain, *et al*. 2004). SSR (36 markers) had been applied to fingerprint 33 medicinal rice and giving 166 polymorphic alleles that were very useful as reference for intelectual property right of the varieties (Behera *et al*. 2012).

The most recent markers developed was SNP. There were 160,000 non redundant SNPs. Introgression patterns of shared SNPs were identified by resequencing 100Mb unique fraction of 20 reference varieties. Some of the SNPs were associated with agronomic traits and it has become milestones in rice improvement (McNally *et al*. 2009). Various techniques to apply SNP markers had been developed, such as enzime based, DNA-hybridization based, and fluorescent based technique. Fluorescent based technique seem to be more efficient, effective, and possible for high throughput running (Takatsu *et al*. 2004). SNP markers could be run more simply on agarose and it was widely used for mapping and for germplasm characterization (Raghavan *et al*. 2006). More modern and high throughput equipments had also been developed, thus encouraging the increase of SNP marker utilization among laboratories.

Various released rice varieties had been widely adopted by farmers. Sun *et al*. (2001) reported that genetic variability among cultivated varieties tended to be lower than the local landraces and wild relatives. It had happened in the field that the rice genotypes planted by farmers showed high degree of similarity and are difficult to be distinguished. The mentioned informal seed distribution had caused changing of the varietal names. It causes some difficulties in tracing the true name of a variety planted by farmer and the seed producers may had violated the property right of the variety. It was suspected that some varieties on farmers'

fields in the rice production center having local names but have similarities with those of released varieties. This research was aimed to distinguish the nine genotypes by using morphological (47 traits), agronomic (9 traits), SSR (12 primer pairs), and 384 SNP markers.

MATERIALS AND METHODS

Differentiation of Rice Genotypes Based on Morphological and Agronomical Characteristics

Nine rice genotypes consisting of four existed rice varieties in farmers' fields in West Java Province and five released varieties as reference were used in this study. The four rice genotypes planted by farmers had no clear history of breeding nor their seed origin. The varietal names were given informally by the farmers, including Manohara, Sidenok Sukra, Sidenok Ciasem, and Ciherang Taiwan. Five varieties or lines had been identified phenotypically matching to the above mentioned varieties, were i.e. Diah Suci, OBS1703, Inpari 10, Ciherang, and Fatmawati (Table 1). The five varieties were used as reference varieties. Manohara is suspected to be similar with Diah Suci. Sidenok Ciasem and Sidenok Sukra with OBS1703 (Inpari Sidenuk), Fatmawati, or Inpari 10. Ciherang Taiwan was suspected to be similar with Ciherang. The seeds of the four farmer's varieties were collected from farmers. The seed of the five reference varieties were obtained from the ICRR Seed Production Unit.

Table 1. The genotypes tested using morphological, SSR, and SNP Markers.

No	Genotype	Remark
1	Diah Suci	A released variety by BATAN (the National Nuclear Energy Agency), derived from gamma radiated mutant of Cilosari
2	Manohara	Variety existed in farmer field without any pedigree information, plant samples collected from framers' field in Bekasi
3	OBS1703	Sinonym to Inpari Sidenuk, a released variety derived from a gamma radiated mutant of Diah Suci
4	Sidenok Ciasem	Variety existed in farmers' field without any pedigree information; plant samples collected from farmers' field in Ciasem, Subang; termed also as Sidenok Kopkarlitan
5	Sidenok Sukra	Variety existed in farmer field without any pedigree information; plant samples collected from farmers' field in Sukra, Indramayu
6	INPARI 10	Released variety; pedigree: S3382-2d-Pn-4-1, cross: S487b-5/2*IR19661// 2*IR64
7	Fatmawati	Released variety; cross: BP68C-MR-4-3-2/Maros
8	Ciherang	Released variety; pedigree: S3383-1D-PN-41-3-1, cross: IR18249-53/IR19661-131-3-1//IR19661-131///IR64////IR64
9	Ciherang Taiwan	Variety existed in farmer field without any pedigree information, plant samples collected from farmers' field in Ciasem

A field expriment was conducted in Ranca Jaya Village, Patok Beusi, Subang, West Java during Wet Season (WS) 2011/2012, using of a randomized complete block design (RCBD) with three replications. Transplanting of 21 day old seedlings were spaced 25 cm x 25 cm on 4 m x 5 m plot size. Plant morphological characters (47 traits) were observed following DUS (Distinct, Unique, and Stable) testing protocol from the Office of Center for Plant Variety Protection and Agricultural Permit, Ministry of Agriculture (PPI, 2006). Agronomic and morphological characteristics were clustered using the program NTSysPc ver. 2.1 (Rohlf, 2000).

Differentiation of Rice Genotypes Based on SSR Markers

The nine rice genotypes were planted in 27 cm diameter pots in green house. DNA from each genotype was extracted from leaf samples of each true genotype by using modified CTAB (*Cetyl Trimethyl Ammonium Bromide*) method (Murray and Thompson 1980). Each of the DNA samples was then splitted into two, one half for SSR reaction in the Plant Breeding Laboratory of the ICRR, Sukamandi and the other half one was sent to IRRI (International Rice Research Institute) for the SNP analysis.

Twelve published SSR markers linked to certain important agronomic traits (Table 2) were chosen and the detail information of the markers were obtained from McCouch *et al*. (2002) and from website of http://www.gramene.org/.

PCR reaction for SSR analysis was conducted using a reaction volume of 10 μl solution containing 50 ng of template DNA, 0.25 μM of each forward and reverse primer, 100 μM of each dNTPs, 1 X reaction buffer (20 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin) and 0.5 unit of Taq DNA polymerase. The profile of PCR amplification was performed as follows: one cycle of 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for one min., 55^{o}C for 1 min. (or according to the specific annealing temperature of the primers), and 72^{o}C for 2 min., and finally 1 cycle of 72^{o}C for 5 min. PCR products were kept at 4^{o}C for short term storage prior to using.

Electrophoresis of the PCR products were done in 8% polyacrilamide gels in 1X TBE buffer at 100 volts for two to four hours depending on the PCR product size.

The gels were then stained using ethidium bromide solution and visualized under UV light using Gel Documentation System. Data were scored as 1 (present) and 0 (not present) for each of the SSR locus. Clustering analysis was conducted using the Power Markers Ver 3.25 (Liu and Muse 2005) based on the distance matrix of Nei (1987).

Differentiation of Rice Genotypes Based on SSR Markers

The DNA used for the SNP analysis was split from the DNA for the SSR analysis. The DNA samples were sent to IRRI for analysis using 384 SNP markers (Thomson *et al*. 2012). The SNP markers were arranged by IRRI to spread evenly at random accross the rice genome. Clustering analysis was conducted using Power Markers Ver 3.25. (Liu and Muse, 2005) based on Nei (1987) distance matrix.

RESULTS AND DISCUSSION

Differentiation of Rice Genotypes Based on Morphological and Agronomical Characteristics

Based on the observation of 47 rice morphological traits, there were only 8 traits that were different among the 9 genotypes, i.e. ligule length, leaf length, leaf width, leaf erectness, length of main axis of panicle, days of senescence, 1000 grain weight, and grain length (Table 3). Varieties Diah Suci and Fatmawati had long ligules, while the others varieties had medium ligules size. Sidenok Sukra and Fatmawati had long leaves, while the other varieties had medium leaves. Fatmawati had a wide leaf blade, while the others had narrow ones. Fatmawati had a semi errect plant stature, while the others had errect ones. Sidenok Sukra and Fatmawati had long panicle main axis, while the others had medium ones. Sidenok Sukra had a low 1000 grain weight, Farmawati had a high one, while the others had medium ones. Sidenok Sukra had a short grain size, while the others had a medium size. The morphological traits could not distinguish among Manohara, OBS1703, Sidenok Ciasem, Inpari 10, Ciherang Taiwan, and Ciherang.

Clustering analysis using the UPGMA method grouped the 9 genotypes into four groups. Manohara, OBS1703, Sidenok Ciasem, Inpari 10, Ciherang Taiwan, and Ciherang were in one group without any differences among them. Diah Suci, Sidenok Sukra, and Fatmawati each was standing alone as separate groups. Fatmawati is the most distinct variety from the others, indicating the highest difference with the other genotypes (Figure 1).

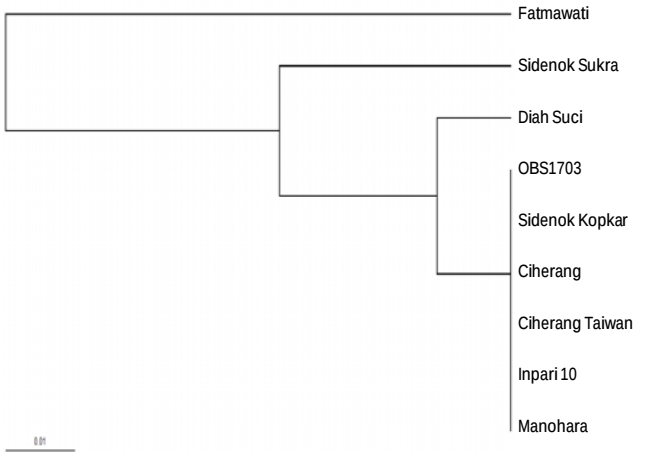

Figure 1. Clustering eight rice genotypes based on 47 morphological traits, using the UPGMA method.

Table 2. SSR Makers (12 primer pairs) used to distinguish eight rice genotypes.

No	Primer	Chromo-some	Gene related	Forward	Reverse	Tm (°C)	Size (bp)
1	RM315	1	*Rf3*	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	55	133
2	RM10852	1	Salt	GAATTTCTAGGCCATGAGAGC	AACGGAGGGAGTATATGTTAGCC		171
3	RM266	2	Seed set	TAGTTTAACCAAGACTCTC	GGTTGAACCCAAATCTGCA	55	127
4	RM282	3	Grain/panicle	CTGTGTCGAAAGGCTGCAC	CAGTCCTGTGTTGCAGCAAG	55	136
5	RM241	4	Plant height	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG	55	138
6	RM164	5	Heading-drought	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC		246
7	RM190	6	Waxi gene	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG	55	124
8	RM510	6	Gel consistency	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC	55	122
9	RM234	7	Maturity date	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	55	156
10	RM248	7	Root development	TCCTTGTGAAATCTGGTCCC	GTAGCCTAGCATGGTGCATG	55	102
11	RM464a	9	*Sub1*	AACGGGCACATTCTGTCTTC	TGGAAGACCTGATCGTTTCC	55	262
12	RM7102	12	*Bph2*	TAGGAGTGTTTAGAGTGCCA	TCGGTTTGCTTATACATCAG	55	168

Tm = melting temperature.

Table 3. Performance of 47 morphological traits, heading date, and maturity of eight rice genotypes.

No	Trait	Mano-hara	Diah Suci	Sidenok Ciasem	Sidenok Sukra	OBS 1703	INPARI 10	Fatma-wati	Cihe-rang	Ciherang Taiwan	Remark
Polymorpic traits											
1	Leaf: Length of ligule (mm)	5 (1.3)	7 (1.8)	5 (1.4)	5 (1.6)	5 (1.3)	5 (1.3)	7 (2.0)	5 (1.3)	5 (1.5)	5 = Medium; 7 = Long
2	Leaf: Length of blade (mm)	5 (48.2)	5 (49.8)	5 (44.5)	5 (52.5)	5 (42.4)	5 (43.2)	7 (56.9)	5 (43.2)	5 (45.8)	5 = Medium; 7 = Long
3	Leaf: Width of blade (mm)	5 (1.6)	5 (1.5)	5 (1.5)	5 (1.7)	5 (1.4)	5 (1.5)	7 (2.0)	5 (1.3)	5 (1.5)	5 = Medium; 7 = Long
4	Flag leaf: Attitude of blade (late observation)	1	1	1	1	1	1	3	1	1	1 = Errect; 3 = Semi Errect
5	Panicle: Length of main axis (mm)	2 (26.9)	2 (29.3)	2 (27.4)	3 (32)	2 (24.1)	2 (26.7)	3 (32)	2 (25.3)	2 (27)	2 = Medium; 3 = Long
6	Leaf: Time of senescence	5	5	5	5	5	5	3	5	5	3 = Early; 5 = Intermediate
7	Grain: Weight of 1000 (g) (fully developed grains)	5 (27.9)	5 (29.2)	5 (28.1)	**3 (25)**	5 (27.3)	5 (28)	7 (29.9)	5 (28.1)	5 (28.6)	3 = Low; 5 = Medium; 7 = High
8	Grain: Length (mm)	5 (9.70)	5 (9.62)	5 (9.49)	**3 (8.81)**	5 (9.26)	5 (9.45)	5 (9.16)	5 (9.26)	5 (9.53)	3 = Short; 5 = Medium
9	Time of heading (days after sowing) (50 % of plants halfway with heads)	102	107	102	115	105	100	100	102	10)	Days after sowing
10	Time of maturity (days after sowing)	129	137	129	146	129	129	129	129	129	Days after sowing
Monomorphic traits											
11	Coleoptile: anthocyanin coloration	1	1	1	1	1	1	1	1	1	1 = Absent
12	Basal leaf: sheath color	1	1	1	1	1	1	1	1	1	1 = green
13	Leaf: Anthocyanin coloration	1	1	1	1	1	1	1	1	1	1 = Absent
14	Leaf sheath: Anthocyanin coloration	1	1	1	1	1	1	1	1	1	1 = Absent
15	Leaf: Auricles	9	9	9	9	9	9	9	9	9	9 = Present
16	Leaf: Anthocyanin coloration of auricles	1	1	1	1	1	1	1	1	1	1 = Absent
17	Leaf: Collar	9	9	9	9	9	9	9	9	9	9 = Present
18	Leaf: Collar color	1	1	1	1	1	1	1	1	1	1 = Green
19	Leaf: Ligule	9	9	9	9	9	9	9	9	9	9 = Present
20	Leaf: Shape of ligule	2	2	2	2	2	2	2	2	2	2 = Cleft
21	Leaf: Color of ligule	1	1	1	1	1	1	1	1	1	1 = Colorless
22	Flag leaf: Attitude of blade (early observation)	1	1	1	1	1	1	1	1	1	1 = Errect
23	Culm: Habit	5	5	5	5	5	5	5	5	5	5 = Open
24	Lemma: Anthocyanin coloration of keel (early observation)	1	1	1	1	1	1	1	1	1	1 = Absent
25	Lemma: Anthocyanin coloration of area below apex	1	1	1	1	1	1	1	1	1	1 = Absent/Very Weak
26	Lemma: Anthocyanin coloration of apex	1	1	1	1	1	1	1	1	1	1 = Absent/Very Weak
27	Spikelet: Color of stigma	1	1	1	1	1	1	1	1	1	1 = White
28	Spikelet: Color of anthers (for A line)	2	2	2	2	2	2	2	2	2	2 = Light Yellow
29	Stem: Thickness	5 (6.2)	5 (5.7)	5 (6.5)	5 (6.0)	5 (6.4)	5 (6.8)	5 (6.5)	5 (6.0)	5 (6.6)	5 = Medium
30	Non-prostrate varieties only: Stem length (excluding panicle)	5 (100.2)	5 (95.1)	5 (100.9)	5 (97.9)	5 (86.8)	5 (91.4)	5 (91.2)	5 (90.9)	5 (91.5)	5 = Medium
31	Stem: Anthocyanin coloration of nodes	1	1	1	1	1	1	1	1	1	1 = Absent
32	Stem: Intensity of anthocyanin coloration of nodes	3	3	3	3	3	3	3	3	3	3 = Weak

Table 3. Continues.

No	Trait	Mano-hara	Diah Suci	Sidenok Ciasem	Sidenok Sukra	OBS 1703	INPARI 10	Fatma-wati	Cihe-rang	Ciherang Taiwan	Remark
33	Stem: Anthocyanin coloration of internodes	1	1	1	1	1	1	1	1	1	1 = Absent
34	Sterile lemma: Color	1	1	1	1	1	1	1	1	1	1 = Straw
35	Panicle: Number per plant	3 (15)	3 (15)	3 (15)	3 (15)	3 (14)	3 (14)	3 (14)	3 (15)	3 (14)	3 = Medium
36	Spikelet: Pubescence of lemma	5	5	5	5	5	5	5	5	5	5 Medium
37	Spikelet: Apiculus color	2	2	2	2	2	2	2	2	2	2 = Straw
38	Panicle: Attitude in relation to stem	2	2	2	2	2	2	2	2	2	2 = Semi Upright
39	Panicle: Presence of secondary branching	9	9	9	9	9	9	9	9	9	9 = Present
40	Panicle: Type of secondary branching	2	2	2	2	2	2	2		2	2 = Strong
41	Panicle: Attitude of branches	3	3	3	3	3	3	3	3	3	3 = Semi Errect
42	Panicle: Exsertion	5 (4.4)	5 (2.1)	5 (3.9)	5 (2.2)	5 (4.7)	5 (5.7)	5 (1.9)	5 (5.6)	5 (5.2)	5 = Just Exerted
43	Lemma: Color Lemma: warna	1	1	1	1	1	1	1	1	1	1 = Straw
44	Lemma: Ornamentation	1	1	1	1	1	1	1	1	1	1 = Absent
45	Lemma: Anthocyanin coloration of keel	1	1	1	1	1	1	1	1	1	1 = Absent or very weak
46	Lemma: Anthocyanin coloration of area below apex	1	1	1	1	1	1	1	1	1	1 = Absent or very weak
47	Lemma: Anthocyanin coloration of apex	1	1	1	1	1	1	1	1	1	1 = Absent or very weak
48	Glume: Length or sterile lemma length (mm)	5 (0.26)	5 (0.28)	5 (0.28)	5 (0.28)	5 (0.25)	5 (0.27)	5 (0.27)	5 (0.24)	5 (0.24)	5 = Medium
49	Grain: Width (mm)	5 (2.28)	5 (2.28)	5 (2.30)	5 (2.31)	5 (2.26)	5 (2.27)	5 (2.31)	5 (2.28)	5 (2.27)	5 = Medium

Remark:
- Polymorphic trait = contains at least one genotypes different to other genotypes.
- Monomorphic trait = all the eight tested genotypes has similar characteristics
- No 9 and 10 are agronomic traits.

Observation on the agronomic traits, especially the heading date, found that Inpari 10, Fatmawati, and Ciherang flowered each at 100 days after sowing. Ciherang Taiwan, Manohara, and Sidenok Ciasem flowered at 102 days, OBS1703 at 105 days, Diah Suci at 107 days, and Sidenok Sukra at 115 days after sowing. Furthermore, Manohara, OBS1703, Sidenok Ciasem, Inpari 10, Fatmawati, Ciherang, and Ciherang Taiwan had a maturing day at 129 days after sowing; Diah Suci at 137 days, and Sidenok Sukra at 146 days. Thus, the heading date data could be used to distinguish the genotypes that could not be distinguished by the morphological traits, i.e. OBS1703 from Inpari 10, Ciherang, Manohara, Sidenok Ciasem, and Ciherang Taiwan. However, it could not distinguish Inpari 10 from Ciherang, and among Manohara, Sidenok Ciasem, and Ciherang Taiwan (Table 2). Furthermore, the heading date and maturing dates were affected by the environment, so that they varied among seasons and locations. Heading date and plant growth duration were longer than in normal condition, this might be due to flood at vegetative stage and frequent heavy rain and cloudy weather during the plant growth.

Differentiation of Rice Genotypes Based on SSR Markers

Application of the 12 SSR markers linked with important agronomic traits was able to devide the nine rice genotypes into four groups (Figure 2). Result was in agreement with the results of clustering based on morphological traits, where Fatmawati and Diah Suci separated from other genotypes, each in a single genotype group. Additionally, SSR markers considered Inpari 10 stood by itself, separated from other groups. Sidenok Sukra together with the rest of the genotypes were clustered as one group. PIC value of the markers were considered very low, ranging from 0 to 0.37. with an average of 0.26. This indicated low variability among the 9 genotypes, with respect to the 12 SSR markers. There were 73 alleles developed ranging from 2 (RM10852) to 10 alleles (RM282) per marker, with an

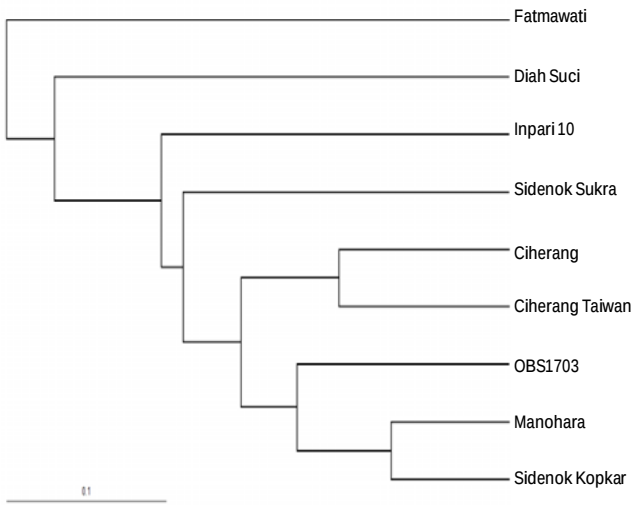

Figure 2. Clustering eight rice genotypes based on 12 SSR markers, using the UPGMA method.

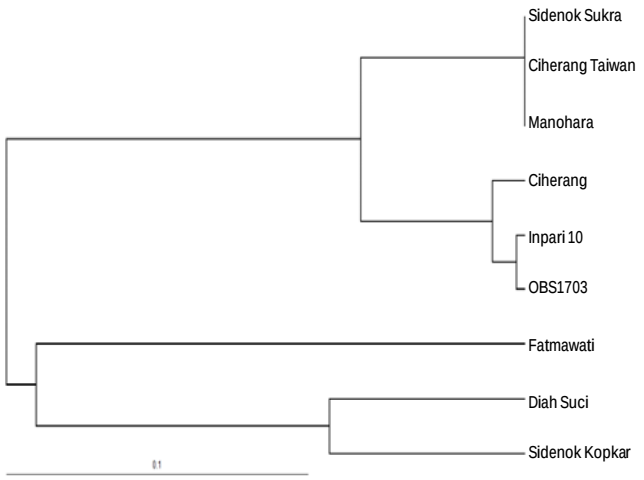

Figure 3. Clustering eight rice genotypes based on 384 SNP markers, using the UPGMA method.

average of 6.01 alleles per markers. The genotypes could be distinguished by the developed alleles.

Differentiation of Rice Genotypes Based on SSR Markers

The application of 384 SNP markers differentiated the 9 genotypes into three groups at 10% genetic distance as threshold (Figure 3). Sidenok Sukra, Ciherang Taiwan, Manohara, Ciherang, Inpari 10, and OBS1703 were in one group, Diah Suci and Sidenok Ciasem in another group, while Fatmawati stood alone as a separate group. There were 91 monomorphic markers out of 384 SNP markers. The SNP markers could not distinguish among Sidenok Sukra, Ciherang Taiwan, and Manohara. The average of PIC value of the 293 polymorphic markers was relatively low (0.248), which indicated the narrow genetic distance among the genotypes, which was in agreement with the SSR markers.

The members of groups developed based on the SNP markers were less than those developed based on morphological and SSR markers. This was surprising, since the number of SNP markers used were more than the number of morphological traits and SSR markers. The SNP markers were located randomly accross the rice genome and were not necessarily linked to certain traits. Thus, they might not be related to either the morphological or agronomical traits of the plant. Therefore, it indicated that the use of gene-trait specific or SSR markers in the functional fragment of the genome was more powerful to distinguish rice genotypes.

Morphological traits, SSR markers, and SNP markers indicated slightly difference in grouping and distinguishing genotypes. Mophological trait alone could not distinguish among OBS1703, Sidenok Ciasem, Ciherang Taiwan, Inpari 10, Manohara, and Ciherang. The agronomic traits, especially the heading date was slightly more powerful than the morphological traits, eventhough it might be affected by the environmental conditions. SSR markers based on 73 alleles could distinguish the 9 genotypes. The SNP markers in this case could not distinguish difference among Sidenok Sukra, Ciherang Taiwan, and Manohara. This indicated that the use of SSR markers were the most effective technique to distinguish rice genotypes among the techniques used. SSR markers could be used efficiently and effectively to distinguish, by carefully choosing the appropriate number of SSR markers applied. Application of SSR markers linked to genes controlling important traits could strongly increase its efficiency due to the possibility of identifying more alleles variability. However, it may not be practical due to its relatively low throughput characteristics. A set of 30 SSR was comparable to 111 SSR markers in distinguishing 40 rice genotypes (Ni *et al*. 2001). This present experiment was able to distinguish the differences among 9 genotypes by using only 12 markers. Increasing the number of markers would undoubtedly increase the power in distinguishing the difference among the genotypes.

The use of SNP markers seemed to be more powerfull, because it gave a high throughput, efficient, and a robust method. However, the SNP markers used in this research were not designed to be linked to a certain gene/genes of traits, they were randomly located accross the rice genome. Application of gene-trait specific SNP markers would probably increase its power to distinguish the rice genotypes.

Results of this research also indicated that molecular markers could be used to distinguish rice genotypes, including the most phenotypically similar genotypes. Nevertheless, SNP or other molecular markers could not determine the ancestors nor the sister lines. It could only differentiate among genotypes based on certain loci, and thus analyze genetic similarity and distance among genotypes.

The research materials used in this study were rice genotypes existed on farmers' fields. Some of the genotypes have the name of released varieties, but others had name of unknown origin, seed source and pedigree history (given name farmers). SSR markers was considered more powerful than the other two techniques. The technique could be used to protect the intelectual property right, through the protection of variety authenticity.

CONCLUSIONS

1. The use of SSR markers was most effective to distinguish rice genotypes as compared to the use of SNP markers, morphological traits, or agronomic traits.
2. Complete data from the morphological and agronomic traits, SSR test, and SNP test were useful to identify authenticity of a rice variety for the purpose of protecting the intellectual property right.

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