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Morpho-agronomic and molecular characterisation of *Oryza glaberrima* germplasm from Mali

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In order to exploit the important agronomic traits that make *Oryza glaberrima* Steud suitable for harsh growing conditions, 79 Riz Africain du Mali (RAM) including four checks of known cultivated rice species (*Oryza sativa* and *O. glaberrima*) were used for both field evaluation over two seasons (2007 and 2008) and assessment of molecular diversity. The molecular characterization showed all the 37 SSR markers used amplified and were polymorphic. The average heterozygosity of the accessions were relatively higher (0.18) than some earlier studies. The population from Mopti and Gao had a higher genetic diversity than other populations. Clustering of individuals showed no clear pattern of grouping based on either location or growing condition; this is due to the higher gene flow ($Nm = 7.83$) that may occur between different growing condition within location. Moreover, populations from different locations were highly differentiated ($f_{st} = 0.06$) as compared to populations from different growing conditions ($f_{st} = 0.03$). The dendrogram based on populations from different growing conditions revealed more similarity among populations with relatively similar growing conditions. Accessions such as, RAM116, RAM100 and RAM122, performed better under drought with a grain weight advantage of 40, 16 and 6.7% respectively over the best performing check.

Key words: Genetic diversity, SSR markers, drought tolerance, *Oryza glaberrima*.

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important human food crop in the world and rice demand is expected to increase dramatically in the developing world. Rice production has to face the difficult challenge to overcome the recurrent problem of obtaining a regular grain weight under variable conditions, notably due to biotic and abiotic stresses. For example, water will become more and more a sparse resource. Upland rice, even needs lots of water to grow and a long way remains to browse to get true drought-tolerant rice varieties. To respond to that challenge,

development of new varieties with more regular and better grain weight, especially under water stress, appears to be the most promising way. To achieve this breeding goal, a promising approach is to search for "new" alleles for stress-response in the genetic diversity available in the nature. It is now well established that:

(1) A lot of genetic diversity was lost during Asian rice domestication that led to the *indica* and *japonica* sub-species.

(2) Rice relatives' species belonging to the *Oryza* AA genome complex contain a high amount of diversity, which is not present in *O. sativa*.

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Abbreviations: SSR, Simple sequence repeat; CTAB, cetyl trimethyl ammonium bromide; RFLP, restriction fragment length polymorphism.

In the genus *Oryza*, the two cultivated rice species belong to the sativa group characterized by the A genome at diploid level ($2n = 24$). *Oryza glaberrima* is a cultivated species to tropical West Africa resulting from independent

domestication; it occupies a wide range of ecosystems from rain fed hilly slopes to deep-water conditions and coastal mangrove areas. Two major agro-ecotypes can be distinguished: a floating photosensitive type and an early erect type cultivated in upland or in moderately inundated lowlands.

The African cultivated rice has been increasingly replaced by Asian cultivated species, *O. sativa*, because of its low grain weight potential (grain shattering, lodging susceptibility), but farmers still favor *O. glaberrima*. In Asian rice, variability in resistance to rice yellow mottle virus (RYMV) and African rice gall midge (AfRGM) is limited. By contrast, abundant sources of resistance are found in *O. glaberrima* as well as several other useful traits such as good weed-competitiveness due to early vigor and excellent ground cover and tolerance to drought, soil acidity and other stresses. The reproductive barriers between the two species make the utilization of *O. glaberrima* in rice improvement difficult.

Genetic diversity refers to the variation of genes within species, that is, the heritable variation within and between populations of organisms. An understanding of the extent and distribution of genetic diversity within and between populations of a species is essential to (i) choose populations for conservation programs, (ii) estimate any possible loss of genetic diversity during conservation programs, (iii) assist the selection of parents for breeding and (iv) offer evidence of the evolutionary forces (mutation, random genetic drift, natural selection and gene flow) shaping natural populations (Thormann et al., 1994).

It is now well established that rice relative species belonging to the *Oryza* AA genome complex contain a high amount of diversity than *O. sativa*. The indigenous African rice, *O. glaberrima*, has acquired adaptive or protective mechanisms against many of the major biotic and abiotic stresses during its evolution. *O. glaberrima* has a low grain weight potential due to the limited number of spikelets per panicle, few panicles due to lack of secondary branches, grain shattering and poor resistance to lodging. However, it represents a rich reservoir of useful genes for resistance and/or tolerance to many of the stresses compared to *O. sativa*. These include (i) the ability to mature early and synchronize maturities towards the end of the wet season; (ii) the ability to withstand and/or escape drought; (iii) high recoverability when water becomes available after a severe drought; and (iv) the presence of high root biomass. *O. glaberrima* thin leaves roll quickly and its thin roots penetrate and make close contact with the soil particles to effectively mine water and nutrients (Dingkuhn et al., 1999). These traits are very useful in breeding for drought tolerance in rice (Babu et al., 2004).

Morphological and agronomic traits have long been the means of studying classification and variability among populations and species (Bretting and Widrlechner, 1995). The study of genetic variation and structure has been greatly facilitated by the advent of DNA marker

technology in the 1980s, which offered a large number of environmentally insensitive genetic markers that could be generated to follow the inheritance of important agronomic traits (Peleman and Van Der Voort, 2003). Microsatellite or simple sequence repeat (SSR) (Akkaya et al., 1992) markers are the most commonly used DNA-based marker types for a wide range of purposes (Olufowote et al., 1997; Temnykh et al., 2001; Semon et al., 2005). Earlier studies using isozymes and RFLP markers revealed the presence of higher level of variability in *O. sativa* (Second, 1982; Wang and Tanksley, 1989) but little allelic diversity among *O. glaberrima* lines (Second, 1982; Ishii et al., 1993). Second (1982) postulated that the low diversity of *O. glaberrima* is due to a bottleneck effect during the domestication process. Recent studies using micro-satellite and retrotransposon probes have detected DNA polymorphism among *O. glaberrima* accessions (Semon et al., 2005). Barry et al. (2007) worked on 26 *O. glaberrima* accessions from Maritime Guinea. The genetic diversity detected was therefore representative of just one region of *O. glaberrima* diversification. Semon et al. (2005) worked on a larger number of accessions (198) collected from 12 different countries in West Africa with 93 SSR markers. A close genetic relationship between *O. sativa* and some *O. glaberrima* accessions has been reported in other studies (Semon et al., 2005; Barry et al., 2007). The long cohabitation of the two cultivated species until the 15 – 17th century may have induced an intro-gression of *O. sativa* into *O. glaberrima* which resulted in the creation of intermediates that cannot be readily distinguished on the basis of phenotypic characters (Semon et al., 2005).

Although the inland valley of the Niger River Delta, which covers different regions of Mali, is known as one of the centers of domestication for *O. glaberrima* rice species (Linares, 2002; Semon et al., 2005), the amount and distribution of genetic diversity in the area has not been properly studied. Therefore, molecular and morphological characterization of the germplasm in this location for use both in conservation and breeding programs is indispensable. Institut d'Economie Rural (IER) scientists, with the support of the Rockefeller Foundation, recently collected 121 *O. glaberrima* accessions from the Niger River Inland Delta (Mopti and Tombouctou), Sikasso and Gao Regions in Mali. These accessions were named Riz Africain du Mali (RAM) or "African Rice from Mali". Preliminary field evaluation of the accessions for different drought tolerant-related traits revealed the presence of a wide range of variation among accessions (Fousseyni Cisse, personal communication). However, the extent of genetic diversity and patterns of relationship among these accessions both at the molecular and agromorphological trait level largely remain unknown. The objectives of the present study are, therefore to investigate (i) the genetic variability and relationship among 83 rice accessions using both microsatellite markers and morphoagronomic data and (ii) the implication of breeding programme.

Table 1. Number, growing condition and description of collection area of *O. glaberrima* (RAM) accessions including varieties used as checks.

Number of accession	Growing condition	Region*	Country of origin	Altitude	Latitude	Longitude
27 RAM	Floating and medium depth	Tombouctou	Mali	266	16°47'N	3°01'W
21 RAM	Floating, medium depth and sub floating	Gao	Mali	271	16°18'N	0°04'W
7 RAM	Floating and sub floating	Sikasso	Mali	443	11°20'N	5°41'W
24 RAM	Floating, medium depth and sub floating	Mopti	Mali	272	13°49'N	4°34'W
TOG7106	Low land	NA	Nigeria	NA	NA	NA
TOG5672	Lowland	NA	Nigeria	NA	NA	NA
IR64	Low land/irrigated	NA	Phillipine	NA	NA	NA
Moroberekan	Lowland	NA	Ivorycoast	NA	NA	NA

* Location where the samples are collected. NA: information not available.

MATERIALS AND METHODS

Study materials

The studies were conducted using 83 accessions (Table 1) which include 79 RAM accessions collected from four regions (Sikasso, Mopti, Tombouctou and Gao) in Mali and four other genotypes obtained from different sources. The geographical distribution of the accessions is shown in Figure 1.

Molecular characterization

DNA was extracted from 2 weeks old seedlings of 83 rice samples using a cetyl trimethyl ammonium bromide (CTAB) protocol as described by Saghai-Marouf et al. (1984). The isolated DNA was dissolved in double distilled water and then quantification and amplification were performed as described by Semagn et al. (2006). The amplified products were separated on 7% non-denaturing polyacrylamide gel and stained with ethidium bromide. Thirty-seven SSR primers chosen for the molecular characterization of these accessions are evenly dispersed on the rice genetic map. They were selected among the 49 used for the genetic diversity of the world rice collection by the Generation Challenge Programme (www.generationcp.org).

Experimental site for field evaluation

For morphological evaluation of the accessions, a field experiment was conducted in 2007 and 2008 at the WARDA station in Cotonou, Benin (21 m above sea level; 6°25'N latitude and 2°19'W longitude).

Split plot design replicated over years was used, with drought and irrigated conditions as main plot and the genotypes as subplot factor. Seventy nine (79) rice accessions, including four checks (Moroberekan, IR64, TOG5672 and TOG7106), were used. Fertilizer was applied at recommended rate of 80 kg N, 30 kg P₂O₅ and 30 kg K, respectively, at final harrowing. Thinning was done two weeks after sowing and frequent weeding was carried out. Drought stress was applied by imposing 38 days drought stress at the vegetative stage, starting 30 days after sowing, while the control treatment was continuously irrigated. Data were collected from four randomly selected plant found in the middle rows following the Standard Evaluation System (SES) of IRRI (1996). Agronomic traits (Plant height, tiller number, Begin germination, 50% germination,

Germination vigor, 50% flowering, Maturity date, leaf length, Leaf width, Leaf number, SPAD and grain weight) and drought related characters (leaf rolling and drying) were measured and analyzed using SAS software (version 6.11) (SAS, 1999). In this model, genotypes and water (drought and irrigated conditions), were considered as fixed whereas year was considered as random. Least square means of the traits in drought and irrigated conditions were used to compute the percent variation, P:

$$P = 100 \frac{T_{irrig} - T_D}{T_{irrig}}$$

Statistical analyses of molecular data

A data set consisted of 83 rows representing accessions and 37 columns corresponding to the total number of polymorphic SSR loci was used for the statistical analyses. POPGENE version 1.32 software (Yeh et al., 1999) were used to calculate genetic diversity for each population as number of polymorphic loci and percent polymorphism, effective number of alleles as $AE = 1/(1-h) = 1/\sum p_i^2$ where p_i is the frequency of the i th allele in a locus and $h = 1 - \sum p_i^2$ refers to heterozygosity in a locus, Shannon information index as a measure of gene diversity, Fixation index were calculated as $F_{ST} = (HT - HS)/HT$ where HT is total diversity and HS is mean within population gene diversity and gene flow estimated from fixation index as $Nm = 0.25 (1 - F_{ST})/F_{ST}$. The unweighted pair group method with arithmetic mean (UPGMA) was used to analyze and compare the regions and then the ecologies based on individual genotypes. Finally phenograms were generated phenogram using NTSYS- pc version 2.02 (Rohlf, 2000).

RESULTS

Genetic diversity of accessions assessed by microsatellites

Table 2 shows superior observed heterozygosity among populations under location (0.36) than growing condition based (0.10). Shannon diversity index showed differences in diversity among populations. The population



Figure 1. Map of Mali and geographic locations (Tombouctou, Gao, Mopti and Sikasso) where RAM accessions were collected.

Table 2. List parameters for genetic analysis of location and growing condition based RAM accessions with their respective mean value.

Location	Number of individuals	Parameters							
		n_e	I	Mean H	NPL	PP	FIS	Fst	Nm
Gao	21	1.96	0.72	0.18	35	94.60			
MPT	24	1.80	0.70	0.17	36	83.78			
SKS	7	1.58	0.51	0.18	31	83.78			
TMB	27	1.40	0.46	0.18	33	89.20			
Overall		1.72	0.67	0.18	37	100.00	0.70	0.06	3.76
Growing condition									
Floating	26	1.48	0.52	0.35	34	91.89			
Medium depth	24	1.80	0.68	0.35	36	97.30			
Subfloating	27	1.84	0.71	0.35	36	97.30			
Overall		1.71	0.67	0.35	37	100.00	0.70	0.03	7.83

*TMB:Tombouctou; SKS: Sikasso; MPT:Mopti; GA:Gao; Phill: Philippines. n_e : Effective number of allele; I: Shannon's information index; Mean H: mean heterozygosity; NPL: Number of polymorphic loci; PP: Percent polymorphic loci; Nm: Gene flow; Fst: Fixation index.

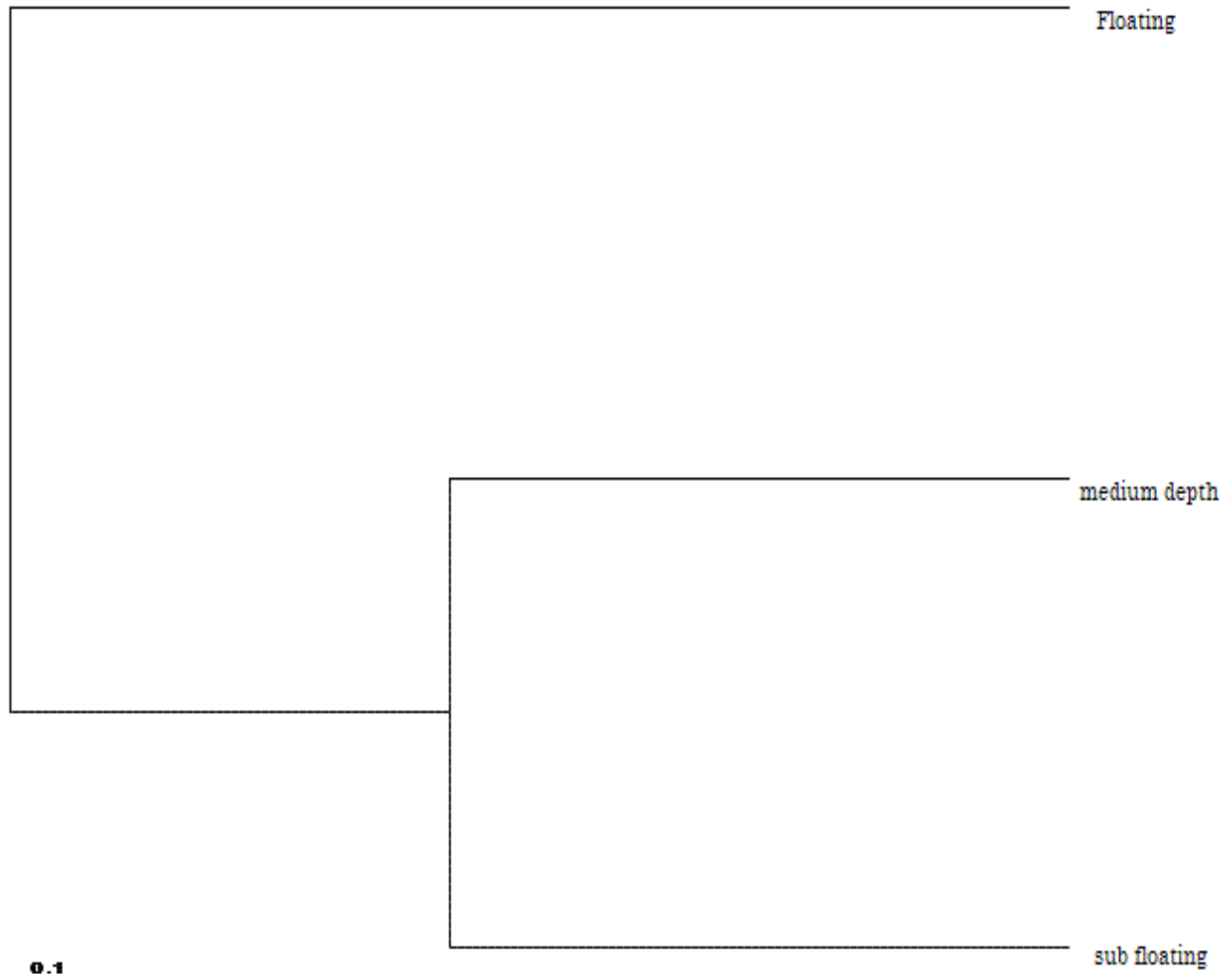


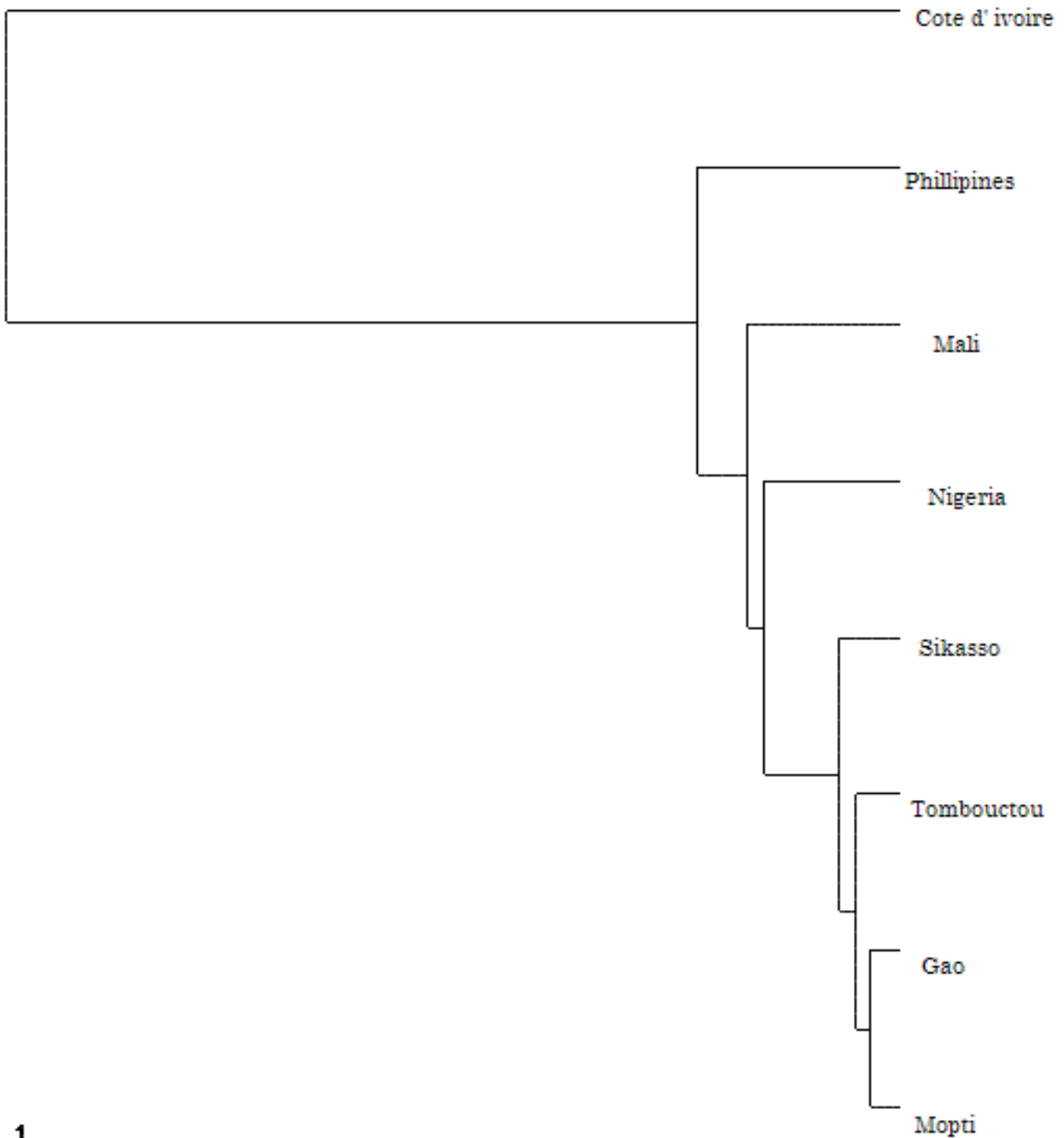
Figure 2. UPGMA based on three growing condition based RAM rice populations using 37 SSR markers.

from the subfloating growing condition had higher Shannon diversity index (0.71) than populations from both floating (0.52) and medium depth (0.68) growing conditions. Among the growing conditions, the subfloating population had more effective number of alleles (1.85) than medium depth (1.80) and floating (1.48) populations. Percent polymorphism was also found higher in medium depth (97.30) and sub floating (97.30) growing conditions than in the floating growing condition (91.89). On the other hand, among the different locations, Gao had the highest effective number of alleles (1.96) followed by Mopti (1.80), Sikasso (1.58) and Tombouctou (1.40). The subfloating and medium depth population had the highest number of polymorphic loci (36) and percent polymorphism (97.30) while the floating population had the lowest (34 and 91.89%, respectively). Mopti and Gao populations had more polymorphic loci (36 and 35 with percent polymorphism of 97.30 and 94.60 respectively than the Sikasso and Tombouctou populations with

number of polymorphic loci 31 and 33 and percent polymorphism 83.78 and 89.20, respectively. There is no difference in the overall effective number of alleles, Shannon diversity index, number of polymorphic loci, percent polymorphism under both discriminant factors, and mean heterozygosity within population based on location and growing condition. .

Genetic similarity and cluster analysis

Medium depth and sub-floating populations show more tendency of similarity (Figure 2) and found distinct from floating population which form separate group. Similarly, from populations based on location the Tombouctou and Sikasso populations were the closest populations whereas the Mopti and Gao populations were found more distant and form separate cluster (Figure 3). Neither location nor growing condition show clear pattern of grouping



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Figure 3. UPGMA based on location based 79 RAM rice populations collected from different regions of Mali including 4 other rice accessions collected from different areas using 37 SSR markers.

for individual based clustering analysis (data not shown).

Performance of *O. glaberrima* accessions under drought conditions

Table 3 summarizes the percent variation of different traits under irrigated and drought conditions. The overall

grain weight performance of accessions under drought conditions was low, with a grain weight loss of up to 23.90% compared to regular watering. In addition to grain weight, percent variation in plant height (10), days to 50% flowering (6.24) and days to maturity (5.40) were significant at $\alpha = 0.05$ and positively correlated with irrigated conditions, while percent variation in SPAD (-9.07) was also significant but with a positive relationship with

Table 3. List of different traits, their respective mean value and percent variation of field evaluation of 79 RAM accessions, including the 4 checks.

Traits	Irrigated		Drought		Variation (%)	Significance ($\alpha = 0.05$)
	Mean	SD	Mean	SD		
Plant height	83.0	10.39	74.7	6.55	10.0	**
Tiller number	11.29	6.23	11.28	3.51	0.09	NS
Begin germination (DAS)	6.39	1.68	6.52	1.68	-2.03	NS
50% germination (DAS)	6.85	0.67	6.98	0.62	-1.86	**
Germination vigor	3.36	0.51	3.24	0.73	3.57	NS
50% flowering (DAS)	86.5	8.52	81.1	6.19	6.24	**
Maturity (DAS)	106.8	3.67	101.0	2.49	5.4	**
Leaf length (cm)	33.5	7.39	35.0	7.42	-4.47	NS
Leaf width (cm)	1.22	0.17	1.19	0.15	2.46	NS
Leaf number	3.67	0.6	3.68	0.57	-0.27	NS
SPAD	30.9	3.51	33.7	2.46	-9.07	**
Grain weight (g/plant)	5.85	2.26	4.45	1.28	23.93	**

** Significant difference at $\alpha=0.05$; NS: no significant difference; SD: standard deviation; DAS: days after sowing; SPAD: chlorophyll meter for leaf greenness

drought conditions. Although the difference in percent variation in tiller number (0.09), germination vigor (3.57) and leaf width (2.46) was not significant, it also showed a positive relation with irrigated conditions. On the other hand, percent variation in beginning of germination date (-2.03), days to 50% germination (-1.86), leaf length (-4.47) and leaf number (-0.27) shows no significant difference but had a positive relation with drought conditions.

In the field evaluation, RAM116, RAM100, RAM122 and Moroberekan gave the best grain weights (9.09, 6.50, 5.80, and 5.40 g/plant, respectively) under drought conditions. RAM10, TOG5672, and RAM1 with 10.10, 9.30, and 8.70 g/plant, respectively (data not known) were the best grain weight under irrigated conditions, whereas RAM122, RAM116 (9.09 and 5.80 g/plant, respectively) were the most stable accessions and gave the highest grain weights under both irrigated and stress conditions.

The average grain weight of all the RAM accessions was 1.29 g/plant. The 14 highest grain weighting RAM accessions under drought conditions are presented in (Table 4). The checks Moroberekan, IR64, TOG7106 and TOG5672 grain weighted 5.45, 3.46, 3.35 and 2.49 g/plant, respectively, and were also considered to have good tolerance to drought.

Although RAM100, RAM122, RAM116 gave higher grain weights than the checks, only RAM116 performed significantly better ($\alpha = 0.05$) than the best check (Moroberekan). Moreover, this accession also had better stability, with good performance under both drought and irrigated conditions.

RAM122 and RAM116 had better leaf rolling ability and better tolerance to leaf drying than the check, Moroberekan (Table 4). Conversely, Moroberekan was more resistant to blast than both RAM122 and RAM116 as resistant as

RAM100, whereas RAM 107 and IR64 were immune to blast.

DISCUSSION

Genetic variability and proximity

Different reports (Second, 1982; Barry et al., 2007) indicated a lower genetic diversity in *O. glaberrima* species than in the Asian rice, *O. sativa*. Although all the genetic diversity parameters showed less variability among populations included in this study, the mean heterozygosity of 0.18 recorded was found higher than 0.03 obtained by Second (1982) and 0.07 reported by Barry et al. (2007). The overall genetic diversity, in terms of both mean heterozygosity (0.17) and Shannon diversity index (0.70), observed at Mopti next to Gao in the inland valley of the Niger River Delta could be related to the history of the area, which is believed to be one of the three *O. glaberrima* domestication centers (Linares, 2002; Semon et al., 2005) and higher genetic diversity at Gao could be explained as seed flow from Mopti area. The population from Sikasso and Tombouctou were found with less heterozygosity and Shannon diversity index. Under different growing conditions, populations with relatively similar growing conditions were more closely connected and had higher gene flow ($N_m = 7.83$) than between populations from different locations ($N_m = 3.76$). Populations were less differentiated ($F_{st} = 0.03$) under different growing conditions than locations ($F_{st} = 0.06$) because different growing conditions could exist in one location but the overall result of F_{st} shows less population differentiation. FIS (inbreeding coefficient) of 0.70 also shows a considerable degree of inbreeding. Dendrogram of individuals

Table 4. List of better performing *O. glaberrima* (RAM) accessions including checks, area of collection, cycle, growing condition, their respective yield, leaf drying, leaf rolling, and recover ability under drought condition.

Accessions	Location	Growing condition	Cycle*	Yield (g/plant)	Leaf drying (e.o.s.)	Leaf Roll ^a	Leaf drying (rec.)	Recover ^b
RAM116	Mopti	Sub floating	3	9.09	3	9	2	1
RAM100	Tombouctou	Medium depth	4	6.49	3	6	3	1
RAM122	Mopti	Sub floating	3	5.84	1	8	1	1
Moroberekan	Ivory coast	upland	4	5.45	3	7	3	1
RAM90	Mopti	Sub floating	3	4.95	3	9	3	1
RAM138	Mopti	Sub floating	3	4.65	3	5	1	1
RAM28	Gao	Floating	5	3.86	2	6	4	1
RAM107	Sikasso	floating	5	3.73	4	9	3	1
RAM137	Mopti	Sub floating	3	3.5	3	9	3	1
IR64	Phillipine	Lowland/irrigated	4	3.47	3	9	2	1
RAM73	Mopti	Sub floating	3	3.44	1	7.5	2	1
TOG7106	Nigeria	Lowland	3	3.35	3	9	2	1
RAM101	Tombouctou	Medium depth	4	3.15	2	9	3	1
RAM8	Tombouctou	Floating	5	3.09	3	9	3	1

*Growth period in months.

e.o.s.: at end of Stress; rec.: after recovery period.

^a Leaf rolling ability at the end of the stress.

^b Recover ability of the accessions 10 days after re watering.

showed no clear cluster grouping both among location and growing condition-based populations, except some tendency for separate grouping shown by floating and Sikasso populations (data not shown). This could also be due to the existence of a higher gene flow among populations.

Drought response of *O. glaberrima* accessions: Effect of water stress on grain weight performance

Although *O. glaberrima* varieties have certain negative features relative to *O. sativa*, they also have some advantages, like good performance under adverse environmental conditions (Linares, 2002; Sarla and Swamy, 2005). In this study, despite the overall grain weight loss of 23.93% due to drought stress, three RAM accessions-RAM116, RAM100 and RAM122 - were more tolerant of drought stress than the best performing check, Moroberekan. RAM116 and RAM 122 from Mopti, sub-floating growing condition where relatively found with higher genetic diversity. These two accessions were also more stable than all the accessions and gave better grain weight under both water stress and irrigated conditions. The better performance of Moroberekan, the so called African *sativa*, over the other checks could also be due to gene admixture with *O. glaberrima* (Semon et al., 2005).

All the best accessions from Mopti were early maturing, which is one of the desirable characteristics for escaping drought by completing the most sensitive developmental stages while water is abundant (Bernier et al., 2008). Early materials sometimes also tend to have a more

stable harvest index than later maturing ones (Lafitte et al., 2003). The mean of different traits from all the locations showed that, under drought conditions, the accessions were less vigorous, matured earlier, had better tillering capacity and narrower leaves than under irrigated conditions. Germination started and attained 50% late; Leaves were longer, while SPAD was higher under drought conditions. Higher yielding accessions from Mopti had better tolerance of leaf drying, high recoverability and resistance to blast. High leaf rolling extent, which can help to reduce water loss in addition to reducing the leaf area exposed to heat and light radiation, was also observed in these accessions (Price and Courtois, 1999). Tolerance of accessions to leaf drying could be used by breeders as criteria for selection (Jongdee et al., 2006; Lafitte et al., 2003). There is also some evidence that enhanced ability to roll leaves confers a grain weight advantage under drought conditions (Singh and Mackill, 1991) and correlated with leaf drying (Price et al., 2002). However, leaf rolling has traditionally been used by breeders to indicate drought severity and used as a negative criterion (Price et al., 2002).

The implications of the results for breeding

The results confirm the existence of a relatively high reservoir of genetic diversity of *O. glaberrima* in Mali especially in the inland valley of the Niger River delta, believed to be one of the domestication centres for the African rice. This area is, therefore, an appropriate place for *in situ* conservation of *O. glaberrima* for future breeding

programs.

In addition to the high genetic diversity offered by accessions from the sub-floating growing condition at Mopti, their early maturity is important for drought escape; this can help breeders, as one of the criterion in selecting parents for rice crop improvement on adaptability to stress and other desirable characteristics. Consequently, the three best performing accessions (RAM116, RAM100 and RAM122) can be selected as parents for transferring drought tolerance to *O. glaberrima* x *O. sativa* interspecific hybrids.

Although identifying grain weight limiting traits and applying them effectively in a breeding program are major challenges, because of the different types of drought and seasonal variations in the severity of drought (Richards et al., 2001), some of the characters observed in better grain weighting accessions - like tolerance to burning/leaf drying, leaf rolling extent and recoverability - could also help breeders in selecting parents.

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