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Full Length Research Paper

Microsatellites and agronomic traits for assessing genetic relationships among 18 New Rice for Africa (NERICA) varieties

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The Africa Rice Center (WARDA) has developed several interspecific rice varieties by crossing the high yielding Asian rice (*Oryza sativa* subsp. *japonica*) with the locally adapted African rice (*Oryza glaberrima*). Eighteen varieties were named with the prefix NERICA (New Rice for Africa) but their genetic difference and patterns of relationship is largely unknown. A total of 102 polymorphic microsatellite markers were used to genotype 18 NERICAs. A subset of seven NERICAs (NERICA 1 to 7) was further characterized for 10 agronomic traits. The microsatellites data revealed no genetic difference between NERICA 8 and 9. The absence of genetic distance and identical SSR haplotype distribution (banding pattern) observed between NERICAs 8 and 9 is highly likely to be due to lack molecular difference at the DNA level but the possibility for seed admixture remains to be explored. This study, however, revealed the presence of a wide range of genetic differences among all other NERICAs, with the highest being between NERICA 6 and 17. Cluster and principal component analyses of the SSR data revealed distinct separation of NERICA 1 to 7 from NERICA 8 to 18. The possible reasons for such separation and the implications for breeding programs are discussed.

Key words: African rice, agronomic traits, microsatellite, NERICA, *Oryza glaberrima*, rice, transgressive segregation.

INTRODUCTION

Rice is the most important food crop in the world and feeds over half of the global population. It consists of the two cultivated species, namely the Asian rice (*O. sativa*) and the African rice (*O. glaberrima*). *O. glaberrima* is traditionally found in diverse West African agro-ecosystems but it is largely abandoned in favor of high yielding *O. sativa* cultivars due to its poor agronomic performance. However, *O. sativa* cultivars are often not sufficiently adapted to various abiotic and biotic conditions in Africa. On the other hand, *O. glaberrima* has been found to have several useful traits: (i) moderate to high level of resistance to blast (Silue and Notteghem, 1991), rice yellow mottle virus (Attere and Fatokun, 1983; John et al., 1985), rice gall midge, insects (Alam, 1988; Sauphanor, 1985) and nematodes (Reversat and Destombes, 1995); (ii) good level of tolerance to abiotic stresses such as acidity, iron toxicity, drought, and weed competition (Sano et al., 1984; Jones et al., 1994).

Crop improvement scientists at the Africa Rice Center (WARDA) evaluated a total of 1721 rice accessions (1130 *O. glaberrima* and 591 *O. sativa* accessions) and selected the best accessions on the basis of morphological characters and agronomic traits for breeding (Jones et al., 1997). One of the *O. glaberrima* (CG 14) and three of the *O. sativa* (WAB 56-104, WAB 56-50 and WAB 181-18) accessions were then used to

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develop interspecific hybrids. F_1 progenies derived from such interspecific hybridization were backcrossed with their *O. sativa* parents. A total of 18 varieties were named by WARDA's Variety Nomination Committee with the prefix NERICA, which is an acronym for New Rice for Africa, followed by a number corresponding to their pedigree. Seven NERICA varieties (NERICA 1 to 7) were named in 2000, and a further 11 (NERICA 8 to 18) were named in March 2005. These varieties combined the best traits of both parents: high yields from the Asian parent and the ability to thrive in harsh environments from the African parent. All the 18 NERICA varieties are suitable for the upland rice ecology of sub-Saharan Africa (SSA). The number of released NERICAs varies from country to country but at least the first named NERICAs were released in one or more West and Central African countries.

Morphological and agronomic traits have long been the means of studying classification and variability among populations and species (Gottlieb, 1984; 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). Microsatellites or simple sequence repeats (SSRs) are are among the most commonly used DNA marker types for a wide range of purposes (e.g. diversity, genome mapping, varietal identification). The first report of microsatellites in plants was made by Condit and Hubbel (1991) who suggested they were abundant in plant systems. Later, Akkaya et al. (1992) reported length polymorphisms of SSRs in soybean, which opened up a new source of PCR-based molecular markers for other plant genomes. At present, SSRs are the most preferred marker types because they are highly polymorphic even between closely related lines, require low amounts of DNA, can be easily automated and allow high-throughput screening, can be exchanged between laboratories, and are highly transferable between populations (Gupta et al., 1999).

Ndjiondjop et al. (2006) have previously used microsatellite and expressed sequence tag (EST) markers to investigate the genetic relationships among rice varieties widely used in breeding programs in Africa. That study revealed the presence of three groups: the *glaberrima* group, *indica* group, and NERICA and *japonica* group. Only four NERICAs (NERICA 1, 4, 5 and 6) were included in that study. Breeders at WARDA are currently using NERICAs as parents for crossing. However, the genetic relationships and distances among NERICAs are unknown at the molecular level but of great interest for breeding programs. The objective of this study was therefore to investigate the genetic variability and relationships among (i) 18 NERICAs developed using microsatellite markers, (ii) a subset of 7 NERICAs using both microsatellite and agronomic traits, and (iii) discuss the implication of the results for breeding.

MATERIAL AND METHODS

Sampling and data collection

Eighteen NERICAs derived through interspecific crossing between *O. glaberrima* and *O. sativa* subsp. *japonica* were used in the present study (Table 1). DNA was extracted from 4-weeks-old seedlings using the CTAB protocol as described by Murray and Thompson (1980). The quality and quantity of the extracted DNA was checked on 1% agarose gel and working dilutions were prepared for PCR reactions. A total of 132 SSR primers (Table 2) were selected to cover all rice chromosomes according to their position on the microsatellite framework map published by Chen et al. (1997), which is available at the Gramene database (http://www.gramene.org).

Amplification reactions were performed in a total reaction volume of 20 µl that consisted of 20 ng DNA, 1X PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 50% glycerol and 10 mM Tris-HCl, pH 8.3), 20 pmol of each of the forward and reverse primers, 200 µM each dNTP (Boerhinger Mannheim) and 2 U Taq DNA polymerase (Perkin-Elmer). Amplifications were carried out in an MJ PTC 100/96 thermal cycler using the following programs: 5 min at 94°C followed by 35 cycles of 1 min 94°C, 1 min 55°C and 2 min at 72°C, with a final extension of 5 min at 72°C. The amplified products were separated on 3% agarose gel using 0.5X TBE buffer and 90 V.

The first named NERICAs (NERICAs 1 to 7) were evaluated for the following 10 agronomic traits: days to heading and maturity, plant height, panicle length, number of primary and secondary branches per panicle, grain shattering, percentage of filled and empty grains, and yield per plant (Table 3). The varieties were sown in plots of 15-20 plants in 3 replicates at the M'bé, Côte d'Ivoire experimental station and assessment was made from 10 plants per variety.

Statistical analyses

Three data matrices were initially used for the statistical analyses but two other modifications were later included as described in the discussion section. DATA SET-1 consisted of 18 x 102 matrix (18 rows representing the 18 NERICAs and 102 columns corresponding to the total number of polymorphic SSR loci); DATA SET-2 contained 7 x 35 matrix (7 rows representing NERICAs 1 to 7 and 35 columns corresponding to the total number of polymorphic SSR loci among these varieties) while DATA SET-3 include 7 x 10 matrix (7 rows representing NERICAs 1 to 7 and 10 columns corresponding to 10 agronomic traits). DATA SET-2 was a modified version of DATA SET-1 by deleting all SSRs that were monomorphic among NERICAs 1 to 7.

Statistical analyses were performed on non-standardized DATA SET-1 and DATA SET-2, and standardized DATA SET-3 (mean of each variable was subtracted from data values and divided by the standard deviation). Because of the multi-state nature of the SSR data and presence of both qualitative and quantitative data points in the agronomic traits, Euclidean distance was calculated for all data sets as a measure of genetic distance between varieties. The distance matrices were used to generate phenograms using the UPGMA method of SAHN clustering. In order to obtain estimates of the magnitudes of differences between phenograms produced for NERICAs 1 to 7 from DATA SET-2 and DATA SET-3, cophenetic matrices were computed for each phenogram and compared using the Mantel matrix-comparison test (Mantel, 1967). Euclidean distance, cluster analyses and Mantel tests were performed using

Table 1: The 18 upland NERICA varieties with their pedigree. WAB 56-50, WAB 56-104 and WAB 181-18 are *O. sativa* japonica varieties whereas CG 14 is an *O. glaberrima* variety.

Variety	Pedigree	Backcross
NERICA 1	WAB 450-I-B-P-38-HB	WAB 56-104/CG 14//WAB56-104
NERICA 2	WAB 450-11-1-P31-1-HB	WAB 56-104/CG 14//WAB56-104
NERICA 3	WAB 450-I-B-P-28-HB	WAB 56-104/CG 14//WAB56-104
NERICA 4	WAB 450-I-B-P-91-HB	WAB 56-104/CG 14//WAB56-104
NERICA 5	WAB 450-11-1-1-P31-HB	WAB 56-104/CG 14//WAB56-104
NERICA 6	WAB 450-I-B-P-160-HB	WAB 56-104/CG 14//WAB56-104
NERICA 7	WAB 450-I-B-P-20-HB	WAB 56-104/CG 14//WAB56-104
NERICA 8	WAB 450-1-BL1-136-HB	WAB 56-104/CG 14//WAB56-104
NERICA 9	WAB 450-B-136-HB	WAB 56-104/CG 14//WAB56-104
NERICA 10	WAB 450-11-1-1-P41-HB	WAB 56-104/CG 14//WAB56-104
NERICA 11	WAB 450-16-2-BL2-DV1	WAB 56-104/CG 14//WAB56-104
NERICA 12	WAB 880-1-38-20-17-P1-HB	WAB 56-50/CG 14//WAB56-50
NERICA13	WAB 880-1-38-20-28-P1-HB	WAB 56-50/CG 14//WAB56-50
NERICA 14	WAB 880-1-32-1-2-P1-HB	WAB 56-50/CG 14//WAB56-50
NERICA 15	WAB 881-10-37-18-3-P1-HB	CG 14/WAB 181-18//WAB181-18
NERICA 16	WAB 881-10-37-18-9-P1-HB	CG 14/WAB 181-18//WAB181-18
NERICA 17	WAB 881-10-37-18-13-P1-HB	CG 14/WAB 181-18//WAB181-18
NERICA 18	WAB 881-10-37-18-12-P3-HB	CG 14/WAB 181-18//WAB181-18

Table 2. Summary of the 10 agronomic traits evaluated in NERICAs 1 to 7.

Traits	Mean	Std. dev	Min	Max
Days to heading	77.14	5.67	70.00	85.00
Days to maturity	103.14	7.58	95.00	117.00
Plant height (cm)	116.00	12.86	100.00	130.00
Panicle length (cm)	26.57	0.79	25.00	27.00
No. of primary branches	11.00	0.58	10.00	12.00
No. of secondary branches	39.43	1.13	37.00	40.00
Grain shattering	2.86	0.38	2.00	3.00
Filled grain number	227.14	28.70	200.00	260.00
Empty grains number	14.71	2.98	10.00	17.00
Yield (kg/ha)	4714.29	393.40	4000.00	5000.00

Table 3. Chromosomal distribution of the total number of screened and polymorphic SSR markers used to study New Rice for Africa (NERICA) varieties.

Chromosome	No. of screened SSRs	No. of polymorphic SSRs among:	
		NERICA 1 to 18	NERICA 1 to 7
1	15	13	5
2	13	9	2
3	12	7	2
4	13	9	7
5	10	8	2
6	11	9	4
7	8	7	2
8	10	8	2
9	11	8	1
10	10	8	2
11	11	10	3
12	8	7	3
Total	132	102	35

Table 4. Euclidean distance matrix among 18 NERICAs using 102 SSR markers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
NERICA1	0.00																	
NERICA2	4.80	0.00																
NERICA3	4.47	5.92	0.00															
NERICA4	4.80	4.24	4.36	0.00														
NERICA5	5.29	4.58	5.66	3.61	0.00													
NERICA6	6.16	7.00	6.71	6.56	7.87	0.00												
NERICA7	5.10	6.08	4.47	4.36	5.48	6.16	0.00											
NERICA8	22.96	23.49	22.41	23.15	23.52	24.19	23.39	0.00										
NERICA9	23.04	23.58	22.49	23.24	23.60	24.27	23.47	0.00	0.00									
NERICA10	22.14	22.69	21.63	22.43	22.41	23.45	22.41	10.25	10.05	0.00								
NERICA11	21.49	22.27	20.81	21.70	21.82	22.83	21.56	9.38	9.59	7.00	0.00							
NERICA12	22.09	22.69	21.45	22.11	22.25	23.26	22.23	7.87	7.87	7.94	6.71	0.00						
NERICA13	22.20	22.80	21.56	22.23	22.38	23.39	22.34	8.12	8.12	7.68	7.28	2.83	0.00					
NERICA14	23.09	23.79	22.38	23.11	23.47	24.39	23.26	7.21	7.21	9.54	9.17	8.60	8.37	0.00				
NERICA15	23.24	24.02	22.54	23.43	23.62	24.54	23.41	10.44	10.44	11.45	9.64	10.86	10.34	9.33	0.00			
NERICA16	22.85	23.64	22.41	23.13	23.24	24.17	23.11	10.95	10.95	11.49	9.38	10.86	10.10	10.30	4.69	0.00		
NERICA17	23.52	24.00	23.22	23.83	23.92	24.82	24.10	9.49	9.70	10.72	10.05	8.94	8.94	9.59	10.25	9.43	0.00	
NERICA18	23.56	24.33	22.87	23.75	23.94	24.80	23.73	10.77	10.77	12.17	10.20	11.23	10.86	9.80	3.32	5.10	10.10	0.00

NTSYS-pc for Windows, version 2.0, Exeter Software (Rohlf, 1998). For our second multivariate analysis, principal component analysis (PCA) was performed using 'THE UNSCRAMBLER' software (Computer-Aided Modelling, CAMO, version 9.2, Oslo, Norway). The first two principal components were plotted for visual examination of the clustering patterns among varieties.

RESULTS

Variation and relationships among 18 NERICAs

A total of 132 SSR primers (Table 2) covering all rice chromosomes were used to genotype the 18 NERICAs. One hundred and two of these primers (77.3%) were polymorphic among the studied materials while the remaining 30 primers (22.7%) were monomorphic. The number of polymorphic markers per chromosome varied from 7 to 13, and the overall average was 8.5 markers. Euclidean distances calculated from DATA SET-1 varied from 0.00 between NERICA 8 and 9 to 24.82 between NERICA 6 and 17 (Table 4), and the average distance among all 18 NERICAs was 15.57. NERICA 8 and 9 showed identical haplotype distribution along all the102 SSR markers.

The genetic relationship among the 18 NERICAs was assessed by a UPGMA cluster analysis of the Euclidean distance matrix shown in Table 4. The phenogram revealed bifurcation patterns (Figure 1A) corresponding to NERICA 1 to 7 (group-1) and NERICA 8-18 (group-2). NERICAs in group-2 were further divided into two subgroups, with NERICA 8 to 14 and 17 forming the first subgroup and the others (NERICA 15, 16 and 18) the second subgroup. The patterns of cluster analysis were also confirmed by principal component analysis (PCA). The first five principal components from principal component analyses of DATA SET-1 explained 87% of the total variation. As shown in Figure 1B, a plot of PC1 (57%) and PC2 (13%) revealed three distinct groups. PC1, which explained 57% of the variation, clearly separated all the group-1 NERICAs from those of group-2 (Figure 1B). PC2 further separated NERICAs within group-2 into two subgroups in the same way as the cluster analysis.

Microsatellite and agronomic traits variation among NERICAs 1 to 7

Thirty-five of the 102 polymorphic SSR in DATA SET-1 were polymorphic among NERICAs 1 to 7 (Table 2). Table 5 shows the Euclidean distance matrix derived from both SSR markers and agronomic traits. The lowest Euclidean distances for SSRs and agronomic traits were obtained between NERICAs 4 and 5 (3.61) and between NERICAs 1 and 2 (2.01), respectively. Euclidean distance was the highest between NERICAs 5 and 6 (7.87) for SSRs and between NERICAs 1 and 5 (6.23) for agronomic traits. These distances were also evident in the cluster (Figure 2) and principal component (Figure 3) analyses. For both molecular and agronomic data, NERICA 6 was the variety the least genetically related to all others within group-1. The patterns of relationship of NERICA 2 with the others appeared to be contradictory in the two phenograms. This resulted to a very low cophenetic correlation (r = 0.47) between the phenograms derived from SSR and agronomic data.

DISCUSSION

Genetic variability and proximity

The microsatellite data from the present study demonstrated the lack of genetic difference between NERICA 8 and 9, which could be due to (1) either no or extremely low difference between the two varieties at the DNA level, and/or (2) admixture of seed during multiplication. These two varieties are also very similar in morphological and agronomic traits that strengthen the former supposition rather than the latter. NERICA 8 and 9 are possibly duplications. There was, however, sufficient amount of molecular variation among all other NERICA varieties. The highest genetic distance was found between NERICA 6 and 17, which is as expected as both were derived from different *japonica* varieties. The distinct separation of NERICAs 1 to 7 from NERICAs 8 to 18 in both cluster and principal component analyses of DATA SET-1 was striking and not in agreement with their pedigree. NERICAs 1 to 11 were all derived from the cross between WAB 56-104 and CG 14, and repeated backcrossing with the former. All these eleven NERICAs were therefore expected to share the highest similarity in most parts of the genome other than the regions associated with traits under selection.

NERICAs 1 to 7 and 8 to 11 were developed at different times and hence differences in the selection criterion used during the development of NERICAs 1 to 7 might have exerted differential selective pressure on closely linked genes or coadapted gene blocks. About 66% of the SSR markers in DATA SET-1 (67 of the 102 SSRs) were completely monomorphic (fixed) among NERICAs 1 to 7, indicating a reduction in diversity among these varieties compared to NERICAs 8 to 11. Such a high proportion of fixed loci (genes) among NERICAs 1 to 7 might have contributed to their distinct separation from the others. In order to evaluate the effects of such fixed loci in the patterns of relationship, cluster analysis was performed using a modified version of DATA SET-1 by excluding all 35 polymorphic SSRs in NERICAs 1 to 7. This analysis revealed a similar bifurcation pattern as DATA SET-1 but NERICAs 1 to 7 appeared to be identical (Figure 4A). Cluster analysis conducted using only the 35 SSR markers showing polymorphism in NERICAs 1 to 7 revealed the same patterns of grouping

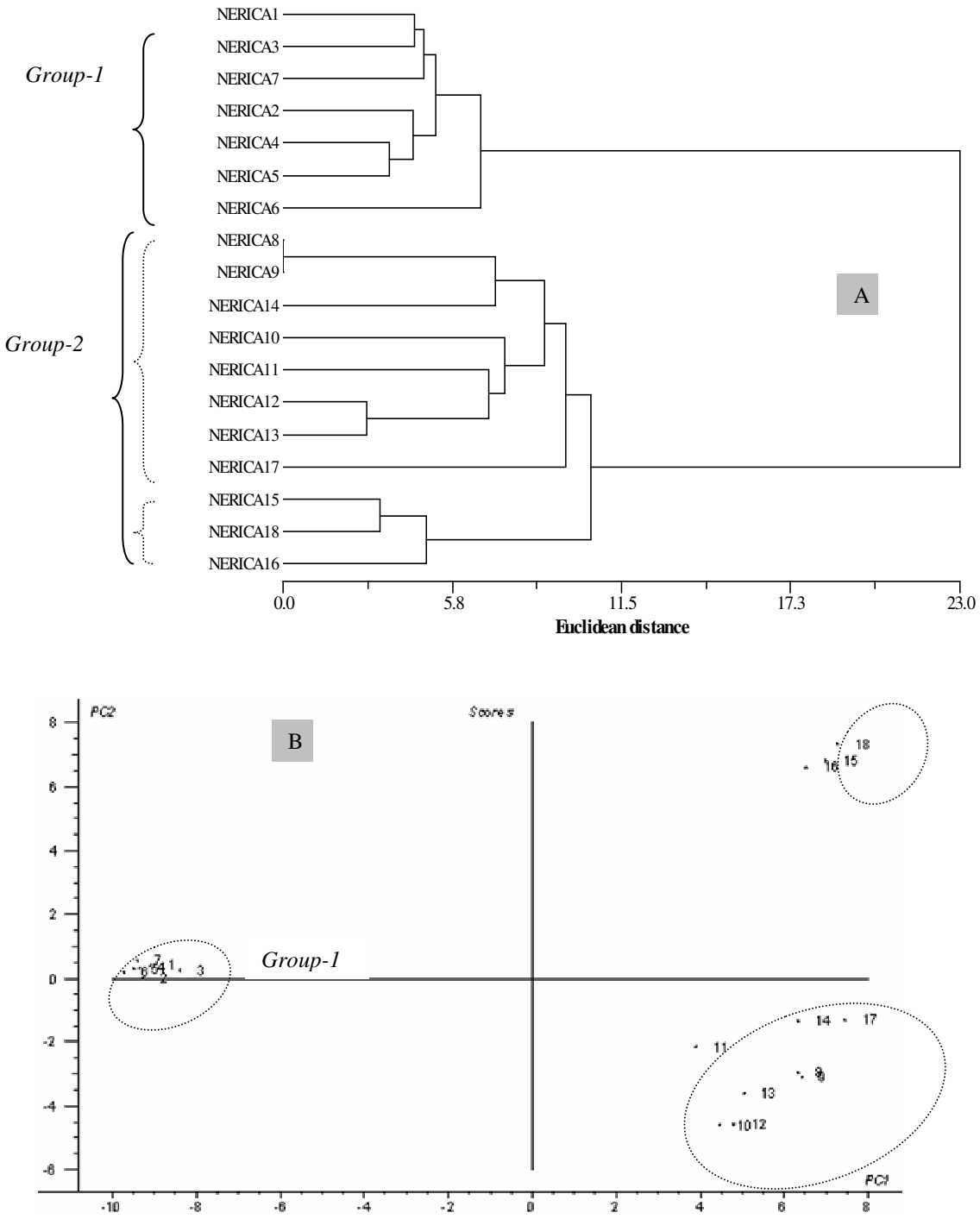

Figure 1. Cluster and principal component analyses performed using 102 SSR markers: (A) UPGMA phenogram of 18 NERICAs based on Euclidean distance matrix, and (B) score plot of NERICAs 1 to 18 from principal component (PC) analysis. PC1 and PC2 explained 57% and 13 %, respectively; numbers in the plot correspond to NERICA's 1 to 18 as shown in Table 1.

as DATA SET-1 (Figure 4B). There were, however, two differences within subgroups: that NERICA 12 and 13 appeared to be identical, and that NERICA 17 clustered with NERICAs 15, 16 and 18, which is in agreement with their pedigree information.

NERICAs 1 to 7 were also developed through selection among transgressive segregants (extreme phenotypes that exceed those of the parental lines) for a wide range of agronomic traits, including yield and weed competitiveness (Jones et al., 1997). We propose that

Table 5. Euclidean distance for NERICAs 1 to 7 using 10 agronomic traits (below diagonal) and 35 SSR markers (above diagonal)

	1	2	3	4	5	6	7
NERICA1	**0.00**	4.80	4.47	4.80	5.29	6.16	5.10
NERICA2	2.01	**0.00**	5.92	4.24	4.58	7.00	6.08
NERICA3	2.54	2.39	**0.00**	4.36	5.66	6.71	4.47
NERICA4	4.61	5.42	3.33	**0.00**	3.61	6.56	4.36
NERICA5	6.23	5.81	4.11	3.21	**0.00**	7.87	5.48
NERICA6	4.97	5.60	4.69	5.15	4.66	**0.00**	6.16
NERICA7	3.56	4.37	2.98	3.59	4.38	3.68	**0.00**

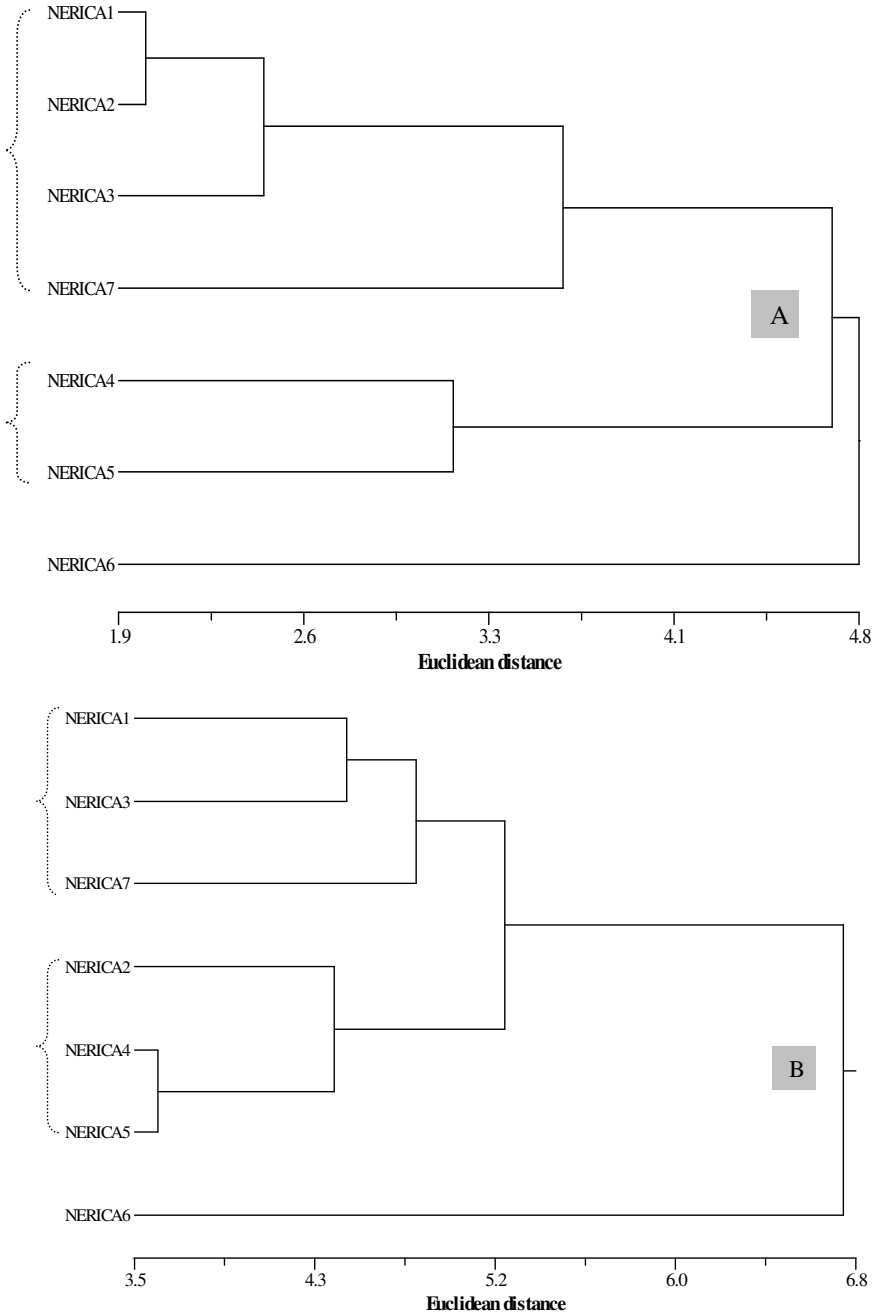

Figure 2. UPGMA phenograms of 7 NERICAs based on Euclidean distance matrices calculated from (A) ten agronomic traits, and (B) 35 polymorphic SSR markers.

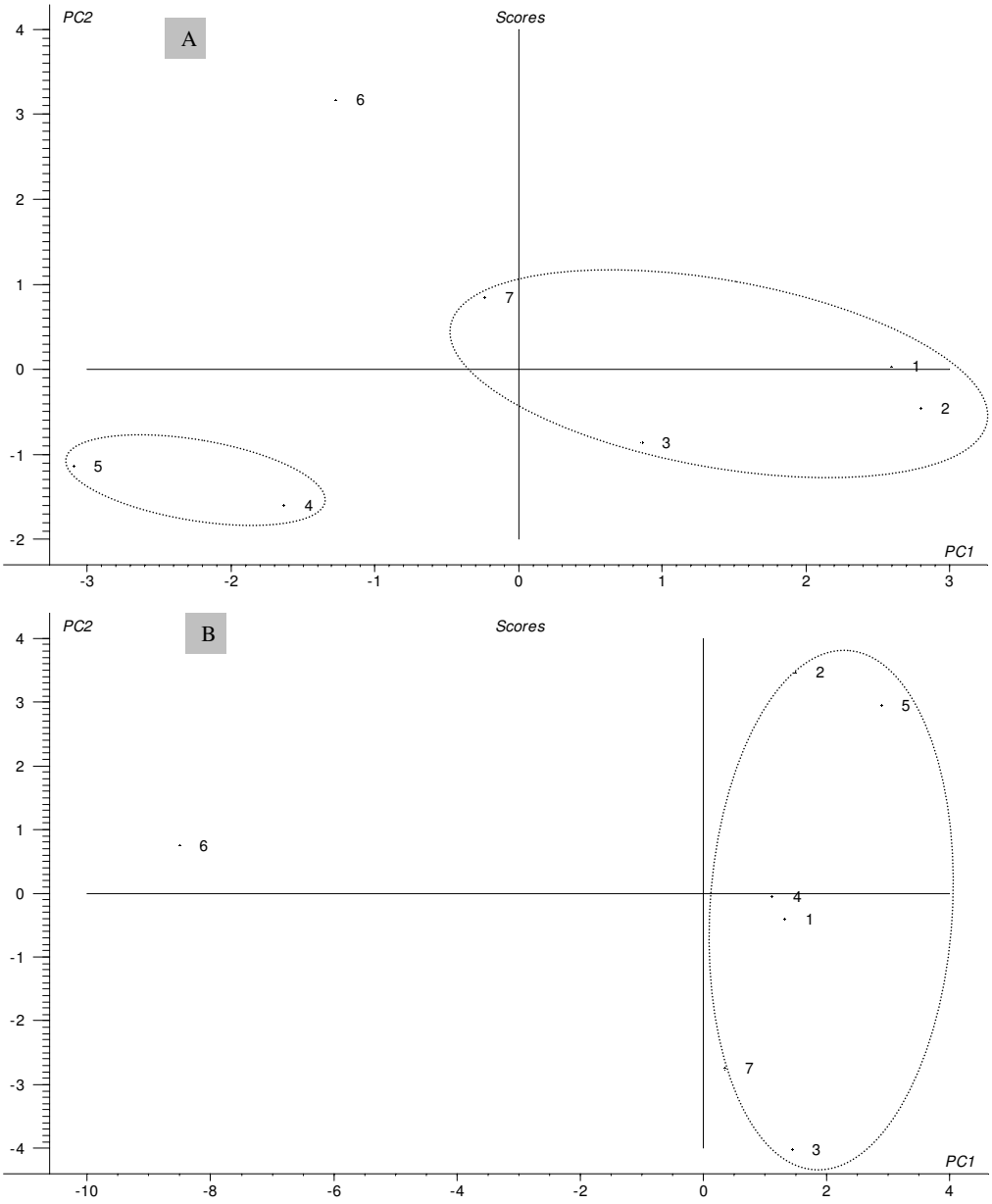

Figure 3. Score plots of NERICAs 1 to 7 using (A) ten agronomic traits, and (B) 35 polymorphic SSR markers. Numbers in the plot correspond to NERICA's 1 to 7.

the interspecific hybridization has contributed to genetic divergence between NERICAs 1 to 7 and NERICAs 8 to 11 largely via transgressive segregation. Transgressive segregation has been reported as a mechanism for large and rapid evolutionary transitions in various plant species because hybridization generates variation at many genes simultaneously and the variant alleles have already been tested by selection (de Vicente and Tanksley, 1993; Rieseberg et al., 1999; Lexer et al., 2003; Rieseberg et al., 2003). Genetic studies indicated that transgressive segregation mostly results from the appearance of combinations of alleles from both parents that have effects in the same direction (complementary gene action; Rick and Smith, 1953; Vega and Frey, 1980; Xu et al., 1998). Directional selection for transgressive genotypes could then shift the mean of phenotypic traits in segregating hybrid populations in a direction that allows them to fit into an extreme habitat or in favor of the trait used for selection (de Vicente and Tanksley, 1993; Rieseberg et al., 1999). Other mechanisms have been proposed for transgressive segregation such as an increased mutation rate, the exposure of recessive alleles in segregating hybrid populations, epistasis and overdominance, but these alternative mechanisms have received little support (Rieseberg et al., 1999).

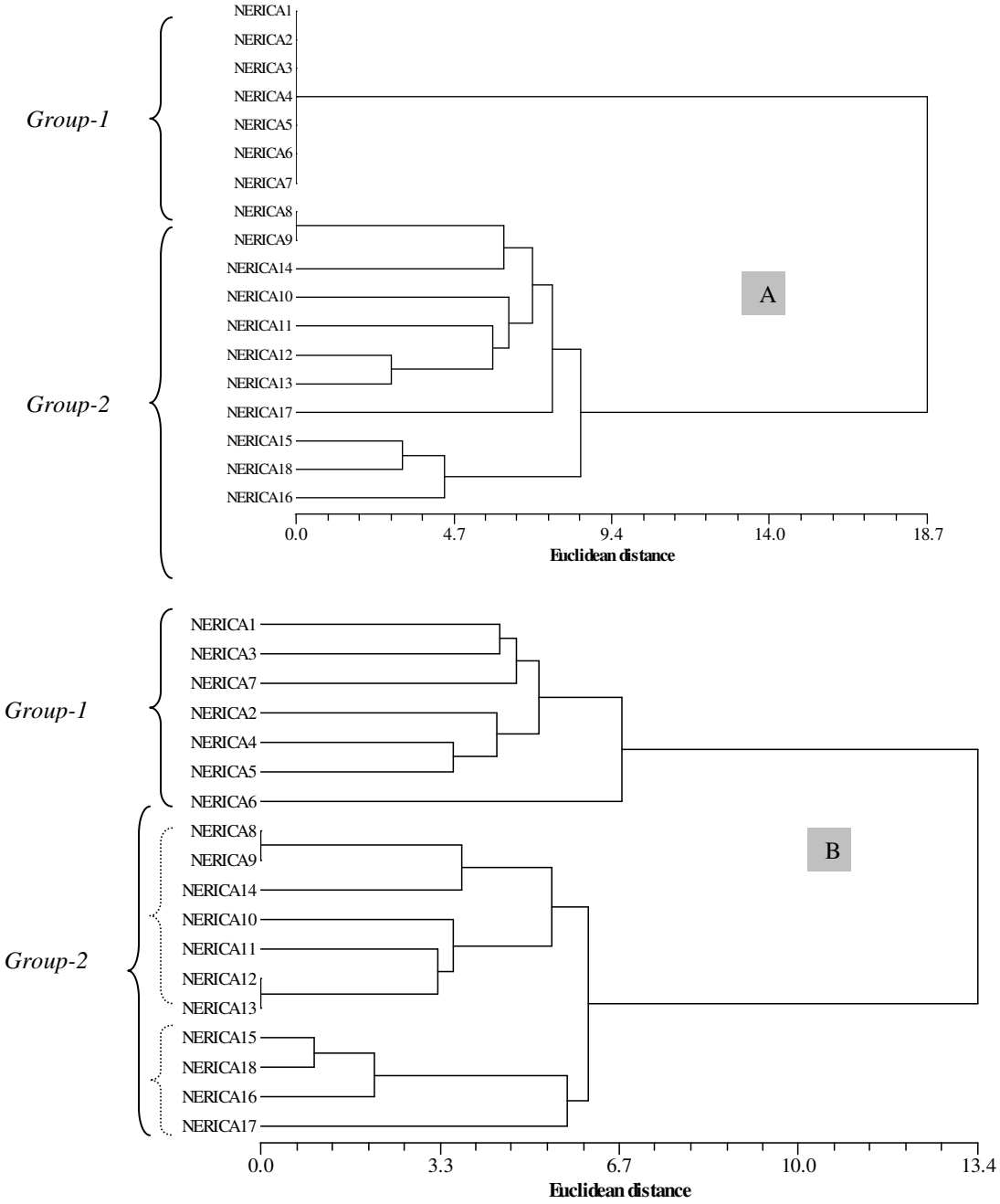

Figure 4. UPGMA phenograms of 18 NERICAs based on Euclidean distance matrices calculated from (A) 67 SSR markers after excluding 35 SSRs which were polymorphic among NERICAs 1 to 7, and (B) 35 SSR markers after excluding all 67 SSRs that were monomorphic among NERICAs 1 to 7.

The implication of the result for breeding

Several authors (e.g. Bhatt, 1970; Ariyo 1987; Peeters and Martinelli, 1989; Souza and Sorrells, 1991) pointed out that crosses designed between genetically distant genotypes should produce higher variances in segregating populations than crosses between related genotypes. The lack of genetic difference between NERICA 8 and 9 and the high genetic distance between NERICA 6 and 17 suggest therefore that crosses made between the latter would produce higher variances in segregating populations than the former. Furthermore, crosses to be made between NERICAs from the two distinct groups might be the best for any rice improve-

ment program in Africa. Cluster and principal component analyses performed on both microsatellite and agronomic data scored for NERICA 1 to 7 revealed the distinct separation of NERICA 6 from the others (Figures 2 and 3). The latter has also previously been reported to be different from others in plant height, grain length, grain width, grain and amylose content (WARDA, unpublished). NERICA 6 could therefore be one of the best varieties to serve as a parent for breeding in order to get higher variances in segregating populations. The use of NERICAs for any breeding programs must, however, be considered with considerable caution. These varieties are interspecific hybrids and the origin of some part of their genome is unknown. Such portions of the genome may contribute to undesirable traits. Furthermore, there have been reports of hybrid sterility in the early progenies of crosses (Jones et al., 1997) and the use of NERICAs as parents in breeding may restore the sterility problem.

Matrix comparison

The Mantel (1967) matrix correspondence test demonstrates that there is low correspondence between the distance matrices generated from SSR and agronomic traits ($r = 0.35$). Rohlf (1993) stated that if one matrix is a cophenetic value and the other matrix is that upon which the clustering was based, the cophenetic correlation to be computed from the two matrices could be used as a measure of goodness of fit for a cluster analysis. The degree of fit can be interpreted subjectively as follows: $r > 0.9$ very good fit; $0.8 < r < 0.9$ good fit, $0.7 < r < 0.8$ poor fit, and $r < 0.7$ very poor fit. According to this interpretation, the phenograms derived from the microsatellite and agronomic traits data showed very poor fit ($r = 0.47$). The patterns of relationships of NERICA 2 with the others were of the obvious contradictory results observed between the SSRs and agronomic data (Figures 2 and 3). Morphological and agronomic traits are known to be less polymorphic, dominant, late in expression, and susceptible to environmental and developmental variations (Gottlieb, 1984; Bretting and Widrlechner, 1995). The authors therefore believe that the patterns of RELATIONSHIPS Obtained from the microsatellite data are more reliable and accurate than those obtained from the agronomic traits in determining genetic proximity.

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