Genetic diversity of American wild rice species

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ABSTRACT: Studies on genetic diversity and genetic structure of natural populations are important in order to define strategies for *in situ* and *ex situ* conservation actions and for plant pre-breeding programs. Aiming to assess the genetic diversity and genetic structure of three wild American *Oryza* species with isozyme markers, 14 populations of the diploid *O. glumaepatula* ($A^{gl}A^{gl}$), 11 populations of the tetraploid *O. grandiglumis* (CCDD) and five populations of the also tetraploid *O. latifolia* (CCDD) were studied. They were all originated from Rio Paraguay hydrographic basin and the Amazon. Four enzymes were used and they gave 40 polymorphic bands. The most polymorphic species was *O. glumaepatula*, followed by *O. latifolia* and *O. grandiglumis*. A cluster analysis with the Jaccard similarity coefficient separated the diploid from the two tetraploid species, and also the two tetraploid species. This separation was also evident on a scatter plot from a principal component analysis, suggesting that they should be treated as two separate species, although further studies are necessary to provide support for this affirmative. The AMOVA analyses showed a high intrapopulational variability for *O. latifolia* (67.6%) and *O. grandiglumis* (52.2%), when compared to their interpopulational variability (32.4% and 47.8%, respectively), which suggests the hypothesis of a higher degree of outcrossing events within these species. When studying the correlation between the Jaccard dissimilarity coefficient and geographic distances, a spatial genetic structure was observed for *O. glumaepatula* only. These results are important for defining strategies of both *in situ* and *ex situ* conservation.
Keywords: *Oryza glumaepatula*, *O. latifolia*, *O. grandiglumis*, Amazon, genetic structure

Introduction

The *Oryza* genus comprises two cultivated species (*Oryza sativa*, native to Southeast Asia, spread throughout tropical and sub-tropical environments, and *O. glaberrima*, limited to Western Africa) and 22 wild species distributed in all tropical and subtropical world regions (Vaughan et al., 2003). Eight wild species occur both in Asia and Oceania, four in Asia, two in Oceania, five in Africa and four in America (IRRI, 2009). The four American species are distributed from Mexico to Argentina and all of them are found in Brazil (Oliveira, 1994).

Genetic and ecological studies with wild rice natural populations are necessary to provide information for both *ex situ* or *in situ* conservation programs for these species (Xie et al., 2010). To date, there are quite a few studies concerning the genetic structure of the diploid *O. glumaepatula* populations (Akimoto et al., 1998; Buso et al., 1998; Brondani et al., 2005; Karasawa et al., 2007a,b; Veasey et al., 2008a), but very little has yet been published on the genetic structure and variability of the tetraploid American species (Agrama and Eizenga, 2008; Arrieta-Espínoza et al., 2005; Quesada et al., 2002; Veasey et al., 2008b). The wild species represent an important genetic reservoir for rice plant breeding for the purpose of introgression of useful genes into the cultivated species *O. sativa* (Brondani et al., 2002; Eisenga et al., 2009; Multani et al., 2003; Yoon et al., 2006).

This study analyzed the genetic diversity and genetic structure of two tetraploid American wild rice species, *O. latifolia* and *O. grandiglumis*, and the diploid *O. glumaepatula* as an outgroup, using isozyme markers. The objectives were to provide useful information for *in situ* or *ex situ* conservation of these species, and also to provide further data in order to elucidate the taxonomic issue of the tetraploid species.

Materials and Methods

A total of 30 populations were assessed from the wild rice germplasm bank of Escola Superior de Agricultura "Luiz de Queiroz", University of São Paulo, consisting of 11 populations of the tetraploid species *O. grandiglumis*, five of the tetraploid *O. latifolia* and 14 populations of the diploid *O. glumaepatula*. The five *O. latifolia* and four *O. glumaepatula* populations originated from the Rio Paraguay hydrographic basin. The 11 *O. grandiglumis* and 10 *O. glumaepatula* populations originated from the Japura, Purus, Tapajós, Solimões, Negro and Xingu hydrographic basins in the Amazon region. The *O. glumaepatula* population originated from Rio Xingu (RX1) was located far from

the other Amazonian populations, in the State of Mato Grosso (Table 1, Figure 1).

A bulk of 50 seeds was formatted using randomly sampled two to three seeds from each plant within each population. After germination in plastic gerboxes on filter paper at 27°C in the dark in germination chambers, the seedlings were planted in pots in the greenhouse. The number of evaluated plants from each population varied from four (ARG6) to 36 (RPG1), according to their germination potential, amounting to a total of 700 individuals (Table 1).

For enzyme extraction, newly expanded leaves (200 mg) were ground in liquid nitrogen in 1.5 mL microtubes using a power homogenizer, adding 1 mL of number 1 extraction buffer (Alfenas et al., 1991), excluding diethyldithiocarbamic acid (DIECA) and 2- mercaptoethanol. The crude extract was then centrifuged at 18,000 *x* g for 20 min at 4°C and 130 µL of the supernatant placed in 0.5 mL microtubes, diluted in 150 µL of a solution containing Tris-HCl pH 6.8 and Coomassie blue, the latter component indicating the protein migration in the gel. Electrophoresis was conducted according to Veasey et al. (2008a).

Six enzyme systems presenting higher band resolution were initially selected for the analyses: glucose-6-phosphate isomerase (GPI; E.C. 5.3.1.9), aspartate aminotransferase (AAT; E.C. 2.6.1.1), shikimate dehydrogenase (SKD; E.C. 1.1.1.25), glutamate dehydrogenase (GDH; E.C. 1.4.1.2), phosphoglucomutase (PGM; E.C. 2.7.5.1) and malate dehydrogenase (MDH; E.C. 1.1.1.37). Due to the difficulty in interpreting, the MDH and PGM systems were discarded. The PGM system was excellent for *O. glumaepatula* popu-

Table 1 – List of 30 *Oryza* populations, including population code, species identification, number of individuals sampled/population, hydrographic basin, river and lake or municipality of the original collection sites, and geographic coordinates.

Nº	Code	Species	Nº samples	Hydrographic Basin	River	Lake (Municip.)	Latitude	Longitude
1	RTAQ1	*O. latifolia*	30	Paraguay	Taquari	Pantanal	19°15' S	57°13' W
2	RPG1	"	32	Paraguay	Paraguay	Pantanal	19°01' S	57°30' W
3	ARG5	"	26	Paraguay	Paraguay	-	27°18' S	59°24' W
4	ARG8	"	17	Paraguay	Paraguay	-	27°26' S	58°52' W
5	ARG6	"	4	Paraguay	Paraguay	-	27°17' S	59°23' W
6	RP2	*O. grandiglumis*	14	Purus	Purus	Bravo	3°51' S	61°26' W
7	RS20	"	30	Solimões	-	-	2°50' S	65°13' W
8	RN32	"	11	Negro	-	-	1°01' S	61°53' W
9	RJP2	"	9	Japurá	Japurá	-	2°57' S	64°50' W
10	RS3	"	30	Solimões	-	Paru	3°18' S	60°35' W
11	RJP3	"	32	Japurá	Japurá	-	2°40' S	64°57' W
12	RS26	"	31	Solimões	-	Caldeirão	3°15' S	60°11' W
13	RS14	"	30	Solimões	-	Jucara	4°02' S	63°08' W
14	RS10	"	26	Solimões	-	Miuá	3°50' S	62°05' W
15	RSJN3	"	32	Solimões	-	Janauacá	3°19' S	60°11' W
16	RP1	"	11	Purus	Purus	-	3°49' S	61°25' W
17	RJP4	*O. glumaepatula*	21	Japurá	Japurá	Cuiucuiú	2°22' S	65°07' W
18	RS14	"	9	Solimões	-	Jucara	4°02' S	63°08' W
19	RP1	"	21	Purus	Purus	-	3°49' S	61°25' W
20	RT1	"	24	Tapajós	Tapajós	-	2°26' S	54°42' W
21	RS17	"	30	Solimões	-	Coari	4°10' S	63°15' W
22	RS6	"	27	Solimões	Solimões	Manacapuru	3°11' S	60°47' W
23	RPG3	"	27	Paraguay	Corumbá	-	18°59' S	57°37' W
24	RPG2	"	23	Paraguay	-	-	19°00' S	57°41' W
25	RPG1	"	36	Paraguay	Paraguay	-	19°01' S	57°30' W
26	RS21	"	23	Solimões	-	Mamiá	4°15' S	63°03' W
27	RPG4	"	23	Paraguay	Taquari	-	19°15' S	57°13' W
28	RX1	"	24	Xingu	Xingu	Piulaga	12°14' S	53°35' W
29	RN18	"	24	Negro	Branco	-	1°53' S	61°22' W
30	RN26	"	23	Negro	Carabinani	-	1°54' S	61°23' W

lations but provided a poor resolution for the tetraploid species.

Due to the tetraploid species (*O. grandiglumis* and *O. latifolia*), the zimograms of all three species were interpreted as presence/absence of bands (binary data), as in the case of dominant markers. Using the UPGMA (unweighted pair group method with arithmetic averages) method and the Jaccard similarity coefficient, a cluster analysis was obtained with NYSYS-pc software (Rohlf, 1992). The reliability of the formed groups in the cluster analyses was verified with a bootstrap analyses using 10000 permutations implemented in the software BOOD-P, version 2.0 (Coelho, 2001). Also, a principal component analysis was conducted by PROC PRINCOMP procedures of SAS program (SAS, 1993) for the two tetraploid species, and a scatter graph was obtained using BioStat 4.0 software (Ayres et al., 2005).

The level of genetic spatial structure was analyzed using the Pearson coefficient of correlation (r) between the Jaccard dissimilarity coefficient matrix and the geographic distances (shortest distance between two given points on the map) matrix between populations, using the NTSYS-pc software (Rohlf, 1992). Mantel's Z statistic (Mantel, 1967) and 10000 random permutations were used to test the significance of these correlations.

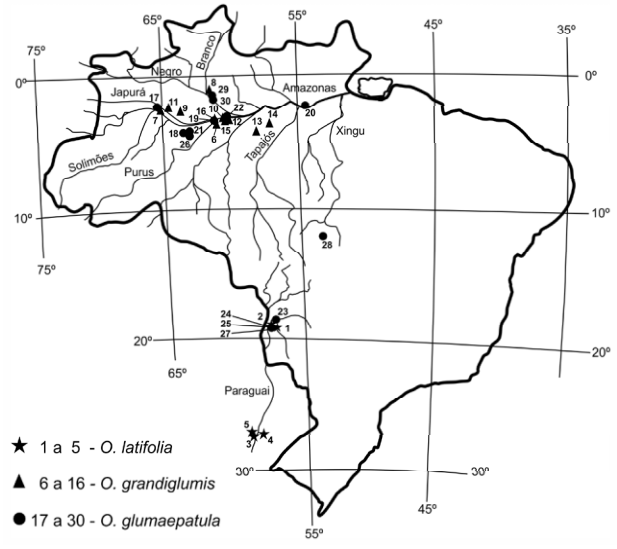

Figure 1 – Map of Brazil showing the location of 30 *Oryza glumaepatula*, *O. latifolia* and *O. grandiglumis* populations.

Using the ARLEQUIN software (Schneider et al., 2004), analyses of molecular variance for dominant markers (AMOVA) (Excoffier et al., 1992) were conducted with binary data to assess the genetic structure of the species/populations under investigation. Four AMOVA analyses were carried out, one considering the two tetraploid species in a total of 16 populations; and three further analyses, one for each species and their respective populations (*O. latifolia* with five populations, *O. grandiglumis* with 11 populations, and *O. glumaepatula* with 14 populations). These three latter analyses allowed us to estimate the parameter ϕ_{ST}, which is analogous to Wright ϕ_{ST}, an interpopulation diversity parameter (Lacerda et al., 2001).

Results and Discussion

A total of 40 isozyme bands were found in 30 populations belonging to the three species evaluated: four bands for SKDH, 13 for AAT, 14 for GPI and nine for GTDH. High level of polymorphism was observed in all 30 populations and in all the species tested (Table 2). The diploid *O. glumaepatula* was the most polymorphic (88%), followed by *O. latifolia* (84%) and *O. grandiglumis* (79%). The same result was observed for morphoagronomic traits in a similar set of populations (Veasey et al., 2008b). Although *O. grandiglumis* was represented by a larger number of populations than *O. latifolia* in both studies, they were all originated from the Amazon region and showed less polymorphism. On the other hand, *O. latifolia* was represented by two populations from the Pantanal ecosystem in Brazil and three populations collected in a more distant area in Argentina. However, these two groups were not separated in the cluster analysis (Figure 2). Based on total genomic hybridization and in agreement with our findings, Aggarwal et al. (1996) showed that *O. latifolia* was the most divergent among the three CCDD species, and that *O. alta* and *O. grandiglumis* were more similar to each other. Bao and Ge (2004) concluded that the CD genome originated from a single hybridization event, and that the C genome species (*O. officinalis* or *O. rhizomatis* instead of *O. eichingeri*) served as the maternal parent while the E genome species (*O. australiensis*) was the paternal donor during the formation of CD genome.

Similar isozyme patterns were shared by the two tetraploid species, but not the diploid *O. glumaepatula*, which exhibited its own particular pattern. Comparing the 30

Table 2 – Number of bands (b), number of polymorphic bands (pb) and percent polymorphism (%P) assessed for each enzymatic system and for each species.

Species	*O. grandiglumis*			*O. latifolia*			*O. glumaepatula*		
System	b	pb	% P	b	pb	% P	b	pb	% P
AAT	7	4	57	11	8	73	6	5	83
PGI	12	12	100	10	10	100	8	8	100
SKDH	2	1	50	2	1	50	2	2	100
GTDH	8	6	75	9	8	89	1	0	0
Total	29	23	-	32	27	-	17	15	-

populations of the three species, a wide range was found for the Jaccard similarity coefficient, varying from 0.05 to 1.0, but most of this variability was due to the separation of the diploid from the tetraploid species. The dendrogram (Figure 2) showed two well defined great groups, one including the two tetraploid species and one including the populations of the diploid species, *O. glumaepatula*. Although minor groups were formed, three groups were noticed within the *O. glumaepatula* great group. The first one classifying the Amazon populations, except for Rio Japura (RJP-4), which had a different pattern for the GPI system in relation to all the other populations, and was, therefore, classified as a second separate group, with 100% reliability. A third group classified the four Rio Paraguay populations together with the Rio Xingu population, with 53% reliability.

When studying morphoagronomic traits, Veasey et al. (2008b) also found an evident separation of *O. glumaepatula* populations, to the right side of the first canonical axis, which explained 64.8% of total variation, from the tetraploid species, scattered towards the left side. Arrieta-Espinoza et al. (2005) also showed that the first and third components, which together explained 55.1% of the variation, separated *O. latifolia* and *O. grandiglumis* (CCDD genomes) from *O. glumaepatula*, *O. sativa*, and other AA genome species in Costa Rica. Also in Costa Rica, Zamora et al. (2003) found that the small ligule and the wide flag leaf characteristic of the two CCDD species separated them from the AA diploid *O. glumaepatula*.

Within the tetraploid great group, the *O. latifolia* populations were clearly separated from *O. grandiglumis*, with 59% reliability as shown by the bootstrap analysis (Figure 2). Within the *O. latifolia* populations, no separate groups were observed between the two areas sampled, the Pantanal in the State of Mato Grosso do Sul, Brazil and Argentina. Although showing less polymorphism, *O. grandiglumis* populations were separated also into different clusters, with two Rio Solimões populations (RS20 and RS10) and one Rio Japura population (RJ3) showing greater genetic similarity to the *O. latifolia* populations. A Rio Negro *O. grandiglumis* population (RN32) was isolated from the others, as it displayed unique isozyme band patterns for the GPI system. Another group classified most of the Amazonian *O. grandiglumis* populations, with 100% reliability.

The scatter graph was done only for the two tetraploid species, in order to show their genetic differences or similarities (Figure 3), which were obtained from the principal component analysis, whose first two components explained 57.6% of total variation, confirmed the results from the cluster analyses. It showed the separation of the *O. latifolia* populations from the *O. grandiglumis* populations, which had a higher dispersion, from what we may suggest that they should be considered as two separate species. Examining the scatter graph, the *O. latifolia* populations were clustered together on the lower right side of the graph and separated from the *O. grandiglumis* populations. RN32 *O. grandiglumis* population was also separated from the other populations of this species, agreeing with the dendrogram, which also separated this population in a single sub-group (Figure 2).

The taxonomic status of the two tetraploid species is more complex. In this study, the two species were grouped in separate clusters, from what we suggest that they be considered as separate species. However, further studies with a higher number of accessions of each species, which could include the third American tetraploid species, *O. alta,* and other molecular markers such as microsatellites or AFLP (amplified fragment length polymorphism), should be conducted to clarify this issue. Buso et al. (2001), Grover and Pental (1992) and Vaughan et al. (2003) suggested that the three American CCDD tetraploid species should be treated as a complex or a single species. But some support the view that these are separate species (Aggarwal et al., 1996; Ge et al., 1999; Veasey et al., 2008b; Zamora et al., 2003), while others suggest that *O. latifolia* should be treated as a single

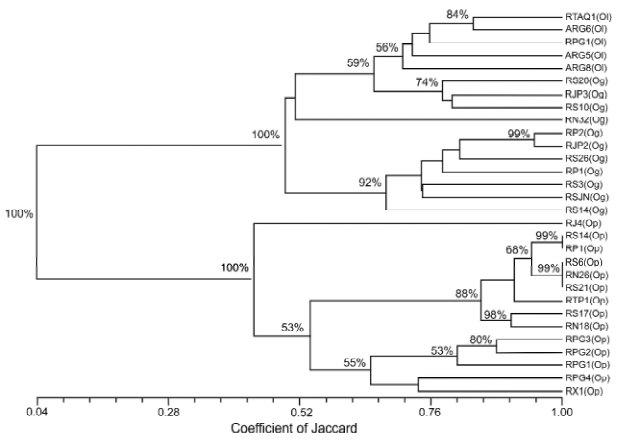

Figure 2 – Dendrogram obtained with the UPGMA method and the Jaccard coefficient for 30 populations of *Oryza glumaepatula* (Op), *O. latifolia* (Ol) and *O. grandiglumis* (Og).

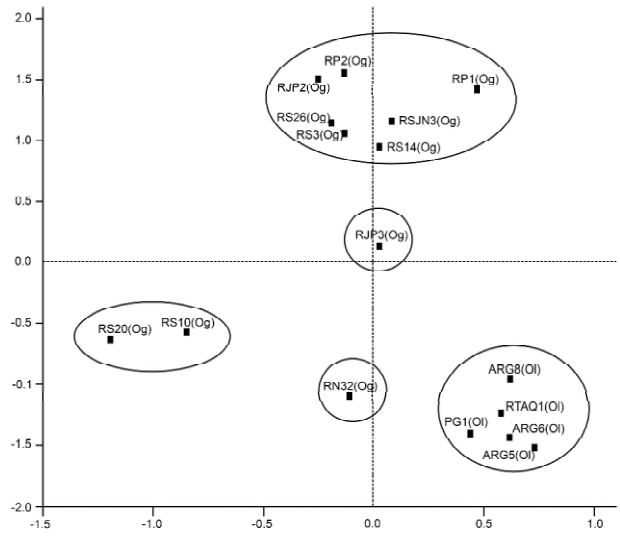

Figure 3 – Scatter graph obtained from the principal component analysis (princomp 1 explaining 44.4% and princomp 2 explaining 13.2% of total variation) for the 16 populations of the tetraploid species: *O. latifolia* (Ol) and *O. grandiglumis* (Og).

species in contrast to *O. alta* and *O. grandiglumis* which share more similarities. Bao and Ge (2004), based on two chloroplast fragments and three nuclear gene fragments, suggested treating *O. alta* and *O. grandiglumis* as a single species, but not *O. latifolia*. Nishikawa et al. (2005) have shown that *O. latifolia* has a different chloroplast genome from *O. alta* and *O. grandiglumis*. Phylogenetic trees using microsatellite markers have placed both *O. latifolia* and *O. alta* in the same cluster within a set of wild rice species, in comparison to *O. sativa* (Agrama and Eizenga, 2008; Eisenga et al., 2009).

Taking into account the genetic structure of these species, the AMOVA analysis conducted for the two tetraploid species showed a relatively low percentage of the variability distributed between species (38.4%), with a similar percentage of the variability distributed within populations (34.1%) (Table 3). When considering each individual species, a higher interpopulational diversity was found for the diploid *O. glumaepatula* populations (ϕ_{ST} = 0.737), compared to the interpopulational diversity observed for *O. latifolia* populations (ϕ_{ST} = 0.324) and *O. grandiglumis* populations (ϕ_{ST} = 0.478) (Table 4). On the other hand, higher intrapopulation diversity was found for the tetraploid species, especially *O. latifolia* (67.6%) followed by *O. grandiglumis* (52.2%), against 26.3% for the diploid *O. glumaepatula* populations.

The interpopulation diversity parameter (ϕ_{ST}) estimated in this study can be compared with Wright F_{ST} interpopulation variability obtained in other studies. A lower F_{ST} value (0.491) was obtained by Karasawa et al. (2007) with microsatellite markers for *O. glumaepatula* populations, although similar values were obtained by Brondani et al. (2005) (F_{ST} = 0.847) and Silva et al. (2007) (F_{ST} = 0.715). The fact that most of the isozymic variability was distributed between rather than within species is in accordance with the predominantly autogamic mating system of these species, especially the diploid *O. glumaepatula* (Akimoto et al., 1998; Buso et al., 1998; Brondani et al., 2005; Karasawa et al., 2007a,b; Vaz et al., 2009). Apparent outcrossing rates were estimated as 0.143 and 0.135 by Karasawa et al. (2007a) and Brondani et al. (2005), respectively, for *O. glumaepatula,* both using microsatellite markers, while multilocus outcrossing rates varying from 0.011 to 0.223 were reported by Karasawa et al. (2007b), justifying the *O. glumaepatula* interpopulation variation found in nature. A higher apparent outcrossing rate (0.30 on average) using 18 microsatellite loci was reported by Vaz et al. (2009) for a single large population of *O. glumaepatula*, occurring in the Paraguay River, Brazil. However, the higher variability found within populations for the two tetraploid species, when compared to the interpopulational variability, suggest the hypothesis of a higher degree of outcrossing events within these species. Further progeny studies are recommended for a better understanding of the mating system of the CCDD tetraploid species.

Quesada et al. (2002) found high levels of interpopulation diversity for *O. latifolia* in Costa Rica, with most populations being monomorphic for at least one genotype, suggesting little gene flow within populations. However, Quesada et al. (2002) observed high frequency of heterozygous-like isozymatic patterns which may suggest that the reproductive system of *O. latifolia* might be more complex. In our study, heterozygous-like isozymatic patterns were also observed, especially for the GPI system. The excess of heterozygous patterns and fixed heterozygotes could be due to polyploidy, and have been observed in autogamous allopolyploids, mainly in colonizing plants (Barret and Shore, 1989). However, the diploid *O. glumaepatula* also presented high frequency of heterozygotes for the dimeric GPI system, which is harder to explain, considering that the other systems showed low frequency of heterozygotes.

Table 3 – Analysis of Molecular Variance (AMOVA) with isozyme markers for 16 populations belonging to the tetraploid species *Oryza latifolia* and *O. grandiglumis*.

Source of variation	DF	SS	Percentage of total variation	*p* value[1]
Between species	1	339.553	38.43	0.0000
Between populations within species	14	460.886	27.43	0.0000
Within populations	349	608.981	34.14	0.0000
Total	364	1409.419	-	

[1]Number of permutations = 1023.

Table 4 – Analysis of Molecular Variance (AMOVA) with isozyme markers for *Oryza latifolia* with 5 populations, *O. grandiglumis* with 11 populations and *O. glumaepatula* with 14 populations.

	O. latifolia			*O. grandiglumis*			*O. glumaepatula*		
Source of variation	DF	SS	Percentage of total variation[2]	DF	SS	Percentage of total variation[2]	DF	SS	Percentage of total variation[2]
Between populations	4	74.873	32.36	10	386.013	47.85	13	417.746	73.67
Within populations	104	179.439	67.64	245	429.542	52.15	321	152.439	26.33
Total	108	254.312	-	255	815.555	-	334	570.185	-

[1]Number of permutations = 1023; [2]$p < 0.0000$.

When correlation analyses between geographic and genetic distances, using the Mantel test, were conducted for each species separately, the tetraploid species (*O. grandiglumis* with 11 populations and *O. latifolia* with five populations) did not show any correlation among these two distances. However, a strong correlation between genetic and geographic distances was found for *O. glumaepatula* ($r = 0.67$; $p \leq 0.0004$), tested with 14 populations. Also, when the analysis was conducted with the 10 Amazon populations only, excluding the more distant ones from the Rio Paraguay hydrographic basin, a high correlation was still observed ($r = 0.66$; $p \leq 0.03$), but at a lower (5%) significance level.

The strong correlation between geographic and genetic distances observed for *O. glumaepatula* agrees with the cluster analysis (Figure 2) where the most distant populations are also genetically differentiated. In this study, the populations are quite distant from each other. However, even among the Amazonian populations, excluding the more distant Pantanal populations, a correlation was found. The cluster analysis separated the Pantanal populations in Rio Paraguay from the Amazonian populations. The Xingu population, which is at an intermediate position in the physical map, was also at an intermediate position in the cluster analysis, confirming the correlation result observed for this species. No correlation, however, was found for genetic and geographic distances, when assessing almost the same group of *O. glumaepatula* populations with microsatellite markers (Karasawa et al., 2007b), which may be explained by the complete absence of selection effect in this neutral marker, while some selection effect could be found in isozymes.

To understand the reasons for this greater space structuring among the diploid *O. glumaepatula* populations, the interpopulational diversity parameter (ϕ_{ST}) was compared between both tetraploids and the diploid populations. The higher interpopulational value found for the diploid populations ($\phi_{ST} = 0.737$) was indicative of a higher genetic structuring among populations, which could provide an explanation for the higher level of genetic spatial structure observed for *O. glumaepatula* populations. Comparable values of F_{ST} were found for *O. glumaepatula* populations by other authors, such as $F_{ST} = 0.85$ (Brondani et al., 2005) with SSR markers, $F_{ST} = 0.763$ (Veasey et al., 2008a) with isozyme markers, although a lower $F_{ST} = 0.491$ was reported by Karasawa et al. (2007a) with SSR markers.

In conclusion, our data showed a high enzymatic polymorphism for the three wild American rice species, which is important considering their possible use in plant breeding programs. The AMOVA results with the two tetraploid species showed high intrapopulation variability, a different result from the diploid species, with higher interpopulational variability, which may suggest a higher level of outcrossing for the two tetraploid species. Further studies on the mating system of the tetraploid species are recommended, considering that this type of information is very important for *ex situ* or *in situ* conservation strategies. The higher outcrossing pattern found in the tetraploid species suggests a mixed or predominantly cross-fertilization breeding system. This would lead to the sampling of a higher number of plants within fewer populations, in comparison with a predominantly inbreeding population. The strategies for collection would include sampling from many plants from several populations, considering the sampling of seeds for *ex situ* conservation. But strategies for maintaining these populations at an *in situ* conservation program are also important and this knowledge allows the definition of the number of populations to be preserved. Xye et al. (2010) have shown the necessity to maintain *in situ* conservation of *O. rufipogon* populations, considering that *ex situ* conservation of this species populations failed to maintain the genetic identity and reduced genetic diversity. Our data also suggests the distinction of *O. latifolia* and *O. grandiglumis* as two separate species, although further studies with a greater number of accessions and other molecular markers could provide a more reliable conclusion concerning the taxonomic status of the tetraploid species.

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