

# GENETIC DIVERSITY ANALYSIS IN RICE MUTANTS USING ISOZYME AND MORPHOLOGICAL MARKERS

Jorge L. Fuentes<sup>1</sup>✉, Alba Alvarez<sup>1</sup>, Juan E. Deus<sup>2</sup>, Miriam C. Duque<sup>3</sup> and Maria T. Cornide<sup>4</sup>

(1) Departamento de Radiobiología, Centro de Estudios Aplicados al Desarrollo Nuclear. Apartado Postal 6122, calle 30, No 502. Esq. 5ta Ave., Playa, C. Habana, Cuba.

(2) Departamento de Genética, Instituto de Investigaciones del Arroz. Bauta, La Habana, Cuba..

(3) Proyecto de Arroz. Centro Internacional de Agricultura Tropical, Rice Project. A.A. 6713, Cali, Colombia.

(4) Departamento de Biotecnología de Plantas, Centro Nacional de Investigaciones Científicas (CNIC). Apartado postal 6990, Ciudad de la Habana, Cuba.

## ABSTRACT

In this work, isozyme and agromorphologic variability of radiation-induced rice mutants with different cytoplasm base was surveyed. Agromorphologic data (plant type, lodging resistance, life cycle and yielding) were transformed into binary data. This markers, along with isozyme (Peroxidases, Esterases, Catalases, Alcohol Dehydrogenases and Polyphenoloxidases) data, were considered for genetic diversity analyses in order to estimate the extent of diversity generated by ionizing radiation.

Genetic Similarity between individuals was obtained based on Dice's Coefficient. The UPGMA phenogram defined three main clusters that clearly corresponded to the different cytoplasm sources. However, further discrimination between control varieties and their mutants could be obtained. Bootstrapping analysis was performed to estimate the robustness of the group in the phenogram. According to their bootstrap *P* value (99.6%), Basmati-370 mutant lines could be considered statistically different from their control. This analysis is suggested as an useful supporting tool for an accurate varietal validation. A Multiple Correspondence Analysis (MCA) showed individuals dispersion around the three principal axis of variation. In general the UPGMA phenogram pattern was corroborated at MCA. Variables such as life cycle, presence of bands Est-a and Prx-m and the absence of Est-i, Prx-h and Prx-i accounted for the higher contribution to variation. The adequacy of morphological and isozyme descriptors for new mutant lines validation is also discussed.

## 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 Dee-geo-woo-gen (3,4). Recent genetic diversity studies in some of these varieties molecular markers evidenced a narrow genetic base (5).

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

Isozymes polymorphism have been the most widely employed genetic marker during the last quarter century (8). In rice, these studies had the major input with the demonstration that considerable variation could be revealed by starch gel electrophoresis (9).

Isozyme analyses of Cuban rice varieties have also been developed (10,11,12). These studies have basically been directed to the characterization of commercial varieties and induced mutants for breeding programs assistance. However, information supporting actual utility of isozyme for genetic diversity characterization of rice induced mutants is not sufficient yet.

Considering the importance of use mutant lines with different cytoplasm sources to broad the genetic base of this crop, the present 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*

In table 1 are presented the group of promising mutant lines obtained from rice genotypes with different cytoplasm (Basmati-370, Gloria and Jucarito-104) using <sup>60</sup>Co-gamma and 14 MeV fast neutron radiation and their previously performance under field conditions (2).

**Table 1.** Principal agromorphological traits of the selected mutant lines and their control varieties.

|                         | PT <sup>a</sup> | Vg <sup>a</sup> | Ldg <sup>a</sup> | Thr <sup>a</sup> | C <sup>a</sup> | Y (t/ha) <sup>a</sup> |      |      | PO <sup>b</sup> |      | W(g) <sup>a</sup> |
|-------------------------|-----------------|-----------------|------------------|------------------|----------------|-----------------------|------|------|-----------------|------|-------------------|
|                         |                 |                 |                  |                  |                | IIA                   | S.J. | Juc. | S.J.            | Juc. |                   |
| 1.Gloria C 10-2-1-7 *** | 3               | 2               | R                | MR               | S              | 5.2                   | 7.9  | 4.0  | 3               | 4    | 24.6              |
| 2.Gloria C 10-2-1-8 *** | 3               | 2               | R                | MR               | S              | 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               | S              | 4.2                   | 4.4  | 5.6  | -               | 3    | 23.0              |
| 5.Basmati 14-2-3 **     | 4               | 3               | R                | MR               | S              | 3.7                   | 4.1  | 5.4  | 2               | -    | 23.0              |
| 6.Basmati               | 9               | 3               | HS               | MS               | S              | 1.9                   | 1.5  | 1.7  | 3               | 3    | 23.0              |
| 7.L-3764 *              | 3               | 3               | R                | MR               | M              | 9.5                   | 9.4  | 6.4  | 2               | -    | 30.0              |
| 8.L-3763 *              | 3               | 3               | HR               | MR               | M              | 8.0                   | 8.7  | 8.1  | 3               | -    | 30.0              |
| 9.L-3263 *              | 3               | 3               | HR               | MR               | M              | 3.9                   | -    | -    | -               | -    | 30.0              |
| 10.L-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; **C:** Life cycle. (**S**, short = 135 days; **M**, medium > 135 days); **Y(t/ha):** Yield; **PO:** Resistance to *Piricularia oryzae* (0-9 IRRI-scale); **W(g):** 1000 grains 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, <sup>60</sup>Co gamma rays (\*\*): 200Gy, <sup>60</sup>Co 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:

**Esterases:** Gels were immersed in a saturated solution of H<sub>3</sub>BO<sub>3</sub> for 30 min at 4°C. Later, they were incubated in 50 ml of a 100mM Na-Phosphate buffer, pH=6.5, (0.5 mg of α-naphtylacetate / 0.5mg of (β-naphtylacetate) 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<sub>2</sub>O<sub>2</sub>) 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 rinse, they were immersed for 1 min in a 10 % H<sub>2</sub>O<sub>2</sub> solution and finally bands were obtained by incubation in a Iodine solution (0.12 M of I<sub>2</sub> 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, 0.5mM of MTT, 0.13mM 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 1mg 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.

### ***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 on field conditions (2) were transformed into binary data as follows: tall plant type (0), semi-dwarf plant type (1); lodging susceptibility (0), logging resistance (1); medium life cycle (0) and short life cycle (1). Average yield (t/ha) at three experimental farms were considered as (0) in control varieties (Basmati-370, Gloria and Jucarito-104) and as (1) in mutant lines with yields values higher than 1.5 t/ha regarding their respective control variety.

### ***Diversity Analysis***

To determine genetic diversity all pairwise Dice's coefficient were estimated (13). 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 (14).

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 (15).

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 (16).

## **RESULTS AND DISCUSSION**

The number of monomorphic and polymorphic bands, Polymorphism index, number of distinct genotype patterns and percentage of genotypes identified, obtained for each isozyme system appears in Table 2.

**Table 2.** Polymorphism level detected at each isozyme system across the 11 studied genotypes.

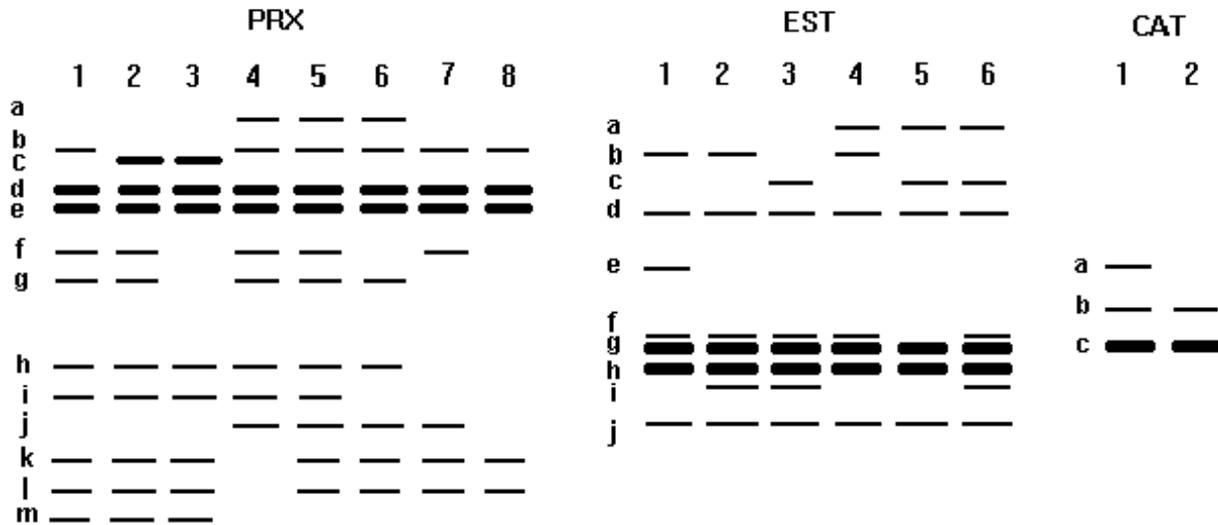
|                                         | <b>Isozyme systems</b> |            |            |            |            |
|-----------------------------------------|------------------------|------------|------------|------------|------------|
|                                         | <b>Prx</b>    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

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

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

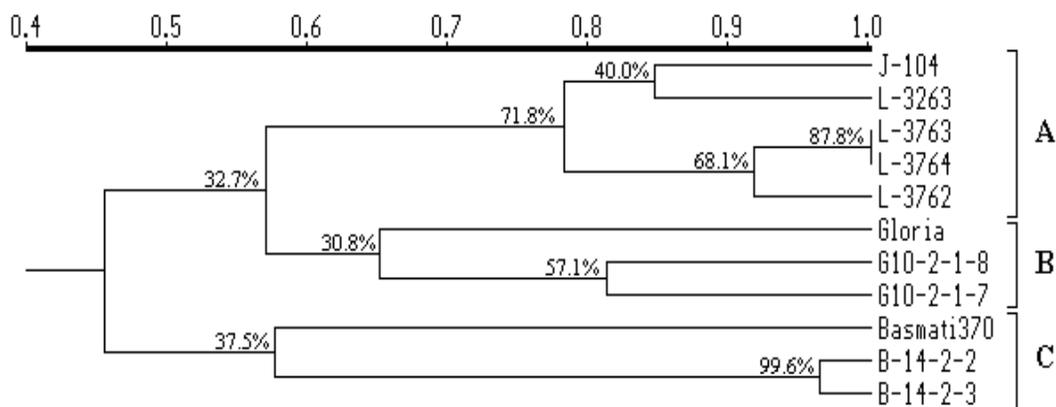
Prx, *Est* and *Cat* resulted polymorphic, while *Adh* and *Pox* systems showed no polymorphic bands. Based on the percentage of polymorphic fragments, isozyme systems detected different levels of polymorphism, ranging from 33 (*Cat*) to 85 % (*Prx*). Eight, six and two distinct isozyme patterns among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

**Figure 1.** Different electrophoretic patterns for each isozyme system.



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 (10,12, have supported these results. In both, previous and the current study, *Prx* and *Est* resulted the most polymorphic isozyme systems. Different authors (17,18) have also reported *Est* and *Prx* as the most polymorphic systems in plants.

**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.



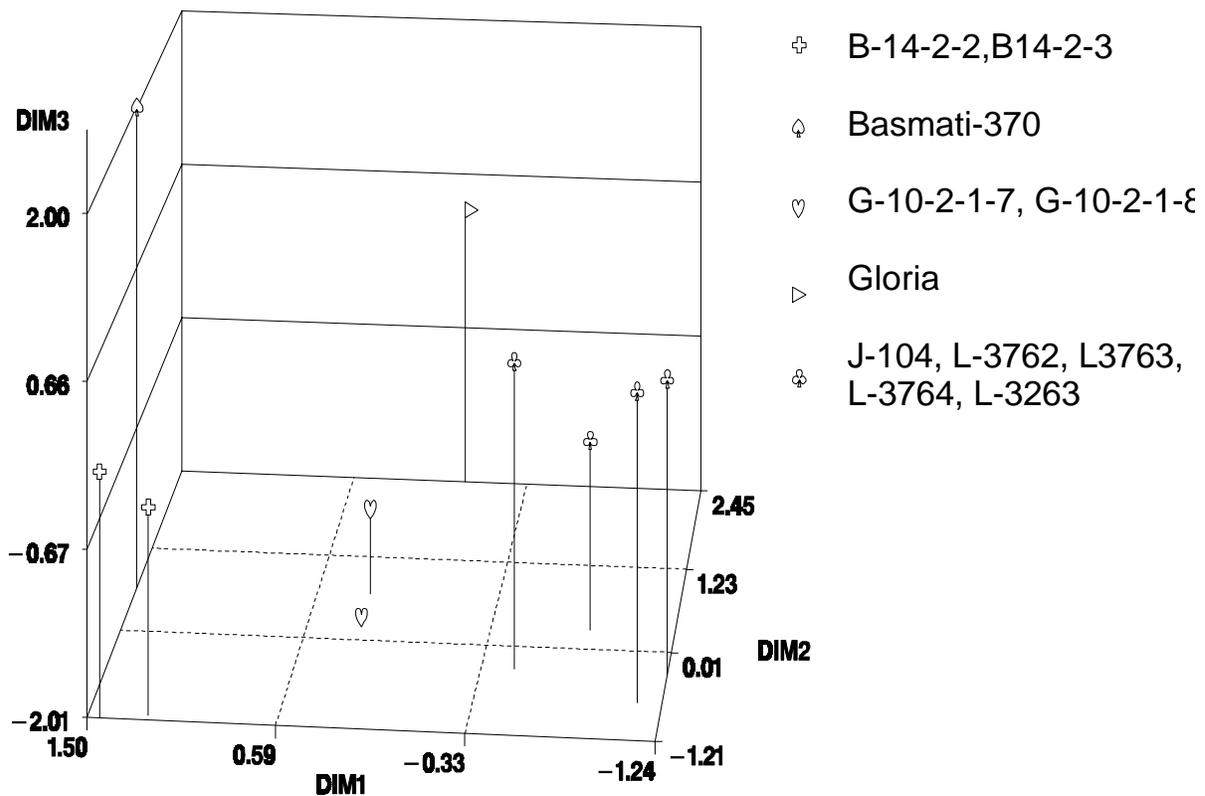
Three genotype groupings (A-C) can be considered in the UPGMA phenogram (Figure 2). A, B and C groups included J-104, Gloria and Basmati related genotypes, with within group average similarity estimates of 0.84, 0.7 and 0.7, respectively. These suggested that higher genetic diversity was induced by ionizing radiation in Gloria and Basmati genotypes. The similarity index between A and B groups was 0.57. Group C resulted the more distant cluster ( $S=0.46$ ). The higher distances between group C and the other ones confirmed previous reports on varieties with intermediate features between indica and japonica cultivars (9).

The robustness of the groups formed in the UPGMA phenogram was evaluated by bootstrapping analysis. According to the obtained majority-rule consensus tree, only one statistically different group was obtained (Basmati-14-2-2 and Basmati-14-2-3). It has been suggested (19) that only groups with bootstrap  $P$  values of 95 % or greater be considered significant. Following this rule, only this group is truly robust, 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 still quite distant from the parental genotype (Jucarito-104). The cluster conformed by Gloria-related mutants, however, was not so robust ( $P$  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, Deus *et al.*, (2) obtained higher frequency of semi-dwarf mutants for Basmati-370 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 higher 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).

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



The MCA (Figure 3) allowed obtaining 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 the 75 % of the variability (Table 3). 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.

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 life cycle, the presence of bands Est-a and Prx-m and the absence of Est-i, Prx-h and Prx-i.

**Table 3.** Matrix of values and vectors of Multiple Correspondence Analysis.

|                                 | Axis 1       | Axis 2       | Axis 3       |
|---------------------------------|--------------|--------------|--------------|
| $\lambda_i$                     | 0.519        | 0.449        | 0.362        |
| Axis -specific contribution (%) | 34           | 25           | 16           |
| Accumulated contribution (%)    | 34           | 59           | 75           |
| Vectors                         |              |              |              |
| Prx-m <sub>1</sub>              | <b>0.961</b> | 0.344        | 0.273        |
| C <sub>1</sub>                  | <b>0.961</b> | 0.145        | 0.344        |
| Prx-h <sub>0</sub>              | <b>1.384</b> | 0.577        | 0.531        |
| Prx-i <sub>0</sub>              | <b>1.121</b> | 0.351        | 0.103        |
| Est-a <sub>1</sub>              | <b>0.895</b> | 0.279        | 0.128        |
| Est-i <sub>0</sub>              | 0.266        | <b>0.874</b> | 0.282        |
| Y-SJ <sub>1</sub>               | 0.826        | 0.340        | <b>0.929</b> |
| Est-f <sub>0</sub>              | 1.502        | 0.634        | <b>1.999</b> |
| Prx-a <sub>1</sub>              | 0.218        | 1.151        | <b>0.987</b> |

$\lambda_i$ : 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 variable higher contributions. C<sub>1</sub>: Short life cycle, Y-SJ<sub>1</sub>: Significantly different yield in the farm Sur del Jibaro.

In general, the different cytoplasm bases surveyed (Gloria, Basmati-370 and J-104) conformed different groups when Dimensions 1 and 2 are 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 the bands Prx-k and Prx-i. Additionally, the tall plant type and medium life cycle characterize the mother variety, while the mutant lines resulted semi-dwarf and earlier plants, contributing to its divergence (Table 1).

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, susceptible to lodging and was 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 to J-104 gene pool. In this case, presence of Est-e band and absence of Prx-a in isozyme profiles of J-104 variety resulted the mainly contributing data.

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

As have 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 for consider them as a rapid and simple criteria for variety discerning. Despite the low genetic diversity present at Cuban rice crop, a good variability level has been shown by the above mentioned descriptors (life cycle, 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 (8). 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 as 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) for a new variety (20). The use such DNA related techniques could be considered for these varieties (J-104 and Gloria, in this case) for which a morphologic and isozyme analyses were not sufficient to establish statistical differences between genotypes.

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