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Genetic diversity analysis of rice cultivars from various origins using simple sequence repeat (SSR) markers

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Genetic diversity is of paramount importance for the success of any plant breeding program. An experiment was conducted to assess the extent of genetic diversity and similarity of 24 rice cultivars from various origins using 29 simple sequence repeat (SSR) markers. A total of 144 alleles were detected at the 29 SSR primer pairs evaluated in the 24 cultivars. The number of alleles per loci ranged from 3 to 8 with a mean of 4.966 alleles per locus. The mean unbiased expected hetrozygosity (UH_e) and expected hetrozygosity (H_e) were 0.674 and 0.659, respectively. Cluster analysis using UPGMA algorism divided the 24 cultivars into two major groups. Group I contained the two NERICA, all japonica, and a tropical japonica cultivar. Group II contained the NPT and all indica cultivars. The genetic diversity analysis showed the presence of higher allelic diversity in the cultivars analyzed. NERICA cultivars, which showed greater dissimilarity from all the other cultivars, could be used as parents in future breeding programs so as to come up with transgresive segregants.

Key words: Genetic diversity, hetrozygosity, NERICA, rice, simple sequence repeat (SSR) markers.

INTRODUCTION

Among the cereals in the world, rice is the most important crop and a primary source of food for more than half of the world's population. More than 90% of the world's rice is grown and consumed in Asia. It accounts for 35 to 75% of the calories consumed by more than 3 billion Asians. The crop is planted on about 11% of the cultivated land worldwide (Khush, 2005). About 11.6 and 164.1 million ha of land is used for rice production in Thailand and worldwide, respectively (FAO, 2013).

The amount of genetic diversity within species is essential for the survival of species and their adaptation to changing environments (Gao, 2003; Rao and Hodgkin, 2002). This knowledge is valuable for germplasm conservation; individual, population and variety identification; and for the improvement of crop plants and for studying the evolutionary ecology of populations (Duran et al., 2009). Plant breeding involves the continuous development and release of improved cultivars of crop plants and the success of this endeavor depends on availability and access to a pool of diverse genetic material (Hoisington et al., 1999; Maxted et al., 2002).

Simple sequence repeats (SSR) or microsatellites are valuable markers because of their multiallelic property, codominance, abundance, ease of detection by PCR and their requirement of small amount of DNA (Powell et al., 1996). These days a number of rice SSR markers are available (Chen et al., 1997; McCouch et al., 1997, 2002; Temnykh et al., 2000, 2001), and these markers have been extensively utilized for different purposes in rice: hybrid parental lines and variety identification and purity testing (Coburn et al., 2002; Singh et al., 2004; Joshi and Behera, 2006; Sundaram et al., 2008), identification of non-pollen type thermo-sensitive genic male sterile gene (Matthayatthaworn et al., 2011), pyramiding of blast disease

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resistance quantitative trait loci (QTLs) (Sreewongchai et al., 2010) and to study genetic diversity (Chakravarthi and Naravaneni, 2006; Faivre-Rampant et al., 2010; Giarrocco et al., 2007; Lapitan et al., 2007; Ndjiondjop et al., 2010; Pervaiz et al., 2009; Saini et al., 2004; Seetharam et al., 2009; Thomson et al., 2007 and Zhao et al., 2009).

In order to combine the important traits of Asian rice (*Oryza sativa*) and African rice (*Oryza glaberrima*), and utilize the subsequent variability, an interspecific cross was made in West Africa Rice Development Association (WARDA) and several fertile progenies were produced by employing backcross and double haploid breeding (Jones et al., 1997). This created a new gene pool and increased the genetic diversity of rice. The interspecific progeny so created were named New Rice for Africa (NERICA). By employing farmers' participatory variety selection, WARDA has released 18 upland NERICAs (Samado et al., 2008). This research was conducted with the objecttive to study the extent of genetic diversity and relationships within rice cultivars using simple sequence repeat (SSR) markers and to investigate whether this variation is good enough to design further breeding program. The similarity/dissimilarity of two NERICA varieties from some Asian varieties was compared.

MATERIALS AND METHODS

In the genetic diversity study by using SSR fingerprinting, a total of 24 rice cultivars were used. The materials included 13 *indica*, four *japonica*, four New Plant Types (NPT), one tropical japonica and two NERICA varieties (Table 1).

DNA was extracted from fresh leaves of young rice plants, using the method described by Doyle and Doyle (1990). Agarose gel electrophoresis was used to estimate DNA concentration. Thirty microsatellite primer pairs were selected based on the published rice microsatellite framework map. Primer sets in the study were random selections from the panel of 50 standard SSR markers recommended for rice diversity analysis by the Generation Challenge Program and from McCouch et al. (2002). After screening of the primer pairs over the 24 rice cultivars, 29 polymorphic markers were used for the diversity analysis. One monomorphic marker (RM428) was excluded from the analysis. The original source and chromosomal positions for these markers can be found in the rice genome database (http://www.gramene.org).

Polymerase chain reaction (PCR) profile

Amplification reactions were carried out in 10 µl reaction mixture containing 20 ng of template DNA, 0.5 µM of each primers, 0.2 µl Phire Hot Start DNA Polymerase and 5 µl of 2x Phire Plant PCR Buffer (FINNZYMES; containing dNTPs and 1.5 mM $MgCl_2$). Reactions were performed using a MULTIGENE thermal cycler (Labnet International, Inc.) programmed as 98°C for 5 min, followed by 40 cycles of 5 s at 98°C, 5 s at 57°C, 30 s at 72°C with a final extension of 1 min at 72°C. Amplification products were separated in 6% denaturing polyacrylamide gel electrophoresis and visualized by silver staining method (Benbouza et al., 2006). A 10 bp DNA ladder was used as a size marker to compare the molecular weights of the amplified products (Figure 1).

Data analysis

Multi state scoring was employed to score clear SSR polymorphic bands. The common diversity parameters estimated for each SSR marker include average number of alleles, effective number of alleles (Ne), allele frequencies, gene diversity or expected heterozygosity (Nei, 1973), unbiased expected heterozygosity (Nei, 1978), Shannon's information index (I) and genetic distances (Nei, 1978). Genetic distance values were used for cluster analysis of rice cultivars using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA). POPGENE 1.31 softwares (Peakall and Smouse, 2006) were used for data analysis. GeneAlEx 6.41 software was used for principal coordinate analysis by using Nei (1978) unbiased minimum distance coefficients as input, and Tools for Population Genetic Analysis (TFPGA) 1.3 was used for dendrogram construction.

RESULTS AND DISCUSSION

Allele frequency and heterozygosity

Plant breeding depends on the correct combination of specific alleles at the genetic loci present in a plants' genome (Hoisington et al., 1999). The knowledge of frequency and distribution of alleles is also important to identify genetically divergent parents; this information in turn assists plant breeders in creating hybrid (in the F_1) and variability in the subsequent generations. Out of the rice SSR markers screened, 29 (21 standard and 8 additional from McCouch et al. (2002)) polymorphic markers were used for the diversity analysis. A total of 144 alleles were detected at the 29 SSR primer pairs evaluated in the 24 rice cultivars. The number of alleles per SSR loci ranged from 3 (RM162, RM284, RM312, RM338 and RM507; all standard markers) to 8 (RM19; standard marker) with a mean of 4.966 alleles per locus. The mean number of alleles estimated in this experiment was smaller than the one reported by Alvarez et al. (2007), Giarrocco et al. (2007), Thomson et al. (2007) and Faivre-Rampant et al. (2010); and closer to that of Lapitan et al. (2007) and Saini et al. (2004). The size of PCR products ranged between 80 and 200 bp depending on the SSR marker and the cultivar. Unbiased expected heterozygosity (UH_e) over loci was 0.674 and it ranged from 0.334 (RM162) to 0.841 (RM552). Similarly, the mean expected hetrozygosity (H_e) was 0.659 and it ranged from 0.327 (RM162) to 0.823 (RM280) (Table 2). When compared with other findings, the mean H_e value is lower than the one reported by Alvarez et al. (2007). However, Thomson et al. (2007) reported a slightly lower H_e value (0.46). Most of the markers used for the study could be regarded as highly informative as 82.76% of them had high (greater than 0.5) H_e values. The genetic diversity of each SSR locus was found to be correlated ($r = 0.648$) with the number of alleles (Na) detected per locus; the higher the H_e value, the higher the Na per locus.

Although, the number of loci sampled from the rice chromosomes were not equal and chromosome 7 was not represented by any polymorphic SSR marker in this study, higher number of alleles were detected on chromosome 4 (6.5), chromosome 11 (6.2) and chromosome 10 (6); the lowest number of allele (3), however, was

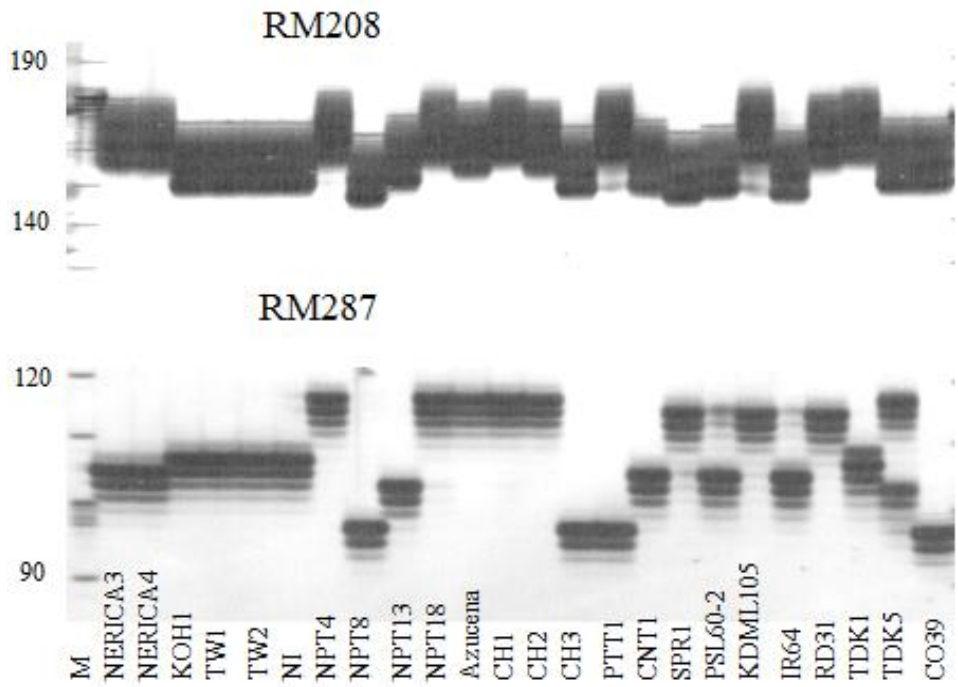

Figure 1. Silver stained polyacrylamide gel of amplification products obtained with SSR markers RM208 and RM287 among 24 rice cultivars (M = size marker).

Table 1. Description of the 24 rice cultivars used for the diversity analysis using SSR markers.

Name	Type	Origin
NERICA 3	Interspecific cross	Côte d'Ivoire
NERICA 4	Interspecific cross	Côte d'Ivoire
KOH1	Japonica	Japan
TW1	Japonica	Taiwan
TW2	Japonica	Taiwan
NI	Japonica	Japan
NPT4	New plant type	Philippines
NPT8	New plant type	Philippines
NPT13	New plant type	Philippines
NPT18	New plant type	Philippines
Azucena	Tropical japonica	Philippines
CH1	Indica	China
CH2	Indica	China
CH3	Indica	China
PTT1	Indica	Thailand
CNT1	Indica	Thailand
SPR1	Indica	Thailand
PSL60-2	Indica	Thailand
KDML105	Indica	Thailand
IR64	Indica	Philippines
RD31	Indica	Thailand
TDK1	Indica	Laos
TDK5	Indica	Laos
CO39	Indica	India

Table 2. Summary of genetic diversity parameters of all loci estimated for 24 rice cultivars.

Chromosome	Locus	Na	Ne	I	He	UHe	Repeat motif
1	RM237	5	4.174	1.507	0.760	0.777	(CT)18
1	RM259	5	2.439	1.175	0.590	0.605	(CT)17
1	RM312	3	1.882	0.762	0.469	0.479	(ATTT)4(GT)9
1	RM5	6	3.000	1.328	0.667	0.681	(GA)14
2	RM208	6	4.114	1.574	0.757	0.773	(CT)17
2	RM452	4	3.612	1.331	0.723	0.740	(GTC)9
2	RM6	5	4.431	1.538	0.774	0.791	(AG)16
3	RM338	3	2.597	1.010	0.615	0.631	(CTT)6
4	RM280	7	5.647	1.830	0.823	0.840	(GA)16
4	RM559	6	3.369	1.409	0.703	0.719	(AACA)6
5	RM161	4	2.880	1.199	0.653	0.667	(AG)20
5	RM178	4	1.869	0.857	0.465	0.475	(GA)5(AG)8
5	RM413	5	3.374	1.365	0.704	0.723	(AG)11
5	RM507	3	2.651	1.028	0.623	0.642	(AAGA)7
6	RM133	4	3.023	1.235	0.669	0.684	(CT)8
6	RM162	3	1.485	0.619	0.327	0.334	(AC)20
6	RM510	4	2.642	1.105	0.622	0.635	(GA)15
8	RM284	3	1.716	0.709	0.417	0.427	(GA)8
9	RM105	5	4.500	1.545	0.778	0.794	(CCT)6
9	RM201	5	2.851	1.253	0.649	0.663	(CT)17
10	RM590	6	4.881	1.679	0.795	0.812	(TCT)10
11	RM144	7	3.310	1.531	0.698	0.713	(ATT)11
11	RM286	5	3.600	1.411	0.722	0.738	(GA)16
11	RM287	6	5.143	1.691	0.806	0.823	(GA)21
11	RM536	7	2.160	1.234	0.537	0.552	(CT)16
11	RM552	6	5.628	1.756	0.822	0.841	(TAT)13
12	RM19	8	5.538	1.871	0.819	0.837	(ATC)10
12	RM270	4	1.882	0.918	0.469	0.479	(GA)13
12	RM277	5	2.909	1.257	0.656	0.670	(GA)11
	Mean	4.966	3.356	1.301	0.659	0.674	
	SE	0.255	0.228	0.062	0.024	0.025	

Na = Number of different alleles, Ne = number of effective alleles, I = Shannon's information index, He = expected heterozygosity, UHe = unbiased expected heterozygosity.

Table 3. Number of alleles, information index and heterozygosity values of SSR loci in rice chromosomes.

Chromosome	Number of loci	Na	Ne	I	He	UHe
1	4	4.75	2.87	1.193	0.621	0.635
2	3	5.00	4.05	1.481	0.751	0.768
3	1	3.00	2.60	1.010	0.615	0.631
4	2	6.50	4.51	1.620	0.763	0.780
5	4	4.00	2.69	1.112	0.611	0.627
6	3	3.67	2.38	0.986	0.539	0.551
8	1	3.00	1.72	0.709	0.417	0.427
9	2	5.00	3.68	1.399	0.714	0.729
10	1	6.00	4.88	1.679	0.795	0.812
11	5	6.20	3.97	1.525	0.717	0.733
12	3	5.67	3.44	1.349	0.648	0.662
Mean	2.64	4.80	3.34	1.28	0.65	0.67

Table 4. Pairwise Nei's (1978) unbiased minimum distance coefficients for all possible combinations of the 24 cultivars using allele frequencies of 29 SSR markers.

	NERICA3	NERICA4	KOH1	TW1	TW2	NI	NPT4	NPT8	NPT13	NPT18	AZUCENA	CH1	CH2	CH3	PTT1	CNT1	SPR1	PSL602	KDML105	IR64	RD31	TDK1	TDK5
NERICA3																							
NERICA4	0.040																						
KOH1	0.680	0.690																					
TW1	0.708	0.654	0.269																				
TW2	0.680	0.690	0.379	0.115																			
NI	0.680	0.655	0.207	0.154	0.207																		
NPT4	0.833	0.741	0.926	0.880	0.926	0.926																	
NPT8	0.880	0.828	0.828	0.808	0.793	0.793	0.407																
NPT13	0.880	0.857	0.821	0.846	0.964	0.929	0.556	0.679															
NPT18	0.917	0.929	0.821	0.885	0.929	0.929	0.539	0.607	0.593														
AZUCENA	0.667	0.714	0.714	0.760	0.786	0.714	0.885	0.857	0.852	0.852													
CH1	0.880	0.893	0.857	0.808	0.857	0.857	0.500	0.536	0.630	0.519	0.889												
CH2	0.917	0.889	0.889	0.885	0.889	0.926	0.280	0.444	0.615	0.407	0.923	0.556											
CH3	0.818	0.769	0.923	0.833	0.846	0.846	0.417	0.539	0.600	0.615	0.960	0.400	0.440										
PTT1	0.957	0.852	0.889	0.792	0.815	0.852	0.440	0.407	0.692	0.577	0.923	0.577	0.440	0.500									
CNT1	0.870	0.889	0.852	0.880	0.926	0.926	0.539	0.333	0.577	0.519	0.885	0.462	0.500	0.480	0.440								
SPR1	0.917	0.857	0.893	0.846	0.821	0.857	0.577	0.464	0.741	0.607	0.852	0.593	0.519	0.500	0.346	0.407							
PSL602	0.905	0.880	0.960	0.913	0.960	0.960	0.652	0.480	0.708	0.560	0.880	0.625	0.583	0.667	0.500	0.292	0.560						
KDML105	0.875	0.857	0.893	0.880	0.857	0.857	0.615	0.571	0.778	0.667	0.821	0.630	0.539	0.480	0.308	0.423	0.370	0.440					
IR64	0.913	0.926	0.815	0.833	0.852	0.889	0.600	0.556	0.692	0.500	0.885	0.615	0.480	0.600	0.654	0.360	0.539	0.458	0.577				
RD31	0.864	0.885	0.923	0.913	0.923	0.962	0.760	0.654	0.600	0.640	0.840	0.680	0.625	0.652	0.640	0.480	0.560	0.609	0.680	0.480			
TDK1	0.957	0.926	0.926	0.875	0.889	0.926	0.615	0.593	0.731	0.654	0.923	0.654	0.560	0.542	0.462	0.500	0.423	0.625	0.539	0.539	0.346		
TDK5	1.000	1.000	0.926	0.917	0.926	0.963	0.680	0.704	0.731	0.654	0.923	0.615	0.640	0.708	0.654	0.680	0.654	0.625	0.692	0.615	0.731	0.615	
CO39	0.875	0.929	0.857	0.840	0.857	0.893	0.667	0.536	0.593	0.519	0.852	0.556	0.577	0.600	0.539	0.482	0.519	0.708	0.630	0.539	0.346	0.407	0.577

estimated for chromosomes 3 and 8 (Table 3). The genetic diversity, as measured by the expected hetrozygosity, was also higher for chromosome 10 (0.795), chromosome 4 (0.763), chromosome 2 (0.751); however, that of chromosome 8 (0.417) was the lowest.

Genetic distance

A total of 276 pairwise unbiased minimum distance values were estimated using the possible pairwise combination of the 24 cultivars (Table 4). From the total combination, 40.94% had greater than 0.8 genetic distance coefficients indicating higher level of dissimilarity within the cultivars analyzed. When these pairwise distance values were compared, the two NERICA cultivars showed the smallest (0.04) distance between them; they shared higher degree of similarity as expected. They are sister lines and developed from same parent (Samado et al., 2008). The next smaller values, 0.115 and 0.154 were for TW1-TW2 and TW1-NI (Nipponbare) paired cultivars; both of them are japonica pairs. The two NERICA cultivars, however, showed the highest (1) genetic distance from the cultivar TDK5 followed by TW2-NPT13 (0.964), NI-TDK5 (0.963), NI-RD31 (0.962) and Azucena-CH3 (0.96) pairwise genetic distances.

Cluster and principal coordinate analyses

The genetic distance values were used for clustering the 24 cultivars and to construct the dendrogram depicted in Figure 2. The diagram showed two main groups with additional sub-clusters within group two. Group I contained NERICA and *japonica* (both temperate and tropical) types, while Group II was made up of new plant type (NPT) and *indica* types. It is interesting to note that

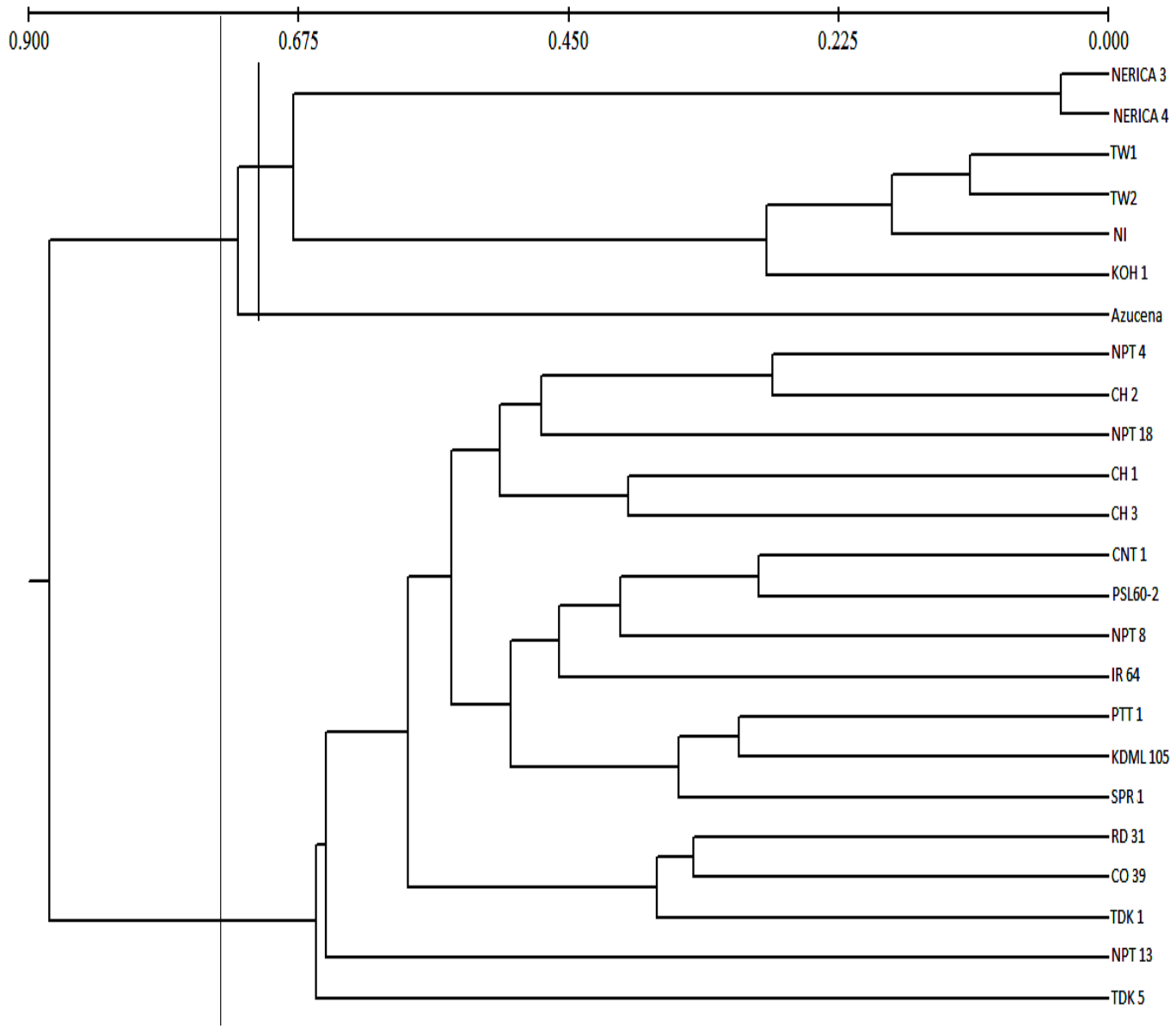

Figure 2. Dendrogram of the 24 rice cultivars based on Nei (1978) unbiased minimum distance and constructed by means of UPGMA algorithm.

the *O. sativa* parent of upland NERICA types is *japonica* (Samado et al., 2008).

Group I was comprised of 7 cultivars. This group is further divided into three subgroups. Subgroup 1 contained NERICA 3 and NERICA 4. These cultivars areimportant in West Africa, where they are developed, and other upland rice growing sub-Saharan Africa countries (Samado et al., 2008). Subgroup 2 was composed of 4 japonica cultivars; TW1, TW2, NI (Nipponbare) and KOH1. However, the third sub-group contained the distinct, aromatic, non-glutinous and traditional tropical japonica cultivar, Azucena.

Group II, a sub-set of 17 cultivars, was composed of 13 *indica* and 4 NPT type cultivars. Most of the *indica* cultivars included in this group are known for different traits of agronomic importance and cultivated in Asia. The 4 NPTs are breeding lines developed by the International Rice Research Institute (IRRI) during implementation of ideotype breeding (Kush, 2005).

Principal coordinate analysis (PCoA) was executed to complement the cluster analysis, where the first three principal coordinate axes explained 40.13, 16.37 and 13.15% of the variance, respectively; and which summed up to 69.65% (Figure 3). The PCoA showed that the cultivars are generally placed to the left and to the right of the two-dimensional plane. Those which are placed to the left are placed close to each other (aggregated), showing similarity among them. The other cultivars which are

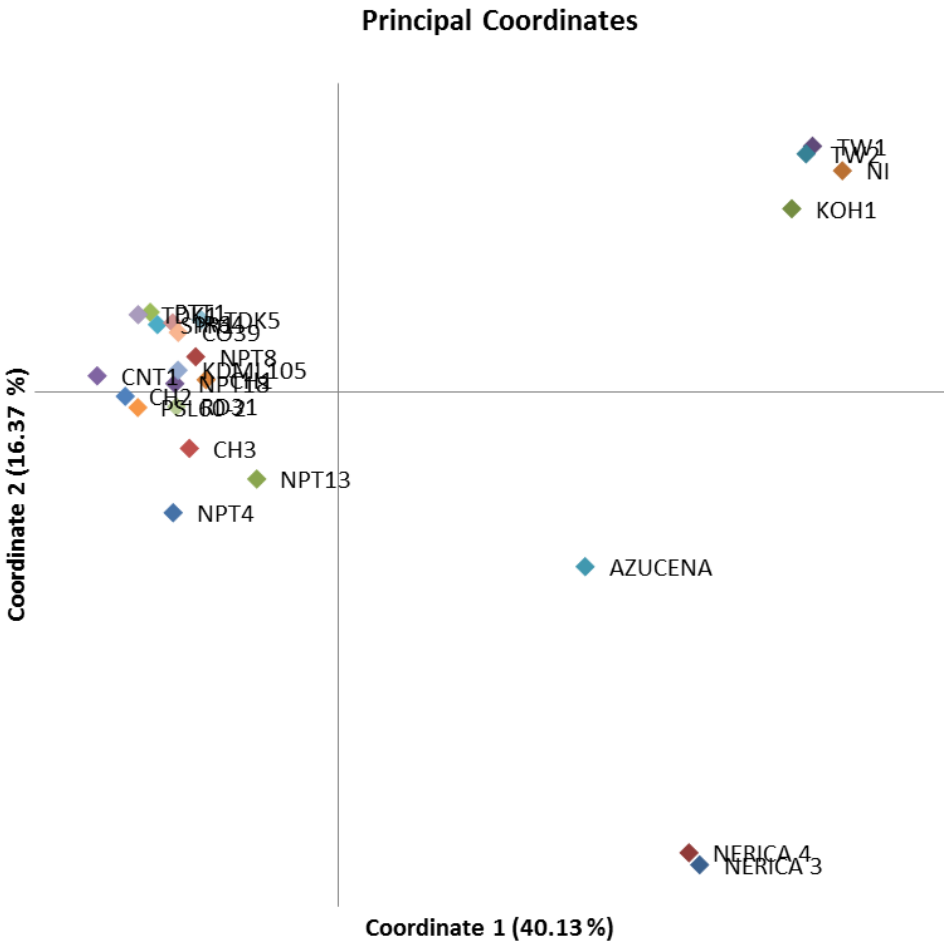

Figure 3. Two-dimensional representation of the 24 cultivars analyzed by using 29 polymorphic SSR markers.

placed to the right are somewhat scattered than the former. This also shows the necessity of further dividing of the member of this cluster into subgroups. The cluster analysis was in general agreement with PCoA in discriminating the cultivars.

Conclusions

Estimation of morphological diversity may provide additional information on the present finding. Nonetheless, the 29 SSR markers provided considerable genetic resolution and this genetic diversity analysis showed higher level of allelic diversity in the cultivars analyzed. Due to the higher amount of heterozygosity in the sample at each marker and the observed allelic diversity, most of the markers used in this study could be used in association and linkage studies. The result could be used for designing effective breeding programs. Cultivar combinations from the two clusters with higher value of dissimilarity coefficient could be used as parents as long as they have desirable combination of traits of agronomic importance; and thus heterotic expression in the F_1 and considerable variability in the subsequent segregating population may be expected. In this study, NERICA cultivars showed higher degree of dissimilarity from all the other cultivars (especially cultivars from the second group). These cultivars could be used as parents for designing further breeding programs so as to come up with transgressive segregants.

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