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Genetic Diversity of Colored Rice Lines Based on Botanical Characteristics and Simple Sequence Repeat (SSR) Markers

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This study aimed to evaluate a genetic diversity by simple sequence repeat (SSR) markers and morphological characteristics among colored rice lines of the developed CNU (Chungnam National University) lines. Development of colored rice cultivars is one of the main breeding goals to meet a consumer's needs in Korea. Total 34 colored rice lines that selected from advanced population improved by crosses and artificial mutation techniques were tested. SSR analysis of colored rice lines showed 20 polymorphic bands using seven SSR primer pairs (RM pairs) and had average three alleles per SSR marker. The average of genetic distance based on SSR marker was 0.56 and ranged from 0.22 to 0.81. The genetic distance was higher in each different pedigree or cross combinations than that in the same pedigree. The mean of polymorphism information content (PIC) were 0.48 and the ranges of PIC reached 0.08 (RM 579) to 0.67 (RM 457), respectively. Genetic classification of 34 colored rice lines by SSR marker analysis were divided into four groups when genetic distance was more than 0.5. The results of cluster analysis of 34 colored rice lines by SSR markers were compared with the results of cluster analysis using morphological characteristics, i.e. color of spikelet and seed coat, and awn existence and its length.

INTRODUCTION

Genetic diversity is an essential element for the development of new line of rice, and has an importance for hybrid combinations in colored rice. It is also useful for genetic improvement and for the development of colored rice for desired traits such as yield, quality, pest and insect resistance, and stress tolerance.

Maintaining genetic diversity and improving the management of genetic resources are important issues among colored rice breeders. Many methodologies exist for the assessment of genetic diversity in colored rice. Since the morphological characteristics are influenced by the environment, they sometimes do not express genetic diversity. In addition the evaluation based on such traits is time and expenses consuming (Dubreuil *et al*., 1996). Nevertheless morphological data are still widely used for selection of genetically diverse parents from a colored rice collection. But the morphological differences are usually determined by a small number of genes and may not be representative of genetic divergence in the entire genome (Singh *et al*., 1999; Brown–Guedira *et al*., 2000). Compared with morphological variation, molecular polymorphism is generally considered to be independence of the environment (Gauthier *et al*., 2002). Molecular genetic techniques can be applied for the evaluation of genetic diversity and to traditional approaches in the conservation and utilization of plant genetic resources (Gauthier *et al*., 2002; Ghebru *et al*., 2002).

DNA based markers provide some useful information on genetic diversity. The information provided by molecular markers can be used in breeding programs to better estimate the genetic value to selection (Hospital *et al*., 1997) Molecular markers allow for the selection of desired traits based on genotype rather than phenotype and can therefore complement and accelerate plant breeding programs. They can also be used for the early selection of traits such as persistence, competitive ability and seed yield, which are not expressed during the juvenile phase. Molecular markers have been successfully used for the construction of genetic linkage maps and for the identification and tagging of economically important genes and quantitative trait loci (QTLs) in a large number of plant species (Rafalski *et al*., 1996; Staub *et al*., 1996; Mohan *et al*., 1997; Kumar, 1999).

Molecular markers can provide an effective tool for efficient selection of desired agronomic traits since they are based on the plant genotypes and thus are independent of environmental variation. Highly informative molecular markers, such as simple sequence repeats (SSRs), can greatly accelerate breeding programs. SSRs are very abundant and dispersed throughout the genome, usually co–dominant inheritance, and can uncover a great number of polymorphisms since multi–allelic loci are very common (Chin *et al*., 1996). The use of molecular markers can facilitate rice breeding by means of marker–assisted selection (MAS) to improve agronomical important traits such as yield, quality and disease resistance. This study aims to evaluate genetic diversity by simple sequence repeat (SSR) markers among colored rice lines of the developed CNU lines.

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MATERIALS AND METHODS

Plant materials and DNA isolation

CNU lines were planted in randomized complete block design (RCBD) on April 30, 2004 at Chungnam National University's Farm (Table 1). CNU lines were planted in a single row with 0.15 m spacing between plants and 0.3 m spacing between rows. Genomic DNA of the 34 CNU lines was isolated from young leaves following the method described by Yu and Pauls (1994). For each sample, four fresh leaf disks (100 mg) were put into 400 μL of DNA extraction buffer in a 1.5 mL of Eppendorf tube and homogenized with a plastic pestle (Mandel Scientific Company Ltd.). Then 400 μL of 24:1 chloroform/isoamyl alcohol was added to the homogenized solution, vortexed and left at room temperature for 30 min. The homogenate was spun in the tube at 10,500 rpm for 2 min and 350 μL of supernatant were transferred in to a new 1.5 mL of Eppendorf tube. For DNA precipitation, an equal volume (350 μL) of isopropanol was added to the tube, left at room temperature for 5 min and then spun at 11,000 rpm for 5 min. Then, the DNA pellet was air–dried at room temperature for 30 to 60 min before it was dissolved in 200 μL of DNase–free dH_2O at 4 ˚C overnight. The supernatant was collected after centrifugation at 1,300 rpm for 2 min, yielded about 25 ng μL^{-1} of DNA.

PCR amplification and product electrophoresis

PCR amplifications were performed in 25 μL of reaction mixture containing 5 μL of genomic DNA (5 ng), 1 μL of dNDP (2.5 mM), 2.5 μL of 10×buffer, 0.5 μL of Tag polymerse of 5 U μL^{-1} (TaKaRa), 0.5 μL of P–primer (50 ng μL^{-1}), 0.5 μL of M–primer (50 ng μL^{-1}) using SSR primer pair shown in Table 2, 15 μL of dH_2O. The PCR amplification conditions were programmed as one cycles of denaturation at 72 ˚C for 5 min followed by 35 cycles amplification with 3 min denaturing at 95 ˚C, 1 min at 95 ˚C, 1 min at 55 ˚C, 2 min at 72 ˚C. PCR reaction was performed using the GeneAmp® PCR System 2700 (ABI, U.S.A).

Genetic analysis

All 34 lines were used to screen the SSR primers

Table 1. Lines and their cross combination of the 34 colored rice used in the study

Lines	Primer	Lines	Primer
Check	Heukjinjubyeo	CNU72	Jekminamgeoma×Ou349, F_7
CNU01	Dohoku149, M_6	CNU73	Jekminamgeoma×Ou349, F_7
CNU02	Dohoku149, M_6	CNU74	Jekminamgeoma×Ou349, F_7
CNU30	Dohoku149, M_6	CNU76	Jekminamgeoma×Ou349, F_7
CNU33	Suwon451×Milyang152, F_7	CNU77	Ou349×Suwon451, F_7
CNU39	Suwon451×Milyang152, F_7	CNU80	Suwon425×Killimheukmi, F_7
CNU40	Heuknambyeo×Heukjinjubyeo, F_7	CNU81	Suwon425×Killimheukmi, F_7
CNU41	Heuknambyeo×Heukjinjubyeo, F_7	CNU83	Suwon425×Killimheukmi, F_7
CNU43	Heuknambyeo×Heukjinjubyeo, F_7	CNU85	Suwon425×Killimheukmi, F_7
CNU44	Heuknambyeo×Heukjinjubyeo, F_7	CNU86	Suwon425×Killimheukmi, F_7
CNU45	Heuknambyeo×Heukjinjubyeo, F_7	CNU87	Suwon425×Killimheukmi, F_7
CNU46	Heuknambyeo×Heukjinjubyeo, F_7	CNU88	Suwon425×Killimheukmi, F_7
CNU48	Heuknambyeo×Heukjinjubyeo, F_7	CNU90	Suwon425×Killimheukmi, F_7
CNU50	Heuknambyeo×Heukjinjubyeo, F_7	CNU91	Suwon425×Killimheukmi, F_7
CNU52	Heuknambyeo×Heukjinjubyeo, F_7	CNU93	Suwon425×Killimheukmi, F_7
CNU54	Heuknambyeo×Heukjinjubyeo, F_7	CNU94	Suwon425×Killimheukmi, F_7
CNU71	Jekminamgeoma×Ou349, F_7	**Total**	**34 Lines**

Table 2. SSR primer pairs, their sequences and repeat sequence used in this study

Primer pair	Sequence	Repeat sequence
RM 503	CACCTTTCACACACACACAC// GCCCCACTAACAAAACCAAG	(CA)59, (TA)26
RM 504	TCTATAATGTAGCCCCCCCC// TTTCAGGGGCTTCTACCAAC	(CA)9
RM 528	GGCATCCAATTTTACCCCTC// AAATGGAGCATGGAGGTCAC	(AGAT)9
RM 577	GCTTTCCCTCTAACCCCTCT// GGATGTACCGCTGACATGAA	(TA)9, (CA)8
RM 579	TCCGAGTGGTTATGCAAATG// AATTGTGTCCAATGGGCTGT	(GA)25
RM 457	CTCCAGCATGGCCTTTCTAC// ACCTGATGGTCAAAGATGGG	(TTAA)5
RM 553	AACTCCACATGATTCCACCC// GAGAAGGTGGTTGCAGAAGC	(CT)10

(Table 2) for PCR amplification and polymorphism. The number of alleles were recorded and the polymorphism information content (PIC) of an SSR locus was calculated as described by Saal and Wricke (1999), based on expected heterozygosity (Hedrick, 1985):

$$PIC = 1 - \sum_{i=1}^{k} P_i$$

Where p_i is the frequency of the i–th allele out of the total number of alleles at an SSR locus, and k is the total number of different alleles for that locus. For phylogenetic analysis, only the data for the polymorphic SSR loci were entered for all DNA samples, and a "1" or "0" was used if an allele was present or absent for a genotype, respectively. The data were analyzed using the computer program TREECON (Van de Peer and De Wachter, 1994). The estimation of genetic distance was based on the method described by Nei and Li (1979).

All 34 lines were clustered by the estimated genetic distance, and phylogenetic tree topology was inferred with the clustering method of the Unweighted Pair Group Method Using Arithmetic Average (UPGMA).

RESULTS AND DISCUSSION

Botanical characteristics of the developed colored rice

The culm length of Heukjinjubyeo as check line was 73.8 cm while that of CNU33 and CNU76 was 93.8 cm and 90.6 cm, respectively, but that of CNU87 was smaller rather than other varieties. Panicle length of check line was 22.3 cm while CNU33 and CNU83 was little longer than check line. The number of spikelet per panicle of check line was 149, that of CNU33 was the highest among 34 CNU lines but that of CNU80 was the lowest as 92. The result of this experiment showed more wide range variation in the spikelet number per panicle. The numbers of effective branches and panicles per plant were more CNU73 and CNU77 than check line but those of CNU74 were smaller than other CNU lines. The check line was the earliest variety compared with other ones in days of heading (Table 3). The 1000 grain weight of the CNU90 and CNU76 was heavier than that of check line, but that of CNU54 was the lightest among 34 CNU lines (Table 4).

Table 3. Agronomic characteristics for the 34 colored rice lines

Lines	Culm length (cm)	Panicle length (cm)	Spikelets per panicle	Secondary rachis branches	Panicles per plant	Effective branches	Days to heading
Check	73.8	22.3	149	11	9	9	91
CNU01	76.8	21.9	97	9	10	9	101
CNU02	79.1	24.6	119	9	11	10	99
CNU30	81.0	22.5	100	11	12	12	115
CNU33	93.8	25.1	169	15	9	9	117
CNU39	81.9	22.9	156	13	9	8	115
CNU40	71.3	19.2	153	12	11	11	117
CNU41	71.2	22.0	142	11	11	11	108
CNU43	66.9	20.0	139	11	12	11	113
CNU44	88.2	22.4	133	13	10	10	107
CNU45	71.9	20.7	138	12	12	12	107
CNU46	74.2	20.8	151	12	10	10	107
CNU48	79.4	22.0	143	12	12	11	112
CNU50	76.8	22.0	126	11	11	9	112
CNU52	73.8	20.7	141	13	10	10	112
CNU54	73.9	23.5	139	12	11	10	112
CNU71	66.1	22.1	165	12	11	11	111
CNU72	74.2	20.8	118	10	10	10	111
CNU73	74.3	25.3	155	13	14	14	111
CNU74	82.3	20.1	162	12	7	7	113
CNU76	90.6	22.2	96	9	9	9	115
CNU77	79.5	21.2	98	9	14	13	108
CNU80	79.2	22.5	92	9	9	8	110
CNU81	79.9	21.7	100	11	9	9	113
CNU83	83.3	25.1	138	13	11	11	113
CNU85	80.4	19.7	137	9	10	10	105
CNU86	66.1	19.3	95	7	8	8	105
CNU87	58.3	21.3	132	11	10	10	108
CNU88	81.9	21.0	103	8	12	12	105
CNU90	72.1	17.9	159	11	9	9	108
CNU91	67.5	22.4	148	10	11	10	108
CNU93	64.2	20.3	149	10	11	10	115
CNU94	80.8	20.0	146	10	9	9	115
Min.	58.3	17.9	92	7	7	7	91
Max.	93.8	25.3	169	15	14	14	117
Mean	76.2	21.7	133	10	10	10	110

Table 4. Yield characteristics in 34 colored rice lines

Lines	1000 grain weight (g)	Ripening Ratio (%)	Grain			Yield (g plant^{-1})
			Length (A)(mm)	Width (B)(mm)	A/B	
Check	22.6	91.4	5.6	2.8	2.0	28.7
CNU01	23.1	94.5	5.8	2.4	2.5	21.9
CNU02	24.8	92.3	6.4	2.7	2.4	29.1
CNU30	24.6	93.6	5.6	3.0	1.9	28.4
CNU33	23.3	89.7	5.7	2.7	2.1	31.8
CNU39	23.9	90.3	5.1	3.0	1.7	30.3
CNU40	21.0	90.1	5.5	2.6	2.1	31.8
CNU41	22.0	89.9	6.0	2.8	2.2	31.0
CNU43	19.2	92.3	5.9	2.6	2.2	29.6
CNU44	21.3	89.8	5.3	2.8	1.9	25.4
CNU45	24.3	91.3	5.6	2.9	1.9	30.6
CNU46	21.5	91.7	5.5	2.5	2.2	29.8
CNU48	22.9	92.3	5.7	2.9	2.0	36.3
CNU50	23.2	93.1	5.4	2.5	2.1	29.1
CNU52	19.0	92.3	5.5	2.5	2.2	25.6
CNU54	19.0	90.3	6.3	2.7	2.4	27.0
CNU71	23.2	90.8	5.8	2.8	2.1	38.2
CNU72	24.9	91.3	5.5	2.8	2.0	26.7
CNU73	22.6	89.9	5.6	2.9	1.9	37.8
CNU74	28.6	92.0	5.4	2.9	1.8	29.8
CNU76	26.9	94.3	5.8	3.0	1.9	23.4
CNU77	27.7	93.3	5.9	2.9	2.0	34.7
CNU80	25.8	91.3	6.1	3.0	2.1	20.2
CNU81	27.0	91.3	6.0	2.9	2.1	23.0
CNU83	25.2	93.5	5.9	3.1	1.9	36.9
CNU85	27.4	95.5	5.7	2.9	1.9	34.7
CNU86	26.0	93.3	5.6	2.9	2.0	18.4
CNU87	27.8	94.4	6.2	2.9	2.2	34.8
CNU88	27.6	92.3	5.6	3.0	1.9	30.6
CNU90	27.1	88.1	5.6	2.9	1.9	34.2
CNU91	25.6	90.3	6.6	2.9	2.3	36.5
CNU93	23.7	90.7	6.0	2.7	2.2	35.2
CNU94	26.2	91.7	5.9	2.8	2.1	31.5
Min.	19.0	87.0	4.8	2.4	1.6	18.4
Max.	27.1	95.7	6.6	3.1	2.5	38.2
Mean	23.6	91.5	5.7	2.8	2.0	32.5

Check line was similar to the mean values of 34 CNU lines in the ripening ratio (%) but CNU85 was little higher than check line, while CNU90 was lowest. Heukjinjubyeo yielded less than CNU71 and CNU73 due to be small in panicles per plant and 1000 grain weight (Moon *et al.*, 1998). One thousand grain weight of CNU74 and CNU87 were 28.6 g and 27.8 g, respectively. However that of CNU52 and CNU54 was lighter than other CNU lines.

Yield per plant in Heukjinju was 28.7 g while that of CNU71 and CNU73 was 38.2 g and 37.8 g, respectively, because these varieties may be higher value in spikelets per panicles and effective branches.

In the ear characteristics, most of colored rice varieties showed easily shattering, while CNU33 was very hard to shattering. Seed shattering is still the major component of yield loss, because shattering before harvest in paddy fields. Understanding the inheritance of the shattering trait is necessary to decrease yield reduction, as the losses have been estimated 26% (Schertz and Boedicker, 1977) to more than 40% (Porter *et al.*, 1994). Simple inheritance trait of one or two dominant gene(s) has been proposed to describe the inheritance of seed shattering (Woods and Clark, 1976; Elliot and Perlinger, 1977).

Awn of almost all these developed varieties was none or short character, while CNU33 and CNU73 have a long awn. The glume color of these developed colored rice lines was brown, black, gold and straw color. The testa color of 34 CNU lines showed three kinds of purple, brown and white, and that of most CNU lines were purple (Table 5).

Number of allelic bands and PIC values

As an indication of polymorphism, the number of allelic band and their frequency at each locus was analyzed. All marker loci were polymorphic and resulted in total of 20 allelic bands among 34 colored rice lines. The number of allelic band per loci ranged from two to five, PIC values for SSR loci ranged from 0.08 (RM457) to 0.67 (RM579) and the average of PIC value is 0.48 (Table 6).

Table 5. Grain characteristics in the 34 colored rice lines

Lines	Shattering	Awn	Glume color	Testa color
Check	Easy	Absent	B F	Purple
CNU01	Easy	Absent	Straw	Purple
CNU02	Easy	Absent	Straw	Purple
CNU30	Easy	Absent	B F	Purple
CNU33	Hard	L & F	Straw	Purple
CNU39	Moderately	Absent	Brown	Brown
CNU40	Moderately	Absent	Black	L B
CNU41	Easy	Absent	Black	Purple
CNU43	Easy	Absent	B S	V P
CNU44	Easy	S & P	Brown	V P
CNU45	Easy	Absent	B F	L B
CNU46	Moderately	Absent	Straw	V P
CNU48	Easy	Absent	Straw	Brown
CNU50	Moderately	S & P	Brown	V P
CNU52	Easy	S & P	Black	Brown
CNU54	Easy	Absent	Brown	V P
CNU71	Easy	S & P	B F	Purple
CNU72	Easy	Absent	Straw	Purple
CNU73	Moderately	L & F	B F	Purple
CNU74	Easy	Absent	B S	V P
CNU76	Moderately	S & P	Straw	Purple
CNU77	Very easy	S & F	Gold	White
CNU80	Very easy	Absent	Straw	Purple
CNU81	Easy	Absent	Brown	V P
CNU83	Moderately	Absent	Black	Brown
CNU85	Easy	S & P	B F	Brown
CNU86	Very easy	Absent	Brown	V P
CNU87	Moderately	S & P	Gold	Purple
CNU88	Easy	S & P	B F	V P
CNU90	Easy	S & P	B F	Purple
CNU91	Moderately	Absent	B F	Purple
CNU93	Easy	Absent	Straw	Purple
CNU94	Easy	S & P	Straw	Purple

S & P: Short and partly, S & F: Short and fully, L & F: Long and fully, B F: Brown furrows, B S: Brown spots on straw, L B: Light brown, V P: Variable purple

Table 6. Numbers of allelic band and PIC values of primers used in SSR analysis in 34 colored rice lines

Primer pair	No. of allelic band	PIC value	Primer pair	No. of allelic band	PIC value
RM 503	3	0.48	RM 579	5	0.67
RM 504	2	0.60	RM 457	2	0.08
RM 528	3	0.52	RM 553	3	0.48
RM 577	2	0.52	Overall mean	3	0.48

Genetic diversity and grouping of 34 colored rice varieties

The presence of genetic diversity in crop populations is not easily detected by morphological characteristics of growing plants. The use of molecular markers is increasingly common method for the detection of differences at the DNA level in crop populations (Meng *et al*., 1998). The primary objective of this study was to assess genetic diversity between CNU lines using SSR markers.

Genetic distance between 34 colored rice lines ranged from 0.28 to 0.67 (Fig. 1). In the UPGMA clustering algorithm, all the 34 colored rice populations were classified into four groups and subdivided into several subgroups (Table 7); in the first group two subgroups were classified (Heukjinju and CNU33 as subgroup I–1,

Table 7. CNU lines classified into groups by SSR analysis

Large group	Subgroup	Lines
I group	I–1	Check (Heukjinju), CNU33
	I–2	CNU80, CNU83
Ⅱ group	Ⅱ–1	4 lines including CNU40
	Ⅱ–2	CNU46, CNU91
	Ⅱ–3	7 lines including CNU45
Ⅲ group	–	CNU1, CNU32, CNU93
Ⅳ group	Ⅳ–1	CNU2, CNU30, CNU44
	Ⅳ–2	CNU43, CNU 76
	Ⅳ–3	5 lines including CNU48
	Ⅳ–4	CNU52, CNU77
	Ⅳ–5	CNU39

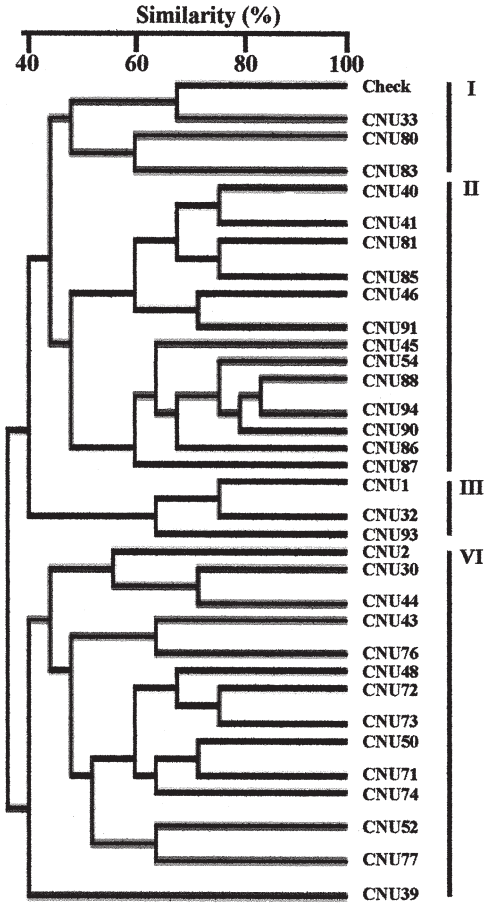

Fig. 1. Cluster analysis according to genetic distance calculated from SSR analysis in 34 colored rice lines.

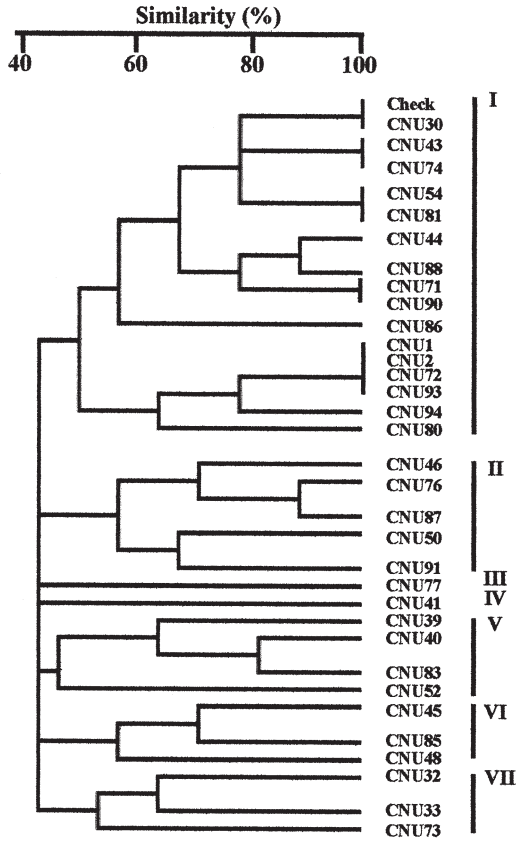

Fig. 2. Cluster analysis according to genetic distance calculated from agronomic characteristics in the 34 colored rice lines.

CNU80 and CNU83 as subgroup I–2); in the second group three subgroups were classified (CNU40, CNU41, CNU81, CNU85, as subgroup II–1, CNU46, CNU91 as subgroup II–2, CNU45, CNU54, CNU88, CNU94, CNU90, CNU86, CNU87 as subgroup II–3); in the third group one subgroup was classified (CNU1, CNU32, CNU93); in the four group five subgroup were classified (CNU2, CNU30, CNU44 as subgroup IV–1, CNU43, CNU76 as subgroup IV–2, CNU48, CNU72, CNU73, CNU50, CNU71, CNU74 as subgroup IV–3, CNU52, CNU77 as subgroup IV–4, CNU39 as subgroup IV–5).

While in cluster analysis according to genetic distance calculated from agronomic characteristics, the 34 colored rice populations were classified into seven groups (Fig. 2); in the first group three subgroups were classified (Check, CNU30, CNU43, CNU74, CNU54, CNU81, CNU44, CNU88, CNU71, CNU90 as subgroup I–1, CNU86 as subgroup I–2, CNU1, CNU2, CNU72, CNU93, CNU94, CNU80 as subgroup I–3); in the second group two subgroups were classified (CNU46, CNU76, CNU87 as subgroup II–1, CNU50, CNU91 as subgroup II–2); in the third group one subgroup was classified (CNU77); in the fourth group one subgroup was classified (CNU41); in the fifth group two subgroups were classified (CNU39, CNU40, CNU83 as subgroup V–1, CNU52 as subgroup V–2); in the sixth group two subgroups were classified (CNU45, CNU85 as subgroup VI–1, CNU48 as subgroup VI–2); in the seventh group two subgroup were classified (CNU32, CNU33 as subgroup VII–1, CNU73 as subgroup VII–2).

The genetic diversity of 34 CNU lines and useful lines

The evaluation of genetic diversity using SSR markers may be very useful not only for breeding programmer, but also for devising strategies for conserving and managing colored rice germplasm. The genetic distance in colored rice lines showed little different between agronomic characteristics and SSR analysis. In SSR analysis, they divided into 4 large groups (Fig. 1), but had 7 large groups according to genetic distance calculated from agronomic characteristics (Fig. 2). Relationships between colored rice will be a good indicator for breeders who are interested in developing new cultivars or improving colored rice populations. Information on genetic diversity within CNU lines may be useful in breeding program for line development and find linkage relationship. CNU71, CNU73, CNU83, CNU91 and CNU48 lines showed useful lines for high yields in 34 colored rice lines.

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