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Genetic Diversity among 18 Accessions of African Rice (*Oryza glaberrima* Steud.) Using Simple Sequence Repeat (SSR) Markers

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Abstract: Studies were carried out on 18 accessions of African rice (*Oryza glaberrima* Steud.) collected from four geographical regions of Ghana to assess genetic diversity and potential of these accessions, towards a thorough exploitation of the species, in a region-wide breeding programme to obtain new rice varieties better adapted to the harsh growing conditions of West and Central Africa (WCA). Simple Sequence Repeat (SSR) markers were used to estimate diversity among the accessions. Out of 24 SSR primers used, 23 (i.e., 95.83%) showed allelic polymorphism in the accessions studied. Overall genetic diversity was high (I = 1.178, He = 0.625 and Nei's He = 0.608) and the Fixation index statistics (Fst) revealed that 51.5% of the total variation exists among populations collected from the four geographical regions. All accessions were identified as separate entries with no duplications.

Key words: African rice, *Oryza glaberrima*, molecular characterisation, genetic diversity, polymorphism, SSR markers

INTRODUCTION

African rice (*Oryza glaberrima* Steud.) is native to West Africa and was domesticated over 3500 years ago (Linares, 2002; Jones *et al*., 1997a; Porteres, 1976). It closely resembles the Asian rice (*Oryza sativa* L.) (Richards, 1996) and interspecific crosses between the two are successful (Diagne *et al*., 2010; Haskins and Mohapatra, 2010; Futakuchi and Sie, 2009; Jones *et al*., 1997b).

Selection and cultivation over several millennia at the hands of indigenous farmers of West Africa without further manipulation has led to the crop developing adaptive and protective mechanisms for survival against both biotic and abiotic stress factors in the environment (Agnoun *et al*., 2012; Jones *et al*., 1997b). Indeed many researchers have alluded to the richness of *O. glaberrima* as a reservoir of useful genes for resistance to insects (Sauphanor, 1985), diseases (Silue and Notteghem, 1991; John *et al*., 1985; Attere and Fatokun, 1983) as well as tolerance to weed competition (Dingkuhn *et al*., 1999; Jones *et al*., 1997b), acid soils, drought, unfavourable temperatures and excess water (Jones *et al*., 1997b) and iron toxicity (Sahrawat and Sika, 2002; Jones *et al*., 1997b). The species also exhibits high tillering ability (Dingkuhn *et al*., 1999; Johnson *et al*., 1998). These characteristics have made *O. glaberrima* a worthwhile source of important traits for developing improved varieties capable of producing up to 5.6 ton ha^{-1} in more favourable rainfed lowland environments (Haskins and Mohapatra, 2010) but also capable of giving appreciable yields under the harsh conditions prevalent in farmers' fields.

Rice scientists have over the years relied heavily on *O. sativa* which is also widely cultivated species in the sub-region for breeding with marginal results. This was attributed to the limited resistance of the latter to many of the stresses that affect upland rice in the region (WARDA, 1992).

However, two key developments in the past few years related to rice production and consumption in Africa have aroused interest in *O. glaberrima*, leading to a paradigm shift in strategies for increasing production of the crop on the continent. First was the identification of

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rice as a continent-level strategic commodity, for food security and poverty reduction and the need to protect and promote its production on the continent towards attainment of self-sufficiency in rice production by 2015 (FARA, 2009). Second was development of the NERICA series which have gained popularity among rice farmers in Africa within a relatively short time due to their improved agronomic performance and resistance to Africa's harsh growth conditions (Diagne *et al*., 2010; Somado *et al*., 2008) as they combine the hardiness of the African species with the productivity of the Asian species (Somado *et al*., 2008; Sie *et al*., 2005; Linares, 2002; Jones *et al*., 1997b).

Consequently, there has been a change in focus of rice breeding efforts in the region, intended to give preference to *O. glaberrima* over *O. sativa* so as to produce varieties better adapted to the harsh growing conditions in Africa, bearing in mind the effects of climatic change (Haskins and Mohapatra, 2010). Closely linked is the recent findings from the African Rice Centre that counter the widely held view that African rice (*Oryza glaberrima* Steud.) is inherently lower yielding than Asian rice (*Oryza sativa* L.) (Haskins and Mohapatra, 2010).

African rice (*O. glaberrima*) is thought to have been domesticated from the wild ancestor *Oryza barthii* (Agnoun *et al*., 2012; Bezancon and Diallo, 2006; Linares, 2002) by people living in the floodplains at the bend of the River Niger. The species is currently grown in the zone extending from the delta of the River Senegal in the West to Lake Chad in the East. Its range of cultivation to the South East is bordered by the river basins of Benue, Logone and Chari. However, intensive cultivation takes place in the floodplains of Northern Nigeria, the inland delta of the river Niger in Mali, parts of Sierra Leone and the hills on the Ghana-Togo border (Bezancon and Diallo, 2006).

Some 1130 accessions of *O. glaberrima* were collected and characterised by Africa Rice Centre (WARDA) leading to the development of the NERICA series (Futakuchi and Sie, 2009) through backcrossing and doubled haploid breeding (Jones *et al*., 1997a), aided by embryo rescue to ensure that the crosses were fertile and matured successfully. The best NERICA varieties combine the multiple stress resistances of *O. glaberrima* with the high yielding potential of *O. sativa* (Somado *et al*., 2008; Sie *et al*., 2005; Jones *et al*., 1997a). A number of these varieties have been adopted by farmers in >20 African countries and the demand for NERICA seed is high across the region (Haskins and Mohapatra, 2010). However, with the rate of introgression of the *O. glaberrima* genome into NERICA series genome estimated at only 7.2-13.2% (Agnoun *et al*., 2012; Ndjiondjop *et al*., 2010a), it is obvious that most of the treasure trove of genes of *O. glaberrima* remain untapped. Indeed, there are 2500 accessions of *O. glaberrima* but NERICA varieties were developed from a few parents of *O. glaberrima* (Haskins and Mohapatra, 2010), especially the CG 14 line. Besides many pockets of native rice growing communities may have been omitted by the earlier expeditions leading to the assembly of the accessions currently under storage.

In view of further challenges yet to be met towards achieving rice self-sufficiency throughout Africa and for the local African rice to become competitive in world markets, there is need for a more thorough collection and characterisation of *O. glaberrima* germplasm throughout the region to better exploit this rich reservoir of genes for tolerance or resistance to environmental stresses (Agnoun *et al*., 2012). New investigations that capture the biodiversity of *O. glaberrima* in potential collection sites that were not covered by previous research done by Africa Rice Centre including the hills of the Ghana-Togo border and Northern Ghana where indigenous accessions still play key roles in the socio-economic lives of the people (Agnoun *et al*., 2012; Linares, 2002) will contribute greatly in attaining this goal.

The objective of this study was to assess genetic diversity among 17 accessions of African rice (*Oryza glaberrima* Steud.) and their populations collected from four geographical locations of Ghana (Asanti region, Northern region, Old Baika and Lolobi both of Volta region), alongside NERICA 1 as check.

MATERIALS AND METHODS

Experimental site: The experiment was carried out at the Biotechnology Centre of the College of Agriculture and Consumer Sciences, University of Ghana, Legon. The research was carried out from February, 2012 to March, 2012.

Extraction of DNA: Total genomic DNA was extracted from 200 mg of 7 days old fresh seedlings of each accession using CTAB (500 μL) according to Doyle and Doyle (1990) protocol with slight modifications. About 24 SSR primer pairs designed from cultivated rice were used to assay genetic variation of all included materials, based on the Rice Genes Database (http://gramene.org). PCR amplification reaction volume of 25 μL was set up separately for each accession containing $MgCl_2$ at a concentration of 1.5 mM, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Fermentas, USA), 0.4 units of taq polymerase, 2.5 μL of 10x PCR buffer, 0.4 mM of each

forward and reverse primer and 1.2 µL of genomic DNA. The amplification was carried out using I thermal cycler from Bio-Rad. The following temperature cycles were used. First denaturation step of 5 min at 94°C, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min followed by a final extension at 72°C for 7 min. The amplicons or PCR products were primarily verified for amplication by electrophoresis on 1% agarose gel. The PCR products were separated on 10% polyacrylamide denaturing gels and then stained in 400 mL of 0.05% ethedium bromide via orbital shaker for 1 h and then visualized under UV light and photographed using BioDoc-it gel documentation system. The molecular size of amplified alleles for each SSR locus was determined based on its migration relative to a 50 bp DNA marker.

Data collection and analysis: The amplified SSR DNA bands representing different alleles were scored as different genotypes or accessions on the co-dominant nature of SSR markers. As a result, the bands were recorded as homozygous genotypes (AA, BB, CC) or heterozygous genotypes (AB, AC, BC). Genetic parameters such as average observed allele number (Na), the percentage of polymorphic loci (P), observed heterozygosity (Ho), expected heterozygosity (He), Nei heterozygosity (Nei He), Shannon's diversity index (I) and gene flow (Nm) were calculated to estimate the level of genetic diversity. The F-statistics (Fis, Fit and Fst) (Wright, 1978) were computed for polymorphic loci to test for the departure from Hardy-Weinberg equilibrium and to estimate genetic differentiation among *Oryza glaberrima* populations under study. The out-crossing rate ($t = (1-F_{it})/(1 + F_{it})$) was calculated based on the F_{it} values to estimate indirectly the mating pattern of *Oryza glaberrima* populations (Wright, 1978). All of the calculations were performed using POPGENE version 1-32 (Yeh *et al*., 1999).

A hierarchical Analysis of Molecular Variance (AMOVA) of the rice populations was calculated to partition genetic diversity within and among regions. For AMOVA, the genotype banding patterns were converted into a 1 (present) and 0 (absent) matrix and subjected to analysis, using Genstat software based on simple matching coefficient using single linked similarity matrix method.

RESULTS

Microsatellite variation statistics for all loci: A total of 107 alleles with a mean of 4.4583 per locus were generated by the 24 SSR primers (Table 1). It was observed that loci RM 1, RM 178 and RM 215 recorded the highest number of alleles whereas the lowest number of alleles was scored at locus RM 55. However, locus RM 178 recorded the highest level of genetic variability with Shannon's Information index (I) of 1.7981 as well as expected heterozygosity (He) and Nei's expected

Table 1: Genic variartion statistics for all loci

Locus	Ch. No.	Repeat motif	na*	Forward primer	Reverse primer	ne*	I*
RM 001	01	(GA) 26	7	gcgaaaacacaatgcaaaaa	gcgttggttggacctgac	05.0625	01.7729
RM 055	03	(GA) 17	1	ccgtcgccgtagtagagaag	tcccggttattttaaggcg	01.0000	00.0000
RM 259	01	(CT) 17	4	tggagtttgagaggaggg	cttgttgcatggtgccatgt	02.6557	01.1175
RM 312	01	(ATTT) 4 (GT)21	4	gtatgcatatttgataagag	aagtcaccgagtttaccttc	03.0000	01.1938
RM 510	06	(GA) 15	5	aaccggattagtttctcgcc	tgaggacgacgagcagattc	03.1765	01.3776
RM 474	10	(AT)13	6	aagatgtacgggtggcattc	tatgagctggtgagcaatgg	04.1274	01.5346
RM 237	01	(CT)18	6	caaatcccgactgctgtcc	tgggaagagagcactacagc	04.7647	01.6441
RM 334	05	(CTT)20	3	gttcagtgttcagtgccacc	gactttgatctttggtggacg	02.0506	00.8277
RM 178	05	(GA)5(AG)8	7	tcgcgtgaaagataagcggcgc	gatcaccgttccctccgcctgc	05.4000	01.7981
RM 284	08	(GA) 8	6	atctctgatactccatccatcc	cctgtacgttgatccgaagc	05.4454	01.7456
RM 277	12	(GA) 11	4	cggtcaaatcatcacctgac	caaggcttgcaagggaag	02.5714	01.1200
RM 489	03	(ATA) 8	4	acttgagacgatcggacacc	tcacccatggatgttgtcag	02.5714	01.1200
RM 025	08	(GA) 18	5	ggaaagaatgatcttttcatgg	ctaccatcaaaaccaatgttc	02.3824	01.1649
RM 118	07	(GA) 8	5	ccaatcggagccaccggagagc	cacatcctccagcgacgccgag	04.2632	01.5051
RM 125	07	(GCT) 8	2	atcagcagccatggcagcgacc	aggggatcatgtgccgaaggcc	01.6701	00.5908
RM 283	01	(GA) 18	2	gtctacatgtacccttgttggg	cggcatgagagtctgtgatg	01.3846	00.4506
RM 307	04	(AT) 14 (GT) 21	6	gtactaccgacctaccgttcac	ctgctatgcatgaactgctc	03.9755	01.5458
RM 005	01	(GA)14	6	tgcaacttctagctgctcga	gcatccgatcttgatggg	05.2258	01.7109
RM 044	08	(GA) 16	5	acgggcaatccgaacaacc	tcgggaaaacctaccctacc	02.7000	01.1945
RM 162	06	(AC) 20	4	gccagcaaaaccagggatccgg	caaggtcttgtgcggcttgcgg	02.9455	01.1858
RM 514	03	(AC) 12	2	agattgatctcccattcccc	cacgagcatattactagtgg	01.9059	00.6682
RM 495	01	(CTG) 7	3	aatccaaggtgcagagatgg	caacgatgacgaacacaacc	01.4087	00.5566
RM 215	09	(CT) 16	7	caaaatggagcagcaagagc	tgagcacctccttctctgtag	03.9512	01.6095
RM 161	05	(AG) 20	3	tgcagatgagaagcggcgcctc	tgtgtcatcagacggcgctccg	02.0506	00.8277
Total	-	-	107			75.6891	28.2623
Mean	-	-	4.4583			03.1537	01.1776
SD	-	-	1.744			01.3692	00.4810

na* = Observed number of alleles; ne* = Effective number of alleles (Kimura and Crown, 1964); I* = Shanon's information index

Table 2: Genetic diversity in 18 rice populations from five location estimated based on polymorphisms of the 24 SSR loci

Regions	N_a	N_e	PL	P (%)	Ho	He	Nei's He	I	F_{is}	F_{it}	F_{st}	t	Nm
Volta (Old Baika)	3.958	2.861	23	95.83	0.019	0.589	0.562	1.071	--	--	--	--	--
Volta (Lolobi)	1.792	1.761	18	75.00	0.042	0.500	0.375	0.529	--	--	--	--	--
Northern	2.125	2.017	19	79.17	0.056	0.506	0.421	0.651	--	--	--	--	--
Ashanti	1.000	1.000	00	00.00	0.000	0.000	0.000	0.000	--	--	--	--	--
WARDA	1.042	1.042	01	04.17	0.042	0.042	0.021	0.029	--	--	--	--	--
Over all	4.458	3.154	23	95.83	0.028	0.625	0.608	1.178	0.881	0.942	0.515	0.029	0.235

Na = Average number of alleles; Ne = Average number of effective alleles; PL = No. of polymorphic Loci; P = Percentage of polymorphic loci; Ho = Observed heterozygosity (Levene, 1949); He = Expected heterozygosity; Nei's He = Nei's expected heterozygosity (Nei, 1978); I = Shannon diversity index (Lewontin, 1972); F_{is}, F_{it} and F_{st} = Estimates of F-statistics of regional populations; t, out crossing rate = $(1-F_{it})/(1+F_{it})$ (Cao *et al*., 2006); Nm = Gene flow estimated from $F_{st} = 0.25 (1 - F_{st})/F_{st}$

heterozygosity (Nei's He) of 0.8381 and 0.8148, respectively. About 23 out of the 24 (i.e., 95.83%) SSR primers showed allelic polymorphism (Fig. 1) in all the accessions studied. This implies that one locus was monomorphic as can be observed in locus 55.

Differentiation of rice populations: The study reveals estimated genetic parameters with overall I being 1.178 and He and Nei's He being 0.625 and 0.608, respectively (Table 2). However, genetic variability was not uniformly distributed across the population. The highest level of genetic diversity was found in the population made up of Old Baika (I =1.071, Nei's He = 0.562) and followed by those comprising the Northern population (I = 0.651, Nei's He = 0.421) then Lolobi population (I = 0.529, Nei's He = 0.375), WARDA population (I = 0.029, Nei's He = 0.021) with the least being the Ashanti population (I = 0.000, Nei's He = 0.00). The F-statistics showed high positive values of F_{is} and F_{it} among the population, indicating the lack of heterozygosity in the populations and a significant departure of allelic frequencies from the Hardy-Weinberg equilibrium. The F_{st} value revealed that 51.5% of the total genetic variations exist among populations. The overall out-crossing rate was very low (0.029).

Genetic similarity and cluster analysis: A dendogram based on Nei (1978)'s genetic distance using Unweighted Pair Group Method with Arithmetic mean (UPGMA) modified from neighbor procedure of Phylip version 3.5 was constructed (Fig. 2). Lolobi and Old Baika populations showed the greatest similarity and were distinct from the other populations which formed separate groups or clusters at distances of 5.85, 15.95 (i.e., 10.09982+5.85222) and 32.23 for Northern, Ashanti and WARDA populations, respectively (Table 3). This further confirms genetic diversity among the populations.

Cluster analysis based on 24 SSR primers: The study also reveals the genetic relationship based on polymorphisms of 24 SSR loci in the 18 accessions of the five populations under study based on simple matching

Table 3: Length of genetic distance betweeen populations

Between	And	Length
4	3	16.27989
3	2	10.09982
2	1	05.85222
1	pop1	15.51111
1	pop2	15.51111
2	pop5	21.36332
3	pop3	31.46314
4	pop4	47.74304

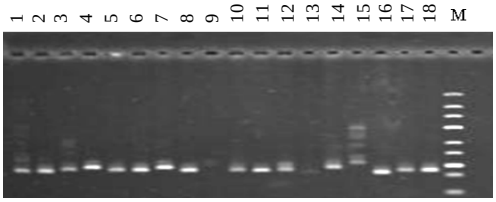

Fig. 1: The banding pattern of primer RM 1 on accessions numbered 1-18: Viwonor tall = 1; Awerema = 2; Guamea = 3; N/4 = 4; Badayi 2 = 5; NERICA 1 = 6; Oloma = 7; Viwotor = 8; Badayi 1 = 9; Balemi 1 = 10; Davi = 11; SARI 1 = 12; Viwonor short = 13; Akotiako = 14; Volta = 15; Adaesi = 16; Balemi 2 = 17; SARI 2 = 18; M = 50 bp ladder

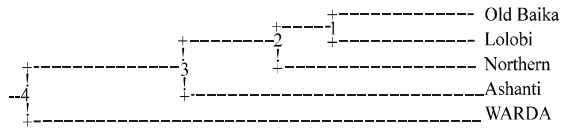

Fig. 2: Genetic distance Dendogram using 24 SSR markers; Old Baika population = Adaesi; Akotiako; Balemi 1; Balemi 2; Badayi 1; Badayi 2; Davi; Oloma; Viwonor tall; Viwonor short and Viwotor; Lolobi population = Awerema; Volta; Northern population = N/4; SARI 1; SARI 2; Ashanti population = Guamea; WARDA population = NERICA 1

coefficient using single linked similarity matrix method (Fig. 3). The dendogram reveals clustering at a Genetic Similarity (GS) of 74.6% for all the accessions studied. The clusterings did not exactly coincide with geographical origin or population groupings of the accessions. At GS

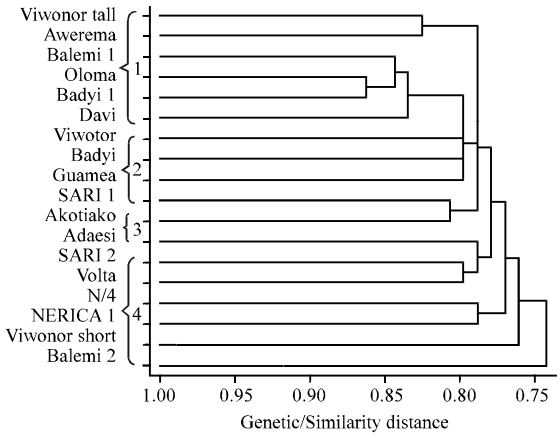

Fig. 3: A dendogram showing genetic relationship revealed by 24 primers among the accessions based on simple matching coefficient using single linked similarity matrix method; the grouping indicated by numbers shows how clusterings did not exactly coincide with geographical origin of the accessions

of 74.6, Balemi 2 was separated from all other accessions which formed the main cluster of 17 accessions.

The main cluster separated at GS 76.4% to form sub-clusters. The accessions that showed closest resemblance among these 17 accessions were Oloma and Badayi 1 at a similarity index of 86.4%. Viwonor tall and Balemi 2 were widely separated.

DISCUSSION

Microsatellite variation statistics for all loci and populations: The high allelic polymorphism (95.83%) revealed by the 24 SSR primers among the accessions indicates that the primers are very informative, especially the primer or locus RM 178 which produced 7 alleles with effective allelic number of 5.40. This polymorphic pattern demonstrated a high overall genetic variability among the accessions with I being 1.178 as well as He and Nei's He being 0.625 and 0.608, respectively. This result is consistent with findings by Ndjiondjop *et al.* (2010b) whose overall mean heterozygosity for 79 *Oryza glaberrima* accessions with four checks were 0.18 and 0.35 for locations and growing conditions, respectively and also produced a Shannon's diversity index of 0.67 for both conditions. It is also similar to findings by Cao *et al.* (2006) whose overall He and I were 0.380 and 0.572, respectively for all the rice weed populations studied but inconsistent with findings by Second (1982) and Barry *et al.* (2007) who obtained mean heterozygosities of 0.03 and 0.07, respectively. The differences for the overall genetic diversity between the present study and among the populations for Ndjiondjop *et al.* (2010a) may be due to the difference in the geographical origin of the accessions and their genetic constitution.

From the SSR analysis, the higher level of genetic variation observed among the individual accessions within populations, compared to genetic variation (F_{st}) among populations according to Muller *et al.* (2001) suggests that populations have a high genetic overlap as a result of probable common ancestry or exchange of germplasm/seed among breeders and growers in different geographical locations.

The low out-crossing rate (≈0.030) and high F_{is}(0.881) and F_{it}(0.942) recorded in the experiment confirms that rice is an autogamous species with an extremely low out-crossing rate and restricted pollen-mediated gene flow (Cao *et al.*, 2006; Chen *et al.*, 2004; Gealy *et al.*, 2003). This is further confirmed by the gene flow of 0.235 recorded among the populations studied. These prove that there is high genetic diversity among most of the accessions and gene flow did not influence diversity.

Genetic divergence: Estimates of Nei's unbiased measures of genetic identity and genetic distance also confirm the wide genetic diversity among the accessions, with the populations of WARDA and Lolobi been the most widely diverse. It also shows that the Lolobi population and that of Old Baika have a higher degree of resemblance. The primary reason for their close identity may be due to the geographical proximity (both populations collected from Volta region). Although, these populations appear to be close, pair-wise comparison of any two accessions from each population at random may either confirm closeness or distant relation. Furthermore, the genetic distance between all the other populations and WARDA, tends to be wide comparably. The reason for this diversity may be due to the genetic constitution of the populations. This is because, the WARDA population (NERICA 1) is a hybrid between WAB 56-104 (Asian accession) and CG 14 accession (*Oryza glaberrima*) while the other populations are natural populations. Secondly, the WARDA population has parents from Asia (WAB 56-106) and Senegal (CG14), producing a hybrid which does not have a source from Ghana (Futakuchi and Sie, 2009; Futakuchi *et al.*, 2008). In principle, the wide variation between the WARDA population and the rest of the populations provides a broad genetic base and hence potential for their adaptation to a wide range of agroecosystems, indicating usefulness of each population (Cao *et al.*, 2006; Holt and Hochberg, 1997), especially the

Old Baika and Lolobi populations which are most diverse from WARDA as a valuable genetic resource for selection of superior genotypes for development of synthetic varieties with high level of heterosis.

Genetic diversity among accessions as revealed by SSR primers: The dendogram generated by the SSR primers further elucidates the genetic relationship among individual accessions or groups. Results obtained from the dendogram reveals the onset of clustering at a GS of 74.6% for all the accessions studied. However, all accessions were identified (separated) at GS 86.4%. This indicates genetic diversity among the accessions as evidenced from the high heterozygosity (He) and Shannon's diversity index (I) obtained (Ndjiondjop *et al*., 2010b; Cao *et al*., 2006). These however, contrast with results obtained by Barry *et al*. (2007) and Second (1982) who concluded that there is low genetic diversity among *Oryza glaberrima* species.

CONCLUSION

The results show that the 18 accessions exhibited great variability with respect to their molecular traits. SSR markers identified all accessions as separate entries with no duplications. The least genetic similarity among the populations was observed between Old Baika and Lolobi.

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