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Genetic diversity of rice *(Oryza sativa)* germplasm from six countries using simple sequence repeats markers

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This experiment was carried out to determine the genotypic variation among rice *(Oryza sativa)* accessions using simple sequence repeats (SSR) markers. In the present study, a total of 12 SSR markers were used across 87 rice accessions from six countries. NTSYS-pc and PowerMarker software were used for data analysis. Six primers out of these 12 primers showed DNA amplification and polymorphism among the 87 rice accessions. The number of alleles detected by these six primers ranged from 2 to 9 with an average of 6.83 while polymorphism information content (PIC) ranged from 0.34 to 0.79 with an average of 0.55. The unweighted pair group method with arithmetic averages (UPGMA) cluster dendrogram generated based on the six SSR markers grouped the accessions into 4 clusters with 41% similarity coefficient. Accessions from these four clusters have late maturity, green basal leaf sheath colour, no awn and fewer tillers, respectively. This experiment has proven that even a small number of SSR markers are effective in assessing genetic diversity in rice. The genetic diversity revealed by the SSR markers in this study would be very important to select potentially good genotypes for future rice improvement programmes.

Key words: Dendrogram, genetic diversity, molecular markers, rice.

INTRODUCTION

Rice (*Oryza sativa* or *Oryza glaberrima*) is consumed by more than 50% of the world's population especially in developing countries. In terms of production levels, it is the third highest cereal after wheat and maize (FAOSTAT, 2012). By the year 2025, global demand for rice will be 880 million tonnes which is 70% more of the present world production (IRRI, 2010). The average growth rate of rice yield was 3.68% annually in the 1980s, but it decreased to 0.75% per year in the late 1990s (Nguyen and Ferrero, 2006). Besides, the plateauing of yields, other challenges that could limit increased rice production include biotic and abiotic stresses, declining productivity in intensive rice production systems, increasing cost of production, and increasing public

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concern for the protection of natural resources (Nguyen and Ferrero, 2006). In years to come, expanding the areas of rice cultivation will be limited because of land and water resource scarcity due to climate change, urbanization and population growth especially in Asia where more than 50% of the world rice is produced (Devi and Ponnarasi, 2009).

One of the major ways of addressing the issues affecting rice production and also increasing the average yield of this rice crop is through breeding for rice varieties that produce higher yields per unit land area, and also meeting the world's rice requirements will depend upon the development of high yielding genotypes that have resistance against biotic and abiotic stresses using conventional and biotechnological approaches (Paterson et al., 2005).

The effectiveness of any rice breeding programme depends on the utilization of different germplasm stock available in research organizations/institutes around the world (Susan et al., 2012). This will enable breeders to evaluate and select desirable varieties for breeding programmes.

There have been extensive efforts by rice breeders around the world to improve the quantity and quality of rice by crossing varieties. These efforts have produced many rice varieties. It is foreseen that more will follow as environmental conditions and consumers' desires are changing. However, for breeding efforts to be successful, the genetic resources available to plant breeders need to be assessed accurately using molecular markers (Meti et al., 2013). In contrast to morpho-agronomic traits, molecular markers can reveal differences existing among genotypes at the DNA level. Molecular markers have been proven to be very objective and independent of environmental conditions (Se-Jong et al., 2012). Therefore, the aim of this study was to evaluate the level of genetic diversity among 87 rice accessions from six countries using simple sequence repeats markers.

MATERIALS AND METHODS

Plant and DNA isolation

A total of 87 accessions of rice germplasm from Ghana (CRI and SARI), Thailand, Mali, Benin (Africarice), Cameroon and Philippines (IRRI) were collected and used in this study (Table 1). Genomic DNA was extracted from young fresh leaves of three weeks old plants of the 87 rice accessions using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990). Samples were stored at 4°C until it was required for use.

Molecular markers and polymerase chain reactions

Twelve simple sequence repeats markers (SSR) covering 10 of the 12 chromosomes of rice were used to detect polymorphism among the rice accessions (Table 2). The SSRs markers were procured from Metabion International AG (Germany). Polymerase chain reaction (PCR) was carried out in Eppendorf mastercycler (Eppendorf, Hamburg, Germany) of 96-well plates. PCR kits (KAPA 3G Fast Ready Mix) procured from KAPA Biosystems (Pty) Ltd (South Africa) was used for the reaction. Total volume of 15 µl with final concentrations of 1 X KAPA Plant PCR Buffer + dNTPs and 1.5 mM $MgCl_2$, 0.3 µM forward and reversed primers, 0.1 X KAPA Plant PCR Enhancer, 1 U/µl KAPA 3G Plant DNA Polymerase and 10 µg/µl of crude DNA.

PCR amplification was subjected to initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 1 min and a final extension at 72°C for 7 min. The reactions (PCR products) were then held at 4°C until electrophoresis.

Gel electrophoresis

PCR products obtained were electrophoresed using 2% agarose gel stained with ethidium bromide solution. The tracking dye in the PCR premix (KAPA 3G) made visual tracking of the PCR products through the gel. Approximately, 10 µl of the amplified products and 5 µl 100 bp molecular ladder (Universal ladder) obtained from KAPA Biosystems (Pty) Ltd (South Africa) were electrophoresed at 120 V for 120 min using Galileo Bioscience (81 to 2325) tank. A control was loaded in the first well and the molecular ladder was loaded into well two and the DNA of the rice accessions were loaded in the adjacent wells. The gel was visualized by illumination on Benchtop UV transilluminator. The gels were photographed under UV light.

Scoring of DNA bands

Amplified polymorphic products from microsatellite analyses were scored qualitatively for absence (0) and presence (1) for each marker allele-genotype combination.

SSR data analysis

The number of alleles per locus, major allele frequency, gene diversity, heterozygosity and polymorphism information content (PIC) values were all calculated using PowerMarker version 3.25 (Liu and Muse, 2005). NTSYS-pc version 2.21q was used to construct unweighted pair group method with arithmetic averages (UPGMA) dendrograms to show the distance-based relationship among the rice accessions.

RESULTS

A total of 12 SSR loci were evaluated for their efficiency of polymorphism across 87 accessions of rice received from different countries. Among the 12 SSR primers used in this study, six yielded scorable amplification products (Table 3). Forty one alleles with a mean of 6.83 alleles per locus were obtained from these six SSR primers. The number of alleles per locus ranged from 2 [Xtxp 284 (1)] to 9 [Xtxp 10 (9)]. Major allele frequency ranged from 0.31 to 0.73 with an average of 0.54. The locus Xtxp 149 (1) was the most informative since it had the highest level of polymorphism with PIC value of 0.79 and gene diversity value of 0.81 (Table 3). Xtxp 149 (1) also had the highest heterozygosity of 0.91 followed by Xtxp 201 (2) 0.87. Xtxp 284 (1) with a scoring of 0.00 had the lowest heterozygosity.

Genetic relationship revealed by the six SSR primers

Table 1. Plant materials and their source.

S/N	Accession	Source	S/N	Accession	Source
1	WAB 2081-WAC B-TGR4-B	AfricaRice, Benin	47	BASMATI 123	CSIR-SARI, Ghana
2	WAB 2125-WAC B-1-TGR3-WAT B1	AfricaRice, Benin	48	CRI-30	CSIR-SARI, Ghana
3	IR 841 (CHECK)	AfricaRice, Benin	49	CRI-2	CSIR-SARI, Ghana
4	DKA-M2	AfricaRice, Benin	50	CRI-45	CSIR-SARI, Ghana
5	JASMINE 85	CSIR-SARI, Ghana	51	CRI-73	Cameroon
6	FAROX 508-3-10-F43-1-1	AfricaRice, Benin	52	CRI-48	Mali
7	FAROX 508-3-10-F44-2-1-1	AfricaRice, Benin	53	NERICA 1	Mali
8	WAB 2098-WAC2-1-TGR2-WAT B2	AfricaRice, Benin	54	AFRK-7	Mali
9	WAB 2056-2-FKR2-5-TGR1-B	AfricaRice, Benin	55	AFRK-8	Mali
10	WAB 2060-3-FKR1-WAC2-TGR4-B	AfricaRice, Benin	56	AFRK-5	Mali
11	TXD 88	AfricaRice, Benin	57	AFRK-13	Mali
12	WAB 2098-WAC3-1-TGR1-4	AfricaRice, Benin	58	NERICA 4	Mali
13	WAB 2076-WAC1-TGR1-B	AfricaRice, Benin	59	AFRK-6	Mali
14	WAB 2081-WAC2-2-TGR2-WAT B3	AfricaRice, Benin	60	AFRK-2	Mali
15	GBEWAA	CSIR-SARI, Ghana	61	AFRK-11	Mali
16	PERFUME IRRIGATED	Thailand	62	NERICA 14	Mali
17	WAS-122-13-WAS-10-WAR	AfricaRice, Benin	63	AFRK-9	Mali
18	LONG GRAIN ORDINARY 2	Thailand	64	AFRK-3	Mali
19	EXBAIKA	CSIR-SARI, Ghana	65	AFRK-1	Mali
20	WAS-163-B-5-3	AfricaRice, Benin	66	AFRK-10	Mali
21	FAROX 15	CSIR-SARI, Ghana	67	AFRK-5	Cameroon
22	PERFUME SHORT	Thailand	68	AFRK- 4	AfricaRice, Benin
23	KATANGA	CSIR-SARI, Ghana	69	IR 74963-2-6-2-5-1-3-3	IRRI, Philippines
24	TOX 3107	CSIR-SARI, Ghana	70	IR 81412-B-B-82-1	IRRI, Philippines
25	ANYOFULA	CSIR-SARI, Ghana	71	IR 55419-04	IRRI, Philippines
26	NABOGU	CSIR-SARI, Ghana	72	IR 79913-B-179-B-4	IRRI, Philippines
27	GR 21	CSIR-SARI, Ghana	73	APO	IRRI, Philippines
28	PHKA RUMDON	Cameroon	74	N22	IRRI, Philippines
29	MLI 20-4-1-1-1	Mali	75	IR 77298-14-1-2-10	IRRI, Philippines
30	DKA-M2	Mali	76	KALIAUS	IRRI, Philippines
31	SIK 353-A-10	Mali	77	UPL RI 7	IRRI, Philippines
32	DK 3	Mali	78	KALIA	IRRI, Philippines
33	MLI 6-1-2-3-2	Mali	79	IR 74371-46-1-1	IRRI, Philippines
34	MLI 25-1-2	Mali	80	IR 74371-54-1-1	IRRI, Philippines
35	DKA 4	Mali	81	IR 80411-49-1	IRRI, Philippines
36	DKA- M8	Mali	82	IR81023-B-116-1-2	IRRI, Philippines
37	SIK 350-A-150	Mali	83	WAY RAREM	IRRI, Philippines
38	DKA-M11	Mali	84	VANDANA	IRRI, Philippines
39	DKA 22	Mali	85	IR 77298-5-6-18	IRRI, Philippines
40	DKA-M9	Mali	86	IR 74371-70-1-1	IRRI, Philippines
41	DKA 1	Mali	87	UPL RI 5	IRRI, Philippines
42	DKA 21	Mali			
43	MLI 20-4-3-1	Mali			
44	SBT 70	Cameroon			
45	BASMATI 113	Thailand			
46	AGRA RICE	CSIR-CRI, Ghana			

using similarity coefficients based on UPGMA is shown in Figure 1. From the figure, the 87 rice accessions were clustered into four major groups at 41% similarity coefficient. Cluster I contained 10 accessions from Benin

Table 2. SSR Primers and their sequences used in DNA fingerprinting.

SSR locus and chromosome location	Primer sequence (5' To 3')	Type of SSRs
Xtxp 149 (1)	F=AGCCTTGCATGATGTTCC R=GCTATGCTTGGTGTGGG	$(CT)_{10}$
Xtxp 284 (1)	F=CCAGATTGGCTGATGCATACACACT R=AAGGGTAATTTATGCACTCCAAGGTAGGAC	$(AAG)_{19}$
Xtxp 201 (2)	F=GCGTTTATGGAAGCAAAAT R=CTCATAAGGCAGGACCAAC	$(GA)_{36}$
Xtxp 197 (2)	F=GCGTCAATTAATCCAAACAGCCTC R=GAGTTCCTATTCCCGTTCATGGTGAT	$(AC)_{10}$
Cba (3)	F=AAAGCTCGGCGTTAGAAATA R=CGTTTAACAACTCGTACCATC	$(TA)_{18}$
Xtxp 51 (4)	F=TCTCGGACTCAAGAGCAGAGG R=GGACAGCAGCGGCTTCAG	$(TG)_{11}$
Xtxp 274 (6)	F=GAAATTACAATGCTACCCCTAAAAGT R=ACTCTACTCCTTCCGTCCACAT	$(TTC)_{19}$
Xtxp 278 (7)	F=GGGTTTCAACTCTAGCCTACCGAACTTCCT R=ATGCCTCATCATGGTTCGTTTTGCTT	$(TTG)_{12}$
Xtxp 295 (7)	F=AAATCATGCATCCATGTTCGTCTTC R=CTCCCGCTACAAGAGTACATTCATAGCTTA	$(CT)_{19}$
Xtxp 258 (9)	F=CACCAAGTGTCGCGAACTGAA R=GCTTAGTGTGAGCGCTGACCAG	$(AAC)_{19}$
Xtxp 10 (9)	F=ATACTATCAAGAGGGGAGC R=AGTACTAGCCACACGTCAC	$(CT)_{14}$
Xtxp 217 (10)	F=GGCCTCGACTACGGAGTT R=TCGGCATATTGATTTGGTTT	$(GA)_{23}$

F, Forward primer; R, Reverse primer (Missihoun et al., 2015).

Table 3. Allele number, major allele frequency, gene diversity, heterozygosity and polymorphism information content (PIC) values generated from six SSR molecular markers.

Marker	Allele no.	Major allele frequency	Gene diversity	Heterozygosity	PIC
Xtxp 149 (1)	8.00	0.31	0.81	0.91	0.79
Xtxp 284 (1)	2.00	0.69	0.43	0.00	0.34
Xtxp 201 (2)	8.00	0.52	0.61	0.87	0.55
Xtxp 197 (2)	8.00	0.73	0.44	0.27	0.43
Xtxp 278 (7)	6.00	0.54	0.57	0.31	0.50
Xtxp 10 (9)	9.00	0.47	0.71	0.42	0.68
Mean	6.83	0.54	0.60	0.46	0.55

(Africa rice), Thailand, Mali and Philippines (IRRI) which are late maturing, cluster II six accessions from Ghana (CSIR-SARI), Thailand and Mali, they have green basal leaf sheath colour. Majority of the accessions (57) studied were grouped into cluster III, they are accessions with no awn and broad leaf width. This cluster contained accessions from all the six countries in which collections were done and cluster IV, contains 14 accessions from Benin (Africa rice), Ghana (CSIR-SARI and CRI), Mali, Thailand and Philippines (IRRI); they have short culm

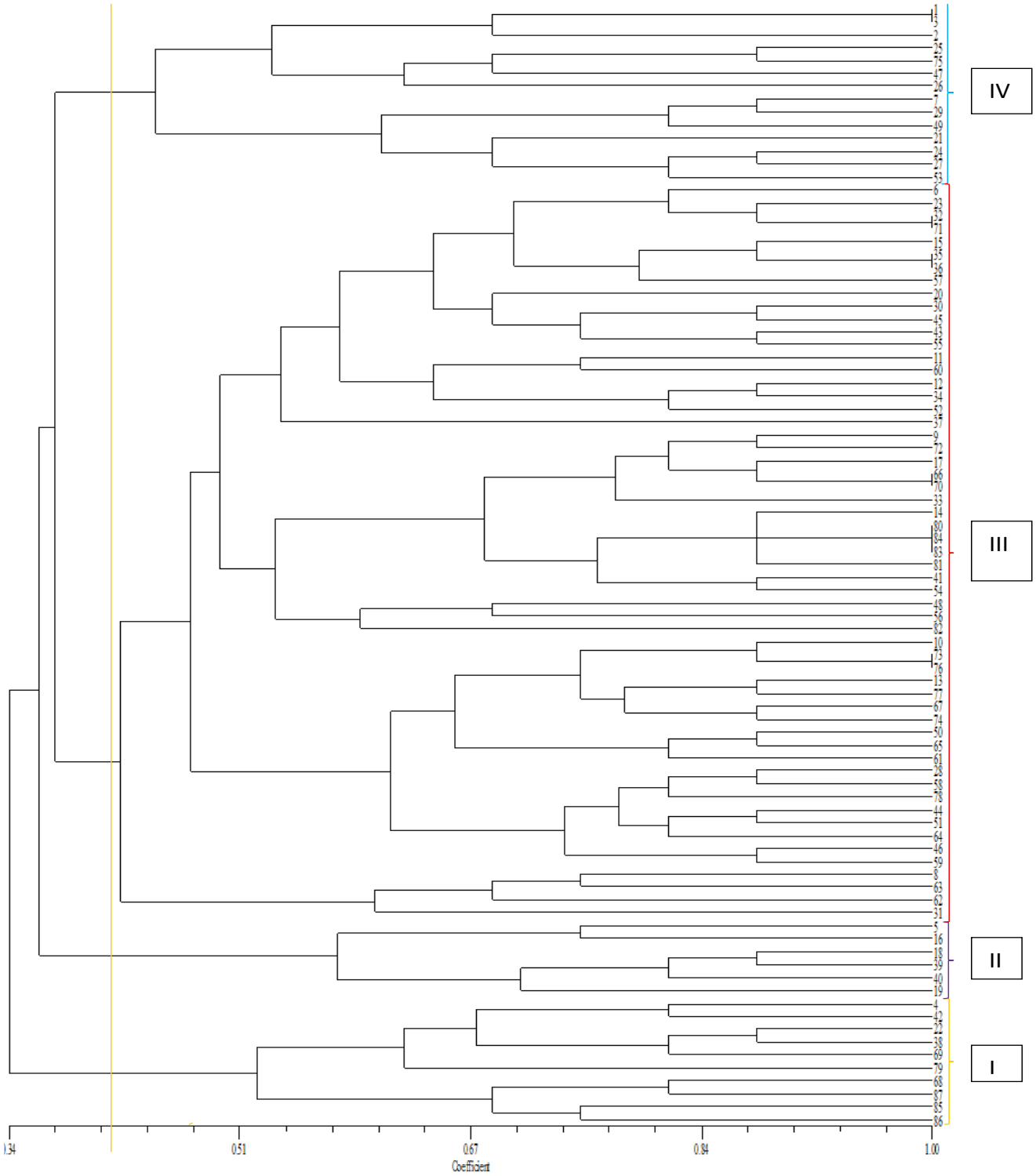

Figure 1. An UPGMA cluster dendrogram showing genetic relationships among 87 rice accessions based on six SSR markers. Number follows details in Table 1.

length and fewer tillers. Further, at 58% similarity coefficient, the four major clusters were divided into sub-clusters. Cluster I (IA and IB), cluster II (IIA and IIB), cluster III (IIIA, IIIB, IIIC, IIID, IIIE, IIIF and IIIG) and cluster IV (IVA, IVB and IVC). More than half of the accessions studied (49) from all six countries showed the

closest resemblance at a similarity coefficient of 88%. Accession [73(APO) and 76(KALIAUS) from Philippines (IRRI)], [66 (AFRK-10) from Benin (Africa Rice) and 70(IR 81412-B-B-82-1) from Philippines (IRRI)], [35(DKA 4) and 36(DKA- M8) from Mali], [32(DK 3) from Mali and 70 (IR 55419-04) from Philippines (IRRI)], [1(WAB 2081-WAC B-TGR4-B) and 3 (WAB 2125-WAC B-1-TGR3-WAT B1) from Benin (Africa Rice)] and finally [80 (IR 74371-54-1-1), 83(WAY RAREM) and 84(VANDANA) all three from Philippines (IRRI)] showed 100% similarity.

DISCUSSION

Success of rice improvement programmes depends on the amount of genetic variability and the degree to which the desirable traits are heritable (Ravi et al., 2003). Hence, assessment of genetic variability among genotypes becomes important in establishing relationships among different cultivars. Characterization using molecular markers is the alternative strategy to overcome the several limitations of morpho-agronomic traits characterization of genetic materials. Morphological characterization affected by environmental condition, requires a longer duration and may be more expensive. In the present investigation, twelve SSR markers were used to characterize and assess the genetic variability among 87 rice accessions collected from six countries. Only six out of these 12 microsatellite markers revealed genetic polymorphism and ensured unambiguous identification of the rice accessions. Small numbers of molecular markers can be used to assess genetic diversity as shown earlier in other studies. Ali et al. (2011) reported that a subset of 36 microsatellite markers gave nearly similar results as using 169 SSR markers for genetic diversity studies. These six SSR primers yielded a total of 41 alleles ranging from 2 [Xtxp 284 (1)] to 9 [Xtxp 10 (9)] with an average of 6.83 alleles per locus and were similar to those earlier reported by Ni et al. (2002). They used Indian quality rice germplasm and reported an average of 6.80 alleles per locus. The number of alleles detected in the present study is lower than those observed by Chakhonkaen et al. (2012), who reported a total of 127 alleles that ranged from 4 to 12 alleles using 19 InDel (Insertion-Deletion) markers to evaluate genetic diversity in 101 rice accessions. The average genetic diversity of 0.60 obtained was higher compared to 0.55 previously reported by Sajib et al. (2012), who used nine SSR markers to study genetic diversity among 12 aromatic landraces of rice. Polymorphism information content (PIC) is a measure of polymorphism among varieties for a marker locus used in linkage analysis (Sajib et al., 2012). It ranged from 0.34 to 0.79 with an average of 0.55 in this study. The PIC range and average observed in this study are similar to those reported earlier by Meti et al. (2013), they reported PIC range of 0 to 0.74 with an average of 0.58 using 12 SSR markers to estimate genetic diversity in 48 aromatic rice genotypes. Higher values of PIC might be the result of diverse genotypes and lower values may be the result of closely related genotypes (Prabakaran et al., 2010).

The dendrogram showed that there was genetic variation among the 87 rice accessions in relation to the SSR primers used. The similarity coefficient of these accessions ranged from 0.34 to 1.00, which is an indication of the genetic variation among the accessions based on the SSR primers. The variation observed among the accessions is an indication that SSR markers can reveal diversity existing between rice accessions. This is in agreement with earlier findings of Pervaiz et al. (2010); they reported that SSR markers are effective tools in discriminating rice genotypes. The accessions were grouped into four main clusters at a genetic similarity coefficient of 0.41. Also, at a similarity coefficient of 0.58, each of the four main clusters were divided into sub-clusters. Accession 73 (APO) and 76 (KALIAUS) both from Philippines (IRRI) showed 100% similarity revealing that no genetic variability exist between these two accessions based on the six SSR primers. The similarity could have risen from informal exchange of seeds (germplasm) among farmers, but given different names because of differences in dialect and ethnic groups. It is important to eliminate duplicates to enable effective management and conservation of germplasm.

Broadening the genetic base of rice in breeding programmes is urgently needed to enhance heterozygosity in crosses and create heterotic progenies. Overall, this study has explained the relevance of employing molecular markers to determine genetic distances and relationships in rice. Moderate level of genetic diversity was observed among the rice accessions.

Conflict of Interests

The authors have not declared any conflict of interests.

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