1 **Introgression of Clearfield™ rice crop traits into weedy red rice outcrosses**

2 Vijay Singh¹, Shilpa Singh¹, Howard Black², Virginia Boyett³, Supratim Basu¹, David Gealy²,
3 Edward Gbur⁴, Andy Pereira¹, Robert C. Scott⁵, Ana Caicedo⁶, Nilda R. Burgos^{1*}

—

* Corresponding author
nburgos@uark.edu (Nilda R. Burgos)

¹Department of Crop, soil, and Environmental Sciences, Plant Sciences, University of Arkansas,
Fayetteville, AR 72704, USA

²USDA-ARS Dale Bumpers National Rice Research Center (DBNRRC), Stuttgart, AR 72160,
USA

³Rice Research and Extension Center, University of Arkansas, Stuttgart, AR 72160, USA

⁴Agricultural Statistics Laboratory, University of Arkansas, Fayetteville, AR 72704, USA

⁵Lonoke Agricultural Center, University of Arkansas, Lonoke, AR, 72086, USA

⁶Biology Department, University of Massachusetts, Amherst, MA 01003, USA

Abstract

Studies were conducted to determine the impact of introgression of crop alleles into weedy rice populations. Seeds of 89 weedy rice accessions collected from Arkansas fields in 11 counties, with a history of ClearfieldTM rice production, were planted in 2011 and sprayed twice with imazethapyr (0.071 kg ai ha⁻¹). Survivors were genotyped using an allele-specific PCR assay to detect the presence of resistance-endowing *als* mutations, S₆₅₃N and G₆₅₄E, in herbicide-resistant (HR) weedy rice that could have originated from ClearfieldTM cultivars planted in the state. Hybridization between ClearfieldTM rice and weedy rice was verified using microsatellite markers RM 215, RM 220, RM 234 and RID12. Two to five HR plants, representing different plant types per accession per replication (698 plants total) were characterized for 16 morphological and biological traits. Seventy-nine percent of accessions were resistant to imazethapyr. The HR weedy rice plants carried the S₆₅₃N mutation and were confirmed as outcrosses with ClearfieldTM rice using microsatellite markers. Ten weedy rice accessions (nearly 25% of confirmed outcrosses) harbored multiple alleles from rice cultivars, indicating previous hybridization events with multiple cultivars or outcrossing with weedy rice. The majority of HR weedy rice accessions were similar to cultivated rice in height, culm angle, flag leaf length and width, days to flower, seed shattering and dormancy. Kernels of 20% of the parent weedy rice accessions were segregating for white and red bran color. Two of the parent weedy rice accessions with gold hull were homozygous for white bran and produced progenies with 100% white-bran kernels; thus, weedy rice was no longer exclusively red. Therefore, HR weedy rice outcrosses generally had acquired crop-like traits, which make it easier to deplete from the soil seedbank, but also difficult to scout.

Keywords: bran color; gene flow; herbicide resistance; hybrid rice; weediness

46

47 **1. Introduction**

48 Hybridization is an important component of plant evolution (Rieseberg and Ellstrand,
49 1993). More than 70% of plant species might have descended from hybrids (Grant, 1981). Gene
50 flow from crop species to wild or weedy species leads to introgression of alleles of one plant
51 population into the gene pool of another, thereby changing the population structure of a species
52 (Anderson, 1949; Pusadee et al., 2013) or even resulting in the evolution of new species.
53 Hybridization between crops and weedy relatives results in genotypes with intermediate
54 characteristics (Burgos et al., 2014; Pusadee et al., 2013; Suh et al., 1997), which in some cases
55 are more weedy in nature perhaps due to increased fitness (Arnold and Hodges, 1995; Langevin
56 et al., 1990). Introgression of crop alleles is favored if it endows fitness advantages (Barton,
57 1993; Haygood et al., 2004). The introgression of single-gene traits such as herbicide resistance
58 may lead to even greater fitness advantages in outcrossed populations compared to introgression
59 of conventional crop traits (Shivrain et al. 2009a; Snow et al., 2003).

60 Owing to genetic and morphological similarities between cultivated and weedy rice,
61 weedy rice could not be controlled selectively in rice fields (Shivrain et al., 2010; Vaughan et al.,
62 2001). The introduction of non-transgenic, herbicide-resistant (HR) ClearfieldTM rice (Tan et al.,
63 2005) in 2002 allowed growers to control selectively 95 to 100% of the weedy rice in rice fields
64 by applying imazethapyr (imidazolinone, IMI) herbicide (Avila et al., 2005; Steele et al., 2002).
65 Despite the success of ClearfieldTM rice technology, 100% control of weedy rice could not be
66 attained sustainably in all fields. Weedy rice can escape control due to herbicide application
67 factors, as well as environmental and biological factors (Burgos et al., 2008). These escaped
68 weedy rice plants can hybridize with ClearfieldTM rice cultivars when flowering periods of the
69 two plant types are synchronized (Shivrain et al., 2008). In the last decade many researchers have

investigated gene flow from cultivated rice to weedy rice populations in North America (Gealy et al., 2003; Shivrain et al., 2006, 2008, 2009b; Zhang et al., 2006). The HR weedy rice outcrosses in the F₂ generation or later are difficult to manage, as these segregate into several diverse weedy plant types, which can compete with rice and produce inferior grain yield and quality.

Hybridization between weedy rice and rice cultivars can range from 0.1% to 3.2%, (Cao et al., 2006; Langevin et al., 1990; Shivrain et al., 2009a; Zhang et al., 2006), but mostly $\leq 1\%$ outcrossing is detected. Once outcrossed, the crop alleles introgress into weedy populations within a few generations (Gealy et al., 2003). The low outcrossing rate and subsequent introgression contribute to the evolution of weedy rice populations (Cao et al., 2006; Gealy et al., 2003; Gealy et al., 2015). The introgression of crop traits into weedy populations can change the population dynamics, morphological characteristics, and genetic structure of weedy populations (Burgos et al., 2014). For a primarily self-pollinated species, weedy rice exhibits high diversity within and among populations (Londo and Schaal, 2007; Shivrain et al., 2010; Tseng et al., 2013). This has been attributed to the low-level natural hybridization between weedy and cultivated rice, which for example, had been documented to produce admixed populations of outcrosses that are taller, with larger leaves, and have more tillers than the historical weedy populations (Langevin et al., 1990; Shivrain et al., 2006). In Thailand, an intermediate population of weedy rice exists at the interface of rice production fields and wild rice (Pusadee et al., 2013). Insight into the consequences of hybridization between weedy rice and HR rice cultivars on the evolution of weedy rice populations is important for the management of this troublesome weed. Sustainable weedy rice management necessitates a thorough understanding not only of weedy rice diversity but also of evolutionary changes in the weedy populations ensuing from crop-weed and weed-weed interactions as impacted by management tactics. This

study was conducted to evaluate the impact of introgression of HR trait and crop alleles into weedy populations, particularly on weedy traits, and its broader implications on the use of other improved crop traits.

2. Materials and Methods

2.1. Screening for herbicide-resistance

Seeds of 89 weedy rice accessions were collected from 26 fields across 11 counties in Arkansas (Fig. 1) in 2010. Each field generally had multiple weedy rice types. Up to 400 g seed was collected from plants representing each plant type. In cases where only a few plants represented a plant type, the amount of seed collected could be less than 50 g depending on the number of panicles in each plant. Seed collection was conducted in a two-week period and only mature panicles were harvested. Panicles were air-dried at room temperature for up to three weeks before the seeds were threshed, cleaned, and stored at room temperature until used. The hull colors of weedy rice accessions were diverse – 43% strawhull, 26% blackhull, 8% brownhull, 11% goldhull, 9% greyhull, and 3% mixed-hull color with striations. These accessions were tested for resistance to imazethapyr in a field experiment at the Rice Research and Extension Center (RREC) Stuttgart, AR in 2011. The soil was a DeWitt silt loam (fine, smectitic, thermic Typic Albaqualfs) with 0.9% organic matter and pH 6.2. Eighty-nine red rice accessions and three ClearfieldTM rice cultivars (CL151, CLXL729, CLXL745) were planted in 6.1-m row per accession at 50 seeds per row on 20 May, 2011. Imazethapyr was applied at 0.5X (35 g ha⁻¹) and 1X (70 g ha⁻¹) of the field use rate when seedlings had two- to three-leaves, followed by a second application 7 d later. Injury and mortality were recorded at 21 d after treatment (DAT) from the 1X rate only because the majority of plants survived this treatment.

Mortality (%) was calculated based on the number of plants that survived out of the total number sprayed.

2.2. Molecular marker analysis

2.2.1 Plant materials and DNA extraction

Leaf tissues were harvested from 89 HR weedy plants, surviving the 1X rate of imazethapyr, each representing a parent accession. Also included were three reference cultivars (CL151, CLXL729, CLXL745). Total genomic DNA was extracted using a modified hexadecyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1990). The genomic DNA was quantified using a spectrophotometer, NanoDrop 2000c V. 1.0 (NanoDrop Technologies, Wilmington, DE, USA), and diluted to 100 ng μl^{-1} with deionized water.

2.2.2 Allele-Specific PCR (AS-PCR)

Polymerase chain reaction (PCR) reactions were conducted for the *ALS* gene SNPs ($S_{653}N$ and $G_{654}E$) using a set of allele-specific forward primers and a common reverse primer as described by Kadaru et al. (2008). For each sample, a 20- μl PCR mix consisting of 3 μl genomic DNA (20 ng μl^{-1}) mixed with 10 μl of 2X Taq Mastermix; EmeraldAMP[®] MAX HS PCR Master Mix (Takara Bio, Madison, WI, USA), 1 μl each of 10 μM forward and reverse primers and 5 μl deionized water. DNA amplification reactions were carried out using thermocycle conditions of 95 °C—2 min; 95 °C—20 s, 60 °C—20 s, and 72 °C—20 s for 28 cycles and 72 °C—5 min. After amplification, 7 μl of each PCR product was loaded onto a 2% OmniPur[®] agarose gel (EMD Chemicals Inc., Gibbstown, NJ, USA), and ran for 60 min in 1X TBE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The PCR products were stained with

GelRedTM Nucleic Acid Stain (Biotium, Hayward, CA, USA), visualized using GelDoc-It TS2 Imager (UVP LCC, Upland, CA, USA) and documented.

2.2.3. Verification of Hybridization

Hybridization between weedy red rice and ClearfieldTM rice cultivars was verified using 10 cultivars as standards. These included nine ClearfieldTM rice cultivars (inbreds and hybrids) and one conventional inbred cultivar, 'Jupiter'. The InDel marker, RID12 (Sweeney et al., 2006), and three microsatellite markers [RM215 (Gealy et al., 2002), RM220 (Akagi et al., 1996), and RM234 (Zhang et al., 2006)] were used to differentiate alleles known to be present in commercial white rice from those known to be present in US weedy red rice. Microsatellite markers are codominant, therefore the outcrosses are expected to show PCR-amplified DNA fragments from both the susceptible weedy rice and the rice cultivars (Gealy et al., 2002). For PCR amplification, 2 μ l of Zymo purified template DNA (80 ng μ l⁻¹) were used in a reaction containing HotStarTaq DNA Polymerase, Qiagen Multiplex PCR Buffer with 6 mM MgCl₂, ultrapure quality dNTP Mix, and 0.25 μ M of each primer. The PCR was performed using Qiagen Multiplex PCR Kit, (Qiagen, Valencia, CA, USA) using the cycling protocol of a 15-min hot start at 95 °C followed by 35 cycles of traditional 3-step thermal cycling starting with denaturation at 94 °C for 30 s, primer annealing at 55 °C for 90 s, and elongation at 72 °C for 60 s. The reactions were then heated to 60 °C and incubated for 30 min before storing the completed reactions at 8 °C. Two microliters of each diluted PCR product were added to the Genescan 400HD [ROX] size standard (Applied Biosystems, Foster City, CA, USA). The PCR products were resolved in an Applied Biosystems 3730 DNA Analyzer using POP-7 polymer on a 36 cm 48-capillary array. The fluorescent signal of the resolved amplicons was analyzed using GeneMapper v4.0 software.

2.3. Morphological characterization

Two- to five plants per accession (total of 698 weedy rice and 21 Clearfield™ rice plants) which survived the 1X rate of imazethapyr, representing different phenotypes among the segregating offspring, were characterized based on 16 morpho-physiological traits according to the guidelines of the International Board for Plant Genetic Resources (IBPGR-IRRI, 1980). The traits evaluated were culm angle; stem color; leaf color and texture; flag leaf length, width, and angle; leaf exertion; days to flower; panicle length; hull color; awn color and awn length; grain yield per plant; seed shattering and seed dormancy. Culm angle was determined relative to the horizontal plane (low value = open canopy). Flag leaf angle was determined with reference to the culm (low value = erect flag leaf). Length and width of flag leaves were measured from five tillers per plant. Leaf width was measured at the widest point of the leaf. Leaf texture was evaluated by rubbing the finger along the adaxial surface of the leaf blade. Plant height was measured from the base of the plant to the tip of the flag leaf on the main culm. Panicle exertion was determined based on the portion of panicle still enclosed by the flag leaf (i.e. small panicle exertion value equates to a 'large' proportion of the panicle still enclosed by the flag leaf). Flower initiation of characterized plants was recorded weekly. A plant was considered flowering when four tillers had at least partially exerted panicles. The length of five panicles was measured per plant. Awn length was measured for five seeds of the same panicle. Awn color and hull color were evaluated visually, after the grains had matured. After characterization, plants were enclosed in Delnet® bags (Delstar Technologies, Middletown, DE, USA) to collect the shattered seeds.

2.4. Seed shattering

Weedy rice (698) and Clearfield™ rice plants (21) were enclosed in Delnet® bags after morphological characterization and were harvested 45 d after flowering. Harvesting spanned a period of 10 wk because of the diversity of flowering dates of weedy rice. At maturity, each plant was cut 10 cm from the ground and seed shattering was recorded within a week from harvest. To determine the level of seed shattering, appropriate sets of weights were attached to the bagged panicle bundles to attain a uniform weight of 2.5 kg. Each bundle was then dropped from a height of 1 m into a container so that each plant was subjected to the same force of 50 kgf. The shattered seeds were weighed. The panicles with remaining seeds were threshed, and the remaining seeds were weighed. The total yield was recorded as shattered seeds + non-shattered seeds. Seed shattering (%) was calculated as:

$$\text{Shattering (\%)} = [\text{shattered seed weight (g)} / \text{total seed yield (g)}] * 100$$

2.5. Evaluation of seed dormancy

Seed dormancy was evaluated at 75 d after harvest (DAH) and at 270 DAH. Seeds of 644 HR offspring and 15 Clearfield™ rice plants were germinated in batches according to the respective harvest date of each plant. Thirty seeds from each offspring were placed in a Petri-dish (9 cm diameter), replicated three times, moistened with 6 ml deionized water, and dark-incubated in a growth chamber at 30 °C. The Petri-dishes were placed in trays and the trays were arranged in randomized complete block design in the growth chamber, with replications serving as blocks. The trays were covered with a plastic sheet to prevent desiccation of seeds and were re-watered every 4 d after each germination evaluation. Germination capacity (GC) was evaluated in a 12-d period. Seeds were considered germinated when the radicle protruded from

the caryopsis. The Petri plates were re-randomized after each evaluation period. Firm, non-germinated seeds were counted as viable, dormant seeds. This approach for evaluating seed viability was supported by a preliminary study comparing the tetrazolium test with the seed-firmness test, using 10 samples. Both tests produced the same viability results (data not shown). The seed-firmness test enabled the evaluation of more than 1,000 samples.

2.6. Kernel characterization

Size and pericarp color of seeds harvested from growers' fields (89 accessions) as well as seeds of progenies (698) and reference cultivars (Clearfield™ rice) characterized at the RREC were evaluated using Graincheck (2312 FOSS TECATOR). Samples were de-hulled and the bran color of 100 kernels per sample was recorded according to the guidelines of IBPGR-IRRI (1980). There were seven categories for bran color in this system – white, light brown, speckled brown, brown, red, variable purple, and purple. Length (L), width (W), L/W ratio and 1000-kernel weight were recorded.

2.7. Data analyses

All data were analyzed using JMP Pro (version 11.0; SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was conducted for all the quantitative plant traits using a completely randomized design. Differences among and within ecotypes in plant traits were determined using one-way ANOVA and means were separated by Fisher's Protected LSD at the 5% probability level. Principal component analyses (PCA) for morphological traits was conducted using six principal weedy traits; based on their contribution and 'Eigen' values. K-means clustering (JMP Pro v. 11) was performed to group the weedy rice offspring (698 plants) based on the six selected traits. Cluster analysis was performed on germination and seed shattering. K-

means cluster analysis was carried out where variables were scaled individually (differences were scaled by an overall estimate of the standard deviation of each variable) by default in JMP Pro (v.11). The numbers of clusters were selected based on fit statistic, with the largest CCC (Cubic Clustering Criterion). PCA and *K*-means cluster analysis were conducted for 679 offspring based on all kernel characteristics to distinguish weedy population groups by type of kernels.

3. Results

3.1. Frequency of herbicide-resistant weedy rice

ClearfieldTM rice had been planted in the 26 sampled fields (Fig. 1) for different numbers of crop seasons. Seventy-nine percent of the accessions from these fields were resistant to imazethapyr (0-79% injury); of these, 98% were highly resistant (0-20% injury) (Fig. 2). However, only the S₆₅₃N mutation was detected in the HR weedy rice accessions (Fig. 3) which was the same mutation harbored by the ClearfieldTM rice cultivars planted in the state. The frequency of resistant offsprings was independent of hull color of mother plants, probably due to multiple outcrossing events that had occurred across multiple years. Using the InDel primer and three microsatellite markers (Fig. 4) we detected 42 weedy rice accessions harboring the same alleles as those of rice cultivars. Twenty-five percent of these 42 accessions harbored multiple alleles that were also present in more than one cultivar. This indicates that these HR weedy rice accessions had hybridized with at least one of the reference ClearfieldTM cultivars used. The remaining 28 HR weedy rice accessions did not contain any of the crop alleles that were amplified by the set of primers. These 28 HR weedy rice might have hybridized with other ClearfieldTM rice cultivars not included in the test. Forty-eight percent of HR weedy rice

accessions were homozygous resistant and 31% were heterozygotes, carrying both mutant and wild alleles (Fig. 3).

3.2. Differentiation of morphological and phenological traits

Hull colors of the remnant weedy rice accessions (89) from fields with history of ClearfieldTM rice, were diverse – 43% were strawhull, 26% blackhull, 8% brownhull, 11% goldhull, 9% greyhull, and 3% mixed-hull color with striations. The offsprings of these 89 accessions collectively segregated into 260 (37%) blackhull, 209 (30%) brownhull, 29 (14%) goldhull and 200 (28%) strawhull ecotypes (Table 1 & 2). Out of 16 plant traits characterized, six contributed significantly to the overall morphological diversity of HR weedy rice including plant height, days to flower, panicle length, hull color, seed shattering and grain yield (Suppl. Fig. 1). Overall, 76% of the characterized progenies were taller than cultivated rice (130 cm) (Suppl. Fig. 2-A).

K-means cluster analysis grouped the 698 HR weedy rice plants into three clusters (Table 1) based on six traits with the largest contribution to population differentiation as determined by PCA. Clusters 1 and 2, representing 55% of the characterized plants, had mean heights of 127-131 cm similar to that of the reference cultivars in the study. The largest group, Cluster 3, consisted of 314 very tall individuals (166 cm). These plants had longer awns (2.3 cm), intermediate seed shattering (56%) and lower yield potential (40 g plant⁻¹) than plants in the other clusters. Seventy-eight percent of weedy rice plants in Cluster 3 had dark hull color (45% blackhull and 33% brownhull).

The range of flowering initiation among HR weedy rice was 70 to 135 d after planting (DAP) with 71% flowering at 96-99 DAP similar to that of ClearfieldTM rice cultivars (97 DAP). Blackhull and brownhull weedy rice flowered later (101 DAP) than strawhull types (96 DAP). Plants in Cluster 1, consisting of about ¼ (26%) of HR weedy rice offspring, were

phenotypically similar to cultivated rice (Table 1). Cluster 2 consisted of 29% HR offspring that were also morphologically similar to the rice cultivars (the same height, upright leaves and closed canopy), but flowered later (+7 DAP), had higher seed shattering (+35%), shorter panicles (-2.3 cm), and lower yield potential (-20.1 g) than Clearfield™ rice. Generally, HR plants exhibited some crop-like characteristics regardless of hull color. The majority of HR weedy rice had upright leaves and culm angles similar to cultivated rice.

3.3. Differentiation of seed shattering and dormancy

K-means cluster analysis of GC at 75 and 270 DAH involving 698 HR weedy rice produced three distinct clusters (Table 3). Cluster 1, comprised of 69% of individuals, had high GC (94 and 97%) similar to cultivated rice (90 and 91%) at 75 and 270 DAH, respectively. Clusters 2 and 3 had lower GC than cultivated rice. Only a small proportion of HR weedy rice (10%, Cluster 3) had up to 50% dormancy after a long afterripening period of 270 DAH. Further, 42% of HR weedy rice offspring showed only 40% seed shattering. Eighteen percent of HR weedy rice offspring had equal or less seed shattering than the cultivars (Suppl. Table 1). Blackhull weedy rice had the highest seed shattering (60%) and goldhull weedy rice had the lowest (39%) (Table 2).

3.4. Differentiation of kernel characteristics

The PCA analysis of kernel characteristics revealed that kernel width, length, and weight of 1000-kernels were major contributors to component 1; bran color was the main contributor to the variation in component 2. Together, these explained 81% of the variation in kernel characteristics of HR weedy rice offspring from field-collected plants. K-means cluster analysis based on bran color, kernel length, width and 1000-kernel weight grouped HR weedy rice offspring into three clusters (Suppl. Table 2). Clusters 1, 2 and 3 had 40, 26 and 13% of kernels

with white bran color, respectively. Kernels in Clusters 2 and 3 were longer and had higher 1000-kernel weight. In general, the kernels of HR weedy rice with red bran were larger than those with white bran and characteristics associated with red bran positively affected yield parameters. Kernels with light brown bran, were the largest (Suppl. Table 3), but the proportion of kernels with light brown bran was very low (<1%) and did not influence the average kernel size and weight.

Of 109 characterized HR offspring from 17 weedy rice parent accessions with mixed-color kernels, 66% had red bran and 33% had white bran (Fig. 5). Out of 89 parent accessions, 60 with red-bran kernels produced offspring that segregated into red (69%) and white (30%) bran. This data was from 477 plants (76% of total characterized HR offspring). Thirty-five percent of these segregating offspring with white-bran kernels were blackhull ecotypes and 28% were strawhull. Two of the parent weedy rice accessions (goldhull types) with white-bran kernels had offspring with 100% white-bran kernels. Collectively, kernels with white bran were observed in about one-third (30%) of the characterized HR weedy rice offspring (Fig. 5, Table 4). Only 6 of 89 parent weedy rice accessions were homozygous for the red bran trait, producing 100% progenies with red-bran kernels (Fig. 5). Heterozygous parent accessions had progenies that produced red- and white-bran kernels. Microsatellite marker analysis of 89 HR offspring, each representing one parent accession, showed that 57% and 20% of progenies were homozygous for red and white bran phenotypes, respectively (Fig. 4 and Fig. 5). Twelve percent were heterozygous, containing alleles for both red and white bran color.

Four parent accessions, with more than 60% of their progenies producing white-bran kernels, were selected for comparison with cultivated rice (Table 5). Two of these parent accessions were homozygous for white-bran kernels. White-kernel progenies of red-kernel

parents were more diverse morphologically and had larger ranges of culm angles, 1000-kernel weight, and GC. These types of outcrosses overlapped 100% in flowering with the reference cultivars.

4. Discussion

4.1. Frequency of herbicide-resistant weedy rice

A survey of rice crop consultants in 2006 estimated that weedy red rice infested up to 60% of the total rice area in Arkansas (Burgos et al., 2008). In recent years, however, weedy red rice infestation has been reduced to roughly 20% due to a generally good adherence by growers to the stewardship program, which strongly promotes practices to minimize escapes and prevent seed production of the escaped plants (R.C. Scott, 2015, personal communication). Weedy rice seed can remain dormant in the soil for up to 10 years (Goss and Brown, 1939); thus, effective management needs to be sustained over a long period to deplete the soil seedbank and prevent reinfestation. The occurrence of resistant offspring from remnant weedy rice sampled across 26 fields in 2010 (Burgos et al., 2014) indicates that gene flow of the HR trait to weedy rice had occurred in accordance with the low-level outcrossing behavior of the species. Clearfield™ rice cultivars in the USA harbor either G₆₅₄E or S₆₅₃N. Clearfield™ cultivars CL121 and CL141 harboring the G₆₅₄E mutation were introduced in 2002 in the USA and were adopted rapidly by growers. However, these were replaced quickly in 2003 by newer cultivars (e.g. CL161) harboring the S₆₅₃N mutation, which endows a higher resistance level (Avila et al., 2005; [Wenefrida et al., 2007](#); McClain, 2003). In Arkansas, 19% of the rice area in 2004 was planted with Clearfield™ inbred rice (CL121, CL141 and CL161) and Clearfield™ hybrid rice (CLXL8) (Wilson and Branson, 2004). With the introduction of Clearfield™ hybrids (e.g. CLXL8,

CLXL745, CLXL729) harboring the S₆₅₃N mutation, adoption of this technology increased even further owing to the higher yield potential of these hybrids. In 2009, 45% of the rice hectareage in Arkansas was planted with ClearfieldTM rice, the majority of these being ClearfieldTM hybrids (Wilson et al., 2010). These ClearfieldTM cultivars and those planted later, harbored the S₆₅₃N mutation (Fig. 3). The weedy rice accessions used in the current study were collected in 2010 and with the first commercial cultivars harboring the G₆₅₄E mutation being planted for just one cropping season, it was not surprising that only the S₆₅₃N mutation was detected among the HR outcrosses. The area planted with ClearfieldTM rice increased to 69% in 2011, which was the peak of technology adoption to date. The recent decline in ClearfieldTM rice area is attributed to the decline in weedy rice-infested fields as a result of generally proper stewardship of the HR technology and, partly due to the appearance of HR weedy rice populations, which forced growers to rotate to other crops (R.S. Scott, pers. communication). Still, ClearfieldTM hybrid cultivars (e.g. CLXL729, CLXL745) are among the most popular in the US ricebelt. In 2015, 44% of total rice area in Arkansas was planted with ClearfieldTM cultivars, >50% of which were ClearfieldTM hybrids (Hardke, 2016).

In Brazil, 82% of the IMI-resistant weedy rice plants carried the same *als* mutations as the predominant rice cultivars planted in the years prior to the weedy rice survey (Roso et al., 2010). ClearfieldTM rice cultivars in Brazil carry at least one of three *als* mutations (S₆₅₃N, G₆₅₄E, A₁₂₂T), which endow resistance to IMI herbicides. . In Brazil, however, some cases of *de novo* mutation in weedy rice *als* might have been selected also by the imidazolinone herbicides used in ClearfieldTM rice.

368 Weedy rice plants are primarily strawhull or blackhull types (Gealy et al., 2002;
369 Vaughan et al., 2001). Differences in morpho-phenological characteristics of these groups are
370 expected to impact their outcrossing rates with rice cultivars. Collectively, strawhull weedy rice
371 ecotypes have a longer flowering overlap with rice cultivars than blackhull weedy rice and the
372 overlap in flowering is positively correlated with outcrossing rate (Shivrain et al., 2008). In the
373 current study, the frequency of HR offsprings was independent of hull color of the mother plants,
374 reflecting the fact that besides synchronization in flowering, other factors impact gene flow
375 between cultivated rice and weedy rice (Gealy et al., 2015; Shivrain et al., 2009b). The similar
376 frequency of resistant offsprings among blackhull and strawhull types in the current study might
377 be due to multiple outcrossing events across years within weedy rice populations and between
378 weedy rice and cultivated rice. The fact that 25% of HR plants that harbored crop alleles
379 shared these alleles with multiple cultivars indicates that these contemporary HR weedy
380 populations had hybridized with multiple cultivars or other earlier-generation weedy outcrosses.
381 Contemporary populations here pertain to HR populations that had evolved during the Clearfield
382 riceTM era. . Homozygous alleles in weedy rice indicate crop-weed hybridization that occurred
383 multiple generations prior and now is introgressed into the weedy populations.

384 *4.2. Differentiation of morphological and phenological traits*

385 Historical weedy rice populations are generally 15-65% taller than cultivated rice (Do
386 Lago, 1982; Shivrain et al., 2006). Historical populations are those that had co-existed with rice
387 through the years of rice production in the US. Height is a major trait that lends weedy rice a
388 competitive advantage over cultivated rice and causes further yield losses as weedy rice lodges
389 severely at high density, bringing the rice crop down with it. The majority of contemporary HR
390 outcrosses were taller than cultivated rice (Suppl. Fig. 2-A) just as reported by Shivrain et al.

(2006) where outcrosses of weedy rice and cultivated rice were taller than either parent in the first generation. The second generation outcrosses segregate in height from 40-180 cm (Shivrain et al., 2006), with the majority still being taller than cultivated rice. In contrast to the historical weedy rice populations, some contemporary populations of HR weedy rice (Clusters 1 and 2, Table 1) are shorter and more similar to the crop. Notwithstanding the potential influence of plant height on gene flow (Shivrain et al., 2009b, 2010; Zhang et al., 2003), these intermediate admix of HR weedy rice populations can serve as conduits for further crop-weed and weed-weed gene exchange. Historical weedy rice populations in general have droopy leaves and an open canopy (Do Lago, 1982; Shivrain et al., 2010) whereas the majority of contemporary HR weedy rice plants have upright leaves and similar culm angles as rice cultivars. The change in gross plant morphology among the HR weedy rice populations is among the consequences of hybridization with cultivated rice (Gealy et al., 2012; Shivrain et al., 2010). Many contemporary HR populations, therefore, can be camouflaged in the rice field. From the standpoint of monitoring and management decision-making, this is a problem as rice scouts and farmers can no longer distinguish between weedy and cultivated rice during the vegetative stage as easily as with historical weedy types.

The overall range of flowering among contemporary HR weedy rice populations was similar to that reported for historical populations (70-130 DAP) in Arkansas in 2002 (Shivrain et al., 2010), but a shift in phenology distribution within this flowering range was observed among HR outcrosses. Out of 20 weedy rice accessions in 2002, 90% of the plants flowered 98 DAP which was similar to that of ClearfieldTM cultivars (97 DAP). In the current study, a lesser proportion (71%) of weedy rice outcrosses had heading dates similar to those of ClearfieldTM cultivars (Table 1) because many of these outcrosses were segregating for heading date, with

some earlier and others later. The shift in phenology is not due to introgression of the HR trait; rather, it emanated from the introgression of flowering-related crop alleles as a consequence of crop-weed hybridization. Thurber et al. (2014) reported a wide range of heading dates of hybrids derived from strawhull or blackhull with tropical *japonica* cultivars. The extended flowering range in recent HR weedy populations would increase the window for outcrossing with cultivated rice. Thus, the risk of gene flow or backcrossing involving contemporary HR weedy rice, cultivated rice, and the natural weedy rice populations is expected to be higher than when involving only historical weedy rice populations.

Contemporary blackhull and brownhull weedy rice still flowered later (101 DAP) than strawhull types (96 DAP), similar to historical populations (Do Lago, 1982; Shivrain et al., 2010; Thurber et al. 2014). Because strawhull weedy rice has longer overlap in flowering with cultivated rice, it was expected that the frequency of HR weedy rice would be higher among strawhull populations than among blackhull or brownhull populations. This was not the case. The frequencies of HR weedy rice progeny were similar across hull types, indicating the role of other factors in modifying effective gene flow and introgression.

Hybrid cultivars such as CLXL8 exhibited longer flowering overlap (+2 to 3 d) with weedy rice versus conventional cultivars such as CL161 (Shivrain et al., 2009b). This extended synchronization in flowering increases the probability of hybridization between weedy rice and hybrid rice. The area planted with hybrid rice in Arkansas is increasing owing to the generally higher yield potential of hybrids (Hardke, 2016). The increasing area under Clearfield™ hybrid rice could accelerate the evolution of HR weedy rice and increase the diversity of weedy populations as hybrid rice cultivars outcross with weedy rice at higher rates than do inbred cultivars (Shivrain et al., 2009b). For example, the out-crossing rate of CLXL8 (Clearfield™

hybrid) with weedy rice was 0.23% while that of CL161 (ClearfieldTM inbred) and weedy rice was 0.07%. With increasing area of hybrid rice comes higher risk of gene flow and potentially increased frequency of HR outcrosses.

The largest group of HR weedy rice plants (Cluster 3, Table 1), consisting of very tall individuals (166 cm), were taller than the rice cultivars planted in the southern US rice belt, which were 95 -105 cm tall (Shivrain et al., 2010). This type of HR plants are expected to be more competitive with rice cultivars than the other weedy rice groups just by sheer plant size. The flowering of these contemporary HR weedy rice synchronized more with rice cultivars than did the flowering of historical accessions where only 60% overlapped in flowering with cultivated rice (Shivrain et al., 2010), indicating a shift toward higher synchronization with cultivars. This means that once the HR trait is introgressed into the weedy population, the risk of gene flow can increase between weed to crop and crop to weed. Therefore, with current or future HR rice technology, whether with inbreds or hybrids, it is very important to ensure complete control of weedy rice and prevent the seed production of escapes in HR rice systems.

Other changes between historical weedy rice and contemporary HR weedy rice were observed also. Among the 2002 weedy rice accessions, 48/215 (22%) were blackhull, 15/215 (7%) were brownhull, and the rest (71%) were strawhull (Shivrain et al., 2010). However, in the current study, the majority of HR weedy rice have darker-colored hulls where parents and progenies had only 43% and 28% strawhulls respectively (Table 2), in contrast to the dominance of straw-colored weedy rice among historical populations. This is possibly because blackhull and brownhull weedy rice have deeper dormancy than strawhull weedy rice (Tseng et al., 2013); thus, the nondormant strawhull seeds would be the cohorts killed by overwinter flooding, winter freeze, chemical fallow, or stale seedbed practice.

460

461 *4.3. Seed shattering and dormancy*

462 Seed shattering and seed dormancy are major weedy traits of weedy rice (Cao et al.,
463 2006; Delouche et al., 2007; Thurber et al., 2010). The shattered seeds remain in the soil and
464 germinate intermittently over many years. Gu et al. (2004) reported that weedy rice can shatter
465 up to 100% of its seeds, whereas their hybrids with cultivated rice exhibit significantly less seed
466 shattering. In the current study, almost half of the HR weedy rice offspring showed <40% seed
467 shattering and 18% of the offspring showed equal or less seed shattering than rice cultivars
468 which showed 31% seed shattering (Suppl. Table 1).

469 It was reported previously that blackhull weedy rice has three times lower germination
470 than strawhull weedy rice (Do Lago, 1982) and requires longer after-ripening time to release
471 dormancy (Tseng et al., 2013). However, the hybrids of weedy rice and rice cultivars have >90%
22 472 germination irrespective of hull-types (Shivrain et al., 2009a), similar to the traits exhibited by
473 HR weedy rice in this current study. The GC of historical weedy rice ranges from 18-42% and
474 historical weedy rice populations have high intra-population variation in seed dormancy, with
475 blackhull populations being more variable than strawhull populations (Do Lago, 1982; Tseng et
476 al., 2013). In contrast, contemporary HR weedy rice showed no variation in seed dormancy
477 between strawhull and blackhull populations and low intrapopulation seed dormancy variation.
478 The loss of dormancy means less persistence in the soil seedbank. This suggests that chemical
479 fallow, winter flooding, and preplant application of nonselective herbicides will effectively
480 reduce HR weedy rice. The reduction or loss of shattering trait means less replenishment of the
481 soil seedbank, which is good for weed management. On the other hand, it also means more
482 contamination of harvested grain in the short term as more weedy rice seeds are harvested with

the rice crop. The seed contamination problem will be eliminated as soon as 100% control of weedy rice is achieved, which is going to be easier with a smaller seedbank size. The reduction or loss of these two major weedy traits favor sustained success in weedy rice management. The difficulty in scouting for weedy rice that are more crop-like can then be countered by the adoption of integrated management practices aimed at reducing the soil seedbank.

4.4. Differentiation of kernel characteristics

Red bran is a dominant trait (Gealy et al., 2003). Weedy rice in North America has red bran, hence the term ‘red rice’ (Delouche et al., 2007), except for a rare report of white-bran weedy rice in Mississippi, USA in the early 1980s (Do Lago, 1982). The occurrence of white bran in weedy rice has been accepted as an outcome of low-level hybridization between crop and weed as supported by several studies including that of Gealy et al. (2006). Weedy rice plants with white kernels (outcrosses) show crop-like traits and are generally not distinguishable from cultivated rice (Delouche et al., 2007; Do Lago, 1982). The current study revealed that remnant weedy rice, regardless of hull color, in fields with multiple years of Clearfield rice production collectively produced a larger proportion of individuals (30%) with white bran (Table 4) than was observed in previous studies. This increased diversification of HR weedy rice kernels relative to the historical populations indicates that where the level of HR weedy rice infestation is high, the harvested grain could be contaminated with a broader array of weedy rice kernels.

White bran (*rc* allele) is the mutant (nonfunctional) version of the ancestral *O. rufipogon* (*Rc*) red allele (dominant). The *Rc* allele can be detected with the RID12 microsatellite primer (Sweeney et al., 2006). *Rc-g* is another dominant, red-bran wild type allele, which was

506 discovered relatively recently, but this cannot be detected with the RID12 microsatellite primers
507 (Brooks et al., 2008). In our study, the detection of *Rc* with the RID12 primer and the allele
508 similarity with the other three microsatellite markers (RM215, RM220 and RM234) confirmed
509 that the white bran phenotype among the HR weedy rice plants (Fig.5, Table 4) is not the result
510 of natural mutation in weedy rice; rather, it is due to gene flow from cultivated rice. Gealy et al.
511 (2003) predicted the effects of a single outcrossing event and subsequent segregation of bran
512 color, along with a dominant HR trait, across multiple years. Considering that the red seed bran
513 (RR) and herbicide resistance (NN) are dominant traits, the population resulting from a single
514 hybrid (single hybridization between HR cultivated white rice; rrNN and herbicide-susceptible
515 weedy red rice; RRnn) is expected to be more than 95% HR (half with white bran) within six
516 generations. This suggests that the weedy rice populations from commercial fields with history
517 of ClearfieldTM rice, which produced segregating red- and white-bran kernels were at least F₂
24
518 generation outcrosses based on the proportion of red and white kernels among the offspring.

519 Gealy et al. (2006) demonstrated the expected segregation patterns of key phenotypic
520 traits in F₂ plants following an outcrossing event between blackhull or strawhull weedy red rice
521 and inbred rice. Nearly 25% of the F₂ crosses produced white seeds, which were expected to be
522 homozygous. All the weedy rice accessions carrying only the white bran (*rc*) allele were
523 homozygous for that allele (*rc* being a recessive trait). Thus, the presence of white-bran kernels
524 in a weedy plant type (such as those in the current study) indicates that this gene, along with
525 others, has already introgressed into these populations, resulting in weedy plants with diverse
526 characteristics. The presence of only the S₆₅₃N polymorphism in the *als* gene of contemporary
527 HR weedy rice indicates that these populations could not be beyond F₇ as ClearfieldTM cultivars
528 harboring S₆₅₃N were commercialized in 2003 and the weedy rice samples were collected in

2010. However, some of the outcrosses were heterozygous for the red bran allele suggesting that these are more recent outcrosses, *Rc* being a dominant trait.

The progenies of four accessions which, produced mostly white-bran kernels (60%), were upright with erect leaves and have many other characteristics similar to those of the reference cultivars (Table 5). These crop-like HR weedy populations could no longer be controlled with Clearfield™ rice technology. One means of managing these evolving populations is with the use of an alternative HR technology. Recently, 'Provisia® rice' has been developed by BASF involving cultivars with resistance to acetyl-CoA carboxylase (ACCase) inhibitors, which selectively control grasses. With this technology the imidazolinone-resistant populations can be controlled with the application of quizalofop-P-ethyl herbicide (Webster et al., 2015). However, for this to be sustainable, it needs to be used in a crop rotation system involving soybean (or other appropriate rotational crop), conventional rice, and Clearfield™ rice. In addition, chemical fallow between seasons and stale seedbed techniques will remove the nondormant component of the seedbank, which could be the majority of outcross seeds. Deviation from these best management practices will result in both HR traits (ALS- and ACCase resistance) being introgressed into contemporary HR populations.

5. Conclusion

Remnant weedy rice in fields with history of HR rice are generally herbicide-resistant and HR plants carry the mutant *als* with S₆₅₃N mutation from Clearfield™ rice. These HR weedy populations are more crop-like than their historical counterparts. This study provided strong support for the stewardship recommendations for herbicide-resistant rice. Controlling weedy rice that escape, or survive, the herbicides used in Clearfield™ rice is imperative; otherwise, the

remnant weedy rice plants would outcross with the crop and eventually result in the evolution of HR weedy rice populations as documented here as well as in Latin America, Europe and Asia. The ClearfieldTM rice technology has served as a model system from which we can glimpse, in real time, how natural crop-weed hybridization and subsequent introgression of crop alleles into the weedy population can impact the population dynamics and evolution of a weedy relative. This is important for the future introduction and adoption of HR technologies, or cultivars with improved traits. Removal of escapes, or prevention of seed production of remnant weedy rice is critical; otherwise, gene flow of HR trait to the weed allows for selection of outcrosses in succeeding crop cycles, evolution of HR weedy rice population, and increase in HR weedy rice population size. The outcrosses serve as conduits for continued gene flow between crop and weed and among weedy populations. The HR weedy rice populations also harbor other crop alleles and are more crop-like. The reduction in seed shattering and loss of seed dormancy do not favor persistence, but would mean higher crop grain contamination in the immediate. Being more crop-like also reduces the utility of weed scouting as the weed becomes indistinguishable from the rice crop in the field. An alternative HR technology based on a different mode of action would help curtail the evolution of HR weedy rice if integrated with cultural practices stipulated in the best management practices for resistance management.

7. Acknowledgement

The authors thank Te Ming Tseng, Leopoldo Estorninos, Jr., Mariccor S.A.B. Batoy, George Botha, Reiofeli Salas, and Ed Allan Alcober for their help in implementation of the experiment in the field and evaluation of seed germination capacity and dormancy in the laboratory. The study was part of a project funded by the National Science Foundation Grant # IOS -1032023.

575

576 **Literature cited**

577 Akagi, H., Yokozeki, Y., Inagaki, A., Fujimura, T., 1996. Highly polymorphic microsatellites
578 of rice consist of AT repeats, and a classification of closely related cultivars with these
579 microsatellite loci. Theor. Appl. Genet. 94:61–67

580 Anderson, E., 1949. Introgressive Hybridization. New York: Wiley. pp.109.

581 Arnold, M.L., Hodges, S.A., 1995. Are natural hybrids fit or unfit relative to their parents?
582 Trends in Ecol. and Evol. 10, 67-71.

583 Avila, L.A., Lee, D.J., Senseman, S.A., McCauley, G.N., Chandler, J.M., Cothren, J.T., 2005.
584 Assessment of acetolactate synthase (*ALS*) tolerance to imazethapyr in red rice ecotypes
585 (*Oryza spp*) and imidazolinone tolerant/resistant rice (*Oryza sativa*) varieties. Pest
586 Manag. Sci. 61, 171-178.

587 Barton, N., 1993. The probability of fixation of a favoured allele in a subdivided population.
588 Genet. Res. 62, 149-157.

589 Brooks, S.A., Yan, W.G., Jackson, A.K., Deren, C.W., 2008. A natural mutation in *rc* reverts
590 white-rice-pericarp to red and results in a new, dominant, wild-type allele: *Rc-g*. Theor.
591 Appl. Genet. 117, 575–580.

592 Burgos, N.R., Norsworthy, J.K., Scott, R.C., Smith, K.L., 2008. Red rice (*Oryza sativa*) status
593 after 5 years of imidazolinone-resistant rice technology in Arkansas. Weed Technol. 22,
594 200-208.

595 Burgos, N.R., Singh, V., Tseng, T.M., Black, H., Young, N.D., Huang, Z., Hyma, K.E., Gealy,
596 D.R., Caicedo, A.L., 2014. The impact of herbicide-resistant rice technology on
597 phenotypic diversity and population structure of United States weedy rice. Plant Physiol.
598 166, 1208-1220.

599 Cao, Q., Lu, B.R., Xia, H., Rong, J., Sala, F., Spada, A., Grassi, F., 2006. Genetic diversity and
600 origin of weedy rice (*Oryza sativa* f. *spontanea*) populations found in north-eastern China
601 revealed by simple sequence repeat (SSR) markers. *Ann. of Bot.* 98, 1241-1252.

602 Delouche, J.C., Burgos, N.R., Gealy, D.R., de San Martin, G.Z., Labrada, R., Larinde, M.,
603 Rosell, C., 2007. Weedy rices: origin, biology, ecology and control. FAO. pp. 45-62.

604 DoLago, A.A., 1982. Characterization of red rice (*Oryza sativa* L.) phenotypes in Mississippi.
605 Ph.D dissertation. Mississippi State University, MS.

606 Doyle, J.J., Doyle, J.L., 1990. Isolation of DNA from small amounts of plant tissues. *BRL*
607 focus 12, 13-15.

608 Gealy, D.R., Agrama, H.A., Jia, M.H., 2012. Genetic analysis of atypical US red rice
609 phenotypes: indications of prior gene flow in rice fields? *Weed Sci.* 60, 451–461.

610 Gealy, D.R., Burgos, N.R., Yeater, K.M., Jackson, A.K., 2015. Outcrossing potential between
28 611 US blackhull red rice and *indica* rice cultivars. *Weed Sci.* 63, 647-657.

612 Gealy, D.R., Mitten, D.H., Rutger, J.N., 2003. Gene flow between red rice (*Oryza sativa*) and
613 herbicide-resistant rice (*O. sativa*): implications for weed management. *Weed Technol.*
614 17, 627-645.

615 Gealy, D.R., Tai, T.H., Sneller, C.H., 2002. Identification of red rice, rice, and hybrid
616 populations using microsatellite markers. *Weed Sci.* 50, 333-339.

617 Gealy, D.R., Yan, W., Rutger, N., 2006. Red Rice (*Oryza sativa*) Plant types affect growth,
618 coloration, and flowering characteristics of first- and second-generation crosses with rice.
619 *Weed Technol.* 20, 839-852.

620 Goss, W., Brown, E., 1939. Buried red rice seed. *J. of the Amer. Soc. of Agron.* 31, 633-637.

621 Grant, V., 1981. *Plant speciation*. 2nd ed. Columbia University Press. New York. pp. 563.

622 Gu, X-Y., Kianian, S.F., Foley, M.E., 2004. Seed dormancy imposed by covering tissues
 623 interrelates to shattering and seed morphological characteristics in weedy rice. *Crop Sci.*
 624 45, 948–955.

625 Hardke, J.T. 2016. Trends in Arkansas rice production, 2015. In Norman, R.J. and Moldenhauer,
 626 K.A.K. (eds.). *B.R. Wells Arkansas Rice Research Studies, 2015. Arkansas Agriculture*
 627 *Experiment Station Res. Ser. 634*, 13-23.

628 Haygood, R.A, Ives, A.R., Andow, D.A., 2004. Population genetics of transgene containment.
 629 *Ecol. Lett.* 7, 213-220.

630 IBPGR-IRRI, 1980. Rice Advisory Committee, and International Board for Plant Genetic
 631 Resources. Descriptors for Rice, *Oryza Sativa* L. International Rice Research Institute
 632 (eds.). International Rice Research Institute. pp. 1-21.

633 Kadaru, S., Zhang, W., Yadav, A., Oard, J.H., 2008. Development and application of allele-
 29 634 specific PCR assays for imazethapyr resistance in rice (*Oryza sativa*). *Euphytica.* 160,
 635 431-438.

636 Langevin, S.A., Clay, K., Grace, J.B., 1990. The incidence and effects of hybridization between
 637 cultivated rice and its related weedy red rice (*Oryza sativa* L.). *Evol.* 44, 1000-1008.

638 Londo, J., Schaal, B., 2007. Origins and population genetics of weedy red rice in the USA. *Mol.*
 639 *Ecol.* 16, 4523-4535.

640 McClain, R., 2003. Clearfield 161 has rice growers buzzing. *Louisiana Agricul.* 46, 23-23.

641 Oard, J., Cohn, M. A., Linscombe, S., Gealy, D., Gravois, K. 2000. Field evaluation of seed
 642 production, shattering, and dormancy in hybrid populations of transgenic rice (*Oryza*
 643 *sativa*) and the weed, red rice (*Oryza sativa*). *Plant Sci.* 157, 13-22.

644 Pusadee, T., Schaal, B.A., Rerkasem, B., Jamjod, S., 2013. Population structure of the primary

645 gene pool of *Oryza sativa* in Thailand. *Genet. Resour. Crop Evol.* 60, 335-353.
 646 Rieseberg, L.H., Ellstrand, N.C., 1993. What can molecular and morphological markers tell us
 647 about plant hybridization. *Crit. Rev. in Plant Sci.* 12, 213-241.
 648 Roso, A.C., Merotto, A., Delatorre, C.A., Menezes, V.G., 2010. Regional scale distribution of
 649 imidazolinone herbicide-resistant alleles in red rice (*Oryza sativa* L.) determined through
 650 SNP markers. *Field Crops Res.* 119, 175-182.
 651 Shivrain, V., Burgos, N., Scott, R.C., Gbur, E.E., Estroninos, L.E., McClelland M.R., 2010.
 652 Diversity of weedy red rice (*Oryza sativa* L.) in Arkansas, USA in relation to weed
 653 management. *Weed Res.* 50, 289-302.
 654 Shivrain, V.K., Burgos, N.R., Gealy, D.R., Moldenhauer, K.A., Baquiereza, C.J., 2008. Maximum
 655 outcrossing rate and genetic compatibility between red rice (*Oryza sativa* L.) biotypes
 656 and Clearfield™ rice. *Weed Sci.* 56, 807-813.
 30 657 Shivrain, V.K., Burgos, N.R., Gealy, D.R., Sales, M.A., Smith, K.L., 2009a. Gene flow from
 658 weedy red rice (*Oryza sativa* L.) to cultivated rice and fitness of hybrids. *Pest Manag.*
 659 *Sci.* 65, 1124-1129.
 660 Shivrain, V.K., Burgos, N.R., Moldenhauer, K.A., Mcnew, R.W., Baldwin, T.L., 2006.
 661 Characterization of spontaneous crosses between Clearfield Rice (*Oryza sativa* L.) and
 662 Red Rice (*O. sativa* L.). *Weed Technol.* 20, 576-584.
 663 Shivrain, V.K., Burgos, N.R., Sales, M.A., Mauromoustakos, A., Gealy, D.R., Smith, K.L.,
 664 Black, H.L., Jia, M., 2009b. Factors affecting the outcrossing rate between Clearfield™
 665 rice and red rice (*Oryza sativa*). *Weed Sci.* 57, 394-403.
 666 Singh, V., Burgos, N.R., Singh, S., Gealy, D.R., Gbur, E.E., Caicedo, A.L. 2016. Impact of
 667 volunteer rice infestation on yield and grain quality of rice. *Pest Manag Sci.* DOI

668 10.1002/ps.4343

669 Snow, A., Pilson, D., Rieseberg, L.H., Paulsen, M., Pleskac, N., Reagon, M., Wolf, D., Selbo, S.,
670 2003. A Bt transgene reduces herbivory and enhances fecundity in wild sunflowers. *Ecol.*
671 *App.* 13, 279-286.

672 Steele, G.L., Chandler, J.M., McCauley, G.N., 2002. Control of weedy red rice (*Oryza sativa*) in
673 imidazolinone tolerant rice (*O. sativa*). *Weed Technol.* 16, 627-630.

674 Suh, H., Sato, Y., Morishima, H., 1997. Genetic characterization of weedy rice (*Oryza sativa* L.)
675 based on morpho-physiology, isozymes and RAPD markers. *Theor. Appl. Genet.* 94,
676 316-321.

677 Sweeney, M.T., Thomson, M.J., Pfeil, B.E., McCouch, S., 2006. Caught red-handed: *Rc* encodes
678 a basic helix-loop-helix protein conditioning red pericarp in rice. *The Plant Cell* 18, 283-
679 294.

31
680 Tan, S., Evans, R.R., Dahmer, M.L., Singh, B.K., Shaner, D.L., 2005. Imidazolinone-tolerant
681 crops: history, current status and future. *Pest Manag. Sci.* 61, 246-257.

682 Thurber, C. S., Reagon, M., Gross, B. L., Olsen, K. M., Jia, Y., Caicedo, A. L., 2010. Molecular
683 evolution of shattering loci in US weedy rice. *Mol. Ecol.*, 19, 3271-3284.

684 Thurber, C.S., Reagon, M., Olsen, K.M., Jia, Y. and Caicedo, A.L., 2014. The evolution of
685 flowering strategies in US weedy rice. *Am. J. of Bot.* 101, 1737-1747.

686 Tseng, T., Burgos, N., Shivrain, V., Alcober, E., Mauromoustakos, A., 2013. Inter and
687 intrapopulation variation in dormancy of *Oryza sativa* (weedy red rice) and allelic
688 variation in dormancy linked loci. *Weed Res.* 53, 440-451.

689 Vaughan, L.K., Ottis, B.V., Prazak-Havey, A.M., Bormans, C.A., Sneller, C., Chandler, J.M.,
690 Park, W.D., 2001. Is all red rice found in commercial rice really *Oryza sativa*? *Weed Sci.*

691 49, 468–476.
 692 Webster, E.P., Linscombe, S.D., Bergeron, E.A., McKnight, B.M., Fish J.C., 2015. Provisia
 693 Rice: A future option in rice. Proceedings of 68th South Weed Sci Soc. 271 p.
 694 Wenefrida, I., Utomo, H.S., Meche, M.M., Nash, J.L., 2007. Inheritance of herbicide resistance
 695 in two germplasm lines of Clearfield[®] rice (*Oryza sativa* L.). Can. J. of Plant Sci. 87,
 696 659-669.
 697 Wilson, C. E., S. K. Runsick, S.K., and R. Mazzanti, R., 2010. Trends in Arkansas rice
 698 production. In B. R. Wells Rice Research Studies 2009. Arkansas Agriculture
 699 Experiment Station Research Ser. 581, 11–21.
 700 Wilson, C.E. Jr., Branson, J.W., 2004. Trends in Arkansas rice production. In B. R. Wells, ed.
 701 Rice Research Studies. Fayetteville, AR: Arkansas Agriculture Experiment Station
 702 Research. Ser. 529, pp. 15-24.
 32 703 Zhang, N., Linscombe, S., Oard, J., 2003. Out-crossing frequency and genetic analysis of hybrids
 704 between transgenic glufosinate herbicide-resistant rice and the weed, red
 705 rice. Euphytica, 130, 35-45.
 706 Zhang, W., Linscombe, S.D., Webster, E., Tan, S., Oard, J., 2006. Risk assessment of the
 707 transfer of imazethapyr herbicide tolerance from Clearfield rice to red rice (*Oryza sativa*).
 708 Euphytica 152, 75-86.
 709

710 **Introgression of Clearfield™ rice crop traits into weedy red rice outcrosses**
711 Vijay Singh¹, Shilpa Singh¹, Howard Black², Virginia Boyett³, Supratim Basu¹, David Gealy²,
712 Edward Gbur⁴, Andy Pereira¹, Robert C. Scott⁵, Ana Caicedo⁶, Nilda R. Burgos^{1†}

[†] Corresponding author
nburgos@uark.edu (Nilda R. Burgos)

713
714
715
716
717
718
719
720
721
722
723
724
34
725
726
727
728
729
730

¹Department of Crop, soil, and Environmental Sciences, Plant Sciences, University of Arkansas,
Fayetteville, AR 72704, USA

²USDA-ARS Dale Bumpers National Rice Research Center (DBNRRC), Stuttgart, AR 72160,
USA

³Rice Research and Extension Center, University of Arkansas, Stuttgart, AR 72160, USA

⁴Agricultural Statistics Laboratory, University of Arkansas, Fayetteville, AR 72704, USA

⁵Lonoke Agricultural Center, University of Arkansas, Lonoke, AR, 72086, USA

⁶Biology Department, University of Massachusetts, Amherst, MA 01003, USA

Abstract

Studies were conducted to determine the impact of introgression of crop alleles into weedy rice populations. Seeds of 89 weedy rice accessions collected from Arkansas fields in 11 counties, with a history of ClearfieldTM rice production, were planted in 2011 and sprayed twice with imazethapyr (0.071 kg ai ha⁻¹). Survivors were genotyped using an allele-specific PCR assay to detect the presence of resistance-endowing *als* mutations, S₆₅₃N and G₆₅₄E, in herbicide-resistant (HR) weedy rice that could have originated from ClearfieldTM cultivars planted in the state. Hybridization between ClearfieldTM rice and weedy rice was verified using microsatellite markers RM 215, RM 220, RM 234 and RID12. Two to five HR plants, representing different plant types per accession per replication (698 plants total) were characterized for 16 morphological and biological traits. Seventy-nine percent of accessions were resistant to imazethapyr. The HR weedy rice plants carried the S₆₅₃N mutation and were confirmed as outcrosses with ClearfieldTM rice using microsatellite markers. Ten weedy rice accessions (nearly 25% of confirmed outcrosses) harbored multiple alleles from rice cultivars, indicating previous hybridization events with multiple cultivars or outcrossing with weedy rice. The majority of HR weedy rice accessions were similar to cultivated rice in height, culm angle, flag leaf length and width, days to flower, seed shattering and dormancy. Kernels of 20% of the parent weedy rice accessions were segregating for white and red bran color. Two of the parent weedy rice accessions with gold hull were homozygous for white bran and produced progenies with 100% white-bran kernels; thus, weedy rice was no longer exclusively red. Therefore, HR weedy rice outcrosses generally had acquired crop-like traits, which make it easier to deplete from the soil seedbank, but also difficult to scout.

Keywords: bran color; gene flow; herbicide resistance; hybrid rice; weediness

1. Introduction

Hybridization is an important component of plant evolution (Rieseberg and Ellstrand, 1993). More than 70% of plant species might have descended from hybrids (Grant, 1981). Gene flow from crop species to wild or weedy species leads to introgression of alleles of one plant population into the gene pool of another, thereby changing the population structure of a species (Anderson, 1949; Pusadee et al., 2013) or even resulting in the evolution of new species. Hybridization between crops and weedy relatives results in genotypes with intermediate characteristics (Burgos et al., 2014; Pusadee et al., 2013; Suh et al., 1997), which in some cases are more weedy in nature perhaps due to increased fitness (Arnold and Hodges, 1995; Langevin et al., 1990). Introgression of crop alleles is favored if it endows fitness advantages (Barton, 1993; Haygood et al., 2004). The introgression of single-gene traits such as herbicide resistance may lead to even greater fitness advantages in outcrossed populations compared to introgression of conventional crop traits (Shivrain et al. 2009a; Snow et al., 2003).

Owing to genetic and morphological similarities between cultivated and weedy rice, weedy rice could not be controlled selectively in rice fields (Shivrain et al., 2010; Vaughan et al., 2001). The introduction of non-transgenic, herbicide-resistant (HR) ClearfieldTM rice (Tan et al., 2005) in 2002 allowed growers to control selectively 95 to 100% of the weedy rice in rice fields by applying imazethapyr (imidazolinone, IMI) herbicide (Avila et al., 2005; Steele et al., 2002). Despite the success of ClearfieldTM rice technology, 100% control of weedy rice could not be attained sustainably in all fields. Weedy rice can escape control due to herbicide application factors, as well as environmental and biological factors (Burgos et al., 2008). These

777 escaped weedy rice plants can hybridize with ClearfieldTM rice cultivars when flowering periods
778 of the two plant types are synchronized (Shivrain et al., 2008). In the last decade many
779 researchers have investigated gene flow from cultivated rice to weedy rice populations in North
780 America (Gealy et al., 2003; Shivrain et al., 2008, 2009b; Zhang et al., 2006). The HR weedy
781 rice outcrosses in the F₂ generation or later are difficult to manage, as these segregate into
782 several diverse weedy plant types, which can compete with rice and produce inferior grain yield
783 and quality.

784 Hybridization between weedy rice and rice cultivars can range from 0.1% to 3.2%, (Cao
785 et al., 2006; Langevin et al., 1990; Shivrain et al., 2009a; Zhang et al., 2006), but mostly $\leq 1\%$
786 outcrossing is detected. Once outcrossed, the crop alleles introgress into weedy populations
787 within a few generations (Gealy et al., 2003). The low outcrossing rate and subsequent
788 introgression contribute to the evolution of weedy rice populations (Cao et al., 2006; Gealy et al.,
37 789 2003; Gealy et al., 2015). The introgression of crop traits into weedy populations can change the
790 population dynamics, morphological characteristics, and genetic structure of weedy populations
791 (Burgos et al., 2014). For a primarily self-pollinated species, weedy rice exhibits high diversity
792 within and among populations (Londo and Schaal, 2007; Shivrain et al., 2010; Tseng et al.,
793 2013). This has been attributed to the low-level natural hybridization between weedy and
794 cultivated rice, which for example, had been documented to produce admixed populations of
795 outcrosses that are taller, with larger leaves, and have more tillers than the historical weedy
796 populations (Langevin et al., 1990; Shivrain et al., 2006). In Thailand, an intermediate
797 population of weedy rice exists at the interface of rice production fields and wild rice (Pusadee et
798 al., 2013). Insight into the consequences of hybridization between weedy rice and HR rice
799 cultivars on the evolution of weedy rice populations is important for the management of this

troublesome weed. Sustainable weedy rice management necessitates a thorough understanding not only of weedy rice diversity but also of evolutionary changes in the weedy populations ensuing from crop-weed and weed-weed interactions as impacted by management tactics. This study was conducted to evaluate the impact of introgression of HR trait and crop alleles into weedy populations, particularly on weedy traits, and its broader implications on the use of other improved crop traits.

2. Materials and Methods

2.1. Screening for herbicide-resistance

Seeds of 89 weedy rice accessions were collected from 26 fields across 11 counties in Arkansas (Fig. 1) in 2010. Each field generally had multiple weedy rice types. Up to 400 g seed was collected from plants representing each plant type. In cases where only a few plants represented a plant type, the amount of seed collected could be less than 50 g depending on the number of panicles in each plant. Seed collection was conducted in a two-week period and only mature panicles were harvested. Panicles were air-dried at room temperature for up to three weeks before the seeds were threshed, cleaned, and stored at room temperature until used. The hull colors of weedy rice accessions were diverse – 43% strawhull, 26% blackhull, 8% brownhull, 11% goldhull, 9% greyhull, and 3% mixed-hull color with striations. These accessions were tested for resistance to imazethapyr in a field experiment at the Rice Research and Extension Center (RREC) Stuttgart, AR in 2011. The soil was a DeWitt silt loam (fine, smectitic, thermic Typic Albaqualfs) with 0.9% organic matter and pH 6.2. Eighty-nine red rice accessions and three ClearfieldTM rice cultivars (CL151, CLXL729, CLXL745) were planted in 6.1-m row per accession at 50 seeds per row on 20 May, 2011. Imazethapyr was applied at 0.5X

823 (35 g ha⁻¹) and 1X (70 g ha⁻¹) of the field use rate when seedlings had two- to three-leaves,
824 followed by a second application 7 d later. Injury and mortality were recorded at 21 d after
825 treatment (DAT) from the 1X rate only because the majority of plants survived this treatment.
826 Mortality (%) was calculated based on the number of plants that survived out of the total number
827 sprayed.

828

829 2.2. *Molecular marker analysis*

830 2.2.1 *Plant materials and DNA extraction*

831 Leaf tissues were harvested from 89 HR weedy plants, surviving the 1X rate of
832 imazethapyr, each representing a parent accession. Also included were three reference cultivars
833 (CL151, CLXL729, CLXL745). Total genomic DNA was extracted using a modified
834 hexadecyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1990). The genomic
39 DNA was quantified using a spectrophotometer, NanoDrop 2000c V. 1.0 (NanoDrop
835 Technologies, Wilmington, DE, USA), and diluted to 100 ng µl⁻¹ with deionized water.
836

837 2.2.2 *Allele-Specific PCR (AS-PCR)*

838 Polymerase chain reaction (PCR) reactions were conducted for the *ALS* gene SNPs
839 (S₆₅₃N and G₆₅₄E) using a set of allele-specific forward primers and a common reverse primer as
840 described by Kadaru et al. (2008). For each sample, a 20-µl PCR mix consisting of 3 µl genomic
841 DNA (20 ng µl⁻¹) mixed with 10 µl of 2X Taq Mastermix; EmeraldAMP[®] MAX HS PCR Master
842 Mix (Takara Bio, Madison, WI, USA), 1 µl each of 10 µM forward and reverse primers and 5 µl
843 deionized water. DNA amplification reactions were carried out using thermocycle conditions of
844 95 °C—2 min; 95 °C—20 s, 60 °C—20 s, and 72 °C—20 s for 28 cycles and 72 °C—5 min.
845 After amplification, 7 µl of each PCR product was loaded onto a 2% OmniPur[®] agarose gel

(EMD Chemicals Inc., Gibbstown, NJ, USA), and ran for 60 min in 1X TBE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The PCR products were stained with GelRedTM Nucleic Acid Stain (Biotium, Hayward, CA, USA), visualized using GelDoc-It TS2 Imager (UVP LCC, Upland, CA, USA) and documented.

2.2.3. Verification of Hybridization

Hybridization between weedy red rice and ClearfieldTM rice cultivars was verified using 10 cultivars as standards. These included nine ClearfieldTM rice cultivars (inbreds and hybrids) and one conventional inbred cultivar, 'Jupiter'. The InDel marker, RID12 (Sweeney et al., 2006), and three microsatellite markers [RM215 (Gealy et al., 2002), RM220 (Akagi et al., 1996), and RM234 (Zhang et al., 2006)] were used to differentiate alleles known to be present in commercial white rice from those known to be present in US weedy red rice. Microsatellite markers are codominant, therefore the outcrosses are expected to show PCR-amplified DNA fragments from both the susceptible weedy rice and the rice cultivars (Gealy et al., 2002). For PCR amplification, 2 µl of Zymo purified template DNA (80 ng µl⁻¹) were used in a reaction containing HotStarTaq DNA Polymerase, Qiagen Multiplex PCR Buffer with 6 mM MgCl₂, ultrapure quality dNTP Mix, and 0.25 µM of each primer. The PCR was performed using Qiagen Multiplex PCR Kit, (Qiagen, Valencia, CA, USA) using the cycling protocol of a 15-min hot start at 95 °C followed by 35 cycles of traditional 3-step thermal cycling starting with denaturation at 94 °C for 30 s, primer annealing at 55 °C for 90 s, and elongation at 72 °C for 60 s. The reactions were then heated to 60 °C and incubated for 30 min before storing the completed reactions at 8 °C. Two microliters of each diluted PCR product were added to the Genescan 400HD [ROX] size standard (Applied Biosystems, Foster City, CA, USA). The PCR products were resolved in an Applied Biosystems 3730 DNA Analyzer using POP-7 polymer on

a 36 cm 48-capillary array. The fluorescent signal of the resolved amplicons was analyzed using GeneMapper v4.0 software.

2.3. Morphological characterization

Two- to five plants per accession (total of 698 weedy rice and 21 ClearfieldTM rice plants) which survived the 1X rate of imazethapyr, representing different phenotypes among the segregating offspring, were characterized based on 16 morpho-physiological traits according to the guidelines of the International Board for Plant Genetic Resources (IBPGR-IRRI, 1980). The traits evaluated were culm angle; stem color; leaf color and texture; flag leaf length, width, and angle; leaf exertion; days to flower; panicle length; hull color; awn color and awn length; grain yield per plant; seed shattering and seed dormancy. Culm angle was determined relative to the horizontal plane (low value = open canopy). Flag leaf angle was determined with reference to the culm (low value = erect flag leaf). Length and width of flag leaves were measured from five tillers per plant. Leaf width was measured at the widest point of the leaf. Leaf texture was evaluated by rubbing the finger along the adaxial surface of the leaf blade. Plant height was measured from the base of the plant to the tip of the flag leaf on the main culm. Panicle exertion was determined based on the portion of panicle still enclosed by the flag leaf (i.e. small panicle exertion value equates to a 'large' proportion of the panicle still enclosed by the flag leaf). Flower initiation of characterized plants was recorded weekly. A plant was considered flowering when four tillers had at least partially exerted panicles. The length of five panicles was measured per plant. Awn length was measured for five seeds of the same panicle. Awn color and hull color were evaluated visually, after the grains had matured. After characterization, plants were enclosed in Delnet[®] bags (Delstar Technologies, Middletown, DE, USA) to collect the shattered

seeds.

2.4. Seed shattering

Weedy rice (698) and ClearfieldTM rice plants (21) were enclosed in Delnet® bags after morphological characterization and were harvested 45 d after flowering. Harvesting spanned a period of 10 wk because of the diversity of flowering dates of weedy rice. At maturity, each plant was cut 10 cm from the ground and seed shattering was recorded within a week from harvest. To determine the level of seed shattering, appropriate sets of weights were attached to the bagged panicle bundles to attain a uniform weight of 2.5 kg. Each bundle was then dropped from a height of 1 m into a container so that each plant was subjected to the same force of 50 kgf. The shattered seeds were weighed. The panicles with remaining seeds were threshed, and the remaining seeds were weighed. The total yield was recorded as shattered seeds + non-shattered seeds. Seed shattering (%) was calculated as:

$$\text{Shattering (\%)} = [\text{shattered seed weight (g)} / \text{total seed yield (g)}] * 100$$

2.5. Evaluation of seed dormancy

Seed dormancy was evaluated at 75 d after harvest (DAH) and at 270 DAH. Seeds of 644 HR offspring and 15 ClearfieldTM rice plants were germinated in batches according to the respective harvest date of each plant. Thirty seeds from each offspring were placed in a Petri-dish (9 cm diameter), replicated three times, moistened with 6 ml deionized water, and dark-incubated in a growth chamber at 30 °C. The Petri-dishes were placed in trays and the trays were arranged in randomized complete block design in the growth chamber, with replications serving as blocks. The trays were covered with a plastic sheet to prevent desiccation of seeds and were

re-watered every 4 d after each germination evaluation. Germination capacity (GC) was evaluated in a 12-d period. Seeds were considered germinated when the radicle protruded from the caryopsis. The Petri plates were re-randomized after each evaluation period. Firm, non-germinated seeds were counted as viable, dormant seeds. This approach for evaluating seed viability was supported by a preliminary study comparing the tetrazolium test with the seed-firmness test, using 10 samples. Both tests produced the same viability results (data not shown). The seed-firmness test enabled the evaluation of more than 1,000 samples.

2.6. Kernel characterization

Size and pericarp color of seeds harvested from growers' fields (89 accessions) as well as seeds of progenies (698) and reference cultivars (ClearfieldTM rice) characterized at the RREC were evaluated using Graincheck (2312 FOSS TECATOR). Samples were de-hulled and the bran color of 100 kernels per sample was recorded according to the guidelines of IBPGR-IRRI (1980). There were seven categories for bran color in this system – white, light brown, speckled brown, brown, red, variable purple, and purple. Length (L), width (W), L/W ratio and 1000-kernel weight were recorded.

2.7. Data analyses

All data were analyzed using JMP Pro (version 11.0; SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was conducted for all the quantitative plant traits using a completely randomized design. Differences among and within ecotypes in plant traits were determined using one-way ANOVA and means were separated by Fisher's Protected LSD at the 5% probability level. Principal component analyses (PCA) for morphological traits was

conducted using six principal weedy traits; based on their contribution and 'Eigen' values. *K*-means clustering (JMP Pro v. 11) was performed to group the weedy rice offspring (698 plants) based on the six selected traits. Cluster analysis was performed on germination and seed shattering. *K*-means cluster analysis was carried out where variables were scaled individually (differences were scaled by an overall estimate of the standard deviation of each variable) by default in JMP Pro (v.11). The numbers of clusters were selected based on fit statistic, with the largest CCC (Cubic Clustering Criterion). PCA and *K*-means cluster analysis were conducted for 679 offspring based on all kernel characteristics to distinguish weedy population groups by type of kernels.

3. Results

3.1. Frequency of herbicide-resistant weedy rice

ClearfieldTM rice had been planted in the 26 sampled fields (Fig. 1) for different numbers of crop seasons. Seventy-nine percent of the accessions from these fields were resistant to imazethapyr (0-79% injury); of these, 98% were highly resistant (0-20% injury) (Fig. 2). However, only the S₆₅₃N mutation was detected in the HR weedy rice accessions (Fig. 3) which was the same mutation harbored by the ClearfieldTM rice cultivars planted in the state. The frequency of resistant offsprings was independent of hull color of mother plants, probably due to multiple outcrossing events that had occurred across multiple years. Using the InDel primer and three microsatellite markers (Fig. 4) we detected 42 weedy rice accessions harboring the same alleles as those of rice cultivars. Twenty-five percent of these 42 accessions harbored multiple alleles that were also present in more than one cultivar. This indicates that these HR weedy rice accessions had hybridized with at least one of the reference ClearfieldTM cultivars used. The

remaining 28 HR weedy rice accessions did not contain any of the crop alleles that were amplified by the set of primers. These 28 HR weedy rice might have hybridized with other Clearfield™ rice cultivars not included in the test. Forty-eight percent of HR weedy rice accessions were homozygous resistant and 31% were heterozygotes, carrying both mutant and wild alleles (Fig. 3).

3.2. Differentiation of morphological and phenological traits

Hull colors of the remnant weedy rice accessions (89) from fields with history of Clearfield™ rice, were diverse – 43% were strawhull, 26% blackhull, 8% brownhull, 11% goldhull, 9% greyhull, and 3% mixed-hull color with striations. The offsprings of these 89 accessions collectively segregated into 260 (37%) blackhull, 209 (30%) brownhull, 29 (14%) goldhull and 200 (28%) strawhull ecotypes (Table 1 & 2). Out of 16 plant traits characterized, six contributed significantly to the overall morphological diversity of HR weedy rice including plant height, days to flower, panicle length, hull color, seed shattering and grain yield (Suppl. Fig. 1). Overall, 76% of the characterized progenies were taller than cultivated rice (130 cm) (Suppl. Fig. 2-A).

K-means cluster analysis grouped the 698 HR weedy rice plants into three clusters (Table 1) based on six traits with the largest contribution to population differentiation as determined by PCA. Clusters 1 and 2, representing 55% of the characterized plants, had mean heights of 127-131 cm similar to that of the reference cultivars in the study. The largest group, Cluster 3, consisted of 314 very tall individuals (166 cm). These plants had longer awns (2.3 cm), intermediate seed shattering (56%) and lower yield potential (40 g plant⁻¹) than plants in the other clusters. Seventy-eight percent of weedy rice plants in Cluster 3 had dark hull color (45% blackhull and 33% brownhull).

The range of flowering initiation among HR weedy rice was 70 to 135 d after planting (DAP) with 71% flowering at 96-99 DAP similar to that of Clearfield™ rice cultivars (97 DAP). Blackhull and brownhull weedy rice flowered later (101 DAP) than strawhull types (96 DAP). Plants in Cluster 1, consisting of about ¼ (26%) of HR weedy rice offspring, were phenotypically similar to cultivated rice (Table 1). Cluster 2 consisted of 29% HR offspring that were also morphologically similar to the rice cultivars (the same height, upright leaves and closed canopy), but flowered later (+7 DAP), had higher seed shattering (+35%), shorter panicles (-2.3 cm), and lower yield potential (-20.1 g) than Clearfield™ rice. Generally, HR plants exhibited some crop-like characteristics regardless of hull color. The majority of HR weedy rice had upright leaves and culm angles similar to cultivated rice.

3.3. Differentiation of seed shattering and dormancy

K-means cluster analysis of GC at 75 and 270 DAH involving 698 HR weedy rice produced three distinct clusters (Table 3). Cluster 1, comprised of 69% of individuals, had high GC (94 and 97%) similar to cultivated rice (90 and 91%) at 75 and 270 DAH, respectively. Clusters 2 and 3 had lower GC than cultivated rice. Only a small proportion of HR weedy rice (10%, Cluster 3) had up to 50% dormancy after a long afterripening period of 270 DAH. Further, 42% of HR weedy rice offspring showed only 40% seed shattering. Eighteen percent of HR weedy rice offspring had equal or less seed shattering than the cultivars (Suppl. Table 1). Blackhull weedy rice had the highest seed shattering (60%) and goldhull weedy rice had the lowest (39%) (Table 2).

3.4. Differentiation of kernel characteristics

The PCA analysis of kernel characteristics revealed that kernel width, length, and weight of 1000-kernels were major contributors to component 1; bran color was the main contributor to

the variation in component 2. Together, these explained 81% of the variation in kernel characteristics of HR weedy rice offspring from field-collected plants. *K*-means cluster analysis based on bran color, kernel length, width and 1000-kernel weight grouped HR weedy rice offspring into three clusters (Suppl. Table 2). Clusters 1, 2 and 3 had 40, 26 and 13% of kernels with white bran color, respectively. Kernels in Clusters 2 and 3 were longer and had higher 1000-kernel weight. In general, the kernels of HR weedy rice with red bran were larger than those with white bran and characteristics associated with red bran positively affected yield parameters. Kernels with light brown bran, were the largest (Suppl. Table 3), but the proportion of kernels with light brown bran was very low (<1%) and did not influence the average kernel size and weight.

Of 109 characterized HR offspring from 17 weedy rice parent accessions with mixed-color kernels, 66% had red bran and 33% had white bran (Fig. 5). Out of 89 parent accessions, 60 with red-bran kernels produced offspring that segregated into red (69%) and white (30%) bran. This data was from 477 plants (76% of total characterized HR offspring). Thirty-five percent of these segregating offspring with white-bran kernels were blackhull ecotypes and 28% were strawhull. Two of the parent weedy rice accessions (goldhull types) with white-bran kernels had offspring with 100% white-bran kernels. Collectively, kernels with white bran were observed in about one-third (30%) of the characterized HR weedy rice offspring (Fig. 5, Table 4). Only 6 of 89 parent weedy rice accessions were homozygous for the red bran trait, producing 100% progenies with red-bran kernels (Fig. 5). Heterozygous parent accessions had progenies that produced red- and white-bran kernels. Microsatellite marker analysis of 89 HR offspring, each representing one parent accession, showed that 57% and 20% of progenies were homozygous for red and white bran phenotypes, respectively (Fig. 4 and Fig. 5) . Twelve percent

were heterozygous, containing alleles for both red and white bran color.

Four parent accessions, with more than 60% of their progenies producing white-bran kernels, were selected for comparison with cultivated rice (Table 5). Two of these parent accessions were homozygous for white-bran kernels. White-kernel progenies of red-kernel parents were more diverse morphologically and had larger ranges of culm angles, 1000-kernel weight, and GC. These types of outcrosses overlapped 100% in flowering with the reference cultivars.

4. Discussion

4.1. Frequency of herbicide-resistant weedy rice

A survey of rice crop consultants in 2006 estimated that weedy red rice infested up to 60% of the total rice area in Arkansas (Burgos et al., 2008). In recent years, however, weedy red rice infestation has been reduced to roughly 20% due to a generally good adherence by growers to the stewardship program, which strongly promotes practices to minimize escapes and prevent seed production of the escaped plants (R.C. Scott, 2015, personal communication). Weedy rice seed can remain dormant in the soil for up to 10 years (Goss and Brown, 1939); thus, effective management needs to be sustained over a long period to deplete the soil seedbank and prevent reinfestation. The occurrence of resistant offspring from remnant weedy rice sampled across 26 fields in 2010 (Burgos et al., 2014) indicates that gene flow of the HR trait to weedy rice had occurred in accordance with the low-level outcrossing behavior of the species. Clearfield™ rice cultivars in the USA harbor either G₆₅₄E or S₆₅₃N. Clearfield™ cultivars CL121 and CL141 harboring the G₆₅₄E mutation were introduced in 2002 in the USA and were adopted rapidly by growers. However, these were replaced quickly in 2003 by newer cultivars (e.g. CL161)

1053 harboring the S₆₅₃N mutation, which endows a higher resistance level (Avila et al., 2005;
1054 Wenefrida et al., 2007; McClain, 2003). In Arkansas, 19% of the rice area in 2004 was planted
1055 with ClearfieldTM inbred rice (CL121, CL141 and CL161) and ClearfieldTM hybrid rice (CLXL8)
1056 (Wilson and Branson, 2004). With the introduction of ClearfieldTM hybrids (e.g. CLXL8,
1057 CLXL745, CLXL729) harboring the S₆₅₃N mutation, adoption of this technology increased even
1058 further owing to the higher yield potential of these hybrids. In 2009, 45% of the rice hectareage
1059 in Arkansas was planted with ClearfieldTM rice, the majority of these being ClearfieldTM hybrids
1060 (Wilson et al., 2010). These ClearfieldTM cultivars and those planted later, harbored the S₆₅₃N
1061 mutation (Fig. 3). The weedy rice accessions used in the current study were collected in 2010
1062 and with the first commercial cultivars harboring the G₆₅₄E mutation being planted for just one
1063 cropping season, it was not surprising that only the S₆₅₃N mutation was detected among the HR
1064 outcrosses. The area planted with ClearfieldTM rice increased to 69% in 2011, which was the
49
1065 peak of technology adoption to date. The recent decline in ClearfieldTM rice area is attributed to
1066 the decline in weedy rice-infested fields as a result of generally proper stewardship of the HR
1067 technology and, partly due to the appearance of HR weedy rice populations, which forced
1068 growers to rotate to other crops (R.S. Scott, pers. communication). Still, ClearfieldTM hybrid
1069 cultivars (e.g. CLXL729, CLXL745) are among the most popular in the US ricebelt. In 2015,
1070 44% of total rice area in Arkansas was planted with ClearfieldTM cultivars, >50% of which were
1071 ClearfieldTM hybrids (Hardke, 2016).

1072 In Brazil, 82% of the IMI-resistant weedy rice plants carried the same *als* mutations as
1073 the predominant rice cultivars planted in the years prior to the weedy rice survey (Roso et al.,
1074 2010). ClearfieldTM rice cultivars in Brazil carry at least one of three *als* mutations (S₆₅₃N, G₆₅₄E,
1075 A₁₂₂T), which endow resistance to IMI herbicides. . In Brazil, however, some cases of *de novo*

1076 mutation in weedy rice *als* might have been selected also by the imidazolinone herbicides used in
1077 ClearfieldTM rice.

1078 Weedy rice plants are primarily strawhull or blackhull types (Gealy et al., 2002;
1079 Vaughan et al., 2001). Differences in morphological and phenological characteristics of these
1080 groups are expected to impact their outcrossing rates with rice cultivars. Collectively, strawhull
1081 weedy rice ecotypes have a longer flowering overlap with rice cultivars than blackhull weedy
1082 rice and the overlap in flowering is positively correlated with outcrossing rate (Shivrain et al.,
1083 2008). In the current study, the frequency of HR offsprings was independent of hull color of the
1084 mother plants, reflecting the fact that besides synchronization in flowering, other factors impact
1085 gene flow between cultivated rice and weedy rice (Gealy et al., 2015; Shivrain et al., 2009b). The
1086 similar frequency of resistant offsprings among blackhull and strawhull types in the current study
1087 might be due to multiple outcrossing events across years within weedy rice populations and
50 1088 between weedy rice and cultivated rice. The fact that 25% of HR plants that harbored crop alleles
1089 shared these alleles with multiple cultivars indicates that these contemporary HR weedy
1090 populations had hybridized with multiple cultivars or other earlier-generation weedy outcrosses.
1091 Contemporary populations here pertain to HR populations that had evolved during the Clearfield
1092 riceTM era. Homozygous alleles in weedy rice indicate crop-weed hybridization that occurred
1093 multiple generations prior and now is introgressed into the weedy populations.

1094 *4.2. Differentiation of morphological and phenological traits*

1095 Historical weedy rice populations are generally 15-65% taller than cultivated rice (Do
1096 Lago, 1982; Shivrain et al., 2006). Historical populations are those that had co-existed with rice
1097 through the years of rice production in the US. Height is a major trait that lends weedy rice a
1098 competitive advantage over cultivated rice and causes further yield losses as weedy rice lodges

severely at high density, bringing the rice crop down with it. The majority of contemporary HR outcrosses were taller than cultivated rice (Suppl. Fig. 2-A) just as reported by Shivrain et al. (2006) where outcrosses of weedy rice and cultivated rice were taller than either parent in the first generation. The F₂ outcrosses segregate in height from 40-180 cm (Shivrain et al., 2006), with the majority still being taller than cultivated rice. In contrast to the historical weedy rice populations, some contemporary populations of HR weedy rice (Clusters 1 and 2, Table 1) are shorter and more similar to the crop. Notwithstanding the potential influence of plant height on gene flow (Shivrain et al., 2009b, 2010; Zhang et al., 2003), these intermediate admix of HR weedy rice populations can serve as conduits for further crop-weed and weed-weed gene exchange. Historical weedy rice populations in general have droopy leaves and an open canopy (Do Lago, 1982; Shivrain et al., 2010) whereas the majority of contemporary HR weedy rice plants have upright leaves and similar culm angles as rice cultivars. The change in gross plant morphology among the HR weedy rice populations is among the consequences of hybridization with cultivated rice (Gealy et al., 2012; Shivrain et al., 2010). Many contemporary HR populations, therefore, would be camouflaged in the rice field. From the standpoint of monitoring and management decision-making, this is a problem as rice scouts and farmers can no longer distinguish between weedy and cultivated rice during the vegetative stage as easily as with historical weedy types.

The overall range of flowering among contemporary HR weedy rice populations was similar to that reported for historical populations (70-130 DAP) in Arkansas in 2002 (Shivrain et al., 2010), but a shift in phenology distribution within this flowering range was observed among HR outcrosses. Out of 20 weedy rice accessions in 2002, 90% of the plants flowered 98 DAP which was similar to that of ClearfieldTM cultivars (97 DAP). In the current study, a lesser

proportion (71%) of weedy rice outcrosses had heading dates similar to those of Clearfield™ cultivars (Table 1) because many of these outcrosses were segregating for heading date, with some earlier and others later. The shift in phenology is not due to introgression of the HR trait; rather, it emanated from the introgression of flowering-related crop alleles as a consequence of crop-weed hybridization. Thurber et al. (2014) reported a wide range of heading dates of hybrids derived from strawhull or blackhull with tropical *japonica* cultivars. The extended flowering range in recent HR weedy populations would increase the window for outcrossing with cultivated rice. Thus, the risk of gene flow or backcrossing involving contemporary HR weedy rice, cultivated rice, and the natural weedy rice populations is expected to be higher than when involving only historical weedy rice populations.

Contemporary blackhull and brownhull weedy rice still flowered later (101 DAP) than strawhull types (96 DAP), similar to historical populations (Do Lago, 1982; Shivrain et al., 2010; Thurber et al. 2014). Because strawhull weedy rice has longer overlap in flowering with cultivated rice, it was expected that the frequency of HR weedy rice would be higher among strawhull populations than among blackhull or brownhull populations. This was not the case. The frequencies of HR weedy rice progeny were similar across hull types, indicating the role of other factors in modifying effective gene flow and introgression.

Hybrid cultivars such as CLXL8 exhibited longer flowering overlap (+2 to 3 d) with weedy rice versus conventional cultivars such as CL161 (Shivrain et al., 2009b). This extended synchronization in flowering increases the probability of hybridization between weedy rice and hybrid rice. The area planted with hybrid rice in Arkansas is increasing owing to the generally higher yield potential of hybrids (Hardke, 2016). The increasing area under Clearfield™ hybrid rice could accelerate the evolution of HR weedy rice and increase the diversity of weedy

populations as hybrid rice cultivars outcross with weedy rice at higher rates than do inbred cultivars (Shivrain et al., 2009b). For example, the out-crossing rate of CLXL8 (ClearfieldTM hybrid) with weedy rice was 0.23% while that of CL161 (ClearfieldTM inbred) and weedy rice was 0.07%. With increasing area of hybrid rice comes higher risk of gene flow and potentially increased frequency of HR outcrosses.

The largest group of HR weedy rice plants (Cluster 3, Table 1), consisting of very tall individuals (166 cm), were taller than the rice cultivars planted in the southern US rice belt, which were 95 -105 cm tall (Shivrain et al., 2010). This type of HR plants are expected to be more competitive with rice cultivars than the other weedy rice groups just by sheer plant size. The flowering of these contemporary HR weedy rice synchronized more with rice cultivars than did the flowering of historical accessions where only 60% overlapped in flowering with cultivated rice (Shivrain et al., 2010), indicating a shift toward higher synchronization with cultivars. This means that once the HR trait is introgressed into the weedy population, the risk of gene flow can increase between weed to crop and crop to weed. Therefore, with current or future HR rice technology, whether with inbreds or hybrids, it is very important to ensure complete control of weedy rice and prevent the seed production of escapes in HR rice systems.

Other changes between historical weedy rice and contemporary HR weedy rice were observed also. Among the 2002 weedy rice accessions, 48/215 (22%) were blackhull, 15/215 (7%) were brownhull, and the rest (71%) were strawhull (Shivrain et al., 2010). However, in the current study, the majority of HR weedy rice have darker-colored hulls where parents and progenies had only 43% and 28% strawhulls respectively (Table 2), in contrast to the dominance of straw-colored weedy rice among historical populations. This is possibly because blackhull and brownhull weedy rice have deeper dormancy than strawhull weedy rice (Tseng et al., 2013);

thus, the nondormant strawhull seeds would be the cohorts killed by overwinter flooding, winter freeze, chemical fallow, or stale seedbed practice.

4.3. Seed shattering and dormancy

Seed shattering and seed dormancy are major weedy traits of weedy rice (Cao et al., 2006; Delouche et al., 2007; Thurber et al., 2010). The shattered seeds remain in the soil and germinate intermittently over many years. Gu et al. (2004) reported that weedy rice can shatter up to 100% of its seeds, whereas their hybrids with cultivated rice exhibit significantly less seed shattering. In the current study, almost half of the HR weedy rice offspring showed <40% seed shattering and 18% of the offspring showed equal or less seed shattering than rice cultivars which showed 31% seed shattering (Suppl. Table 1).

It was reported previously that blackhull weedy rice has three times lower germination than strawhull weedy rice (Do Lago, 1982) and requires longer after-ripening time to release dormancy (Tseng et al., 2013). However, the hybrids of weedy rice and rice cultivars have >90% germination irrespective of hull-types (Shivrain et al., 2009a), similar to the traits exhibited by HR weedy rice in this current study. The GC of historical weedy rice ranges from 18-42% and historical weedy rice populations have high intra-population variation in seed dormancy, with blackhull populations being more variable than strawhull populations (Do Lago, 1982; Tseng et al., 2013). In contrast, contemporary HR weedy rice showed no variation in seed dormancy between strawhull and blackhull populations and low intrapopulation seed dormancy variation. The loss of dormancy means less persistence in the soil seedbank. This suggests that chemical fallow, winter flooding, and preplant application of nonselective herbicides will effectively reduce HR weedy rice. The reduction or loss of shattering trait means less replenishment of the

soil seedbank, which is good for weed management. On the other hand, it also means more contamination of harvested grain in the short term as more weedy rice seeds are harvested with the rice crop. The seed contamination problem will be eliminated as soon as 100% control of weedy rice is achieved, which is going to be easier with a smaller seedbank size. The reduction or loss of these two major weedy traits favor sustained success in weedy rice management. The difficulty in scouting for weedy rice that are more crop-like can then be countered by the adoption of integrated management practices aimed at reducing the soil seedbank.

4.4. Differentiation of kernel characteristics

Red bran is a dominant trait (Gealy et al., 2003). Weedy rice in North America has red bran, hence the term ‘red rice’ (Delouche et al., 2007), except for a rare report of white-bran weedy rice in Mississippi, USA in the early 1980s (Do Lago, 1982). The occurrence of white bran in weedy rice has been accepted as an outcome of low-level hybridization between crop and weed as supported by several studies including that of Gealy et al. (2006). Weedy rice plants with white kernels (outcrosses) show crop-like traits and are generally not distinguishable from cultivated rice (Delouche et al., 2007; Do Lago, 1982). The current study revealed that remnant weedy rice, regardless of hull color, in fields with multiple years of Clearfield rice production collectively produced a larger proportion of individuals (30%) with white bran (Table 4) than was observed in previous studies. This increased diversification of HR weedy rice kernels relative to the historical populations indicates that where the level of HR weedy rice infestation is high, the harvested grain could be contaminated with a broader array of weedy rice kernels.

White bran (*rc* allele) is the mutant (nonfunctional) version of the ancestral *O. rufipogon* (*Rc*) red allele (dominant). The *Rc* allele can be detected with the RID12 microsatellite primer

(Sweeney et al., 2006). *Rc-g* is another dominant, red-bran wild type allele, which was discovered relatively recently, but this cannot be detected with the RID12 microsatellite primers (Brooks et al., 2008). In our study, the detection of *Rc* with the RID12 primer and the allele similarity with the other three microsatellite markers (RM215, RM220 and RM234) confirmed that the white bran phenotype among the HR weedy rice plants (Fig.5, Table 4) is not the result of natural mutation in weedy rice; rather, it is due to gene flow from cultivated rice. Gealy et al. (2003) predicted the effects of a single outcrossing event and subsequent segregation of bran color, along with a dominant HR trait, across multiple years. Considering that the red seed bran (RR) and herbicide resistance (NN) are dominant traits, the population resulting from a single hybrid (single hybridization between HR cultivated white rice; rrNN and herbicide-susceptible weedy red rice; RRnn) is expected to be more than 95% HR (half with white bran) within six generations. This suggests that the weedy rice populations from commercial fields with history of ClearfieldTM rice, which produced segregating red- and white-bran kernels were at least F₂ generation outcrosses based on the proportion of red and white kernels among the offspring.

Gealy et al. (2006) demonstrated the expected segregation patterns of key phenotypic traits in F₂ plants following an outcrossing event between blackhull or strawhull weedy red rice and inbred rice. Nearly 25% of the F₂ crosses produced white seeds, which were expected to be homozygous. All the weedy rice accessions carrying only the white bran (*rc*) allele were homozygous for that allele (*rc* being a recessive trait). Thus, the presence of white-bran kernels in a weedy plant type (such as those in the current study) indicates that this gene, along with others, has already introgressed into these populations, resulting in weedy plants with diverse characteristics. The presence of only the S₆₅₃N polymorphism in the *als* gene of contemporary HR weedy rice indicates that these populations could not be beyond F₇ as ClearfieldTM cultivars

harboring S₆₅₃N were commercialized in 2003 and the weedy rice samples were collected in 2010. However, some of the outcrosses were heterozygous for the red bran allele suggesting that these are more recent outcrosses, *Rc* being a dominant trait.

The progenies of four accessions which, produced mostly white-bran kernels (60%), were upright with erect leaves and have many other characteristics similar to those of the reference cultivars (Table 5). These crop-like HR weedy populations could no longer be controlled with ClearfieldTM rice technology. One means of managing these evolving populations is with the use of an alternative HR technology. Recently, 'Provisia® rice' has been developed by BASF involving cultivars with resistance to acetyl-CoA carboxylase (ACCase) inhibitors, which selectively control grasses. With this technology the imidazolinone-resistant populations can be controlled with the application of quizalofop-P-ethyl herbicide (Webster et al., 2015). However, for this to be sustainable, it needs to be used in a crop rotation system involving soybean (or other appropriate rotational crop), conventional rice, and ClearfieldTM rice. In addition, chemical fallow between seasons and stale seedbed techniques will remove the nondormant component of the seedbank, which could be the majority of outcross seeds. Deviation from these best management practices will result in both HR traits (ALS- and ACCase resistance) being introgressed into contemporary HR populations.

5. Conclusion

Remnant weedy rice in fields with history of HR rice are generally herbicide-resistant and HR plants carry the mutant *als* with S₆₅₃N mutation from ClearfieldTM rice. These HR weedy populations are more crop-like than their historical counterparts. This study provided strong support for the stewardship recommendations for herbicide-resistant rice. Controlling weedy rice

that escape, or survive, the herbicides used in Clearfield™ rice is imperative; otherwise, the remnant weedy rice plants would outcross with the crop and eventually result in the evolution of HR weedy rice populations as documented here as well as in Latin America, Europe and Asia. The Clearfield™ rice technology has served as a model system from which we can glimpse, in real time, how natural crop-weed hybridization and subsequent introgression of crop alleles into the weedy population can impact the population dynamics and evolution of a weedy relative. This is important for the future introduction and adoption of HR technologies, or cultivars with improved traits. Removal of escapes, or prevention of seed production of remnant weedy rice is critical; otherwise, gene flow of HR trait to the weed allows for selection of outcrosses in succeeding crop cycles, evolution of HR weedy rice population, and increase in HR weedy rice population size. The outcrosses serve as conduits for continued gene flow between crop and weed and among weedy populations. The HR weedy rice populations also harbor other crop alleles and are more crop-like. The reduction in seed shattering and loss of seed dormancy do not favor persistence, which is good for soil seedbank reduction, but would mean higher crop grain contamination in the immediate. Being more crop-like also reduces the utility of weed scouting as the weed becomes indistinct from the rice crop in the field. An alternative HR technology based on a different mode of action, such as the upcoming ACCase-resistant rice, would help curtail the evolution of HR weedy rice if integrated with cultural practices stipulated in the best management practices for resistance management.

7. Acknowledgement

The authors thank Te Ming Tseng, Leopoldo Estorninos, Jr., Mariccor S.A.B. Batoy, George Botha, Reiofeli Salas, and Ed Allan Alcober for their help in implementation of the experiment

1283 in the field and evaluation of seed germination capacity and dormancy in the laboratory. The
1284 study was part of a project funded by the National Science Foundation Grant # IOS -1032023.

Literature cited

- Akagi, H., Yokozeki, Y., Inagaki, A., Fujimura, T., 1996. Highly polymorphic microsatellites of rice consist of AT repeats, and a classification of closely related cultivars with these microsatellite loci. *Theor. Appl. Genet.* 94:61–67
- Anderson, E., 1949. *Introgressive Hybridization*. New York: Wiley. pp.109.
- Arnold, M.L., Hodges, S.A., 1995. Are natural hybrids fit or unfit relative to their parents? *Trends in Ecol. and Evol.* 10, 67-71.
- Avila, L.A., Lee, D.J., Senseman, S.A., McCauley, G.N., Chandler, J.M., Cothren, J.T., 2005. Assessment of acetolactate synthase (*ALS*) tolerance to imazethapyr in red rice ecotypes (*Oryza spp*) and imidazolinone tolerant/resistant rice (*Oryza sativa*) varieties. *Pest Manag. Sci.* 61, 171-178.
- Barton, N., 1993. The probability of fixation of a favoured allele in a subdivided population. *Genet. Res.* 62, 149-157.
- Brooks, S.A., Yan, W.G., Jackson, A.K., Deren, C.W., 2008. A natural mutation in *rc* reverts white-rice-pericarp to red and results in a new, dominant, wild-type allele: *Rc-g*. *Theor. Appl. Genet.* 117, 575–580.
- Burgos, N.R., Norsworthy, J.K., Scott, R.C., Smith, K.L., 2008. Red rice (*Oryza sativa*) status after 5 years of imidazolinone-resistant rice technology in Arkansas. *Weed Technol.* 22, 200-208.
- Burgos, N.R., Singh, V., Tseng, T.M., Black, H., Young, N.D., Huang, Z., Hyma, K.E., Gealy, D.R., Caicedo, A.L., 2014. The impact of herbicide-resistant rice technology on phenotypic diversity and population structure of United States weedy rice. *Plant Physiol.* 166, 1208-1220.

- Cao, Q., Lu, B.R., Xia, H., Rong, J., Sala, F., Spada, A., Grassi, F., 2006. Genetic diversity and origin of weedy rice (*Oryza sativa* f. *spontanea*) populations found in north-eastern China revealed by simple sequence repeat (SSR) markers. *Ann. of Bot.* 98, 1241-1252.
- Delouche, J.C., Burgos, N.R., Gealy, D.R., de San Martin, G.Z., Labrada, R., Larinde, M., Rosell, C., 2007. Weedy rices: origin, biology, ecology and control. FAO. pp. 45-62.
- DoLago, A.A., 1982. Characterization of red rice (*Oryza sativa* L.) phenotypes in Mississippi. Ph.D dissertation. Mississippi State University, MS.
- Doyle, J.J., Doyle, J.L., 1990. Isolation of DNA from small amounts of plant tissues. *BRL focus* 12, 13-15.
- Gealy, D.R., Agrama, H.A., Jia, M.H., 2012. Genetic analysis of atypical US red rice phenotypes: indications of prior gene flow in rice fields? *Weed Sci.* 60, 451–461.
- Gealy, D.R., Burgos, N.R., Yeater, K.M., Jackson, A.K., 2015. Outcrossing potential between US blackhull red rice and *indica* rice cultivars. *Weed Sci.* 63, 647-657.
- Gealy, D.R., Mitten, D.H., Rutger, J.N., 2003. Gene flow between red rice (*Oryza sativa*) and herbicide-resistant rice (*O. sativa*): implications for weed management. *Weed Technol.* 17, 627-645.
- Gealy, D.R., Tai, T.H., Sneller, C.H., 2002. Identification of red rice, rice, and hybrid populations using microsatellite markers. *Weed Sci.* 50, 333-339.
- Gealy, D.R., Yan, W., Rutger, N., 2006. Red Rice (*Oryza sativa*) Plant types affect growth, coloration, and flowering characteristics of first- and second-generation crosses with rice. *Weed Technol.* 20, 839-852.
- Goss, W., Brown, E., 1939. Buried red rice seed. *J. of the Amer. Soc. of Agron.* 31, 633-637.
- Grant, V., 1981. *Plant speciation*. 2nd ed. Columbia University Press. New York. pp. 563.

- Gu, X-Y., Kianian, S.F., Foley, M.E., 2004. Seed dormancy imposed by covering tissues interrelates to shattering and seed morphological characteristics in weedy rice. *Crop Sci.* 45, 948–955.
- Hardke, J.T. 2016. Trends in Arkansas rice production, 2015. In Norman, R.J. and Moldenhauer, K.A.K. (eds.). *B.R. Wells Arkansas Rice Research Studies, 2015. Arkansas Agriculture Experiment Station Res. Ser. 634*, 13-23.
- Haygood, R.A, Ives, A.R., Andow, D.A., 2004. Population genetics of transgene containment. *Ecol. Lett.* 7, 213-220.
- IBPGR-IRRI, 1980. Rice Advisory Committee, and International Board for Plant Genetic Resources. Descriptors for Rice, *Oryza Sativa* L. International Rice Research Institute (eds.). International Rice Research Institute. pp. 1-21.
- Kadaru, S., Zhang, W., Yadav, A., Oard, J.H., 2008. Development and application of allele-specific PCR assays for imazethapyr resistance in rice (*Oryza sativa*). *Euphytica.* 160, 431-438.
- Langevin, S.A., Clay, K., Grace, J.B., 1990. The incidence and effects of hybridization between cultivated rice and its related weedy red rice (*Oryza sativa* L.). *Evol.* 44, 1000-1008.
- Londo, J., Schaal, B., 2007. Origins and population genetics of weedy red rice in the USA. *Mol. Ecol.* 16, 4523-4535.
- McClain, R., 2003. Clearfield 161 has rice growers buzzing. *Louisiana Agricul.* 46, 23-23.
- Oard, J., Cohn, M. A., Linscombe, S., Gealy, D., Gravois, K. 2000. Field evaluation of seed production, shattering, and dormancy in hybrid populations of transgenic rice (*Oryza sativa*) and the weed, red rice (*Oryza sativa*). *Plant Sci.* 157, 13-22.
- Pusadee, T., Schaal, B.A., Rerkasem, B., Jamjod, S., 2013. Population structure of the primary

gene pool of *Oryza sativa* in Thailand. *Genet. Resour. Crop Evol.* 60, 335-353.

Rieseberg, L.H., Ellstrand, N.C., 1993. What can molecular and morphological markers tell us about plant hybridization. *Crit. Rev. in Plant Sci.* 12, 213-241.

Roso, A.C., Merotto, A., Delatorre, C.A., Menezes, V.G., 2010. Regional scale distribution of imidazolinone herbicide-resistant alleles in red rice (*Oryza sativa* L.) determined through SNP markers. *Field Crops Res.* 119, 175-182.

Shivrain, V., Burgos, N., Scott, R.C., Gbur, E.E., Estroninos, L.E., McClelland M.R., 2010. Diversity of weedy red rice (*Oryza sativa* L.) in Arkansas, USA in relation to weed management. *Weed Res.* 50, 289-302.

Shivrain, V.K., Burgos, N.R., Gealy, D.R., Moldenhauer, K.A., Baquiereza, C.J., 2008. Maximum outcrossing rate and genetic compatibility between red rice (*Oryza sativa* L.) biotypes and Clearfield™ rice. *Weed Sci.* 56, 807-813.

Shivrain, V.K., Burgos, N.R., Gealy, D.R., Sales, M.A., Smith, K.L., 2009a. Gene flow from weedy red rice (*Oryza sativa* L.) to cultivated rice and fitness of hybrids. *Pest Manag. Sci.* 65, 1124-1129.

Shivrain, V.K., Burgos, N.R., Moldenhauer, K.A., Mcnew, R.W., Baldwin, T.L., 2006. Characterization of spontaneous crosses between Clearfield Rice (*Oryza sativa* L.) and Red Rice (*O. sativa* L.). *Weed Technol.* 20, 576-584.

Shivrain, V.K., Burgos, N.R., Sales, M.A., Mauromoustakos, A., Gealy, D.R., Smith, K.L., Black, H.L., Jia, M., 2009b. Factors affecting the outcrossing rate between Clearfield™ rice and red rice (*Oryza sativa*). *Weed Sci.* 57, 394-403.

Singh, V., Burgos, N.R., Singh, S., Gealy, D.R., Gbur, E.E., Caicedo, A.L. 2016. Impact of volunteer rice infestation on yield and grain quality of rice. *Pest Manag Sci.* DOI

- Snow, A., Pilson, D., Rieseberg, L.H., Paulsen, M., Pleskac, N., Reagon, M., Wolf, D., Selbo, S., 2003. A Bt transgene reduces herbivory and enhances fecundity in wild sunflowers. *Ecol. App.* 13, 279-286.
- Steele, G.L., Chandler, J.M., McCauley, G.N., 2002. Control of weedy red rice (*Oryza sativa*) in imidazolinone tolerant rice (*O. sativa*). *Weed Technol.* 16, 627-630.
- Suh, H., Sato, Y., Morishima, H., 1997. Genetic characterization of weedy rice (*Oryza sativa* L.) based on morpho-physiology, isozymes and RAPD markers. *Theor. Appl. Genet.* 94, 316-321.
- Sweeney, M.T., Thomson, M.J., Pfeil, B.E., McCouch, S., 2006. Caught red-handed: *Rc* encodes a basic helix-loop-helix protein conditioning red pericarp in rice. *The Plant Cell* 18, 283-294.
- Tan, S., Evans, R.R., Dahmer, M.L., Singh, B.K., Shaner, D.L., 2005. Imidazolinone- tolerant crops: history, current status and future. *Pest Manag. Sci.* 61, 246-257.
- Thurber, C. S., Reagon, M., Gross, B. L., Olsen, K. M., Jia, Y., Caicedo, A. L., 2010. Molecular evolution of shattering loci in US weedy rice. *Mol. Ecol.*, 19, 3271-3284.
- Thurber, C.S., Reagon, M., Olsen, K.M., Jia, Y. and Caicedo, A.L., 2014. The evolution of flowering strategies in US weedy rice. *Am. J. of Bot.* 101, 1737-1747.
- Tseng, T., Burgos, N., Shivrain, V., Alcober, E., Mauromoustakos, A., 2013. Inter and intrapopulation variation in dormancy of *Oryza sativa* (weedy red rice) and allelic variation in dormancy linked loci. *Weed Res.* 53, 440-451.
- Vaughan, L.K., Ottis, B.V., Prazak-Havey, A.M., Bormans, C.A., Sneller, C., Chandler, J.M., Park, W.D., 2001. Is all red rice found in commercial rice really *Oryza sativa*? *Weed Sci.*

49, 468–476.

Webster, E.P., Linscombe, S.D., Bergeron, E.A., McKnight, B.M., Fish J.C., 2015. Provisia

Rice: A future option in rice. Proceedings of 68th South Weed Sci Soc. 271 p.

Wenefrida, I., Utomo, H.S., Meche, M.M., Nash, J.L., 2007. Inheritance of herbicide resistance

in two germplasm lines of Clearfield[®] rice (*Oryza sativa* L.). Can. J. of Plant Sci. 87, 659-669.

Wilson, C. E., S. K. Runsick, S.K., and R. Mazzanti, R., 2010. Trends in Arkansas rice

production. In B. R. Wells Rice Research Studies 2009. Arkansas Agriculture Experiment Station Research Ser. 581, 11–21.

Wilson, C.E. Jr., Branson, J.W., 2004. Trends in Arkansas rice production. In B. R. Wells, ed.

Rice Research Studies. Fayetteville, AR: Arkansas Agriculture Experiment Station Research. Ser. 529, pp. 15-24.

Zhang, N., Linscombe, S., Oard, J., 2003. Out-crossing frequency and genetic analysis of hybrids

between transgenic glufosinate herbicide-resistant rice and the weed, red rice. Euphytica, 130, 35-45.

Zhang, W., Linscombe, S.D., Webster, E., Tan, S., Oard, J., 2006. Risk assessment of the

transfer of imazethapyr herbicide tolerance from Clearfield rice to red rice (*Oryza sativa*). Euphytica 152, 75-86.

List of Figures

Figure 1. Geographic distribution of red rice accessions collected from eleven counties in 2010, Arkansas, USA.

Figure 2. Resistance profile of remnant weedy rice (*Oryza sativa* L.) offspring collected from Arkansas, USA rice fields with history of Clearfield rice. Three- to four-leaf seedlings were treated with imazethapyr (two applications @ 70 g ha⁻¹). Susceptible plants= 80 to 100% injury, moderately resistant plants= 21 to 79% injury, highly resistant plants= 0 to 20% injury.

Figure 3. Representative electrophoresis gel image of S₆₅₃N and G₆₅₄E *als* mutation assays of 89 weedy rice accessions. Allele-specific PCR products (1=wild allele, 2= mutant allele; 134 bp band size) for the S₆₅₃N mutation are shown on the top panel; products for the G₆₅₄E mutation assay (1=wild allele, 2 = mutant allele; 131 bp band size) are on the bottom panel. Note that all of the weedy rice alleles for G₆₅₄E carry the wild type allele, and thus could not have been donated by cultivars such as CL141 or CL121. The S₆₅₃N mutation in the *als* gene is at 1880 bp and G₆₅₄E is at 1883 bp. Cultivar: JUP='Jupiter' (susceptible standard); CL=ClearfieldTM cultivars and CLXL=ClearfieldTM hybrids (resistant standards). Figure represents gel image of few selected offspring.

Figure 4. Hybridization test: Array view (screen shot) of (ABI 3730) microsatellite and InDel DNA markers. Red [ROX] peaks/lines are the GENESCAN 400HD [ROX] size standard consisting of 21 DNA fragments of known sizes ranging from 50-400 nt (left to right).

RM215 (Blue [FAM]; 136-162 nt size), RM220 (Blue [FAM]; 100-130 nt size), RID12 (Green [HEX]; 151 and 165 nt size), and RM234 (Green [HEX]; 128-157 nt size).

Tabular panel on the right: Spaces in the upper section (red rice) with the same color as those of a cultivar in the lower section indicate that the allele size for that marker was the same between the red rice sample and cultivar. Uncolored spaces in the red rice section indicate accessions with different alleles from the cultivars used in this test. Blue color represents alleles belonging to more than one cultivar.

Figure 5. Graphical representation of bran color segregation in HR weedy red rice (2010-2011), Arkansas, USA. The number of parent accessions in each group is in parenthesis. Rb = red bran, Wb = white bran, Brb = brown bran. Group 4, comprised of parents with a mixture of white and red kernels were not included in the segregation analysis. Parental data were not available for 3 accessions and were excluded from analysis.

Counties	Fields	Accessions
Chicot	2	4
Clay	1	2
Cross	1	1
Desha	1	2
Greene	2	8
Jackson	3	16
Lincoln	2	10
Mississippi	3	7
Monroe	2	13
Poinsett	6	10
Randolph	3	16
Total	26	89

Total accessions (*N*): 89

S₆₅₃N (Serine to Asparagine)

G₆₅₄E (Glycine to Glutamic acid)

Table 1.

K-means cluster analysis of herbicide-resistant weedy rice (*Oryza sativa* L.) offspring (698) based on six traits with the largest contribution to principal components analysis, Arkansas, USA.

Clusters ^a	N	Plant height	Culm angle	Leaf texture ^b		Flag leaf length	Days to flower ^c	Panicle length	-----Hull color ^d -----				Awn length	Shattering	Yield plant ⁻¹
		(cm)	(°)	S	R	(cm)	(DAP)	(cm)	S	G	Br	B	(cm)	(%)	(g)
1	182	131	73	144	38	34	96	23.5	103	11	33	35	1.7	40	95.7
2	202	127	74	137	65	32	104	20.8	40	7	74	81	1.8	67	32.7
3	314	166	76	188	125	34	99	24.5	57	11	102	144	2.3	56	40.3

^aClusters based on plant height, days to flower, panicle length, hull color, seed shattering, and seed yield per plant

^bLeaf texture: S=smooth, R=rough

^cDAP = days after planting

^dHull color; S=strawhull, G=goldhull, Br=Brownhull, B=Blackhull

Table 2.

Characteristics of herbicide-resistant weedy red rice offspring (698) by hull color, Arkansas, USA.

Hull color ^a	N ^b	Plant height	Culm angle	Flag leaf color ^c			Flag leaf length	Flowering	Panicle length	Awn length	Shatter-ing	Yield plant ⁻¹
		(cm)	(°)	G	PM	P	(cm)	(DAP)	(cm)	(cm)	(%)	(g)
straw	200	137 b	74.6 a	144	27	29	33.1 a	96 c	23.2 a	1.6 b	52.7 b	64.1 a
gold	29	147 a	74.8 a	19	6	5	32.8 a	97 bc	23.8 a	1.7 b	39.3 c	65.7 a
brown	209	148 a	74.6 a	151	33	26	33.7 a	102 a	23.1 a	2.2 a	53.6 b	47.5 b
black	260	149 a	75.2 a	179	36	45	33.4 a	101 ab	23.0 a	2.1 a	60.0 a	46.1 b

^aMeans followed by different letters are significantly different at $P=0.05$

^bNumber (N) of different hull types among 89 parent accessions, strawhull: 38, blackhull: 23, brownhull: 7, goldhull: 10, greyhull: 8, and 3 mixed-hull color with striations.

^cFlag leaf color; G=green, PM=Purple margin, P=purple

Table 3.

Cluster analysis (*K*-means) of germination capacity (GC) of herbicide-resistant weedy rice offspring (644) at two afterripening periods: 75 and 270 days after harvest (DAH), Arkansas, USA.

Clusters ^b	N	Germination capacity (%), 75 DAH					Germination capacity (%), 270 DAH				
		S ^a	G ^a	Br ^a	B ^a	Cluster ^d mean	S ^a	G ^a	Br ^a	B ^a	Cluster ^d mean
		Mean (Std dev) ^c	Mean (Std dev)	Mean (Std dev)	Mean (Std dev)		Mean (Std dev)	Mean (Std dev)	Mean (Std dev)	Mean (Std dev)	
1	478	95 (5)	94 (6)	94 (5)	93 (5)	94	96 (5)	99 (3)	97 (4)	97 (5)	97
2	101	67 (12)	64 (11)	63 (13)	66 (14)	66	84 (10)	88 (7)	86 (10)	86 (10)	85
3	65	70 (22)	56 (39)	76 (13)	82 (13)	77	54 (12)	49 (13)	50 (14)	52 (14)	52

^aHull colors: S=Strawhull; G=Goldhull; Br=Brownhull; B=Blackhull

^bAverage germination of three reference cultivars (CL 151, CLXL 729, CLXL 745) at 75 DAH = 90%; and 270 DAH = 91%

^cNumbers in parentheses are standard deviation

Table 4.

Frequency of bran color of kernels with various hull colors from herbicide-resistant weedy rice (*Oryza sativa* L.) offspring (698), Arkansas, USA.

		Bran color				Total offspring ^a
		White	Light Brown	Brown	Red	
Hull color
		(%)	(%)	(%)	(%)	
Straw		40	1	0	59	200
Gold		47	0	0	53	29
Brown		22	1	1	77	209
Black		26	0	1	74	260

^aNumber of plants based on hull color

Table 5.

Vegetative, reproductive, and grain traits of weedy rice (*Oryza sativa* L.) offspring with white bran and reference cultivars.

Vegetative, reproductive, and grain traits of weedy rice (<i>Oryza sativa</i> L.) offspring with white bran and reference cultivars.															
Accession ID ^a	N ^b	Parent bran color	Hull color ^c				Plant height (cm)		Culm angle (°) ^f		Flag leaf length (cm)		Flag leaf width (cm)		
			S	G	Br	Bl	M ^d	R ^e	M	R	M	R	M	R	
AR10-03	5	white	3	1	0	1	141	119-170	79	75-90	30	26-33	1.4	1.2-1.7	
AR10-100	5	Red	3	1	0	1	136	126-141	68	45-80	35	30-47	1.6	1.5-1.9	
AR10-102	4	Red	2	0	2	0	147	112-169	68	45-75	35	28-41	1.5	1.2-1.7	
AR10-32	8	white	3	3	1	1	136	105-157	74	70-75	36	28-59	1.4	1.2-1.5	
CL151	3	white					119	97-130	75	75	30	22-35	1.4	1.1-1.4	
CLXL729	3	white	S					126	116-131	82	75-90	30	28-34	1.2	1.1-1.3
CLXL745	3	white					126	113-134	77	75-80	33	32-34	1.3	1.2-1.3	
	Flowering (DAP)		Kernel length (mm)		Kernel width (mm)		1000-kernel weight (g)		Shattering (%)		Germination capacity (%)				
	M	R	M	R	M	R	M	R	M	R	M	R			
AR10-03	91	85-98	6.4	6.0-7.3	2.2	2.0-2.4	16.6	14.9-18.9	55	30-86	84	52-99			
AR10-100	97	88-105	6.1	5.9-6.4	2.4	2.1-2.5	17.4	15.6-18.4	64	53-80	61	31-94			
AR10-102	102	90-109	6.6	6.0-7.4	2.5	2.4-2.6	20.2	15.6-23.4	56	24-75	98	97-99			
AR10-32	90	78-108	6.6	6.0-7.5	2.3	2.1-2.3	17.7	15.3-21.3	50	20-92	97	95-99			
CL151	96	93-97	7.0	6.5-7.5	2.4	2.0-2.6	19.1	18.6-22.9	9	10-44	95	89-97			
CLXL729	96	93-97	6.9	6.6-7.2	2.2	2.1-2.4	18.6	17.3-20.9	31	10-34	96	94-98			
CLXL745	91	87-96	6.9	6.7-7.5	2.3	2.1-2.5	20.5	19.7-21.7	33	22-45	91	82-97			

^aCL151=ClearfieldTM inbred rice; CLXL729 and CLXL745= ClearfieldTM hybrid rice; AR10-03, 100, 102, 32 = Weedy rice^bN= Number of characterized offspring^cHull color: S=Strawhull; G=Goldhull; Br=Brownhull; Bl=Blackhull^dM=Mean; ^eR=Range^fCulm angle relative to the horizontal axis; 75-90° = upright or erect