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Research Article

Genotype frequency of black hull locus *(Bh4)* in weedy rice (*Oryza sativa* f. *spontanea*) populations

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Abstract- Black hull 4 (*Bh4*), is a hull color candidate gene that causes a black pigmentation in the ripened hulls of rice. A 22-bp deletion within exon 3 of the *Bh4* variant disrupted the *Bh4* function, which lead to a straw-white hull in Asian cultivated rice and African cultivated rice. A survey for the *Bh4* locus in a collection of weedy rice from Thailand and Laos was performed. The data showed that 37.5% of the samples were heterozygous, 15.5% with the black hull genotype (BB) and 5.5% with yellow genotype (YY). The allele frequencies were 0.71 and 0.29 for the Y and B alleles, respectively. Based on the Hardy-Weinberg principle, this *Bh4* locus of weedy rice is in Hardy-Weinberg equilibrium (HWE).

Keywords: *Oryza sativa* f. *spontanea*, weedy rice, genetic diversity, black hull color

1. Introduction

Weedy rice (*Oryza sativa* f. *spontanea*) is found in cultivated rice fields worldwide. It is prolific in Asian countries, and in China it has spread rapidly in areas where direct seeding or related technologies have been adopted and accompanied by less weed management (Yu *et al.,* 2005). It causes yield reduction and affects the quality of the rice grains (Olofsdotter *et al.,* 2000). Weedy rice is classified as the same species as Asian cultivated rice (*O. sativa*), but it has morphological characteristics typical of wild rice species (e.g. seed dormancy and shattering) and cultivated rice (e.g. rapid growth and high selfing rate) (Reagon *et al*., 2010). Weedy rice is classified into two categories: (1) occurring together with common wild rice and (2) distributed in regions where no wild rice occurs (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 shows different origins. In China, weedy rice in the lower Yangtze valley may be the result of natural hybridization between a japonica cultivar and japonica-like wild weedy rice (Tang and Morishima, 1997) and some weedy rice populations most probably originated from local rice varieties by mutation and inter-varietal hybrids (Cao *et al*., 2006). In Thailand, 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, 2009). 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, 2009) as shown in Figure 1. Knowledge of weedy rice genetic diversity in these areas is still limited. Methods for weedy rice management should be considered on genetic data. Therefore, it is necessary to study weedy rice populations occurring in Thailand and Laos. These results will enable us to understand the level of genetic diversity and the evolution of the weedy rice in some areas of Laos plain and the northeastern and central regions of Thailand.

Among rice genes, some loci have been used for studying the genetic basis of evolution such as the seed shattering gene (*sh4*) in weedy rice populations (Zhu *et al*., 2012). Based on the *sh4* locus of a weedy rice sample, the result of a study showed that rice accessions with the seed-shattering phenotype had the mutational type “T” nucleotide instead of the wide-type seed shattering “G” nucleotide at *sh4*’s functional nucleotide polymorphism (FNP) site, whereas all cultivated rice accession samples with the non seed-shattering phenotype had the “T” nucleotide at *sh4*’s FNP. The recessive allele “T” for the non-seed shattering found in weedy rice samples may be the result of gene transfer from cultivated rice to weedy rice.

The hulls of cereal are considered to play a role in the protection of the seeds from physical and oxidative damages (Ramarathnam *et al*., 1987). In US and Thai weedy rice populations, two main morphological groups, including straw-hull (SH) type and black- hull (BH) type, have been reported (Thurber *et al.,* 2010; Prathepha, 2009).

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The genetic mechanism involved in the transition from the black-colored seed hull of the ancestral wild rice to the straw-white seed hull of cultivated rice during grain ripening was reported by Zhu *et al.* (2011). They reported that the black hull of *O. rufipogon* was controlled by the *Bh4* gene, which was fine-mapped to an 8.8-kb region on rice chromosome 4. The *Bh4* gene encodes an amino acid transporter. A 22-bp deletion within exon 3 of the *Bh4* variant disrupted the *Bh4* function, leading to the straw -white hull in cultivated rice. The black hull gene has been studied in terms of the parallel evolution of hull color variation in the domesticated and weedy rice systems (Vigueira *et al.*, 2013). In the current study the results show that independent *Bh4* coding mutations have arisen in African and Asian rice that were correlated with the straw hull phenotype and this indicated that the *Bh4* gene is responsible for parallel trait evolution. The most important part of this study is that the weed forms can draw on both ancestral genes and crop genes as these genes respond to the selection pressures exerted by factors of modern agriculture.

Recently, the most important finding about genes correlated with black hull of rice was reported by Fukuda *et al.* (2012). They found that only the plants that had both functional *Bh4* and *Phr1* genes produce black hull, which resulted from the analysis of segregating populations in crosses between *indica* and *japonica* rice.

As a notorious weed occurring in rice fields, weedy rice commonly causes yield reduction and affects the quality of the 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, 2009). Longterm sympatric distribution has led to similarities between weedy rice and cultivated rice through natural hybridization and introgression that make the control of weedy rice very difficult when compared with other weeds. This adds to the complexities of managing weedy rice.

Evolution of hull color in weedy rice populations was reported by (Vigueira *et al.*, 2013). They found that test for selection indicated that the straw hull of weedy rice deviates from neutrality at this gene. This means that there was possible selection on the hull color gene (*Bh4*) during both rice domestication and de-domestication. The black hull seeds of weedy rice fall off easily from the panicle of the rice plant at the maturation stage and a black hull color can protect them from being targeted by birds, as such the allele frequency of black hull color would increase in the next generation. Recent studies show that birds around the rice field play an important role in the selection of straw-white seed hulls in rice (Zhu *et al.*, 2011).

In this study, weedy rice was sampled from paddy fields in the northeastern and central regions of Thailand (Fig. 1) and from the central plain of Vientiane, Laos to determine how hull color variation in Thai and Laos weedy rice is based on the deletion of 22-bp in the third exon of the *Bh4* gene. The results will yield data on the genetic background and insights into the adaptive mechanisms of weedy rice in the paddy fields, which would have valuable implications for the evolution of weedy rice.

2. Materials and methods

2.1 Plant materials for genotype analysis

Rice samples consisted of a total of nine cultivated rice accessions, 112 weedy rice accessions, and four wild rice (*O. rufipogon*) accessions. The weedy rice samples were selected from the accessions used in previous studies. Flag leaves of weedy rice samples and their progeny were collected and seed morphological characters were identified and reported previously (Prathepha, 2009; Prathepha, 2011) (Table 1).

Table 1. Description of weedy rice (*Oryza sativa f. spontanea*) populations used in this study (Prathepha, 2011).

Population/code (N)	Location (N/E)	Habitat and population status
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)
Central region, Thailand/ PCH (no. 23-50)	Pluak Suk section (16° 31/100° 08’), Phichit Province	Co-exist in rice fields (large population, > 500 m^2)
North-eastern region, Thailand/ MSK (no. 51-90)	Thungkula Ronghai (15° 30’/103° 32’), Mahasarakham Province	Co-existed in rice fields (large population, >500 m^2)
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)

2.2 Genomic DNA extraction, PCR amplification and sequencing

DNA samples from flag leaves of each weedy rice plant were extracted using 1% CTAB following a modification to the method of Doyle and Doyle (1987). For the genotype analysis of the *Bh4* gene, the region including the 22 –bp deletion in the third exon was amplified using PCR with the forward primer: 5’-CTGCGCTTCACGTACCAGG-3’ and reverse primer: 5’-CGTCGAACTTGACGTACGT-GG-3’, as reported by Zhu *et al.* (2011) and Fukuda *et al.* (2012). The PCR reactions were carried out in a volume of 20 μL containing 1x buffer; 1 mM each of dATP, dCTP, dTTP; 2 mMMgCl$_2$; 10 mM of each forward and reverse primer; 50 ng of genomic DNA; and 1 unit of *Taq* polymerase (Promega). The polymerase chain reactions were performed using the following profile: 4 min at 94 °C for denaturation, followed by 36 cycles of 40 s at 94 °C, 30s at 55 °C and 40 s at 72 °C; and 10 min at 72°C for the final extension. The PCR products were separated within 2 % agarose gel electrophoresis and stained with ethidium bromide. The DNA size marker was a commercial 100 bp ladder (BioLabs, MA). Using these primer pairs, DNA templates from the yellow hull of Khao Dawk Mali 105 (KDML105) and black hull of wild rice (*O. rufipogon*) were used as negative (yellow hull) and positive (black hull) controls for the PCR product of this locus in the comparison of the bands resulting from PCR between black and yellow hull rice accessions. After electrophoresis, PCR electrophoresis bands were scored as YY (yellow type), BB (black type) and YB (heterozygote) for the *Bh4* loci. The genotypic and allele frequencies were computed based on Hardy-Weinberg formulations. To estimate the frequency of B and Y alleles in weedy rice populations, according to the Hardy-Weinberg equation: p = the frequency of the Y allele q = the frequency of the B allele. For a population in genetic equilibrium:

$$p^2 + 2pq + q^2 = 1$$

The three terms of this binomial expansion indicate the frequencies of the three genotypes:

p^2 = frequency of *YY*
$2pq$ = frequency of *YB*
q^2 = frequency of *BB*

After the PCR products were electrophoresed and scored, the DNA fragments were cut from the gel and purified using a Gel extraction kit. Sequences were separated on an ABI 3730 automated sequencer (Applied Biosystems, USA) at the Macrogen DNA Sequencing Service (Macrogen Korea, Seoul, Rep. of Korea). The PCR products of two accessions of weedy rice which carried the YY genotype and BB genotype were selected to determine DNA sequences of *Bh4* locus.

3. Results, discussion and conclusion

3.1 Genotype analysis of *Bh4* in weedy rice

A PCR assay was used to determine the genotype of each of the 112 weedy rice accessions used in this study. An example of three genotypes (BB/YB/YY) was shown in Figures 2, 3. DNA sequences of the *Bh4* locus of black hull (BB) weedy rice accessions and cultivated rice cv. KDML 105 with yellow hull (YY) are shown in Figure 4. A total number of 112 weedy rice accessions were genotyped for YY (58) (non-functional allele Y, 22-bp deletion), BB (12) (functional allele B, non-deletion 22 bp), and YB (42) (heterozygote). The data showed that 37.5% of the samples were heterozygous. The allele frequencies were 0.71 and 0.29 for Y and B alleles, respectively. Based on the Hardy-Weinberg principle, when a population meets all of the Hardy-Weinberg conditions, it is said to be in Hardy-Weinberg equilibrium (HWE). How far a population deviates from HWE can be measured using the "goodness of fit"or Chi-squared test (χ^2). Since the Chi-squared value (0.442) falls below the 0.05 (degree of freedom=2) significance cut off, the weedy rice population does not differ significantly from what we would expect for the Hardy-Weinberg equilibrium of the *Bh4* locus. A previous study reported that birds may play an important role in the selection process during the evolution of the *Bh4* gene (Zhu *et al*., 2011). They suggested that when a black-hull seed mutated to straw white, it would be protected from bird predation, and the allele frequency of mutated allele would increase over generations of the cultivation. They also found that birds almost equally favored eating straw-white hull and black hull rice grains while they were ripening in the field.These findings support the result from this present study that weedy rice populations might maintain equilibrium of the alleles of the *Bh4* gene and meet all of the Hardy-Weinberg conditions (i.e., no immigration, no genetic drift and no selection). If these conditions are met, the frequencies of this gene will be maintained from one generation to the next. Weedy rice was found in many rice planting areas in Thailand and possibly originated from hybridization between cultivated rice and wild rice (Prathepha, 2009). In addition, weedy rice exhibited fitness-related traits that make it highly competitive with cultivated rice. These traits included rapid growth, deep roots, high seed production and production of more offspring (Basu *et al*., 2004). The mutated allele of the *Bh4* locus showing more frequency than the wild type allele may be caused by gene flow (or introgression) from cultivated rice to its weedy and wild relative. Several studies have shown that gene flow occurred with a noticeable frequency from cultivated rice to its weedy relative (Chen *et al*., 2004; Xia *et al*., 2011).

Figure 1. Weedy rice populations found in cultivated rice fields in Thailand.

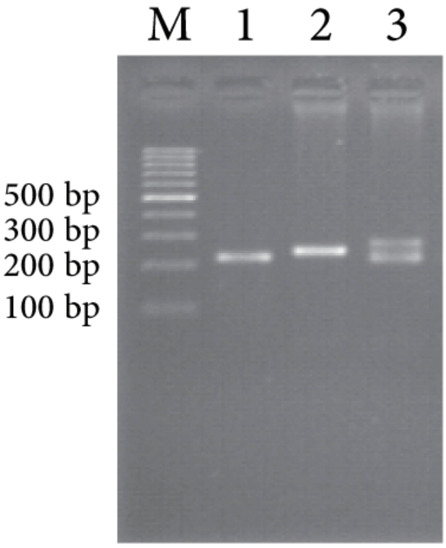


Figure 2. Example of amplified DNA of weedy rice accessions after resolution in agarose gel by using the primer pairs: forward primer: 5'-CTGCGCTTCACGTACCAGG-3' and reverse primer: 5'-CGTCGAACTTGACGTACGTGG-3'. M= DNA marker. Lane 1,2,3 = weedy rice accession with genotype YY, BB and YB, respectively.

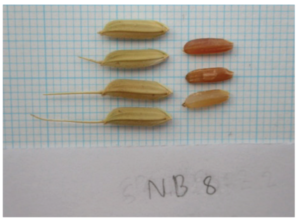
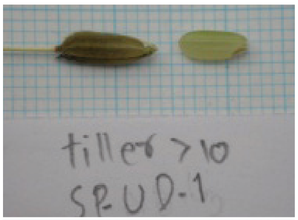
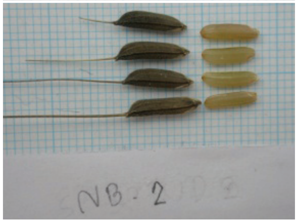
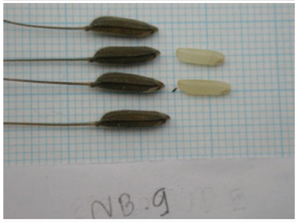
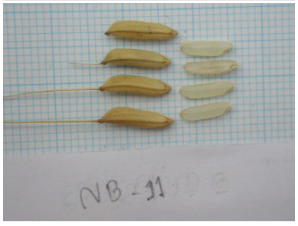
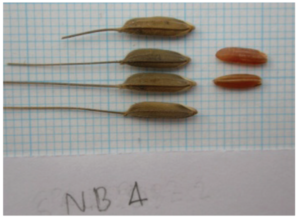
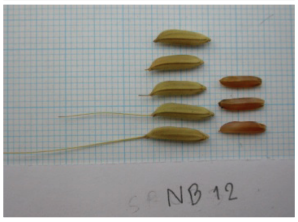
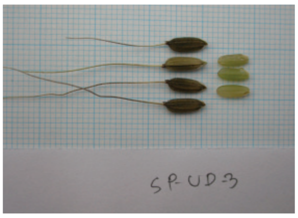
Weedy rice accessions	Genotypes	Weedy rice accessions	Genotypes	Weedy rice accessions	Genotypes
	BB		YY		YB
	YB		YB		YB
	YB		YY		YY
	YB		YY		BB
					BB

Figure 3. Seed morphology and genotype of *Bh4* gene determined by PCR assay of some weedy rice accessions used in this study.

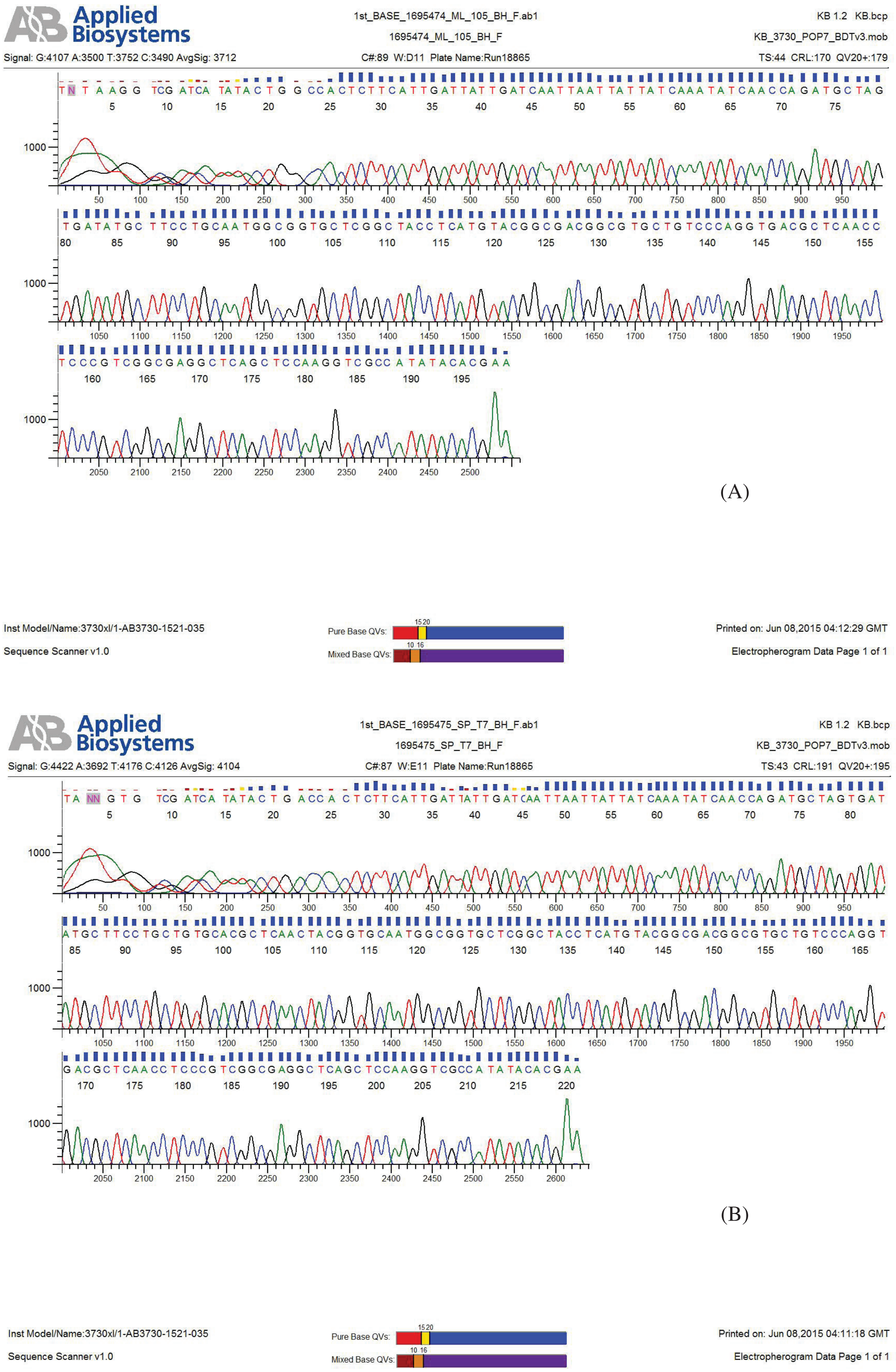


Figure 4. Chromatogram of *Bh4* locus for (A) KDML 105 carried BB genotype (yellow hull) and (B) for a weedy rice accession YY genotype (black hull). The BB genotype showed 22-bp deletion and YY genotype had the 22 bp (5'-TGTGCACGCTCAACTACGGTGC-3', position 95th-116th) in exon 3 of *Bh4* locus.

3.2 Genotype variation and hull color among weedy rice accessions

Studies have reported that there is a relationship between the two loci (*Bh4* and *Phr1*) that causes a black pigmentation in the ripening hulls of rice (Fukuda *et al*., 2012). They found that only rice plants that had both functional alleles of *Bh4* and *Phr1* genes could produce a black hull at ripening time. In the present study, weedy rice accessions showed a relationship between the functional allele and/or non-functional allele of the *Bh4* locus and hull color (Fig. 3). In addition, the genotype of weedy rice accessions was examined and the phenotype of the seeds could be expected for the other locus (i.e. *Phr1*). For example, the weedy rice accessions that showed black pigmentation had a genotype of heterozygote (YB), this implies further that these rice plants had a genotype with a functional allele (B, non-deletion of 22 bp on the third exon) of the *Bh4* locus, and carried a functional allele (non-deletion of 18 bp on the third exon) of the *Phr1* locus. In this study, seed hull color of weedy rice samples with a different genotype or the same genotype of the *Bh4* gene showed a variation in black hull (Fig. 3). For example, accession SPUD-4, NB-3 and NB-4, which had the heterozygous genotype (i.e. YB), showed a variation of black hull seed. Recently, Fukuda *et al*. (2012) reported a complementary gene (i.e., *Phr1* gene) that caused black pigmentation in the ripened hulls of rice. Results from their experiment indicated that only rice plants that had both functional genes *Bh4* and *Phr1* showed the black hull phenotype. Therefore, these weedy rice accessions (SPUD-4, NB-3 and NB-4) should carry a functional allele of the gene *Phr1* in their genomes. This observation supports the idea that the phenotype is in accordance with the expression pattern of both the *Bh4* gene and *Phr1* gene, and this must be something that is transported from the grain to the hull for the formation of the black hull, as suggested by Zhu *et al*. (2011). They also found that rice seeds began to turn black about 15 days after pollination and seeds did not turn black if they were not pollinated. Pericarp and hull color are maternal tissue, so hull color and pericarp color of progeny depend on the maternal genotype (Sweeney *et al*., 2006). The weedy rice accessions in Figure 3 showed different pericarp color (white, slight red, red), this evidence reflects the genetic variation in the maternal weedy rice plants used in this study.

In addition, weedy rice exhibited fitness-related traits that make it highly competitive with cultivated rice. These traits include rapid growth, deep roots, high seed production and production of more offspring (Basu *et al*., 2004). The fact that the mutated allele showed a greater frequency than the wild type allele may be caused by gene flow (or introgression) from cultivated rice to weedy rice and wild relative. Studies have shown that gene flow occurred with a noticeable frequency from cultivated rice to its weedy rice (Chen *et al*., 2004; Xia *et al*., 2011). Weedy rice seeds with black hulls may survive better than seeds with yellow hulls due to the black hull pigmentation screening UV radiation to protect the seeds and acting as an antioxidant (Huang *et al*., 2011; Fukuda *et al*., 2012). Results from the present study would shed a light on the weedy rice population in terms of evolution and they are probably of interest for evolutionary biologists to investigate other aspects.

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