

GENETIC DIVERSITY ANALYSIS IN RICE MUTANTS USING ISOZYME AND MORPHOLOGICAL MARKERS

Alba Alvarez, J. L. Fuentes✉, J. E. Deus, Miriam C. Duque and María T. Cornide

ABSTRACT. In this work, isozyme and agromorphological variability of radio-induced rice mutants with different cytoplasm bases was surveyed. Agromorphological data (plant type, lodging resistance, maturity and yield) were transformed into binary data. These markers, along with isozyme (*Peroxidasas, Esterases, Catalasas, Alcohol Dehydrogenases* and *Polyphenoloxidasas*) data, were considered for genetic diversity analyses in order to estimate the extent of diversity generated by ionizing radiation in rice. The genetic similarity between genotypes was obtained based on Dice's Coefficient. The UPGMA phenogram defined three main clusters that clearly corresponded to the different rice cytoplasm sources. A bootstrapping analysis was performed to estimate the strength of the groups in the phenogram. According to their bootstrap value, Basmati-370 mutant lines could be considered statistically different from their mother variety. A Multiple Correspondence Analysis (MCA) showed a genotype dispersion around the three principal axes of variation. In general, the UPGMA phenogram pattern was corroborated at MCA. Variables such as: maturity, presence of *Est-a* and *Prx-m* bands and the absence of *Est-i*, *Prx-h* and *Prx-i* accounted for the highest contribution to variation. The adequacy of morphological and isozyme descriptors for the new mutant line validation is also discussed.

Key words: isozymes, agromorphological traits, genetic diversity, rice

RESUMEN. En el presente trabajo, se estudió la variabilidad isoenzimática y agromorfológica de mutantes de arroz con diferentes fuentes citoplasmáticas. Los datos agromorfológicos (tipo de planta, resistencia al acame, madurez y rendimiento) se transformaron en datos binarios, que unidos a datos isoenzimáticos (*Peroxidasas, Esterasas, Catalasas, Alcoholdehidrogenasas* y *Polifenoloxidasas*) fueron utilizados para estimar la diversidad genética inducida por las radiaciones ionizantes en arroz. Se calculó la similitud genética (Índice de Dice) para cada par de genotipos estudiados. El fenograma UPGMA mostró tres grupos principales que claramente correspondieron con las diferentes fuentes citoplasmáticas de arroz estudiadas. Se desarrolló un análisis de remuestreo para conocer la fortaleza de los grupos formados en el fenograma, mostrando que las líneas mutantes de Basmati formaron un grupo significativamente diferente de su variedad madre. Se desarrolló un Análisis de Correspondencia Múltiple (ACM), que mostró la dispersión de los genotipos en relación con los tres ejes principales de la variación. En general, el patrón de agrupamiento del fenograma UPGMA fue corroborado por el ACM. Variables tales como: madurez, presencia de las bandas *Est-a* y *Prx-m* y ausencia de las bandas *Est-i*, *Prx-h* y *Prx-i* mostraron las mayores contribuciones a la variación. En el trabajo se discute la efectividad de los descriptores isoenzimáticos y morfológicos para la validación de nuevos mutantes de arroz.

Palabras clave: isoenzimas, caracteres agromorfológicos, diversidad genética, arroz

INTRODUCTION

One of the major limitations of Latin American rice breeding programs is their narrow genetic source. The use of a narrow source of germplasm has become a quite common trend in breeding programs. A recent study based on pedigree analysis indicated that only a group of 14 landraces accounts for nearly the 70 % of the genes

present in the cultivars released in Latin America (1). The 98 % of cultivated rice varieties in Cuba carry the semi-dwarfing genes (2) from the dwarf Chinese variety Deewoo-wo-gen (3,4). Recent genetic diversity studies in some of these varieties using molecular markers evidenced a narrow genetic base (5).

Rice mutation breeding could be considered especially successful to obtain new cultivars and to broaden the genetic base of this crop. Thus in 1991, 251 new varieties were introduced in rice production (6). Recently, 322 new rice varieties have been reported, 215 of them were directly released (7). Among them, cultivars improved for grain quality, earliness, salt tolerance, resistance to rice blast and *Hoja Blanca* virus, and semi-dwarf genotypes from different cytoplasm sources can be mentioned (2,8).

Alba Alvarez and J. L. Fuentes, Researchers from the Radiobiology Department, Centro de Estudios Aplicados al Desarrollo Nuclear (CEADEN), apartado postal 6122, Playa, Havana; J. E. Deus, Senior Research Assistant from the Instituto de Investigaciones del Arroz. Bauta, Havana, Cuba; Dr. Miriam C. Duque, Researcher from the Centro Internacional de Agricultura Tropical (CIAT), Proyecto de Arroz, apartado aéreo 6713, Cali, Colombia and Dr. María T. Cornide, Senior Researcher from the Centro Nacional de Investigaciones Científicas (CNIC), apartado postal 6990, Havana, Cuba.

✉ Corresponding author: Fax: 53-7-221518,

e-mail: fuentes@ceaden.edu.cu

Isozyme polymorphism has been the most widely employed genetic marker during the last quarter of the century (9). In rice, these studies had the major input with the demonstration that considerable variation could be revealed by starch gel electrophoresis (10).

Isozyme analyses of Cuban rice varieties have also been developed (11, 12, 13). These studies have been basically directed to the characterization of commercial varieties and induced mutants for breeding programs assistance. However, information supporting the actual utility of isozyme markers for genetic diversity characterization of rice induced mutants is not yet sufficient.

Considering the importance of using mutant lines with different cytoplasm sources to broad the genetic base of this crop, this work was aimed at determining the genetic diversity present in rice mutants obtained by gamma rays and fast neutrons using isozyme and agromorphological markers.

MATERIALS AND METHODS

Rice genotypes. The group of promising mutant lines obtained from rice genotypes with different cytoplasm (Basmati-370, Gloria and Jucarito-104) is presented in Table I. Their previous performance under field conditions (2) is also included.

Esterases: Gels were immersed in a saturated solution of H_3BO_3 for 30 min at 4°C. Later, they were incubated in 50 mL of a 100 mM Na-Phosphate buffer, pH=6.5 (0.5 mg of α -naphthylacetate / 0.5mg of β -naphthylacetate) during 25 min. in darkness. After rinsed, gels were stained with 0.2 % Fast Blue RR salt at 40°C.

Peroxidases: Gels were incubated in 100 mL containing 0.25 g of Benzidine 2HCl, 0.3 % of Hydrogen Peroxide (H_2O_2) and 5 % of acetic acid until bands appeared.

Catalases: For reverse staining, gels were previously incubated in a starch solution (10 % of hydrolyzed starch, 10 mM of Na-Acetate) for 30 min. After rinsed, they were immersed for 1 min in a 10 % H_2O_2 solution and finally bands were obtained by incubation in an Iodine solution (0.12 M of I_2 and 0.18 M of KI).

Alcohol dehydrogenases: Gels were immersed in a 50 mM Tris-HCl buffer solution, pH=8, also containing 0.3 mM of NAD, 0.5 mM of MTT, 0.13 mM of PMS and 0.4 % of Ethanol until bands appeared.

Polyphenol oxidases: Gels were stained in 100 mL of 0.1 M Tris-HCl buffer, pH=7.2 which contained 1 mg of 3,4-L-dihydroxyphenyl alanine (L-DOPA) and 1 mg of L-Proline.

In all cases, gels were fixed in a 10 % Acetic Acid solution. Electrophoresis runs were repeated at least three times and only consistent and reproducible bands were taken into account for visual band scoring.

Table I. Main agromorphological traits of the selected mutant lines and their control varieties

	PT ^a	Vg ^a	Ldg ^a	Thr ^a	Mat ^a	Y (t.ha ⁻¹) ^a			PO ^b		W(g) ^a
						IIA	S.J.	Juc.	S.J.	Juc.	
1. Gloria C 10-2-1-7 ***	3	2	R	MR	E	5.2	7.9	4.0	3	4	24.6
2. Gloria C 10-2-1-8 ***	3	2	R	MR	E	4.1	5.5	4.4	3	3	24.8
3. Gloria	7	2	HS	MR	M	3.1	3.0	2.7	3	2	25.0
4. Basmati 14-2-2 **	4	3	R	MR	E	4.2	4.4	5.6	-	3	23.0
5. Basmati 14-2-3 **	4	3	R	MR	E	3.7	4.1	5.4	2	-	23.0
6. Basmati	9	3	HS	MS	E	1.9	1.5	1.7	3	3	23.0
7. 3764 *	3	3	R	MR	M	9.5	9.4	6.4	2	-	30.0
8. 3763 *	3	3	HR	MR	M	8.0	8.7	8.1	3	-	30.0
9. 3263 *	3	3	HR	MR	M	3.9	-	-	-	-	30.0
10. 3762 *	3	3	HR	MR	M	6.7	8.4	4.6	7	-	30.0
11. J-104	3	3	HR	MR	M	8.6	9.0	9.3	7	7	30.5

PT: Plant type (1-9 IRRI scale); Vg: Vigor (1-9 IRRI scale); Ldg: Lodging resistance; Thr: Threshing resistance; Mat: Maturity (E, early=135 days; M, medium > 135 days); Y(t.ha⁻¹): Yield; PO: Resistance to *Piricularia grisea* (0-9 IRRI-scale); W(g): 1000-grain weight. R: Resistant; HR: Highly Resistant; MR: Moderately Resistant; HS: Highly Susceptible, MS: Moderately Susceptible. IIA: Cuban Rice Research Institute, S.J.: Sur del Jíbaro. Juc.: Jucarito (a): Dry season. (b): Wet season. (***): 300Gy, ⁶⁰Co gamma rays (**): 200Gy, ⁶⁰Co gamma rays, (*): 20Gy, 14meV fast neutrons

Isozyme assays. Plumules and coleoptiles were collected from fifteen-day-old seedlings and ground in liquid nitrogen. The extraction was performed in a 20 % sucrose solution (1/1 weight/volume). Homogenate samples were centrifuged and stored at -20°C. Vertical electrophoresis was performed in polyacrilamide gels, overnight, at 70 V, in a Tris-Glycine buffer. Five isozyme systems were assayed: *Esterases (Est)*, *Peroxidases (Prx)*, *Catalases (Cat)*, *Alcohol dehydrogenases (Adh)* and *Polyphenol oxidases (Pox)*. The staining techniques were applied as follows:

Isozyme and agromorphological data. To obtain a synthetic representation of genetic diversity in rice genotypes, isozyme and agromorphological data were included into a simple binary data matrix. For this purpose, isozyme patterns were binary coded by visual scores for each genotype: presence (1) or absence (0).

Four agromorphological traits previously assessed under field conditions (2) were transformed into binary data as follows: tall plant type (0), semi-dwarf plant type (1); lodging susceptibility (0), lodging resistance (1); medium

maturity (0) and early maturity (1). Average yield ($t\cdot ha^{-1}$) at three experimental farms were considered as (0) in control varieties (Basmati-370, Gloria and Jucarito-104) and as (1) in mutant lines with higher yield values than $1.5 t\cdot ha^{-1}$ regarding their respective control variety.

Diversity analysis. To determine genetic diversity, all pairwise Dice's coefficients were estimated (14). A cluster analysis using Unweighted Pair Group Method with Arithmetic Average (UPGMA) was performed on the similarity matrix employing SAHN program of NTSYS-pc package (15).

Confidence limits (95 %) of the phenogram genotype groupings were established by the bootstrapping non-parametric method. The binary data matrix was reassembled with replacement 2000 times using the WinBoot program (16). A multiple correspondence analysis (MCA) was also performed on binary data matrix to determine the main contributing variables to total genetic diversity and to corroborate genotype grouping using CORRESP/SAS package version 6.09 (17).

RESULTS AND DISCUSSION

The number of monomorphic and polymorphic bands, polymorphism index, number of distinct genotype patterns and percentage of genotypes identified for each isozyme system appears in Table II. *Prx*, *Est* and *Cat* resulted polymorphic, while *Adh* and *Pox* systems showed no polymorphic bands. Based on the percentage of polymorphic fragments, isozyme systems detected different levels of polymorphism, ranging from 33 (*Cat*) to 85 % (*Prx*). Eight, six and two distinct isozyme patterns among the eleven screened genotypes were obtained for *Prx*, *Est* and *Cat* systems, respectively (Figure 1).

Table II. Polymorphism level detected at each isozyme system across the 11 studied genotypes

	Isozyme systems				
	<i>Prx</i>	<i>Est</i>	<i>Cat</i>	<i>Adh</i>	<i>Pox</i>
Bands scored	13	10	3	4	2
Monomorphic bands	2	4	2	4	2
Polymorphic bands	11	6	1	0	0
Polymorphism index ^a	85 %	60 %	33 %	0	0
Distinct genotype patterns ^b	8	6	2	0	0
% genotypes identified	73	54	18	0	0

a: Percentage is based on polymorphic fragments divided by total number of fragments scored

b: Distinct genotype patterns in relation to the total genotype number (11)

The high number of different isozyme patterns among all genotypes strongly evidences that a high genetic variability was generated by ionizing radiation in rice. Previous studies developed in our laboratory (11,13) support these results. In both, previous and the current study, *Prx* and *Est* resulted the most polymorphic isozyme systems.

Different authors (10,18) have also reported *Est* and *Prx* as the most polymorphic systems in plants.

Three genotype groupings (A-C) can be considered in the UPGMA phenogram (Figure 2). A, B and C groups included J-104, Gloria and Basmati related genotypes, with within group average similarity estimates of 0.84, 0.7 and 0.7, respectively. These suggest that a higher genetic diversity was induced by ionizing radiation in Gloria and Basmati genotypes. The similarity index between A and B groups was 0.57. Group C resulted the most distant cluster ($S=0.46$). The highest distances between group C and the other ones confirmed previous reports on varieties within intermediate features between indica and japonica cultivars (19).

The strength of the groups formed in the UPGMA phenogram was evaluated by bootstrapping analysis. According to the majority-rule consensus tree, only one statistically different group was obtained (Basmati-14-2-2 and Basmati-14-2-3). It has been suggested (20) that only the groups with bootstrap *P* values of 95 % or greater can be considered significant. Following this rule, only this group is truly strong, with a bootstrap *P* value of 99.6 %. Although the group including L-3763 and L-3764 mutant lines has a relatively low *P* value 87.7 %, it is still quite distant from the parental genotype (Jucarito-104). The cluster conformed by Gloria-related mutants, however, was not so strong (*P* value of 57.1 %).

It could be suggested that, among the screened genotypes, Basmati-370 genetic background responded with a higher variability to ionizing radiation. In fact, some authors (2) obtained a higher frequency of semi-dwarf mutants for Basmati-370 rather than for Gloria with a radiation dose of 200 Gy.

According to their corresponding bootstrap *P* value, Basmati-14-2-2 and Basmati-14-2-3 can be considered statistically different and, thus, different genotypes in relation to their control variety. It is important to point out that the highest genetic variability was obtained when agromorphological markers were considered. These resulted determinant for the statistical significance level obtained for this group; in fact, its bootstrap *P* value decreased to 85.3 % when only isozyme markers were considered (data not shown).

Likewise, for Gloria gene pool a clear differentiation between the control and mutant lines could be obtained by including agromorphological data. On the contrary, isozyme data resulted crucial for the optimal discerning between J-104 and their mutants (data not shown).

The MCA (Figure 3) allowed to obtain the three-dimensional distribution of genotypes around the principal axes of variation. The analysis of the values and vectors of MCA revealed that the three considered axes extracted 75 % of the variability (Table III). In this picture, five clusters were defined considering an *R*-squared value of 0.94; these are J-104 related varieties, Basmati-370, Basmati-370 mutant lines, Gloria and Gloria mutant lines.

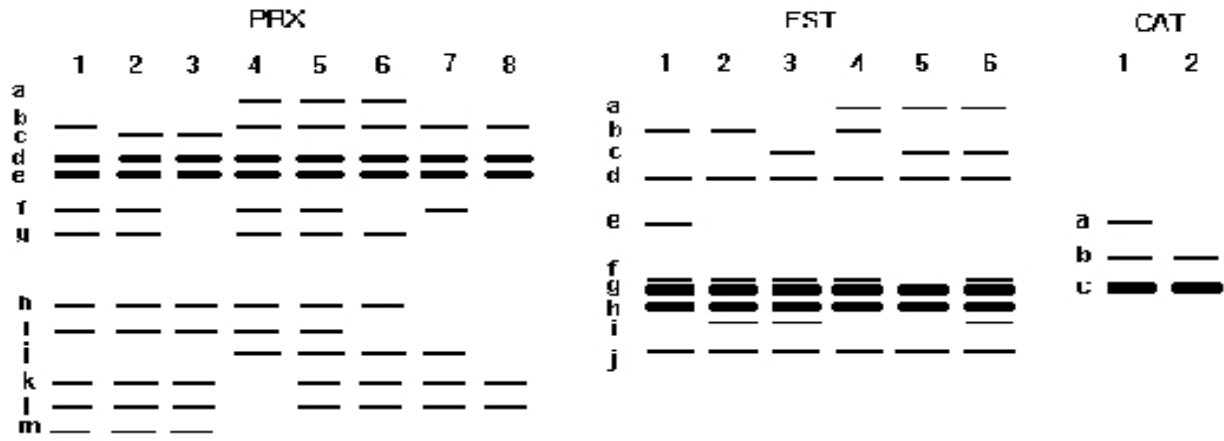


Figure 1. Different electrophoretic patterns for each isozyme system

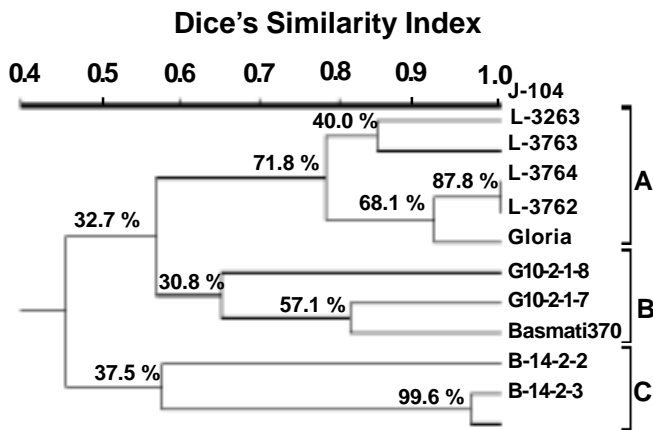


Figure 2. UPGMA phenogram based on isoenzymatic and morphological data for mutant and control varieties. Bootstrap *P* values are indicated at the corresponding node for each cluster

Table III. Matrix of values and vectors of Multiple Correspondence Analysis

	Axis 1	Axis 2	Axis 3
λ_i	0.519	0.449	0.362
Axis-specific contribution (%)	34	25	16
Accumulated contribution (%)	34	59	75
Vectors			
Prx- m_1	0.961	0.344	0.273
Mat ₁	0.961	0.145	0.344
Prx- h_0	1.384	0.577	0.531
Prx- i_0	1.121	0.351	0.103
Est- a_1	0.895	0.279	0.128
Est- i_0	0.266	0.874	0.282
Y-SJ ₁	0.826	0.340	0.929
Est- f_0	1.502	0.634	1.999
Prx- a_1	0.218	1.151	0.987

λ_i : singular values. Letters from a to m were assigned to the different bands in the electrophoretic profiles (see Fig. 1). Absence (0) or presence (1) of bands are modalities of each variable. Bold values represent higher variable contributions. Mat₁: early maturity, Y-SJ₁: Significantly different yield in the farm Sur del Jibaro

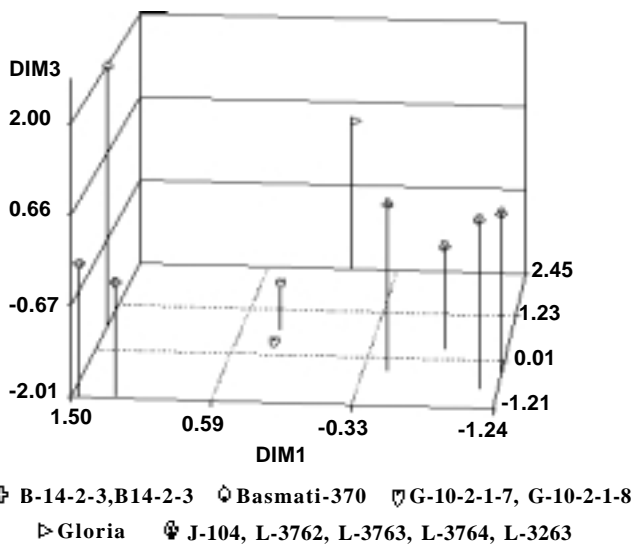


Figure 3. Genotype dispersion in the Multiple Correspondence Analysis, based on isoenzymatic and agromorphological data

Dimensions 1 and 2 were determinant for the grouping pattern. They accounted for the largest part of the whole variability (59 %). It was mainly due to the contribution of the variables maturity, presence of *Est-a* and *Prx-m* bands and absence of *Est-i*, *Prx-h* and *Prx-i*. In general, the different cytoplasm bases surveyed (Gloria, Basmati-370 and J-104) conformed different groups when Dimensions 1 and 2 were considered. At this point, the MCA analysis strongly corroborates the UPGMA phenogram grouping.

However, additional partitioning within Gloria and Basmati-370 gene pools could be obtained. Dimension 2 accounted for the greatest discerning between Gloria and its mutant lines. It was particularly due to the presence of *Prx-k* and *Prx-i* bands. Additionally, the tall plant type and medium maturity characterize the mother variety, while mutant lines resulted semi-dwarf and earlier plants contributing to its divergence (Table I).

On the other hand, the main division within Basmati-370 gene pool was obtained in Dimension 3, where a significantly higher yield value characterized the semi-dwarf

mutant lines. On the contrary, Basmati-370 is a tall variety, which is susceptible to lodging and identified by the absence of *Est-f* band.

Few differences within J-104 gene pool were detected in relation with the remaining cytoplasm sources. While the semi-dwarf plant type, lodging resistance and earliness were the main selective breeding criteria for Gloria and Basmati-370 genotypes, they were not considered for J-104 gene pool. In this case, the presence of *Est-e* band and absence of *Prx-a* in isozyme profiles of J-104 variety resulted the main contributing data.

In the present work, the genetic diversity analysis based on isozyme and agromorphological data allowed to confirm the adequacy of ionizing radiation for inducing high genetic variability in rice.

As it has been largely discussed, genetic descriptors for varietal validation must be highly stable and reproducible. Agromorphological traits are highly heritable and, in some cases, they result polymorphic enough to be considered as a rapid and simple criterium for variety discerning. Despite the low genetic diversity present in Cuban rice crop, a good variability level has been shown by the above mentioned descriptors (maturity, plant height, lodging resistance and yield) in mutation breeding programs. Therefore, we suggest that they can be considered as varietal validation criteria of newly released mutant lines.

Concerning the use of isozyme markers for variety validation, several constraints such as the few enzyme systems that can be visualized and, therefore, the limited number of loci detected have been largely discussed (9). However, in regard with Basmati-370 and its mutant genotypes, our descriptors (see UPGMA and Multiple Correspondence analysis) allowed to measure a genetic similarity value with a statistical significance enough to affirm that the screened mutant lines are different from their original variety. Bootstrapping analysis could be considered as a helpful tool for these aims.

Several advanced DNA-based techniques have arisen with a sort of advantages over biochemical methods for demonstrating Distinctiveness, Uniformity and Stability (DUS criteria) to a new variety (21). The use of such DNA-related techniques could be considered for these varieties (J-104 and Gloria, in this case) to which morphologic and isozyme analyses were not sufficient to establish statistical differences between genotypes.

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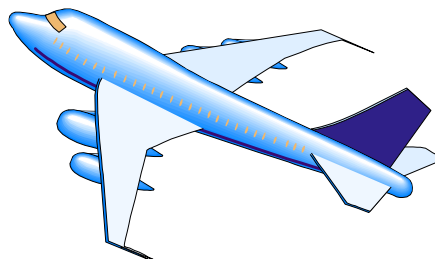
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