RESEARCH

Genetic diversity, identification, and certification of Chilean rice varieties using molecular markers

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It takes approximately 14 yr to produce a new rice (*Oryza sativa* L.) variety, that is, from initial hybridization to its commercial release. Currently, new varieties are identified based on morphological descriptors, which have been efficient over time. However, due to the main constraints on seed type impose to other breeding objectives and the pressure of continuous release of varieties, high degree of parentage, and genetic and morphological uniformity has been observed in the breeding populations. The objectives of this study were: to determine the genetic variability of Chilean and foreign commercial rice varieties, and determine, identify, and certify the genetic relationships among varieties, using simple sequence repeat (SSR) markers. A total of 16 commercial varieties, some of them closely related, were included in the study, which were genetically analyzed using 54 microsatellites. The 54 microsatellite loci allowed the discrimination among the 16 varieties. The number of alleles ranged between 2 and 8 with a mean of 3.54 alleles per locus, while the polymorphism information content (PIC) presented a mean of 0.44. The genetic distance and diversity parameters between pairs of varieties indicate a limited diversity among these genotypes. The cluster analysis indicated that varieties were grouped according to their grain type and pedigree. Results demonstrate that the identification and certification of varieties using microsatellite markers could be a good complement to existing agro-morphological data when varieties are closed related.

Key words: Genetic diversity, *Oryza sativa*, SSR, variety certification and identification.

INTRODUCTION

Rice (*Oryza sativa* L.) is an important crop worldwide since more than 3.5 billion people depend on it and supplements more than 20% of their daily calories requirement (Seck et al., 2012). Demand for rice has steadily increased with population growth; rice uses one fifth of the area sown with cereals and it is the species with the second largest planted area (Kshirsagar et al., 2013). Fortunately, world rice production has been duplicated in the last 25 yr using high-yield varieties and improved agronomic practices (Kshirsagar et al., 2013).

In Chile, rice is one of the basic foods and its production represents the main source of revenue for small- and medium-sized farmers and it is produced in "paddy soils" where very few production alternatives are available (Paredes et al., 2013).

Rice was introduced to Chile in the 1920s and used 3000 ha that were unsuitable for the production of other crops. At first, yield was 4.5 t ha^{-1}, but this yield decreased to 2.5 t ha^{-1} due to the lack of rotations, use of fertilizer, and inefficient weed control (Paredes et al., 2013). Currently, a total of 21 000 ha are sown between Maule and Biobío Regions in Chile with a mean yield of 6.2 t ha^{-1} (ODEPA, 2013). It is important to note that new varieties of Instituto de Investigaciones Agropecuarias (INIA) can achieve yields over 7 t ha^{-1} under farmer´s conditions and 10 or 12 t ha^{-1} under experimental fields (Paredes et al., 2013).

Identification through morphological traits has been one of the first tools to identify species and varieties (IRRI, 1965). For instance, seed color, germination response, leaf color, ligule shape apex, stigma color, growth habit, plant height, flag leaf length and width, grain length and width, and yield components (grain weight, number of grains per panicle, number of panicles, and percentage of filled grains) (Ali et al., 2011).

Morphological characterization has been efficient until now for identifying genetic material; however, this method has become inefficient for differentiation of commercial varieties (Rahman et al., 2009) because of the greater uniformity in morphological traits of the commercial varieties and the low number of morphological traits available (Rahman et al., 2009; 2010; Islam et al., 2011).

A complementation to the morphological identification of the varieties is their genetic characterizations, using molecular markers (Rahman et al., 2009). Both methods are complementary for describing varieties (Becerra and Paredes, 2000; Lapitan et al., 2007).

Simple sequence repeat (SSR) markers have been widely used in genetic diversity studies and to identify

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rice varieties (McCouch et al., 2002; Garris et al., 2004; Lu et al., 2005; Chuang et al., 2011; Islam et al., 2011; Kanawapee et al., 2011). In rice, genomic SSRs form part of coding or noncoding regions of the genome (Varshney et al., 2005) and they are also called neutral or anonymous because they do not consider the degree of phenotypic expression of the sequences (Gupta and Rustgi, 2004). SSRs are distributed in its 12 chromosomes (Temnykh et al., 2001; Rahman et al., 2009) and widely available in the rice genome. For instance, there are more than 20 000 microsatellites from the genome of the *japonica* varieties (Lin et al., 2012). The main advantages of SSRs are their high degree of polymorphism, codominant genetic nature, multiallelism, abundance, locus specificity, transferability between genera, and high reproducibility among laboratories. These advantages allow developing unique profiles known as fingerprinting or genetic fingerprint from the alleles detected by one or more SSR loci (Rahman et al., 2009; 2010) and identify closely related genotypes (Xin et al., 2005; Zhu et al., 2012) and adulteration of rice from seed lots (Kumari et al., 2011) and polished rice sold in the domestic market of protected rice varieties (Chuang et al., 2011).

In Chile, identification of rice varieties using molecular tools is scarce. There are only a couple studies that have determined low genetic diversity among Chilean varieties by analyzing seed storage proteins, and AFLP markers (Hinrichsen et al., 1996; Aguirre et al., 2005).

It is crucial for a breeding program to identify and certify varieties and determine their relationships as a way to protect their quality and plant breeder rights. The use of molecular markers in this context is still under discussion. The issue is partially due to the definition of "variety". A variety is defined as the expression of traits resulting from a genotype or the combination of genotypes and can be differentiated from any other group of plants because at least one of these traits is expressed according to the 1991 Act of the UPOV Convention (UPOV, 1991).

Therefore, the objectives of this research study were: 1) to determine the genetic diversity of Chilean and foreign commercial rice varieties, and 2) to identify, and certify the genetic relationships among them, using simple sequence repeat (SSR) markers.

MATERIALS AND METHODS

Plant material

Sixteen temperate *japonica* genotypes were used in the present study, which correspond to old and current Chilean and foreigner commercial varieties, including an *indica* genotype as an out-group (Table 1). Seeds were provided by INIA's Rice Breeding Program at Chillán, Chile.

Germination and plant vegetative growth were carried out under greenhouse conditions. Young leaves of each variety at the 4-leaf stage were harvested to extract DNA. Harvested leaves were maintained at -96 °C until DNA

Table 1. Varieties, origin, agronomic description and type of grain for *Oryza sativa* analyzed by microsatellites (SSR).

N°	Variety name	Origin	Subspecies	Flowering	Spikelet fertility (0-9)	Panicle exsertion (0-9)	Shattering (0-9)	Lodging (0-9)	Plant height	Grain yield	Grain whiteness (5-70%)	Grain transparency (0.01-8.0%)	Milling degree points (0-199)	Grain length	Grain width	Length/ width Ratio
				d					cm	$t\,ha^{-1}$				mm	mm	
1	Oro	Old commercial variety	*japonica*	101	2.5	1.9	2.4	4.7	97	79.2	41.9	2.5	101.7	5.4	3.0	1.8
2	Ámbar-INIA	Commercial variety	*japonica*	105	2.2	3.4	1.6	0.1	73	90.2	49.9	1.1	134.7	4.9	2.8	1.7
3	Quella-INIA	Commercial variety	*japonica*		3.0	1.0	3.0	0.0	92	95.0	41.9	3.7	110.0	5.1	2.8	1.8
4	Brillante-INIA	Commercial variety	*japonica*	115	3.7	3.7	4.0	1.0	90	98.3	39.1	3.7	98.6	7.1	2.5	2.8
5	Buli-INIA	Commercial variety	*japonica*	113	4.3	3.9	2.7	0.6	96	78.7	36.9	3.6	87.5	7.3	2.2	3.3
6	Diamante -INIA	Commercial variety	*japonica*	110	3.5	3.5	3.3	1.8	95	90.4	39.3	4.0	101.0	7.3	2.4	3.0
7	Cuarzo-INIA	Commercial variety	*japonica*	107	3.4	3.5	3.2	0.3	90	91.7	38.4	4.1	97.5	7.2	2.6	2.8
8	Zafiro-INIA	Commercial variety	*japonica*	76	3.8	3.6	3.3	0.2	92	113.1	39.9	4.1	104.4	7.1	2.5	2.9
9	Guadiamar	Spain	*japonica*	110	1.4	3.4	1.4	0.1	75	98.9	36.7	3.3	83.8	5.5	2.6	2.2
10	Guara	Spain	*japonica*	110	1.9	2.3	2.9	0.0	80	101.1	42.8	2.8	108.4	5.7	3.0	1.9
11	Hispagran	USA	*japonica*	113	2.4	3.1	2.0	0.6	79	110.7	43.5	2.8	111.2	5.8	3.0	1.9
12	Susan	Spain	*japonica*	113	1.6	5.2	2.2	0.0	64	91.4	43.8	2.9	113.3	6.0	3.1	2.0
13	Euro	Europe	*japonica*	112	1.9	5.0	2.1	0.0	74	90.2	35.9	3.3	79.1	6.2	2.3	2.7
14	Ranballi	Bulgaria	*japonica*	107	2.2	5.0	2.0	0.0	71	73.2	40.1	2.9	96.8	5.5	2.9	1.9
15	Karolina	Hungary	*japonica*	95	2.8	3.0	3.7	3.5	86	40.7	42.0	3.2	107.2	7.2	2.6	2.8
16	Basmati	India	*indica*	128	7.5	7.0	2.3	0.0	63	10.8	36.5	3.2	82.5	6.8	1.8	3.8

was extracted. Leaves were then macerated in a mortar with liquid nitrogen. Samples were incubated with 400 μL extraction buffer (100 mM Trizma; 1.4 M NaCl; 20 mM EDTA; 1% polyvinylpyrrolidone; 2% CTAB; 1% 2-mercaptoethanol; pH 8.0) at 65 °C for 45 min and gently mixed for homogenization.

Subsequently, samples were cooled to room temperature and two protein extractions were carried out with chloroform:isoamyl alcohol (24:1). The emulsion was centrifuged at 4500 rpm for 15 min for phase separation. The aqueous supernatant was transferred to a clean tube and DNA was precipitated with isopropanol (2/3 of the volume) and then incubated at -20 °C all night. The precipitate was washed with ethanol (76%; 10 mM NH_4Ac) and dried at room temperature. Finally, DNA was resuspended in TE buffer (pH 8.0) and treated with RNAse. The quality of the DNA was verified by electrophoresis in 1% agarose gels and its concentration was measured in a spectrophotometer UV-Vis (NanoDrop 2000, Thermo Scientific, Wilmington, Delaware, USA).

SSR amplification

A set of 200 primers distributed among the 12 chromosomes were initially screened for selection and 54 out of 200, exhibiting higher levels of polymorphism were selected (Table 2). These primer sequences were obtained from the Gramene database (http://www.gramene.org/markers). Reaction conditions were performed in a 25 μL

Table 2. Set of evaluated microsatellite primers for identification and certification of rice varieties.

SSR	Chromosome	Forward sequence	Reverse sequence	Annealing temperature (°C)
RM8068	1	AAACCTCTCGCTGTAATTAG	TGAACATTTATTGATATGGTAAA	55
RM129	1	TCTCTCCGGAGCCAAGGCGAGG	CGAGCCACGACGCGATGTACCC	55
RM24	1	GAAGTGTGATCACTGTAACC	TACAGTGGACGGCGAAGTCG	55
RM315	1	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	55
RM243	1	GATCTGCAGACTGCAGTTGC	AGCTGCAACGATGTTGTCC	55
RM292	1	ACTGCTGTTGCGAAACGC	TGCAGCAAATCAAGCTGGAA	55
RM283	1	GTCTACATGTACCCTTGTTGGG	CGGCATGAGAGTCTGTGATG	55
RM5807	2	CTGCTGTTGCGTTGGAGTAC	TGCTCCGCCATGCCTAAC	55
RM525	2	GGCCCGTCCAAGAAATATTG	CGGTGAGACAGAATCCTTACG	55
OSR17	2	GCTGGTTGATTCAGCTAGTC	GCCTCGTTGTCGTTCCACAC	55
RM208	2	TCTGCAAGCCTTGTCTGATG	TAAGTCGATCATTGTGTGGACC	55
RM263	2	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG	55
OSR16	3	AAAACTAGCTTGCAAAGGGGA	TGCCGGCTGATCTTGTTCTC	55
RM1164	3	CGTTTCTCCGAGAAAAGTCG	CAAGGTGGTCGTTGAGGC	55
RM5903	3	TGTCTAGGTTCGTAGCATGTGTTCC	CCACTGAAAGCACCGTTAAATCC	55
RM1230	3	GGGTGGTGTGAGCTTTTCTC	TTCCACTTCGACAACCCTTC	55
RM130	3	TGTTGCTTGCCCTCACGCGAAG	GGTCGCGTGCTTGGTTTGGTTC	55
RM231	3	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG	55
RM22	3	GGTTTGGGAGCCCATAATCT	CTGGGCTTCTTTCACTCGTC	55
RM148	3	ATACAACATTAGGGATGAGGCTGG	TCCTTAAAGGTGGTGCAATGCGAG	55
RM143	3	GTCCCGAACCCTAGCCCGAGGG	AGAGGCCCTCCACATGGCGACC	65
RM241	4	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG	55
RM252	4	TTCGCTGACGTGATAGGTTG	ATGACTTGATCCCGAGAACG	55
RM5608	4	GTATCTTTGATCGCGCGC	ACTGGTAGAGAGCCCTGCTG	55
RM413	5	GGCGATTCTTGGATGAAGAG	TCCCCACCAATCTTGTCTTC	53
RM164	5	TCTTGCCCGTCACTGCAGATATCC	GCAGCCCTAATGCTACAATTCTTC	55
RM31	5	GATCACGATCCACTGGAGCT	AAGTCCATTACTCTCCTCCC	55
RM153	5	GCCTCGAGCATCATCATCAG	ATCAACCTGCACTTGCCTGG	55
RM5907	5	TGCTGTCTCCACTTCCCTTC	AAGGAGGCGTGCTTAACAGG	55
RM510	6	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC	55
RM276	6	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA	55
RM162	6	GCCAGCAAAACCAGGGATCCGG	CAAGGTCTTGTGCGGCTTGCGG	65
RM5463	6	ACCCTTGCAGACAACGTACC	ATATACCAGCAGCTGCATGC	55
RM2	7	ACGTGTCACCGCTTCCTC	ATGTCCGGGATCTCATCG	55
RM234	7	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	55
RM1253	7	CTGAACTTGCCTGAGAACTC	GACGACCTCTCCATGCTCG	55
RM10	7	TTGTCAAGAGGAGGCATCG	CAGAATGGGAAATGGGTCC	55
RM182	7	TGGGATGCAGAGTGCAGTTGGC	CGCAGGCACGGTGCCTTGTAAG	55
RM433	8	TGCGCTGAACTAAACACAGC	AGACAAACCTGGCCATTCAC	55
RM310	8	CCAAAACATTTAAAATATCATG	GCTTGTTGGTCATTACCATTC	55
RM447	8	CCCTTGTGCTGTCTCCTCTC	ACGGGCTTCTTCTCCTTCTC	55
RM44	8	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC	55
RM502	8	GCGATCGATGGCTACGAC	ACAACCCAACAAGAAGGACG	55
RM547	8	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATCCTCGTAGCG	55
RM201	9	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	55
RM219	9	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG	55
RM23654	9	CTCCGATGCCTTCTTCCTCTTGC	AAAGGGAGTAGCAAGCCGAGTGG	55
OSR28	9	AGCAGCTATAGCTTAGCTGG	ACTGCACATGAGCAGAGACA	55
RM311	10	TGGTAGTATAGGTACTAAACAT	TCCTATACACATACAAACATAC	55
RM222	10	CTTAAATGGGCCACATGCG	CAAAGCTTCCGGCCAAAAG	55
RM21	11	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	55
RM286	11	GGCTTCATCTTTGGCGAC	CCGGATTCACGAGATAAACTC	55
RM17	12	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	55
RM309	12	GTAGATCACGCACCTTTCTGG	AGAAGGCCTCCGGTGAAG	55

total volume made up of 0.1 µM each primer, 1 unit *Taq* DNA polymerase, 0.2 µM each dNTP, 10 mM Tris-HCl pH 7.2, 50 mM KCl, 1.5 mM $MgCl_2$, DMSO (50%), and 120 µg DNA. The reaction was amplified in DNA Engine Dyad™ thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) programmed for one cycle at 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 55-65 °C (in accordance with the primer) for 2 min, and an extension period at 72 °C for 7 min.

A 1.5 μL aliquot of PCR products was loaded onto 6% denaturing polyacrylamide gels and run in 0.5× TBE buffer at 1800 V for about 2 h. Gels were stained with silver nitrate to visualize DNA fragments according to Promega's protocol (Madison, Wisconsin, USA). Allele sizes were estimated based on Perfect DNA 50 bp (EMD Chemicals, Madison, Wisconsin, USA) and PCR marker 50-2000bp (EMD Chemicals) ladders.

Data analysis

PowerMarker program v.3.25 (Lui and Muse, 2005) was used to analyze the polymorphic alleles detected by the 54 simplified SSRs; this allowed the calculation of the following diversity parameters: allele number, major and minor frequency and standard deviation of alleles, genetic diversity (He), heterozygosity, polymorphism information content (PIC), and inbreeding coefficient of microsatellites used in rice.

The genetic similarity between pairs was estimated by Roger coefficient to transform values into genetic distance, which was defined as the square root of the one-complement of the similarity ($\sqrt{1-S}$). The distance matrix was analyzed by metric multidimensional scaling (MDS) as the grouping technique. A hierarchical clustering technique using the unweighted pair group method with arithmetic mean (UPGMA) method was used (Weir, 1990). Statistical analysis of data was performed with the InfoGen version 2013 FCA statistical program (Balzarini and Di Rienzo, 2013).

RESULTS AND DISCUSSION

Genetic diversity parameters of varieties

A total of 191 alleles, with a mean of 3.54 alleles per locus, were detected in the 16 commercial rice varieties that were analyzed by 54 polymorphic SSR primers. All 54 primers showed polymorphism between the 16 rice varieties and a large number of loci (30) detected 2 or 3 alleles per locus (Table 3). A higher number of alleles, with an average of 14.7 alleles per locus, were found in Brazilian varieties (Brondani et al., 2006).

The most polymorphic loci were RM547 (8 alleles), RM44 (7 alleles), and RM8068 (6 alleles) (Table 3, Figure 1). The genetic diversity mean was 0.50 for all the analyzed loci, while the highest genetic diversity was detected by SSR RM547 (0.84). The heterozygosity mean of the evaluated microsatellites was 0.002, which was expected for a self-pollinating species. The RM5807 and RM143 loci were the only ones that detected heterozygotes in the analyzed varieties even though they detected only three alleles in the genotypes. The mean PIC value was 0.44, which indicated the appropriate polymorphism content for the whole sample, the highest PICs were observed for the most informative SSRs (Table 3). A total of 72 unique alleles were detected in certain varieties, defining as unique alleles those that exhibited a frequency less than or equal to 0.07. Since polymorphic microsatellites markers show different banding pattern among varieties, those which show different bands could be used as identifiers for a specific variety.

This is the first molecular analysis of genetic material generated by the INIA's Rice Breeding Program (RBP) with a large number of microsatellites because the previous studies used only dominant markers (Hinrichsen et al., 1996; Aguirre et al., 2005).

Of the total 200 SSRs evaluated, 54 were able to genetically differentiate the 16 rice varieties. The selection of these SSRs was based on their reproducibility, multiallelism, and sharpness of their bands. In this analysis, only 27% of the amplified primers were able to efficiently identify genetic variants. It could be two reasons to explain this result; the first is a high degree of parentage of the varieties generated by the INIA's Rice Program. The number of detected alleles was generally lower than that reported in the literature. The set of microsatellites used in the study exhibited a lower mean of alleles (3.5) than the 4.4 alleles reported by Bonow et al. (2009) and even lower than the 11.7 reported by Rahman et al., 2010 on local Indian varieties. In a preliminary analysis of varieties included in this study, the use of 12 SSRs did not allow the genetic discrimination among the 16 varieties (data not shown). This result could confirm that genetic diversity is low among the analyzed commercial varieties. For example, 'Diamante-INIA' and 'Zafiro-INIA' showed the lowest genetic distance (0.27) although they come from two different genetic selection systems, that is, pedigree and recurrent selection, respectively. The RM547 and RM1164 primers were very important for the genetic differentiation of both varieties, which corroborates the usefulness of microsatellites in identifying varieties exhibiting a narrow genetic base (Zhu et al., 2012).

The second reason could be due to the low reproducibility of a large number of amplified primers, which would indicate that SSR transferability generated from these primers in different genotypes was not always effective even though the amplification reaction conditions were modified. Then, those primers were excluded from the analysis.

Polymorphism information content (PIC) is a good indicator of the polymorphism level of the genotypes under study; values near 1 indicate a high degree of genetic diversity and are associated with the high number of alleles, while values less than 0.5 indicate a low

Table 3. Allele number, major and minor frequency and standard deviations, genetic diversity, heterozygosity, unique alleles, polymorphism information content (PIC), and inbreeding coefficient determined by SSR in rice varieties.

SSR marker	Allele number	Major frequency	SD	Minor frequency	SD	Genetic diversity	Heterozigosity	Unique alleles	PIC	F
RM10	3	0.50	0.125	0.06	0.061	0.55	0	1	0.46	1
RM44	7	0.44	0.124	0.06	0.061	0.73	0	5	0.69	1
RM502	2	0.63	0.121	0.38	0.121	0.47	0	0	0.36	1
RM547	8	0.25	0.108	0.06	0.061	0.84	0	3	0.83	1
RM1164	3	0.56	0.124	0.06	0.061	0.54	0	1	0.45	1
RM5807	3	0.67	0.115	0.06	0.061	0.49	0.063	1	0.41	0.879
RM8068	6	0.63	0.121	0.06	0.061	0.56	0	3	0.53	1
RM510	3	0.50	0.125	0.06	0.061	0.55	0	1	0.46	1
RM286	5	0.38	0.121	0.13	0.083	0.76	0	0	0.72	1
RM525	5	0.50	0.125	0.06	0.061	0.66	0	2	0.62	1
OSR28	5	0.44	0.124	0.06	0.061	0.69	0	2	0.64	1
RM276	4	0.69	0.116	0.06	0.061	0.49	0	1	0.46	1
OSR17	2	0.88	0.083	0.13	0.083	0.22	0	0	0.19	1
OSR16	2	0.93	0.064	0.07	0.064	0.12	0	1	0.12	1
RM413	3	0.75	0.108	0.06	0.061	0.40	0	1	0.35	1
RM433	3	0.56	0.124	0.06	0.061	0.54	0	1	0.45	1
RM5903	5	0.53	0.129	0.07	0.064	0.63	0	3	0.58	1
RM1230	3	0.63	0.121	0.31	0.116	0.51	0	1	0.43	1
RM1253	3	0.53	0.129	0.07	0.064	0.55	0	1	0.46	1
RM129	3	0.81	0.098	0.06	0.061	0.32	0	1	0.29	1
RM130	3	0.88	0.083	0.06	0.061	0.23	0	2	0.21	1
RM162	5	0.31	0.116	0.06	0.061	0.76	0	1	0.72	1
RM164	3	0.50	0.125	0.19	0.098	0.62	0	0	0.54	1
RM201	3	0.50	0.125	0.06	0.061	0.55	0	1	0.46	1
RM208	3	0.56	0.124	0.06	0.061	0.54	0	1	0.45	1
RM21	3	0.88	0.083	0.06	0.061	0.23	0	2	0.21	1
RM222	3	0.88	0.083	0.06	0.061	0.23	0	2	0.21	1
RM231	4	0.56	0.124	0.06	0.061	0.58	0	2	0.51	1
RM234	2	0.56	0.124	0.44	0.124	0.49	0	0	0.37	1
RM2	3	0.75	0.108	0.06	0.061	0.40	0	1	0.35	1
RM17	2	0.56	0.124	0.44	0.124	0.49	0	0	0.37	1
RM22	4	0.40	0.127	0.07	0.064	0.68	0	1	0.62	1
RM24	5	0.69	0.116	0.06	0.061	0.50	0	3	0.47	1
RM148	2	0.75	0.108	0.25	0.108	0.38	0	0	0.30	1
RM31	3	0.56	0.124	0.06	0.061	0.54	0	1	0.45	1
RM311	5	0.38	0.121	0.06	0.061	0.74	0	1	0.70	1
RM315	3	0.81	0.098	0.06	0.061	0.32	0	1	0.29	1
RM143	3	0.47	0.121	0.06	0.061	0.56	0.063	1	0.46	0.894
RM182	3	0.63	0.121	0.06	0.061	0.51	0	1	0.43	1
RM153	3	0.87	0.088	0.07	0.064	0.24	0	2	0.23	1
RM241	5	0.38	0.121	0.06	0.061	0.72	0	2	0.67	1
RM243	3	0.56	0.124	0.06	0.061	0.54	0	1	0.45	1
RM219	4	0.75	0.108	0.06	0.061	0.41	0	2	0.39	1
RM252	3	0.5	0.125	0.06	0.061	0.55	0	1	0.46	1
RM263	3	0.88	0.083	0.06	0.061	0.23	0	2	0.21	1
RM310	5	0.69	0.116	0.06	0.061	0.50	0	3	0.47	1
RM292	2	0.88	0.083	0.13	0.083	0.22	0	0	0.19	1
RM309	2	0.75	0.108	0.25	0.108	0.38	0	0	0.30	1
RM447	3	0.81	0.098	0.06	0.061	0.32	0	1	0.29	1
RM5463	4	0.44	0.124	0.06	0.061	0.61	0	2	0.53	1
RM5608	3	0.63	0.121	0.06	0.061	0.51	0	1	0.43	1
RM283	4	0.63	0.121	0.06	0.061	0.55	0	1	0.51	1
RM23654	3	0.50	0.125	0.06	0.061	0.55	0	1	0.46	1
RM5907	5	0.53	0.129	0.07	0.064	0.63	0	3	0.58	1
Mean	3.54	0.61	0.114	0.10	0.069	0.50	0.002	1.3	0.44	0.996

polymorphism level. Results showed a mean PIC of 0.5, which indicates that the materials evaluated were quite similar. Another rice study showed a higher PIC value (0.86) with 7 SSRs on Indian rice varieties (Rahman et al., 2010) and 0.73 using 12 SSR on 192 Brazilian rice varieties (Brondani et al., 2006). It is known that genetic material from breeding programs is more uniform because of high selection pressure and selection method. In extreme cases, the repeated use of parents has contributed even more to decreasing the diversity of genetic material. Given this situation, it is important to introduce germplasm from other regions to increase the genetic diversity of the rice breeding program.

Genetic distance of rice varieties

The Roger coefficient was used to determine genetic distance among varieties based on the alleles detected by the 54 SSRs. Genetic distance between pairs of genotypes ranged from 0.27 to 0.97% (Table 4) with a 0.76 mean value.

The whole mean genetic distance for the Chilean varieties was 0.64 (Table 4). 'Diamante-INIA' compared with 'Zafiro-INIA' exhibited the nearest genetic distance

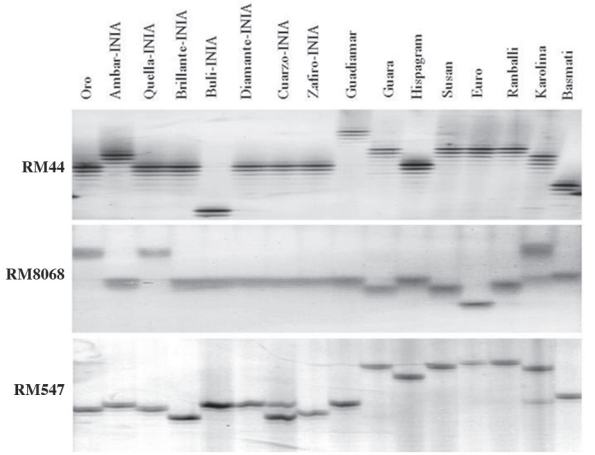

Figure 1. Banding pattern detected in 16 rice varieties with RM44, RM8068, and RM547.

(0.27), while 'Buli-INIA' compared with 'Ámbar-INIA' showed the highest genetic distance (0.81). The comparison between mostly sown varieties in Chile ('Zafiro-INIA', 'Brillante-INIA', and 'Diamante-INIA') showed a genetic distance between 0.60 and 0.27. Mean genetic distance was 0.72 for the foreign *japonica* varieties, and the lowest genetic distance was 0.56 between 'Guara' and 'Ranballi' varieties (Table 4).

'Basmati' exhibited the highest genetic distance with all the *japonica* varieties because it is an *indica* genotype. Its mean genetic distance with japonica varieties was 0.87. In general, no two genetically identical accessions were found in the 16 varieties analyzed with the 54 SSRs.

Clustering and identification of varieties based on genetic distances

The 16 analyzed varieties were separated into two major clusters, 15 *japonica* varieties and one *indica* genotype (Basmati) rice with a general mean of 0.76. This fact, confirms that there are two large groups in *O. sativa*, *indica*, and *japonica*. The *indica* subspecies has a different agroclimatic domestication center than *japonica* subspecies (Huang et al., 2012). Also, this confirms that microsatellites are potentially effective when differentiating *japonica* and *indica* varieties (Zhu et al., 2012).

The general average of the mean genetic distance of all *japonica* varieties compared with the *indica* genotype Basmati was 0.87 using Roger coefficient and ranged from 0.82 ('Basmati'-'Karolina') to 0.97 ('Basmati'-'Oro'). No identical genotypes were found within the 16 varieties (Figure 2, Table 4). A similar general mean genetic distance (0.77) was obtained in 192 traditional rice varieties from Brazil (Brondani et al., 2006). But, if only Chilean varieties are analyzed, a mean genetic distance of 0.64 was observed. This comparison showed a lower diversity for the Chilean varieties.

Cluster analysis by the UPGMA method led to a dendrogram with a cophenetic correlation index of 0.93, which indicates that dendrogram distances reflect a real situation of the sample of varieties studied (Figure 2). This dendrogram showed two main subgroups within the

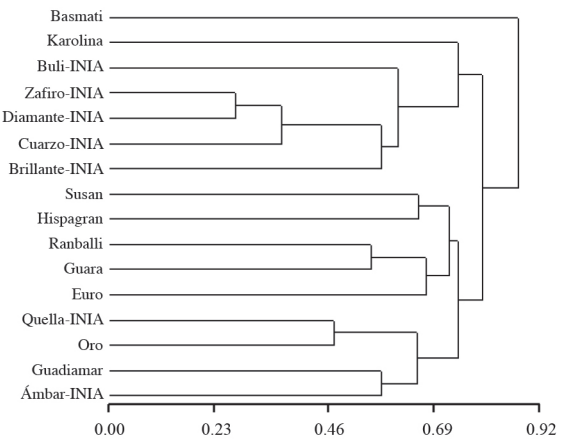

Figure 2. Dendrogram of 16 rice varieties based on SSR analysis (Roger coefficient).

Table 4. Genetic distances among 16 *Oryza sativa* varieties detected by SSR marker analysis (Roger coefficient).

Cultivar	Ámbar-INIA	Basmati	Brillante-INIA	Buli-INIA	Cuarzo-INIA	Diamante-INIA	Euro	Guadiamar	Guara	Hispagran	Karolina	Oro	Quella-INIA	Ranballi	Susan	Zafiro-INIA
Ámbar-INIA	0.00															
Basmati	0.87	0.00														
Brillante-INIA	0.80	0.86	0.00													
Buli-INIA	0.81	0.83	0.62	0.00												
Cuarzo-INIA	0.76	0.87	0.58	0.60	0.00											
Diamante-INIA	0.76	0.86	0.6	0.58	0.37	0.00										
Euro	0.75	0.85	0.81	0.78	0.78	0.76	0.00									
Guadiamar	0.58	0.90	0.76	0.82	0.76	0.77	0.77	0.00								
Guara	0.71	0.85	0.80	0.79	0.80	0.81	0.68	0.64	0.00							
Hispagran	0.75	0.86	0.86	0.87	0.84	0.85	0.73	0.68	0.70	0.00						
Karolina	0.82	0.82	0.74	0.77	0.73	0.72	0.79	0.81	0.78	0.80	0.00					
Oro	0.66	0.97	0.76	0.74	0.77	0.80	0.84	0.67	0.75	0.75	0.82	0.00				
Quella-INIA	0.68	0.95	0.75	0.71	0.74	0.75	0.83	0.66	0.75	0.75	0.80	0.48	0.00			
Ranballi	0.66	0.87	0.83	0.77	0.79	0.78	0.66	0.64	0.56	0.74	0.78	0.74	0.75	0.00		
Susan	0.76	0.85	0.88	0.92	0.88	0.89	0.78	0.69	0.69	0.66	0.82	0.80	0.81	0.75	0.00	
Zafiro-INIA	0.77	0.85	0.60	0.58	0.41	0.27	0.75	0.78	0.81	0.85	0.71	0.81	0.75	0.78	0.89	0.00

japonica varieties. At the bottom of the *japonica* varieties were located those that have short/wide grain type such as 'Quella-INIA', 'Oro', 'Ámbar-INIA' and most of the European variety with medium small grain such as 'Guadiamar', 'Euro', 'Guara', 'Ranballi', 'Hispagran', and 'Susan'. On the contrary, in the other subgroup were included most INIA commercial long-wide seeded type varieties, such as 'Buli-INIA', 'Zafiro-INIA', 'Diamante-INIA', 'Cuarzo-INIA', and 'Brillante-INIA', as well as the Hungarian medium seed size variety 'Karolina'.

In order to visualize genetic relationships among varieties, a metric multidimensional scaling (MDS) analysis was done by using the Roger coefficient (Figure 3). The two-dimensional spatial location of varieties for this molecular marker, explains 39.3% of total variability.

The principal coordinate analysis, specifically, PC1 and PC2, explained 23.1% and 16.2% of the total variability, respectively, for the 54 SSR. PC1 showed the separation between commercial *japonica* varieties. The group on the right consisted of Chilean commercial varieties, defined as long-wide grain varieties (Figure 3). Most of these varieties were generated by INIA Rice breeding program. On the other hand, 'Quella-INIA', 'Oro' and 'Ámbar-INIA' were connecting the first group to the varieties on the left, which exhibit a different molecular profile. The latest varieties corresponded to European genotypes and they were evenly distributed along the PC2. Far away from these two varieties groups was the *indica* genotype 'Basmati'.

Clustering of varieties was related to the type of grain (Figures 2 and 3). In general, short-grain varieties were observed in the lower branch: 'Quella-INIA', 'Oro', 'Guadiamar', 'Ámbar-INIA', 'Susan', 'Hispagran', 'Ranballi', and 'Guara', with length/width ratio values between 1.8 and 2.2 (Table 1). For instance, 'Oro' corresponds to the first short/wide grain variety introduced and used in Chile since 1960 up to day. It was initially used as parent in the Rice Breeding Program and it is one of the parents of the no longer used 'Quella-INIA' short-grain variety created in 1979. This explains the proximity of these varieties in both genetic distance analyses.

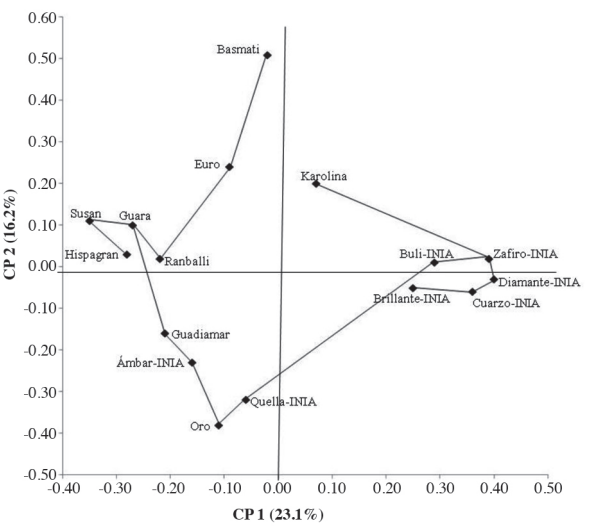

Figure 3. Metric multidimensional scaling (MDS) analysis grouping on 16 rice varieties analyzed using 54 SSR markers (Roger coefficient).

On the other hand, varieties with long/wide grain or higher length/width ratio (2.8 to 3.3) (Table 1) are located in the upper branch: 'Zafiro-INIA', 'Diamante-INIA', 'Cuarzo-INIA', and 'Brillante-INIA', and that correspond to the type of grain mostly consumed in Chile. Long/narrow grain 'Buli-INIA' variety is grouped with varieties with higher length/width ratio. For this group, all current INIA commercial varieties included 'Diamante-INIA' in their genetic background; this explains why they were clustered together. The long/narrow grain 'Basmati' was also located in the upper branch of the dendrogram (Table 1).

These results agreed with those found by Kumari et al. (2011), where microsatellites were able to differentiate medium/narrow grain Indian varieties and domestic and foreign rice varieties in Taiwan (Chuang et al., 2011). It has already been reported that some SSRs are associated with regions of DNA that determine the grain type (Huang et al., 2013).

The need to use 54 SSRs to discriminate among 16 varieties, mainly of the *japonica* type indicated a high degree of parentage, especially among Chilean varieties. This is unlike other studies where few sets of SSRs primers were used to discriminate a set hybrid (He et al., 2012) and varieties (Brondani et al., 2006; Rahman et al., 2010). On the other hand, the DNA profiles obtained with the 54 SSRs will be considered as a genetic fingerprint of the commercial rice varieties to complement agro-morphological information.

CONCLUSIONS

The polymorphism level detected by simple sequences repeats (SSR) is generally medium to low among INIA commercial rice *Oryza sativa* varieties. This is supported by the diversity parameters, such as the number of alleles (3.50) and polymorphism index content (PIC, 0.50). The highest PIC was positively associated with the highest number of alleles detected by SSRs. Given this situation, it is important to continuously introduce germplasm from other regions to increase the Rice Breeding Program's genetic base.

Genetic clustering of varieties using SSR with Roger coefficient determined the separation of *japonica* and *indica* rice. This clustering was also related to the type of grain; thus, varieties were clustered according to grain length/width: short/wide, long/wide, and long/narrow.

A set of SSRs was defined to allow the identification and certification of *O. sativa* varieties, including INIA and foreign varieties.

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