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RESEARCH ARTICLE

Morphological and Genetic Diversity Analysis of Rice Accessions (*Oryza sativa* L.) Differing in Iron Toxicity Tolerance

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Abstract

A major emphasis in breeding for iron toxicity tolerance in rice is to identify differences that are associated with resistance and harness them for genetic improvement. In this study, thirty accessions, including IRRI gene bank accessions, two varieties from Brazil, 8 cultivars from West Africa and 10 cultivars from Uganda were analyzed for sensitivity to iron toxicity, and genetic diversity using morphological and SSR markers. Two genotypes, IR61612-313-16-2-2-1 and Suakoko 8 showed significantly high resistance with an average score of ≤ 3.5 on 1 - 9 scale. The SRR markers were highly informative and showed mean polymorphism information content (pic) of 0.68. The PIC values revealed that RM10793, RM3412, RM333, RM562, RM13628, RM310, RM5749, and RM154 could be the best markers for genetic diversity estimation of these rice cultivars. Diversity at the gene level showed an average of 4.61 alleles ranging from 2 to 12 per locus. Mean gene diversity (H) value for all SSR loci for the 30 genotypes evaluated was 0.69 but was decreased to 0.53 when analysis was performed on Ugandan accessions. The low genetic diversity found among the Ugandan accessions is the evidence of a narrow genetic base, and such a scenario has a potential vulnerability for resistance break down. A low correlation was detected between the observed molecular and morphological datasets. This means that a combination of morphological traits and SSR analysis would be required when assessing genetic variation under iron toxic conditions, and could be a practical strategy for breeders when planning crosses. A distinction between the resistant and susceptible accessions in both phenotyping and SSR datasets suggests the presence of unique alleles that could be harnessed for improvement of rice against iron toxicity.

Key words: genetic diversity, iron toxicity, phenotypic variation, Phospho-Molybdate dye assay, SSR

Introduction

Rice is the world's most important food crop, serving as staple food for more than half of the world's population (Khush 2005). Genetic diversity of rice plays an important role in sustainable development and food security, as it allows the cultivation of crops in the presence of various biotic and abiotic stresses. It is also important for the selection of parents that can be used in plant breeding programs. Several biotic and abiotic stresses threaten replace with productivity in rice-growing areas. Iron (Fe) toxicity is one of the most important yield-limiting abiotic stresses in flooded lowland rice production areas in Africa Since the first report of its occurrence (Ponnamperuma et al. 1955), iron toxicity in rice has been reported in several countries in Asia, South America, West and Central Africa, and Uganda (De Datta et al. 1994; Onaga et al. 2012; Sahrawat 2004; van Breemen and Moormann 1978; Yoshida 1981). Injuries due to iron toxicity are estimated to reduce overall rice grain yield by 30 - 60% (Majerus et al. 2007b; Sahrawat 2000). Toxicity at seedling and early vegetative stages can strongly affect plant growth, and may result in complete yield loss (Abifarin 1988). Many cultivars are

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hypothesized to employ tolerance rather than avoidance or exclusion mechanisms (Becker and Asch 2005; Yamanouchi and Yoshida 1981). Storage in the apoplast and vacuole, and detoxification of Fe-induced reactive oxygen species (ROS) by antioxidant enzymes are the mostly reported tolerance mechanisms (Dufey et al. 2009). These mechanisms are important in the selection of tolerant rice genotypes but have not been directly targeted in breeding programs because of the difficulties involved in precise measurements (Shabala 2010). Efforts to identify QTLs have focused on easily measurable traits such as leaf bronzing index, shoot and root dry weight, tiller number, plant height, root length, and Fe accumulation in the shoot (Dufey et al. 2009; Wan et al. 2003). Studies have demonstrated that these traits could potentially complement molecular markers in characterizing a germplasm collection (Majerus et al. 2007a; Shimizu et al. 2009). On the other hand, successful crop improvement depends on genetic variability that arises from genetic diversity (Rana and Bhat 2004). Lack of genetic diversity may limit breeding progress and gain from selection (Cornelius and Sneller 2002). Thus, understanding both morphological and genetic diversity of a germplasm collection provides a basis for improvement of crops and development of resistant varieties. Simple sequence repeats (SSR) are the most widely-used DNA marker type to characterize germplasm collections of crops (Van Inghelandt et al. 2010). The desirable features that make SSRs suitable for assessing the genetic diversity include co-dominance, multiallelism (Vignal et al. 2002), locus specificity, and high reproducibility (Jones et al. 2007; Smith et al. 1996). SSRs have been effectively used to identify genetic variation among rice cultivars (Caicedo et al. 2007; Garland et al. 1999; Garris et al. 2005; Giarrocco et al. 2007; Thomson et al. 2007, 2009). Hence, SSRs possess considerable potential not only for genetic fingerprinting and genome mapping within species but also for comparative studies (Bandopadhyay et al. 2004; Cordeiro et al. 2001; Thiel et al. 2003; Varshney et al. 2004). The purpose of this study was to identify sources of resistance to iron toxicity and to understand the genetic diversity among Ugandan cultivars and compare them with the IRRI and Brazilian tolerant genotypes.

Materials and Methods

Plant materials, iron treatment, and evaluation of iron toxicity tolerance

A collection of 10 rice accessions from Uganda, eight genotypes from West Africa, two genotypes from Brazil, and 10 International Rice Research Institute (IRRI) gene bank accessions were used for phenotypic and genotypic analysis. Rice seeds were soaked in water, incubated, and pre-germinated on filter papers in Petri dishes in the dark at 32°C until the seeds sprouted at about 48 h. The germinating seeds were transferred to polystyrene floating plates in a plastic container filled with deionized water and the entire setup was transferred to the phytotron. After 3 d, the seedlings were transferred to half-strength Yoshida solution (Yoshisa et al.1976) for 4 d, and later changed to full-strength nutrient solution for 5 d. Seedlings with uniform growth were selected and introduced into a nutrient solution (pH 4.0) containing 300 ppm Fe^{2+} in 40 L plant growing tubs. The nutrient solution was initially low in phosphorus (0.045 mM). The control solution contained 60 ppm of Fe^{2+} with EDTA in approximately 1:1 concentration (molar base). The pH, Fe^{2+}, and P were checked and adjusted every 2 d to ensure availability of phosphorus (P) for plant growth and Fe^{2+} for toxicity induction. Iron toxicity symptoms were evaluated at 14 d after subjecting the seedlings to iron toxicity and the data on plant height, root length, toxicity score, shoot and root dry weight was recorded. Plant traits were presented as 'relative values', which is the growth in $FeSO_4$-treated seedlings relative to that in the control (%). Leaf bronzing types 1 to 3 were considered as resistant and 5 to 9 as susceptible in the iron toxicity evaluation scale of 1 - 9 (International Rice Research Institute 1996). All of the values for each parameter were means of three independent replications. An ANOVA was performed on growth variables using the GLM procedure of SAS 9.3 (SAS Institute 2012). Tukey's multiple range test was used for the comparisons of the growth parameters measured among the genotypes. To identify the trait that effectively differentiated the 30 genotypes, Principal Component Analysis (PCA) was carried out using MINITAB release 15 version 15.0.0.1, 2007 (Minitab Inc., Pennsylvania, USA), based on mean values of the five morphological traits measured on the 30 genotypes.

DNA extraction and SSR analysis

Sample DNA was extracted using the modified CTAB method (Thomson et al. 2007). Briefly, leaf samples were collected from 21-day-old seedlings, frozen, and ground in liquid nitrogen. The fine powder was allowed to thaw in 2mL Eppendorf tubes and 800 µL of pre-heated extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 1.25% (w/v) SDS, 3.8 M NaBisulfite) was added. The mixture was vortexed and incubated at 65°C for 20 min. A total of 10% CTAB was added and incubated at 65°C for 15 min, followed by centrifugation at 13,000 ×g in a microcentrifuge. The aqueous phase containing DNA was transferred to a new 1.5 mL Eppendorf tube. Chloroform extraction was performed with 24:1 chloroform:isoamyalcohol solution. DNA was precipitated by adding 800 mL of ice cold isopropanol. The pellets were rinsed twice with 70% ethanol, air-dried, and dissolved in 100 µL of Tris-EDTA buffer [10 mM Tris-HCl (pH 8) and 1 mM EDTA]. The DNA concentration was checked by the use of NanoDrop ND-100 spectrophotometry and adjusted accordingly.

PCR and gel electrophoresis

Each PCR reaction contained 1.5 µL of 10 × buffer (200 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 30 ng of DNA template, 1 mM dNTP, 5 µM of forward and reverse primers, and 4 U μL^{-1} of *Taq* DNA polymerase. SSR

Table 1. Relative plant height, root length, shoot dry weight, and root dry weight of the genotypes exposed to 300 ppm Fe^{2+}. Values are the means of three replicate experiments

Genotype/cultivar	Relative plant height		Relative root length		Relative shoot dry wt		Relative root dry wt	
WAC 117	0,50	b - d	0,48	b - d	0,88	ab	0,92	ab
WAC 116	0,49	c - e	0,38	c - e	0,84	a - c	0,92	a - c
IR 47686-9-2-B	0,51	b - d	0,49	b - d	0,80	bc	0,89	bc
GIGANTE	0,41	ef	0,33	de	0,79	bc	0,84	b - d
PNA	0,35	fg	0,45	cd	0,59	g	0,62	g
SUPA	0,58	ab	0,60	b - c	0,91	a	0,94	a
K85	0,45	d - f	0,54	bc	0,78	b - d	0,85	b - d
K5	0,40	ef	0,24	e	0,69	ef	0,75	de
K98	0,52	bc	0,65	ab	0,88	ab	0,89	bc
NS4	0,39	ef	0,67	ab	0,67	ef	0,76	c - e
KAYISO	0,46	c - e	0,40	c - e	0,70	d - f	0,70	e - g
KYABUKOOLI	0,57	ab	0,45	cd	0,79	b - d	0,74	d - f
KIBUYU	0,47	c - e	0,48	b - d	0,71	c - f	0,73	d - f
WITA 3	0,52	bc	0,53	bc	0,82	bc	0,84	b - d
WITA 4	0,47	c- e	0,53	bc	0,78	b - d	0,85	b - d
SEBAGALA	0,47	c - e	0,40	c - e	0,79	b - d	0,83	cd
JAGARI	0,61	a	0,56	bc	0,88	ab	0,93	ab
IR 73678-20-1-B	0,44	d - f	0,45	cd	0,72	c - f	0,77	c - e
IR 80310-12-B-1-3-B	0,51	b - d	0,52	bc	0,88	ab	0,92	ab
BRIRGA 409	0,48	c - e	0,66	ab	0,74	c - e	0,63	g
IRGA 420	0,40	ef	0,64	ab	0,71	c - f	0,69	e - g
SUAKOKO 8 (IRGC 50405)	0,39	ef	0,51	bc	0,86	a - c	0,89	bc
AZUCENA (IRGC 52992)	0,45	d - f	0,26	de	0,76	b - d	0,82	cd
AZUCENA 2005DS (IRGC 113515)	0,51	b - d	0,49	b - d	0,75	c - e	0,86	b - d
AZUCENA 2001DS (IRGC 97853)	0,36	f	0,53	bc	0,77	b - d	0,84	b - d
AZUCENA 2008WS (IRGC 117264)	0,49	c - e	0,29	de	0,77	b - d	0,84	b - d
IR61612-313-16-2-2-1	0,36	f	0,68	a	0,72	c - e	0,84	cd
AZUCENA 2001DS (IRGC 52861)	0,57	ab	0,45	cd	0,85	a - c	0,89	bc
BAO THAI (IRGC 61181)	0,37	f	0,45	cd	0,74	b - e	0,75	de
IR64	0,48	c - e	0,40	c - e	0,79	c - d	0,82	cd
ANOVA (model)	***		***		***		***	
CV	10,01		22.03		7,82		7,03	

***indicates significance of the F test at P >0.001. Means followed by the same letters are not significantly different at 5% probability error.

primer sequences were chosen on the basis of the published rice microsatellite framework map (McCouch et al. 2002; Temnykh et al. 2000). The motifs for these markers can be found in a public domain (http://www.gramene.org/markers/microsat/). Details of the SSR loci are presented in Table 3. The PCR cycles consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 5 min. PCR products were resolved in 1% agarose gels, stained with Sybr Safe, and viewed on a UV transilluminator (AlphaImager gel documentation system).

Data analysis

The alleles of each SSR locus were scored (bp) with reference to a known size standard followed by transformation to binary codes as presence (1) or absence (0) of the respective fragment size. A 50 bp DNA ladder (Fermentas) was used as standard for scoring. Bands that were diffuse or too difficult to score were considered as missing data ('9'). In cases where multiple bands of varying intensity were found, the most intense band was scored as '1', and the others considered as missing in the statistical analysis. The polymorphic information content (PIC) was calculated for each SSR according to the formula described by Anderson et al. (1993). Allelic diversity of the SSRs was calculated according to the diversity index, H, described by Nei (1987), in the following formula

$$H_E = \frac{2N}{2N-1}\left(1-\sum_{i=1}^{n} P_i^2\right)$$

where Pi is the frequency of the i^{th} of k alleles. Genetic similarity between the genotypes was estimated according to Dice coefficient, i.e. Dc = 2nxy/(nx + ny), where nx and ny represent the putative number of SSR alleles for materials X and Y, respectively, and nxy represents the number of putative SSR alleles shared between X and Y (Dice 1945). UPGMA clustering was performed following the Sequential Agglomerative Hierarchical and Nested (SAHN) method of the software NTSYS-pc (Rohlf 2000). To determine if the dendrogram obtained was a good fit to the similarity matrix, the cophenetic coefficient was computed and tested using the Mantel matrix correspondence test (MXCOMP program) (Mantel 1967). A Mantel test was also used to assess the significance of correlation between SSR data and morphological data. Principal component analysis (PCA) was performed to better visualize differences between the genotypes and discern groups using GenAlex software version 6.41.

Results

Phenotypic variation under iron toxic conditions

Exposure of rice plants to 300 ppm Fe^{2+} inhibited growth and development of roots and shoots (Fig. 1). Shoot and root biomass were reduced by 78 and 82.0, respectively (Table 1). Plant height and root length were reduced by 47 and 48%, respectively. A high susceptibility to iron toxicity was recorded in Supa and WAC117 with bronzing scores of 8.2 and 8.0, respectively (Fig. 2). IRRI accessions IR61612-313-16-2-2-1 (3.0) and Suakoko 8 (3.5) had the lowest bronzing scores among all the tested genotypes. PNA and IRGA420 had the lowest percentage reduction in both shoot and root dry weight while Supa and Jagari had the greatest reduction in these traits under iron toxicity. Significantly less percentage reductions in plant height and root length were recorded in PNA, IR61612-313-16-2-2-1, and Suakoko 8. Among the AZUCENA accessions evaluated, AZUCENA (IRGC 52992) and AZUCENA 2008WS (IRGC 117264) were the most tolerant. However, all the accessions showed large reductions in root and shoot dry weights (Table 1). Principal component analysis indicated that four principal components accounted for most of the variation (Table 2). The first two principal components accounted for 77.8% of the total variation.

Fig. 1. Effect of iron toxicity on rice shoot and root development of 28-day-old seedlings cultivated in solution culture containing 300 ppm Fe^{2+} (+300 ppm Fe^{2+}) and 60 ppm Fe^{2+} (control). IR61612-313-16-2-2-1 and Suakoko 8 (tolerant, three plants each), Supa (susceptible, two plants).

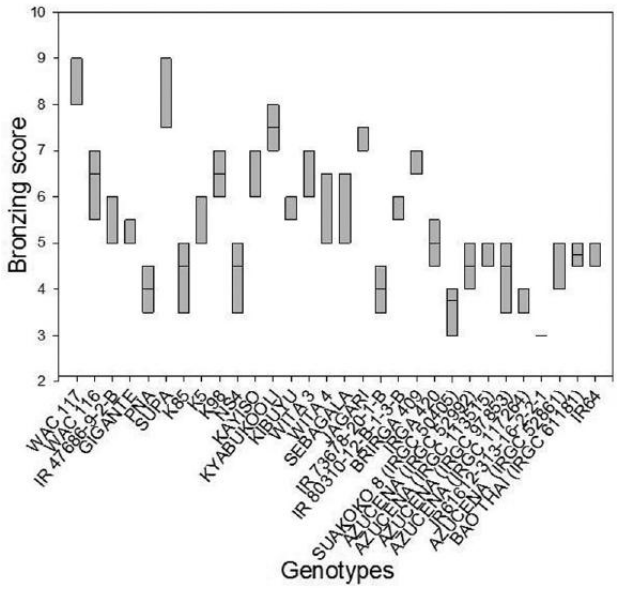

Fig. 2. Severity of leaf bronzing (scores) exhibited by 30 genotypes of rice after exposure to 300 ppm of Fe^{2+} in hydroponic cultures.

Table 2. Correlation matrix of four principal components, Eigen values, relative and cumulative proportion of total variance on traits of genotypes evaluated

Trait	Common principle component coefficients			
	PC1	PC2	PC3	PC4
Plant height (cm)	0.51	0.26	-0.30	0.76
Root length (cm)	0.19	0.53	0.82	0.02
Shoot dry weight	0.55	-0.36	0.10	-0.11
Root dry weight	0.47	-0.53	0.25	-0.13
Iron toxicity score	0.43	0.49	-0.40	-0.63
Eigen value observed	2.77	1.11	0.86	0.21
Relative proportion of total variance	55.50	22.20	17.30	4.20
Cumulative proportion of total variance	55.50	77.80	94.90	99.20

Table 3. List of the 42 polymorphic SSR markers used in this study, their chromosome location and PIC values

SSR Marker	Chr	Core motif	Anealing temp	PCR product size	PIC
RM31	5	(GA)15	55	140	0.71
RM154	2	(GA)21	61	183	0.80
RM169	5	(GA)12	67	167	0.74
RM477	8	(AATT)5	55	223	0.57
RM300	2	(GTT)14	55	121	0.61
RM333	1	(TAT)19(CTT)19	55	191	0.76
RM505	7	(CT)12	55	199	0.65
RM6364	10	(GAA)13	55	163	0.53
RM336	7	(CTT)18	55	154	0.73
RM346	7	(CTT)18	55	175	0.69
RM5806	10	(AGG)9	55	163	0.62
RM5749	4	(ACT)8	55	162	0.75
RM341	2	(CTT)20	55	172	0.72
RM6334	1	(GAA)8	61	147	0.74
RM309	12	(GT)13	55	169	0.63
RM279	2	(GA)16	55	174	0.67
RM224	11	(AAG)8(AG)13	55	157	0.62
RM206	11	(CT)21	55	147	0.68
RM19199	5	(CT)21	-	158	0.47
RM562	1	(AAG)13	55	243	0.75
RM334	7	(CTT)20	55	182	0.62
RM349	4	(GA)16	55	136	0.56
RM310	8	(GT)19	55	105	0.76
RM257	9	(CT)24	55	147	0.60
RM119	4	(GTC)6	67	166	0.59
RM110	2	(GA)15	55	156	0.74
RM515	8	(GA)11	55	211	0.60
RM410	9	(TA)13	55	183	0.56
RM400	6	(ATA)63	55	321	0.71
RM10793	1	(ATAG)7	-	123	0.78
RM5	1	(GA)14	55	113	0.61
RM536	11	(CT)16	55	243	0.68
RM13628	2	(CT)22	-	282	0.77
RM10655	1	(GA)40	-	281	0.69
RM3412	1	(CT)17	55	211	0.78
RM219	9	(CT)17	55	202	0.68
RM149	8	(AT)10	55	253	0.58
RM574	5	(GA)11	55	155	0.59
RM340	6	(CTT)8T3(CTT)14	55	163	0.74
RM232	3	(CT)24	55	158	0.60
RM228	10	(CA)6(GA)36	55	154	0.59
RM5378	2	(TC)14	55	145	0.61

Principal component 1 was positively and strongly associated with shoot dry weight, plant height, and root dry weight. This means that accessions with high values of component 1 had greater reduction in these traits (upper right quadrangle, Fig. 3). Root length and bronzing score were the most important contributors to PC2, which accounted for 22.2% of the total variation. The major characters that contributed to PC3 were root length and root dry weight. Principle component four was mainly contributed by plant height. The rest of the principal components accounted for only 0.08% of the total variation, thus not included in Table 2.

Allelic variability and polymorphism information-content of the SSR markers

Out of the 46 SSRs analyzed on 30 accessions, 42 turned out to be polymorphic and four monomorphic (RM118, RM455, RM429, and RM3683). The number of alleles detected and PIC values of SSRs are given in Table 3. The 42 polymorphic SSRs resulted in 189 alleles averaging 4.61 alleles per locus. The highest number of alleles (12) in polymorphic SSRs was scored for RM154 and the lowest (2) for RM536, RM410, RM346, RM5378, and RM477. The highest PIC value was estimated for RM154 (0.80) and the lowest for RM19199 (0.47) with a mean of 0.68 over the 42 polymorphic loci. The markers RM10793, RM3412, RM333, RM562, RM13628, RM310, RM5749, and RM154 demonstrated a high level of allelic diversity and were found to be highly informative on the set of accessions analyzed (PIC > 0.75). A strong positive correlation (r = 0.74) was found between the PIC values and the number of alleles detected at each marker locus. Marker RM10793 was useful in distinguishing the susceptible cultivars, Supa and WAC117, from Suakoko 8 and AZUCENA accessions. Mean gene diversity (*H*) value for all SSR loci for the 30 genotypes evaluated was 0.69 but decreased to 0.53 when the analysis was performed with only the 10 Ugandan accessions.

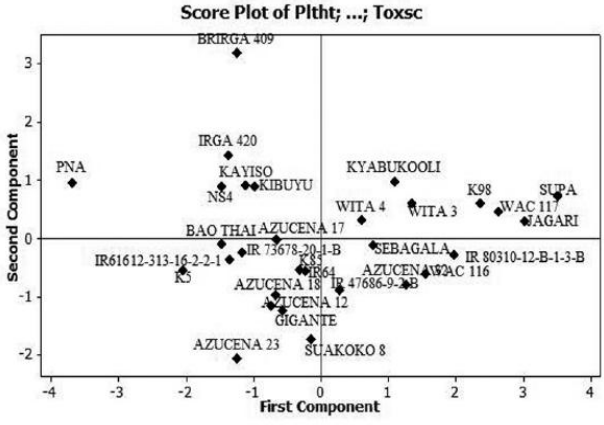

Fig. 3. Principal component analysis of 30 accessions based on morphological traits.

Genetic relatedness among the accessions

The genetic similarity (GS) between accessions varied from 0.15 to 0.92. Minimum similarity was observed between Gigante and AZUCENA 2001DS (IRGC 52861) and Gigante versus Suakoko 8 (0.17). Comparing the mean genetic similarity between accessions within the geographic region, a high genetic similarity was observed between four cultivars from Uganda : Kyabukoli, Kibuyu, Kayiso, and Sebagala (mean GS = 0.81, range 0.64 - 0.92). Accessions K5, K85, and K98 from the same region had a mean similarity of 0.48, with K5 more similar to K85 (0.53) than K98 (0.40). Supa was genetically distant from all the accessions evaluated with the highest similarity to WITA3 (0.51).

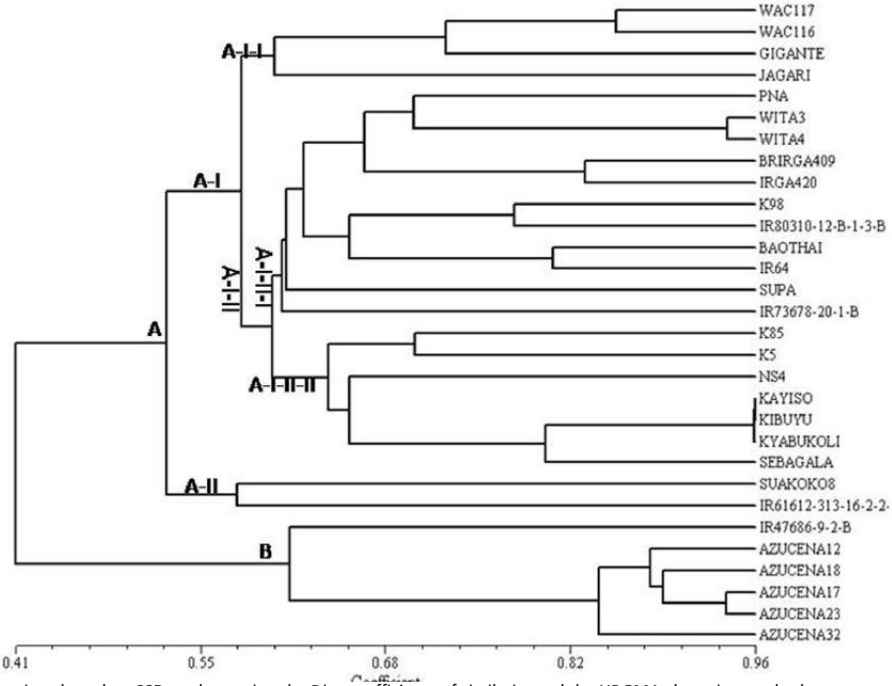

Fig. 4. Cluster analysis of 30 rice accessions based on SSR markers using the Dice coefficients of similarity and the UPGMA clustering method.

Accessions WITA3 and WITA4 from West Africa had a high similarity coefficient of 0.88, followed by WAC116 versus WAC117 (GS = 0.74) from the same region. Accession PNA was only closely similar to WITA3 (0.53). AZUCENA accessions from Asia exhibited high similarity (mean GS = 0.75, range 0.70 - 0.80) but were clearly distant from Ugandan accessions. The two Brazilian accessions included in this study, BRIRGA409 and IRGA420, had a similarity of 0.71, with BRIRGA409 highly distant from all the other accessions than IRGA420. The UPGMA cluster analysis revealed two broad groups of genotypes (Fig. 4). A small cluster (B) predominantly consisted of AZUCENA accessions from IRRI, While all the other 24 accessions were grouped in a big cluster (A). However, cluster A could be seen in two distinct groups at the sub-cluster level. The largest sub group (A-I) consists of 22 accessions representing genotypes from different origins (IRRI, Africa, and Brazil) while sub-group A-II grouped two accessions, Suakoko 8 and IR61612-313-16-2-2-1. Within subgroup A-I, two sub-clusters A-I-I and A-I-II, were obtained. Three accessions from West Africa, WAC116, WAC117, Gigante, and one from Uganda (Jagary) were included in sub-cluster A-I-I. In sub-cluster A-I-II, genotypes tended to aggregate according to their origin. Seven accessions from Uganda were clearly separated as cluster A-I-II-II, whereas cluster A-I-II-I contained a mixed group of accessions. Although genetically most accessions were significantly different ($P < 0.05$) from each other, a group of three possible duplicate accessions (Kyabukoli, Kibuyu, and Kayiso) was identified, which shared more than 90% of their banding pattern. UPGMA clustering also gave a high cophenetic correlation score (r = 0.94) indicating a very good fit between the similarity coefficients and the clustering method. However, the relationship between morphological and molecular distances was low and insignificant (r = 0.106). PCA based on Nei's genetic distance (Nei 1972) was used to visualize the genetic relationship among the accessions (Fig. 5). The PCA analysis supports the results obtained from UPGMA cluster analysis. Azucena accessions are clearly separated from other accessions in a PCA scatter plot. In agreement with UPGMA cluster analysis, the PCA plot showed that accessions Kyabukoli, Kibuyu, and Kayiso are closer to each other and are located in the same group with Sebagala, K5, K85, and NS4. On the other hand, cultivars Supa and Jagary from Uganda grouped with IRRI, Brazil, and West African accessions.

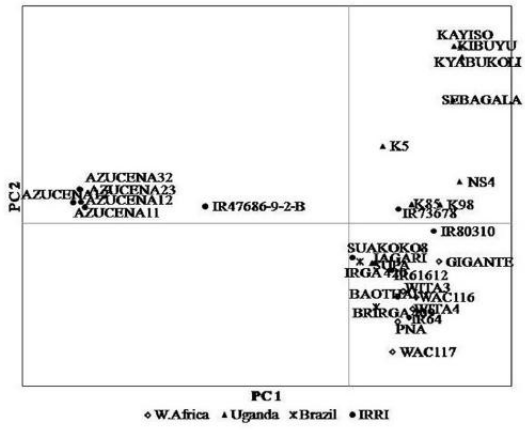

Fig. 5. Principal component analysis of 30 genotypes based on SSR genotyping.

Discussion

Phenotypic variation under iron toxic conditions

Evaluation of accessions against iron toxicity provided an insight into the genotypic differences associated with iron toxicity tolerance. Based on the analysis of the results obtained, the study revealed seven genotypes: Suakoko 8, IR61612-313-16-2-2-1, PNA, AZUCENA (12) IRGC 52992), AZUCENA 2008WS (23) (IRGC 117264), IRGA 420, and IR 73678-20-1-B that showed appreciable levels of tolerance to iron toxicity. Particularly promising were IR61612-313-16-2-2-1 and Suakoko 8, which showed consistently high resistance with an average score of ≤ 3.5 on a scale of 1-9 when compared with other accessions. Previous studies have shown deferential response of rice genotypes to iron toxicity (Gunawardena et al. 1982; Mohanty and Panda 1991; Virmani 1977). However, most of the cultivars identified in the screening experiments have not been consistently tolerant when tested using different methods (Becker and Asch 2005). In culture solution methods, a major difficulty has been to maintain the iron concentration at levels that induce toxicity. Two factors that lower the dissolved Fe^{2+} concentration include: auto-oxidation by water-dissolved oxygen and Fe^{2+}-phosphorus precipitation. Auto-oxidation is less pronounced in waterlogged conditions, and can be overcome by using EDTA-chelated iron (Fe-EDTA) as a source of Fe and/or minimizing the exposure of the culture solution to atmospheric oxygen. Phosphorus and Fe precipitation (Dalton et al. 1983) is a major challenge in screening experiments. Plaques of Fe form and act as a barrier to movement of P into the roots on the root surfaces (Zhang et al. 1998) causing indirect iron toxicity. The protocol used in the present study takes into consideration both factors to ensure that excess iron (II) and optimum P is available in solution. We maintained iron stress levels by monitoring available iron (II) in solution on a daily basis and adjusting the concentration to 300 ppm every 2 d. Similarly, phosphorus quantification was monitored and adjusted using Phospho-Molybdate dye assay. Maintaining a concentration of 300 ppm proved to be sufficient to induce severe iron toxicity (Fig. 1) and clearly differentiates the genotypes tested. Earlier breeding and screening experiments identified rice cultivars for growing in iron-toxic soils (De Datta et al. 1994; Sahrawat 2004). Azucena and Suakoko 8 were among the cultivars recommended as sources of tolerance genes (Audebert and Sahrawat 2000; Sahrawat et al. 2000). AZUCENA has also been used in Quantitative Trait Loci (QTLs) analysis (Wu et al. 2003). PNA and IR 73678-20-1-B were recently reported as tolerant cultivars in Uganda under both greenhouse and field condi-

tions (Onaga et al. 2012). All these genotypes showed appreciable levels of tolerance to iron toxicity in the present study. However, such resistance levels may be deployable only in mild iron toxic environments. Even though less leaf bronzing was observed in Suakoko 8 and IR61612-313-16-2-2-1, both genotypes had significant percentage reductions in root and shoot dry weights indicating that these genotypes are not likely to prove useful in severe iron toxic environments. New genotypes with stable resistance across all the indicative traits may need to be identified. In the meantime, Suakoko 8 and IR61612-313-16-2-2-1would serve as parental lines for iron toxicity improvement based on their root growth potential and reduced bronzing. However, the type and mechanisms of resistance in these genotypes remain unknown. Contrary to our previous findings, this study found that K98 is susceptible to iron toxicity suggesting that the screening method used in the present study prevented errors in evaluating resistance due to 'escapes'. The PCA plot (Fig. 3) showed a clear separation of highly susceptible accessions (Supa, Jagary, WAC117) from resistant accessions suggesting efficiency of the screening procedure in discriminating between the resistant and susceptible accessions. The susceptibility of Ugandan accessions to iron toxicity confirms the need for varietal improvement through breeding.

Genetic diversity analysis using SSRs

We evaluated 46 SSR markers on 30 rice accessions. Out of the 18 accessions grown in Uganda, 10 of them represent *indica* varieties commercially cultivated. The other eight cultivars represent accessions from West Africa and IRRI (Table 1). Forty-two SSRs were polymorphic across the 30 genotypes. A total of 189 alleles were detected with an average number of alleles of 4.61 per locus (range of 2-12). This value is low when compared with other large scale studies (range = 3 - 17, mean = 7.4) (Olufowote et al. 1997; Yu et al. 2003) but comparable to values reported for the worldwide collection (range 2 -11, mean = 6.3) and for studies performed on smaller germplasm sets (Cho et al. 2000; Hashimoto et al. 2004; Siwach et al. 2004). Differences in polymorphism information content (PIC) values were observed (Table 3). The SSRs RM10793, RM3412, RM333, RM562, RM13628, RM310, RM5749, and RM154 were highly informative, with a PIC value of ≥ 0.75, while RM19199 had the lowest PIC value (0.47). The PIC of an SSR marker, which is also defined as its capacity to discriminate genotypes, depends on the allelic diversity (Ribeiro-Carvalho et al. 2004). In this respect, we estimated a correlation coefficient of r = 0.74 between the PIC-values and the number of alleles detected. A strong positive correlation between gene diversity of an SSR locus and the number of alleles detected is also reported by Yu et al. (2003). This supports previous studies that SSRs analysis has a considerable potential for studying the genetic diversity of rice (Bligh et al. 1999; Jeung et al. 2005; Xu et al. 2004). A high level of polymorphism has been reported with more repeats in the structure of SSRs (Cho et al. 2000; Innan et al. 1997; Panaud et al. 1996; Schug et al. 1998). Highly informative SSR markers in this study are in concordance with this general pattern and exhibited higher *H* values, higher number of alleles, and larger size differences among alleles. Mean gene diversity (*H*) value for all SSR loci for the 30 genotypes evaluated was 0.69 but was decreased to 0.53 when the analysis was performed with only the 10 Ugandan accessions. This value is lower than the estimates for the rice accessions (*H* = 0.68) from eight major rice-growing regions of the world (Yu et al. 2003), suggesting that lowland rice cultivars grown in Uganda are not sufficiently diverse. Some of the SSRs were able to uniquely produce differentiating alleles for the genotypes tested. More specifically, eight SSR markers (RM31, RM3412, RM333, RM562, RM341, RM309, RM224, and RM336) were able to discriminate the 10 Ugandan rice cultivars. These markers, when combined with the highly informative RM10793, RM13628, RM310, RM5749, and RM154, have a potential for molecular characterization of rice germplasm. Data of genetic distances between Ugandan cultivar pairs (mean = 0.59) indicated a high degree of relatedness. Exceptions were observed for the cultivars Supa and K98 for which the original source is unknown. Low genetic diversity has been reported for Japanese, Korean, and Venezuelan rice germplasm (Ghneim et al. 2008; Hashimoto et al. 2004; Song et al. 2002). References to the narrow genetic base of cultivated rice varieties are also available from other regions, including Latin America (Aguirre et al. 2005; Cuevas-Pérez et al. 1992; Fuentes et al. 1999; Guimaraes et al. 1995), USA (Dilday 1990; Xu et al. 2004) and Taiwan (Lin 1991). There are no records indicating genetic studies on Ugandan cultivars. In fact, since the introduction of rice in Uganda a century ago(1904), the rice breeding program was not functional until the late 1990s. The recent advances have mainly occurred in upland rice research. The low genetic base observed in this study is not surprising because only a few varieties are available in the country. Several of the cultivars named locally could have arisen through field outcrossing and farmer selection over the years. Hence, one might expect that genetic diversity was on one hand, enhanced by mutation and meiotic recombination, and on the other curtailed by genetic drift and natural and artificial selection as suggested by Hartl and Clark (1997). UPGMA cluster analysis showed that Suakoko 8 and IR61612-313-16-2-2-1 grouped together at 60% similarity. These genotypes were the most tolerant in iron toxicity screening experiments. A clear distinction between the resistant IR61612-313-16-2-2-1, Suakoko 8, and Ugandan accessions indicates the presence of unique alleles that may be useful in marker-assisted selection. A low correlation was detected between the observed molecular and morphological variation patterns indicating a low congruence between the two datasets generated in this study. This suggests that a combination of morphological traits and SSR analysis could be useful when assessing genetic variation under iron toxic conditions, and could be a practical strategy for breeders when planning crosses. SSR markers cover a larger propor-

tion of the genome than the morphological markers and are less influenced by the environment than morphological markers (Kolliker et al. 2001; Zhang et al. 2010). Molecular markers also originate from different parts of the genome including coding and non-coding regions, and can cover either the full genome or large genomic segments, while morphological traits are controlled by a relatively small number of loci (Bruschi et al. 2003). Phenotypic plasticity can evolve independent from genetic variability, and it may bias the genetic variability based on morphological traits (Kölliker et al. 1999; Zhang et al. 2010). Such differences could be the cause for insignificant correlation between morphological and molecular markers. The limited number of morphological markers examined could have also contributed to a low correlation between the two markers.

Conclusions

All the Ugandan cultivars used in this study succumbed to iron toxicity indicating that there is currently no known source of resistance to iron toxicity among the lowland cultivars grown in the country. Based on the analysis of the data obtained, the study revealed two promising genotypes, IR61612-313-16-2-2-1 and Suakoko 8, which showed relatively high resistance. However, both genotypes had significant percentage reductions in root and shoot dry weights indicating that they are not likely to prove useful in severe iron toxic environments. New genotypes with stable resistance across all the indicative traits may need to be identified. The UPGMA cluster analysis showed that all 30 rice accessions could be easily distinguished based on the information generated by the 42 polymorphic SSR markers. The PIC values revealed that RM10793, RM3412, RM333, RM562, RM13628, RM310, RM5749, and RM154 might be the best markers for identification and diversity estimation of rice varieties. Higher similarity among the three Ugandan accessions (Kyabukoli, Kibuyu, and Kayiso) could be a result of duplicated accessions. Analyzing genetic diversity in a large germplasm collection will be relevant for the successful implementation of the various breeding approaches.

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