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Genetic Variability Assessment in Irrigated Rice (*Oryza sativa* and *Oryza glaberrima*) by PCR-SSR in Mali

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Abstract: Rice species (*Oryza sativa, L.* and *Oryza glaberrima, Steud*) provide 20% energy of world's food. Knowledge of genetic variability is important to remove duplicate materials for gene bank management and conservation. In this study, 59 microsatellite markers were used to assess the genetic diversity of 54 intraspecific (*Oryza sativa*) and interspecific (*Oryza sativa* X *Oryza glaberrima*) irrigated rice varieties by PCR-SSR. A total of 250 alleles were detected with an average of 4.24 alleles per SSR. Genetic diversity was ranged from 0.0713 (RM333, RM3744) to 0.8937 (RM251) to with an average of 0.4325. Polymorphism Information Content (PIC) varied from 0.0688 (RM333, RM3744) to 0.8854 (RM251) with an average of 0.3940. Rice genotypes were shared between five Groups based on their similarity with majority of them (64.81%) under Group V. Variety SK 7-8 within Group III was highly dissimilar to other varieties. Malian varieties were shared out between Groups II, III, VI and V. Strong genetic variability was observed within cluster V_2 of Group V among the most cultivated and appreciated rice varieties such as Kogoni 91-1, Adny11 and BG90-2. Allelic variability observed among rice varieties could help breeding programs to identify cultivars with good agronomic traits for crop improvement.

Keywords: Genetic Diversity, Irrigated Rice, PCR-SSR, Mali

1. Introduction

Rice, species (*Oryza sativa*, L. and *Oryza glaberrima*, Steud) belonging to the family Graminae and subfamily Oryzoidea, is the staple food crop for one third of the world's population and occupies almost one-fifth of the total land area covered under cereals [1] and constitute one of the most calories sources in Africa [2]. In Mali, its consumption is 81.61 kg/person/year and represents approximately more than 30% of total cereal consumption [3]. Rice benefits a high genetic diversity [2] with more than 150 000 cultivated varieties worldwide and approximately 107 000 accessions kept within the gene bank of the International Rice Research Institute (IRRI) [4]. This diversity is based on natural or artificial crosses of *Oryza sativa* with wild species *Oryza rufipogon*, *Oryza sativa* (intra crosses) and *Oryza glaberrima* (inter crosses) [4; 5]. *Oryza sativa* can be divided into two main groups: *a* group, mainly grown in temperate and tropical highlands zones and *indica* group, particularly well adapted to tropical conditions [6]. The last group constitutes irrigated or lowland rice [7]. Since the beginning of rice cultivation in hydro-agricultural areas conditions, *indica* group is used in Malian breeding program to create irrigated rice varieties [8] related to yield, grain quality and earliness [9]. Recently, some important agronomic and genetic traits on *indica* irrigated rice varieties were selected for water supply regime [10], organoleptic quality and amylose content (20 - 25%) [10], physiological characteristics and tolerance or resistance to biotic and abiotic stress [8; 9]. The irrigated *indica* group represents more than 80% of Malian irrigated rice varieties [8]. Genetic diversity in plants has been traditionally assessed using morphological or physiological

traits [11]. Knowledge of genetic diversity allows better conservation and parsimonious use of raw materials in a perspective of varietal innovation [12]. On the other hand, identified genetic variations based on DNA polymorphism are abundant and independent of environmental factor. DNA markers that differentiate genotype are more reliable and convenient than physiological or morphological characters in the identification and characterization of genetic variation [13]. The genetic variation, through morphological and molecular markers, may be useful in breeding for abiotic stress [14].

According to Demol *et al.* [15], high genetic diversity is essential for plant improvement. Consisting of a few base pairs repeated in tandem on the genomes of most eukaryotic species [16], SSR markers were realized to be the suitable one for genetic variability assessment [17-19]. In contrast to other molecular markers such as Allozymes, Amplified Fragment Length Polymorphisms (AFLPs), Sequential Loci and Single Nucleotide Polymorphisms (SNPs), SSRs were found to be the choice of rice genetic diversity markers due to their multiallelic, co-dominant and widely distributed throughout the genome and their reproducibility [16]. In addition, they are widely used in molecular maps construction, gene mapping and genetic purity test [20; 21; 22]. McCouch *et al.* [23] developed and mapped 2 240 SSR markers for *Oryza sativa* L. and more than 20 000 SSR markers were extracted from the genomic sequence of *Oryza sativa* L., *japonica* cv. *Nipponbare* and are accessible to the public [24]. Several studies of genetic diversities were assessed around the world using microsatellites: 35 SSRs exploited to analyze the variability among 30 rice varieties in India [25], 80 rice genotypes including 52 *japonica* and 28 *indica* evaluated with 114 microsatellite primers in Taiwan [26], 150 varieties in the Ting rice germplasm collection genotyped with 274 SSR markers for identification of the *indica* and *japonica* subgroups [27]. Similar works have been done by Watanabe *et al.* [28] and Vazirzanjani *et al.* [29], respectively in Myanmar and Iran. GOITA [14] used 10 SSRs to analyze the genetic diversity of 26 rice genotypes from Mali for alkalinity tolerance. The objective of this study is to reveal the genetic relationship between 54 irrigated rice varieties based on phylogenetic tree through 59 SSR markers.

2. Methods and Materials

Fifty-four (54), composed of 47 intraspecific (*Oryza sativa*) and 7 interspecific (*Oryza sativa* and *Oryza glaberrima*) irrigated rice varieties from the gene bank of irrigated rice Program of Institute of Rural Economy were evaluated (Table 1). Among them, 31 were registered and described in Official Catalog of Species and Varieties in 2013, whereas 23 are still under process for being approved. Varieties were randomly selected within the rice germplasm and seeds were kept at - 4°C at the Research Laboratory in Microbiology and Microbial Biotechnology.

Table 1. *List of irrigated rice varieties.*

No	Name	Varietes types	Sources
1	BG 90-2	*O. sativa indica*	SRILANKA
2	Kogoni 91-1	*O. sativa indica*	Mali
3	Adny11	*O. sativa indica*	SIERA LEONE
4	SK 20-28	*O. sativa indica*	Mali
5	SK 16-25	*O. sativa indica*	Mali
6	NERICA-L-1-IER	*O. sativa X O. glabberima*	Mali
7	NERICA-L-2-IER	*O. sativa X O. glabberima*	Mali
8	Sambalamalo	*O. sativa indica*	Mali
9	Sutura	*O. sativa indica*	AFRICA RICE
10	WAPMO	*O. sativa indica*	AFRICA RICE
11	WAS 127 12-1-2-3	*O. sativa indica*	AFRICA RICE
12	ARICA 10	*O. sativa indica*	AFRICA RICE
13	WAS 203B-B-1	*O. sativa indica*	AFRICA RICE
14	Saku	*O. sativa indica*	AFRICA RICE SAINT LOUIS
15	WAS 73-B-B-231-1-4	*O. sativa indica*	AFRICA RICE SAINT LOUIS
16	IR 76345-B-B-1-1-1	*O. sativa indica*	AFRICA RICE SAINT LOUIS
17	ARICA 9	*O. sativa indica*	AFRICA RICE
18	Yiriwamalo	*O. sativa indica*	AFRICA RICE
19	Nenekala	*O. sativa indica*	AFRICA RICE
20	Wassa	*O. sativa indica*	IRRI/PHILLIPINES
21	Nio 1-14-34-2	*O. sativa X O. glabberima*	Mali
22	Nio 1-31-29-2	*O. sativa X O. glabberima*	Mali
23	Nio 2-31-37-4	*O. sativa indica*	Mali
24	Nio 1-14-32-1	*O. sativa X O. glabberima*	Mali
25	Nio 1-12-62-1	*O. sativa X O. glabberima*	Mali
26	Nio 2-19-30-1	*O. sativa indica*	Mali
27	Nio 1-39-15-1	*O. sativa X O. glabberima*	Mali
28	Gambiaka Kokoun	*O. sativa indica*	Mali

No	Name	Varietes types	Sources
29	BH2	*O. sativa indica*	Mali
30	Telimani	*O. sativa indica*	CHINE
31	FROX 521-146-H1	*O. sativa indica*	AFRICA RICE SAINT LOUIS
32	WAB2081-WAC-2-2-	*O. sativa indica*	AFRICA RICE SAINT LOUIS
33	Sahel 317	*O. sativa indica*	AFRICA RICE SAINT LOUIS
34	Sahel 328	*O. sativa indica*	AFRICA RICE SAINT LOUIS
35	Sahel 329	*O. sativa indica*	AFRICA RICE SAINT LOUIS
36	CT117130-M-1-2-4-1-2-M	*O. sativa indica*	AFRICA RICE SAINT LOUIS
37	IR 77542-520-1-1-1-1-3	*O. sativa indica*	AFRICA RICE SAINT LOUIS
38	IR 78585-64-2-4-3	*O. sativa indica*	AFRICA RICE SAINT LOUIS
39	IR 78944-B-8-B-B-B	*O. sativa indica*	AFRICA RICE SAINT LOUIS
40	IR 82489-7-2-2-2	*O. sativa indica*	AFRICA RICE SAINT LOUIS
41	NSIC RC 152	*O. sativa indica*	AFRICA RICE SAINT LOUIS
42	UPL RI 7	*O. sativa indica*	AFRICA RICE SAINT LOUIS
43	YN 2610 -2-2-2-1-2-1	*O. sativa indica*	AFRICA RICE SAINT LOUIS
44	IR 82589 –B-63-2	*O. sativa indica*	AFRICA RICE SAINT LOUIS
45	IR 78937 –B-B-B	*O. sativa indica*	AFRICA RICE SAINT LOUIS
46	HHZ5-Y 3-S A L3-DT1	*O. sativa indica*	AFRICA RICE SAINT LOUIS
47	IR78877-048-B-B-3	*O. sativa indica*	AFRICA RICE SAINT LOUIS
48	RP 4075-135-35-5	*O. sativa indica*	AFRICA RICE SAINT LOUIS
49	Nionoka	*O. sativa indica*	INDE-Mali
50	Shwetasoké	*O. sativa indica*	South America
51	Sahelika	*O. sativa indica*	CUBA-Mali
52	SK 7-8	*O. sativa indica*	Mali
53	Gigante	*O. sativa indica*	AFRICA RICE
54	Seberang MR 77	*O. sativa indica*	MALAYSIA

2.1. DNA Extraction

Different genotypes were sown into the pots filled with 10 kg sterilized mold and their young leaves were collected two weeks after sowing and stored at -20°C. Five centimeters (5 cm) of the leaves from each sample were crushed using a pestle in a 1.5 ml Eppendorf tube containing 750 µl of extraction buffer (6.5 ml of PanReac AppliChem pure water, 1 ml of Tris base 1M pH8, 1.4 ml of 5M NaCl, 1 ml of 0.5M EDTA pH8 and 0.4 g of HTAB Merck). The mixture was incubated at 65°C for 30 minutes (min) in the GRANT GD 120 water bath before adding 400 µl of chloroform. The mixture was centrifuged with the EPPENDORF 5424 centrifuge for 20 minutes at 12 000 rpm. The supernatant was recovered and transferred into a new 1.5 ml Eppendorf tube and precipitated with 1 ml absolute ethanol (100%). The mixture of supernatant-ethanol was incubated at -20°C for 10 min in the LIEBHERR freezer and then centrifuged with the Eppendorf 5424 centrifuge at 12 000 rpm for 4 min. The resulting pellets (DNA) were washed twice with 70% ethanol by centrifugation at 10 000 rpm for 30 seconds and dried it for two (2) hours under room temperature. Fifty microliters (50 µl) of 0.5 M Tris EDTA (TE) were added to dissolve the DNA. One microliter (1 µl) Promega RNase was mixed in the diluted DNA and incubated at 37°C for 15 min. The DNA concentration was determined using the Eppendorf spectrophotometer. The DNAs were diluted into 20 ng / µl and stored them at -20°C.

2.2. DNA Amplification: PCR (Polymerase Chain Reaction)

Extracted DNA samples were amplified using 59 pairs of SSR selected according to their position on the 12 pair's chromosomes molecular weight and PIC [26] markers (Table 2). Additional information on these SSRs can be found on Gramene site (http://www.gramene.org) and from article published by McCouch *et al.* [23]. PCR mixture made up to 15 µl was composed of 7.5 µl Go Taq G2 Green Start Green Master Mix 2X (Promema), 1 µl (100pmol / µl) of the primer forward, 1 µl (100pmol / µl) of the reverse primer, 2µl (20ng / µl) and 3.5µl of Nuclease-Free Water. Amplification was done using the 2720 applied thermocycler System programmed as follows:

a) Initial denaturation: 94°C for 5:00 min;
b) Final denaturation: 94°C for 1:00 min;
c) Hybridization: 55°C for 0:45 seconds;
d) Initial elongation: 72°C for 2:00 min;
e) Final elongation: 72°C for 10:00 min; and
f) Conservation: 4°C forever.

The denaturation, hybridization and elongation steps were repeated 35 times.

2.3. Electrophoresis and Visualization of PCR Products

Ten microliters (10µl) of PCR products were run on 3% (w/v) of CONDA MS-4 agarose (recommended for separation of DNA fragments smaller than 500pb) at 80V for 150 minutes. The gel was prepared with 0.5X TBE AppliChen Buffer Solution (Tris, Acid Borate, EDTA), 30µl of ethidium bromide (1mg/ml). Gel visualization was done

using UV photographed with E-BOX VX2 Gel Chamber Black Gel Analyzer version 15.06.

2.4. Data Analysis

Band (DNA fragments) sizes were determined in base pair with the E-Capt software version 15.06 compared to the standard Promega 50 bp DNA Step Ladder marker. Most polymorphic markers were selected after screening and scored based on the presence (1) and absence (0) of the band. Diversity among rice collection was assessed with Power Marker software version 3.25 [30; 26; 31] to determine allele frequency, number of alleles, genetic diversity and Polymorphism Information Content (PIC) [30; 32]. Dendrogram was generated with Power Marker Version 3.25 [33] using UPGMA (Un-weighted Pair-Group Method with Arithmetic mean) method based on Nei's genetic distance. MEGA software Version 7 was applied to edit the resulting map of the dendrogram [34].

3. Results and Discussion

3.1. Polymorphism Level of Molecular Markers

High genetic variability was observed between Irrigated rice genotypes. The 59 pairs (forward and reverse) primers generated a total of 250 alleles across the 54 rice genotypes with an average of 4.24 per locus (Table 2). Number of alleles per locus ranged from 2 to 9 and 25.42% of the SSR markers amplified 3 alleles per locus.

Table 2. *Genetic diversity parameter values of SSR (simple sequence repeat) loci.*

Marker	Nomber of alleles	Allele No	Major Allele Frequence	Gene Diversity	PICa
RM243	6	3	0.5926	0.5219	0.4321
RM246	2	3	0.463	0.5919	0.3311
RM3252	6	8	0.3148	0.8107	0.7869
RM1	5	3	0.7593	0.3807	0.3391
RM580	6	4	0.9259	0.1406	0.1371
RM009	2	3	0.7963	0.3409	0.309
RM5	3	2	0.9259	0.1372	0.1278
RM5780	4	5	0.537	0.6159	0.5569
RM211	6	5	0.4444	0.6948	0.6464
RM341	6	6	0.6111	0.583	0.5493
RM5356	5	2	0.7407	0.3841	0.3103
RM324	5	4	0.463	0.6509	0.586
RM6038	2	2	0.9074	0.168	0.1539
RM251	9	19	0.2037	0.8937	0.8854
RM218	3	4	0.5556	0.5988	0.5376
RM551	3	5	0.4444	0.6907	0.6398
RM255	3	2	0.7222	0.4012	0.3207
RM252	8	7	0.6481	0.5439	0.5131
RM518	4	2	0.8704	0.2257	0.2002
RM5687	8	6	0.463	0.6626	0.6035
RM1359	6	7	0.7222	0.4547	0.4298
RM273	5	5	0.6852	0.4842	0.4403
RM1024	4	4	0.8704	0.2359	0.226
RM430	5	3	0.9259	0.1399	0.1352
RM3838	3	2	0.9259	0.1372	0.1278
RM584	3	4	0.463	0.6104	0.5322
RM508	3	4	0.6111	0.5569	0.5026
RM276	3	4	0.4259	0.6646	0.5991
RM190	3	4	0.6852	0.4966	0.464
RM225	4	3	0.5926	0.5631	0.4997
RM253	6	3	0.963	0.072	0.0707
RM125	5	5	0.4815	0.6269	0.557
RM214	3	4	0.9259	0.1406	0.1371
RM010	6	3	0.7593	0.3738	0.3164
RM18	4	3	0.9259	0.1399	0.1352
RM025	3	7	0.3519	0.7538	0.7167
RM072	5	7	0.463	0.6818	0.6321
RM152	6	6	0.5556	0.62	0.5744
RM331	5	6	0.8519	0.2689	0.2609
RM515	3	4	0.7593	0.3985	0.3693
RM223	2	2	0.9074	0.168	0.1539
RM257	2	3	0.9444	0.1063	0.1033
RM242	3	3	0.9259	0.1399	0.1352
RM3744	6	2	0.963	0.0713	0.0688
RM3912	2	2	0.8889	0.1975	0.178
RM278	2	2	0.8519	0.2524	0.2205
RM258	9	3	0.5926	0.4973	0.3907

Marker	Nomber of alleles	Allele No	Major Allele Frequence	Gene Diversity	PICa
RM222	4	5	0.4444	0.6893	0.638
RM1375	6	3	0.7593	0.3807	0.3311
RM333	3	2	0.963	0.0713	0.0688
RM496	2	2	0.8148	0.3018	0.2562
RM167	4	3	0.963	0.072	0.0707
RM286	4	6	0.3889	0.7435	0.7056
RM206	8	10	0.4815	0.7167	0.6911
RM287	2	3	0.7222	0.4355	0.3902
RM21	2	3	0.4815	0.6337	0.5624
RM101	3	5	0.5185	0.5631	0.4721
RM17	3	5	0.4815	0.6715	0.6218
RM235	2	4	0.7963	0.3512	0.3323
Mean	4.24			0.4325	0.394

a.: Polymorphic Information Content

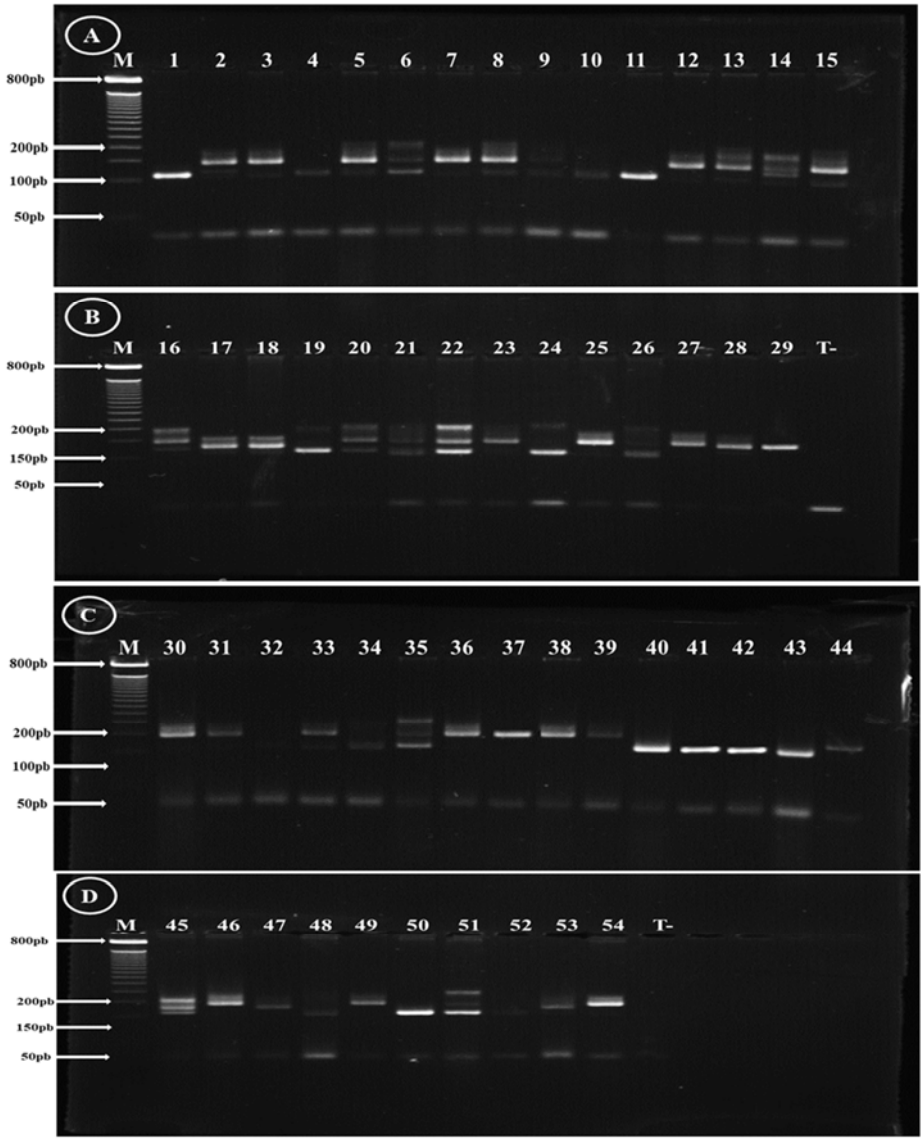

Figure 1. *PCR product profils from Irrigated rice varieties with RM251 SSR makers on Agarose gel MS-4. A (1: BG 90-2, 2: Kogoni 91-1, 3: Adny11, 4: SK 20-28, 5: SK 16-25, 6: NERICA-L-1-IER, 7: NERICA-L-2-IER, 8: Sambalamalo, 9: Sutura, 10: WAPMO, 11: WAS 127 12-1-2-3, 12: ARICA 10, 13: WAS 203B-B-1, 14: Saku, 15: WAS 73-B-B-231-1-4), B (16: IR 76345-B-B-1-1-1, 17: ARICA 9, 18: Yiriwamalo, 19: Nenekala, 20: Wassa, 21: Nio 1-14-34-2, 22: Nio 1-31-29-2, 23: Nio 2-31-37-4, 24: Nio 1-14-32-1, 25: Nio 1-12-62-1, 26: Nio 2-19-30-1, 27: Nio 1-39-15-1, 28: Gambiaka Kokoun, 29: BH2, T-: témoin négatif) C (30: Télimani, 31: FROX 521-146-H1, 32: WAB 2081-WAC-2-2-TGR2-WAT1-1, 33: Sahel 317, 34: Sahel 328, 35: Sahel 329, 36: CT 117130-M-1-2-4-1-2-M, 37: IR 77542-520-1-1-1-1-3, 38: IR 78585-64-2-4-3, 39:IR 78944-B-8-B-B-B, 40: IR 82489-7-2-2-2, 41:NSIC RC 152, 42:UPL RI 7, 43:YN 2610 - 2-2-2-1-2-1, 44: IR 82589 –B-63-2), D (45: IR 78937 –B-B-B, 46: HHZ5-Y 3-S A L3-DT1, 47: IR78877-048-B-B-3, 48: RP 4075-135-35-5, 49: Nionoka, 50: Shwetasoké, 51: Sahelika, 52: SK 7-8, 53: Gigante, 54: Seberang MR 77: T-: témoin négatif.).*

Markers RM251 (Figure 1) and RM258 revealed the highest number of alleles (9 alleles) respectively on chromosome 3 and 10. Allele frequency was between 0.2037 (RM251) and 0.9630 (RM253, RM3744, RM333 and RM167). The Polymorphic Information Content (PIC) representing allele diversity for specific locus ranked from 0.0688 (RM333, RM3744) to 0.8854 (RM251) with an average of 0.3940. According to Botstein *et al.* [32], the marker is highly informative if the PIC value is > 0.5, reasonably informative if it is between 0.25 and 0.5 (0.5> PIC> 0.25) and slightly informative if the PIC value is < 0.25. Based on that, 28.81% of study's SSR markers were highly informative, 40.68% reasonably informative and 30.51% slightly informative. Markers RM333 and RM3744 scored the highest genetic diversity (0.8937) whereas the lowest (0.0713) was attributed to marker RM251 with an average of 0.4325. Diversity study with Marker Assisted Selection (MAS) is important since it allows removing duplicate materials for gene bank management and conservation and also selection of raw materials for improvement of landraces susceptible to constraints [16; 10].

The average PIC and allele values obtained were significantly lower than that of Kanawapee *et al.* [35] who evaluated the genetic diversity of 30 *indica* varieties from Thai, Indian and IRRI with 20 SSRs. However, 99% of SSR markers used in that study was highly informative including the four markers (RM1, RM5, RM025 and RM206) used in common. The difference between both studies could be linked to the large rice samples (30 rice varieties) and markers (20) used in Kanawapee *et al.* [35] work. Zhang *et al.* [27] obtained an average of genetic diversity (0.484) close to our study by assessing 118 *indica* rice varieties collected in 20 provinces of China with 274 SSRs. These different works confirmed the diversity of irrigated rice varieties according to breeding programs. Other genetic diversity studies were conducted by Lin *et al.* [26] and Zhu *et al*. [36] on both groups of *Oryza sativa* (*indica* and *japonica*) revealing mean PICs of 0.43 and 0.31, respectively. However, seven interspecific varieties were included in the samples studied. Majority (96%) of SSR markers in this work were used by Lin *et al.* [26]. These markers showed different PIC except RM225. The RM1 and RM17 markers also revealed the same number of alleles with current study and the assessment done by Pervaiz *et al.* [37] with 75 rice varieties in Pakistan.

3.2. Genetic Distance

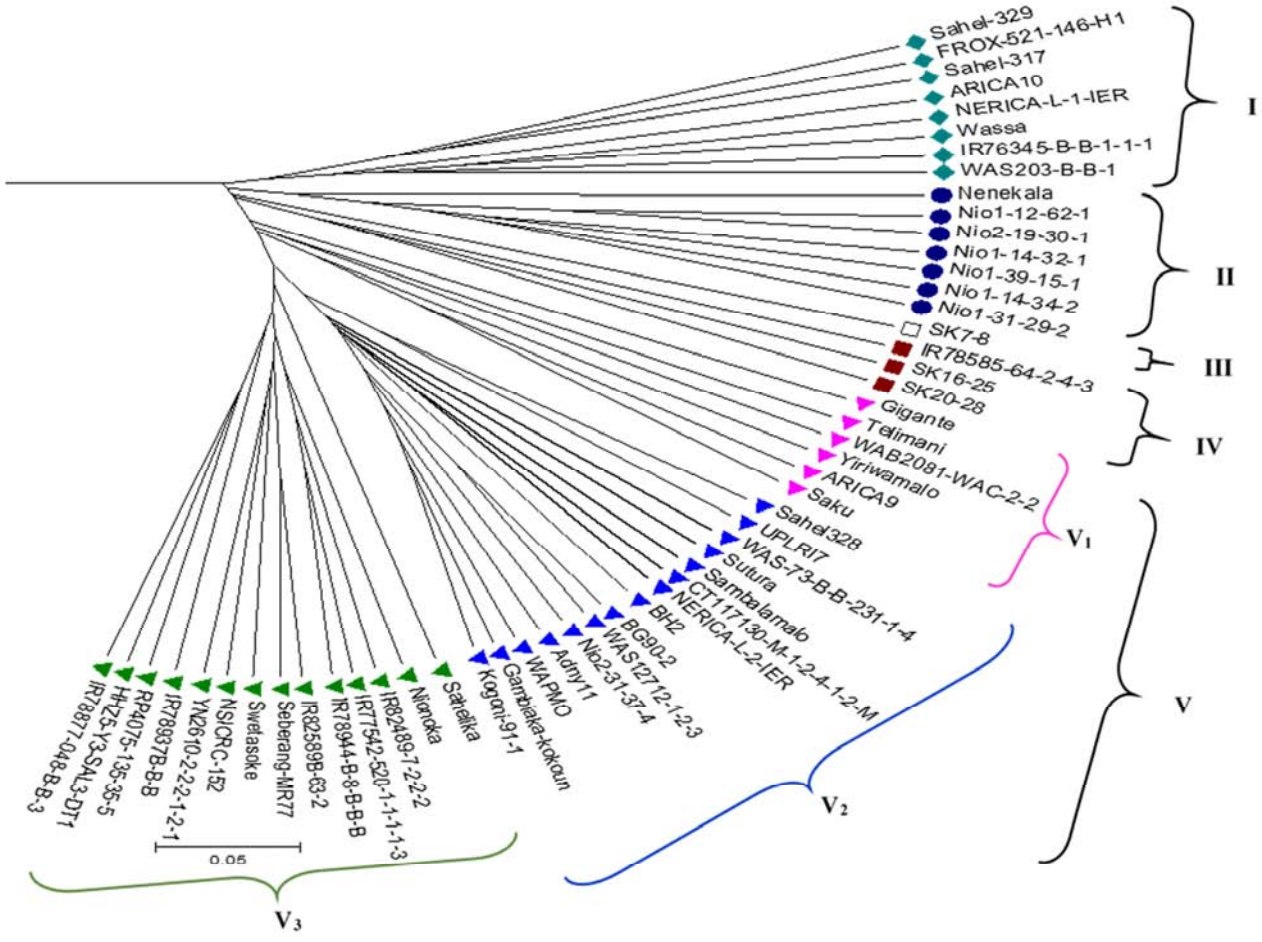

Figure 2. *Dendrogram generated from 59 SSR markers amplified 54 irrigated rice varieties based on UPGMA (unweighted pair-group method with averages).*

Dendrogram generated from 59 SSR markers shared out the 54 Irrigated rice varieties into 5 major Groups (Figure 2). Most study materials (64.81%) were under Group V which was divided into 3 clusters (V_1, V_2 and V_3). The variety SK 7-8, under Group III, recorded the highest degree of dissimilarity compared to the whole study materials. The most cultivated rice varieties in "Office du Niger" zone such as Kogoni91-1, BG90-2, Adny 11 showed strong genetic similarity between them and were within cluster V_2 of Group V. Varieties from Institute of Rural Economy showed high genetic diversity and were shared between Groups II, III, IV and V sometimes with their parents. For instance, Kogoni 91-1 generated from cross between Gambiaka kokoum x IR 36 and Gambiaka Kokoum have strong genetic similarity. Varieties SK 20 28 from cross Kogoni91-1 x Gigante x IR and SK16 25 from between cross Kogoni91-1 x Gigante were highly dissimilar to their donor parents Kogoni 91-1 and Gigante. However, these two varieties had high genetic similarity (cluster V1) due to two common parents. More than 40% of Malian rice varieties were within Group II, which consisted of five inter-specifics and two intra-specifics rice varieties with both donor parents 1AR7 and 2AR6. The results of current study corroborate with those of by Lin *et al.* [26] who indicated strong similarity within *indica* group. Likewise, Zhang *et al.* [17] obtained five subgroups (I, II, III, IV and V) based on genetic similarity analysis of 137 *indica* genotypes.

4. Conclusion

Current study revealed low diversity level among Malian rice landraces using SSR markers especially those developed by rice breeding program of Institute of Rural Economy. High level of dissimilarity was between SK-7-8 variety and the remaining of study materials based such medium maturing, high yielding and resistant to rice yellow mottle virus (RYMV); and also between varieties SK 16-25 and SK 20-28 and most of rice genotypes based on 1000 seed weight and plant height. These varieties could be used in breeding program to improve varieties with low yielding, late maturing, low height and low number of panicle characters. This study has backed up previous information recorded on morphological traits of the same rice varieties by rice breeding program of Institute of Rural Economy.

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