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Molecular Analysis of Utility of a Retrotransposon, p-SINE1-r2 in the Asian Wild Rice and Weedy Rice Populations

Preecha Prathepha
Rice Genetics Research Unit, Department of Biotechnology, Faculty of Technology,
Mahasarakham University, Khamriang Sub-District, Kantarawichai District,
Maha Sarakham Province 4400, Thailand

Abstract: The distribution of a retrotransposon, p-SINE1-r2 located at the waxy locus was analyzed by the PCR assay in the perennial wild rice (*Oryza rufipogon*) which inhabited in four isolated and six disturbed populations and in the weedy rice population. The level of clonality of the wild rice species was determined in populations subject to level of water supply and another disturbance. The results showed that all four isolated populations carried the genotype (-/-) and (-/+), while three genotypes (-/-), (-/+) and (+/+) was found on the six populations which grown near by rice fields. This finding was strongly supported the idea that the original wild rice populations of *O. rufipogon* exhibited prominent genotype (-/-) and (-/+) and mainly propagated by vegetative reproduction and the allele (+) which found in the wild rice plant with the genotype (+/+) may originated from gene flow from cultivated rice to wild rice. Weedy rice accessions used in this study showed the three genotypes based on this DNA locus. The distribution of this DNA locus in wild rice and weedy rice populations were deviated from the Hardy-Weinberg equilibrium. The perennial wild rice populations were annually under season drought (March to May of the year in Thailand, Laos and Cambodia), they tended to have small size clones with relatively high clonal diversity (i.e., number of genotypes), except for the population from Cambodia, which carried only the genotype (-/+). Although DNA maker used to detect genetic variation at population levels is too small, but this locus is very sensitive enough to be a useful indicator for genetic variation at the population level.

Key words: Wild rice, weedy rice, retrotransposon, clonality, gene flow

INTRODUCTION

Retrotranspons include long terminal repeat (LTR) and non-LTR retrotranspons. LTR retrotransposons are divided into two major superfamilies, Copia and Gypsy and include terminal-repeat retrotransposons in miniature (TRIM) elements which lack the coding regions required for mobility (Witte *et al*., 2001). Non-LTR retrotransposons include long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) (Kumar and Bennetzen, 1999). The short interspersed nuclear elements (SINEs) replicate via., RNA by RNA polymerase III and are distributed widely in higher eukaryotes (Deininger, 1989). SINEs are abundant in higher plant genomes and therefore these elements were considered effective tools for investigating the evolution of eukaryotes, such as rice (Xu and Ramakrishna, 2008; Krom *et al*., 2009) foxtail millet (Kawase *et al*., 2005).

The short interspersed elements (SINEs) are retrotransposons and are considered as effective tools for investigating the evolution of eukaryotes. The p-SINE1 family was found as a plant SINE in rice (Umeda et *al*., 1991; Mochizuki *et al*., 1992). The p-SINE1-r2, one of this SINE family, was found in the 10th intron of *waxy* gene of *O. sativa* (Umeda *et al*., 1991). In a earlier study, p-SINE1-r2 was integrated into the wx locus after *O. sativa* and *O. rufipogon* had diverged from other species with the AA genome (Hirano *et al*., 1994). The p-SINE1-r2 showed polymorphisms among the genus *Oryza*, but in terms of *O. rufipogon*, no tendency associated with annual-perennial differentiation was found (Hirano *et al*., 1994).

To explain the different patterns of presence and absence of p-SINE1-r2 in the *waxy* gene among populations of the two Asian wild rice and weedy rice which possessing AA genome (*O. nivara*, *O. rufipogon* and *O. sativa f. spontanea*), correlation of insertion polymorphism of this element with rice species was interested to investigate. Since, the wild rice found in mainland Southeast Asia has an annual (i.e., *O. nivara* and *O. sativa f. spontanea*) or perennial (*O. rufipogon*) life-history cycle that includes seed reproduction for *O. nivara* and *O. sativa f. spontanea*), clonal-

generation/seed-reproduction in *O. rufipogon*. Since, these rice species showed different breeding systems, *O. nivara* is a predominantly autogamous/self-pollination crop, which limits gene flow. While the other two species is predominantly outcrossing species. In a earlier study, as suggested that genetic differentiation is expected to be derived from self-pollination species as compared to out-crossing species (Garris *et al*., 2005; Londo *et al*., 2006). This pattern raises the question of the genotype diversity in wild rice and weedy rice populations which possessing different mating systems.

The perennial *O. rufipogon* possess a capacity for sexual reproduction by vegetative propagation (or clonality) and seed production. In Thailand, this wild rice species propagates mainly by asexual means (ratooning) (Barbier, 1989). Xie *et al.* (2001) reported that the populations under serious disturbance or seasonal drought tended to have small clones with relative high clonal diversity caused by sexual reproduction, whereas the populations with little disturbance and sufficient supply of water were prone to have large clone with relative low clonal variation and low sexual reproduction. Based on results of this study they recommend that an interval of >12 m should be required for collecting samples for *ex situ* conservation and for population genetic studies to capture possible genetic diversity for *O. rufipogon* in China.

In Thailand, no studies have been carried out to clarify of clonality of the perennial wild rice. As suggestion by Xie *et al.* (2001) is that an understanding of clonality is critical for implementation of the most appropriate conservation management of threatened clonal plant. Hence, in this study, co-dominant DNA marker, p-SINE1-r2 was used to detect clonal diversity in the perennial wild rice at population levels and to clarify and compare the tendency of the distribution of this element in natural wild rice populations in both isolated populations (original wild rice populations) and wild rice populations grown near by rice fields.

MATERIALS AND METHODS

During the 2006-2008 field survey, ten populations of wild rice, *O. rufipogon* were random collected of their leaves from each of ten populations studied, a total of 201 strains of the wild rice species were collected and used in this study. Among the ten populations, four populations are typical or original wild rice population. There are two populations from Laos were located in forested swamps of the Northern Vientiane Plain, the Lao PDR. One population located in a forested swamp of Savannaket Province, the Lao PDR and one population was found in a swamp of Siem Reap Province, Cambodia. The other five wild rice populations grown near by rice fields. These collection sites are shown in Table 1. Due to the perennial type *O. rufipogon*, grown in swamps that it was obviously the dominant plant species throughout the year. Therefore, to avoid the risk of collecting two samples from the same mother plant (genet), a distance of approx. 3 to 5 m was left between samples. This caution was taken for

Table 1: Collection sites of *Oryza rufipogon* and weedy rice (*O. sativa f. spontanea*) and distribution of p-SINE1-r2 in each population

			Genotype		
Population (N)	Habitat and population status	Locality (N/E)	(-/-)	(-/+)	(+/+)
O. rufipogon					
SWK (36)	Forested swamp, isolated from rice fields	Savannakhet Province, Laos (16° 45.25'/105° 28.9')	18	18	0
VTP1 (34)	Forested swamp, isolated from rice fields	The Vientiane Plain, Vientiane, Laos (18° 10.25'/102° 37.9')	12	22	0
VTP2 (12)	Forested swamp, isolated from rice fields	The Vientiane Plain, Vientiane, Laos (18° 10.25'/102° 25.7')	12	0	0
VTP3 (13)	Paddy canal, <5 m apart from rice fields	The Vientiane Plain, Vientiane, Laos (8° 15'/102° 24.8')	1	6	6
PPH (24)	Mountain marsh, <10 m apart from rice fields	Sakon Nakhon Province, Thailand (17° 03'/103° 42')	4	16	4
SRP (6)	Marsh, isolated from rice fields	Siem Reap Province, Cambodia (13° 41'/103° 81')	0	6	0
MSK (19)	Pond, <10 m apart from rice fields	Maha Sarakham Province, Thailand (15° 27'/103° 24')	14	2	3
CHR (23)*	Swamp, <5 m apart from rice fields	Chiang Rai Province, Thailand (19° 30'/099° 48')	0	23	0
NKP (13)*	Swamp, <5 m apart from rice fields	Nakhon Phanom Province, Thailand (17° 23'/104° 44')	7	6	0
SKN (21)*	Swamp, <20 m apart from rice fields	Sakon Nakhon Province, Thailand (17° 07'/104° 13')	7	14	0
O. sativa* f. *spontanea					
TKR (82)	Co-existing with cultivated rice	Maha Sarakham Province, Thailand (15° 30'/103° 32')	6	26	50

*From a earlier results by Prathepha (2008)

each population sampled. Fresh leaves of the perennial type were sampled at 3 or 5 m intervals along one or two transects. For weedy rice sample, flag leaves of 82 accessions of weedy rice (*O. sativa f. spontanea*) from six different paddy rice fields in the northeastern Thailand as described by Prathepha (2009) were collected and used in this study.

DNA extraction and PCR assay: Total genomic DNA was extracted from fresh leaves using a CTAB procedure according to Doyle and Doyle (1987). Polymerase chain reaction was performed to detect the presence or absence of p-SINE1-r2 at the *waxy* locus followed protocols described by a earlier report (Prathepha, 2008). The primer pair for a PCR assay (forward: 5'-GGAGGACGTGCAGATCGTTC-3' and reverse: 5'-ACGAGTCCACCGGTGGACGC-3') was used. This primer pair was made based on nucleotide sequence flanking p-SINE1-r2 and was expected to amplify the fragment including p-SINE1-r2. Some earlier reports have shown that this polymorphism can be detected by PCR assay (Hirano *et al*., 1994; Yamanaka *et al*., 2003; Prathepha, 2008). The PCR was run on a Thermocycler in a reaction volume of 20 µL containing 10 pg of each primer, 10 ng DNA template, 0.2 mM of each dNTPs, 2 mM $MgCl_2$, 0.5 units of Tag DNA polymerase. The PCR amplification of 30 cycles were run with the program: 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C and a final 5 min extension at 72°C followed. A negative control, in which template DNA was omitted, was included with every run in order to verify the absence of contamination. The amplified DNA fragments corresponding to the expected size (ca. 650 and 450 bp) were electrophoresed through 2% agarose gel (Promega Corporation, Madison, Wisconsin, USA) containing 0.5 µl mL^{-1} ethidium bromide and were visualized and photographed on an UV transilluminator. The molecular weight of amplified DNA fragments were estimated by using a 100 bp DNA Ladder (Pharmacia Biotech, SE-751 84, Uppsala, Sweden).

Statistical analysis

Clonal diversity analysis: Two different measures (G/N and D) of clonal diversity were used in this study. The number of genotypes was determined for each population of wild rice. Each genotype was regarded as representing one genet. The G/N was calculated for each population, where G is the number of genets and N is the number of ramets sampled (McClintock and Waterway, 1993). Genet size was defined as the number of ramets per genet in the population samples. Simpson's diversity index (D) corrected for finite sample sizes (Pielou, 1969) was calculated for each population as:

$$D = 1-\Sigma [N_j(N_j-1)/N(N-1)]$$

where, N_j is the number of samples of the *j*th genotype and N is the sample size. This index was originally developed as a measure of species diversity and evenness and has also been employed to measure the clonal diversity within a population (McClintock and Waterway, 1993; Ge *et al*., 1999).

Frequency distribution of p-SINE1-r2 at population levels: Individual genotype of rice samples were scored for the locus. The distribution of the p-SINE1-r2 element in wild rice populations and weedy rice was calculated. Deviations from Hardy-Weinberg expectation were analyzed by the chi-square test. In a diploid organism like the perennial wild rice and weedy rice with the AA genome, at a given locus (i.e., p-SINE1-r2), there are three possible genotypes: *absence/absence* (-/-), *absence/presence* (-/+) and *presence/presence* (+/+). In this study, *p* represents the frequency of *absence* and *q* represents the frequency of *presence*. Hence the genotype frequencies as follows: $(p)(p)$ or p^2 for (-/-), $(q)(q)$ or q^2 for (+/+) and $2(p)(q)$ for (-/+). The equation for genotype frequencies is $p^2+2pq+q^2 = 1$.

RESULTS

Presence and absence of a retrotransposon (p-SINE1-r2) in *waxy* gene of wild rice and weedy rice: Among the 201 perennial wild rice plants examined for presence of the p-SINE1-r2 in their genome, three genotypes (i.e., non-presence homozygous (-/-), heterozygote (-/+) and presence homozygous (+/+) were detected (Fig. 1). Frequencies of the presence of this element showed

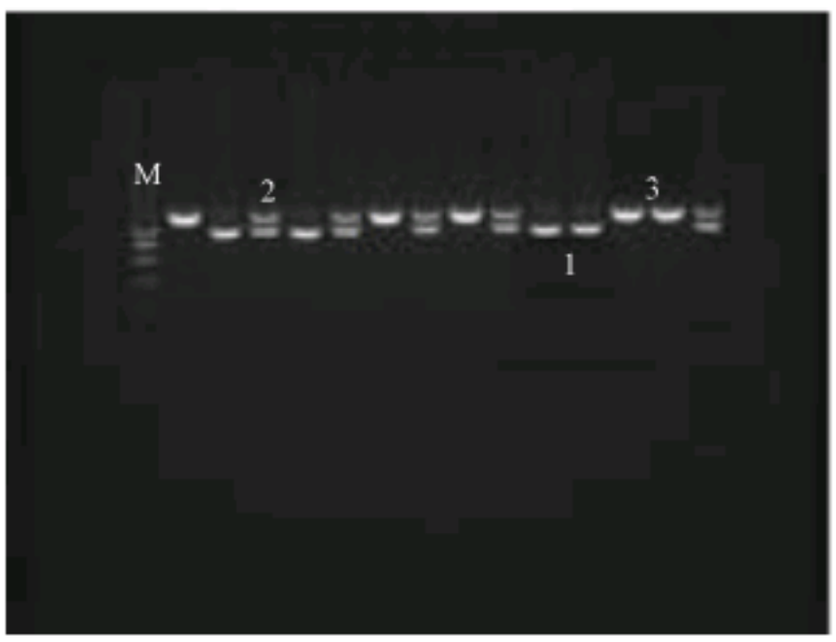

Fig. 1: The presence or absence of p-SINE1-r2 at the waxy gene in the perennial wild rice *O. rufipogon* strains. Three genotypes, 1: absence/absence (450/450); 2: absence/presence (450/650) and 3: presence/presence (650/650) were indicated in photo. The leftmost lane (M) is DNA molecular weight markers

Table 2: Clonal diversity within ten populations of the perennial wild rice *O. rufipogon* and weedy rice population

Populations	N	G	G/N	D
O. rufipogon				
SVK	36	2	0.056	0.52
VTP1	34	2	0.058	0.48
VTP2	12	1	0.083	0.00
VTP3	13	3	0.231	0.62
PPH	24	3	0.125	0.53
SRP	6	1	0.167	0.00
MSK	19	3	0.158	0.02
CHR	23	1	0.043	0.00
SKN	23	2	0.095	0.55
O. sativa* f. *spontanea				
TRK	82	3	0.037	0.55

N: No. of ramets sampled, G: No. of different genotypes, G/N: Clonal diversity, D: Simpson diversity index

values of 0.35 and 0.77 for populations of *O. rufipogon* and *O. sativa* f. *spontanea*, respectively. Test of goodness-of-fit was performed to assess the evolutionary processes influencing this DNA locus. The test indicated the evolutionary processes did have a significant influence ($\chi^2 = 14.6$, $p<0.01$ and $\chi^2 = 5.6$, $p<0.05$). This can be indicative that this DNA locus of wild rice and weedy rice populations were deviated from Hardy-Weinberg equilibrium (HWE).

Clonal diversity: *Oryza rufipogon*, the spatial pattern of clonal diversity (G/N) varied little (0.04 to 0.17) among ten populations studied (Table 2). All populations had a low levels of clonal diversity. The number of genet per sampled ramets was very low, on average 0.10, although there were greatly differences between ramet-level (ranged from 6 to 36) and genet-level (ranged from 1 to 3) calculations. Furthermore, Simpson diversity index (*D*), ranging from 0 to 0.62 where high values indicate high levels of genotype diversity.

Oryza sativa f. *spontanea*, three genotypes were detected among the 82 weedy rice plants examined. The weedy rice population had a low levels of clonal diversity ($G/N = 0.037$), while genotype diversity value was of 0.55.

DISCUSSION

Among the ten populations of the perennial wild rice, four populations were found in forested swamps in the Lao PDR (SVK, VTP1, VTP2) and Cambodia (SRP). These populations were distributed in swamps where isolated from rice fields. It is therefore these populations seem to be lacking gene flow between wild rice and cultivated rice due to their habitats were recognized as isolated populations. Interestingly, two genotypes (-/-) and (-/+) were found in these four populations. In contrast, the remaining six populations (VTP3, PPH, MSK, CHR, NKP and SKN) which growing adjacent to rice paddy fields showed three genotypes (-/-), (-/+) and (+/+). Regardless of the problems associated with collection methods, the perennial wild rice that carried the genotype (+/+) may have been caused by a gene flow from cultivated rice to wild rice. In Southeast Asia, gene flow from cultivated rice to wild rice had been reported in Thailand and the Lao PDR (Akimoto *et al*., 1999; Kuroda *et al*., 2005). The evidence is that the six populations carried the genotype (+/+). From earlier studies, rice cultivars examined for the p-SINE1-r2 polymorphism showed only the genotype (+/+) (Prathepha, 2003). Taken together, this finding was strongly supported the idea that the original wild rice populations of *O. rufipogon* exhibited prominent genotype (-/-) and (-/+) and mainly propagated by vegetative reproduction as proposed by Prathepha (2008). Whereas, the populations which are adjacent to rice paddy fields carried the three genotypes. Hence, the (+) allele found in the wild rice plants with the genotype (+/+) was originated from gene flow from cultivated rice to wild rice and/or gene flow between wild rice plants which carried the genotype (-/-) and (-/+), resulting in wild rice progenies with only both genotypes (-/-) and (-/+) in populations. Historically, populations of the wild rice species *O. rufipogon* sampled from Thailand were characterized by a predominant to exclusive use of asexual reproduction and the outcrossing rate are very high based on isozyme analysis (Barbier, 1989).

Based on the insertion of p-SINE1-r2, the three genotypes observed in weedy rice population pose an intriguing question about the source of the underlying phenotypic variation found in these weedy rice plants as previous reported by Prathepha (2009). Based on different genotypes in weedy rice population, variation in the weedy rice may have been used to classified between cultivated rice population and weedy rice population. As described above that all cultivated rice carried only the genotype (+/+), whereas weedy rice population showed all three genotypes.

Implications for conservation of the perennial of wild rice populations: The perennial wild rice *O. rufipogon* reproduced by both seeds and horizontal stems. In Thailand, it propagates mainly by asexual reproduction (ratooning) (Sano and Morishima, 1982). In China, forming new stem and adventitious roots the horizontal stems of this wild rice species extended in water or underground for up to 5 m (Xie *et al*., 2001). Based on filed observations in Thailand and the Lao PDR, in the dry season (from February to April of a year) the ten wild rice populations surveyed and leaves collection for used in this study were disturbed by grazing of cattle of local people that living near by the wild rice habitats. In

Fig. 2: A natural habitat of the perennial wild rice *Oryza rufipogon* Griff. located on Sakon Nakhon Province, during the dry season (a) and the rainy season (b)

addition, seasonal fluctuation of water level between dry season and wet season (from March to October of a year) as shown in Fig. 2. Thus, it is difficult for them to produce large dominant clones. This wild rice may have prominent seed producing rather than vegetative reproduction. In the present study, an interval of 3 to 5 m be used for collecting sample result in collection of complete distinct three genotypes (-/-), (-/+) and (+/+), although there are small sample size such as population PPH (n = 24) and MSK (n = 19). The population SRP (n = 6) found only one genotype (-/+), this may reflect to low genetic diversity in this population. According to results of this study, implication for conservation of *O. rufipogon in situ*. These populations showed distinct genotypes based on the insertion of p-SINE1-r2 in intron 10 of the *waxy* gene of rice, although a locus used is too small to be detected by examining the population level of genetic variation, the different number of genotypes of this locus may be sensitive enough to be a useful indicator for genetic variation at the population level. This idea is supported the suggestion by Yamanaka *et al.* (2003) is that polymorphism of p-SINE1-r2 is useful for evaluation of both inter and intra population diversity, because the heterozygotes are clearly distinguishable.

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