

Microsatellite marker-based genetic variability in Spanish rice cultivars and landraces

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Abstract

The genetic relationships among *japonica* rice cultivars and landraces were studied. Most of them are of Spanish origin, and were developed and cultivated for varying time periods over more than a century. To uncover genetic diversity within each cultivar, we analysed 30 plants per cultivar or accession using 10 fluorescently labelled primer pairs for SSR markers. Six cultivars were included in the study, with accessions from four different regions of Spain of the traditional cultivar Bomba. A total of 37 alleles were detected with a mean of 3.7 alleles per locus. Polymorphism information content (PIC) ranged from 0 to 0.78 with an average of 0.51 per locus. Genetic diversity for cvs. Albufera, Bahia, Taipei 309 and Jsendra ranged between 0.008 and 0.062. Cultivar Bomba accessions displayed higher number of alleles and genetic diversity, with Tarragona the lowest (0.08) and Valencia the highest (0.16) genetic diversity. Although infrequent, some heterozygous individuals were detected in cvs. Bomba, Bahia and Taipei 309. Cluster analysis enabled to categorize four accessions of cv. Bomba and cv. Albufera in one group and cvs. Bahia, Taipei 309, Jsendra and Senia in a second group supporting previously known relationships among varieties. These results show the utility of SSR markers for characterization of rice cultivars. In addition, the use of high number of individuals per cultivar, enabled to evaluate both inter- and intra-varietal genetic diversity. Therefore, this investigation should be useful in rice breeding programmes for genotype identification and for the evaluation of germplasm purity.

Additional key words: *japonica* rice; molecular characterization; *Oryza sativa* L.; SSR markers.

Resumen

Variabilidad genética de los cultivares y razas locales de arroz español mediante marcadores microsatélite

Se estudian las relaciones genéticas entre cultivares y razas locales de arroz de tipo *japonica*. La mayoría de ellas son de origen español, desarrolladas y cultivadas durante más de un siglo. La diversidad genética se estudió en 30 individuos de cada cultivar usando 10 pares de cebadores para microsatélites (SSR). Se estudiaron seis cultivares con muestras de cuatro regiones diferentes de España para el cultivar tradicional Bomba. El número total de alelos fue de 37 con una media de 3,7 alelos por locus. El polimorfismo de dichos loci varió entre 0 y 0,78, siendo 0,51 el promedio por locus. La diversidad genética de los cultivares Albufera, Bahia, Taipei 309 y Jsendra varió entre 0,008 y 0,062, mientras que el cv. Bomba presentó valores más elevados (0,12). Aunque poco frecuentes, se encontraron individuos heterocigóticos en los cultivares Bomba, Bahia y Taipei 309. El análisis cluster situó las muestras de las cuatro procedencias de cv. Bomba y el cv. Albufera en un mismo grupo, mientras que los cultivares Bahia, Taipei 309, Jsendra y Senia se situaron en otro grupo de acuerdo con las relaciones previamente conocidas entre variedades. Estos resultados muestran la utilidad de los marcadores SSR para la caracterización de cultivares así como la utilidad de estudiar números relativamente elevados de individuos por cultivar, lo cual permite evaluar la diversidad genética intere intra-varietal del arroz. Esta investigación resultará útil para el desarrollo de programas de mejora así como para la identificación de genotipos y la evaluación de la pureza del germoplasma.

Palabras clave adicionales: arroz *japonica*; caracterización molecular; marcadores SSR; *Oryza sativa* L.

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Abbreviations used: bp (base pair), CTAB (cetyl trimethyl ammonium bromide), dNTP (deoxyribonucleotide triphosphate), FAM (6-carboxyfluorescein), HEX (4, 7, 2', 4', 5', 7'-hexachloro-6-carboxyfluorescein), NED (2'-chloro-5'-fluoro-7',8'-fused phenyl-1.4-dichloro-6-carboxyfluorescein), PCR (polymerase chain reaction), PIC (polymorphism information content), SSR (simple sequence repeat).

Introduction

Rice (*Oryza sativa* L.) is a major cereal crop of high agronomic and nutritional importance as global rice production is over 650 million tonnes (<http://www.fao.org/>). It is highly polymorphic with wide geographical and genetic differentiation (Sarla *et al.*, 2005). Rice landraces, maintained through traditional farming practices, possess high genetic diversity and specific traits such as disease resistance, environmental constraint tolerance and nutritional quality which are often used in crop improvement (Camacho-Villa *et al.*, 2005). Furthermore, landraces are adapted to local agro-environmental conditions which contributes to yield stability and hence, they continue playing an important role in traditional and subsistence farming (Camacho-Villa *et al.*, 2005). However, extensive efforts to improve rice productivity have led to large-scale cultivation of high-yielding genetically uniform varieties, the replacement of local cultivars and the concomitant decrease in rice genetic resources that created a widespread concern to promote conservation of traditional cultivars/landraces (Kohli *et al.*, 2004; Camacho-Villa *et al.*, 2005; Barry *et al.*, 2007).

Conventionally, genetic diversity has been estimated on the basis of the morphological and physiological markers. Molecular markers offer additional advantages such as high polymorphism and independence from effects related to environmental conditions and the physiological stage of plant. Molecular characterization can be assessed again after several years of maintenance and new accessions can be related to existing collections which provides useful information for different breeding programmes (Bolaric *et al.*, 2005). In addition, molecular markers provide information on possible genetic mechanisms for observed evolutionary patterns (Bautista *et al.*, 2001).

Research on genetic diversity and phylogenetic relationships within both cultivated and wild rice species is necessary for better understanding of rice evolution and for aiding plant breeders in selecting appropriate materials for further genetic improvement of cultivars. In this respect, microsatellites or simple sequence repeat (SSR) markers are abundant and well-distributed throughout the 12 rice chromosomes. More than 2,200 SSR markers had been described in rice by McCouch *et al.* (1997, 2002a,b).

Microsatellite markers has been used to study inter-specific relationships between *Oryza sativa* and either

wild (*O. rufipogon* and *O. nivara*) or cultivated (*O. glaberrima*) species (Ni *et al.*, 2002; Barry *et al.*, 2007). Ni *et al.* (2002) also determined the pattern of diversity in *indica* and *japonica* rice subspecies and, within the *japonica* group, in both *temperate japonica* and *tropical japonica* types. Additionally, the genetic diversity of a number of rice cultivars and landraces from several countries/regions have been studied by the use of SSR markers (Luce *et al.*, 2001; Brondani *et al.*, 2006; Ram *et al.*, 2007; Tu *et al.*, 2007).

Although rice is a major food crop in Europe, with an annual production of over 3.5 million tonnes, its cultivation is restricted to few countries. In this respect, efforts are directed to exploit and characterize the European and international rice germplasm from the temperate rice area for their conservation and sustainable use in agriculture (<http://www.eurigen.net>). Spain is the second European rice producer with estimations for 2008 of over 660 thousand tonnes harvested in approximately 96 thousand hectares (<http://www.mapa.es>). Some rice cultivars of agronomic importance in Spain have been developed in different time periods that range from the end of the XIX to the XXI century. Thus, the traditional cv. Bomba was one of the only two cultivars extensively used by the 1890s. Although the high plant size hinders productivity, cv. Bomba is still highly appreciated due to the grain organoleptic properties. Cultivars Bahia and Senia (Bahia × Sequial) have been the most widely cultured in the 80s and 90s, respectively. Cultivars Jsendra (Senia × M202) and Albufera (Bomba × Senia) were registered during 2005 and 2007, respectively, and Jsendra is already extensively cultivated.

Cell lines and seedlings of cvs. Bomba and Bahia were used in a number of experiments related to abiotic stress tolerance in our previous research (Perales *et al.*, 2005; Wankhade *et al.*, 2010), and Taipei 309, a cultivar with high competence for genetic transformation, has long been utilized in our laboratory as a source of cell lines (Moukadiri *et al.*, 1999, 2002; Bahaji *et al.*, 2002; Perales *et al.*, 2008).

In this research SSR markers were used to evaluate in detail the genetic variability and relationships among five *japonica* rice cultivars (Bomba, Bahia, Senia, Jsendra and Albufera) that have been extensively cultivated in Spain. Cultivar Taipei 309 was also analyzed for comparative purposes. The application of SSR markers to a large number of individuals per cultivar or accession has allowed the reliable characterization of both varieties and landraces.

Material and methods

Plant materials

The following six *japonica* rice cultivars were used in this investigation: cvs. Bahia, Senia, Bomba, Jsendra, Albufera and Taipei 309. Cultivars Bahia, Senia, Albufera and Jsendra were developed, maintained and kindly provided by the Rice Department of the *Instituto Valenciano de Investigaciones Agrarias* (IVIA) at Sueca (Valencia). Due to phenotypic variability, four accessions of cv. Bomba from different Spanish rice growing regions were evaluated: Murcia, Sevilla, Tarragona and Valencia. They were kindly provided by cooperatives *Virgen de la Esperanza*, Calasparra, Murcia; *Veta la Mora*, Isla Mayor, Sevilla; and the Rice Department, IVIA (Tarragona and Valencia accessions).

Rice plants were grown in the greenhouse with day/night temperatures of 27/18°C and relative humidity of 60-80%. Leaf samples were collected when seedlings were in their vegetative growth stage and stored at -80°C.

DNA extraction, fragment amplification and analysis

Genomic DNA extractions from a total of 270 plants, 30 plants per cultivar and accession, were performed according to CTAB protocols described by Doyle and Doyle (1987). Leaf fragments of approximately 5 cm in length from each genotype were ground in liquid nitrogen in 1 mL extraction buffer using mortar and pestle. The purity and concentration of extracted DNA was evaluated by horizontal electrophoresis on 0.8% (w/v) agarose gel in 1x TBE buffer using a known ladder of Lambda DNA/*EcoRI*+*HindIII* (Fermentas Life Sciences). The stock DNA samples were stored at -20°C and working DNA samples, diluted with sterile distilled water (1:100 v:v) at 4°C for PCR use.

Primer sequences were obtained from Chen *et al.* (1997) and McCouch *et al.* (2002a,b). They were selected according to data on variability reported by Coburn *et al.* (2002). Initial SSR amplifications were performed with 11 non-labelled primers and based on satisfactory amplification results, 10 fluorescently labelled primers (Applied Biosystems) were selected (Table 1).

DNA amplification was carried out in a 25 µL reaction mixture containing 2.5 µL 10x reaction buffer with 1 to 2 mM MgCl₂, 0.625 U of Taq DNA polymerase

(Netzyme Molecular Netline Bioproducts), 0.2 µM forward and reverse primer (Applied Biosystems), 20 mM dNTP mix (Fermentas Life Sciences) and 10 µL DNA. The Polymerase Chain Reaction (PCR) was performed in PTC-100 (MJ Research) as follows: 4 min at 95°C, 35 cycles of 30 sec at 94°C, 45 sec at 55°C, 1 min at 72°C, and 5 min at 72°C for the final extension. Annealing temperature was adjusted for each primer according to melting temperature (Chen *et al.*, 1997; McCouch *et al.*, 2002a,b). The amplified PCR products were subjected to electrophoresis in 2% (w/v) agarose gel in 1x TBE buffer at 100-130 V along with a known 100 bp DNA ladder (Fermentas Life Sciences). DNA bands were stained with ethidium bromide for 5 min and visualised under UV light (Gibco BRL UV Transilluminator). The 1.5 µL of PCR products were dissolved in 30 µL of sterile distilled water for fragment analysis.

PCR products were mixed avoiding overlapping sizes before fragment analysis into 4 panels with RM333(FAM, 6-carboxyfluorescein), RM225(NED, 2'-chloro-5'-fluoro-7',8'-fused phenyl-1.4-dichloro-6-carboxyfluorescein) and RM400(HEX, 4, 7, 2', 4', 5', 7'-hexachloro-6-carboxyfluorescein) in panel I; RM171(FAM), RM484(NED) and RM224(HEX) in panel II; RM206(NED) and RM234(HEX) in panel III; RM248 and RM464(HEX) in panel IV. Fragment analyses were performed by capillary electrophoresis in an automated ABI Prism 3700 DNA analyser (Applied Biosystems). Peak sizes were scored with GeneScan vs. 3.1.2 and Genotyper vs. 2.5 softwares (Applied Biosystems) at Servicio Central de Soporte a la Investigación Experimental (SCSIE) of University of Valencia.

Data analysis

Different size of amplification products from the same primer pair were considered as different alleles. As RM248 did not show variability, a matrix with 270 individuals and 18 character states was generated. Polymorphism Information Content (PIC) or expected heterozygosity (*H*) for each SSR marker was calculated based on the formula:

$$H = 1 - \sum p_i^2$$

where p_i is the allele frequency for the i -th allele (Nei, 1973). Three different pairwise distances (F_{ST} , Slatkin, 1995) among cultivars implemented in ARLEQUIN vs. 3.00 (Excoffier *et al.*, 2005) were computed and

Table 1. Chromosome location (CL), size range (SR), fluorescent marker, primer sequences, motifs, number of alleles (NA) and polymorphism information content (PIC) for ten simple sequence repeat (SSR) markers used in the study

SSR loci	CL	SR (bp)	Fluorescent marker ¹	Primer sequences (forward and reverse)	Motif	NA	PIC
RM171	10	327-343	FAM	AACGCGAGGACACGTATTAC ACGAGATACGTACGCCTTTG	GATG	2	0.49
RM206	11	172-196	NED	CCCATGCGTTTAACTATTCT CGTTCCATCGATCCGTATGG	CT	8	0.77
RM224	11	229-233	HEX	CTTCGATCCATCATCCATGG ATTGCTATACGCAGTTTCGG	AT	3	0.49
RM225	6	132-142	NED	TGCCCATATGGTCTGGATG GAAAGTGGATCAGGAAGGC	CT	2	0.40
RM234	7	130-140	HEX	ACAGTATCCAAGGCCCTGG CACGTGAGACAAAGACGGAG	CT	3	0.51
RM248	7	81	NED	TCCTTGTAATCTGGTCCC GTAGCCTAGCATGGTGCATG	CT	1	0
RM333	10	171-198	FAM	GTACGACTACGAGTGTACCAA GTCTTCGCGATCACTCGC	TAT, CTT	7	0.78
RM400	6	243-331	HEX	ACACCAGGCTACCCAAACTC CGGAGAGATCTGACATGTGG	ATA	4	0.64
RM464	9	260-262	HEX	AACGGGCACATTCTGTCTTC TGGAAGACCTGATCGTTTCC	AT	2	0.49
RM481	7	125-167	NED	TAGCTAGCCGATTGAATGGC CTCCACCTCCTATGTTGTTG	CAA	5	0.52

¹ FAM, 6-carboxyfluorescein; HEX, 4, 7, 2', 4', 5', 7'-hexachloro-6-carboxyfluorescein; NED, 2'-chloro-5'-fluoro-7',8'-fused phenyl-1.4-dichloro-6-carboxyfluorescein.

different clustering methods (single, complete, weighted and un-weighted pair-group method arithmetic average) were checked using NTSYS 2.2 (Rohlf, 2002) to select the best quality indicator (the cophenetic value). The combination of F_{ST} and un-weighted pair-group method arithmetic average (UPGMA) was selected.

Results

All SSR loci amplified consistently size fragments, yielding a total of 37 alleles in the six cultivars and accessions studied. The number of alleles per locus varied widely among 10 primer pairs, ranging from 1 (RM248) to 8 (RM206) with an average of 3.7 alleles per locus. The overall size of amplified PCR products ranged from 81 to 343 bp, with most of the sizes between 125 and 260 bp (Table 1).

No relationships were found among the number of alleles and nucleotide motif composition or the number of repetitions in the motifs (Table 1). The only invari-

able, monomorphic locus (RM248) showed the lowest size of 81 bp, while the most variable loci (RM206 with 8 alleles and RM333 with 7 alleles) ranged between 172 to 196 and 171 to 198 bp respectively. On the other hand, only two alleles were found in RM171, a locus with the largest bp size. Two loci, RM224 and RM464 with bi-nucleotide (AT) repeat motifs detected low number of alleles *i.e.* 3 and 2 respectively, while RM206, RM225, RM234 and RM248 with CT repeat motifs ranged from 1 to 8 alleles. RM333, RM400 and RM481, loci with tri-nucleotide repeat motifs detected 7, 4 and 5 alleles respectively; while the number of alleles was 2 for the only tetra-nucleotide motif locus (RM171).

Similarly, no relationship was found between the number of alleles and chromosome in which each particular locus is placed as different loci on the same chromosome widely varied in the number of alleles (Table 1). Several alleles were in frequencies lower than 10% within each sample, meaning that they were scored less than 6 times in total. These low frequency

Table 2. Data on the alleles of the different simple sequence repeat (SSR) for each cultivar and source. Alleles with less than 10% frequencies are indicated in bold digits (3 individuals or 6 copies)

Cultivar	RM171		RM206		RM224		RM225		RM234		RM248		RM333		RM400		RM464		RM481	
	NA	SR	NA	SR	NA	SR	NA	SR	NA	SR	NA	SR	NA	SR	NA	SR	NA	SR	NA	SR
Bomba Valencia	1	343	2	192,194	1	229	2	132-142	2	132-140	1	81	4	171, 174, 173, 186	1	243	1	260	2	137-140
Bomba Tarragona	1	343	5	176, 190, 192, 194, 196	1	229	2	132-142	1	140	1	81	1	183	1	243	1	260	2	140- 143
Bomba Murcia	1	343	4	190, 192, 194, 196	2	229-231, 233	2	132-142	1	140	1	81	2	183-186	1	243	1	260	2	140- 143
Bomba Sevilla	1	343	5	178, 190, 192, 194, 196	2	229-231	2	132-142	1	140	1	81	4	183, 186, 189, 192	1	243	1	260	3	125, 137-140
Albufera	1	343	1	190	1	229	1	132	1	140	1	81	2	171-174	1	318	1	260	1	140
Bahia	1	327	2	174- 176	1	231	1	132	1	132	1	81	2	186-189	2	309-331	1	262	1	167
Taipei 309	1	327	1	174	1	231	1	142	2	130-132	1	81	1	192	1	331	1	262	1	167
Jsendra	1	327	1	172	1	231	1	132	1	132	1	81	2	192-198	1	318	1	262	1	167
Senia	1	327	1	174	1	231	1	132	1	132	1	81	1	198	1	318	1	262	1	167

NA: number of alleles. SR: size range. Alleles separated by «-» indicate all intermediate sizes are present.

alleles were widespread among cultivars and loci, being cv. Bomba the variety with higher number of them and RM206 and RM333 the loci showing the higher number of alleles (Table 2). Low frequency alleles were observed in 7 out of 10 SSR loci studied. Loci RM206, RM333, RM481 and RM224 showed 6, 4, 3, and 2 rare alleles respectively; while 1 allele each was detected in loci RM225, RM234 and RM400. As expected, loci showing greater number of alleles detected more low frequency alleles too. Frequencies of alleles are available upon request.

Genetic diversity among varieties

Genetic diversity was estimated through total number of alleles, average number of alleles and average genetic diversity (Table 3). All these parameters ranged widely as a function of the cultivar. Cultivar Senia showed no variation in any of the loci. Four cultivars (Albufera, Taipei 309, Bahia and Jsendra) showed low levels of variation with total number of alleles ranging between 11 to 13 with an average of 1.11 to 1.33 alleles per locus and average genetic diversity between 0.008 and 0.062. Conversely, high levels of variation were found in cv. Bomba whose total number of alleles among accessions ranged between 17 to 21 with an average of 1.66 to 2.22 alleles per locus. Within cv. Bomba accessions,

Tarragona showed the lowest number of alleles and levels of diversity, while Sevilla had the highest number of alleles and Valencia the highest levels of genetic diversity. A total of 127 alleles were estimated for ten microsatellite markers across six rice varieties including accessions of cv. Bomba from four different regions (Table 3).

Ten individuals, representing a 2.7% of the total, showed different alleles in heterozygosis. Two heterozygous individuals belonged to cvs. Bahia and Taipei 309, while the remaining eight were detected in accessions of cv. Bomba, *i.e.* 4 in Tarragona, 2 in Sevilla and 1 each in Valencia and Murcia. Heterozygotes were detected in five out of ten SSR loci (RM206, RM224, RM234, RM333 and RM481) representing five heterozygous individuals for locus RM333, two for RM481 and one each for loci RM206, RM224 and RM234.

Cultivars relationships and characterization

Affinities among cultivars and accessions are shown in the dendrogram obtained using F_{ST} distances, forming two different groups (Fig. 1). The first cluster included four accessions of cv. Bomba and cv. Albufera where cv. Bomba accessions were closer among them than any of them to cv. Albufera. The second cluster was composed of cvs. Bahia, Taipei 309, Jsendra and Senia

Table 3. Total number of alleles (T_{NA}) for 10 simple sequence repeat (SSR) loci, average number of alleles (A_{NA}), average genetic diversity and alleles allowing cultivars and source identification (indicated by the primer name followed by allele size). Low frequency alleles were not considered for cultivar characterization purposes

	T_{NA} (10 loci)	A_{NA}	Average genetic diversity	Alleles allowing cultivars/source identification
Bomba Valencia	17	1.88	0.16 ± 0.215	RM206-192,194 RM333-174,183
Bomba Tarragona	16	1.66	0.08 ± 0.146	RM206-190
Bomba Murcia	17	1.88	0.11 ± 0.149	RM206-190,194
Bomba Sevilla	21	2.22	0.15 ± 0.218	RM206-192,194,196
Bomba	17.75	1.91	0.12 ± 0.180	RM333-183 RM400-243
Albufera	11	1.11	0.01 ± 0.023	RM333-174
Bahia	13	1.33	0.06 ± 0.132	RM333-189
Taipei 309	11	1.11	0.04 ± 0.117	RM234-130, RM333-192
Jsendra	11	1.22	0.02 ± 0.029	RM206-172
Senia	10	1	0	

where cvs. Bahia and Taipei 309 were closer than any of them to cvs. Jsendra or Senia (Fig. 1).

With the exception of cv. Bomba accession from Sevilla, the other three accessions showed high fre-

quencies for allele RM225-132 which is shared by cvs. Albufera, Bahia, Jsendra and Senia while cv. Taipei 309 showed allele RM225-142. Cultivar Albufera (Bomba \times Senia), shared common alleles (RM171-343,

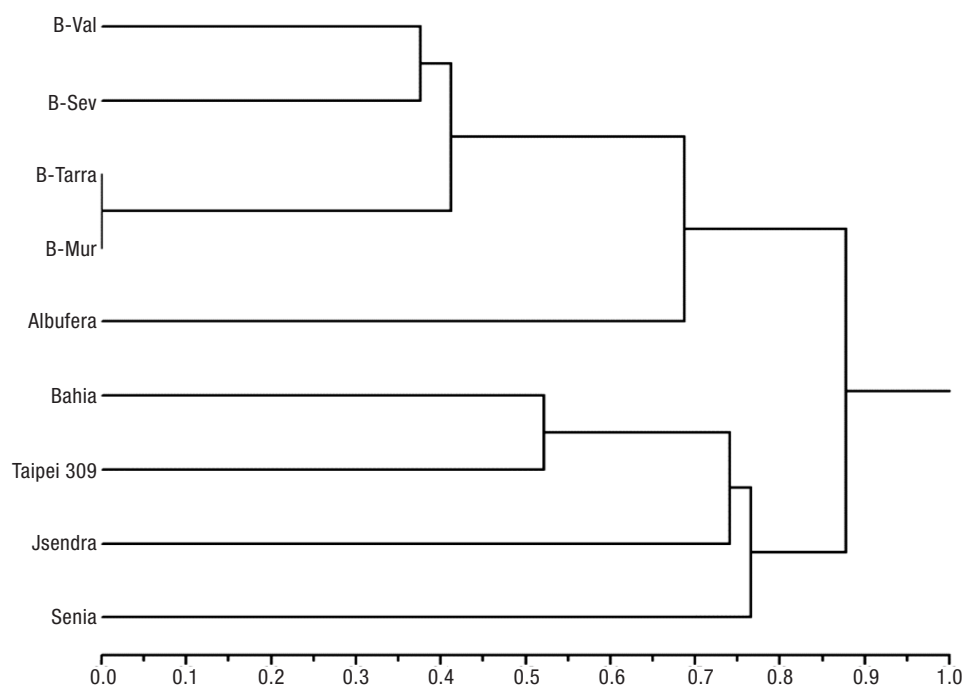


Figure 1. Dendrogram based on F_{ST} distances among the studied cultivars and sources using UPGMA. Coefficient of cophenetic correlation $r = 0.98$. B-Val, B-Sev, B-Tarra and B-Mur = cv. Bomba Valencia, Sevilla, Tarragona and Murcia accessions, respectively.

RM224-229, RM234-140, RM464-260 and RM481-140) with cv. Bomba, while two (RM400-318 and RM225-132) with cv. Senia, all together indicating its hybrid origin. Several other low frequency alleles RM224-231 and RM234-132 were also shared by cv. Bomba and other cultivars (Table 2).

All the studied cultivars except Senia had exclusive alleles (Table 3). Alleles RM333-183 and RM400-243 were characteristics of cv. Bomba as they were shared by all four Bomba accessions. Allele RM333-174, although shared by Bomba-Valencia, characterized cv. Albufera as the more frequent allele, while in Bomba-Valencia RM333-174 and RM333-183 were more frequent alleles (Table 3). Cultivar Bahia was characterized by allele RM333-189 and the high frequency of RM333-186. Cultivar Jsendra was characterized by an allele RM206-172, while alleles RM234-130 and RM333-192 were characteristic for cv. Taipei 309 (Table 3).

Cultivar Senia did not show any exclusive allele as it shared most alleles with cultivars of the second group (Fig. 1). Alleles of locus RM206 allow to differentiate cv. Senia (RM206-174) and cv. Jsendra (RM206-172) (Table 2).

Taking together the combinations of alleles from all nine variable loci, all the studied cultivars can be differentiated as follows: cvs. Jsendra and Senia, varieties that shared all alleles except for locus RM206. Similarly, cvs. Bahia and Taipei 309 showed different more frequent alleles for loci RM225 and RM333. Likewise, cvs. Bomba and Albufera shared most of the alleles in the studied loci except an allele RM400-318 which was shared with Senia (Table 2), showing its hybrid origin. Cultivar Bomba show higher similarity than cv. Senia with respect to cv. Albufera (Bomba × Senia). Among cv. Bomba accessions different combinations of more frequent alleles, mainly at locus RM206, allowed the identification of their origin (Table 3).

Discussion

The genetic relationships among five Spanish rice cultivars that were developed within a long timeframe as well as their genetic diversity were investigated. In this respect, we focused on the diversification of the traditional cultivar Bomba into several landraces.

Our results were obtained with relatively few primers, showing the ability of SSR markers to characterize these Spanish cultivars, as well as cv. Taipei 309.

Therefore, they corroborate previous investigations (Nagaraju *et al.*, 2002; Ravi *et al.*, 2003; Sarla *et al.*, 2005; Brondani *et al.*, 2006; Lapitan *et al.*, 2007; Singh *et al.*, 2010) in which SSR markers had been able to discriminate among cultivars.

The size variations of alleles are within the range reported in previous research (Temnykh *et al.*, 2000; Coburn *et al.*, 2002). The PIC values across the variable loci ranged between 0.40 (RM225) and 0.78 (RM333) (Table 1), being lower than those reported by several other authors (Coburn *et al.*, 2002; Nagaraju *et al.*, 2002; Pessoa-Filho *et al.*, 2007). Thus, high levels of heterogeneity of PIC values indicate clear differences among the studied cultivars.

Among cultivars, relationships had been shown through a cluster analysis based on F_{ST} distances, showing that cvs. Bomba and Albufera are genetically closer than any of the studied varieties, while the remaining varieties are in a second group where cvs. Bahia and Taipei 309 are closer between them than to cvs. Jsendra and Senia. Cultivar Albufera is a hybrid of cvs. Bomba and Senia that was generated with the aim of maintaining the grain properties of cv. Bomba but with increased productivity (Carreres *et al.*, 2006), therefore, the short genetic distance between cvs. Bomba and Albufera should be expected. On the other hand, the close relation between cvs. Taipei 309 and Bahia is very interesting at the cellular level as both show higher morphogenic ability *in vitro* than a number of *japonica* cultivars we tested (unpublished observations).

Among cv. Bomba accessions, the distance between Tarragona and Murcia was very low (0.008) therefore it appears as 0 in the dendrogram. Interestingly, Valencia and Sevilla were closer in the cluster while farther geographically. This might be related to the migration of Valencian rice growing farmers, during 1930s-40s, to Sevilla. The high number of alleles in cv. Bomba accessions together with the fact that several of them were shared with other varieties agrees with a low fixation of characters of this traditional cultivar of unknown origin.

Self-pollination (autogamy) results in restricted gene flow between landraces of rice and hence, heterozygosity is considered to be rare in these plants (Garris *et al.*, 2005; Wang and Lu, 2006). However, Kohli *et al.* (2004) reported that low rates of heterozygosity sometimes exist in rice varieties. Our results suggest that five out of the six varieties studied, were highly homogeneous and the heterozygosity found in cvs. Bahia and Taipei 309 should be considered as residual.

On the contrary, the levels of heterozygosity detected in cv. Bomba accession were relatively high, representing a 6.66%. Since this traditional cultivar was originated more than a century ago the possibility of cross-pollination prior to proper *ex situ* conservation cannot be excluded.

Although Spain is the second rice producer in Europe, few rice varieties have been developed. Identification of each of the studied cultivars as well as different provenances of cv. Bomba is possible through alleles and characteristic combinations for each one of them, which has implications in germplasm conservation. This is particularly important for cv. Bomba accessions which should be studied deeply to gain a better knowledge on the genetic composition of its landraces for an accurate preservation. The hybrid origin of cv. Bomba is supported by the fact that it shares two alleles for locus RM225 as well as several others in low frequency present within other varieties. Presence of low frequency alleles among different rice varieties were also observed by several other researchers and can be useful for breeding purposes (Jain *et al.*, 2004; Siwach *et al.*, 2004; Lapitan *et al.*, 2007; Luan *et al.*, 2008). Among European cereals, the diversification of traditional cultivars into landraces had been frequent (Zeven, 1998) as there were no individual institutions in charge of their preservation. As far as we know, all the cv. Bomba collections maintain the grain properties highly appreciated by the consumers and the high size of plants that hinders their productivity. These findings are useful tools to detect adulteration of rice varieties, particularly of cv. Bomba and can support their utility in quality assurance of agro-economically important traits (Siwach *et al.*, 2004). However, some diversity has been noticed in additional phenotypic characteristics and they appear to be reflected in the data presented here. At present, cereal landraces and traditional varieties are highly valued in Europe as the source of ingredients for traditional food and drinks (<http://www.niab.com/research/pgbe/genetics/diversity-genomics-roup/research/projects/landraces.html>) and their preservation in germplasm collections or *in situ* is organized by Biodiversity International (<http://www.biodiversityinternational.org>).

This is the first report on the genetic variability of cv. Bomba, a traditional cultivar, as well as on the genetic characterization of rice cultivars cultured along time periods covering more than a century. The results obtained should also be useful in the development of breeding programmes as they provide a valuable tool

for the precise identification of rice varieties and landraces.

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