Comparison of microsatellites and isozymes in genetic diversity studies of *Oryza glumaepatula* (Poaceae) populations

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Abstract: The study of the genetic structure of wild plant populations is essential for their management and conservation. Several DNA markers have been used in such studies, as well as isozyme markers. In order to provide a better comprehension of the results obtained and a comparison between markers which will help choose tools for future studies in natural populations of *Oryza glumaepatula,* a predominantly autogamous species, this study used both isozymes and microsatellites to assess the genetic diversity and genetic structure of 13 populations, pointing to similarities and divergences of each marker, and evaluating the relative importance of the results for studies of population genetics and conservation. A bulk sample for each population was obtained, by sampling two to three seeds of each plant, up to a set of 50 seeds. Amplified products of eight SSR loci were electrophoresed on non-denaturing polyacrylamide gels, and the fragments were visualized using silver staining procedure. Isozyme analyses were conducted in polyacrylamide gels, under a discontinuous system, using six enzymatic loci. SSR loci showed higher mean levels of genetic diversity (A=2.83, p=0.71, A_P=3.17, H_o=0.081, H_e=0.351) than isozyme loci (A=1.20, p=0.20, A_P=1.38, H_o=0.006, H_e=0.056). Interpopulation genetic differentiation detected by SSR loci (R_{ST}=0.631, equivalent to F_{ST}=0.533) was lower than that obtained with isozymes (F_{ST}=0.772). However, both markers showed high deviation from Hardy-Weinberg expectations (F_{IS}=0.744 and 0.899, respectively for SSR and isozymes). The mean apparent outcrossing rate for SSR ($\bar{t}_a$=0.14) was higher than that obtained using isozymes ($\bar{t}_a$=0.043), although both markers detected lower levels of outcrossing in Amazonia compared to the Pantanal. The migrant number estimation was also higher for SSR (Nm=0.219) than isozymes (Nm=0.074), although a small number for both markers was expected due to the mode of reproduction of this species, defined as mixed with predominance of self fertilization. No correlation was obtained between genetic and geographic distances with SSR, but a positive correlation was found between genetic and geographic distances with isozymes. We conclude that these markers are divergent in detecting genetic diversity parameters in *O. glumaepatula* and that microsatellites are powerful for detecting information at the intra-population level, while isozymes are more powerful for inter-population diversity, since clustering of populations agreed with the expectations based on the geographic distribution of the populations using this marker. Rev. Biol. Trop. 60 (4): 1463-1478. Epub 2012 December 01.

Key words: genetic structure, gene flow, isozymes, mating system, SSR.

Oryza glumaepatula Steud. (Poaceae) is one of the four wild rice species originated in America, the others being *O. latifolia*, *O. grandiglumis* and *O. alta* (Morishima 1994), and occurs widely in Latin America from 23°N in Cuba to 23°S in Brazil (Vaughan *et al.* 2003). It is the only diploid wild American species and its importance relies on its use in crosses with

O. sativa in plant breeding programs (Brondani *et al.* 2001, Yoshimura *et al.* 2010) aiming at introgression of important traits from the wild species and amplifying the genetic basis of the cultivated crop.

The Brazilian *O. glumaepatula* populations are found in the extensive river basins of the Amazon and Pantanal Matogrossense and the smaller river basins which occur in the states of Goiás and Tocantins (Oliveira 1994, Brondani *et al.* 2005). This species presents annual, bi-annual or perennial populations, depending on its geographical location (Oliveira 1993, Akimoto *et al.* 1998), growing along the riverbeds and margins, presenting behavior typical of weeds or colonizing plants. As limited portions of their culms rot and their bodies are released to float on the surface, they are dispersed on the rivers by the force of wind and water, mostly downstream (Akimoto *et al.* 1998), but sometimes upstream (Black 1950), founding new populations or clustering to those already existing.

The *Oryza* genus presents different reproductive systems such as outcrossing, inbreeding, intermediate crossing rates and vegetative reproduction. Several studies have indicated that *O. glumaepatula* is a self-fertilizing species (Akimoto *et al.* 1998, Buso *et al.* 1998, Ge *et al.* 1999), but recently studies have reported that this species has different outcrossing rates in different populations, with values ranging from 9.3% to 30% (Brondani *et al.* 2005, Karasawa *et al.* 2007a,b, Vaz *et al.* 2009). The breeding system was classified as mixed with predominance of inbreeding by Karasawa *et al.* (2007b).

The knowledge of genetic diversity and genetic structure of populations is essential for understanding species evolution, the genetics of natural populations, as well as the adoption of collecting strategies aiming at *in situ* or *ex situ* management, restoration and conservation practices (Slatkin 1987, Vigouroux *et al.* 2008). Several molecular techniques are available for the detection of genetic variability in natural populations (Agarwal *et al.* 2008). Among the different molecular markers, the first to be established were the isozymes in the 60s (Lewontin & Hubby 1966). Isozyme markers have been widely used in different genetic studies in the genus *Oryza*, including studies of population genetics aiming at characterizing the diversity and genetic structure (Glaszmann 1988, Barbier 1989, Morishima & Barbier 1990, Akimoto *et al.* 1998, Gao *et al.* 2000, Gao *et al.* 2002a, Veasey *et al.* 2008). This marker is relatively simple and cheap, and presents a codominant nature and known genetic control. However, its use is limited due to the low number of loci and alleles per locus detected, to post-translational modifications, tissue-specific forms, modifications in response to environmental conditions and to the developmental stage of the individual (Murphy *et al.* 1990).

With the advent of PCR in the late 1980s, which made the analysis and genotyping straightforward, microsatellites, characterized by high heterozygosity and the presence of multiple alleles, became the marker of choice in genome mapping and also in population genetics studies and related areas (Ellegren 2004). Microsatellites or simple sequence repeats (SSRs) consist of repeated tandem sequences with motifs of one to four base pairs. The advantages of this marker are the codominant nature, high frequency and random distribution in genomes and the high polymorphism it usually shows (Schlötterer 2004). Microsatellites have been used in several conservation and genetic diversity studies in wild rice species (Gao *et al.* 2002b, Zhou *et al.* 2003, Gao 2004, Gao 2005, Gao *et al.* 2006, Xu *et al.* 2006, Wang *et al.* 2008), and specifically in studies of *Oryza glumaepatula* natural populations, including the genetic structure and diversity (Brondani *et al.* 2005, Karasawa *et al.* 2007a, Silva *et al.* 2007, Vaz *et al.* 2009), the mating system determination (Karasawa *et al.* 2007b, Vaz *et al.* 2009), genetic mapping (Brondani *et al.* 2001) and phylogeny (Bautista *et al.* 2001).

Studies comparing genetic structure of natural populations with these two markers (isozymes and microsatellites) have been conducted for quite a few species, such as *Sorghum bicolor* (Djé *et al.* 1999), *Elymus caninus*

(Sun *et al.* 2001), oaks (*Quercus* spp.) (Curtu *et al*. 2007), and *Euterpe edulis* (Conte *et al.* 2008). But the only report to-date comparing isozymes and microsatellite markers in the genus *Oryza* was conducted for *O. rufipogon*, a predominantly cross-pollinated species, studying genetic structure and genetic diversity parameters of natural populations (Gao *et al.* 2002b). Therefore, in order to provide a better comprehension of the results obtained and a comparison between codominant markers which will help choose tools for future studies in natural populations of *O. glumaepatula*, this study used both isozyme and microsatellite markers to assess (1) the level and distribution of genetic diversity; (2) the distribution of this diversity within and among populations, pointing to similarities and divergences; and (3) evaluating the relative importance of the results for studies of population genetics and conservation, considering the same set of populations and individuals of *O. glumaepatula*.

MATERIALS AND METHODS

Populations studied and sampling: Thirteen *O. glumaepatula* populations were assessed in this study, belonging to the wild rice collection in the Genetics Department of the Luiz de Queiroz College of Agriculture, University of São Paulo. The populations were selected from three regions in Brazil: (I) Amazon region, in Amazonas and Roraima States, with eight populations originating from the Purus, Solimões, Japurá, Tapajós, Negro and Branco River basins; (II) Xingu region, in Goiás State, which also belongs to the Amazon region but will be referred to in this study as the Xingu region, with one population collected at the Xingu River basin, located far from the other Amazonian populations; (III) Pantanal region, in Mato Grosso do Sul State, with four populations from the Paraguay River basin and one from Taquari River basin, which belong to the Pantanal ecosystem (Table 1, Fig. 1). These populations were sampled during two expeditions to the Rio Negro basin in 1992 and of the Rio Solimões basin in 1993 (Ando 1994), except for the Xingu population included in the collection more recently. During field collection, populations were sampled on an individual plant basis (Ando 1994). Since then these population samples have been maintained in refrigerators at -4ºC, inside plastic boxes with

TABLE 1
Oryza glumaepatula populations assessed in this study and details of their origins

Population	Individuals per population (Mean)[1]	Hydrographic basin	River	Lake	Geographic location
XI-1	24 Isoz; 27.4 SSR	Xingu	Xingu	Piulaga	12º14' S - 53º35' W
PG-1	30 Isoz; 35.0 SSR	Paraguay	Paraguay	Pantanal	19º01' S - 57º30' W
PG-2	21 Isoz; 16.6 SSR	Paraguay	Paraguay	Pantanal	19º00' S - 57º41' W
PG-3	27 Isoz; 10.3 SSR	Paraguay	Corumbá	-	18º59' S - 57º37' W
PG-4	22 Isoz; 29.9 SSR	Paraguay	Taquari	-	19º15' S - 57º13' W
JA-4	19 Isoz; 35.4 SSR	Japurá	Japurá	Cuiucuiú	02º02' S - 65º07' W
SO-6	25 Isoz; 16.7 SSR	Solimões	Solimões	Manacapuru	03º11' S - 60º47' W
SO-17	30 Isoz; 16.1 SSR	Solimões	-	Coari	04°02' S - 63°15' W
SO-21	23 Isoz; 12.0 SSR	Solimões	-	Mamiá	04º15' S - 63º03' W
PU-1	21 Isoz; 29.7 SSR	Purus	Purus	-	03º49' S - 61º25' W
NE-18	24 Isoz; 29.1 SSR	Branco	Branco	-	01º53' S - 61º22' W
NE-26	23 Isoz; 21.6 SSR	Negro	Carabinani	-	01º54' S - 61º23' W
TA-1	18 Isoz; 32.4 SSR	Tapajós	Tapajós	-	02º26' S - 54º42' W

1. Isoz: isozyme markers; SSR: microsatellite markers.

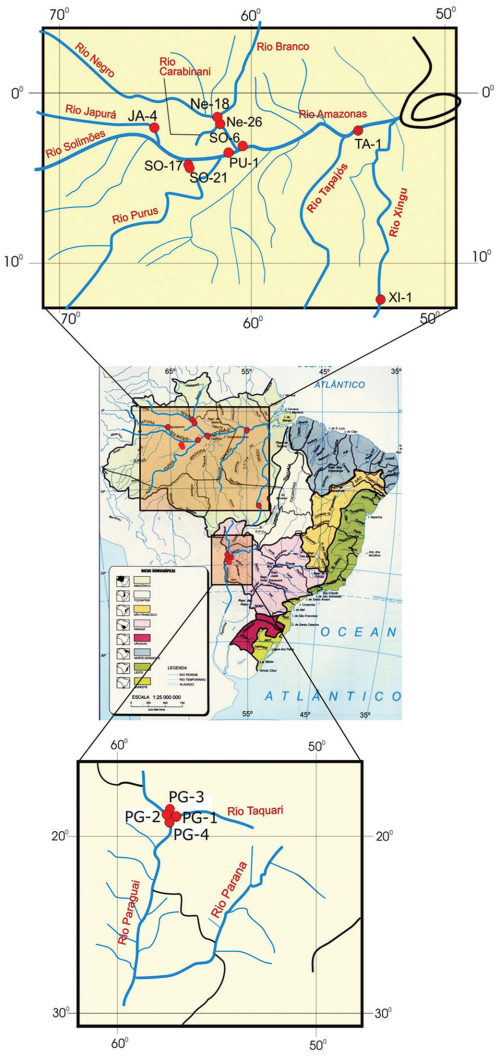

Fig. 1. Brazil showing the localization of the 13 *Oryza glumaepatula* populations, described in table 1.

silica to avoid humidity, and have not been previously multiplied.

Each population sample in the collection is maintained as a set of maternal progenies, each progeny having been collected from an open-pollinated panicle of an individual plant in the field. For the analysis in this study, a bulk sample was obtained from each population, by sampling two to three seeds of each individual plant, up to a set of 50 seeds. These seeds were germinated in square plastic Gerboxes (11x11cm) with damp tissue paper at a temperature of 27 ± 5ºC in the dark. Germinated seeds were transplanted to pots in the greenhouse where they were grown up to adult stage. Each population was composed, on average, of 30 individuals.

DNA extraction and amplification of SSR loci: Total genomic DNA was extracted from adult lyophilized leaves of individual plants using the CTAB method according to Hoisington *et al.* (1994), modified by Karasawa *et al.* (2007a). DNA was quantified on 4% (w/v) polyacrylamide gels and bands were revealed using the silver nitrate staining procedure (Bassam *et al.* 1991). Eight SSR loci (OG-22, OG-26, OG-27, OG-29, OG-36, OG-39, OG-42 e OG-63), developed by Brondani *et al.* (2001), were used in this study (Table 2).

For each PCR reaction, 30ng of genomic DNA from individual plants were used in a 12µL volume containing 0.3µM of each primer, 0.25mM of each dNTP, 1.5mM of $MgCl_2$, 10mM Tris-HCl, and 0.6 unit of Taq DNA polymerase enzyme (Invitrogen, Eugene, Oregon, USA). The reactions were performed in a Primus 96 Thermocycler with 4min initial denaturation at 94ºC, 30 subsequent cycles (1 min at 94ºC, 1 min at 54ºC, 56ºC or 60ºC, 1 min elongation at 72ºC), followed by a final elongation of 5min at 72ºC. Amplified products were electrophoresed on 6% non-denaturing polyacrylamide gels (4cm/V, for three hours). Amplified fragments were visualized using silver staining procedure (Bassam *et al.* 1991).

Isozyme analyses procedures: Isozyme analyses were conducted in polyacrylamide gels, under a discontinuous system. The gel and electrode buffers used were a basic buffer (Hames 1996), with pH 8.8 for the 5.5% resolving gel and pH 6.8 for the 3.5% stacking gel.

The newest expanded leaves (200mg) were used for enzyme extraction of each plant. The leaves were ground in liquid nitrogen in microcentrifuge tubes using a power homogenizer, adding 1mL of extraction buffer 1 (Alfenas

TABLE 2
Primers developed for *Oryza glumaepatula* (Brondani *et al.* 2001) used in this study

Loci	Primer sequences	Chrom.	bp	T_a(ºC)	*A*	H_o	H_e
OG 22	(F) GCCATCCATTCTTACCAG (R) CACAGGTGTGGTGCTCA	12	165-265	56	21	0.101	0.804
OG 26	(F) CATGGTGCCGATTACGGT (R) CATCTCCATCGCGGTCAT	10	95-120	60	5	0.025	0.751
OG 27	(F) TCGGACGTGGCATATGA (R) CTGTTCCGAGCGAGAGT	9	120-210	54	16	0.083	0.882
OG 29	(F) GACCAGTTCACCATGCAG (R) GAGTGAGGCAGCAAGACA	1	80-125	60	10	0.121	0.814
OG 36	(F) AACGTTCATCGGTTCTGG (R) TGCTTGCCAGGTTATTCC	4	160-190	56	8	0,106	0.793
OG 39	(F) GCGTACTAGGCCATGATA (R) TCCACGTAAGAACACTCG	3	250-280	56	6	0.042	0.742
OG 42	(F) TGCAGGCTCTGAGCTAC (R) AGAACAGATCTTGCCGTC	5	420-440	56	6	0.043	0.536
OG 63	(F) CAGGGGACAAGCACATA (R) TAGACGATGTCGAGAAGG	2	100-150	56	9	0.155	0.766
Mean				-	10.12	0.085	0.761

Chrom.: chromosome location; pb: fragment size; $T_{a:}$ annealing temperature; *A* - number of alleles per locus; H_o - observed heterozygosity; H_e - gene diversity.

et al. 1991), leaving out diethyldithiocarbamic acid (DIECA) and 2-mercaptoethanol. The extract was centrifuged at 18 000g for 20min at 4ºC. Afterwards, 130µL of the supernatant were diluted in 150µL of a solution containing Tris-HCl pH 6.8 and Coomassie blue, the latter component indicating the protein migration in the gel. This amount of extract was sufficient for four gels, which allowed the assessment of 38 individuals each. The same control plant was added to each gel as a marker. The voltage was set at 50V during three hours, adjusted to 100V for the next 13 hours, usually staying overnight at 4ºC.

From a total of 10 promising enzymatic systems (Veasey *et al.* 2008), four were selected in this study due to the presence of higher band resolution: aspartate aminotransferase (AAT; E.C. 2.6.1.1, with three loci), phosphoglucomutase (PGM; E.C. 2.7.5.1), shikimate dehydrogenase (SKD; E.C. 1.1.1.25) and glutamate dehydrogenase (GDH; E.C. 1.4.1.2).

For the statistical analysis, the GDA program (Lewis & Zaykin 2000) was used to estimate allelic and genotypic frequencies, number of alleles per locus (*A*), observed heterozygosities (H_o), gene diversity (H_e), and fixation indices (*f*). Genotypic frequencies obtained from both markers were submitted to Fisher's exact test considering the Hardy-Weinberg equilibrium, as defined by Weir (1996), using the TFPGA software (Miller 1997).

Wright's F statistics were estimated considering a random model, defined according to Weir (1996), where the sampled populations were considered representative of the species and with a common evolutionary history. These estimates (F_{IS}, F_{ST} e F_{IT}) were obtained using the software F_{STAT} (Goudet 1995). Confidence intervals (95%) were also obtained for each of these estimates. Private alleles were also identified with the GDA program (Lewis & Zaykin 2000).

Considering that the mutation process in microsatellite loci is not in line with the expectations under the infinite alleles model with low rates, the analogue of F_{ST} parameter (Slatkin 1995) developed specifically for

microsatellite data (R_{ST}) was also estimated. Parameters R_{ST} and gene flow (Nm) were estimated using the R_{ST}Cal program (Goodman 1997). Dendrograms were constructed from Nei's genetic distances matrix and the UPGMA clustering criteria, using the TFPGA software (Miller 1997).

Patterns of spatial variation were analyzed using Pearson's coefficient of correlation (r) between Nei's genetic distances matrix (Nei 1978) and the matrix of geographic distances (shortest distance between two given points on the map) between populations, using NTSYS-pc (Rohlf 1992). Significance of these correlations was tested by Mantel's statistic Z (Mantel 1967), using 1000 random permutations. Average apparent outcrossing rate was estimated considering the relation $\bar{t}_a = (1\text{-}F_{IS})/(1+F_{IS})$. The parameter $\bar{t}_a$was also estimated for each population [$\bar{t}_a = (1\text{-}f)/(1+f)$] (Weir 1996)

RESULTS

Genetic diversity levels: All SSR loci showed polymorphism (Table 2) while isozymes were monomorphic for two of the six loci, *Aat3* and *Gdh* (Table 3). A total of 81 alleles were found for the SSR markers, varying from five to 21 alleles per locus (Table 2), while 11 alleles were found for the isozyme markers, varying from one to three alleles per locus (Table 3). For both markers, the mean expected heterozygosity or gene diversity was higher than the mean observed heterozygosity (H_o=0.085 and H_e=0.761 for SSR; H_o=0.007 and H_e=0.247 for isozymes) (Tables 2 and 3).

TABLE 3
Isozyme loci used in this study with their respective number of alleles per locus (A), observed heterozygosity (H_o) and gene diversity (H_e)

Loci	A	H_o	H_e
Aat1	2	0.012	0.481
Aat2	2	0.007	0.393
Aat3	1	0.000	0.000
Skdh	2	0.007	0.283
Pgm	3	0.012	0.326
Gdh	1	0.000	0.000
Mean	1.83	0.007	0.247

Considering each population, JA-4 was monomorphic for all eight SSR loci, while populations PG-3, SO-6, PU-1 and NE-26 were monomorphic for the six isozyme loci. The average polymorphism rate was 20% for isozymes and 71% for microsatellites (Table 4). The average number of alleles per locus per population ($\bar{A}$) was 1.20 and 2.83, with means over loci of 1.83 and 10.12, respectively, and the average number of alleles per polymorphic locus ($\bar{A}_p$) of 1.83 and 3.17, respectively, for isozymes and microsatellites (Table 4).

No private alleles were detected for the isozyme markers, probably due to the small number of alleles and small number of enzyme loci, whereas the SSR markers were effective in detecting private alleles (Fig. 2). There was only one private allele detected in the Xingu region, 19 in the Amazon and eight in the Pantanal region (Fig. 2B). At the population level, the number of private alleles detected with SSR varied from 0 to 8 (Fig. 2A).

The average observed heterozygosity (H_o) and gene diversity (H_e) assessed with isozymes and SSR markers for the 13 populations were 0.006 and 0.056, and 0.081 and 0.351, respectively (Table 4). On the other hand, when considering H_o and H_e as the mean over loci, we detected H_o=0.007 and 0.085, and H_e=0.247 and 0.766 for isozymes and SSR, respectively. The tendency for the microsatellites to detect higher H_o and H_e levels, within each of the three regions, was maintained (Table 5). The apparent outcrossing rate (t_a) estimated by these markers showed surprising differences at the populational (Table 4) and regional (Table 5) levels, but both markers showed lower levels in the Amazon region (1.9% and 2.8%), while the highest levels occurred in the Pantanal region, with 5.2% and 10.5%, respectively, for isozymes and microsatellites (Table 5). However, the markers were not in agreement in the case of the Xingu region, represented by the XI-1 population, which registered absence of outcrossing when assessed with isozymes but showed outcrossing rates of 54.1% when

TABLE 4
Genetic diversity parameters for 13 *O. glumaepatula* populations using isozymes (Isoz) and SSR markers

Populations	N		$\overline{A}$		*P (%)*		$\overline{A}_p$		H_o		H_e		*f*		$\overline{t}_a$	
	Isoz	SSR	Isoz	SSR	Isoz	SSR	Isoz	SSR	Isoz	SSR	Isoz	SSR	Isoz	SSR	Isoz	SSR
XI-1	24.00	27.38	1.18	2.13	17.0	87.0	2.00	2.29	0.000	0.282	0.085	0.397	1.000	0.298	0.000	0.541
PG-1	35.00	30.00	1.50	3.87	50.0	100.0	2.00	3.87	0.024	0.100	0.164	0.569	0.858	0.827	0.076	0.095
PG-2	21.00	16.63	1.33	4.00	33.0	87.0	2.00	4.43	0.007	0.127	0.059	0.562	0.877	0.780	0.066	0.126
PG-3	27.00	10.33	1.00	2.67	0.0	50.0	-	4.33	0.000	0.057	0.000	0.234	-	0.767	-	0.132
PG-4	22.00	29.88	1.40	4.38	40.0	100.0	2.00	4.37	0.018	0.176	0.066	0.585	0.727	0.703	0.158	0.174
JA-4	19.00	35.38	1.33	1.00	33.0	0.0	2.00	-	0.000	0.000	0.071	0.000	1.000	-	0.000	-
SO-6	25.00	16.75	1.00	2.50	0.0	75.0	-	3.00	0.000	0.136	0.000	0.364	-	0.634	-	0.224
SO-17	30.00	16.14	1.17	2.00	17.0	57.0	2.00	2.75	0.000	0.000	0.071	0.277	1.000	1.000	0.000	0.000
SO-21	23.00	12.00	1.17	2.17	17.0	67.0	2.00	2.75	0.000	0.000	0.014	0.225	1.000	1.000	0.000	0.000
PU-1	21.00	29.75	1.00	4.00	0.0	87.0	-	4.43	0.000	0.071	0.000	0.328	-	0.787	-	0.119
NE-18	24.00	29.13	1.33	3.13	33.0	100.0	2.00	3.13	0.021	0.073	0.132	0.526	0.845	0.863	0.084	0.074
NE-26	23.00	21.63	1.00	1.13	0.0	13.0	-	2.00	0.000	0.011	0.000	0.030	0.000	0.628	-	0.229
TA-1	18.50	32.38	1.17	3.87	17.0	100.0	2.00	3.87	0.007	0.018	0.060	0.464	0.887	0.961	0.000	0.020
Mean over population	24.04	23.64	1.20	2.83	20.0	71.0	1.38	3.17	0.006	0.081	0.056	0.351	0.910	0.771	0.043	0.145
Mean over loci			1.83	10.12					0.007	0.085	0.247	0.761				

N - Mean number of plants analyzed; $\overline{A}$ - mean number of alleles per locus; *P* - proportion of polymorphic loci; $\overline{A}_p$ - mean number of alleles per polymorphic locus; H_o - observed heterozygosity; H_e - gene diversity; *f* - intrapopulation fixation index; and $\overline{t}_a$ - apparent outcrossing rate.

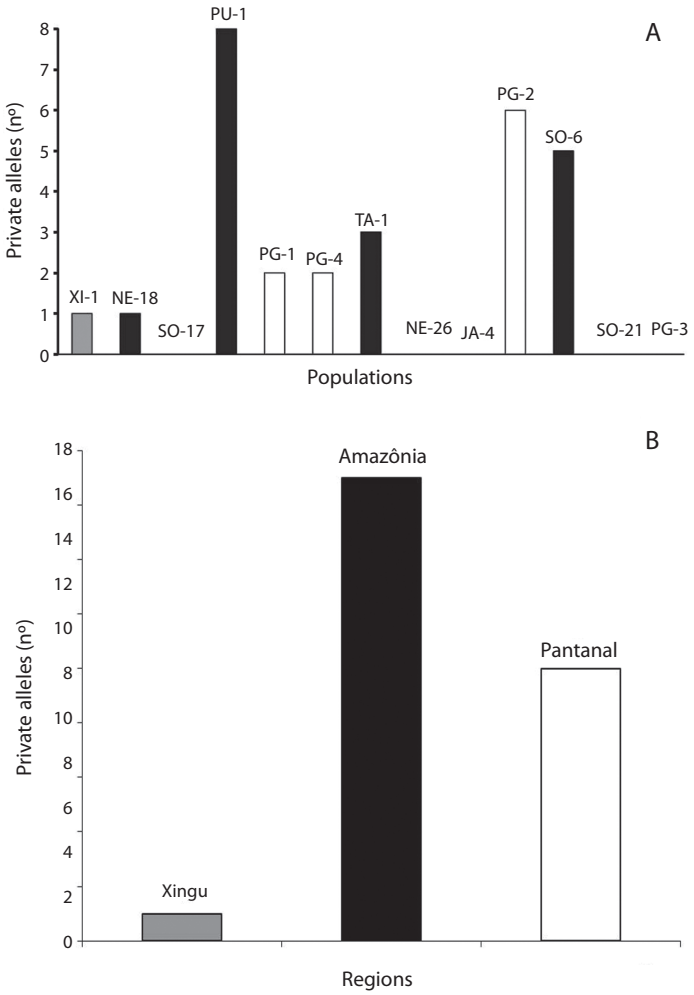

Fig. 2. Number of private alleles for 13 *Oryza glumaepatula* populations (A) and three regions (B) assessed with microsatellite markers.

TABLE 5
Genetic diversity parameters for each of the three regions considered in this study using isozymes (Isoz) and SSR markers

Region	H_o		H_e		f		$\bar{t}_a$	
	Isoz	SSR	Isoz	SSR	Isoz	SSR	Isoz	SSR
Xingu	0.000	0.282	0.085	0.397	1.000	0.298	0.000	0.541
Amazon	0.004	0.039	0.099	0.720	0.963	0.945	0.019	0.028
Pantanal	0.014	0.133	0.147	0.693	0.902	0.810	0.052	0.105

H_o - observed heterozygosity; H_e - gene diversity; f - fixation index; $\bar{t}_a$ - apparent outcrossing rate

analyzed with microsatellites. These results showed that, in general, SSR markers are more efficient than isozymes to detect outcrossing events both at the populational and regional levels and that, except for the Xingu region, with only one population assessed, this species presents a mixed mating system with predominance of self-fertilization.

Genetic population structure: Mean inbreeding registered within each population (*f*) was higher, in general, for the isozymes (0.910) when compared to microsatellites (0.771) (Table 4). This tendency was maintained for both markers also at the regional level (Table 5). The estimates of F statistics revealed that total inbreeding coefficient (F_{IT}) in this species is very high, 0.974 for isozymes and 0.895 for microsatellites (Table 6), and that the main factor promoting the deviation from Hardy-Weinberg equilibrium is the mating system (F_{IS}=0.899 and 0.774, respectively, for isozymes and microsatellites). However, although of a lower magnitude, but also expressive, the genetic differentiation among populations (F_{ST}) contributed for the total inbreeding levels observed, with the isozyme markers showing a value of 0.772 for this parameter, while the level registered for the SSR markers was 0.533 (or 0.631 for the corrected value of R_{ST}). On the other hand, the number of migrants per generation (Nm = ¼ [(1/F_{ST} or R_{ST})-1]) observed for both markers was small, only 0.074 for the isozymes and 0.146 for the SSR marker (Table 6).

The cluster analysis, based on Nei´s genetic distances (Nei 1978) and the UPGMA method, showed that isozyme data tended to exhibit clustering according to the origins of the populations, with two clear groups: the Amazon populations in the first group and the Pantanal populations as well as the Xingu population (as a sub-group) in the second group. For *Aat-1* and *Aat-2* loci, different alleles predominated in each of the two regions, Pantanal and the Amazon (Fig. 3A). The Xingu population (XI-1) and the Pantanal populations shared the same allele frequencies for these two loci, which explains its proximity to the Pantanal populations in the dendrogram. However, it was classified in a sub-group within the second group, differing from the Pantanal populations for presenting a fixed *a2* allele at *Skd-1* locus and from all the others for presenting a high frequency of the *a3* allele at *Pgm-1* locus.

However, data from microsatellites tended to exhibit a random clustering, not in agreement with the geographic origins (Fig. 3B). These results were confirmed in the correlation tests between genetic and geographic distances obtained for each marker. In this sense, correlations between genetic and geographic distances showed a positive and significant result (r=0.6594, p<0.05) when estimated with isozymes and an absence of correlation (r=-0.1789, p>0.05), when estimated with microsatellites. On the other hand, no correlation (r=-0.0005, p>0.05) was found between the two markers, when their genetic distances were analyzed.

TABLE 6
Wright's F statistics estimates, number of migrants per generation using F_{ST} (Nm_1) or R_{ST} (Nm_2) for isozymes and microsatellites, for the *Oryza glumaepatula* populations

	Isozymes				Microsatellites					
	F_{IT}	F_{IS}	F_{ST}	Nm_1	F_{IT}	F_{IS}	F_{ST}	R_{ST}	Nm_1	Nm_2
Under all loci	0.974	0.899	0.772	0.074	0.895	0.774	0.533	0.631	0.219	0.146
Upper (CI 95%)	0.981	0.923	0.875	-	0.929	0.833	0.597	-	-	-
Lower (CI 95%)	0.966	0.813	0.619	-	0.860	0.704	0.472	-	-	-

Nm_1 = Based on F_{ST}; Nm_2 = Based on R_{ST}; CI 95% = 95% confidence interval.

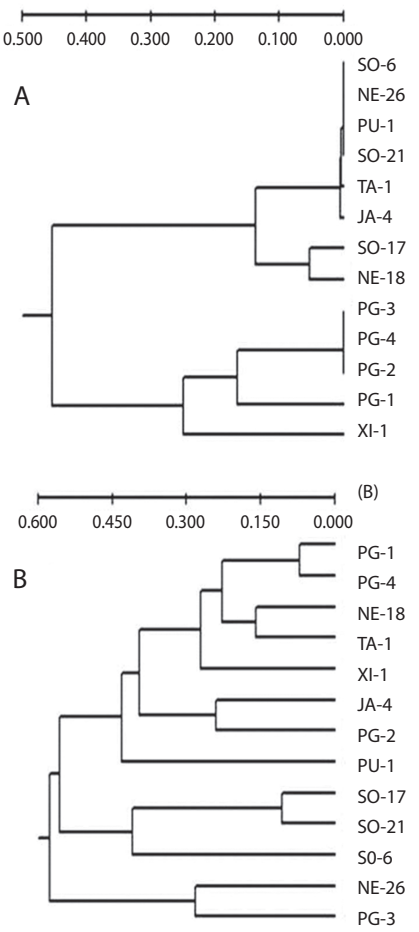

Fig. 3. Pattern of genetic divergence among 13 *Oryza glumaepatula* populations based on the UPGMA clustering method and Nei's genetic distances (Nei, 1978) for both isozyme (A) and microsatellite (B) markers.

DISCUSSION

Genetic diversity levels: This study compared the levels of diversity assessed with isozymes and microsatellite markers in 13 *O. glumaepatula* populations in order to establish the potential of each marker for a predominantly inbreeding wild rice species. The results of the analysis of microsatellite and isozyme loci are consistent with the principles of each marker, considering the fact that microsatellites showed a wide range of alleles at each locus whereas isozymes showed only one to three alleles per locus. Possibly, the adaptive nature of some genes that govern the production of proteins, when under environmental effect, tends to express monomorphism within populations (Schlötterer 2004, Lowe *et al.* 2007). Another reason for the relatively fewer apparent alleles in isozymes is the ineffectiveness of most mutations in changing the electrophoretic mobility of the bands (i. e., the visualization of different alleles), since only a few aminoacid changes modify the net electric charge of the proteins, whereas most length changes in SSRs cause their band speed to vary. Additionally, the types of mutation process that give rise to SSR alleles and to isozyme alleles are different and the former is more common. On the other hand, microsatellites are usually found in non-coding repetitive DNA regions and the high mutation rate through gain and/or loss of repeats (Goldstein & Schlötterer 2000) can be explained by their usual neutrality. Recently, however, increasingly more microsatellites have been found and characterized within protein-coding genes and their untranslated regions (UTRs), which subjects them to stronger selective pressure than other genomic regions because of their functional importance (Li *et al.* 2004). In our study, however, most of the SSR loci used (six out of eight loci) were not located within genes (Karasawa *et al.* 2007b). But irrespective of their location in the genome, polymorphism is common in SSR markers while the presence of monomorphism tends to be a rare condition (Panaud *et al.* 1995).

In this study, isozymes showed only 42.4% to 43.5% of the microsatellites' diversity detected in the parameters average number of alleles ($\overline{A}$) and average number of alleles per polymorphic locus ($\overline{A}_p$), respectively. Similarly, we found that microsatellites detected private alleles, whereas no private alleles were found with isozyme markers, which may be due to a lower number of apparent alleles per locus and a lower number of loci used in this study.

The levels of observed heterozygosity (H_o) and gene diversity (H_e) assessed with isozymes represented, on average, 7.4% and 15.9% of those observed with microsatellites,

respectively. However, when gene diversity was considered over loci we could verify that the amount is higher than at the population level. This fact occurred because the split of the ancestral population led to a fixation of specific alleles in each population. This reduction in the number of alleles in each population, from 1.83 to 1.20 and from 10.12 to 2.83, respectively for isozymes and microsatellites, led to lower gene diversity at the population level. Microsatellites are considered genetically more informative than other markers, especially when dealing with populations with low genetic diversity content at the DNA level (Paetkau *et al.* 1995). The low gene diversity (H_e) levels in this species has already been shown in previous studies with isozymes, revealing variations from 0.044 to 0.060 (Akimoto *et al.* 1998, Buso *et al.* 1998, Veasey *et al.* 2008), as compared with microsatellites with higher levels, ranging from 0.113 to 0.491 (Brondani *et al.* 2005, Karasawa *et al.* 2007a, Silva *et al.* 2007). Thus, considering the information obtained in this study with isozymes and microsatellites, we can conclude that microsatellite markers are in fact more informative as they were able to detect higher levels of intra-population diversity in this species. Gao *et al.* (2002b) also found higher levels of genetic variation with microsatellite loci than isozyme loci when studying *O. rufipogon* populations from China. Similarly, higher levels of polymorphism revealed by microsatellites when compared to isozymes were found in natural populations of *Euterpe edulis* Mart. in Brazil (Conte *et al.* 2008), oaks (*Quercus* spp.) in West-Central Romania (Curtu *et al*. 2007), in a natural *Elymus caninus* (L.) L. population from Denmark (Sun *et al*. 2001) and in sorghum (*Sorghum bicolor* L.) landraces in North-Western Morocco (Djè *et al.* 1999).

Population genetic structure: Isozyme markers showed similar inbreeding levels (*f*) as microsatellites for populations, regions and for total inbreeding of the species (F_{IT}), except for the Xingu population. The existence of inbreeding within populations showed, with both markers, that this species has an inbred maternal family structure within populations and that this structure was established primarily by the reproductive system (F_{IS}), and that most of the total diversity is located among families within populations. In fact, the reproductive system of this species, classified as mixed with predominance of self-fertilization (Karasawa *et al.* 2007b, Vaz *et al.* 2009), seems to have a predominant effect on differentiation within and between populations. Both markers are also congruent in that the fragmentation of the species led to a considerable and important amount of genetic diversity among populations (F_{ST}). Gao *et al.* (2002b) also found an agreement in this type of result with both markers, but in *O. rufipogon* the greatest effect was in the formation of genetic structure due to fragmentation (F_{ST}). Measures of genetic structure, such as Wright´s F statistics, or Nei's coefficient of gene differentiation (G_{ST}) (Nei 1973), were similar for the two sets of markers (isozymes and SSR) assessed in natural populations of *Sorghum bicolor* (Djé *et al.* 1999), *Elymus caninus* (Sun *et al.* 2001) and *Euterpe edulis* (Conte *et al.* 2008).

Previous studies on genetic structure conducted in the genus *Oryza* have recorded several F_{ST} (G_{ST}) values, but largely agree that the mode of reproduction of the species has generated prominent effect on the differentiation observed. In *O. glumaepatula*, studies using allozymes showed F_{ST} values of 0.346 (Akimoto *et al.* 1998), 0.310 (Buso *et al.* 1998) and 0.763 (Veasey *et al.* 2008), while those using microsatellites showed values such as 0.847 (Brondani *et al.* 2005), 0.491 (Karasawa *et al.* 2007a) and 0.715 (Silva *et al.* 2007). In progeny studies, the value recorded with microsatellites was 0.451 for the parental populations, and 0.284 for the families of these populations (Karasawa *et al.* 2007b).

O. rufipogon studies with isozymes showed genetic structure levels (F_{ST}) dependent on the developmental stages (seed, young and adults plants) and the life cycle (annual or perennial). Annual populations showed F_{ST} values varying from 0.085 to 0.208 for seeds, from 0.087 to 0.145 for young plants and 0.193

for adults, while among the perennial populations higher values were found, ranging from 0.327 to 0.366 at the seed level, from 0.129 to 0.356 for young plants and from 0.360 to 0.390 for the adults (Barbier 1989). Morishima & Barbier (1990) obtained F_{ST} values of 0.289 for intermediate populations, 0.396 for perennials and 0.600 for annuals. Gao *et al.* (2000) and Gao *et al.* (2002a) reported values such as 0.310 and 0.254 in perennial populations with isozymes. With microsatellites, Gao (2004) obtained an F_{ST} value of 0.246, whereas when comparing isozymes and microsatellites, Gao *et al.* (2002b) obtained values of 0.468 for isozymes and 0.388 for microsatellites. Among Chinese *O. officinalis* perennial populations, an F_{ST} value of 0.442 was reported (Gao 2005).

The results in our study with *O. glumaepatula* populations, comparing microsatellites (F_{ST}=0.533) with isozymes (F_{ST}=0.772), are in accordance with the results obtained by Gao *et al.* (2002b) for perennial outcrossing *O. rufipogon* populations, although these values were lower than those found in *O. glumaepatula*. It is difficult to conclude whether the heterogeneity of the F_{ST} values found with isozyme loci compared with microsatellites was caused by stochastic processes or as an indirect effect of selection. According to a survey by Frankel *et al.* (1995), the genetic diversity among populations (G_{ST}) with isozymes should be lower in cross-pollinating and perennial plants, and higher in autogamous and annual plants, which may explain the results discussed above.

Due to the mode of reproduction of this species, the number of migrants (*Nm*) estimated promoting gene flow among populations was small, only 0.074 for isozymes and 0.146 for microsatellites. Gene flow is important in the evolution of plant populations, because the geographical variation observed in morphology and gene frequencies of a species results from the balance between forces that act to cause local differentiation counterbalanced by forces that tend to produce homogeneous populations (Slatkin 1987).

Cluster analysis grouped the populations according to their geographical origin with isozymes, whereas a pattern of random clustering of populations not consistent with the geographical origin was observed for microsatellites. Djè *et al.* (1999) also found no concordance between the cluster analysis based on allozymes and the cluster based on microsatellites for sorghum landraces. Gao *et al.* (2002b), comparing data obtained with isozymes and microsatellites in *O. rufipogon*, found that information obtained by both markers were not completely correlated. The grouping of maternal plants of *Elymus caninus* based on isozymes and microsatellites were also not completely consistent with each other (Sun *et al.* 2001).

In our study, we verified a positive correlation between genetic and geographical distances for isozymes and a negligible negative correlation when estimated with microsatellites. A positive correlation between genetic and geographical distances, when the genetic markers are neutral, is frequently assumed as a result of limited gene flow between contiguous populations, while a lack of correlation may result from long-distance dispersal, geographic barriers or unlimited gene flow between contiguous populations. The positive correlation found with isozymes may thus be explained by gene flow dependent on geographic distance; the lack of correlation with microsatellites is more difficult to explain and could be due to the rapid allele turnover caused by the high mutation rate in the loci, which would have blurred the geographic pattern still visible with isozymes. When comparing the genetic distances obtained from both markers, the result was an absence of correlation. Therefore, inferences made from one of the markers cannot be considered for the other marker.

Our results showed that microsatellites generate a higher volume of intra-population genetic diversity information compared to those obtained with isozymes in this species. Microsatellites showed 130% more alleles and 3.5 times higher rate of polymorphism, being more effective, therefore, in the analysis of diversity in a predominantly inbreeding species. Therefore, the superiority of microsatellites

in detecting intra-population diversity, private alleles and the relative agreement with isozymes considering the migrant levels between populations, make this an important marker in the study of population genetics and conservation. However, isozymes had the advantage in this study of clustering the populations according to their origins, which was not observed for microsatellites. As no correlation between genetic distances was obtained between the two markers, inferences made from one of the markers cannot be considered for the other marker.

In summary, it can be said that SSR markers showed more power for investigating neutral intra-population diversity, as expected. At the inter-population level, however, despite the relatively small number of loci used, isozymes led to better results, since clustering of populations agreed with the expectations based on the geographic distribution of the populations.

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RESUMEN

El estudio de la estructura genética de poblaciones de plantas silvestres es esencial para su manejo y conservación. Varios marcadores de ADN e isoenzimas se han utilizado en este tipo de análisis. Con el fin de proporcionar una mejor comprensión de los resultados obtenidos y saber que marcador codominante elegir para futuros estudios en poblaciones naturales de *Oryza glumaepatula*, este trabajo busco evaluar y comparar dos marcadores de ADN, isoenzimas y microsatélites, en la diversidad y estructura genética de 13 poblaciones, destacando las similitudes y divergencias de cada marcador, así como la importancia relativa de los resultados en genética de poblaciones y conservación. Para los SSR, ocho loci SSR fueron evaluados, y los fragmentos se visualizaron utilizando el procedimiento de coloración con plata. Los análisis de isoenzimas se realizaron en geles de poliacrilamida, en los seis loci enzimáticos. Los loci SSR mostraron mayores niveles de diversidad genética que los loci isoenzimáticos, en promedio. La diferenciación genética entre los loci SSR (R_{ST}=0.631, equivalente a F_{ST}=0.533) fue inferior a la obtenida con las isoenzimas (F_{ST}=0.772). Ambos marcadores mostraron alta desviación del equilibrio de Hardy-Weinberg (F_{IS}=0.744 y 0.899, respectivamente, para SSR e isoenzimas). La tasa media aparente de cruzamiento para SSR ($\bar{t}_a$=0.14) fue mayor que la obtenida con isoenzimas ($\bar{t}_a$=0.043), aunque ambos marcadores detectaron niveles más bajos en la tasa de fecundación cruzada para la Amazonia, en comparación con la región del Pantanal. La estimación de número de migrantes también fue mayor para los SSR (Nm=0.219) que en isoenzimas (Nm=0.074). No se obtuvo ninguna correlación entre las distancias genéticas y geográficas para los SSR, y para las isoenzimas se obtuvo una correlación positiva entre las distancias genéticas y geográficas. Llegamos a la conclusión de que estos marcadores son divergentes en la detección de los parámetros de la diversidad genética en *O. glumaepatula* y que los microsatélites son más eficientes para detectar la información a nivel intrapoblacional, mientras que las isoenzimas son más potentes para detectar la diversidad entre poblaciones.

Palabras clave: estructura genética, flujo de genes, isoenzimas, SSR, sistema de cruzamiento.

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