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GENETIC DIVERSITY ANALYSIS IN CUBAN TRADITIONAL RICE GERMPLASM USING MICROSATELLITE MARKERS.

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ABSTRACT

The screening of traditional and wild germplasms for different plant genepools, valuable agronomical traits and alternative cytoplasmic bases is among the most important strategies not only for conservative aims but also to support breeding purposes to wide the genetic background of the cultivated varieties. In Cuba, traditional material is held in the Cuban Rice Germplasm Bank. However, a complete agro-morfological characterization of this material has not been accomplished and neither biochemical nor molecular analysis has been performed to date. At the present study, the potential of microsatellite markers to assess the extent of genetic variability in Cuban traditional rice varieties was proved. The work was aimed at identifying alternative genetic diversity pools in this material in comparison to the most important commercial cultivars used in Cuban rice breeding program. For this, 52 traditional accessions, eleven cultivars representing the most planted Cuban material during the last decades and two parent cultivars were studied. Ten SSR primer pairs were used, which mapped to 11 loci at 7 chromosomes of the rice genome. A genetic similarity matrix, based on Dice´s coefficient was used for cluster analysis and the UPGMA dendrogram was obtained by means of the NTSYS-pc program. An average of 12 SSR patterns per primer pair was obtained. Our results corroborate that a high number of alleles can be detected by SSR markers in rice. Additionally, the profit of SSR markers for genotype identification was confirmed for traditional germplasm: the 94% of traditional varieties could be identified with the set of 10 SSR primers. Only three genotypes showed identical patterns, which could indicate for the presence of germplasm duplications or very close related genotypes. Most of the patterns found in the commercial cultivars (30) were present in the traditional varieties, while 85 patterns (70% of the total) were unique for the traditional gene pool. According to this, it could be suggested that traditional genepools were poorly used in Cuban breeding programs during the last decades. Finally, the UPGMA dendrogram allowed for a clear discerning between commercial and traditional varieties. A high genetic diversity within both clusters was evidenced. However, a clear sub-grouping for traditional germplasm could no be established. According to our results, traditional germplasm (mainly varieties into cluster I) can be considered as a very useful source for rare alleles and

alternative backgrounds for both conservative and breeding purposes in Cuban rice breeding programs.

INTRODUCTION

In spite of breeders worldwide have successfully incorporated specific genes for pest and disease resistance from wild relatives, most of the cultivated rice is still based on the same semi-dwarfism genes and Cina cytoplasm [1]. This so-called genetic erosion can lead to the crop vulnerability for responding to pests, pathogens and environmental hazards. Genetic erosion of rice crop has been reported in China, the Philippines, Malaysia, Thailand and Kenya [2].

The screening of traditional and wild germplasms for different plant genepools, valuable agronomical traits and alternative cytoplasmic bases is among the most important strategies not only for conservative aims but also to support breeding purposes to wide the genetic background of the cultivated varieties.

In Cuba, the main efforts for this aim, have been limited to the conservation and maintaining traditional material in the Cuban Rice Germplasm Bank at the Cuban Rice Research Institute (IIA) as result of several collections in different regions between 1975 and 1985. However, a complete agro-morfological characterization of this material has not been accomplished and neither biochemical nor molecular analysis has been performed to date.

A sort of molecular techniques have arisen to support plant genetic resources management, [3,4]. For rice crop, several studies have been performed to determine the genetic characteristics of weedy rice using molecular, isozyme and morpho-physiological markers [5,6]. Also, the genetic relatedness among wild and cultivated rice have been determined [7].

Simple sequence repeats (SSR) or microsatellite arrays are abundant and widely distributed along the rice genome. SSR are co-dominant DNA markers that detect higher levels of allelic variation than restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers. They are therefore highly informative and easily and economically assayed by the polymerase chain reaction (PCR) [8].

In the last decade, SSR markers have been developed and characterized for rice [8-11]. Since then, they have been applied to genetic diversity studies [12], valuable genes tagging [13-16], quantitative trait loci (QTL) analyses [17], to genotype identification [18] and to predict hybrid performance and heterosis based on molecular diversity [19-22]. However, there have been very few reported studies designed to assess the relative richness of genetic variability in landraces in comparison to modern cultivars, especially to the few most popular elite lines [12].

At the present study, the potential of microsatellite markers to assess the extent of genetic variability in Cuban traditional rice varieties was proved. The work was aimed at identifying alternative genetic diversity pools in this material in comparison to the most important commercial and parent cultivars used in Cuban rice breeding program.

MATERIALS AND METHODS

Rice materials:

Fifty-two accessions from de traditional rice germplasm bank at the IIA were studied. Additionally, eleven cultivars (obtained through either traditional or mutation breeding) were studied. These are representative of the most planted Cuban material during the last decades

(Fuentes *et al*., in preparation). Also, two parent cultivars (Pokkali and IR-36), used in Cuban breeding programs were included (Table I).

Microsatellite assays:

Ten SSR primer pairs (Map Pairs, Research Genetics, USA), were used, which mapped to 11 loci at chromosomes 1, 3, 5, 6, 7, 11 and 12 of rice genome. (Table II).

Seeds were planted in greenhouse and leaf material from 20-days old seedlings was collected. DNA extraction was performed according to Dellaporta *et al*.[23]. Polimerase chain reaction (PCR) was conducted in a final volume of 20 µl containing 20 ng of template DNA, 0.1 µl each primer, 250 µM each dNTP, 1.8 mM $MgCl_2$ and 1 unit of Taq polymerase. The reaction was processed at 94^{o}C for 3 min, followed by 34 cycles consisting of 94^{o}C for 30 seg, 54^{o}C for 30 seg and 72^{o}C for 1 min and a final extension step of 72^{o}C for 5 minutes. The annealing temperature varied from 52^{o}C to 54^{o}C depending on the primer sequence in order to obtain the as less background as possible in profiles. After the reaction, 5 µl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene-cianol) was added to the amplification product and 3 µl per sample were loaded on a 6% polyacrilamide denaturing gel containing 6M of urea. A silver-staining procedure [24] was used to reveal bands after electrophoresis.

Statistical analysis:

Genetic diversity analysis was conducted using band variants (patterns). The presence or absence of each SSR pattern was scored as 1 and 0, respectively for every genotype. Binary data was used to compute a similarity matrix for all combinations of individuals based on Dice´s coefficient [25] in the Windist program [26]. The Similarity matrix was then used for cluster analysis and the UPGMA dendrogram was obtained by means of the NTSYS-pc program [27].

RESULTS AND DISCUSSION

SSR markers polymorphism:

In this survey, the 10 screened microsatellite primer pairs showed polymorphic for the studied sample. From 6 (RM-18) to 17 (RM-11) different SSR patterns were found, for an average of 12 SSR patterns per primer pair (Figs. 1 and 2). Our results come close to those of Wu and Tanksley [9] that found from 5 to 11 alleles per primer in 20 rice accessions. Xiao [22] found a lower value (4 alleles/primer pair) while surveying 10 elite inbred lines. On the contrary, higher average values have also been found (19 SSR alleles/primer pair) [12]. However, in the last case, a larger and diverse sample (238 accessions including *indica* and *japonica* cultivars, landraces and elite cultivars) which could basically explain the differences. Yet, the existence of a high number of alleles that can be detected by SSR markers in rice [22] is confirmed in our study.

The 94% of the traditional varieties could be identified with the set of 10 SSR primers. Twenty-three traditional genotypes showed unique patterns with at least one of these primers (with the only exception of RM-18). In general, the profit of microsatellite markers for genotype identification has been suggested for several crops [28], including rice [18]. In our study, only three genotypes (Blanquito, Espiritista and Sel. Tres Provincias) showed identical patterns with all the screened primer pairs. This could indicate these are germplasm duplications or very close related genotypes. However, additional screening with SSR markers with different chromosomic

location as well as morfo-agronomic characterization becomes necessary in order to corroborate the idea.

Comparison of polymorphisms between traditional and commercial germplasm:
Out of 123 total patterns, 115 were found in the traditional varieties and only 33 appeared in the commercial varieties. Most of the patterns found in the commercial cultivars (30) were present in the traditional varieties. On the contrary, 85 patterns (70% of the total) were unique for the traditional gene pool. The higher number of both unique and total allelic variants found for the traditional germplasm could indicate the presence of alternative gene pools in this germplasm in relation to the commercial rice varieties planted in Cuba.

Genetic Diversity Analysis:
The Dice´s similarity-based UPGMA dendrogram, allowed for a clear discerning between commercial and traditional varieties (Fig.3). Two main variety clusters are formed. Cluster I included only traditional varieties and group II grouped traditional and commercial cultivars. A high genetic diversity within both clusters was evidenced.
No apparent genotype sub-clustering could be established within Cluster I. In general, SSR markers allowed for a clear traditional genotypes discerning, but a clear grouping pattern for this germplasm could no be established from our results. Different kinds of genetic markers need to be assayed in order to suggest the main gene pools and for the assembling of a germplasm core collection.
Commercial cultivars grouped into Cluster II and two subgroups, with relative higher genetic similarity (60%), are formed. Jucarito-104 (J-104), the most extensively planted cultivar in Cuba, its related genotypes and IR-36 grouped together and the remaining cultivars clustered within the other sub-group. Pokkali, an Indian cultivar, used as salt tolerance source for breeding programs, appears quite separate (12 % of genetic similarity) from the commercial germplasm. Previous studies in Cuban rice cultivars using isozyme, RAPD and AFLP markers corroborate the existence of a quite different genetic background in this genotype [29,30].
In general, certain differentiation between traditional and commercial sub-groups was observed in Cluster II. However, traditional accessions highly closed to commercial varieties could be identified. Thus, some accessions in the traditional set (accessions 27, 33, 38, 42 and 51) clustered very close to some commercial varieties. From this result, it could be suggested that a local selection from commercial cultivars was made by farmers and that these cultivars were latter given different denominations. However, considering that no sufficient information is available on the origin, morphological and agronomic traits of this germplasm, further morpho-physiologic and molecular characterization becomes necessary to corroborate this idea.

CONCLUSIONS

Several conclusions arise from our results. First, a high allelic variability could be detected for traditional varieties with SSR markers. Also, high genetic diversity levels were found for this germplasm and a clear discerning between traditional and commercial germplasm could be obtained. According to this, it could be suggested that traditional genepools were poorly used in Cuban breeding programs during the last decades.

Traditional germplasm (mainly those grouped into cluster I) can be considered as a very useful source for rare alleles and alternative backgrounds, for both conservative and breeding purposes. Finally, microsatellite markers allowed for a quite efficient genotype identification in traditional germplasm. However, similar results could not be achieved for very close related commercial cultivars. It is important to mention that our SSR primers set covered only 11 loci at 7 chromosomes of the rice genome. Further screening including SSR markers from different loci and chromosome location becomes necessary to obtain more efficient genotype identification within commercial genotypes.

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Table I. List of the traditional and commercial rice material.

Traditional Germplasm			Traditional Germplasm		
No.	**Name**	**No. of Catalog**	**No.**	**Name**	**No. of Catalog**
1	Blanquito	2	**35**	Selección Blanquito	1518
2	Blue Bonnet	3	**36**	Matahambre Blanco	1520
3	Cuba C-65	5	**37**	Matancero	1521
4	Jabao	6	**38**	Caña Verde I	1522
5	Siete Estrellas Blanco	7	**39**	Blue Bonnet Amarillo II	1524
6	Nira	8	**40**	Rexoro	1527
7	90 días	9	**41**	Selec. Tres Provincias	1528
8	Negrón	11	**42**	Arroz de los campos ¾	1744
9	Gloria	12	**43**	Nira II	1746
10	Cuba C-103	13	**44**	M-1	1747
11	Botón de Oro	17	**45**	M-2	1748
12	Amarillón Grande	19	**46**	Pati Prieto II	1753
13	Espiritista	20	**47**	Amarillo II	1754
14	Brasileño	21	**48**	Cristal II	1756
15	Arroz Millo	22	**49**	Selección en IR-880 (I)	1757
16	Matahambre	24	**50**	Selección en IR-880 (II)	1760
17	Arroz tres cuartos	25	**51**	M-4	1761
18	Mezcla 69	1390	**52**	Selección INIFAT	1800
19	Caña Verde	1393	**Comercial Germplasm**		
20	Blue Bonnet Amarillo	1394	**No.**	**Name**	**Origin**
21	Selección 28	1398	**53**	Pokkali	India
22	Selección 29	1399	**54**	Perla	Unknown
23	Selección 30	1400	**55**	IACuba14	CPI-CP8 / ECIA22-8-103
24	Selección 31	1401	**56**	IACuba16	PNA46-110 / CP1-CP8
25	Negrin	1420	**57**	IACuba19	Jucarito-104 // Jucarito-104 / CICA-8
26	Arroz Bolito	1422	**58**	IACuba20	IACuba10 / ECIA31-104-2-1-1
27	Selección 97	1426	**59**	IACuba21	Fast neutons mutant line of Jucarito-104
28	Selección 132	1446	**60**	IACuba23	Fast neutons mutant line of Jucarito-104
29	Selección 135	1449	**61**	IACuba24	J-104 / ICA10 // J-104 / Siguaraya
30	Selección 138	1452	**62**	IACuba26	Somaclon of Jucarito-104
31	Selección 142	1453	**63**	IR36	IR1561-228-1-2 / IR1737 // CR94-13
32	Selección 143	1454	**64**	Amistad-82	IR1529ECIA / VNIIR3223
33	Jorge Valladares	1508	**65**	Jucarito-104	IR480-5-9-2 / IR930-16-1
34	Selección tres cuartos	1509			

Table II. SSR primer pairs used in the study, including chromosomal location, primer sequence, size of the PCR product and number of perfect repeats.

Marker	Chr. Loc.	Primer pairs *	Size (bp)**	Number of perfect repeats**
RM-4A	12	TTGACGAGGTCAGCACTGAC AGGGTGTATCCGACTCATCC	159	16
RM-4B	11	“”	“”	“”
RM-5	1	TGCAACTTCTAGCTGCTCGA GCATCCGATCTTGATGGG	113	14
RM-7	3	TTCGCCATGAAGTCTCTCG CCTCCCATCATTTCGTTGTT	180	19
RM-11	7	TCTCCTCTTCCCCCGATC ATAGCGGGCGAGGCTTAG	140	17
RM-13	5	TCCAACATGGCAAGAGAGAG GGTGGCATTCGATTCCAG	141	23
RM-18	7	TTCCCTCTCATGAGCTCCAT GAGTGCCTGGCGCTGTAC	157	21
RM-167	11	GATCCAGCGTGAGGAACACGT AGTCCGACCACAAGGTGCGTTGTC	128	16
RM-168	3	TGCTGCTTGCCTGCTTCCTTT GAAACGAATCAATCCACGGC	116	29
RM-202	11	CAGATTGGAGATGAAGTCCTCC CCAGCAAGCATGTCAATGTA	189	30
RM-225	6	TGCCCATATGGTCTGGATG GAAAGTGGATCAGGAAGGC	140	18

*** Forward primer listed on the first line and Reverse primer listed underneath**

**** The size of the PCR product and number of perfect repeats are referred to IR36.**

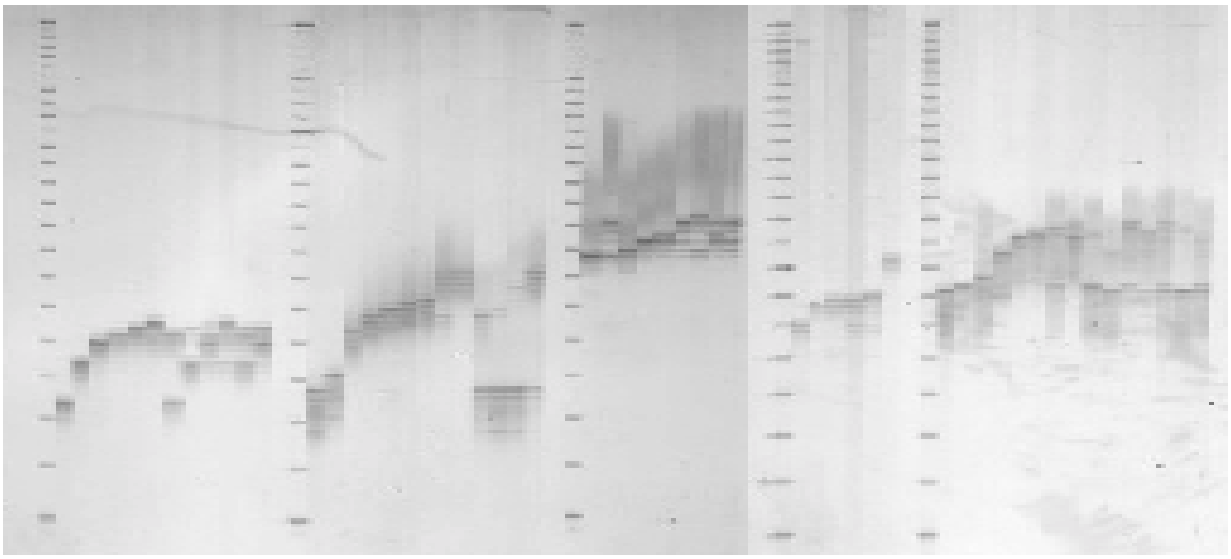

Figure 1. Representative profiles for 5 SSR primer pairs (from left to right: RM-225, RM-167, RM-7, RM-18 and RM-202). Lanes between SSR profiles contain size-marker (10 pb DNA ladder).

Figure 2. Different patterns obtained at one SSR loci, RM-225, among the studied genotypes. Genotype 1 was loaded in lanes 1, 31 and 65 (for better scoring of patterns).

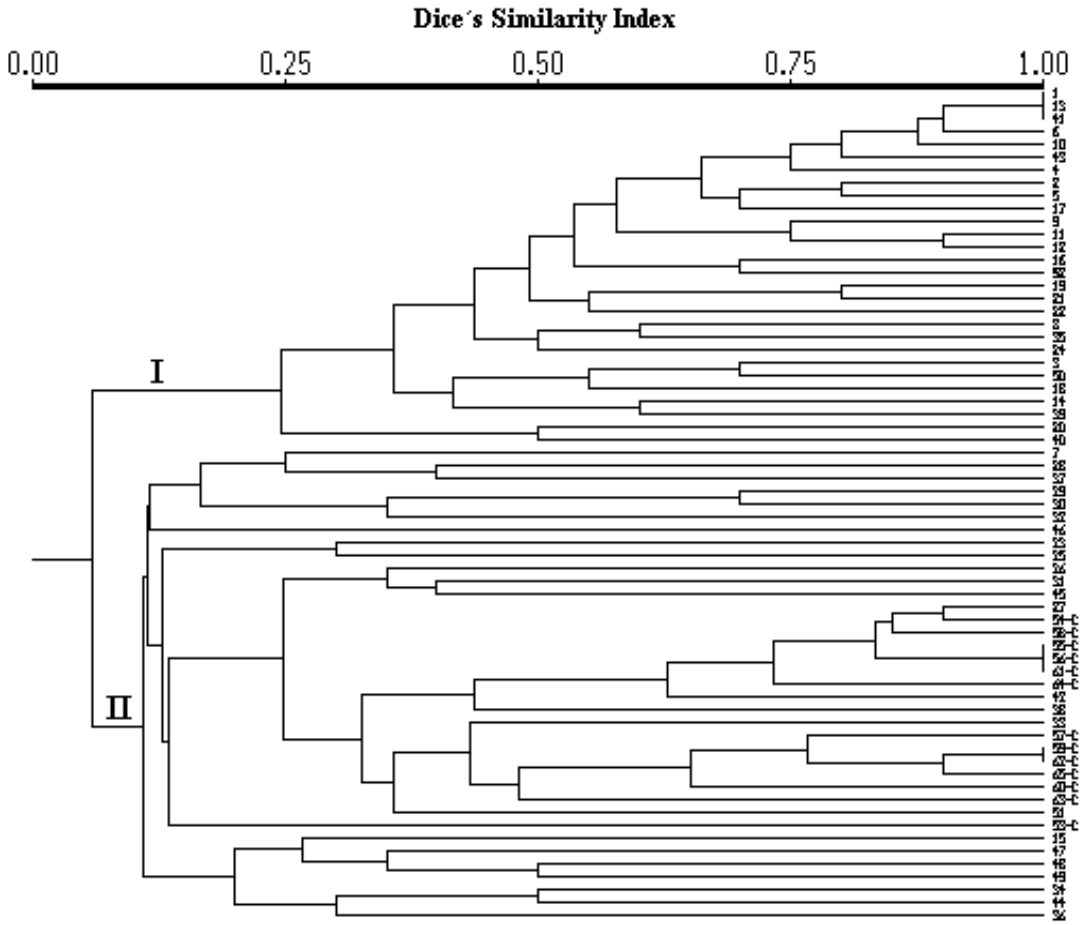

Figure 3. UPGMA dendrogram based on Dice´s Similarity index for the studied sample. Commercial varieties are specified with letter (C).