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Microsatellite analysis of weedy rice (*Oryza sativa* f. spontanea) from Thailand and Lao PDR

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Abstract

Weedy rice (*Oryza sativa* f. spontanea) is one of the most notorious weeds occurring in the rice fields of Thailand and Laos. Genomic research can be applied to improve understanding of weedy rice biology. The purpose of this study was to evaluate the genetic diversity of a collection of weedy rice from Thailand and Laos. Ninety-nine weedy rice accessions from four populations were evaluated by means of four SSR markers. A total of 49 alleles were detected. The number of alleles per locus ranged from 3.6 to 8.7, with an average of 7.6. The overall genetic diversity of weedy rice populations was relative high (H_s=0.619). The genetic differentiation among the four populations showed that genetic variability mainly existed among weedy rice individuals rather than among populations in all four populations. Results from the present study are presenting a clearer understanding of the genetic diversity of weedy rice in Thailand and Laos.

Keywords: *Oryza sativa* f. spontanea, weedy rice, microsatellite, genetic diversity.
Abbreviation: *SSR*, Simple Sequence Repeat, *MCMC*, Markov Chain Monte Carlo, *PCR*, Polymerase Chain Reaction. *RM*, Rice Microsatellite.

Introduction

Weedy rice (*Oryza sativa* f. *spontanea*) distributes over a wide area in rice-growing regions around the world (Suh et al., 1997). Weedy rice is taxonomically classified as the same species as cultivated rice (*O. sativa*), but is strongly characterized by its seed shattering and dormancy, which apparently increase the distribution of this species. As a notorious weed occurring in rice fields, it commonly causes yield reduction and affects the quality of rice grains (Hoagland and Paul, 1978). Most weedy rice strains possess seeds with red pericarps; thus it is also referred to as red rice (Gealy et al., 2003) although some strains have white pericarps (Arrieta-Espinoza et al., 2005; Prathepha, 2009a). Long-term sympatric distribution has led to similarities between weedy rice and cultivated rice through natural hybridization and introgression, making the control of weedy rice very difficult when compared with other weeds, resulting in difficulties in managing weedy rice. Genomics research will improve our understanding of the biology of weed populations and help us to predict and document the results of gene transfer between species (Weller et al., 2001; Basu et al., 2004). In addition, information generated in genomics studies coupled with molecular biology will allow an increase in the understanding of weeds, including the population biology of weeds and the development of novel approaches to weed management. From the viewpoint of weedy rice management, it is preferable to identify genetic diversity. Appropriate management of weeds occurring in agroecosystems will harmonize the systems and enhance the sustainable crop production. A central characteristic of weeds, which contributes to their success in agroecosystems, is the genetic variability and plasticity found within and among weed populations (Green et al., 2001). This enables weeds to infest a wide range of diverse habitats. Thus, a full understanding of the genetic diversity of weeds is a major prerequisite for their effective management. In addition, elucidating the origin and evolutionary processes of weeds is helpful for designing effective management strategies for weed control (Pysek and Prach, 2003). The excellent attributes of DNA markers and the availability of 2240 microsatellite markers in rice (McCouch et al., 1997; McCouch et al., 2002) make it possible to obtain this information. In addition, DNA markers were applied to evaluate genetic diversity of plant germplasm (Zarkti et al., 2010; Keivani et al. 2010).

Simple sequence repeats (SSRs) or microsatellites are stretches of DNA, consisting of tandemly repeating mono-, di-, tri-, tetra- or penta-nucleotide units that are widely distributed across all eukaryotic genomes (Powell et al., 1996). In addition, the uniqueness and value of microsatellites arises from their multiallelic nature, codominant transmission, and ease of detection by PCR. Microsatellite markers for cultivated rice have been developed which were available of 2240 microsatellite markers (McCouch et al., 2002). The utility of microsatellites arises from two main factors: their high information content (i.e., the number and frequency of alleles detected); and ease of genotyping. To date, an evaluation of the amount of diversity detected with microsatellites has revealed more polymorphism compared with other assay procedures (Powell et al., 1996). For *Oryza* AA genome species, microsatellite markers have been applied to detect genetic variation in the perennial wild rice (*Oryza rufipogon*) (Kuroda et al., 2007; Gao, 2004; Song et al., 2003), in cultivated rice (*O. sativa*) (Jayamani et al., 2007; Giarrocco et al., 2007; Garris et al., 2005; Ni et al., 2002;), in weedy rice (*O. sativa f. spontanea)* (Yu et al., 2005; Cao et al., 2006).

Weedy rice are classified into two categories: (i) one occurring together with common wild rice and (ii) the other

Table 1. Description on of weedy rice (*Oryza sativa* f. spontanea) populations analyzed using microsatellite loci. A_R, allele richness;

Population/code (N)	Location (N/E)	Habitat and population status	No. of alleles per locus	A_R	H_s	F_{is}
The Vientiane plain, Laos LAOS (no. 1-22)	Na Phaeng village (18°21.74'/102° 38'), Vientiane Province	Marsh, < 5 m apart from rice fields (large population, >500 m^2)	4.6	4.55	0.579	-0.067*
Central region, Thailand PCH (no. 23-51)	Pluak Suk section (16° 31/100° 08'), Phichit Province	Co-exist in rice fields (large population, > 500 m^2)	7.6	7.05	0.715	0.001*
North-eastern region, Thailand MSK (no. 52-90)	Thungkula Ronghai (15° 30'/103° 32'), Mahasarakham Province	Co-existed in rice fields (large population, >500 m^2)	8.7	5.22	0.589	0.618
UDN (no. 91-99)	Muang Udon Thani, Road no. 2, Udon Thani Province (17° 31'/102° 48')	Canal, < 5 m apart from rice fields(small population, <25 m^2)	3.6	2.82	0.594	0.518

*H*s, genetic diversity; F_{is}, Wright's inbreeding coefficient
* Significant at the 5% level

distributed in regions where no wild rice occurred (Oka, 1988). A major hypothesis for the mechanism of production of weedy rice in South and Southeast Asia is hybridization between cultivated rice and wild rice *(O. rufipogon*), however, weedy rice can often be found outside the range of *O. rufipogon* , such as in North and South America (Londo and Schaal, 2007). The weedy rice in the Mekong River regions showed different origins. In China, weedy from the lower Yangtze valley may be the result of natural hybridization between japonica cultivar and japonica-like wild rice weedy rice (Tang and Morishima, 1997), and some weedy rice populations most probably originated from local rice varieties by mutation and intervarietal hybrids (Cao et al., 2006). In Thailand, the weedy rice might originate from the introgression between cultivated rice and *O. rufipogon*, which often takes place in nature, mostly in a one-way process from cultivated rice to *O. rufipogon* (Prathepha, 2009a). The emergence of weedy rice in the rice growing areas of the Mekong River Basin has already been reported (Yu et al., 2005; Isshiki et al., 2005; Kuroda et al., 2007; Prathepha, 2009a) as shown in Fig. 1, knowledge of its genetic diversity along with procedures and mechanisms of its reoccurrence in these areas is still limited, which obstructs methods for weedy rice management. In order to fill the knowledge gaps, it is necessary to study weedy rice populations occurring in Thailand and Laos. This will enable us to understand the level and distribution of the genetic diversity of weedy rice samples which were collected from the central plain of Vientiane, Laos , and the northeastern and central regions of Thailand.

Materials and methods

Plant materials for SSR analysis

Weedy rice samples were gathered between March to October 2008-2009. The weedy rice samples were collected from the central plain of Vientiane of Laos and the northeastern and central regions of Thailand (Table 1). The precise collection sites were documented with Global Positioning System (GPS) from GARMIN (iQue 3600), Garmin Co. Ltd.). Leaf samples of the weedy rice samples were collected from a cultivated rice field in the Vientiane plain of Laos. In the northeastern region of Thailand, three rice fields (infested by weedy rice) with a distance of at least 50 km from each other (Mahasarakham, Roi Et and Udon Thani province) were randomly selected for leaf sampling within each rice field. Weedy rice individuals were randomly sampled from a cultivated rice field of Phichit province in the central region of the country. All individuals from the same field were regarded as members of one population. A total number of 99 individuals were sampled from different populations and four microsatellite loci were used to analyze the samples. Our hypothesis is that weedy rice from different populations could be genetically structured along its area of distribution.

Genomic DNA extraction and microsatellite genotyping

Genomic DNA was extracted by 1% CTAB with a modification of the method of Doyle and Doyle (1987), based on their relatively high allelic polymorphism of microsatellites as suggested by Song et al. (2006). A total of four microsatellite primer pairs (RM84, RM167, RM180 and RM211) (Table 2) were selected as a subset of SSR markers previously used to assay genetic diversity of wild rice and weedy rice by specific polymerase chain reaction (PCR) conditions (Song et al., 2003; Song et al., 2006; Cao et al., 2006). Detailed information of primer sequences is available at http://www.gramene.org/microsat/ssr.txt. The PCR reactions were carried out in a volume of 20 µL containing 1x buffer, 1 mM each of dATP, dCTP, dGTP and dTTP, 2 mM$MgCl_2$, 10 mM of SSR primer, 50 ng of genomic DNA and 1 unit of *Taq* polymerase (Promega). The polymerase chain reactions were performed using the following cycle: 94 °C for 4 min and followed by 36 cycles of 40 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C, and 10 min at 72 °C for the final

Table 2. SSR primer pairs used for weedy rice (*O. sativa* f. spontanea) DNA amplification in this study. The annealing temperatures for all were 55˚C

Primer code	Chr[a]	SSR motif	No. of alleles	Forward	Reverse
RM84	1	(TCT)10	9	*5'-taagggtccatccacaagatg-3'*	*5'-ttgcaaatgcagctagagtac*-3'
RM167	11	GGAA(GA)16GGGG	11	*5'-gatccagcgtgaggaacacgt*-3'	*5'-agtccgaccacaaggtgcgttgtc*-3'
RM180	7	(ATT)10	22	*5'-ctacatcggcttaggtgtagcaacacg*-3'	*5'-acttgctctacttgtggtgagggactg*-3'
RM211	2	(GA)18	7	*5'-ccgatctcatcaaccaactg*-3'	*5'-cttcacgaggatctcaaagg-3'*

[a]Chromosome number

extension. The PCR products were separated on 6 % polyacrylamide denaturing gels and silver stained. Migration distance of each allele was determined by comparing with a known molecular-weight standard (PUC 19 DNA digested by *Msp*I) after performing electrophoresis.

SSR genotyping score and statistical analysis

For each SSR primer, the amplified SSR DNA bands representing different alleles were scored as different genotypes (Fig. 2). As a result, the bands were recorded as homozygous genotypes or heterozygous genotypes. Genetic polymorphisms for each locus were computed. These parameters include mean number of alleles per locus (*A*), the percentage of polymorphic loci (*P*), mean number of alleles per polymorphic locus (A_p), observed heterozygosity (H_o), expected heterozygosity (H_e). The Wright's *F* statistics (F_{is}, F_{it} and F_{st}) (Wright, 1978) were computed for polymorphic loci to test for the departure from Hardy-Weinberg equilibrium and to estimate genetic differentiation among weedy rice populations. The testing of the F_{is} value to determine whether each population was in deficit or excess in heterozygote was calculated (iterations=1000 and 95% confidence interval). All calculations were performed using FSTAT version 2.9.3.2 (Goudet, 2001).

For structuring population, a model-based method was used for delineating clusters of weedy rice individuals on the basis of their genotypes at multiple SSR loci. The Bayesian approach is implemented in the program STRUCTURE version 2.1 (Pritchard et al., 2000) (http:// www. pritch.bsd.uchicago.edu). A series of tests was performed using different numbers of population clusters (*K*=1 to 6), each with five independent runs to guide an empirical estimate of numbers of identifiable populations without consideration of sampling origins. For each run, the admixture model, without prior population information, was applied under the condition of 5000 Markov Chain Monte Carlo (MCMC) replication followed by a 10000 burn-in (iteration) period. Number of clusters (*K*) was inferred when a consistent result was obtained among five independent runs of the admixture analysis. Each test yielded a log-likelihood value of the data (in probability), with the highest indicating which test was closest to the actual number of genetically distinct populations. Individuals were assigned probabilistically to a population or to multiple populations if their genotype profile indicate admixture (Weckworth et al., 2005).

Results and discussion

Microsatellite diversity within populations

In this study, three weedy rice populations from Thailand (the central plain of Thailand (Phichit province, Thungkularonghai, Mahasarakham province and Udonthani province) and one population from the Vientiane plain of Laos were determined for genetic variation by detecting the 99 samples over four SSR loci (RM84, RM167, RM180 and RM211). A total of 49 alleles were detected from these samples. Average over the five loci, the number of alleles per locus was 4.6 - 7.6 at the populations level (Table 1). Allelic richness (A_R) was at a maximum value of 7.05 in the population from the central region of Thailand. Coincidently, the population from the central region of Thailand carried the highest genetic diversity (*H*) value (0.715). The fixation index (F_{is}), a measure of heterozygote deficiency, statistically deviated from zero in the Vientiane plain, Laos and the central region, Phichit province, Thailand (Table 1), which indicates that these two populations deviated from the Hardy-Weinberg expectation. The number of alleles observed per locus in samples of 99 individuals ranged from 7 to 22, with an overall total of 49 alleles scored over the four SSR loci. The four SSR loci determined in this study were polymorphic in all four populations (Table 3). The SSR locus RM 180 showed the most polymorphic locus among the four loci. Genetic diversity of the four populations (*Hs)* ranged from 0.493 to 0.778. The average genetic diversity within population (*Hs* =0.598) was higher than that of the observed heterozygosity (*Ho*=0.445). The number of alleles generated by each of the four SSR primers used in this study (RM84, RM167, RM180 and RM211) showed a higher number of alleles than that previously reported by Song et al. (2003); Song et al. (2006) and Cao et al. (2006). In addition, all SSR loci demonstrated that weedy rice populations used in this study possessed relative high genetic diversity (H_s) , ranged from 0.493 to 0.778 (Table 3).

Table 3. Alleleic diversity of microsatellite loci scored in weedy rice. H_o, observed heterozygosity; H_t, genetic diversity in all populations; H_s, genetic diversity within population

Locus	*Ho*	*Hs*	*Ht*
RM84	0.493	0.577	0.658
RM167	0.441	0.542	0.647
RM180	0.488	0.778	0.965
RM211	0.359	0.493	0.695
Overall	0.445	0.598	0.742

This result agreed with that of a previous study by Cao et al. (2006), where high genetic diversity was found in weedy rice from Liaoning Province, China. This finding implies that these weedy rice populations come from areas in which genetic diversity existed. Weedy rice populations used in this study showed that high genetic diversity might be supportive of the viewpoint of Cao et al. (2006) as follows: 1) different farming practice (e.g. the strength of weedy rice control), seed sources (e.g. company-supplied or self-maintained seeds) and the number of rice varieties used in different

Fig 1. Sympatric and allopatric weedy rice populations found in cultivated rice fields in Thailand.

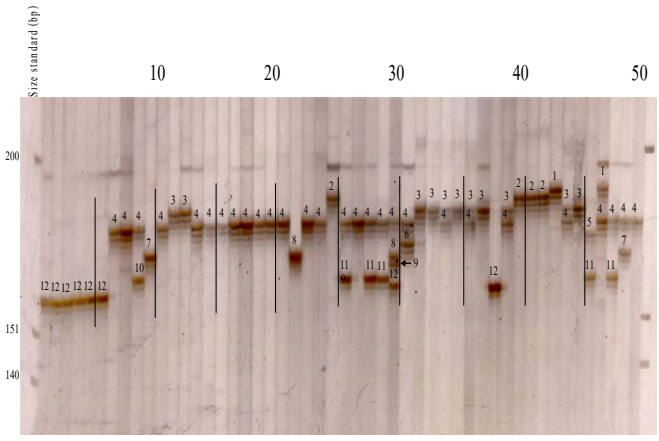

Fig 2. Example of amplified DNA of weedy rice accessions using microsatellite primer RM167 after resolution in 6% polyacrylamide gel and scored for each genotype.

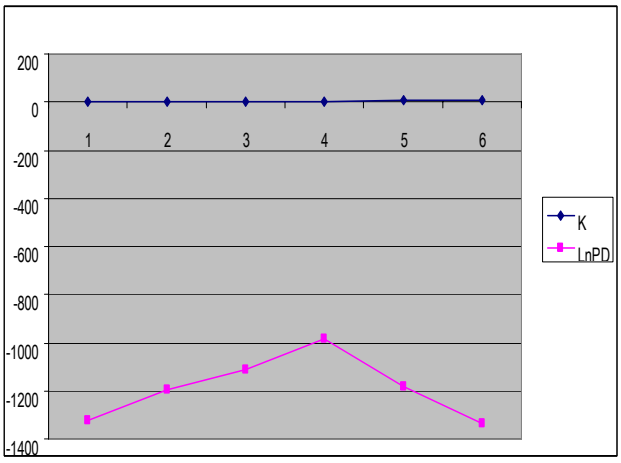

Fig 3. Plot of the log probability of the data at different values of number of six populations (*K*=6) using STRUCTURE software. At *K=3* was selected as the smallest value of *K* that captures most of population structure.

regions; and 2) the origin of weedy rice from different procedures (e.g. mutation or hybridization segregation).

Genetic differentiation among weedy rice populations

The F_{st} measures the genetic differentiation among the four populations and showed that genetic variability mainly existed among weedy rice individuals rather than among populations in all four populations (UDN-MSK, *Fst*=0.224, UDN-PCH, *Fst*=0.177, UDN-LAOS, *Fst*=0.26, MSK-PCH, *Fst*=0.164, MSK-LAOS, *Fst*=0.137 and PCH-LAOS, *Fst*=0.195). The overall loci investigated, a high variation in the genetic differentiation among populations (F_{st} = 0.179) indicates that 17.9% of the total variation existed among populations. The F_{is} values of weedy rice populations ranges from -0.067 to 0.618. Weedy rice populations from the central Vientiane plain and central region of Thailand showed negative and low positive values, which were close to zero, indicating these two populations' excess of heterozygosity and showing significant departure of allelic frequencies from the Hardy-Weinberg equilibrium. Whereas, the two populations from north-east region of Thailand showed high positive values (close to 1), indicating these populations lack of heterozygosity.

Cluster analyses

Using STRUCTURE software, this analysis tested the number of populations which best described the distribution of data into population clusters (*K*). The number of *K* ranged from 1 to 6, the average *Log*- likelihood values quickly decreased between *K*=3 (-1113.7) and *K=4* (-986.5), then the values moderately increased up to *K=5 (-1185.3)* and *K*=6 (-1332.9). Based on a "more-or-less plateaus" rule as previously reported by Thomson et al. (2009), because the *Log*- likelihood values began to plateau at *K=3* and *K=5* (Fig 3), the population number of *K=3* was selected as the smallest value of *K* that captures most of the population structure (Evanno et al. 2005). Underlying the STRUCTURE model used (Fig 4), at *K=3*, the weedy rice samples reveals three clusters/populations. Each of three clusters was largely composed of individuals from its original sampling. For example, cluster 2 (Fig 4A) consisted largely of weedy rice accessions from the central region (Phichit province) of Thailand, while clusters 1 and 3 were composed mostly of weedy rice accessions from the north-east region of Thailand (Mahasarakham and Udon Thani provinces) and the Vientiane plain of Laos. The analysis of the population structure of the 99 weedy rice accessions showed close genetic relationships among weedy rice samples from the north-east region of Thailand and Laos, when compared to those of the central plain of Thailand. Moreover, each cluster exhibited a highly admixed genetic constitution, probably derived from introgression (gene flow) from cultivated rice to sympatric weedy rice population that were actually found in Thailand and Laos (Prathepha, 2009b; Kuroda et al., 2007). According to the results of this study, Bayesian analysis of weedy rice population structure, using the model-based approach of Pritchard et al. (2000), provided support for the existence of a genetic structure in the weedy rice samples used in this study. The results revealed a stratification pattern mostly consistent with the observation of F_{is} values derived from *Wright's F* statistical analysis. In addition, weedy rice appears to have been derived from hybridization between domesticated *O. sativa indica* and wild *O. rufipogon* accessions in Thailand or Myanmar (Londo and Schaal, 2007). This viewpoint provides support for the 'hybrid origin',

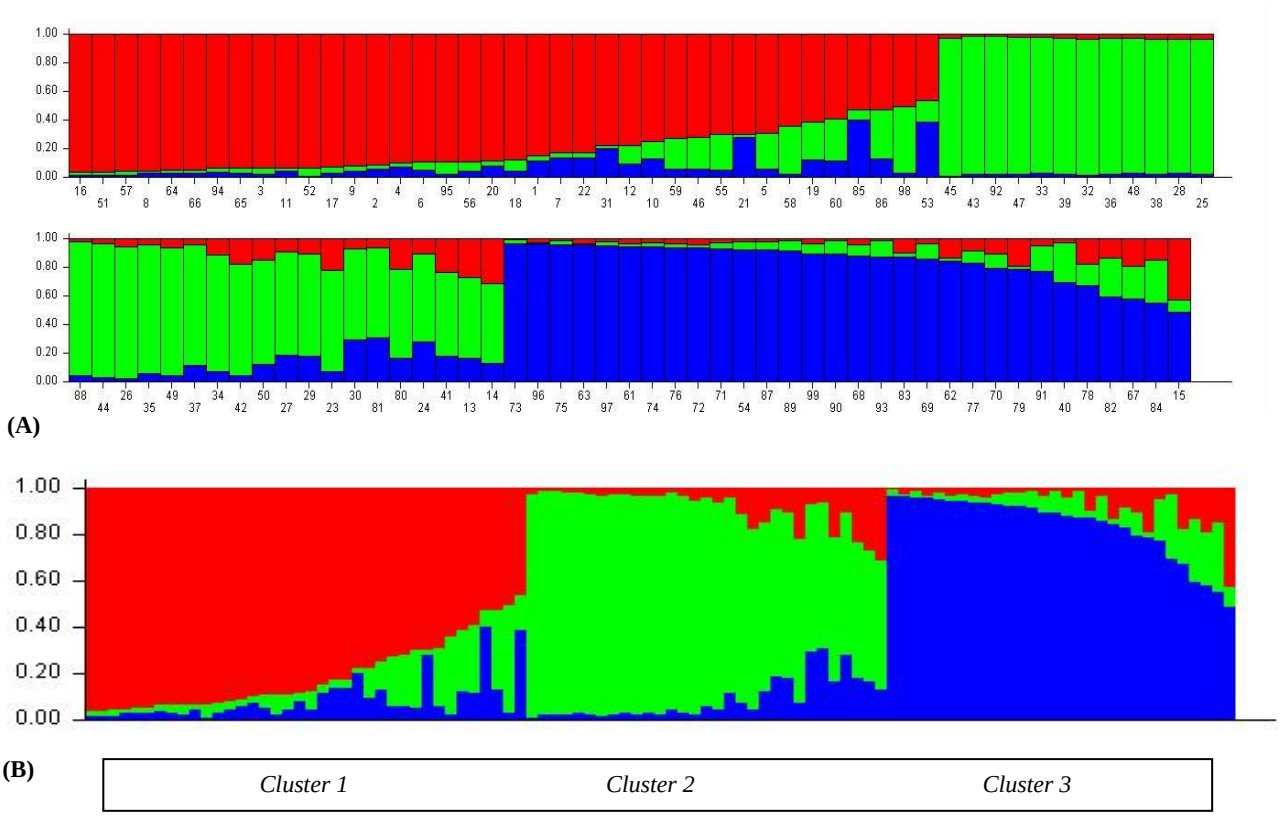

Fig 4. Histograms of STRUCTURE assignment test (A and B) at *K* =3. Each individual is presented by a thin vertical bar and individual code (A), which can be partitioned into *K* colored segments that represent the individual estimated membership to the *K* cluster. Each vertical bar has more than one color indicates admixed genetic constitution. Individuals are arranged in order by populations. Assignment tests for all 99 individuals of weedy rice, in which *K*=3 was selected that illustrates most of the weedy rice population structure.

hypothesis of weedy rice. Perennial wild rice, *O. rufipogon* can still be found at all the weedy rice sampling sites used in this study. Introgression from domesticated rice to wild rice may have resulted in weedy rice and allowed this weed rice to gain some traits from cultivated rice such as the *BADH2* gene from Hom Mali rice now widely spread in Thungkularonghai, northeastern Thailand (Prathepha, 2009b). The high potential for introgression into weedy varieties should be carefully considered when new transgenic genotypes of rice are generated. Transgenes may persist and disseminate within the weed rice or wild rice populations through sexual reproduction and/or vegetative propagation. If the transgenes are responsible for biotic and abiotic stresses, they then can enhance the ecological fitness of weedy rice and wild rice populations (Chen et al., 2004). In addition, gene flow between different weedy varieties may allow these advantageous traits to spread and combine into potentially more problematic phenotypes. Additionally, gene flow from wild rice may be important for introducing new traits into weedy rice (Londo and Schall, 2007). Improved knowledge of gene flow between weedy species and crops (introgression) and the effect on weed management will allow better understanding of the entire weed-crop agroecosystem and the genetic factor controlling the interactions that occur. Results from the present study should be interesting to evolutionary biologists, and uncovering the origins of weedy rice in Thailand and Laos is crucial to preventing the evolution of these weedy rice.

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