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Genetic Diversity of Seed Dormancy and Molecular Evolution of Weedy Red Rice

Te Ming Tseng
University of Arkansas, Fayetteville

GENETIC DIVERSITY OF SEED DORMANCY AND MOLECULAR EVOLUTION OF WEEDY RED RICE

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By

Te-Ming Tseng
Bangalore University
Bachelor of Science in Chemistry, Zoology & Biotechnology, 2005
Allahabad Agricultural Institute – Deemed University
Masters of Science in Biotechnology, 2007

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University of Arkansas

ABSTRACT

Rice is the grain with the third-highest global production. In the US, Arkansas is the largest rice producing state; however, an estimated 62% of the rice fields in the state are infested with red rice, and can cause up to 80% yield reduction in rice. Among its weedy traits, seed dormancy plays an important role in its persistence, and helps red rice escape weed management techniques thereby increasing the red rice soil seedbank. Red rice also has the potential to hybridize among themselves and with cultivated rice, thus resulting in diverse phenotypes and genotypes. In this study we measured variation in seed dormancy at different after-ripening times, and incubation temperatures; determined the genetic diversity of dormant and non-dormant red rice populations; measured diversity in phenological and morphological traits among and within red rice populations collected across Arkansas; and, determined the genotype-phenotype relationship and population structure of old and recent red rice collections using sequence tagged site (STS) markers.

The germination response of red rice to three temperatures (1°C, 15°C, and 35°C) and four after-ripening periods (0, 30, 60, and 90 d), was evaluated. Germination varied among and within red rice populations in response to different temperatures and after-ripening period. Highest variation in germination was observed at 15°C incubation (44-97%). Among the after-ripening periods, the optimum time to release primary dormancy was 90 d. Blackhull red rice ecotypes was more dormant and also showed higher intrapopulation variation in dormancy compared to strawhull ecotypes. To determine the genetic diversity of dormant and non-dormant red rice populations, 25 simple sequence repeat (SSR) markers associated with seed dormancy loci were used. A considerable amount of genetic variation among red rice accessions was found (Nei's gene diversity (h) = 0.355), and blackhull populations (h = 0.398) were more diverse than

strawhull populations (h = 0.245). Higher genetic diversity was observed within and among dormant populations than non-dormant red rice populations.

Phenological and morphological characteristics were found to significantly vary among 113 strawhull, 71 blackhull, and 24 brownhull red rice accessions. Greater variation was observed among blackhull red rice, the tallest, late flowering, and highly tillering among the ecotypes. Strawhull red rice generally tillered less, but produced higher grain yield. Sequence analysis of 27 old (2002-2003 collection) and 52 recent (2008-2009 collection) red rice accessions, using 48 STS markers revealed a total of 447 SNPs. Recent blackhull red rice accessions had higher nucleotide diversity (Pi = 2.43 per Kb) than the old blackhull accessions (Pi = 1.21 per Kb). Old strawhull had lower sequence polymorphisms than old blackhull red rice. Genetic and phenotypic diversity among and within red rice ecotypes suggests the adoption of diverse weed management techniques in order to successfully control this troublesome weed.

This dissertation is approved for recommendation
to the Graduate Council.

Dissertation Director:

_____
Dr. Nilda R. Burgos

Dissertation Committee:

_____
Dr. Pengyin Chen

_____
Dr. James C. Correll

_____
Dr. Amy Lawton-Rauh

_____
Dr. Richard D. Cartwright

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DEDICATION

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Rice is a cereal with third highest production around the world, and is an important source of energy for majority of world's population (FAO, 2012). In the US, Arkansas produces the highest amount of rice among the rice producing states, and represents about 47% of the country's total rice production (USDA, 2012). However, numerous hurdles exist in Arkansas rice production, and one of them is weed management. Weedy red rice is a major problem in rice, infesting about 60% of the rice fields in Arkansas. Competition of red rice with rice results in up to 80% yield reduction, and also deprives rice of essential nutrients by having a larger root system (Estorninos et al., 2005; Burgos et al., 2006). Rice is shown to have originated from its wild relative, *Oryza rufipogon*, after numerous domestication events, and hybridization of wild rice with cultivated rice further gave rise to weedy rice (Londo et al., 2006). Since weedy rice belongs to the same species as rice, herbicide options are limited, as herbicides effective on red rice would also affect rice. Also, since gene flow between rice and red rice occurs at a considerable level, it is important to study the weedy traits of red rice so as to understand the origin of these traits.

Some of the unfavorable traits possessed by red rice are long culm lengths, high tillering capacity, light leaf color, weak culms, red pericarp, highly shattering seeds, and high degree of seed dormancy. Seed dormancy is one of many factors that contribute to red rice persistence. It is a state in which the seeds are prevented from germination even under favorable conditions (Bewley, 1997). Seed dormancy is manifested in diverse types of red rice, thus allowing the seeds to persist in soil for years under various field conditions (Teekachunhatean, 1985). It is thus important to study seed dormancy in red rice so as to forecast the germination potential of the seeds and also the dynamic of red rice soil seedbank. The possibility of predicting dormancy

changes of weed seed banks, and consequently, timing and extent of weed emergence, is of paramount importance to improve weed control strategies (Ghersa et al., 1997; Grundy et al., 1999; Grundy and Mead, 2000). Moreover, the possibility of predicting the emergence potential of the different weed species, allows farmers to make more rational decisions regarding the degree and type of weed management required (Forcella, 1998).

Red rice seed dormancy varies depending on the environmental factors such as temperature and after-ripening period, and the red rice ecotype (Roberts, 1961; Delatore, 1999; Vidotto & Ferrero, 2000; Gianinetti & Cohn, 2008; Do Lago 1982; Diarra *et al.*, 1985; Noldin *et al.*, 1999). Dormancy of red rice seeds was induced when pre-incubated at mid-temperatures of 15°C, but was released on decreasing the pre-incubation temperatures to 1°C (Gianinetti & Cohn, 2008); thus, indicating the induction of secondary dormancy at temperatures around 15°C. Furthermore, on increasing the pre-incubation temperatures from 15°C to 25°C, primary dormancy of red rice seeds was released more effectively than at 1°C. Dormancy in red rice seeds was also completely released when seeds were after-ripened for 28-42 d (Moreno, 1990; Gianinetti & Cohn, 2008). This vast diversity in seed dormancy can allow red rice to escape weed management and thus contribute to the soil seedbank.

Besides the seed dormancy trait, red rice in general shows high variation in morphological, phenological characteristics in addition to high genetic diversity (Cho et al., 1995; Estorninos et al., 2002; Chauhan & Johnson, 2010; Shivrain et al., 2010a, 2010b). Considerable variation in culm lengths, panicle lengths, leaf lengths, maturity and flowering dates, hull color, and grain parameters among red rice populations in Arkansas have been reported. Red rice is also highly diverse genetically which is due to hybridization among itself and with cultivated rice (Langevin et al., 1990; Londo and Schaal, 2007). This tremendous

diversity in red rice contributes to its persistence, as it can escape various management techniques, resulting in evolution of diverse red rice types. It is thus important to determine the genetic difference among and within the red rice populations, and correlate this difference with the diversity of weedy traits. It is also necessary to determine the variability in dormancy in red rice populations and diversity of the dormancy–linked loci in order to better understand the evolution of red rice.

General Objectives

The objectives of this study were to determine the variation in dormancy among and within red rice populations collected from different rice production zones in Arkansas and representing different red rice ecotypes; determine genetic diversity of dormancy-linked loci among and within dormant and non-dormant red rice populations; characterize phenological and morphological traits of red rice populations and determine if phenotypes are associated to particular ecotype, production zone, county, or cropping practice in Arkansas; and access the genetic diversity of red rice populations and relate this diversity to weedy traits of red rice.

Review of Literature

Origin of rice

Numerous studies have shown the cultivated rice (*Oryza sativa*) to have originated from its wild relative *Oryza rufipogon* (Khush, 1997; Londo et al., 2006). However, because *O. rufipogon* is a perennial while *O. sativa* is an annual, it is believed that *O. sativa* was derived from the annual, *O. nivara*, which itself originated from *O. rufipogon* (Khush, 1997; Yamanaka et al., 2003). After numerous domestication events of different *O. nivara* populations,

O. sativa indica and *O. sativa japonica* evolved, which are also the most common subspecies of rice cultivated worldwide. Throughout the domestication process, *O. sativa* has lost numerous wild traits such as long awns, short culm lengths, high degree of dormancy and shattering in seeds, pigmented pericarp, dark colored hull, and low grain yield (Hoshikawa, 1989). Although *O. sativa* is placed under two subspecies, *O. sativa* indica and *O. sativa japonica*, there are about 120,000 different cultivars of rice throughout the world (Khush, 1997). Some of the major differences between these two subspecies of rice are seed size, culm lengths, leaf color, differences in drought tolerance, differential reaction to phenol, respectively (Oka, 1988). Londo et al. (2006) showed the *O. sativa* indica subspecies to have been domesticated in India, Myanmar, and Thailand; whereas the *O. sativa japonica* subspecies was domesticated in China and Vietnam. In the US, *O. sativa japonica* is more commonly cultivated (Gealy et al. 2005).

Rice today

Today, rice is one the most important staple food that is consumed by the majority of people in the world and is the major source of nutrition for Asian countries. Rice provides about 20% of the daily calorie requirements of about 3.5 billion people worldwide (Zeigler & Barclay, 2008). It also ranks third in production among the grains, after wheat and corn (FAO, 2012). Since the sharp decline in global rice production in 2002, production has steadily increased and has reached a record high of 694 million ton in 2011, a 2.5% increase from the previous year. According to 2012 estimates, China and India are the largest rice producers representing about 31 and 21% of the global rice production, respectively. The US is ranked 12th in global rice production, contributing about 1.3% (9.2 million ton) of the world's rice grain (FAO, 2012); 60% of this volume is exported, representing 9% of the global rice export market. Rice provides

about $1.5 billion in US farm gate revenue, and is currently priced at $13.9-14.5 per hundredweight (cwt). The major rice growing states include Arkansas, Louisiana, Texas, Mississippi, Missouri, and California. Arkansas accounts for about 47% of the US rice production, thus playing an important role in the economy of the state. Almost all classes of rice are produced in Arkansas, but long grain rice is more dominantly grown (USDA, 2012). However, rice production in Arkansas faces numerous hurdles, and one of these is efficient and sustainable weed management. A survey by Burgos et al. (2008) reported about 62% of the rice fields in Arkansas infested with red rice and this infestation remains today, albeit at reduced levels due to the Clearfield® rice technology (Burgos, N. R., personal observation). The density of red rice plants in rice fields was estimated to be from 25,364 plants/ha to 104,029 plants/ha, according to growers and consultants (Burgos et al., 2008). Chemical options for controlling red rice are limited since herbicides would equally affect red rice and rice. With the commercialization of imidazolinone-resistant rice in 2002, red rice management has drastically improved (Ottis et al. 2003; Levy 2006). Greater than 90% control can be achieved with the use of imidazolinone-resistant rice (Burgos et al. 2008); however, the low-level natural hybridization between cultivated rice and weedy rice has resulted in the occurrence of imidazolinone-resistant red rice in rice fields (Burgos et al., 2008). If this trend continues, the imidazolinone-resistant rice technology may not be sustained as an option for weedy rice management.

Red rice a troublesome weed

Weedy rice, because of its red pericarp color, is also called red rice, and belongs to the same species of rice that is cultivated in the US and worldwide, *Oryza sativa* (Langevin *et al*. 1990; Parker and Dean 1976). It is a major weed occurring in rice growing regions, not only in

the US but throughout the world (Webster 2000; Dodson 1900; Nelson 1907; Burgos et al. 2008). The appearance of red rice in rice fields is primarily due to the use of rice seeds contaminated with red rice (Noldin and Corbucci, 1999, Dodson, 1900; Knapp, 1899). Infestation of red rice was shown to reduce rice yield up to 80%, depending on the red rice ecotype, and rice cultivar (Estorninos et al., 2005). Losses incurred by red rice infestation in the US, as reported by Smith (1981), was estimated to be $50 million annually, and when adjusted for inflation, this loss at present would be about $158 million annually (Sahr, 2011). Numerous molecular studies have shown US red rice to have originated from Asia (Londo & Schaal, 2007; Vaughan et al. 2001). Red rice is further classified into numerous ecotypes based on its hull color, such as strawhull, blackhull, brownhull, and goldhull. In Arkansas, the major ecotypes found are strawhull and blackhull (Shivrain et al. 2004). Strawhull red rice has a pale yellow seed coat and is usually awnless. Blackhull red rice has a black colored seed coat and is mostly awned. Strawhull red rice ecotypes are closely related to the cultivated rice in the US, while the blackhull ecotypes are more similar to wild rice, *O. rufipogon* (Vaughan *et al*. 2001; Gealy 2005).

Although of the same species as cultivated rice, it possesses numerous unfavorable characteristics such as high level of seed dormancy, high seed shattering potential and pigmented pericarp which would result in reduced quality of harvested rice grains (Craigmiles 1978; Kwon et al. 1992; Noldin et al. 1999; Gealy 2005; Shivrain et al., 2010a). Red rice can grow taller, produce more tillers, and consume more nutrients than rice (Kwon et al. 1992). A study by Burgos et al. (2006) found that red rice can accumulate more nitrogen, and is more efficient in utilizing nitrogen for biomass production than compared to Drew rice (Burgos et al. 2006). Red rice also accumulates more sucrose and shows increased growth characteristics than cultivated

rice (Sales et al., 2011). Among all the weedy traits in red rice, seed dormancy is the most important for its persistence

Seed dormancy in weedy red rice

Seed dormancy is the inability of viable seeds to germinate even when conditions are favorable for germination (Finch-Savage & Leubner-Metzger, 2006). It is an adaptive trait manifested in weedy species which allows them to escape weed management techniques and persist in the soil seedbank (Simpson, 1990). Seed dormancy is not a favorable trait in crops as intensity of seed dormancy in crops can lead to adjustment of sowing time (Ringlund, 1993). Complications may also arise after sowing, if crop seeds are dormant, such as non-uniform germination, thus leading to differences in maturity dates, and varying levels of competition with the weeds. On the other, hand, the absence of dormancy in crop seeds can lead to pre-harvest sprouting. Red rice exhibits a high degree and variability in seed dormancy (Perreto et al., 1993; Teekachunhatean, 1985; Noldin 1995, 1999b; Vidotto and Ferrero, 2000; Gianinetti & Cohn, 2008), and can remain viable in the soil for up to 10 yrs (Goss & Brown, 1939). Variability in seed dormancy is regulated mainly by environmental factors such as temperature and after-ripening time (Roberts, 1961; Delatorre, 1999; Vidotto & Ferrero, 2000; Gianinetti & Cohn, 2008). At the same environmental condition, genotypes can vary from highly dormant with no germination at all, to non-dormant with 100% germination (Li et al., 2006; Gu et al., 2004; Ye et al., 2010). Information on the timing of germination of red rice seeds can help the farmers estimate the emergence potential of these weeds, and thus adjust the crop planting date so as to give a competitive advantage to the crops. An understanding of the seed dormancy behavior in red rice can also help us identify their adaptation to a particular habitat.

Variability of seed dormancy with temperature

A study by Do Lago (1982), investigated the germination behavior of rice and red rice seeds that were after-ripened at room temperature for about 12 d, followed by an additional few weeks of after-ripening at 10°C. Seed dormancy in rice ranged from 26 to 77%, while for the red rice it ranged from 26 to 100%. However, among the red rice accessions, seed dormancy of the 10 blackhull accessions was much higher, ranging from 94 to 98%, compared to the 18 strawhull accessions with dormancy ranging from 26 to 100%. Dormancy was also found to vary among the 19 red rice accessions collected from four states in Italy (Noldin, 1995). More than half of the accessions exhibited greater than 90% dormancy, with a minimum of 63%. Cultivated rice varieties were only 10% dormant. Alternating low temperatures (5-10°C) with high temperatures (20-30°C) was more effective in releasing primary dormancy in rice (Teekachunhatean, 1985). Stratification of rice seeds at low temperatures (3°C) released primary dormancy (Roberts, 1962), but in red rice it induced secondary dormancy (Cohn, unpublished data). In red rice, incubation at sub-optimum temperatures of 25-35°C, effectively released primary dormancy (Gianinetti and Cohn, 2008). In addition, pre-incubation at 1°C was better in releasing dormancy than at 15°C pre-incubation.

Variability of seed dormancy with after-ripening time

After-ripening is known to release seed dormancy in both wild (Oliveira, 1992; Shimamoto et al., 1994) and cultivated rice (Roberts, 1961; Kalita et al., 1994). Wild rice was shown to completely release seed dormancy on after-ripening for 60-120 d at room temperature. In cultivated rice, the optimum after-ripening time to release seed dormancy was found to be 90-

120d, depending on the cultivar (Franco et al., 1997). In red rice, dormancy was about 100% when they were harvested at physiological maturity and dried to about 14% moisture content (Moreno, 1990). However, the level of dormancy gradually decreased on after-ripening the seeds at 30°C, and was almost completely released after 25 d of after-ripening (Moreno, 1990; Gianinetti and Cohn, 2008). Initially when the seeds were harvested, the germination of the Labelle and Nato rice varieties were 8 and 4%, respectively; while for the strawhull and blackhull red rice populations were 7 and 5%, respectively (Moreno, 1990). At just 5 d of after-ripening, the germination of Labelle and Nato reached 94 and 9%, respectively; while for the two red rice populations were about 92%. After 25 days of after-ripening, both the red rice populations and the Nato rice variety reached germination of about 99%, while the germination of Labelle increased to 57%. Dormancy in red rice seeds were shown to vary when initially harvested (Do Lago, 1982). When seeds of 54 strawhull red rice were harvested, majority of the accessions (33/54) were 90-100% dormant, eight of the accessions showed 60-89% dormancy, seven of them were 30-59% dormant, and two of the accessions had dormancy lower than 30%. Teekachunhatean (1985) studied the germination behavior of dormant and non-dormant strawhull and blackhull red rice seeds at different after-ripening periods, and observed that seeds of the dormant red rice phenotypes were protected from decay as compared to the non-dormant. Among the non-dormant accessions, all the blackhull and about 50% of the strawhull red rice decayed after four months of after-ripening at 30°C. In contrast, among the dormant accessions, about 84 and 76% of the blackhull and strawhull red rice, respectively, were dormant and viable even after 12 months of after-ripening at 30°C.

Molecular studies on seed dormancy

Seed dormancy is a hereditary trait that varies from one genotype to another, and this variation is dependent on the environment (Baskin and Baskin, 1998; Lee et al., 2005; Li et al., 2006; Hori et al., 2010; Gu et al., 2004; Ye et al., 2010). Numerous studies have used molecular approaches to investigate seed dormancy mechanisms in various species, including wild and cultivated rice, and red rice (Bewley and Black, 1994; Lang, 1996; Li and Foley, 1997; Cai and Morishima, 2000; Gu et al., 2004, 2011). Most of these studies involved identification of QTLs associated with seed dormancy. QTLs, also known as quantitative trait loci, are regions in the genome that are associated with complex traits, such as in this case, seed dormancy. A study using 245 RFLP markers was able to detect five QTLs linked to seed dormancy in cultivated rice (Lin et al., 1998). These QTLs accounted for about 48% of the total phenotypic variation, and were located on chromosome 3, 5, 7, and 8. A similar study by Dong et al. (2003) identified six QTLs associated with pre-harvest sprouting resistance in cultivated rice by using RFLP markers. Two of these QTLs (*q*PHS-1-1 and *q*PHS-1-2) were located on chromosome 1, while the rest (*q*PHS-4, *q*PHS-5, *q*PHS-7, *q*PHS-8) were located each on chromosome 4, 5, 7, and 8. These QTL regions were able to explain about 10-25% of the total phenotypic variations. Furthermore, *q*PHS-7 was found to be associated with after-ripening time, whereby increasing the after-ripening period released the *q*PHS-7-linked dormancy in rice. In wild rice, three QTLs, namely grm 1.1 (in chromosome 1), grm 4.1 (in chromosome 4), and grm 6.1 (in chromosome 6) were identified to be associated with decreased seed germination (Thomson et al., 2003). Gu et al. (2004) used 151 rice microsatellite markers, distributed across 12 chromosomes, to identify seed dormancy QTLs in a EM93-1/SS18-2 cross, where EM93-1 is a non-dormant rice cultivar while SS18-2 is a dormant red rice accessions from Thailand. Four of the QTLs, qSD-4, qSD-6, qSD-8,

and qSD-12, were located on chromosome 4, 6, 8, and 12, respectively; while qSD-7-1, and qSD-7-2, were located on chromosome 7. The locus qSD-12 was associated with after-ripening period; whereas locus qSD-7-1, was found to be linked to red pericarp color and was later found to be responsible for increasing abscisic acid (ABA) production, which in turn induces seed dormancy (Gu et al., 2011). Additional four QTLs for seed dormancy in red rice were located on chromosome 1, 2, and 6, contributing 4.5-7.1% of the total phenotypic variance (Jing et al., 2008).

Diversity of weedy red rice

Weedy rice exhibits high genetic and phenotypic diversity and this diversity is dependent on the ecotype and habitat (Estorninos et al., 2002; Shivrain et al., 2010a, 2010b; Chauhan and Johnson, 2010). Weedy rice from different regions of Asia (Malaysia, Philippines, Thailand, and Vietnam) varied in terms of grain characteristics and growth response under competition with cultivated rice (Chauhan and Johnson, 2010). Philippines weedy rice produced the highest grain yield, while a higher growth potential was observed in weedy rice from Vietnam. Weedy rice from Thailand was the shortest. In Arkansas, morphological characteristics varied among the two red rice ecotypes and also within each ecotype (Shivrain et al., 2010a). Blackhull red rice ecotype showed greater variation in traits than strawhull red rice. Plant height among blackhull accessions ranged from as short as 75 cm to as tall as 190 cm. The range of plant height in strawhull was greater (46-189 cm), but on an average, they were slightly shorter than blackhull red rice. Flag leaf lengths were longer in strawhull, 38 cm, than in blackhull red rice, 34 cm. Tillering capacity of blackhull red rice (mean = 105 tillers per plant) were higher than strawhull (mean = 95 tillers per plant). Also, red rice accessions from north-eastern region of Arkansas

flowered earlier than other regions in Arkansas. Molecular studies confirmed the differentiation of the two red rice ecotypes (Shivrain et al., 2010b). Strawhull red rice were genetically distant than blackhull red rice (genetic distance (D) = 0.55). A higher genetic diversity within blackhull red rice (D = 0.76) was estimated compared to strawhull red rice (D = 0.68). Reagon et al. (2010) reported high genetic diversity (Pi = 1.48 per Kb; theta = 1.36 per Kb) among and within the US weedy red rice populations, thus indicating a higher potential to evolve. Within weedy rice populations, the blackhull group showed higher nucleotide diversity (Pi = 0.66) compared to strawhull group (Pi = 0.56). Weedy rice was found to be closely related to *O. sativa* indica and *O. sativa* aus, instead of the commonly cultivated rice in the US, *O. sativa japonica*. Similar results were also observed by Londo and Schaal (2007) where most of the weedy rice clustered together with *O. sativa* aus. These results suggest that the US weedy rice evolved from *O. sativa* indica and aus, rather than *O. sativa japonica*.

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CHAPTER II

INTER- AND INTRA-POPULATION VARIATION IN DORMANCY OF WEEDY RED RICE (*ORYZA SATIVA*) AS INFLUENCED BY AFTER-RIPENING PERIOD AND GERMINATION TEMPERATURE

Abstract

Weedy red rice is a serious problem in rice production worldwide. Seed dormancy contributes to its persistence. We determined the effect of germination temperature and after-ripening period on germination capacity (GC) of blackhull and strawhull red rice seeds from Arkansas rice fields in three production zones, Grand Prairie, Delta, and White River. The germination behavior was evaluated at three temperatures (1°C, 15°C, and 35°C) and four after-ripening periods (0, 30, 60, and 90 d) in two independent experiments. Twenty five seeds from each plant sample was placed in Petri dishes lined with filter paper, and moistened with 6 ml of deionized water. At daily evaluations, seeds were considered germinated when radicle emergence was noted. Germination was recorded over a 12 day period. Germination response to temperature and after-ripening time differed among and within populations in each production zone. Overall, populations from the Delta and Grand Prairie were more dormant than those from White River. Regardless of ecotype or production zone, incubation at 35°C (mean GC=84-100%) most favored the germination of seeds after-ripened for 60 d. Germination was most variable at suboptimal temperature (15°C) with mean GC ranging from 44-97%; at 1°C, none of the seeds germinated. Primary dormancy was released in the majority of populations after 90 d of after-ripening. Blackhull populations generally had lower mean GC than strawhull populations regardless of temperature; required longer after-ripening time to release dormancy; and, showed a higher inter- and intrapopulation variation in germination and after-ripening than strawhulls.

Introduction

Rice supplies 20% of the world's total caloric intake (Clay, 2004). In North America, Arkansas is the largest rice-growing state with about 47% of the total US rice production (USDA, 2012). About 60% of rice fields in Arkansas are infested with weedy red rice (Burgos *et al.*, 2008). Although it is of the same species as that of cultivated rice (Hoagland & Paul, 1978), it possesses many unfavorable characteristics including high seed dehiscence and seed dormancy (Noldin, 1995; Suh *et al.*, 1997). The red bran or pericarp is the principal distinguishing feature of red rice and makes it more troublesome because red rice contamination of white rice reduces the quality and price of grain (Menezes *et al.*, 1997; Ottis *et al*., 2005). Red rice competes with cultivated rice by growing faster and taller than rice, producing more tillers, and taking up more nutrients owing to its larger root system (Burgos *et al.*, 2006; Sales *et al.* 2011). Among its weedy traits, seed dormancy is of major importance to its persistence.

Red rice has co-existed with cultivated rice for centuries. Seed dormancy is a trait manifested in all the diverse types and variants of red rices (Teekachunhatean, 1985; Noldin 1995; Noldin *et al.*, 1999; Vidotto & Ferrero, 2000; Gianinetti & Cohn, 2008). Understanding the dormancy behavior of red rice populations and, consequently, the possibility of predicting timing and extent of weed emergence, is of paramount importance in improving weed control strategies (Cardina *et al.*, 1997; Ghersa *et al.*, 1997; Grundy *et al.*, 1999; Grundy & Mead, 2000). Predicting the emergence potential of different weed species allows farmers to make better decisions regarding the intensity of weed management required (Forcella *et al.*, 2000). Knowledge of red rice dormancy behavior in different production areas, and its response to major environmental cues such as temperature, would ultimately allow us to predict the potential impact of crop production practices (fallow tillage, stale seedbed, crop rotation) on the red rice

seed bank and the proportion of the seed bank that could emerge. This would permit farmers to design better tactics to avoid or minimize red rice problems during crop growth. It would also enable us to predict the proportion of the seed bank that could emerge. In some instances, rice planting could be timed to first allow the largest flush of red rice emergence for the most effective preplant reduction of weed population by nonselective herbicide application.

Weed seed dormancy stages fluctuate with the season as a means of survival (Karssen, 1982; Karssen *et al*., 1988). In temperate climates, as in North America, red rice dormancy is released in early spring or late autumn and induced when exposed to extreme temperatures in the summer or winter. Red rice seeds dehisce before and during rice harvest; thus, replenishing the soil seed bank. The seed can remain dormant in the soil for up to 10 yr (Goss & Brown, 1939). Previous studies have shown that red rice seed dormancy type is modified by after-ripening period, storage conditions, and genotype (Roberts, 1961; Delatore, 1999; Vidotto & Ferrero, 2000; Gianinetti & Cohn, 2008). Seed dormancy can be overcome in several ways; effective methods vary between species. A sequence of low temperature (5-10°C) alternating with a warm temperature (20-30°C) was more effective in releasing red rice seed dormancy than incubating at low temperature alone (Teekachunhatean, 1985). In some cultivated rice varieties, stratification of seeds at 3°C released primary dormancy (Roberts, 1962), but in red rice, stratification at 5°C induced secondary dormancy (Cohn, unpublished data). Gianinetti and Cohn (2008) observed higher germination of red rice seeds when pre-incubated at 1°C than at 15°C; incubation at 25-35°C was more effective in releasing dormancy than incubation at 15-20°C. This suggests that in red rice, primary dormancy is overcome when incubation temperature is increased from 15 to 35°C. On the other hand, the degree of secondary dormancy increases when incubation temperature is increased from 1 to 15°C for which there is no clear explanation. After-ripening is

known to release primary seed dormancy in most plant species, including red rice (Steadman *et al.*, 2003; Veasey *et al.*, 2004; Bair *et al.*, 2006; Gianinetti & Cohn, 2008; Li *et al.*, 2009). After-ripening is the period that a mature seed requires to release dormancy. In Louisiana, USA, the germination of freshly harvested strawhull red rice seed was <5%; after-ripening at 30°C for 120 days increased germination to ≥90% (Gianinetti & Cohn, 2008). The hull also plays an important role in imposing seed dormancy. Red rice seed dormancy is linked with black pigmentation of the hull (Gu *et al.*, 2005). Blackhull red rice is generally more dormant than strawhull red rice (Do Lago 1982; Diarra *et al.*, 1985; Noldin *et al.*, 1999). However, there is great diversity in morphology, phenology, and genotype among blackhull red rice as well as among strawhull red rice populations (Shivrain *et al.*, 2010a; 2010b). This is a challenge to farmers because such diversity allows red rice to escape weed management tactics. Rice fields are generally infested with multiple types of red rice (Burgos, N. R., personal observation). Besides dormancy variation between red rice ecotypes, we expect that dormancy varies significantly among populations of the same ecotype even within a small localized area (i.e. County) and among mother plants within a population as observed in other weedy species (Andersson and Milberg, 1998). Diversity in dormancy behavior would enable many red rice genotypes not only to escape weed management tactics but also to persist in the soil seedbank. To be useful in modeling red rice population dynamics, the extent of variability in seed dormancy among populations of red rice ecotypes in different production zones and the variability within populations need to be evaluated. Therefore, we conducted experiments to: 1) determine inter- and intrapopulation variation in dormancy of strawhull and blackhull red rice seeds collected from fields in different production zones, in response to duration of after-ripening period and germination temperature,

and 2) determine the genetic diversity among and within red rice populations with respect to dormancy-linked loci.

Materials and Methods

Experiment 1. Effect of temperature on red rice germination

Red rice seeds were harvested between July and August 2008 from 17 fields across three rice production zones in Arkansas, USA (Grand Prairie, Delta, and White River) (Fig. 1.1). A total of nine blackhull (BH) and nine strawhull (SH) populations were harvested (Table 1.1.). For each population, four mother plants were sampled. Panicles of only those red rice plants bearing at least 90% mature seeds were harvested, air-dried in paper bags for 2 mo at room temperature (27 ± 2 °C), hand-threshed, and passed through a blower to collect filled seeds. Twenty-five seeds of each mother plant were placed in Petri dishes (9 cm diameter) lined with Whatman No.1 filter paper and moistened with 6 mL deionized water. The Petri dishes were arranged on plastic trays, covered with a clear plastic bag to prevent desiccation, and incubated at 1 °C, 15 °C, and 35 °C for 12 d. Seeds that failed to germinate at 1 °C and 15 °C, were transferred to 30 °C (denoted as 1C_30C and 15C_30C, respectively) for an additional 12 d of incubation. Treatments were replicated three times. The number of germinated seeds was recorded and removed daily. Seeds were considered germinated when the radicle protruded from the caryopsis. At the end of the incubation period, seeds that did not germinate and were firm, with no signs of decay, were considered viable and dormant.

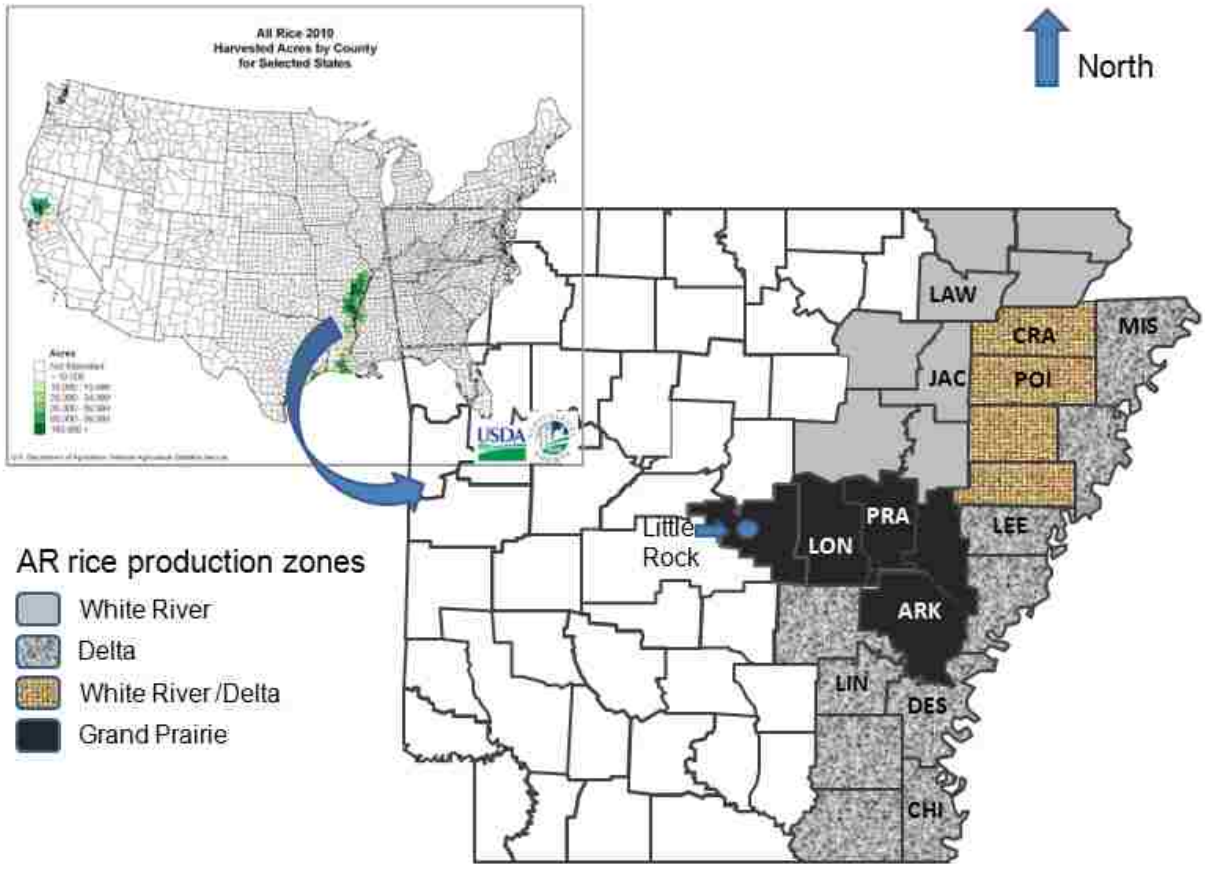

Figure 1.1. Rice production zones in Arkansas, USA. County codes: ARK = Arkansas, CHI = Chicot, CRA = Craighead, DES = Desha, JAC = Jackson, LAW = Lawrence, LEE = Lee, LIN = Lincoln, LON = Lonoke, MIS = Mississippi, POI = Poinsett, PRA = Prairie

Table 1.1. Red rice populations used to study the effect of temperature on germination. Red rice seeds were collected from three rice production zones in July and August 2008, including nine strawhull, and nine blackhull populations.

Production zone	County	Field	Ecotype	Population[a]	Number of mother plants
Delta	CHICOT	CHI08D	Blackhull	CHI08D-BH	4
Delta	DESHA	DES08B	Blackhull	DES08B-BH	4
Delta	LEE	LEE08C	Strawhull	LEE08C-SH	4
Delta	LINCOLN	LIN08A	Strawhull	LIN08A-SH	4
Delta	LINCOLN	LIN08C	Blackhull	LIN08C-BH	4
Delta	MISSISSIPPI	MIS08D	Strawhull	MIS08D-SH	4
Grand Prairie	ARKANSAS	ARK08B	Strawhull	ARK08B-SH	4
Grand Prairie	ARKANSAS	ARK08C	Blackhull	ARK08C-BH	4
Grand Prairie	LONOKE	LON08B	Strawhull	LON08B-SH	4
Grand Prairie	LONOKE	LON08F	Blackhull	LON08F-BH	4
Grand Prairie	PRAIRIE	PRA08B	Strawhull	PRA08B-SH	4
Grand Prairie	PRAIRIE	PRA08C	Blackhull	PRA08C-BH	4
White River	CRAIGHEAD	CRA08B	Blackhull	CRA08B-BH	4
White River	CRAIGHEAD	CRA08B	Strawhull	CRA08B-SH	4
White River	JACKSON	JAC08A	Strawhull	JAC08A-SH	4
White River	JACKSON	JAC08B	Blackhull	JAC08B-BH	4
White River	LAWRENCE	LAW08A	Strawhull	LAW08A-SH	4
White River	POINSETT	POI08C	Blackhull	POI08C-BH	4

[a]Population is defined as group of plants of same ecotype within a particular field.

Experiment 2. Effect of after-ripening period on red rice germination

For this experiment, red rice seeds were harvested between September and October 2009 from three rice production zones in Arkansas, USA (as in experiment 1), representing nine SH, and seven BH populations (Table 1.2.). Six mother plants were sampled from each population. Plants were harvested only when the panicles had at least 90% mature seeds. Harvested panicles were after-ripened in paper bags at room temperature (27 ± 2 °C) for 0, 30, 60, and 90 d. At the end of each after-ripening period, panicles were hand-threshed and cleaned using a blower to remove unfilled grains. The germination test was conducted following the same procedure as in experiment 1, except that all accessions were incubated at one temperature, 30 °C, in the dark for 12 d only. Germinated seeds were counted and removed daily, and dormant seeds were counted after 12 d of incubation.

Data Analysis

All statistical analyses were performed using JMP for Windows software (version 10.0.0; SAS Institute, Cary, NC). For the temperature experiment, the GC (germination capacity, cumulative germination at the end of incubation period) was used as the index of dormancy. The mean GC per mother plant is the average GC across three replications. The average germination estimate of each mother plant from each population was analyzed with a two-way factorial model that included the main effects of temperature treatments, population, and interaction terms. To quantify the variation in germination rates for each effect (zone, county, field, ecotype, plant), we selected the 15 °C temperature environment and conducted an analysis using a random effects nested model. For the after-ripening experiment, the DAR_{50} (after-ripening time to reach 50% germination) (Roberts, 1961) was used as an index of primary dormancy loss, and was

Table 1.2. Red rice accessions used to study the effect of after-ripening periods on germination. Red rice seeds were collected from three rice production zones in September and October 2009, including nine strawhull, and seven blackhull populations.

Production zone	County	Field	Ecotype	Population[a]	Number of mother plants
Delta	DESHA	DES09G	Strawhull	DES09G-SH	6
Delta	DREW	DRE09E	Blackhull	DRE09E-BH	6
Delta	DREW	DRE09E	Strawhull	DRE09E-SH	6
Delta	LEE	LEE09E	Strawhull	LEE09E-SH	6
Delta	LINCOLN	LIN09G	Strawhull	LIN09G-SH	6
Delta	LINCOLN	LIN09H	Blackhull	LIN09H-BH	6
Delta	LINCOLN	LIN09H	Strawhull	LIN09H-SH	6
Grand Prairie	LONOKE	LON09E	Blackhull	LON09E-BH	6
Grand Prairie	LONOKE	LON09E	Strawhull	LON09E-SH	6
Grand Prairie	LONOKE	LON09F	Blackhull	LON09F-BH	6
Grand Prairie	LONOKE	LON09F	Strawhull	LON09F-SH	6
Grand Prairie	MONROE	MON09A	Strawhull	MON09A-SH	6
Grand Prairie	MONROE	MON09B	Strawhull	MON09B-SH	6
White River	CROSS	CRO09A	Blackhull	CRO09A-BH	6
White River	LAWRENCE	LAW09E	Blackhull	LAW09E-BH	6
White River	POINSETT	POI09F	Blackhull	POI09F-BH	6

[a]Population is defined as group of plants of same ecotype within a particular field.

estimated using inverse prediction after fitting a logistic model to percentage GC. The DAR_{50} data for each mother plant per population was subjected to analysis of variance to quantify differences among populations.

Results

Population variation in response to temperature

Incubation at 35 °C was most favorable for germination of red rice seeds that have been after-ripened for 60 d (Table 1.3.). After 1 d of incubation at 35 °C, 1/3 of BH and 2/3 of SH populations attained >90% mean GC (data not shown). After 12 d of incubation at 35 °C, all but two BH populations (CHI08D-BH, LON08F-BH) attained at least 93% mean GC with the majority of populations attaining 99–100% germination. At 1 °C, none of the populations germinated within 12 d of incubation. However, when seeds were transferred from 1 °C to 30 °C for another 12 d, all populations rapidly attained mean GC of 90-100% (data not shown). At 15 °C, seeds started to germinate at 4 d of incubation with mean GC of 9 and 19%, respectively, for the BH and SH populations. After 12 d of incubation at 15 °C, two BH populations had <50% mean GC, while the rest of BH and all SH populations germinated 73-94%. When non-germinated seeds were transferred from 15 °C to 30 °C for another 12 d, the mean GC among populations increased at a range of 0-28%; however, none of the populations reached 100% mean GC in contrast to seeds subjected to the 1C_30C treatment.

The mean GC of BH populations was equal to or lower than that of the SH populations at 15 °C and 35 °C. At 15 °C, the mean GC of BH populations was 11% lower than that of the SH populations, and remained numerically lower than that of SH at 35 °C, averaged over

Table 1.3. Mean germination capacity of red rice populations from Arkansas, USA at 35 °C incubation, for 12 d.

Production zone	County	Field	Ecotype	Population[a]	Gerrnination capacity (%)	
					Mean[b]	Std Err[c]
Delta	CHICOT	CHI08D	Blackhull	CHI08D-BH	84	4
Delta	DESHA	DES08B	Blackhull	DES08B-BH	96	1
Delta	LEE	LEE08C	Strawhull	LEE08C-SH	100	0
Delta	LINCOLN	LIN08A	Strawhull	LIN08A-SH	93	2
Delta	LINCOLN	LIN08C	Blackhull	LIN08C-BH	99	1
Delta	MISSISSIPPI	MIS08D	Strawhull	MIS08D-SH	100	0
Grand Prairie	ARKANSAS	ARK08B	Strawhull	ARK08B-SH	99	1
Grand Prairie	ARKANSAS	ARK08C	Blackhull	ARK08C-BH	97	3
Grand Prairie	LONOKE	LON08B	Strawhull	LON08B-SH	99	1
Grand Prairie	LONOKE	LON08F	Blackhull	LON08F-BH	88	3
Grand Prairie	PRAIRIE	PRA08B	Strawhull	PRA08B-SH	98	2
Grand Prairie	PRAIRIE	PRA08C	Blackhull	PRA08C-BH	99	1
White River	CRAIGHEAD	CRA08B	Blackhull	CRA08B-BH	95	3
White River	CRAIGHEAD	CRA08B	Strawhull	CRA08B-SH	97	2
White River	JACKSON	JAC08A	Strawhull	JAC08A-SH	99	1
White River	JACKSON	JAC08B	Blackhull	JAC08B-BH	100	0
White River	LAWRENCE	LAW08A	Strawhull	LAW08A-SH	100	0
White River	POINSETT	POI08C	Blackhull	POI08C-BH	100	0

[a]Population is defined as group of plants of same ecotype within a particular field; Seeds of mother plants within each population were after-ripened for 60 d.

[b]Averaged over four mother plants per population, three replications, and 25 seeds per replication.

[c]Standard errors indicate the degree of variation in germination capacity (GC). A higher value suggests greater variation in GC among plants within the population.

populations (Table 1.3.). Two BH populations (LON08F-BH and CHI08D-BH) showed the lowest mean GC at 15 °C (<50%), and at 35 °C (<90%). These were the most dormant among the 18 populations. Of the six counties with equal number of BH and SH populations, five counties showed differences in mean GC between BH and SH ecotypes at 15 °C (Figure 1.2.). At 35 °C, three counties showed differences in mean GC between the two ecotypes (Table 1.3.). In the majority of cases where ecotypes within the same county differed in mean GC, the BH ecotype showed a lower mean GC than the SH ecotype.

At 35 °C, the mean GC of all three zones was similar; however, at 15 °C, the mean GC of the Delta and Grand Prairie populations was 11% lower than that of the White River populations (Figure 1.2.). In the Delta, a blackhull from Chicot County (CHI08D-BH) had the lowest average GC (49 ± 12%) among six populations, at 15 °C incubation; one blackhull and two strawhull populations showed the highest average GC of at least 89%. In the Grand Prairie as well as in the White River zones, the lowest average GC was observed with a blackhull population, although some blackhulls had similar average GC as the highest germinating SH populations.

Among the two temperatures (15 °C and 35 °C), 15 °C caused higher variation in GC among mother plants within population. CHI08D-BH (49 ± 12%), DES08B-BH (78 ± 9%), LON08F-BH (44 ± 8%), JAC08B-BH (83 ± 8%), and ARK08B-SH (79 ± 7%), showed the highest intrapopulation variation in GC at 15 °C (Fig. 1.2). Mother plants within population contributed the most to the variance (45% of total variance) in GC at 15 °C incubation. The second largest proportion in variance was attributed to fields (29%). County, zone, and ecotype had minimal contribution (11, 9, and 6%, respectively) to variation in germination behavior of red rice.

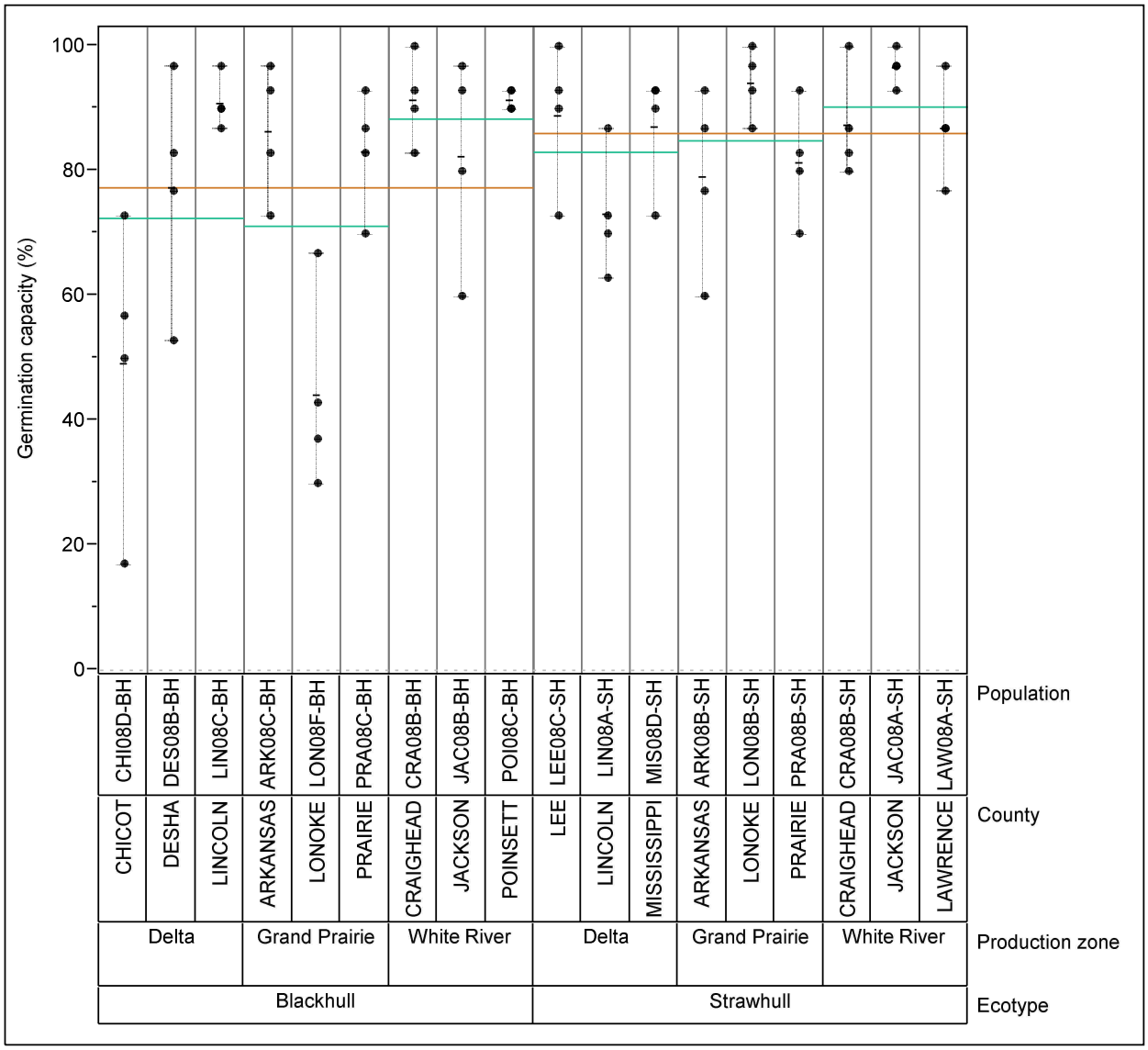

Figure 1.2. Variation in germination capacity (GC) within and among strawhull and blackhull populations when incubated at 15 °C for 12 d. Bars reflect the range of mean GC of four mother plants (indicated as points on the bar) within each population, averaged over three replications. Twenty-five seeds were used per replication. Mean GC of each population is represented by a horizontal line on each vertical bar, and means of production zone and ecotype are presented as green and orange horizontal lines, respectively.

Response to after-ripening period

At 0 DAR, the mean GC was 4%, but rapidly increased to 64% at 30 DAR, averaged over all mother plants and populations (Fig. 1.3). Germination increased further to 84% overall when after-ripened for 90 d. Because the mean GC between 60 DAR, and 90 DAR was similar, after-ripening was not carried longer than 90 d.

Blackhull populations from the Delta (mean DAR_{50} = 69 ± 12 d) and Grand Prairie (mean DAR_{50} = 65 ± 12 d) zones required a longer after-ripening time to release dormancy than the White River BH populations (mean DAR_{50} = 54 ± 7 d) (Fig. 1.4). Also, within the Delta and Grand Prairie zones, the BH populations had 3 times longer mean DAR_{50} than SH populations. The majority of the variance in DAR_{50} (70%) was attributed to mother plants within a population. The second highest source of variance was ecotype (18%). Zone (5%), county (5%), and field (2%) had the least impact on afterripening period. Among the populations, DRE09E-BH (60 ± 20 d), LON09E-BH (86 ± 20 d), and LAW09E-BH (57 ± 21 d) showed the highest intrapopulation variation in DAR_{50}.

A geographical distribution of populations with their respective GCs and DAR_{50} values is presented in Figure 1.5. There was no clear effect of production zone on GC, but it appeared that populations from the northern part of Arkansas had longer afterripening periods than those from the southern half of the state. This may be a latitudinal effect on seed physiology during maturation. Overall, BH populations showed a higher mean DAR_{50} (61 ± 6 d) than the SH populations (23 ± 3 d). Among the BH and SH populations, LON09E-BH (86 ± 20 d) and LON09E-SH (35 ± 5 d) showed the highest DAR_{50}, respectively (Table 1.4.). Both of these populations were from Lonoke county in the Grand Prairie region. Among the twelve fields

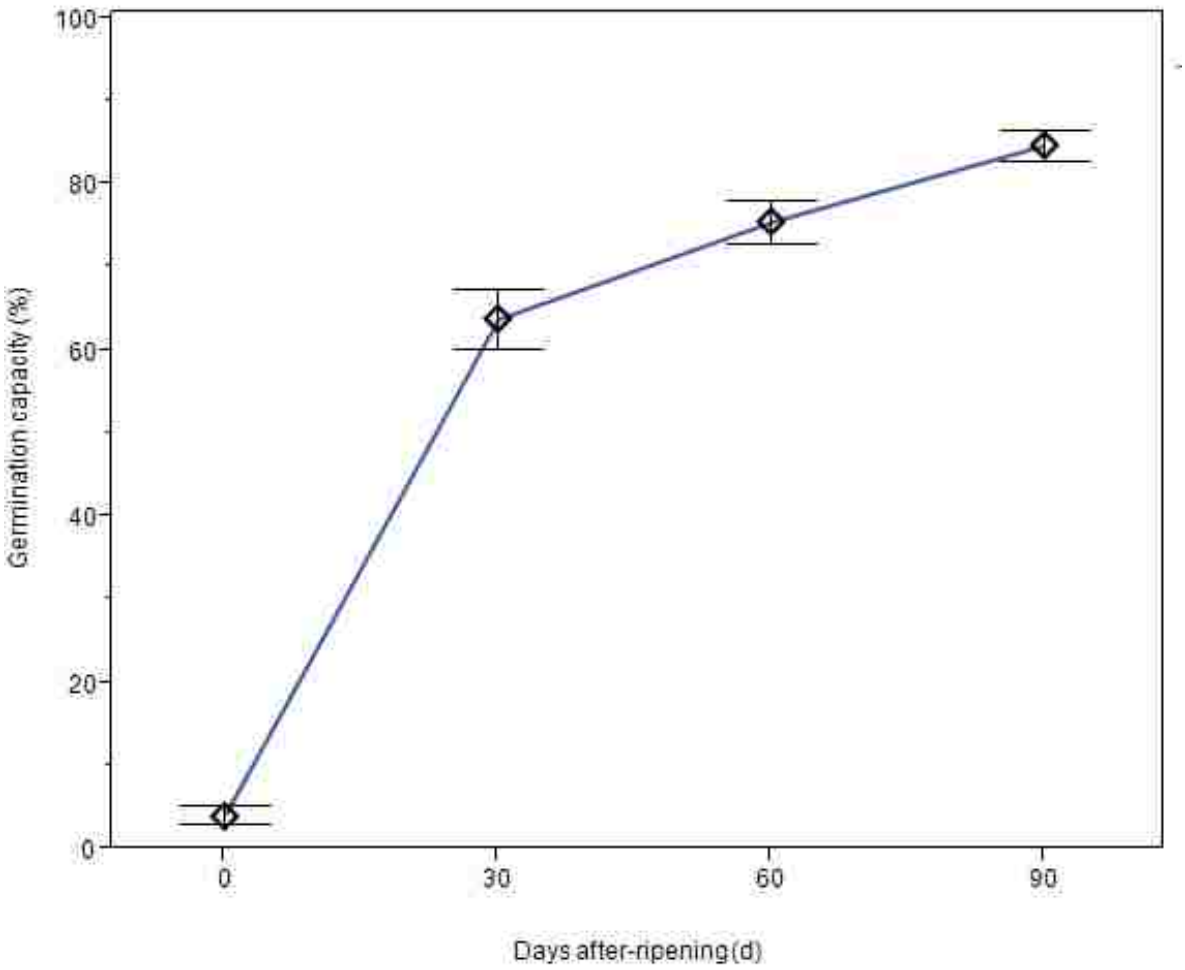

Figure 1.3. Mean (◊) germination capacity at 0, 30, 60, and 90 DAR (days after-ripening), after 12 d of incubation at 30 °C. Data averaged over 16 populations, 6 mother plants per population, and 3 replications. Bars reflect standard errors of the mean.

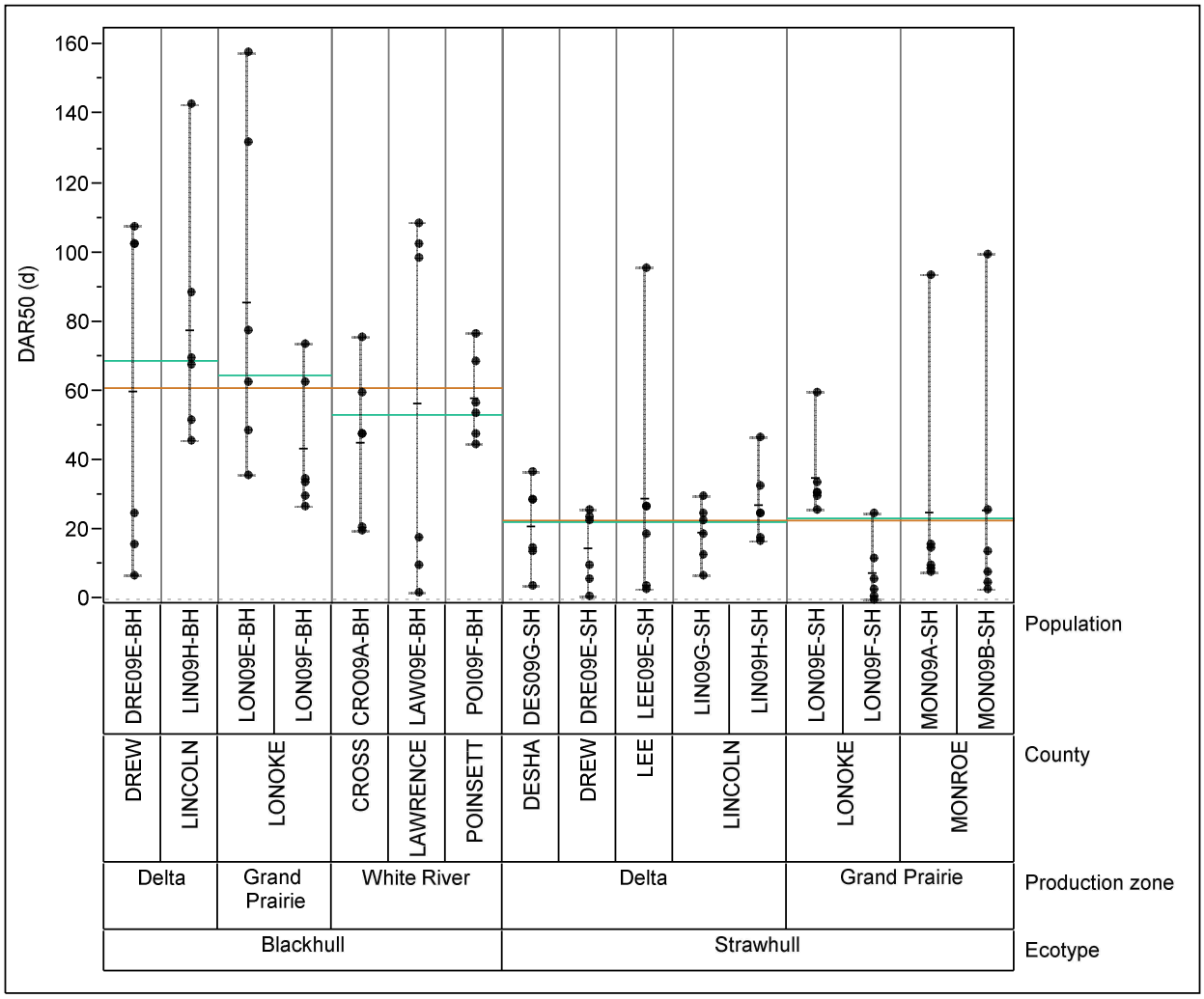

Figure 1.4. Variation in DAR_{50} (lays after ripening to reach 50% germination) among and within strawhull and blackhull populations. Germination assay was conducted at 30 °C for 12 d. Bars reflect the range of mean DAR_{50} of six mother plants (indicated as points on the bar) within each population, averaged over three replications. Twenty-five seeds were used per replication. Mean DAR_{50} of each population is represented by a horizontal line on the vertical bar, while means of production zone and ecotype are presented as green and orange horizontal lines, respectively.

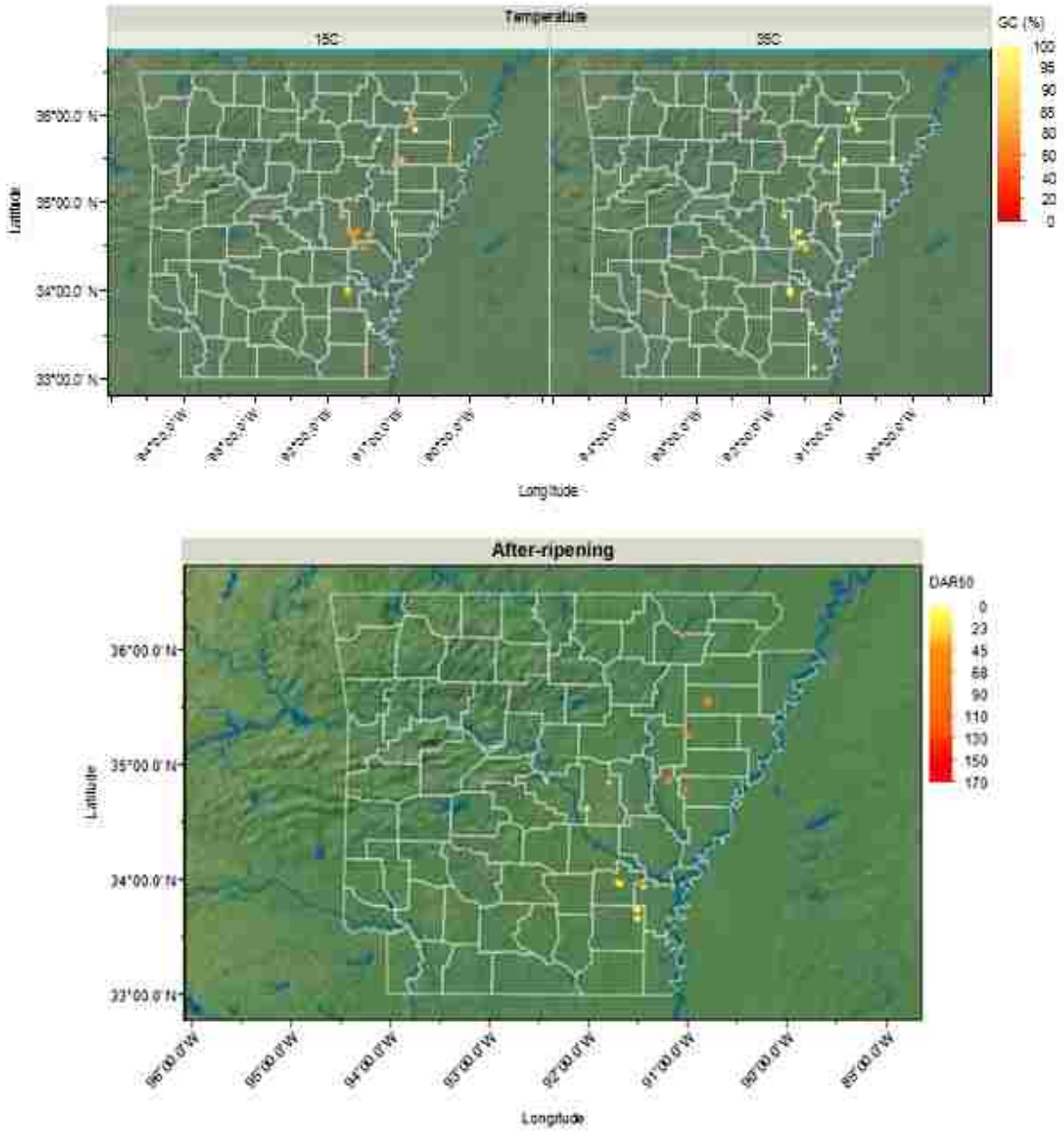

Figure 1.5. Clustering of 8 red rice populations (2 plants per population) based on 13 SSR loci using Nei's unbiased genetic distance coefficients (Nei, 1978). Codes for dormancy: D = dormant, ND = non-dormant; ecotype: BH = blackhull, SH = strawhull; county: CHI = Chicot, CRA = Craighead, JAC = Jackson, LON = Lonoke, MIS = Mississippi. Numbers in boxes indicate the subclusters.

Table 1.4. DAR_{50} of red rice populations from Arkansas, USA, at 30 °C incubation, for 12 d.

Production zone	County	Field	Ecotype	Population[a]	DAR_{50} (d)	
					Mean[b]	Std Err[c]
Delta	DESHA	DES09G	Strawhull	DES09G-SH	21	5
Delta	DREW	DRE09E	Blackhull	DRE09E-BH	60	20
Delta	DREW	DRE09E	Strawhull	DRE09E-SH	15	4
Delta	LEE	LEE09E	Strawhull	LEE09E-SH	29	14
Delta	LINCOLN	LIN09G	Strawhull	LIN09G-SH	20	3
Delta	LINCOLN	LIN09H	Blackhull	LIN09H-BH	78	14
Delta	LINCOLN	LIN09H	Strawhull	LIN09H-SH	28	5
Grand Prairie	LONOKE	LON09E	Blackhull	LON09E-BH	86	20
Grand Prairie	LONOKE	LON09E	Strawhull	LON09E-SH	35	5
Grand Prairie	LONOKE	LON09F	Blackhull	LON09F-BH	44	8
Grand Prairie	LONOKE	LON09F	Strawhull	LON09F-SH	8	4
Grand Prairie	MONROE	MON09A	Strawhull	MON09A-SH	25	14
Grand Prairie	MONROE	MON09B	Strawhull	MON09B-SH	26	15
White River	CROSS	CRO09A	Blackhull	CRO09A-BH	46	9
White River	LAWRENCE	LAW09E	Blackhull	LAW09E-BH	57	21
White River	POINSETT	POI09F	Blackhull	POI09F-BH	58	5

[a]Population is defined as group of plants of same ecotype within a particular field.

[a]Averaged over six mother plants per population, three replications, and 25 seeds per replication.

[b]Standard errors indicate the degree of variation in $DAR_{50.}$ A higher value suggests greater variation among plants within the population.

sampled, four fields were infested with both ecotypes and in all these fields BH populations exhibited 2-6 times longer mean DAR_{50} than SH populations.

Discussion

Effect of germination temperature

The release of dormancy observed at 30 °C when seeds were pre-incubated at 1 °C demonstrated a stratification effect. The germination-promoting effect of cold stratification at 1-5 °C has been observed also in other species, such as *Echinacea angustifolia* (Baskin *et al.*, 1992), *Solidago spp.* (Walck *et al.*, 1997), *Rhodotypos kerrioides* (Flemion, 1933), and *Sorbus aucuparia* (Flemion, 1931). Thus, in the field, when red rice seeds are exposed to freezing temperatures during the winter followed by soil temperatures of around 30-35 °C during late spring to early summer, secondary dormancy in most of the red rice seeds are released, promoting emergence. On the other hand, incubation at 15 °C induced secondary dormancy in most accessions (mother plants), and this secondary dormancy was retained in the majority of accessions when transferred to 30 °C. The induction of secondary dormancy by intermediate low temperature of 15 °C is also reported in cultivated rice varieties (Roberts, 1962; Miura & Araki, 1996). In these past experiments, the loss of secondary dormancy was favored by a reduction in stratification temperature from 12 - 2 °C. The mechanism (genetic controls) of breaking dormancy through exposure to a certain level of low temperature is not yet fully understood. How the minimum stratification temperature also varies across species is not fully understood.

The optimum germination temperature in this study was 35 °C, as was reported in other red rice studies (Eastin, 1978; Gianinetti & Cohn, 2008). The fastest and highest germination

response at 35 °C, compared with that of lower temperatures, among all red rice populations reflects the tropical nature of this weed. However, our data indicate that this optimum temperature would have lesser impact on red rice population dynamics than the suboptimal soil temperatures around rice planting season in the spring. The largest variation in GCs occurred at suboptimal temperature (15 °C) across production zones and ecotypes, except when the temperature hovered around freezing (1 °C) when no physiological activity could happen. Because the principal weed management tactics are done around planting time and into the early vegetative stage of rice, high variability in germination behavior at suboptimal temperatures eventually results in partial weed control and variable reduction of the soil seed bank. By the time the soil reaches an optimum temperature in the summer, the rice crop would have already fully covered the ground area and any red rice germinating in the paddy at this time will be choked. Also during summer when red rice seeds mature and dehisce, some seeds of mother plants that do not require a long after-ripening period can germinate, resulting in late-emerging red rice seedlings that may still be able to produce seeds in late fall. The majority of new seed deposits, however, will not germinate in the immediate because of the after-ripening requirement. Oftentimes, there is insufficient after-ripening time between seed dehiscence in the summer and the onset of cold temperatures in the winter. The rampant variation in after-ripening time among mother plants within a population, or field, creates several opportunities for the weed to reproduce within various lengths of time after crop harvest. Thus, postharvest weed management is critical in red rice-infested fields, most especially in tropical areas.

Different levels of seed dormancy in red rice have been previously reported, where germination differed among 19 red rice populations and among four plants within each population (Perreto *et al.*, 1993). Understanding the dormancy behavior of red rice populations is

important for farmers to appreciate the impact of farming practices and climatic fluctuations on seed longevity and infestation fluctuations. Seed dormancy is a quantitative trait and the expression of multiple dormancy-linked genes is significantly affected by environmental conditions during seed development (Chang & Tagumpay, 1973) as well as after separation from the mother plant. Thus, even just within Arkansas, rice growers in different zones are confronted with differing germination/emergence and persistence behaviors of red rice. In some cases, the genotypic influence was apparent where BH and SH populations from the same county (minimal environmental difference) differed in their degree of dormancy. Where dormancy differences existed among ecotypes, the BH populations were more dormant in most cases than the SH populations. The higher level of dormancy and higher intrapopulation variation in seed dormancy among BH populations relative to SH populations is consistent with earlier observations on red rice populations from Mississippi (Do Lago, 1982). In Do Lago's experiment with 20 red rice phenotypes, 7 of 8 BH phenotypes were three times as dormant as the 11 SH phenotypes.

Effect of after-ripening period

Little research has been conducted on germination and length of after-ripening period of various red rice ecotypes (Gianinetti & Cohn, 2008; Do Lago, 1982). In this experiment, all red rice accessions (mother plants) exhibited primary, or innate, seed dormancy at harvest, a common phenomenon observed in red and wild rices (Cohn & Hughes, 1981; Veasey *et al.*, 2004). Gianinetti and Cohn (2008) reported that the primary dormancy of red rice was released after 45 days after-ripening, in contrast to 90 days or more for the Arkansas red rice accessions. This was most likely because the red rice they tested was a less dormant SH ecotype. There was

a wide variability in after-ripening time requirement to overcome primary dormancy, both among populations and among plants within a population.

Cultivated rice in the USA showed 75 – 96% primary dormancy while 94 – 100% of 20 red rice phenotypes were primarily dormant (Do Lago 1982). After storing for 180 d at 10 °C, primary dormancy was completely released for most rice varieties and reduced to less than 40% for most red rice phenotypes. Weedy rices, therefore, exhibit deeper dormancy than cultivated rice as the crop is intentionally selected and bred for uniform, fast, and high level of germination. In accordance with what was observed in Experiment 1, BH populations showed deeper primary dormancy than SH populations, and primary dormancy among BH populations appeared to be influenced by production zones. The depth of primary dormancy among BH populations from the Delta zone was greater than that of the Grand Prairie and White River zones. This could be an adaptation of the BH populations to the Delta zone. Zoning of rice production areas in Arkansas is primarily based on soil characteristics. The Delta region is mainly composed of clay (USDA NRCS, 2011); thus having high water-holding capacity. In contrast, soil in the Grand Prairie is silty clay, and has limited water-holding capacity; thus becoming extremely dry during the summer months. In the Delta, the red rice seeds probably evolved longer dormancy periods to survive the prolonged anaerobic conditions due to water logging during winter and spring. In flooded conditions, the absence of oxygen induces secondary dormancy in some species (Baskin & Baskin, 1998; Baskin *et al.*, 2000; Insausti *et al.*, 1995; Crawford, 2003). Red rice seeds become more dormant under flooded conditions than in non-flooded conditions (Goss & Brown, 1940; Fogliatto *et al.*, 2010).

Whereas intrapopulation variation in germination was low in red rice seeds that have been after-ripened for 60 d and incubated at optimum temperature, variation in after-ripening

period among mother plants within a population was high and common, especially in BH populations. Three BH populations showed significant plant-to-plant variation in after-ripening period. This occurred even though seeds were harvested from mother plants of uniform maturity stage. Visual inspection of maturity may not entirely reflect the physiological stage of the seed; there are genetic nuances on seed maturation that cannot be delineated by visual inspection. Intrapopulation variation in after-ripening period has a significant impact on red rice population dynamics.

Although not a part of our experiment, it is reasonable to believe that tillage during the off-rice crop cycles exposes some of the deeply buried seed and help reduce the soil seedbank. However, tillage needs to be done only after the freshly deposited red rice seeds on the soil surface have sprouted or deteriorated. Tillage can bury the red rice seeds deeper where conditions are unfavorable for germination; thus, contributing to longer persistence of red rice seed in the soil seed bank. Personal observation in rice fields and interactions with rice growers in Arkansas revealed several cases where fields were thought to be red rice-free for many years, until such time when the field was leveled. Then, suddenly, a new vigorous infestation of red rice appeared. Understanding the dormancy trait of weedy red rice is beneficial not only with respect to improving weed management strategies, but also in rice crop improvement. Because weedy red rice is most genetically similar to cultivated rice, red rice lines with high level of seed dormancy can be selected for breeding of improved pre-harvest sprouting tolerance in cultivated rice in world regions where pre-harvest sprouting is a problem. Genetic approaches can be used to isolate genes involved in dormancy and cloned into rice and other cereals to minimize PHS. Sugimoto *et al.* (2010) were able to clone the rice QTL, Sdr4, which is associated with seed

dormancy. Rice mutants containing the Sdr4 region showed lower germination than the non-mutants. Experiments are on-going to further understand the mechanisms underlying seed dormancy in red rice.

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CHAPTER III

POPULATION STRUCTURE OF DORMANT AND NON-DORMANT WEEDY RED RICE IN ARKANSAS, USA

Abstract

Seed dormancy allows weedy red rice (*Oryza sativa* L.) to persist in rice production systems. Weedy and wild relatives of rice exhibit different levels of dormancy. This high variation in dormancy allows red rice to escape weed management tactics and increases the potential for flowering synchronization, and therefore gene flow, between weedy and cultivated rice. In this study we determine the genetic diversity and differentiation of representative dormant and non-dormant red rice groups from Arkansas. Twenty five simple sequence repeat (SSR) markers, distributed across 4 chromosomes and closely associated with seed dormancy, were used. Four populations were included, dormant blackhull, dormant strawhull, non-dormant blackhull, and non-dormant strawhull. A total of 102 alleles with a mean value of 4.1 alleles per locus were detected. Marker RM28656 and RM5672 were unique to blackhull and strawhull populations, respectively. No markers were unique to dormant or non-dormant populations. The overall gene diversity was 0.355, indicating considerable genetic variation among the populations in these dormancy-related loci. Gene diversity among blackhull populations (0.398) was higher than among strawhull populations (0.245). Higher genetic diversity was also observed within and among the dormant populations than in the non-dormant populations. Cluster analysis of the 16 populations, based on Nei's genetic distance, showed four clusters. Cluster I, III, and IV consisted of all blackhull populations, whereas cluster II was comprised of all strawhull populations. These four clusters did not clearly separate into dormant and non-dormant populations, indicating that not all markers were tightly linked to dormancy. The strawhull groups were most distant from the blackhull red rice groups. These data indicate complex genetic control of the dormancy trait as dormant individuals exhibited higher genetic diversity than non-dormant individuals. Seed dormancy trait contributes to population structure

of weedy red rice, but this influence is lesser than that of hull color. Markers unique to the dormant populations are good candidates for follow-up studies on the control of dormancy in weedy red rice.

Introduction

Weedy red rice is widespread in the southern U.S. rice-producing states and continues to be a major constraint to production wherever it occurs. It belongs to the same species as the cultivated rice (*Oryza sativa* L.) and is highly competitive with the crop (Burgos et al. 2006). Competition results in significant yield losses and reduction of rice grain quality (Shivrain et al. 2009; Ottis et al. 2005). Hence, to make weedy rice management sustainable, it is important to understand the weed's physiological characteristics.

Among its weedy traits seed dormancy is of major importance to red rice persistence. Seed dormancy can allow red rices to persist in the soil for even up to 10 yr (Teekachunhatean 1985; Goss and Brown 1939). The possibility of predicting dormancy is therefore important for improved weed management, as it can allow us to estimate the germinability of different weed seeds thereby letting the farmers adopt appropriate weed management techniques (Grundy and Mead 2000). In addition, seed dormancy trait can be used in breeding of pre-harvest sprouting resistance in rice varieties, to eliminate pre-harvest sprouting problems in South East Asia (Bewley and Black 1982).

The length of dormancy in red rice is affected by storage temperature and after-ripening time. In previous experiments on red rice germination, the optimum after-ripening time in red rice to release dormancy was found to be 90 d, and the optimum germination temperature is 35C (Tseng et al. unpublished). The hull also plays an important role in imposing seed dormancy. In red rice, the blackhull ecotype is generally more dormant than strawhull ecotype (Do Lago 1982; Tseng et al. unpublished). In our previous study on variation in red rice seed dormancy (Tseng et al. unpublished), after-ripening time and germination response to incubation temperature differed both among and within red rice populations. The mean germination capacity (GC) of red rice

populations at 35°C, and 15°C was 84-100%, and 44-97%, respectively. Blackhull populations showed 3-11% lower germination than strawhull populations at all storage temperatures and took at least 30 d longer after-ripening time to release dormancy. In addition, blackhull populations showed a higher inter- and intrapopulation variation in dormancy than strawhull. Knowing this, we also evaluated the genetic diversity of dormancy-related loci among and within red rice populations**.** The phenotypic diversity, specifically maturation period and seed dehiscence, among blackhull and among strawhull red rice populations is high (Shivrainet al. 2010a). Because of the observed large range of seed dormancy variation among weedy rice populations, we hypothesized that the genetic diversity of seed dormancy-linked loci may also be high even among populations of the same hull color. Therefore, the objective of this study was to determine the genetic diversity among and within the Arkansas weedy red rice populations with respect to selected loci linked to seed dormancy in rice and weedy rice.

Materials and Methods

Plant materials

From our previous study on red rice germination (Tseng et al. unpublished), 32 non-dormant and 26 dormant red rice accessions, equally representing blackhull and strawhull ecotypes, were selected. An accession represents a red rice plant, of a particular ecotype, collected from a rice field. A population represents a rice field containing three red rice plants of same ecotype. To confirm their dormancy type, seeds of all these accessions were incubated at 30°C for 28 d. Germination assay was conducted in a similar manner as mentioned in Tseng et al. 2012. From this germination assay, 8 non-dormant (≥80% germination capacity) and 8 dormant (≤20% germination capacity) populations, equally representing strawhull and blackhull

Table 2.1. List of red rice populations used in this study showing its respective group, zone, and county of collection.

Group[a]	Population[b]	Accessions per population	Awn type	Ecotype[a]	Dormancy trait[a]	County	Zone
D-BH	CHI08D-DBH	3	Awned	BH	D	CHICOT	Delta
	LON08F-DBH	3	Awned	BH	D	LONOKE	Grand Prairie
	CRA08B-DBH	3	Awned	BH	D	CRAIGHEAD	White River
	ARK08C-DBH	3	Awned	BH	D	ARKANSAS	Grand Prairie
D-SH	ARK08B-DSH	3	Awnless	SH	D	ARKANSAS	Grand Prairie
	CRA08B-DSH	3	Awnless	SH	D	CRAIGHEAD	White River
	LEE08C-DSH	3	Awnless	SH	D	LEE	Delta
	MIS08D-DSH	3	Awnless	SH	D	MISSISSIPPI	Delta
ND-BH	CRA08B-NDBH	3	Awnless	BH	ND	CRAIGHEAD	White River
	JAC08B-NDBH	3	Awned	BH	ND	JACKSON	White River
	PRA08C-NDBH	3	Awned	BH	ND	PRAIRIE	Grand Prairie
	LIN08C-NDBH	3	Awned	BH	ND	LINCOLN	Delta
ND-SH	CRA08B-NDSH	3	Awnless	SH	ND	CRAIGHEAD	White River
	JAC08A-NDSH	3	Awnless	SH	ND	JACKSON	White River
	LON08B-NDSH	3	Awnless	SH	ND	LONOKE	Grand Prairie
	PRA08B-NDSH	3	Awnless	SH	ND	PRAIRIE	Grand Prairie

[a]BH = blackhull, SH = strawhull, D = dormant, ND = non-dormant.
[b]County codes: CHI = Chico, CRA = Craighead, JAC =Jackson, LON = Lonoke, MIS = Mississippi; letter before hyphen indicates field code.

ecotypes, were selected (Table 2.1.). Three accessions per population were included to access the genetic diversity within populations. The number of accessions was selected since majority of the populations had at least three accessions. The sixteen populations can be assigned to four groups, dormant blackhull (D-BH), non-dormant blackhull (ND-BH), dormant strawhull (D-SH), and non-dormant strawhull (ND-SH).

DNA extraction

From the germination assay, a germinated seed from each accession, for non-dormant population were planted in the greenhouse and leaf tissues were harvested from each plant at three-leaf stage; while for dormant populations, DNA was extracted from a firm and non-germinated seed from each accession. Total genomic DNA was extracted from these harvested tissues, and seeds, using a modified hexadecyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1990). Briefly, 0.05 g of leaf tissue, or a single dehulled seed, was placed in 2-mL collection microtubes (Qiagen) containing two stainless steel beads (Qiagen). To each collection microtube, 500 µL of CTAB extraction buffer (containing 100 mM Tris-HCl, 20 mM EDTA, 2 M NaCl, 2% CTAB, 2% polyvinylpyrrolidone-40, 1 mMphenanthroline, and 0.3% b-mercaptoethanol) was added. The sample was then homogenized using an MM400 mixer mill (Retsch) at 30 Hz for 2 min. After adding an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) to each tube, the mixture was incubated at 55 °C for 45 min, followed by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a new 1.5-mL centrifuge tube (Eppendorf) containing an equal volume of absolute isopropanol, mixed by inverting, and incubated overnight at -80 °C. DNA was then pelleted by centrifuging at 12,000

Table 2.2 List of 25 rice SSR markers used for DNA amplification in this study.

Marker	Locus	Chr[a]	Forward (5' to 3') and reverse (5' to 3') sequence	SSR start (bp)	SSR end (bp)	Predicted product size (bp)	Anneal temp (ºC)	Reference
RM220	qSD1	1	F-gaaatgcttcccacatgtct R-ggaaggtaactgtttccaac	4424458	4424495	127	55	Akagi et al., 1996
RM252	qSD4	4	F-atgacttgatcccgagaacg R-ttcgctgacgtgataggttg	8997573	8997594	216	55	Temnykh et al., 2000
RM564	qSD4	4	F-atgcagaggattggcttgag R-catggccttgtgtatgcatc	4966447	4966488	228	55	Temnykh et al., 2000
RM118	qSD7	7	F-cacatcctccagcgacgccgag R-ccaatcggagccaccggagagc	24488142	24488167	156	67	Temnykh et al., 2000
RM5672	qSD7-1	7	F-tgcccaatatagaggcaacc R-caccctacaaggaaacaagc	6413197	6413217	209	50	McCouch et al., 2002
RM180	qSD7-1	7	F-accttgctctacttgtggtgagggactg R-ctacatcggcttaggtgtagcaacacg	5036853	5036876	110	55	Temnykh et al., 2000
RM270	qSD12	12	F-tgcgcagtatcatcggcgag R-ggccgttggttctaaaatc	22181892	22181957	108	55	Akagi et al., 1996
RM28595	qSD12	12	F-gcccaatcatcttgcatctt R-tacaacgcaccccatctgta	24625121	24625154	240	55	Gu et al., 2008
RM28603	qSD12	12	F-caccaatctccgccattact R-cattggactcacctggaagg	24750565	24750588	207	55	Gu et al., 2008
RM28607	qSD12	12	F-ggcagctcaacccttttcatag R-agaactagagagatgagaaaggaaag	24833611	24833632	250	55	Gu et al., 2008
RM28608	qSD12	12	F-actacaatatggggcggatg R-tgtgtattagtttccatatggtcttca	24834751	24834770	248	55	Gu et al., 2008
RM28621	qSD12	12	F-gccaaaaggtcagggttaca R-cacagtcgaattgcaaagga	25009516	25009541	249	55	Gu et al., 2008
RM28638	qSD12	12	F-ctgaagagctgcgagaatcc R-ccatcctgcctctagcatgt	25137026	25137052	288	55	Gu et al., 2008

RM28642	qSD12	12	F-gtacctctccacccatcgac R-agctgctgagaacacaatcg	25188806	25188827	237	55	Gu et al., 2008
RM28643	qSD12	12	F-ccgatgttgagacaaggtga R-tgggggttgtactcttctcc	25192137	25192156	190	55	Gu et al., 2008
RM28645	qSD12	12	F-acgcagcatgtaggagaggt R-gggcgccagtattagtgttg	25234768	25234788	231	55	Gu et al., 2008
RM28651	qSD12	12	F-cggaactgccgtttattcat R-cttcctggcttcaactctgg	25310459	25310488	250	55	Gu et al., 2008
RM28652	qSD12	12	F-tctcaattgcactccatcca R-aacaacattctctgcaattttcc	25343437	25343456	219	55	Gu et al., 2008
RM28656	qSD12	12	F-tccgattataccattgtattcgtt R-gcacacagtggaagtacgtttg	25420162	25420227	363	55	Gu et al., 2008
RM28659	qSD12	12	F-ccatcgaaagatgtgtggaa R-aaacgcatgcagaagaacct	25459387	25459407	220	55	Gu et al., 2008
RM28661	qSD12	12	F-cgcgtgttgtatggtttcac R-acagtgactttggccgtgtt	25497465	25497486	169	55	Gu et al., 2008
RM28662	qSD12	12	F-gttttaaggcccccatcatt R-ttggagctgattttggagtttt	25502723	25502770	195	55	Gu et al., 2008
RM28664	qSD12	12	F-tgggaagcagaagagtttttg R-gccttagcttctccctgctt	25510147	25510170	243	55	Gu et al., 2008
RM28665	qSD12	12	F-ctcaaggacgtgtggaacg R-tgcagatggtgaggaagttg	25522353	25522373	220	55	Gu et al., 2008
RM28682	qSD12	12	F-tctcctctgcatcacaatcaa R-tctccgagagggtacgtgtc	25804426	25804503	192	55	Gu et al., 2008

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rpm for 10 min. The DNA pellet was washed with absolute ethanol, air dried, and resuspended in 30 mL of 1 x TE (containing 10 mM Tris-HCl, and 1 mM EDTA). The genomic DNA was quantified using NanoDrop 2000c spectrophotometer (NanoDrop Technologies), diluted to 100 ng/µL with deionized water, and used as template in PCR.

Microsatellite DNA amplification

For PCR, 26 SSR primers distributed across 4 chromosomes were used (Table 2.2). PCR was carried out in 25 µl reaction mixtures containing 100 ng/µL DNA, 0.4mM dNTPs each; 25 u/mL Taq DNA polymerase (New England Biolabs), 3 mM MgCl2, and 1 µM each of forward and reverse primers. The PCR profile starts with 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension of 72°C for 7 min was included. The PCR products were electrophoresed in a 6% denaturing polyacrylamide gel at 180V for 70 min. Gels were stained with ethidium bromide and bands were photographed.

Data Analysis

The individual bands were scored as co-dominant markers using Cross Checker 2.91 (Buntjier, 1999). Since number of bands produced by the SSR markers ranged from 1 to 9, the bands were scored as binary characters, so as to maintain the allele information. Thus, data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the band and this data matrix was used to compute the allele frequencies, observed alleles (n_a), effective alleles (n_e), number of alleles per locus (A), percentage of polymorphic loci (P), genetic distance (D), Shannon's index (I), and Nei's gene diversity (h), using POPGENE software version 1.32

Table 2.3. Allele number, gene diversity, and Shannon's index of the 25 SSR markers.

Marker	Locus	Chr.	Allele number (A)	Gene diversity (h)	Shannon's information index (I)
RM220	qSD1	1	6	0.349	0.528
RM252	qSD4	4	7	0.289	0.459
RM564	qSD4	4	9	0.380	0.563
RM118	qSD7	7	7	0.418	0.606
RM5672	qSD7-1	7	3	0.307	0.478
RM180	qSD7-1	7	8	0.243	0.404
RM270	qSD12	12	2	0.354	0.536
RM28595	qSD12	12	2	0.274	0.444
RM28603	qSD12	12	8	0.410	0.595
RM28607	qSD12	12	5	0.349	0.529
RM28608	qSD12	12	3	0.386	0.571
RM28621	qSD12	12	7	0.383	0.568
RM28638	qSD12	12	4	0.337	0.506
RM28642	qSD13	12	3	0.431	0.619
RM28643	qSD14	12	3	0.377	0.562
RM28645	qSD15	12	2	0.336	0.517
RM28651	qSD16	12	4	0.296	0.468
RM28652	qSD17	12	2	0.305	0.483
RM28656	qSD18	12	2	0.153	0.287
RM28659	qSD19	12	2	0.352	0.537
RM28661	qSD20	12	2	0.411	0.600
RM28662	qSD21	12	1	0.458	0.650
RM28664	qSD22	12	7	0.438	0.625
RM28665	qSD23	12	3	0.486	0.679
RM28682	qSD24	12	1	0.187	0.334

[a]Chromosomal location of markers with respect to rice.

(Yeh et al. 1997). The values of genetic distance were used to conduct cluster analysis with UPGMA algorithm and a dendrogram was constructed using the program TREEVIEW ver. 1.52 (Page, 1996). A one-way ANOVA and LSD t-test ($p<0.05$) were applied to compared gene diversity values of SSR markers among and within red rice populations and groups.

Results and discussion

Marker analysis

A total of 102 alleles with an average of 4.12 alleles per locus (ranging from 60 to 650bp) were generated by the 25 SSR primers (Table 2.3.). The highest number of alleles was scored at the locusRM564 (9 alleles) and the lowest number of allele was scored at the locus RM28662 and RM28682 (1 allele).The highest level of genetic diversity was detected at the locus RM28665 (h = 0.486, Table 2.3.), and the lowest level of genetic diversity was detected at locus RM28656 (h = 0.153, Table 2.3.). The allele frequency data indicated two (RM28656, and RM28682) monomorphic loci in the D-BH group, while loci RM5672 showed a monomorphic pattern in both the D-SH and ND-SH group (Table 2.4.). The ND-BH group, however, showed the highest polymorphism with all loci being polymorphic.

We found two markers that were able to discriminate between blackhull and strawhull red rices. Marker RM28656 was unique to blackhull, while marker RM5672 was unique to strawhull red rices (Table **2.5**). However, none of the markers were found to be unique to dormant or non-dormant red rices alone. This is contrary to findings of Gu et al. (2004), who reported that all these markers were tightly linked to dormant red rices. This is probably because the materials we used were from field populations, while Gu et al. (2004) used a pure line derived by crossing a non-dormant strawhull rice (EM93-1) with dormant blackhull weedy rice

Table 2.4. Genetic diversity among red rice populations by groups based on polymorphisms of the 25 SSR markers.

Group[a]	Populations per group	Ecotype[a]	Dormancy Trait[a]	Observed alleles (n_a)	Effective alleles (n_e)	Gene diversity (h)	Shannon's information index (I)	Monomorphic marker	Percent polymorphic loci (P)
D-BH	4	BH	D	1.90	1.66	0.364	0.529	RM28656, RM28682	92
D-SH	4	SH	D	1.67	1.42	0.239	0.356	RM5672	96
ND-BH	4	BH	ND	1.93	1.63	0.366	0.539	-	100
ND-SH	4	SH	ND	1.67	1.36	0.217	0.331	RM5672	96
Blackhull	8	BH	-	2.00	1.69	0.397	0.583	RM28656	96
Strawhull	8	SH	-	1.75	1.42	0.245	0.370	RM5672	96
Dormant	8	-	D	1.98	1.60	0.353	0.526	-	100
Non-dormant	8	-	ND	1.96	1.55	0.331	0.500	-	100

[a]BH = blackhull, SH = strawhull, D = dormant, ND = non-dormant.

Table 2.5. Number of alleles within each population, produced by 25 SSR markers linked to seed dormancy

Population[a]	Observed number of alleles																								
	RM220	RM225	RM256	RM118	RM516720	RM180	RM270	RM285959	RM286037	RM286038	RM286048	RM286216	RM286233	RM286244	RM286243	RM286245	RM286251	RM286256	RM286265	RM286269	RM286276	RM286291	RM286662	RM286664	RM286682
CHI08D-DBH	3	4	8	5	2	3	1	2	6	3	2	5	2	2	2	1	3	2	0	2	1	1	5	3	1
LON08F-DBH	3	3	8	6	3	3	2	0	6	5	3	6	3	2	3	2	3	2	0	2	1	0	6	3	1
CRA08B-DBH	1	3	4	5	2	1	1	0	2	1	1	3	3	3	3	2	2	2	0	2	1	1	0	2	1
ARK08C-DBH	4	2	4	7	2	3	1	1	2	1	1	3	2	2	2	1	2	2	0	2	1	1	0	1	1
ARK08B-DSH	3	1	3	6	0	2	0	0	4	1	0	5	1	2	2	1	4	2	1	2	2	1	4	3	1
CRA08B-DSH	2	1	5	4	0	2	1	0	6	1	0	3	1	2	2	1	4	2	1	2	2	1	6	3	1
LEE08C-DSH	2	2	2	5	0	2	0	0	5	3	2	4	1	2	2	1	3	2	1	2	2	1	4	2	1
MIS08D-DSH	2	1	3	5	0	2	0	1	5	1	1	3	1	1	2	1	3	2	1	2	2	1	4	2	1
CRA08B-NDBH	1	3	5	1	1	0	1	1	3	2	2	5	2	0	2	1	3	2	0	2	2	0	6	3	1
JAC08B-NDBH	2	4	7	7	2	5	2	1	5	4	3	6	3	2	3	2	3	2	0	2	1	1	5	3	1
PRA08C-NDBH	5	1	3	3	1	2	0	0	2	0	0	1	2	2	2	0	4	2	0	2	1	1	0	1	1
LIN08C-NDBH	3	2	4	4	1	2	0	2	7	1	1	5	2	1	2	1	3	2	0	2	1	1	4	2	1
CRA08B-NDSH	1	1	3	5	0	0	0	1	4	1	1	4	1	2	2	1	3	2	1	2	1	1	6	2	1
JAC08A-NDSH	1	1	5	4	0	0	1	0	5	1	0	4	1	1	2	1	3	2	1	2	2	1	5	2	1
LON08B-NDSH	4	2	6	5	0	0	0	0	6	3	2	5	2	1	2	1	3	2	1	2	1	0	5	3	1
PRA08B-NDSH	4	1	6	4	0	2	0	1	4	4	2	4	0	1	2	1	3	2	1	2	1	1	4	3	1

[a]County codes: CHI = Chico, CRA = Craighead, JAC =Jackson, LON = Lonoke, MIS = Mississippi. Letter before the hyphen indicates field code. Ecotype codes: BH = blackhull, SH = strawhull, D = dormant, ND = non-dormant.

(SS18-2) originating from Thailand.

Unique alleles

Among the dormant blackhull (D-BH) populations, 92% of loci were polymorphic (Table 2.4.). In addition, one rare allele was observed exclusively in D-BH populations, RM25 (140bp) (data not shown). Among the non-dormant blackhull (ND-BH) populations, 100% of loci were polymorphic (Table 2.4.), and one rare allele RM12 (290bp) (data not shown), was exclusive to the ND-BH populations. Twenty-four out of 25 loci were polymorphic among the dormant strawhull (D-SH) populations, and among the non-dormant blackhull (ND-SH) populations (Table 2.4.). No rare alleles were observed exclusively in D-SH and ND-SH populations.

Overall genetic diversity

A positive correlation of genetic diversity with red rice groups, ecotype, dormancy trait, populations, and county of collection ($P < 0.05$; Figure 2.1., Figure 2.2., Table 2.4., Table 2.7.) was observed. The geographical zones, however, had no significant impact on genetic diversity of the red rice populations, implying that the evolution of dormancy trait is not localized to a particular zone in Arkansas. Overall, a substantial level of genetic diversity was detected among red rice populations across the dormancy loci (mean $h = 0.355$, $I = 0.534$). Similar genetic diversity ($h = 0.407$) among red rice accessions with respect to overall genome was reported by Shivrain et al. (2010b). These results complement previous observations on high variability in seed dormancy among and within red rice populations (Tseng et al. unpublished; Veasey et al. 2004; Do Lago 1982).

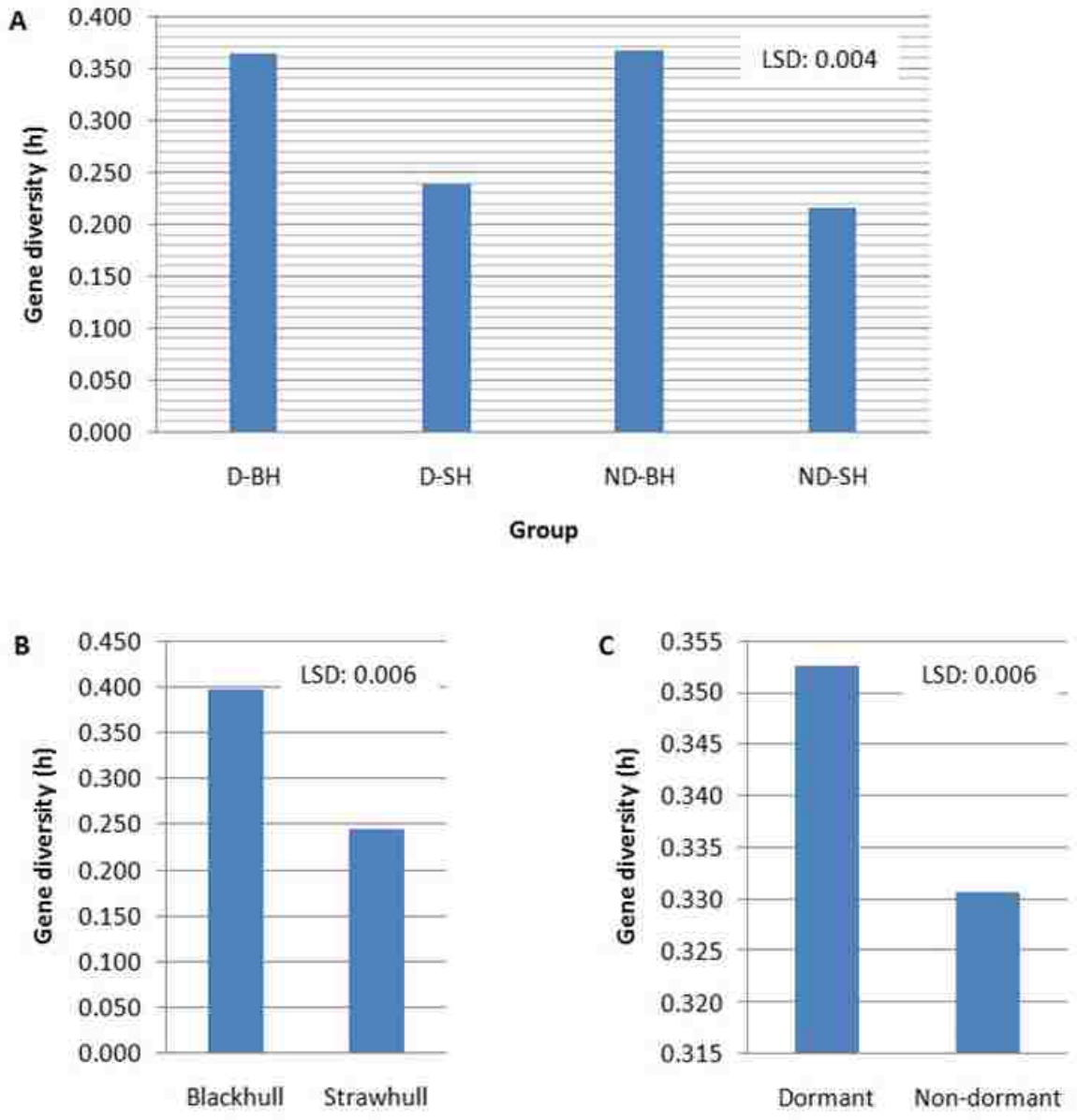

Figure 2.1. Variation in genetic diversity among (A) red rice groups, (B) ecotypes, and (C) dormancy type. Abbreviations: D-BH = dormant blackhull, D-SH = dormant strawhull, ND-BH = non-dormant blackhull, ND-SH = non-dormant strawhulll. Gene diversity values are among all red rice accessions within each group.

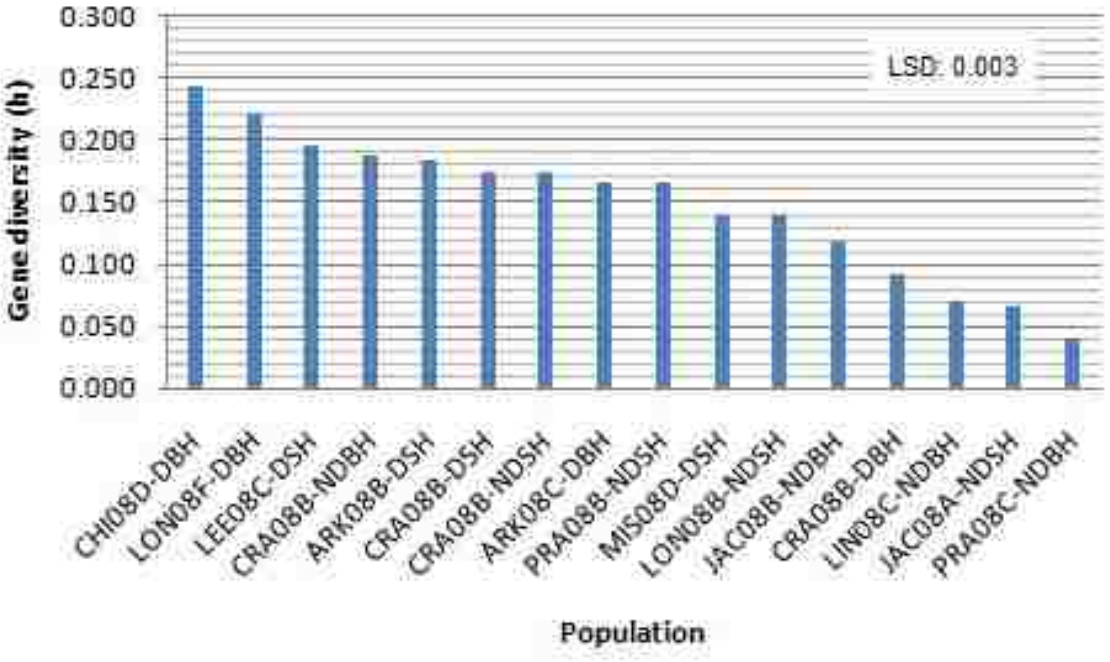

Figure 2.2. Variation in mean gene diversity among red rice populations based on 25 SSR markers linked to four dormancy loci. Gene diversity values are among three red rice accessions within each population.

Table 2.6. Genetic diversity within the 16 populations based on polymorphisms of 25 SSR markers.

Population	Ecotype[a]	Dormancy trait[b]	County	Zone	Gene diversity (h)[c]	Mean gene diversity	Shannon's information index (I)	Effective alleles (n_e)	Percent polymorphic loci (P)
CHI08D-DBH	BH	D	CHICOT	Delta	0.244	0.181	0.350	1.439	72
LON08F-DBH	BH	D	LONOKE	Grand Prairie	0.222		0.318	1.400	72
CRA08B-DBH	BH	D	CRAIGHEAD	White River	0.092		0.131	1.165	48
ARK08C-DBH	BH	D	ARKANSAS	Grand Prairie	0.166		0.237	1.298	76
ARK08B-DSH	SH	D	ARKANSAS	Grand Prairie	0.183	0.173	0.262	1.329	68
CRA08B-DSH	SH	D	CRAIGHEAD	White River	0.174		0.250	1.314	68
LEE08C-DSH	SH	D	LEE	Delta	0.196		0.281	1.353	76
MIS08D-DSH	SH	D	MISSISSIPPI	Delta	0.139		0.200	1.251	68
CRA08B-NDBH	BH	ND	CRAIGHEAD	White River	0.187	0.103	0.268	1.337	76
JAC08B-NDBH	BH	ND	JACKSON	White River	0.118		0.169	1.212	64
PRA08C-NDBH	BH	ND	PRAIRIE	Grand Prairie	0.039		0.056	1.071	20
LIN08C-NDBH	BH	ND	LINCOLN	Delta	0.070		0.100	1.126	36
CRA08B-NDSH	SH	ND	CRAIGHEAD	White River	0.174	0.136	0.250	1.314	72
JAC08A-NDSH	SH	ND	JACKSON	White River	0.065		0.094	1.118	40
LON08B-NDSH	SH	ND	LONOKE	Grand Prairie	0.139		0.200	1.251	56
PRA08B-NDSH	SH	ND	PRAIRIE	Grand Prairie	0.166		0.237	1.298	68

[a]BH = blackhull, SH = strawhull
[b]D = dormant, ND = non-dormant
[c]Gene diversity values are among three plants within each population.

Genetic diversity among red rice ecotypes

Gene diversity (h), and Shannon's index (I) are methods most commonly used for measuring genetic variation (Nei, 1978). Gene diversity is a measure of expected heterozygosity, while Shannon's index is a measure of degree of uncertainty in determining which species an individual would belong to if randomly picked from a group of species. Higher values of gene diversity and Shannon's index would indicate higher genetic diversity. Among the two ecotypes, the blackhull group of populations possessed the highest level of gene diversity and Shannon's index ($h = 0.398$, $I = 0.584$) when compared among themselves (Figure 2.1., Table 2.4.). In contrast, the strawhull group showed lower level of genetic diversity ($h = 0.245$, $I = 0.370$) among populations. Also, among the four dormancy categories, the blackhull groups, D-BH and ND-BH, showed the highest genetic diversity ($h = 0.367$, $I = 0.539$, and $h = 0.365$, $I = 0.530$, respectively), while the ND-SH group of populations were least diverse ($h = 0.218$, $I = 0.332$) (Figure 2.1., Table 2.4.).

Among the blackhull groups, gene diversity within populations ranged from 0.092 to 0.244 for the D-BH group, and from 0.039 to 0.187 for the ND-BH group (Figure 2.2., Table 2.3.). The highest level of gene diversity was found within CHI08D-DBH and CRA08B-NDBH population of D-BH and ND-BH group respectively, whereas the lowest gene diversity was found within the CRA08B-DBH and PRA08C-NDBH population of D-BH and ND-BH group respectively. The Gene diversity within populations ranged from 0.139 to 0.196 for the D-SH group, and from 0.065 to 0.174 for the ND-SH group. The highest level of gene diversity was found within LEE08C-DSH and CRA08B-NDSH population of D-SH and ND-SH group

respectively, whereas the lowest GD was found within the MIS08D-DSH and JAC08A-NDSH population of D-SH and ND-SH group, respectively.

The high level of gene diversity among blackhull red rice compared with the strawhull red rice may be because blackhull is more closely related to wild rice (Londo and Schaal 2007; Vaughan et al. 2001), and wild rice group displays higher gene diversity compared with cultivated and weedy rice groups (Londo and Schaal 2007). Also, blackhull red rice, in general, shows higher phenotypic and genetic variation compared with strawhull red rice (Shivrain et al., 2010a, 2010b). Thus, it is not surprising that the blackhull red rices are more genetically diverse than strawhull types. Since the majority of our accessions were awned blackhull and awnless strawhull, our data conforms to previous findings of Shivrain et al. (2010b), where the awned blackhull group had higher gene diversity (0.337) than the awnless strawhull group (0.239) with respect to genome-wide markers. These findings also support the high intrapopulation variation in dormancy in blackhull red rice, observed in our previous studies (Tseng et al. unpublished).

Genetic diversity among dormant and non-dormant red rice

The mean gene diversity and Shannon's index among individuals for the dormant populations was higher (mean h = 0.177, I = 0.254), than for the non-dormant populations (mean h = 0.120, I = 0.171) (Figure 2.1.). Also, when comparing among populations, the dormant group showed higher diversity (h = 0.353, I = 0.526) than the non-dormant group (h = 0.331, I = 0.500) (Table 2.4.). Higher genetic diversity is known to increase the longevity or fitness of many plant and animal species (Wills, 1981; Danzmann et al., 1986; Ledig, 1986). Greater levels of genetic

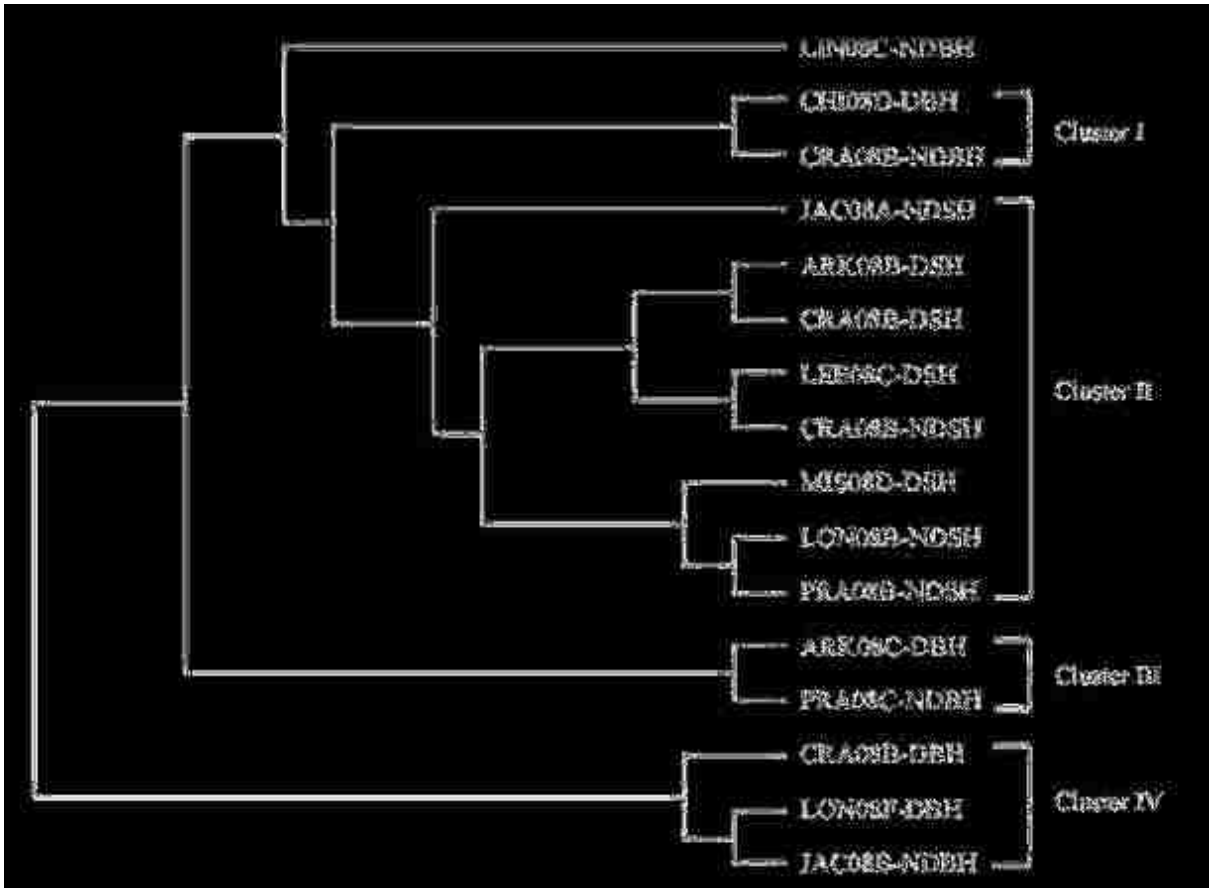

Figure 2.3. UPGMA-based dendrogram of 16 red rice populations based on polymorphisms of 25 SSR markers, using Nei's (1972) genetic distance.

diversity help a population adapt to a wide range of environmental changes, thus allowing them to persist or dominate. The dormant red rice populations which show higher gene diversity than the non-dormant populations are expected to persist longer in the soil. It is therefore very important to adopt an intensive weedy rice management strategy in locations infested with a dormant red rice ecotype.

Effect of county of origin on genetic diversity

Among the nine counties represented by the populations, Chicot county populations showed the highest genetic diversity among individuals (h = 0.244, I = 0.350), while Jackson and Lincoln county populations showed lowest genetic diversity among its individuals (mean h = 0.092, I = 0.131, and h = 0.070, I = 0.100, respectively) (Table 2.6.).The reason why Chicot county red rice population has higher gene diversity is not clear.

Cluster analysis

The UPGMA-based dendrogram obtained from this data is shown in Figure 2.3.. Populations were grouped into four clusters consisting of 15 out of 16 populations. The remaining one population, LIN08C-NDBH shared less similarity with other populations and was therefore not included in any cluster. Cluster I, III, and IV, comprised of all blackhull ecotype, and included two, two, and three populations, respectively. Cluster II was the largest comprising of all the 8 strawhull populations. In this large cluster, there were two sub-clusters, one comprising of mostly dormant populations, and the other comprising of mostly non-dormant populations. One population, JAC08A-NDSH, did not cluster with any population as it was most

Table 2.7. Genetic distance (D) (Nei, 1972) within and between red rice groups based on 25 SSR markers.

Group description	Group name[a]	D[b]
Dormant-blackhull	D-BH	0.357
Dormant-strawhull	D-SH	0.112
Nondormant-blackhull	ND-BH	0.507
Nondormant-strawhull	ND-SH	0.133
Dormant-blackhull vs. Nondormant-strawhull	D-BH vs. D-SH	0.323
Dormant-blackhull vs. Nondormant-blackhull	D-BH vs. ND-BH	0.417
Dormant-blackhull vs. Nondormant-strawhull	D-BH vs. ND-SH	0.353
Dormant-strawhull vs. Nondormant-blackhull	D-SH vs. ND-BH	0.339
Dormant-strawhull vs. Nondormant-strawhull	D-SH vs. ND-SH	0.137
Nondormant-strawhull vs. Nondormant-blackhull	ND-SH vs. ND-BH	0.340
Dormant vs. Nondormant	D vs. ND	0.311
Blackhull vs. Strawhull	BH vs. SH	0.339

[a]BH = blackhull, SH = strawhull, D = dormant, ND = non-dormant, D-BH = dormant blackhull, D-SH = dormant strawhull, ND-BH = non-dormant blackhull, ND-SH = non-dormant strawhulll.
[a]Abbreviations: D, Genetic distance

distant from the other strawhull populations. Among the two populations in Cluster I and III, one was dormant and the other was non-dormant representing different counties. Among the three populations in Cluster IV, two populations, LON08F-DBH and JAC08B-NDBH, were closely related to each other and thus clustered together. Since the four major clusters were grouped mainly based on ecotype, it indicates that the red rice ecotype had a bigger influence on genetic clustering than their dormancy trait.

The genetic distance between the dormant and non-dormant strawhull groups appeared to be lower (D = 0.137) than between the dormant and non-dormant blackhull groups (D = 0.417) (Table 2.7.); thus indicating that dormant and non-dormant red rice populations of blackhull ecotype are more distinct than if they were of strawhull ecotype. Genetic diversity was higher within the two blackhull groups (D = 0.357 and 0.507, respectively) than within the two strawhull groups (D = 0.112 and 0.133, respectively). Similar results were reported by Gealy et al. (2002) where the genetic distance of blackhull group was higher (D = 0.33) than the strawhull group (D = 0.20). The strawhull and blackhull are genetically diverse groups with a genetic distance of 0.339. Similar findings have been reported in other studies where the two major ecotypes, blackhull and strawhull, were shown to be genetically distinct from each (Shivrain et al. 2010b; Londo and Schaal 2007; Gealy et al. 2002). However, the genetic distance values reported in previous studies on red rice genetic diversity are generally higher than those reported in our study. This is because most of these studies used SSR markers non-specific to dormancy loci and were distributed across the genome, compared to dormancy linked loci on just four chromosomes used in this study. This, however, indicates that the dormancy loci used in this study are less polymorphic compared with the genome-wide loci used for genetic diversity studies of red rice.

Red rice populations used in this study exhibit high genetic diversity with respect to seed dormancy loci. This genetic diversity depends on the ecotype, dormancy level, and the county of collection. Blackhull red rice group is most dormant and possess higher level of genetic diversity than the strawhull red rice group. Genetic diversity is also higher in the dormant group than the non-dormant group. Variation in genetic diversity was also observed among the nine counties, and populations from Chicot county showed the highest diversity. The high genetic variability of seed dormancy among and within red rice populations presents a challenge for effective weedy rice management in Arkansas. Rice producers should not grow rice consecutively for more than a year, especially if dormant red rice has been detected in the field. This could prevent the dormant red rice seeds from spreading and also reduce the red rice soil seed-bank. Moreover, the two alleles unique to dormant and non-dormant blackhull populations, respectively, can be used in the identification of dormant and non-dormant phenotypes among blackhull red rice. These unique alleles can also be used in follow-up studies on molecular mechanisms involved in red rice seed dormancy.

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CHAPTER IV

PHENOLOGICAL AND MORPHOLOGICAL VARIATION OF CONTEMPORARY WEEDY RED RICE POPULATIONS IN ARKANSAS

Abstract

Red rice is very successful weed in the southern United States. It can hybridize with cultivated rice resulting in diverse red rice types. Knowledge on the variability among red rice in terms of its phenological and morphological traits is important in understanding the evolution and management of this noxious weed. In this study, the phenological and morphological diversity of 113 strawhull, 71 blackhull, and 24 brownhull red rice accessions collected from different rice production zones in Arkansas was investigated. Results indicate high variation in traits among and within each ecotype, especially for blackhull. Blackhull red rice in general were taller, awned, long grained, late flowering, produced more tillers, but had low grain yield. Strawhull red rice were similar in height compared to blackhull, generally awnless, short grained, early flowering, produced lesser number of tillers, but had high grain yield. Brownhull red rice showed traits similar to blackhull red rice. K-means clustering grouped the accessions into 7 clusters. Accession in cluster 1 were mainly strawhull, cluster 3 and 5, were composed of mostly blackhull, and cluster 4 consisted of mostly brownhull red rice. Cluster 2 contained a mix of strawhull and blackhull red rice, while cluster 6 and 7 consisted of a blackhull and brownhull red rice, respectively. Production zones did not show strong correlation with any traits, implying that red rice in Arkansas is not localized to any cropping practice, or geographical region. Variation in weedy traits among and within red rice ecotypes suggests the need of diverse weed management techniques in order to successfully control diverse red rice types.

Introduction

Red rice is among the most problematic and uncontrollable weeds in rice growing areas in the southern United States (Gealy, 2005). Red rice is similar to cultivated rice in terms of its phenological and morphological traits, and this makes it difficult to identify in the rice field. This similarity also makes it difficult to find herbicides that would kill red rice and not cultivated rice. The red bran or pericarp is the principal distinguishing feature of red rice and makes it more troublesome because red rice contamination of white rice grain reduces grain quality and, the market of rice (Olofsdotter et al., 2000). Even though it is of the same species as that of cultivated rice (Diarra et al., 1985) it possesses many other unfavorable characters such as seed shattering, red pericarp and seed dormancy (Noldin et al., 1999).

Some of the weedy traits that make red rice a successful weed are differences in maturity compared with cultivated rice; high seed production with different levels of dormancy and shattering, thus maintaining the red rice soil seed bank; fast-growing and taller plants than cultivated rice; high tillering capacity; and higher consumption of nutrients because of its large root system (Burgos et al., 2006; Delouche et al., 2007; Tseng et al., unpublished). Red rice highly competes with cultivated rice and this competition results in significant yield reduction up to 80 % (Shivrain et al., 2009; Diarra et al., 1985).

There is vast diversity among and within red rice populations and among red rice and cultivated rice. (Dodson, 1898; Knapp, 1899; Londo and Schaal, 2007). In the Southern US, the two major ecotypes of red rice are strawhull and blackhull, based on hull color (Shivrain et al., 2010). Strawhull ecotypes originated from hybridization between *O. rufipogon* and *O. sativa* indica, while blackhull ecotypes originated from hybridization between rufipogon and *O. sativa* indica var. aus (Londo and Schaal, 2007). Some strawhull and blackhull ecotypes are also

closely related to cultivated rice (*O. sativa japonica*) (Vaughan et al., 2001). These two ecotypes are phenotypically and genotypically different among and within itself in terms of numerous vegetative and reproductive traits such as culm height, number, and angle; leaf length and width; flowering time; panicle length; awn length; grain yield; and, grain length, width, and thickness (Shivrain et al., 2010). Majority of the red rice ecotypes in Arkansas are strawhull (82%) followed by blackhull (12%) and brownhull (6%). Strawhull and brownhull ecotypes are usually awned, while blackhull are awnless. Blackhull ecotypes are taller and took the longest time to flower compared to strawhull ecotypes, thus the blackhull ecotypes are more competitive while the strawhull ecotypes have the ability to mature earlier than cultivated rice and shatter seeds into the soil seed bank, allowing it to persist in the field for a longer time.

Knowledge on the variability among the red rice in terms of its phenological and morphological traits, is thus important in understanding the evolution and management of this noxious weed. The objective of this study was to characterize the phenological and morphological diversity of 88 blackhull and 125 strawhull accessions collected from major rice growing areas in Arkansas, and provide answers to the following questions: what is the magnitude of this diversity?; and how is this diversity distributed among and within ecotypes, and rice growing zones of Arkansas?

Materials and Methods

Sample collection

Red rice seeds were harvested between July to August 2008, and September to October 2009 from fifty-one red rice-infested rice fields, across twenty counties, representing three rice growing zones (Grand Prairie, Delta, and White River) in Arkansas, USA. Panicles of a single

red rice plant bearing at least 90% mature seeds were designated as an accession. Four to twenty-six accessions, at least 100 m apart, were collected from each field, and the geographical coordinates of each accession was recorded. A field, containing numerous accessions, was designated as a population. A total of 228 strawhull, 173 blackhull, and 41 brownhull accessions were collected. Morphological features, such as culm length, number and angle; leaf length and width; awn length and color; basal leaf sheath color; and, hull color, of the sampled plants were recorded. Together with the red rice sampling, the rice growers were interviewed with respect to red rice infestation, field history, and crop management practices. Harvested panicles were air-dried in paper bags for 90 d at room temperature (27 ± 2°C), hand-threshed, and passed though a blower to collect filled seeds.

Field experiment

A subset of 113 strawhull, 71 blackhull, and 24 brownhull accessions was selected (Table 3.1.), representing different culm length, leaf length, number of years in rice production, and weather leveled or non-leveled land. Cleaned seeds of three accessions from each population was planted into 5 cells (two seeds per cell) filled with 3:1 mixture of silt loam field soil and Sunshine Mix #1soil (Sun Gro Horticulture, Inc., Bellevue, WA.), in a 50-cell tray on 13 May 2010 and 28 April 2011, in the greenhouse. Two rice cultivars, a short grain (Nortai), and a long grain (Wells) commonly grown in Arkansas, were used for comparing with red rice. Plants were manually transplanted to the field in Rice Research and Extension Center, Stuttgart (silt loam soil), Arkansas on May 12, 2011 with 1.5 m spacing between plants and 5 replications. Fourteen days prior to transplanting, the fields were tilled and a burndown application of glyphosate (1.12 kg/ha) and halosulfuron (0.03 kg/ha) was made. Seven days prior to the establishment of

Table 3.1.. Ecotype and production zone information of the red rice accessions used to evaluate morphological and physiological variation. Red rice seeds were collected from three rice production zones in Arkansas in 2008, which included a total of 113 strawhull, 71 blackhull, and 24 brownhull accessions.

Ecotype	Production zone	No. of accessions	Sum of accessions
Strawhull	Delta	34	
Strawhull	Grand Prairie	34	113
Strawhull	White River	45	
Blackhull	Delta	22	
Blackhull	Grand Prairie	20	71
Blackhull	White River	29	
Brownhull	Delta	7	
Brownhull	Grand Prairie	9	24
Brownhull	White River	8	

permanent flood, propanil (4 kg/ha), and bensulfuron methyl (0.04 kg/ha) was applied. Date of flowering initiation was recorded when at least four tillers per plant had exerted its panicles, with weekly visits to the field. Thirty-six vegetative and twenty reproductive traits were evaluated in accordance with rice descriptors published by the International Rice Research Institute (1980). Vegetative traits measured in field were, culm length, number, diameter, angle, and strength; color, length, width, texture and angle of five leaves per plant; length of three ligules per plant; basal leaf sheath color; leaf blade color; and, internode and node color. Reproductive traits measured in field were, flowering time; length, type, exertion, axis, secondary branching, and shattering of five panicles per plant; awn color and length of five seeds in a randomly selected mature panicle; and, spikelet fertility of five mature panicles. The degree of grain shattering was evaluated prior to cutting the panicles by lightly tapping the panicles with a meter stick, three times and estimating the percentage of dropped grains in relation to the whole panicle. Spikelet fertility was obtained from counts of well-developed spikelets in proportion to total number of spikelets on each panicle. Culm strength was rated after panicle emergence by gently pushing the tillers back and forth a few times to determine culm stiffness. The colors for each trait were assigned based on the Methuen Handbook of Colour (Kornerup and Wanscher, 1984). Panicles were bagged at the milk stage using Delnet bags (Delstar Technologies, Middletown, DE, USA), to collect seeds as they mature and shatter. Panicles were harvested at maturity, air-dried for 60 d, and threshed. Seeds were cleaned and weighed. One hundred seeds from each accession were selected to determine grain length, grain width, bran color, grain L/W ratio, and thousand kernel weight (TKW) using an automated grain image analyzer (2312 Grain Check, Foss North America, Eden Prairie, MN).

Statistical analysis

All statistical analyses were performed using JMP for Windows software (version 10.0; SAS Institute, Cary, NC). Analysis of variance (ANOVA) was conducted for all the quantitative plant traits using a randomized complete block design with five replicates. Differences among and within ecotypes in plant traits were determined using one-way ANOVAs and means separated by Fisher's Protected LSD Test at the 5% probability level. Principal component analyses was carried out with all the plant traits, and a K-means clustering was performed to group the red rice accessions based on the traits.

Results and Discussion

Vegetative characteristics

Culm length varied within each ecotype, similar to findings of Shivrain et al. (2010) (Table 3.2.). Strawhull red rice were 92-148 cm tall, blackhull red rice were 90-143 cm tall, and brownhull red rice were 91-133 cm tall. The average culm length of strawhull and blackhull was similar (120 and 119 cm, respectively). Brownhull red rice was shortest (114 cm) among the ecotypes. Shivrain et al. (2010) reported the mean culm lengths of strawhull, blackhull, and brownhull to be 133, 139, and 138 cm, which are slightly more than what we found. Moreover, in their study, strawhull was the shortest among the ecotypes; the reason for this difference in result could be due to the low number of blackhull accessions used in their study. Both the long and short grain rice cultivars were shorter than red rice, with a mean culm length of 95 cm for both. Similar results have been reported where red rice was 15-45% taller than rice cultivars (Do Lago, 1982). Plant height is an important aspect in competition. A taller plant is more competitive as it can shade shorter plants, make resources unavailable to its neighboring plants,

Table 3.2. Minimum, maximum, and mean of 11 different quantitative characteristics of each red rice ecotype, averaged across accessions, and five replications per accession.

Characteristics	Ecotype									LSD[b]
	Strawhull			Blackhull			Brownhull			
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	
Culm length (cm)	92	148	120	90	143	119	91	133	114	30
Culm number	33	103	64	35	130	72	57	119	78	48
Leaf length (cm)	29	47	37	25	47	35	27	44	35	11
Leaf width (cm)	0.9	1.6	1.3	0.8	1.5	1.1	1.1	1.4	1.3	0.3
Ligule length (cm)	0.4	1.3	0.8	0.3	1.2	0.7	0.3	0.9	0.7	5.1
Flowering (DAP)	78	111	97	91	130	108	85	121	102	27
Panicle length (cm)	20	30	25	20	30	24	20	29	24	5.4
Awn length (cm)[a]	0.2	4.2	1.4	0.3	3.7	1.4	0.3	2.1	1.7	27
Grain yield (g/plant)	61	154	122	51	158	97	51	158	119	84
Grain L/W ratio	2.2	3.1	2.5	2.1	3.7	2.7	2.5	3.2	2.8	0.6
Thousand kernel weight (g)	12	21	17	12	21	16	13	18	15	4.7

[a]Awns were present only in 13% of strawhull, 55% of blackhull, and 29% of brownhull accessions. Minimum, maximum, and mean awn lengths were calculated based on awned accessions only.
[b]Fisher's LSD test (P = 0.05) was used for means separation.

and increase its rate of photosynthesis by increased light interception (Schwinning and Weiner, 1998; Berntson et al., 2000). Red rice is thus more competitive than rice as it is taller, and blackhull is most competitive among the red rice ecotypes.

The majority of red rice accessions had intermediate culm strengths, however, 13 and 10% of the strawhull and blackhull accessions, respectively, had moderately strong culms. Most of the red rice accessions, had small culms (diameter <5mm), while 18% and 21% of blackhull and brownhull, respectively, had large culms (diameter >=5mm). An intermediate culm angle (~45°) was common in all the red rice ecotypes (Table 3.3.). However, 14% and 11% of strawhull and blackhull accessions had an erect culm (<30°), and about 7% of blackhull had a spreading type culm (>60°). Lodging of red rice is a major problem in rice fields as it can cause yield reduction up to 60 and 90% of tall and short grain rice, respectively (Kwon et al., 1991). In wheat, greater diameter and strength of the culm was shown to increase lodging resistance (Atkins, 1938; Zuber et al., 1999). In addition, thick culms were shown to increase the physical strength of the plant as it accumulates more lignin and cellulose making the culm rigid (Kokubo et al., 1989; Taylor et al., 1999; Tanaka et al., 2003). Results from our study suggest that a few blackhull and brownhull red rice accessions have higher resistance to lodging than strawhull. Although culms of some blackhull red rice are spreading, their seed weight prevents them from lodging.

High tillering capacity is an important competitive trait, as it can produce higher number of panicles, thus higher grain yield. In addition, excessive number of tillers causes a dense canopy, thus contributing to shading of neighbor plants. Although, the highest numbers of culms were seen in a few blackhull plants, on an average, brownhull red rice showed the highest tillering capacity (78 mean culm number) compared with blackhull and strawhull red rice (72

Table 3.3. Proportion of red rice accessions within each ecotype with 11 qualitative characteristics

Characteristics	Category	Proportion of individuals		
		Strawhull	Blackhull	Brownhull
		----------%----------		
Culm angle	Intermediate	72	63	75
	Open	12	18	13
	Erect	14	11	8
	Spreading	3	7	4
Flag leaf angle	Intermediate	75	89	79
	Horizontal	23	7	13
	Erect	2	4	8
Basal leaf sheath color	Green	100	79	96
	Purple	0	21	4
Leaf blade color	Green	81	28	58
	Light green	18	66	33
	Dark green	1	4	4
	Purple	1	1	4
Awn color	Awnless	96	48	63
	Black	0	37	0
	Straw	4	13	33
	Red	0	3	4
Bran color	Red	100	99	96
	Brown	0	1	0
	Light brown	0	0	4
Panicle exsertion	Moderately exerted	42	35	8
	Well exerted	47	13	0
	Just exerted	8	28	33
	Partly exerted	2	11	42
	Enclosed	2	13	17
Panicle type	Intermediate	60	80	75
	Open	36	3	0
	Compact	4	17	25

Panicle axis	Droopy	97	65	54
	Straight	3	35	46
Panicle branching	Light	61	66	63
	Heavy	35	25	25
	Absent	4	8	13
Panicle shattering	Very low (<1%)	68	56	54
	High (>50%)	12	30	17
	Moderate (6-25%)	19	14	29
	Low (1-5%)	1	0	0

and 64 mean culm number, respectively). Number of culms for strawhull, blackhull, and brownhull red rice ranged from 33 to 103, 35 to 130, and 57 to 119, respectively.

The area and leaf angle of the plant is related to its ability to intercept sunlight, thus affecting yield. On an average, strawhull red rice had the longest and widest leaves. Leaves of strawhull red rice was 29-47 cm long, blackhull red rice was 25-47 cm long, and brownhull red rice was 27-44 cm long (Table 3.2.). The flag leaf angle was intermediate (30-79°) in most of the red rice; while, 23 and 13% of strawhull and brownhull, respectively, had horizontally oriented flag leaves (80-100°). The two main functions of ligules in plants are to exclude water, dust and spores from entering the leaf sheath, and to protect the buds (Hackel, 1887; Goebel, 1905). Ligules of strawhull red rice were longest (0.8 cm) among the ecotypes, thus indicating strawhulls to be healthier than other ecotypes (Table 3.2.). Ligule lengths in strawhull were 0.4-1.3 cm, blackhull were 0.3-1.2 cm, and brownhull were 0.3-0.9 cm.

All of the strawhull, and the majority of brownhull and blackhull had green basal leaf sheath (Table 3.3.). Twenty-one percent and four percent of blackhull and brownhull accessions, respectively, had purple basal leaf sheath. Most of the strawhull (81%) and brownhull (58%) had green leaf blade, while the majority of blackhull (66%) had light green leaf blade. Almost all of the red rice accessions had a hard textured leaf. Overall, the strawhull and blackhull accessions had green nodes and light-green internodes, while brownhull accessions had green nodes and internodes. A few blackhull (1%) and brownhull (4%) accessions had purple internode. The intense purple color of the stem and leaves suggests higher anthocyanins. Anthocyanins have antioxidant functions, protecting the cells from harmful superoxides, hydroxyl radicals and hydrogen peroxides (Yamasaki et al., 1996; Tsuda et al., 1996; Bors et al., 1994). Anthocyanins in plant organs help protect the plant from stress, and attack from insects and pathogens (Gould

et al., 2002). Thus, some blackhull and brownhull plants may be able to survive in much adverse environments than strawhull plants.

Reproductive characteristics

Flowering highly differed within each red rice ecotype, and ranging from 78-111 DAP for strawhull, 91-130 DAP for blackhull, and 85-121 DAP for brownhull. In general, the earliest to flower was strawhull red rice (96 days after planting (DAP)), followed by brownhull red rice (102 DAP). Blackhull red rice took the longest time to flower (108 DAP), and flowered at the same time as the two rice cultivars, thus indicating the possibility of gene flow from red rice to rice and vice versa (Shivrain et al., 2007). Although low outcrossing rates (0.003-0.008%) were detected between imidazolinone-herbicide-resistant rice and red rice, a large number of herbicide-resistant red rice plants (about 170 plants/ha) could be produced as a result of gene flow. The range of panicle length among ecotypes was similar (20-30 cm); however, the average length of panicles was longer in strawhull red rice (26 cm) (Table 3.2.). Similar observations were made by Shivrain et al. (2010), where panicles of strawhull red rice were longest. Panicle exsertion is linked to degree of cold tolerance in rice (Nanda & Seshu, 1979). Two cold-tolerant rice cultivars, Quilla64117 and Diamante, showed well exerted panicles as well as higher number of well-developed spikelets (da Cruz et al., 2008). Half of the strawhull accessions showed well exerted panicles while the rest of the accessions had moderately-exerted panicles (Table 3.3.). Blackhull, on the other hand, had 35% accessions with moderately-exerted panicles, 28% of accessions with just-exerted panicles, and only 13% accessions with well-exerted panicles, the rest of the blackhull accessions had poorly-exerted panicles. None of the panicles of brownhull accessions were well-exerted, instead, 42% accessions were poorly-exerted and 33%

of accessions had just-exerted panicles. Strawhull red rice may thus show a higher tolerance to cold than blackhull and brownhull ecotypes since rice phenotypes with well-exserted panicle are also associated with cold tolerance. The majority of red rice accessions had either compact or intermediate type panicle branching, with light secondary branches (Table 3.3.). Some strawhull accessions (36%) showed a more open-type panicle branching. Panicle axis could be classified as straight, or droopy. Most of the strawhull and blackhull, and 54% of brownhull had straight panicles; while, 35% and 46% of blackhull and brownhull red rice had droopy panicles. More than 50% of the red rice accessions in each ecotype had low level of shattering (<1%); while, 12, 30, and 17% of strawhull, blackhull, and brownhull accessions were highly shattering (>50%). Spikelet fertility is based on the number of well-developed spikelets in proportion to total number of spikelets on five panicles. The average range of spikelet fertility of all red rice accessions were 50-74%. About 80% of the strawhull accessions had >50% fertile spikelets, compared to about 50 and 60% of blackhull and brownhull accessions, respectively, with >50% fertile spikelets. Awns were present only in 13% of strawhull accessions, compared with 55% in blackhull, and 29% in brownhull accessions (Table 3.3.). When present, the awns in strawhull red rice were straw-colored, with a mean length of 1.4 cm; awns in blackhull red rice were black-colored and as long as strawhull red rice (Table 3.2.). Brownhull red rice showed straw-colored awns and was longest among the ecotypes (1.7 cm mean awn length). Moreover, 3% and 4% of blackhull and brownhull, respectively, showed red colored awns. In wild rices, awns serve as a mode of seed dispersal, and protection from animal attack (Takahashi et al., 1986). In barley, the awns were shown to be photosynthetically active until maturity, and also played a role in transpiration and respiration (Johnson et al., 1973). Thus, majority of blackhull and few

brownhull spikelets have higher physiological activity, escape predation, and have a higher dispersal rate, than compared to spikelets of strawhull red rice.

Variation in grain yield for each ecotype was similar (Table 3.2.). The average grain yield was lowest in blackhull ecotypes (97 g per plant), compared with strawhull (122 g per plant) and brownhull (119 g per plant) ecotypes. Long grain rice cultivar produced grain yield lower than blackhull red rice (61 g per plant), while the short grain rice cultivar produced grain yield similar to brownhull red rice (107 g per plant). To determine the grain shape, a length/width ratio of 100 grains per accession was measured. Red rice accessions within each ecotype varied in grain shape from medium grain (grain L/W ratio of 2.1 to 3.0) to long grain (grain L/W ratio of 3.1 and more). On an average, blackhull and brownhull grains were longer (grain L/W ratio of 2.7 and 2.8, respectively) than strawhull grains (grain L/W ratio of 2.5). Long grain rice had grains longer than red rice (grain L/W ratio of 3), while the short grain rice produced the shortest grains (grain L/W ratio of 2.4). The weight of thousand dehulled kernels was higher in strawhull (17 g) than blackhull and brownhull ecotypes (16 and 15 g, respectively) (Table 3.2.). Long grain and short grain rice had TKW of 15 and 16 g, respectively. All of the red rice accessions had red colored bran, except for two brownhull and one blackhull accession that had light brown and brown colored bran, respectively. Many of the red rices in China and Central America (Delouche et al., 2007), and some in Mississippi (Do Lago, 1982), were shown to possess white colored bran, thus mimicking cultivated rice. The brownhull and blackhull with light brown and brown bran might therefore be the result of an outcross between rice and red rice.

Cropping system variables

The majority of red rice accessions within each ecotype (about 75%), were collected from fields with silt loam soil, and about 20% collected from fields having clay soil. Also, most of the sampled fields with clay soil belonged to White River production zones. There were just three red rice accessions collected from a sandy loam field, in Poinsett County, and all of these were brownhull ecotypes. Most of the rice grown in Arkansas (55%) is produced on silt loam soil, followed by clay soil (35%), and sandy loam soil (9%), thus the reason why the majority of red rices were collected from fields with silt loam soil. Also, in general, rice grain yield is higher in silt loam soil than in clay soil (Norman et al., 1999), which might be the reason why farmers prefer silt loam soil for rice production. Among the strawhull and blackhull accessions, 57 and 68%, respectively, were collected from fields where rice was cultivated for a single year followed by rotation with soybean or corn. On the other hand, 19% of strawhull and only 9% of blackhull were collected from fields where rice was consecutively grown for 3 years; 3% of strawhull, and 5% of blackhull were collected from fields growing rice consecutively for 4 years. There was an equal number of strawhull and blackhull collected from leveled and non-leveled fields, in contrast to 70% and 30% of brownhull accessions collected from leveled and non-leveled fields, respectively. Most of the red rice accessions (about 80%) were sampled from fields that were conventionally tilled, the remaining were either fields with minimum tillage or no tillage. Studies in Brazil (Noldin and Corbucci, 1999) showed that conventional tillage favors the emergence of red rice because it excavates the viable red rice seeds buried deep in the soil onto the surface.

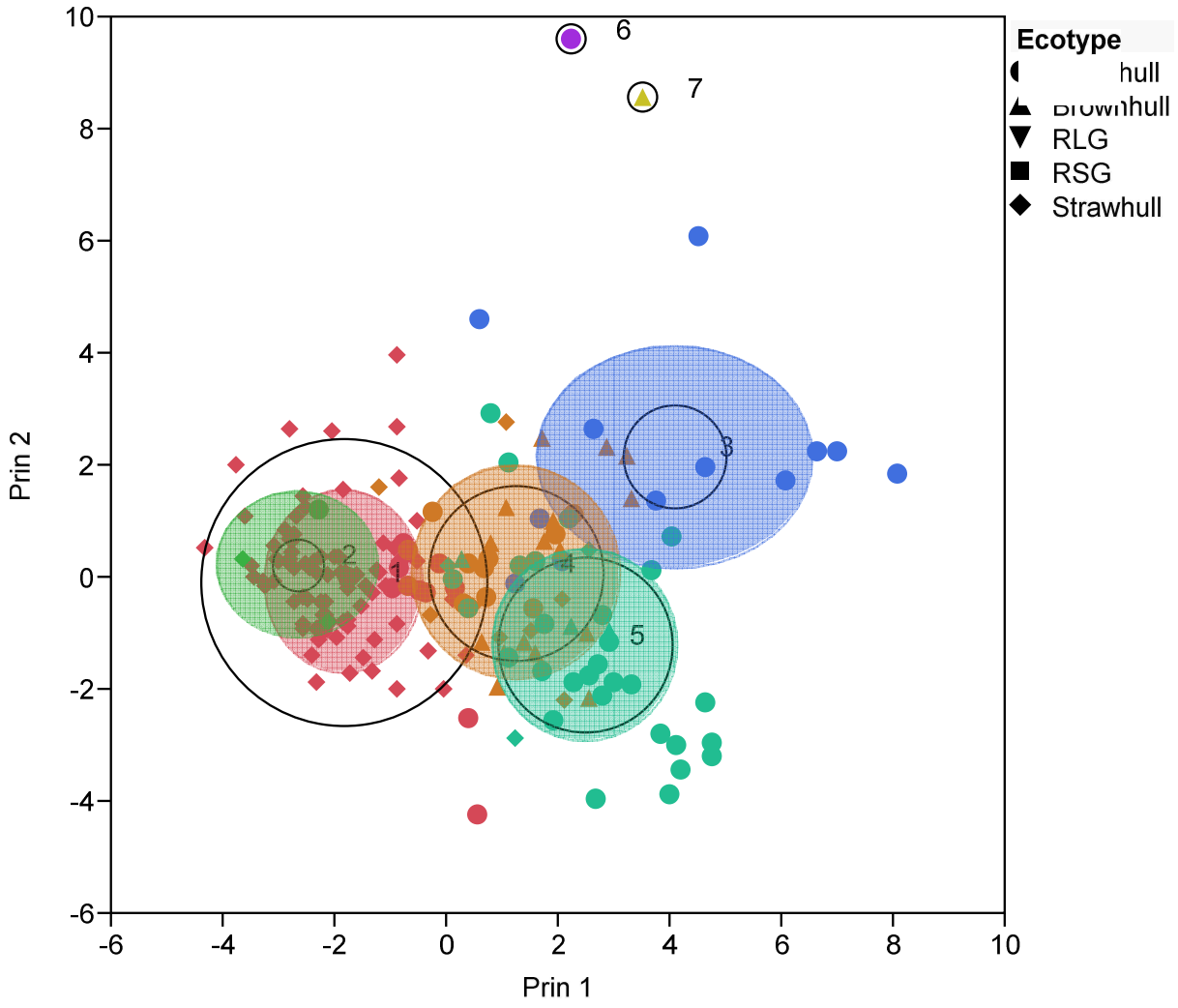

Figure 3.1. K-means clustering of the red rice accessions, combined with principal component analyses. Each point on the graph represents a red rice accession. Clusters are numbered and represented by different colors, and symbols represent each ecotype. Circles around the clusters are drawn proportional to the count inside the cluster.

Cluster analysis

After running cluster analysis of K=2 to K=9, the best or largest cubic clustering criteria (CCC) was selected, and based on this, K = 7 cluster was chosen (Figure 3.1.). Cluster 1 is the largest cluster with 84 strawhull and 11 blackhull red rice accessions, and the majority (41%) were from White River production zone. Accessions in this cluster had intermediate culm length (120 cm), intermediate culm angle of about 45°, intermediate tillering capacity (64 culms per plant), long ligules (0.8 cm), intermediate grain weight (TKW of 16.7 g), were awnless, and early flowering (97 DAP).

Cluster 2 consists of two strawhull, one from White River and other from Grand Prairie production zone, and a blackhull accession from White River production zone. These accessions had intermediate culm lengths (119 cm), widest culm angle (almost flat), low tillering capacity (58 culms per plant), long ligules (0.8 cm), intermediate grain weight (TKW of 16.7 g), were awnless, and early flowering (94 DAP). Moreover, these accessions also showed well exerted open-type panicle, short grain (grain length of 0.6 cm, and lowest grain L/W of 2.5), erect flag leaf angle, and high level of shattering (>50%).

Cluster 3 consists of only 12 blackhull accessions; six accessions from the Delta and three accessions each from the White River and Grand Prairie production zones. In general, the accessions in this cluster were short (110 cm), had an open culm angle of about 60°, highest tillering capacity (92 culms per plant), long ligules (0.8 cm), light grains (TKW of 15.3 g), were awnless and flowered late (116 DAP). These accessions also had long and red awns (1.2 cm). Blackhull accessions in this cluster resemble traits of red rice-rice hybrids. Late flowering and pink awned phenotypes are expected outcrosses between weedy and cultivated rice (Gealy et al., 2006).

Cluster 4 consists of 16 brownhull, 11 blackhull, and 8 strawhull accessions; nine accessions from the Delta and 13 accessions each from the White River and Grand Prairie production zones. In general, the accessions in this cluster are short (113 cm), have an intermediate culm angle of about 45°, high tillering capacity (76 culms per plant), intermediate ligules (0.7 cm), light grains (TKW of 15 g), awnless, and intermediate flowering (104 DAP).

Cluster 5 consists of 29 blackhull, 3 brownhull, and 3 strawhull accessions; 2, 17, and 16 accessions from Delta, White River, and Grand Prairie production zones, respectively. Accessions in this cluster were generally tall (125 cm), have an open culm angle of about 60°, intermediate tillering capacity (69 culms per plant), short ligules (0.5 cm), light grains (TKW of 15 g), awnless, and intermediate flowering (104 DAP).

Cluster 6 and 7, each consist of a single accession, blackhull and brownhull, respectively. The blackhull accession is tall (121 cm), have an erect culm angle (less than 30° from the perpendicular), low tillering capacity (57 culms per plant), intermediate ligules (0.7 cm), long and heavy grains (grain length of 7.8 mm, highest grain L/W of 3.7, and TKW of 21.1 g), long awns (1.5 cm), and late flowering (110 DAP). In addition, this accession also produced the lowest grain yield (51 g per plant), had longest leaves (47 cm), longest awn (1.5 cm), brown seed coat. This blackhull accession resembles hybrids derived from a cross between blackhull red rice and cultivated rice as reported by Gealy et al. (2006). Traits in this blackhull accession that were similar to blackhull/rice crosses are tall height, late flowering, long awned, and presence of light colored pericarp. The brownhull accession in cluster 7 is short (113 cm), have an intermediate culm angle of about 45°, intermediate tillering capacity (72 culms per plant), short ligules (0.6 cm), intermediate grain weight (TKW of 17 g), awned, and early flowering (96 DAP). This accession also had the long panicles (29 cm); purple colored basal leaf sheath, leaf blade, and

internode; red awns; highly sterile spikelets; and light brown seed coat. At least 50% of the crosses between brownhull red rice and rice produced plants that were short, early flowering, with intermediate grain weight, purple colored basal leaf sheath and leaf blade, and presence of red awns; thus, indicating this brownhull red rice to be an outcross between brownhull red rice and cultivated rice.

The results of this study indicate that there is considerable variation in phenological and morphological traits among and within the red rice ecotypes collected from Arkansas. Overall there were more traits that varied in blackhull red rice, than strawhull and brownhull red rice. Blackhull red rice were usually awned, long grained, late flowering, with high tillering capacity, low grain yield, and some accessions showing lodging resistant (large culms) and purple stem. Strawhull red rice were usually awnless, short grained, early flowering, with low tillering capacity, high grain yield, long and wide leaves, long ligules, long panicles, thin culms and green stem. Brownhull red rices were similar to blackhull red rices except that they had shorter culm lengths. Although Shivrain et al. (2010) conducted a similar study on red rice trait diversity; our study used an improved and extensive sampling technique, so as to get a finer picture of red rice diversity in Arkansas. Sampling in this study was targeted to achieve four to twenty-six accessions per field, with a minimum of four fields per county; compared to the variable number of accessions within a field, and fields within a county covered by Shivrain et al. (2010). Our study had greater number of blackhull and brownhull (34 and 11%, respectively), in contrast to 22 and 7%, respectively, in Shivrain et al. (2010). Among these blackhull and brownhull accessions, 48 and 0 %, respectively, were awnless. Additional traits such as culm strength, ligule length, spikelet fertility, and panicle exsertion, type, axis, branching, and shattering were studied. Together with the traits, a rice grower's survey was also conducted to understand the

effect of certain field characteristics or management practices on red rice diversity. With all these data we were able to find great diversity among and within red rice populations. This diversity was among red rice accessions collected from same field or county, thus indicating the existence of different phenotypes and genotypes although exposed to similar management techniques and field conditions. This further suggests genetic introgression among red rice and between red rice and rice, resulting in diverse red rice types. An interesting follow up study would be to look at the sequence diversity among and within red rice populations, and relating this sequence diversity with phenotype diversity.

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CHAPTER V

POPULATION GENETICS AND EVOLUTION OF WEEDY RED RICE IN ARKANSAS

Abstract

Red rice is a troublesome weed in all the southern US rice growing states and can cause up to 80% yield reduction in rice. High variability in phenological and morphological traits assures its persistence as a weed in rice. This, along with high genetic diversity, suggests the red rice populations are continuously evolving. In this study we studied the population structure of 14 strawhull and 13 blackhull collected in 2002-2003 and 52 blackhull red rice accessions collected in 2008-2009. Twenty-two sequence tagged site (STS) fragments were examined for all the 79 red rice accessions, and patterns of single nucleotide polymorphism (SNP) were used to estimate the diversity within and among red rice groups. Phenotypic data from our previous study was combined with the sequence data to find relation between red rice haplotypes and weedy traits. A total of 19,241 bp sequence was analyzed across all the 48 STS fragments, and overall, 447 SNPs were detected. Nucleotide diversity (Pi) across the 79 red rice accessions was 2.01 per Kb. The recent blackhull (Pi = 2.43 per Kb) were more diverse than the old blackhull group (Pi = 1.21 per Kb). Among the two red rice ecotypes, blackhull has higher level of polymorphism than the strawhull accessions. Two genotypic clusters were generated based on Structure analysis using sequences. On combining the genotypic cluster data with phenotypic data, three clusters were identified. The first cluster consisted of all recent blackhull individuals and rice cultivar, mainly belonging to genotypic cluster 1. The second cluster consisted of old blackhull and strawhull individuals, belonging mainly to genotypic cluster 1 and 2, respectively. The third cluster consisted of mostly strawhull individuals, all belonging to genotypic cluster 2. Individuals in cluster 1 were mostly short, late flowering (mean of 107 DAP), had low grain yield, and highly shattering. Cluster 2 individuals were generally tall, early flowering, had intermediate grain yield, and moderately shattering; while cluster 3 individuals in general were

intermediate in height, early flowering, had high grain yield, and highly shattering panicles. Thus, there is vast genotypic and phenotypic diversity among and within red rice ecotypes and also between blackhull individuals collected five years apart. To battle against this highly evolving noxious weedy red rice, it is recommended to adopt diverse weed management techniques in order to successfully control this troublesome weed.

Introduction

The domestication of rice started in Asia, and gradually spread to other parts of the world (Khush, 1997). The ancestor of cultivated rice and weedy rice is said to be wild rice, *O. rufipogon* (Khush, 1997; Londo et al., 2006). Numerous domestication processes of *O. rufipogon* resulted in the evolution of cultivated rice, *O. sativa* ssp. *Indica* and *Oryza sativa* ssp. *Japonica* (Londo et al., 2006). Hybridization between *O. rufipogon* and *O. sativa* ssp. *Indica* further gave rice to weedy rice (Londo and Schaal, 2007). Weedy rice came as contaminants when rice seeds were introduced into the United States in 1685 (Stubbs et al., 1904; Craigmiles, 1978). The major ecotypes of red rice in US are strawhull, and blackhull; strawhull being more common (Shivrain et al., 2010a; Tseng et al., unpublished). A few weedy rice ecotypes in the southern US were found to be related to *O. rufipogon*, while some were found to be related to *O. sativa* ssp. *Japonica* (Vaughan et al., 2001). Red rice hybrids from cross between rice and red rice was also reported by Gealy et al. (2002).

Arkansas represents about 46% of the total US rice production (USDA, 2012), and of the total rice fields in Arkansas, about 60% of them are infested with red rice (Burgos et al., 2008). Because of its morphological and phenological similarity with cultivated rice (Shivrain et al., 2010), identification of this weed is difficult in rice fields, and also not easy to find herbicides specific to red rice and not rice. Red rice has the potential to reduce yield up to 80%, and also decrease the grain quality and market value of rice (Olofsdotter et al., 2000; Estorninos, 2005; Ottis et al., 2005; Shivrain et al., 2009). Although red rice is of the same species as that of cultivated rice (Diarra et al., 1985) it has many other unfavorable weedy traits (Noldin et al., 1999). Some of the weedy traits that make red rice a successful weed are differences in maturity compared to cultivated rice; high seed production with different levels of dormancy and

shattering, thus maintaining the red rice soil seed bank; fast growing and taller plants than cultivated rice; high tillering capacity; and, higher consumption of nutrients because of its large root system (Burgos et al., 2006; Delouche et al., 2007; Tseng et al., unpublished).

There is vast diversity among and within red rice populations and between red rice and cultivated rice. (Dodson, 1898; Knapp, 1899; Londo and Schaal, 2007). In Arkansas, strawhull and blackhull ecotypes are phenotypically and genotypically different among and within itself in terms of numerous vegetative and reproductive traits (Shivrain et al., 2010a; Tseng et al., unpublished). In our previous study (Tseng et al., unpublished) we investigated the phenological and morphological diversity of 113 strawhull, 71 blackhull, and 24 brownhull red rice accessions collected from Arkansas, from different rice production zones. High variation in traits among and within each ecotype, especially for blackhull was observed. Blackhull red rice in general were taller, awned, long grained, late flowering, produced more tillers, but had low grain yield. Strawhull red rice were similar in height compared to blackhull, generally awnless, short grained, early flowering, produced lesser number of tillers, but had high grain yield. Brownhull red rice showed traits similar to blackhull red rice. K-means clustering grouped the accessions into 7 clusters. Accession in cluster 1 were mainly strawhull, cluster 3 and 5, were composed of mostly blackhull, and cluster 4 consisted of mostly brownhull red rice. Cluster 2 contained a mix of strawhull and blackhull red rice, while cluster 6 and 7 consisted of a blackhull and brownhull red rice, respectively. Production zones did not show any strong correlation with any traits, implying that the red rice in Arkansas is not localized to any cropping practice, or geographical region.

Along with high variability in morpho-physiological traits, several studies have also reported high genetic variability in red rice (Quereau, 1920; Londo and Schaal, 2007; Shivrain et al., 2010b). This high genetic diversity is mainly due to hybridization among red rice, and

between red rice and cultivated rice. Molecular studies are considered to be complementary to morphological characterization and provide genetic information of direct value in different areas of plant studies, including conservation genetics (Karp et al. 1997). Various molecular markers have been developed to study variation among the red rice populations at the DNA level (Cho *et al.*, 1995; Suh *et al.*, 1997; Kubo et al. 2000; Shinmura et al., 2005). One of the most widely used markers is microsatellite or simple sequence repeat (SSR) marker. DNA fingerprinting experiments using SSR markers showed that strawhull and blackhull red rices were genetically distinct and clustered separately (Vaughan et al. 2001; Gealy et al. 2002; Estorninos et al. 2006; Shivrain et al., 2010b; Tseng et al., unpublished;). Federici *et al.* (2001) investigated Uruguayan red rice using AFLP markers and found red rice distributed into three clusters; short or no awned strawhull, long awned blackhull, and red rice closely related to cultivated rice. Numerous studies using RAPD, and RFLP markers were able to group red rice into *japonica* and *indica* subspecies (Cho *et al.*, 1995; Suh *et al.*, 1997). Another type of markers, similar to microsatellites, is sequence tagged site (STS) markers. These markers target short known DNA sequences (200-500 base pair) that can be easily PCR-amplified (Olson et al., 1989). Unlike RAPD and RFLP markers, the PCR-based STS assay provides results that are accurate, fast, and easily shared among scientists (Olson et al., 1989). STS sequences containing polymorphic information can be used to distinguish red rice individuals. STS markers have been used to study genetic diversity in rice (Lin et al., 2012), and wheat (Benson et al., 2012; Chen et al., 1994), and for genotyping of citrus cultivars (Fujii et al., 2012). Using 111 STS markers, Caicedo et al. (2007) studied the origin of cultivated rice by examining polymorphisms in DNA sequences. Reagon et al. (2010) selected 48 STS markers from the 111 STS markers used by Caicedo et al. (2007), and studied the population structure of US weedy rice. Both strawhull and blackhull red rice ecotypes were

found to have an origin different from the US commercial rice. The study also showed that distinct weedy rice populations evolved as a result of hybridization among red rice and between weedy rice and cultivated rice.

Studying the genetic differences in red rice populations in relation to phenological and morphological traits will help us better understand the biology and physiology of red rice and the evolution of weedy traits. It can also help us understand the rate of gene flow between red rice and rice. The nucleotide sequence data from the 48 STS loci, together with the phenotypic data can help us understand the evolution of weedy traits in red rice populations. The objectives of this study were to: (1) assess the sequence diversity of blackhull red rice populations in Arkansas using STS markers; and (2) relate the sequence diversity to phenological and morphological traits.

Materials and Methods

Plant material

Fifty-two blackhull red rice accessions collected in 2008 and 2009 (Tseng et al., unpublished), together with 14 strawhull and 13 blackhull red rice accessions, collected in 2002 and 2003 (Shivrain et al., 2008), were selected based on different plant height and maturity groups. A long grain rice cultivar, Wells, commonly grown in Arkansas, was also included. Three seeds, one seed per cell, from each red rice accession and rice cultivar were planted in 50-cell trays in greenhouse. Cells were filled with 3:1 mixture of silt loam field soil and Redi-earth potting mix (Sun Gro Horticulture, Inc., Bellevue, WA.). Leaf tissues were harvested from one plant per accession at three-leaf stage.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from the harvested tissues using a modified hexadecyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1990). Briefly, 0.05 g of leaf tissue was placed in 2-mL collection microtubes (Qiagen) containing two stainless steel beads (Qiagen). To each collection microtube, 500 µL of CTAB extraction buffer (containing 100 mMTris-HCl, 20 mM EDTA, 2 M NaCl, 2% CTAB, 2% polyvinylpyrrolidone-40, 1 mMphenanthroline, and 0.3% b-mercaptoethanol) was added. The sample was then homogenized using an MM400 mixer mill (Retsch) at 30 Hz for 2 min. After adding an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) to each tube, the mixture was incubated at 55 °C for 45 min, followed by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a new 1.5-mL centrifuge tube (Eppendorf) containing an equal volume of absolute isopropanol, mixed by inverting, and incubated overnight at -80 °C. DNA was then pelleted by centrifuging at 12,000 rpm for 10 min. The DNA pellet was washed with absolute ethanol, air dried, and resuspended in 30 mL of 1 x TE (containing 10 mMTris-HCl, and 1 mM EDTA). The genomic DNA was quantified using NanoDrop 2000c spectrophotometer (NanoDrop Technologies), diluted to 100 ng/µL with deionized water, and used as template in PCR. The polymerase chain reaction was performed using 22 of the 48 oligonucleotide STS primer pairs used by Reagon et al. (2010) (Table 4.1.). The PCR was carried out in 25 µL reaction mixtures and includes the following: 100 ng DNA, 0.2 mM dNTPs each; 1 u Taq DNA polymerase (New England Biolabs), 1.5 mM MgCl2, and 0.4 µM each of forward and reverse primers. The PCR profile starts with 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. A final extension of 72°C for 5 min was included. The PCR products were resolved in 1% agarose gel, stained with

ethidium bromide, and visualized under UV to check the success of amplification. For sequencing, PCR products were purified using an equal volume of ExoAP mixture containing 0.5 μL of Exonuclease I, 1 μL of Antarctic phosphatase, and 0.5 μL of distilled water. Sequencing of DNA was carried out in Clemson University Genomics Institute (Clemson, SC).

Data analyses

Contigs were assembled using PHRED/PHRAP program (University of Washington). Bases are identified and quality scores are assigned using PHRED, while PHRAP assembled the contigs. Contig sequences for each locus were aligned in Biolign and examined to identify the presence of multiple peaks in the chromatogram, and indels. If the case of more than one heterozygous sites within a sequence, the haplotype probabilities were resolved using PHASE (Stephens and Donnelly, 2003). Haplotype information was then used in BioEdit where a sequence for each haplotype was created. Sequences alignment of all the 48 fragments was then combined in Excel using the concatenate function. This long sequence for all the 74 red rice accessions, including a rice cultivar, was used in DnaSP for analyzing haplotype diversity (h), nucleotide diversity per site (π), polymorphic loci (P), Tajima's D (Tajima, 1989), number of polymorphic sites (S), polymorphism and divergence, and polymorphic sites, for the total accessions, for each ecotype in each sampling year. Population structure was determinded using Structure (Pritchard et al., 2000). Structure was run from K=1 to K=22, with five runs, 100,000 burn-in period, and 500,000 replications. The best fit K value was determined using Structure Harvester (Earl & vonHoldt, 2012). A graphical representation of the population structure was generated using Distruct (Rosenberg, 2004).

To test the association between genotype and phenotypes, we used the groupings generated from Structure, and analyzed together with seven vegetative and nine reproductive traits in JMP (version 10.0; SAS Institute, Cary, NC). A principal component analyses was performed based on K-means clustering on the highest CCC. Vegetative traits used together with the Structure grouping information were culm height, leaf length, leaf width, flag leaf angle; while the reproductive traits used were bran color, flowering time, panicle length, awn length, grain yield, grain length/width ration (grain L/W), and panicle shattering.

Results and Discussion

The aligned fragment lengths of the 48 STS loci ranged from 235 to 504 bp across all 73 red rice accessions and a rice cultivar. The combined length of all the 48 STS fragments was 19,241 bp. A total of 447 single nucleotide polymorphisms (SNPs) were detected, of which 266 were singleton variable sites, and 181 were parsimony informative sites. Reagon et al. (2010) were able to detect similar number of SNPs in the weedy red rice based on the same 48 STS fragments. There were 2,655 sites containing indels or missing data, and these sites were excluded from the analyses. Total nucleotide diversity (π) in the red rice accessions is 2.01×10^{-3}, while Watterson's estimate of theta is 5.48×10^{-3}, across all 73 accessions (Table 4.2.). Similar values of Pi and theta in weedy rice were also found by Reagon et al. (2010), using the same 48 STS fragments, where Pi and theta for their weedy rice accessions were 0.75×10^{-3} and 0.93×10^{-3}, respectively. However, estimates of theta were found to be higher in this study, indicating higher number of segregating sites in our red rice data set than compared to the weedy rice accessions used by Reagon et al. (2010).

Table 4.1. List of markers used to amplify 22 gene regions across the rice genome.

STS	Chromosome	Forward	Locus name
sts_007	10	GCAGTCAATATTTCTTGCCACAGC AAGGTCTCTTGAGGACTTGATGC	LOC_Os10g40580
sts_011	5	GGTCAAACATTGCACCTCTTCG ACTCGAGTATGCTTGCTGGAAGG	LOC_Os05g49180
sts_012	9	GCTGCACTGGAGAAGTTAGAGTGG TCTGTTACTGCCTTCAAGAACTCG	LOC_Os09g29970
sts_021	4	GTAGCCAAGATTGGGCTGTGG GATACCAAAAGCGTCCACGTACC	LOC_Os04g30040
sts_023	3	TACCTGTCAGACCATCCCAAAGC GGATTGAATGTCATGGGATCAGG	LOC_Os03g40540
sts_025	5	GATGTGATCGTTGAACTGGATGC TCGCACATACTTCACATTTGCAC	LOC_Os05g06440
sts_031	6	TCCTGAGCTGTTTGAAAGTCTTGG GTGCGTTGAACCTCACTATCACC	LOC_Os06g39870
sts_040	4	GCAGTGGATTTTCCAGCTCTCC CACGACTACATCAGGGTTGAATGG	LOC_Os04g44560
sts_046	2	GTGTTCGCGCCCTACTTCAGC GGCGACGTCGTCCTTGAAGC	LOC_Os02g57490
sts_060	4	CGTAGAGGTCAAGAGCGTACTTCG TTGCTTTGACTCGACAGCTTCC	LOC_Os04g05650
sts_061	3	TGAGGGATACAGATGCAATGACC AGAGTGTTTCCCGTGTGTGAAGC	LOC_Os03g49730
sts_063	8	TCTTGTTGGAGATGGTCGTCTAGG GGTACAAGATCATGCTGAGTGAAAGG	LOC_Os08g14770
sts_065	1	GTTTGTGCGAAAGCTTCAATGC CTAGAAGGGCTTGTTCCATCTGC	LOC_Os01g37832
sts_070	10	GAAATGTTCAAGGCTATGGACACC TCCATGCAAGCCTTTTGAAGC	LOC_Os10g39420
sts_080	9	CGCAGGACTGCATTAAACATAGC CTCGGATTGTTGAGTCAGTGTGG	LOC_Os09g31230
sts_086	10	TCAGTGAATACCCCATTGTCTCG ACAGCCAAATCAGCAATGAAGG	LOC_Os10g08550
sts_087	9	ATGTGATGGCGAATCCTAAGGTG ATACATGAACTCCCCACCCCTGAC	LOC_Os09g26390
sts_099	3	GTACGGGAATGCAAATGTGTGG CCAATTGAATCCCTCCATGACC	LOC_Os03g59060

sts_102	6	AGTGAGCTTGCGGATGTTGC AGTACTGGGTCTTGCGCTCACC	LOC_Os06g02380
sts_104	2	CAGCTGGTGCAGCAATCAGG CAAGGTGTCAAGCTCAACAATGC	LOC_Os02g02550
sts_108	7	CCTGAGTACCGAATGTTGGAAGG CCTGAGTCAGCCAAAACATAGGC	LOC_Os07g08120
sts_125	11	CAAAGCCACCAAAGGGTTCG ATGTTGCCAGTGCATTCAACG	LOC_Os11g10480

Table 4.2.. Average diversity values across 48 STS fragments for 74 red rice accessions.

Statistic	All red rice	Old red rice	Recent red rice / Recent Blackhull	Old Blackhull	All Blackhull	Old Strawhull
Number of sequences	74	27	49	13	58	14
Number of variable sites	447	78	446	60	440	68
Number of haplotypes (h)	49	23	37	12	44	11
Haplotype diversity (Hd)	0.976	0.986	0.979	0.987	0.982	0.956
θ_π per Kb [a]	2.01	1.43	2.43	1.21	2.20	1.14
k [b]	33.28161	26.31339	41.51616	22.76923	37.17483	21.26374
θ_W per Kb [c]	5.48	1.10	5.94	1.03	5.70	1.15
Tajima's D[d]	-2.20332 S	0.00110 NS	-2.15546 S	0.79991 NS	-2.19868 S	0.00115 S

[a]Average nucleotide diversity per Kb.
[b]Average number of nucleotide diversity for the total site.
[c]Average Watterson's estimate of theta per Kb.
[d]S, significant; NS, non-significant

Overall, the recent blackhull red rice accessions show higher polymorphism compared to blackhull red rice accessions five years ago. Nucleotide diversity and Watterson's estimates for the recent red rice accessions were 2.43 and 5.94 per Kb, respectively, compared to 1.21 and 1.03 per Kb, respectively, for the old blackhull red rice. When comparing the two ecotypes, strawhull accessions show lower nucleotide diversity both compared to old and new blackhull accessions. Similar polymorphisms in US red rice accessions were reported by Reagon et al. (2010). Although, the nucleotide diversity values in this study were higher than what they reported, similar observations of higher polymorphisms in blackhull compared to strawhull, were found. Previous studies also report a higher diversity in blackhull red rice compared to strawhull red rice (Gealy et al. 2002; Shivrain et al., 2010b; Reagon et al., 2010). Tajima's D statistic was negative for the recent blackhull accessions (-2.16), while a positive value of Tajima's D statistic was obtained from the old blackhull and strawhull accessions. A negative value of Tajima's D indicates population expansion, while a positive value of Tajima's D indicates balancing selection. A high negative value of Tajima D (-2.20) was estimated for the overall red rice accessions used in this study. This value is higher than previous reported Tajima D values for *O. rufipogon*, *O. sativa*, and weedy rice groups, thus indicating red rice accessions in Arkansas are expanding and have a higher evolving potential than wild and cultivated rice. High negative values of Tajima D of the recent blackhull red rice compared to the old red blackhull red rice suggests the recent blackhull to be more capable of diversifying than old blackhull red rice. Blackhull have phenotypes closely related to wild rice, *O. rufipogon* and *O. nivara*, however, study by Reagon et al. (2010) did not show any relation of blackhull red rice with wild rice, as it clustered separately. This is probably because of the intense evolution of blackhull sequences which resulted in high diversification of blackhull from wild rice.

A total of 49 haplotypes were generated in the combined analysis of 74 red rice accessions and one rice cultivar. A haplotype is a combination of nucleotide sequences at all the 48 loci included in this study. Rice cultivar represented a separate haplotype (Hap_1), different from red rice. When analyzed by ecotype and year, the old red rice accessions could be grouped under 23 haplotypes while recent red rice accessions represented 37 haplotypes. Within the old accessions, the blackhull and strawhull set of accessions represented 12 and 11 haplotypes, respectively. The most frequent haplotypes Hap_8 and Hap_9, occurred in 7/74 and 6/77 red rice accessions and consisted of all recent blackhull ecotypes. The rest of the haplotypes were observed in less than five red rice accessions. The total haplotype diversity for all the 74 red rice accessions was 0.98. Interestingly, the old blackhull accessions showed higher haplotype diversity than the recent blackhull accessions.

Population structure analysis using Structure determined K=2 as the best fit K value (Figure 4.1.). Genotypic cluster 1 consisted of nine old blackhull, two old strawhull, and 39 recent blackhull. Rice cultivar also belonged to this genotypic cluster. Genotypic cluster 2 comprised of four old blackhull, twelve old strawhull, and eight recent blackhull. PCA was conducted using the phenological and morphological variables. The eigen values for the first two principal components shows that they provide a good picture of the data set. The first principal component accounts for greatest variance in the data set, accounting for about 31% of the overall phenotypic variance (Figure 4.2.). Traits such as leaf length, bran color, grain length, grain width, grain L/W, and panicle shattering, showed high positive relation with the first component; while culm height, leaf color, flag leaf angle, awn length, and grain yield, were negatively associated. The second principal component accounted for about 16% of the total variance in phenotypic traits, and variables that were positively related to this component were leaf width,

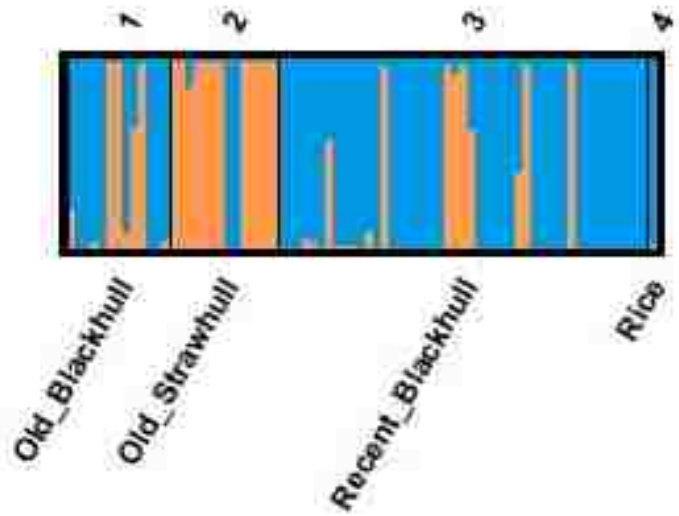

Figure 4.1. Population structure of 74 red rice individuals belonging to different ecotypes (blackhull and strawhull) and collection times (old, 2002-2003; recent, 2008-2009), together with a rice cultivar.

flag leaf angle, grain yield, grain width, and panicle length. Flowering time, bran color, grain L/W, panicle shattering, and basal leaf sheath color showed high negative relation with the second principal component.

Based on the PCA graph (Figure 4.2.), three clusters were identified. The first and the biggest cluster consist of 47 individuals, with 46 of them being recent blackhull red rice types and a short grain rice cultivar. Among the 46 recent blackhull red rice, 38 of them belonged to genotypic cluster 1, same as the rice cultivar, while the rest belonged to genotypic cluster 2 (Table 4.3.). The second cluster consisted of 15 individuals; nine old blackhull, and six old strawhull red rice. Among the nine old blackhull individuals, seven and two belonged to genotypic cluster 1 and 2, respectively; while two and four of the old strawhull red rice belonged to genotypic cluster 1 and 2, respectively. None of the recent blackhull individuals were present in this cluster. The third cluster comprised of four, and eight old blackhull and strawhull red rice, respectively. The four old blackhull red rices consisted of an equal number of individuals from each genotype cluster, while all of the old strawhull in this group belonged to genotypic cluster 2. One recent blackhull of genotypic cluster 1 also belonged to this group. Traits that were commonly associated with individuals in cluster 1 were short culm length, long leaves, intermediate flag leaf angle (about 45º from perpendicular), late flowering (average of 107 DAP), short panicle length, short awns, low grain yield, long grain, and moderately high shattering panicles (Table 4.4.). Individuals in cluster 2 were mostly tall, with short leaves, horizontal flag leaf angle (about 90º from perpendicular), early flowering (average of 97 DAP), intermediate panicle lengths, long awns, intermediate grain yields, short grains, and moderately shattering panicles. Cluster 3 individuals were mostly with intermediate plant heights, long

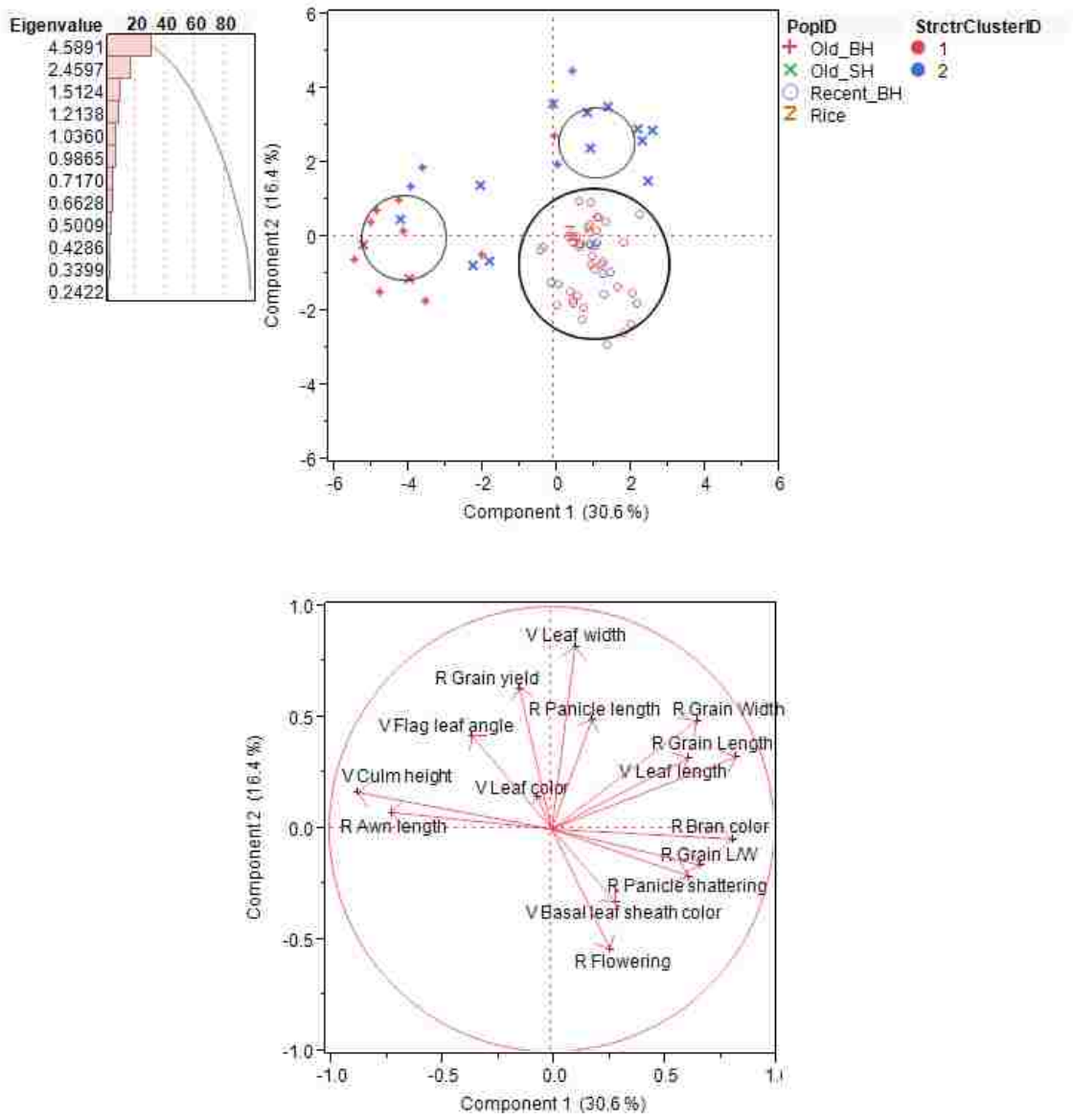

Figure 4.2. Biplot representation of principal component analysis of (A) 74 red rice individuals and a rice cultivar, and (B) selected red rice vegetative (V) and reproductive (R) traits. Traits are represented by rays extending from the plot origin, and traits that point in the same direction are positively correlated. Vegetative and reproductive traits are indicated by letters V and R, respectively, at the beginning of each trait. The genotypic clusters (StrctrClusterID) each individual belongs to are represented by color (red for genotypic cluster 1 and blue for genotypic cluster 2). Clusters are indicated by circles, and the diameter of the circle is drawn proportional to the count inside the cluster.

Table 4.3. Composition of each cluster generated using seven vegetative and nine reproductive traits of 74 red rice individuals and one rice cultivar. Number of individuals representing old blackhull, old strawhull, recent blackhull, or rice, and whether assigned to genotypic cluster 1 and 2, are indicated for each cluster.

Cluster	Old blackhull		Old strawhull		Recent blackhull		Rice	Total
	Genotypic cluster		Genotypic cluster		Genotypic cluster		Genotypic cluster	
	1	2	1	2	1	2	1	
1	0	0	0	0	38	8	1	47
2	7	2	2	4	0	0	0	15
3	2	2	0	8	1	0	0	13

Table 4.4. Number of individuals, and mean values of selected vegetative and reproductive traits represented by each cluster.

Cluster	# of indv.	Vegetative traits				Reproductive traits						
		Culm height (cm)	Leaf length (cm)	Leaf width (mm)	Flag leaf angle	Bran color	Flowering (DAP)[a]	Panicle length (cm)	Awn length (cm)	Grain yield (g/plant)	Grain L/W[b]	Panicle shattering
1	47	120.0	35.2	1.1	interm -ediate	red	107.4	24.4	0.9	98.0	2.7	moderately high (26-50%)
2	15	292.7	29.7	1.1	horizo n-tal	red to brown	96.9	25.1	3.7	122.3	2.1	moderate (6-25%)
3	13	162.2	36.8	1.4	horizo nt-al	red	98.8	26.5	1.7	149.5	2.6	moderately high (26-50%)

[a]Days after planting
[l]Length and width ratio of the grain

leaves, horizontal flag leaf angle (about 90º from perpendicular), early flowering (average of 99 DAP), long panicles, intermediate awn lengths, high grain yield, long grain, and moderately high shattering panicles. On an average, individuals in cluster 3 showed broad leaves, while cluster 2 comprised of individuals with red to brown colored bran.

Evidence of hybridization between red rice and cultivated rice has been well documented by Londo and Schaal (2007) and Shivrain et al. (2010), and this hybridization is responsible for evolution of diverse red rice phenotypes and genotypes even within the same ecosystem, as seen in this study. Results from this study show high nucleotide diversity among the red rice accessions, higher than previously reported by Reagon et al. (2010) for the same genomic regions. Moreover, among the red rice ecotypes, blackhull has the highest diversity, which was also observed by Reagon et al. (2010). The vast growing diversity in red rice populations is an emerging concern for the rice producers as they will need to adopt intense or new management techniques, especially since imadizolinone-herbicide resistant red rices have been documented.

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CHAPTER VI

CONCLUSION

Blackhull red rice was shown to be more dormant than the strawhull ecotypes. Inter- and intrapopulation variation in after-ripening time was observed among and within the 16 populations. The length of time needed to rotate out of rice culture to deplete red rice will depend on the type of red rice and the soil temperature fluctuations with time. Red rice populations exhibit high level of genetic diversity with respect to seed dormancy loci. In addition to high level of seed dormancy, blackhull red rice group possess higher level of genetic diversity than the strawhull red rice group. Dormant red rice group has higher genetic diversity than non-dormant group. The high genetic diversity of seed dormancy among and within red rice populations makes it harder to manage this troublesome weed in Arkansas.

There is also significant variation in phenological and morphological traits detected among and within the red rice ecotypes collected from Arkansas. Blackhull, in general, showed higher variation in traits than the other two red rice ecotypes. Results from sequence analysis show high nucleotide diversity among the red rice individuals and this diversity is higher than previously reported for the same genomic regions. Blackhull shows the highest genetic diversity among the red rice ecotypes. There is thus emerging concerns among rice producers, because of the high evolving capacity of red rice populations, and its ability to remain dormant in soil seedbank. It is very crucial to adopt integrated or new management techniques of weedy red rice, particularly when herbicide resistant red rices have been reported.