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Genetic Analyses of Male Sterility and Wide Compatibility in U.S. Hybrid Rice Breeding Lines

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GENETIC ANALYSES OF MALE STERILITY AND WIDE COMPATIBILITY IN U.S. HYBRID RICE BREEDING LINES

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To my loving wife ***Jennifer***,

my children ***Dathan*** and ***Nasya***

This dissertation is humbly dedicated….

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ABSTRACT

Two line hybrid rice breeding is widely used in Asia and involves the use of environmentally-sensitive male sterile lines to achieve high grain yields compared to pure line varieties. Currently, there is limited information concerning the performance and inheritance of two *indica* male sterile lines 2008S and 2009S under U.S. field conditions. The first objective of the research herein was to characterize genetic male sterility of lines 2009S and 2008S under controlled environment conditions. Results showed that 2009S exhibited a thermosensitive response whereas little or no response was observed with changes in daylength. Furthermore, high pollen fertility was achieved at the proper developmental stage (flag leaf not fully exposed) under low temperature treatment for 10 days. On the other hand, 2008S showed partial fertility when subjected to short daylength and high temperature conditions. Both lines were completely male sterile at high temperature and long daylength under Louisiana field conditions.

The second objective was two-fold: to investigate the inheritance of photoperiod, thermosensitive genetic male sterility (PTGMS) under field conditions and to evaluate SNP-based markers for male sterility. A three-year study of 2008S F_2 and BC_1F_2 populations from 2012 to 2014 revealed a two-locus segregation model indicating two recessive genes acting on male sterility. Inheritance of 2009S in a two-year study from 2013 and 2014 displayed single locus segregation using F_2 and BC_1F_2 populations demonstrating that male sterility was controlled by a single recessive gene. SNP markers at LOC_Os07g12130 and LOC_Os12g36030 in the 2008S background showed significant interactions with up to 65% variance explained in one population, while a SNP marker at LOC_Os02g12290 identified 90-100% of male sterile lines segregating in F_2 and BC_1F_2 populations derived from 2009S.

The third objective was to conduct QTL mapping for male sterility in populations derived from 2008S using a selective genotyping approach. A total of five major QTLs including those previously identified in LOC_Os07g12130 and LOC_Os12g36030 and eight new minor QTLs were detected using single marker analysis. Four QTL intervals were detected with a LOD score of greater than 3.0 using inclusive composite interval mapping (ICIM). QTLs identified will be useful in future studies to fine-map additional markers associated with male sterility and increase prediction accuracy for marker assisted selection.

The fourth objective was to investigate the effect of the S5n wide compatibility gene on grain yield and spikelet fertility in hybrids derived from the SB5 RIL mapping population. Results from this study revealed that heterozygous S5n S5j hybrids produced significantly higher spikelet fertility and yield versus the hybrids carrying S5i S5j genotypes. Indel and SNP markers developed for the S5 locus successfully differentiated the three unique genotypes (S5n, S5i and S5j).

Overall results from this study demonstrated that male sterile lines 2008S and 2009S were useful for two-line hybrid rice breeding under Louisiana conditions. New QTLs discovered and markers developed for male sterility and wide compatibility will help facilitate marker assisted breeding in developing male sterile lines for the LSU hybrid rice breeding program.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Hybrid Rice Breeding in Asia and the United States

Rice is a staple crop that is cultivated worldwide producing ~ 715 million tons annually, with 90 percent of the world's production contributed by Asia (Muthayya et al. 2014). In the United States, rice production in 2015 was approximately 10.7 million tons from 1.04 million hectares that yielded an average of 9.23 tons/hectare (http://www.ers.usda.gov/data-products/rice-yearbook.aspx#57007). With the projected increase in population of 9.7 billion by 2050 (http://www.un.org/), the need to increase food production utilizing the same amount of cultivated area is critical to attain food security. In Asia alone, consumption is expected to increase by 70% over the next 30 years primarily because of population growth (Muthayya et al. 2014). To address the issue of food security, hybrid rice is a key technology that would meet the increasing global consumption of rice, with a proven 20% increase in yield and substantial return of investments (FAORAP and APSA 2014).

Hybrid rice technology was shown to increase rice production by up to 30% under irrigated conditions compared to inbred lines (Virmani et al. 2003). China, the pioneer in hybrid rice research, has released many hybrids that occupied some 58% of their 30 million hectares planted in 2012 (Jiang 2012).

In the United States, hybrid seeds are currently produced by RiceTec, Inc., Alvin, Texas. Their germplasm originated in China, and two decades of breeding have produced commercial hybrid varieties for the southern U.S. (Yan et al. 2010). RiceTec hybrids were associated with an increase in yield of up to 1.6 tons/hectare over conventional lines while the estimated increase in production from adoption of hybrids in AR, LA, and MS was 3.15 million tons from 2003 to 2013 (Nalley and Tack 2015). Owing to this success, the LSU AgCenter and other breeding programs in Arkansas, Mississippi, Texas, and Missouri have established a U.S. public Hybrid

Rice Consortium in 2010 to develop high yielding hybrid varieties with excellent milling and cooking properties.

1.2 Applied Aspects of Male Sterility in Hybrid Rice Production

Hybrid rice breeding utilizes a male sterility system that consists of either a three-line system that uses cytoplasmic male sterility (CMS) or a two-line system that uses photoperiod and/or thermosensitive genetic male sterility (PTGMS) (Xu et al. 2011). The CMS hybrid technology requires three breeding lines: the CMS line that has a male sterile cytoplasm, the maintainer line with normal cytoplasm, but contains the same genetic background as CMS for seed production, and the restorer line that possesses a functional *Rf* gene that restores fertility in the hybrid (Chen and Liu 2014). In contrast, the two-line hybrid system uses male sterile lines that respond to environmental stimuli such as changes in daylength and temperature that allow conversion to male sterility or reversion to fertility. Certain advantages have been realized with the use of the two-line system. Pollen sterility normally develops under high temperature and long daylength which is compatible for hybrid seed production (Yuan 1990). The plant reverts to fertile pollen under low temperature and short day conditions to produce its own seeds as a maintainer line for parental seed increases (Zhou et al. 2011). Furthermore, there is an increased chance of selecting the best hybrid by testing more hybrid combination because virtually any fertile variety can be used as a restorer to produce seeds in the F_1 generation (Xu et al., 2010). Indeed, the two-line system simplified hybrid rice breeding and seed production, and decreased operational costs overall. Moreover, it was reported to increase yields by 5-10% compared to the three-line hybrid system (Chen et al., 2011).

1.3 Characterization of Photoperiod and Thermo-Sensitive Genetic Male Sterility

Studies conducted with Nongken 58S, the first photoperiod genetic male sterile line (PGMS) discovered (Mei et al. 1990), indicated that fertility was regulated by photoperiod. The male sterility was induced when exposed to 14 hours of daylength beginning from the secondary branching primordial stage up to pollen mother cell development (Yuan et al. 1988). The plant restored pollen fertility when exposed to less than 10 hours of light (short day). Seed setting ranged from 0% to 71% when the photoperiod varied from 10 to 15 hours (Zhu and Yang 1992). Liang et al. (2008) classified the male sterile line Changguan S as photoperiod genetic male sterile (PGMS) due to the production of sterile pollen under long (13 hrs.) daylength and fertile pollen at short (12 hrs.) photoperiod coupled with lack of response under high and low temperature conditions when treated in the growth chamber.

The spontaneous mutant Annong-1S discovered in 1988 displayed characteristics of a typical thermosensitive male sterile. Regardless of the length of exposure to light, it was pollen sterile when exposed to temperatures above 33°C while pollen fertility was restored when subjected to temperatures below 24°C (Chen et al. 1994). Zhou et al. (2014) classified to additional lines Zhu1S and Ans-1S, as complete thermosensitive male sterile lines because they did not respond to changes in daylength and displayed pollen sterility at high temperature and reversion to fertility at low temperature. A similar study by Qi et al. (2014) of the thermosensitive male sterile line HengnongS-1 showed pollen fertility when subjected to low temperature and sterility at high temperatures, both at 14 hours of daylength. A “no-pollen type” of male sterility was observed when the temperature reached 32°C.

A study conducted by He et al. (1999) classified male sterile lines Peiai64S and 8902S as both photoperiod and thermosensitive genetic male sterile lines. Both lines became fertile under long daylength (>14.5 hrs.) and low temperature (24°C) or short daylength (10 hrs.) and high

temperature (28°C) conditions, but were consistently sterile at long daylength (14 hrs.) and high temperature conditions (28°C).

These studies suggest that environmentally-sensitive genetic male steriles can be classified into three types depending on the response to photoperiod and temperature. The three classifications were (1) photoperiod genetic male sterile (PGMS), (2) thermosensitive genetic male sterile (TGMS) and (3) photoperiod and thermosensitive genetic male sterile (PTGMS) (Cheng et al. 1996; Virmani et al. 2003).

1.4 Quantitative Trait Locus Mapping and Candidate Genes for Photoperiod and Thermosensitive Genetic Male Sterility

Several studies have described rice photoperiod/thermosensitive genes on different chromosomes. The *pms1*, *pms2*, *pms3* and *p/tms12-1* genes have been identified on chromosomes 7, 3, 12, and 12 respectively (Zhang et al. 1994; Mei et al. 1999a; Zhou et al. 2012). Nine candidate genes associated with thermosensitive male sterility were mapped on different chromosomes. These were *tms1* (Wang et al. 1995) on chromosome 8, *tms2* (Lopez et al. 2003) on chromosome 7, *tms3* (Subudhi et al. 1997), *tms4* (Dong et al. 2000) and *ptmgs2-1* (Xu et al. 2011) on chromosome 2, *tms5* (Wang et al. 2003) on chromosome 5, and *tms6* (Lee et al. 2005) on chromosome 9. Although these genes were mapped using different male sterile lines, it was reported that some of these genes were allelic to each other. For example, studies by Zhang et al. (2014) demonstrated that *tms5, ptmgs2-1* and *tms9* were allelic that mapped to chromosome 2 containing the identical ribonuclease Z (RNZ) gene. Additional research showed that LOC_Os02g12290 contained the SNP responsible for the RNZ mutation following a recessive, single- locus model (Xu et al. 2011; Zhou et al. 2014). The genes *pms3* and *p/tms12-1* were also found to be allelic to each other containing a G/C substitution in the long, non- coding RNA (LNCRNA) found at LOC_Os12g36030. This mutation was believed to be responsible for

altering the structure of the LNCRNA which leads to methylation of the promoter region and reduced transcription under long day conditions resulting in male sterility (Zhou et al. 2012; Ding et al. 2012b). The gene *pms1 (t)* (Zhou et al. 2011) was previously mapped to chromosome 7 that encoded a MYB-like transcription factor (http://rice.plantbiology.msu.edu/).

QTL Mapping using the bulk segregant analysis (BSA) approach was used to identify several candidate genes for male sterility such *as pms1(t), pms1, pms3* and *ptgms 2-1* (Zhang et al. 1994; Mei et al. 1999a; Xu et al. 2011; Zhou et al. 2011). Although the approach involved less genotyping because of DNA pooling, the technique may result in false positives because the allele frequency estimates were dependent on intensity of bands in a gel-based system (Sanabria 2015).

An alternative strategy is to use selective genotyping (SG) that uses samples from extreme contrasting phenotypes that are genotyped individually (Sun et al. 2010). SG is considered a cost-saving method that reduces genotyping work, but still produces results nearly equivalent to whole population genotyping (Lee et al. 2014). The method is also compatible with PCR-based markers run on standard agarose gels. SG was successfully used in identifying QTLs in rice for yield (Vikram et al. 2012), drought resistance (Subashri et al. 2009), salt tolerance (Rana et al. 2009) kernel fissure (Pinson et al. 2013) and markers for resistance to sheath blight in rice (Sanabria 2015).

1.5 The Effect of Wide Compatibility Gene S5n on Spikelet Fertility in Rice Hybrids

Hybrids of *indica x japonica* exhibit greater heterosis compared to the *indica x indica* and *japonica x japonica* counterparts due to apparent differences in morphology and genetic distance (Virmani et al. 2003). However, breeding for *indica x japoni*ca hybrids is difficult due to inter-subspecific hybrid sterility (Mi et al. 2015). Hybrid sterility is known as one of the reproductive

barriers for species isolation that plays an important role in maintaining species identity (Chen et al. 2008). To break this fertility barrier, wide compatible varieties were discovered having the neutral allele at the *S5* locus (LOC_Os06g11010). *Indica* lines generally carry the *S5i* locus while *japonica* lines have the *S5j* locus. When wide compatible varieties having the *S5n* locus were crossed to either *indica* or *japonica* in intersubspecific crosses, spikelet fertility was increased (Ji et al. 2012). Studies showed that heterozygous *S5i S5j* formed aspartyl protease that formed homodimers and heterodimers while the presence of *S5n* prevented dimerization presumably resulting in an increased rate of spikelet fertility (Ji et al. 2010). Further investigation by Ji et al. (2012) showed that the SNP in the heterozygous A/C site in locus LOC_Os06g11010 was associated with embryo sac abortion leading to low spikelet fertility in the hybrid. PCR-based markers targeting the 136 bp deletion were used to survey germplasm and to successfully identify genotypes containing the S5n allele. (Yang et al. 2009b; Sundaram et al. 2010)

1.6 Research Objectives

1. Investigate the effect of photoperiod on male sterility in 2008S and 2009S and identify developmental stage for fertility reversion.
2. Determine inheritance of male sterility and evaluate candidate SNP markers in LOC_Os02g12290 (Xu et al. 2011), LOC_Os07g12130 (Zhou et al. 2011), and LOC_Os12g36030 (Ding et al. 2012a).
3. Conduct QTL mapping using selective genotyping to identify additional QTLs in 2008S that will increase prediction for marker assisted selection.
4. Investigate the effect of wide compatibility gene *S5n* and its association with yield and spikelet fertility in the F1 of inter-subspecific SB5 RIL mapping population.

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CHAPTER 2. EVALUATION OF ENVIRONMENTALLY-SENSITIVE RICE UNDER CONTROLLED PHOTOPERIOD AND TEMPERATURE CONDITIONS

2.1 Introduction

Hybrid rice seed production involves the use of the CMS (cytoplasmic male sterile) three-line system and the PTGMS (photoperiod thermo-sensitive genetic male sterile) two-line system. Since the discovery of PTGMS mutants, several studies have evaluated the potential to complement or replace the CMS system in the production of hybrid rice (Zhang et al. 1994). After the spontaneous PTGMS mutant Nongken 58S was first discovered, utilization of the two-line system for hybrid development has become a major objective in many rice breeding programs in China (Yuan 1992). The two-line system can be used to produce hybrid seed from male sterile lines under long-day conditions or when the environmental temperature is above a critical point (Yuan 1990). Pollen sterility is normally expressed under high temperature and long daylength (HT/LD) and reverts to fertile pollen under low temperature and short day conditions (LT/SD) (Zhou et al. 2011). The plants can also be used as maintainer lines under short-day conditions or below a critical point during sensitive growth stages. Furthermore, there is an increased possibility of finding better hybrid combinations vs. the three-line system because various fertile varieties can be used as parents to restore fertility in the F_1 (Xu et al. 2011). The two-line system has increased yields by 5-10% over those of the three-line approach with planting of PTGMS-based hybrids increasing each year since the early 1990's (Chen et al. 2011b). Since 2007, total planting area of two-line hybrid rice was more than three million hectares, accounting for approximately 25% of the total rice planting area (Chen et al. 2011b). A total of 78 dual-purpose genic male sterile lines in China have been used in commercial production, and 243 two-line hybrid rice combinations have been released in rice growing regions (Yang et al. 2009a).

2.1.1 Photoperiod and Temperature Effects on Male Sterility

Different rice male sterile lines may show different reactions to both photoperiod and temperature (Chen et al. 2011b). Rice genetic male sterile classifications were based on the response to photoperiod and temperature. These are: photoperiod genetic male sterile (PGMS) lines that respond to photoperiod but not to temperature; thermosensitive genetic male sterile (TGMS) lines that respond to temperature but not to photoperiod; and photoperiod-thermosensitive genetic male sterile (PTGMS) lines that respond to both photoperiod and temperature for their sterility-fertility reversion (Cheng et al. 1996; Virmani et al. 2003). Changguan S showed partially fertile pollen when exposed to short day (12 hrs.) and high temperature (26°C) conditions. When exposed to long daylength (13 hrs.) and low temperature conditions (22°C), complete sterility was observed which is an indication of a strict PGMS line (Liang et al. 2008). In contrast, complete thermosensitive male sterile line Zhu1S and Ans-1S were 100% pollen sterile under high temperature conditions (26-30°C) and partially fertile at low temperatures (22-24°C) whether exposed to either short (12 hrs.) or long (13.5 hrs) daylength (Zhou et al. 2014). Male sterile lines Peiai64S and 8902S differed in response to fertility, but both became partially fertile either under long daylength (>14.5 hrs.) and low temperature (24°C) or short daylength (10 hrs.) and high temperature (28°C) conditions in which they were classified as PTGMS lines (He et al. 1999).

The objective of this study is to characterize the effect of photoperiod under high temperature conditions on the pollen sterility of male sterile lines 2008S and 2009S, and to identify the stage of pollen fertility reversion in 2009S as a parent of possible two-line hybrids for breeding and seed production purposes.

2.2 Materials and Methods

2.2.1 Plant Materials

The plant materials evaluated in this study included two *indica* genetic male sterile lines 2009S and 2008S (Figure 2.1) obtained from the Guangxi Academy of Agricultural Sciences through a material transfer agreement (MTA) with the LSU AgCenter. Normal tropical fertile *japonica* breeding lines and varieties 10HHB020, CL131 (U.S. Pat. No. 7,786,360) (Linscombe 2013), Cypress (Reg. no. CV-91, PI561734) (Linscombe et al. 1992) and Catahoula (Reg. No. CV-130, PI 654462) (Blanche et al. 2009) were used as checks.

A B

Figure 2.1 Male sterile lines obtained from Guangxi Academy of Agricultural Sciences planted during the summer of 2013. (A) Left: 2008S at LSU greenhouse. (B) Right: 2009S at H. Rouse Caffey Research Station (courtesy of Dr. Weike Li).

2.2.2 Photoperiod and Temperature

Seeds of the male sterile lines 2009S and 2008S, and fertile lines 10HHB020, CL131, Cypress, and Catahoula varieties were planted in pots in the greenhouse at Louisiana State University, Baton Rouge, in October 2014. Plants were transferred to a culture room at the early booting stage when the flag leaf of the primary or secondary tiller completely emerged from the leaf sheath exposing about one to two cm of internode from the flag leaf collar to the second leaf node. Light inside the culture room was produced from eight 160W cool white fluorescent lamps (Sylvania, Wilmington, MA) at plant level intensity 300 $\mu E\ s^{-1}\ m^{-2}$. Treatments included ten day exposure to long daylength (14 hrs.) and short daylength (10 hrs.) inside the culture room under high temperature conditions (28-30°C) with three replicates in each treatment for a total of 36 plants. For short daylength treatment, a dark chamber was constructed inside the culture room using an adjustable plastic shelf covered with three layers of 90 x 132 inch polyester black tablecloth. Plants in the short day treatment were treated in the culture room under ten hours of light (Figure 2.2, A) then manually transferred to the dark chamber for the remaining hours (14 hrs. dark) (Figure 2.2, B). After ten days of treatment, plants were subsequently transferred to the greenhouse for seed production.

2.2.2.1 Greenhouse Treatment

A separate study was made in the greenhouse. The male sterile lines 2009S and 2008S and fertile lines 10HHB020, CL131, Cypress, and Catahoula varieties were grown in a completely randomized design with three replicates. Plants were exposed at the early booting stage during the natural short days (~10 hrs.) in January 2015. Greenhouse temperatures were set at high temperature (28°C) during the day and low temperature (22°C) at night.

A B

Figure 2.2 Daylength treatment inside the culture room at M.B. Sturgis Hall School of Plant Environmental and Soil Sciences, LSU 2015. The temperature were set to 28-30°C and daylength to 14 hour light and 10 hour dark. (A) For the long day treatment, plants were treated for 14 hrs. light. (B) For the short day treatment, plants were treated for 10 hrs. Plastic shelf with three layers of black cloth were constructed inside the culture room and plants were first exposed for 10 hours light then transferred in the dark chamber for 14 hrs..

2.2.2.2 Stage and Duration for Pollen Fertility Reversion of 2009S

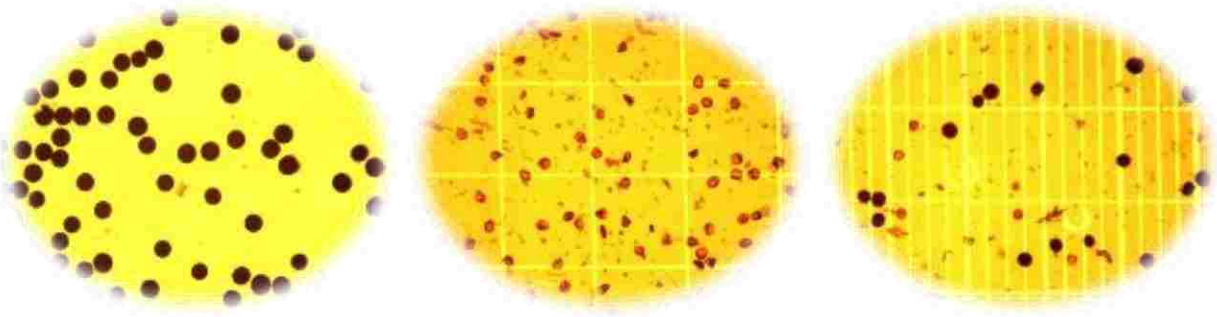
A second group of plants consisted of 14 2009S plants with varying stages of secondary and tertiary panicles were selected for the stage and duration of pollen fertility reversion. A total of 48 panicles were identified and tagged based on two categories: Partially exposed flag leaf where flag leaf length was between eight to twenty cm (Figure 2.3, A), and fully exposed flag leaf where the size of the internode of the flag leaf collar to the second leaf node was one to five cm (Figure 2.3, B). Twelve tillers of partially exposed and fully exposed flag leaves were subjected to ten and 15 day treatment duration under short daylength (12 hrs.) and low temperature (22°C) conditions inside the culture room.

Figure 2.3 Partial and fully exposed flag leaf of 2009S. (A) Left shows flag leaf is not fully exposed and leaf collar is still inside the leaf sheath. (B) Right shows fully exposed flag leaf and leaf collar is visible. In this photo, the distance from the flag leaf collar to the 2nd leaf node is about 1.3 cm.

2.2.3 Phenotypic Data

Pollen and spikelet fertility were evaluated using the modified protocol of Shan et al. (2009). Pre-flowering spikelets were collected and stored in 70% ethanol for pollen staining. The anthers of each sample were removed from the spikelet and were placed in tubes inside an 8 x 12 PCR microtiter plate with 150 µL of 1% I_2KI in deionized water and smashed with a wooden toothpick. A small volume (~20 µL) of the sample solution with smashed anthers was removed and placed on a haemocytometer. The number of fertile and sterile pollen for each plant was determined by manual counting up to 200 stained (fertile) and unstained (sterile) pollen grains using a light microscope at 40x magnification (Figure 2.4). Number of days to heading from planting was recorded when panicles first emerged from the boot in each plant. Height (cm) was measured from soil surface to the tip of the longest panicle. Length (cm) of panicle exerted was measured from tip of panicle down to the exposed panicle or panicle base. Number of spikelets was obtained by counting the total number of spikelets per panicle per plant. Percent (%) seed set

was calculated by dividing the number of filled grains by the total number of spikelets in each panicle. Mean percent (%) pollen fertility as judged by I_2KI staining (Shan et al. 2009) was analyzed using PROC GLM in SAS ver. 9.4 (SAS Institute Inc. Cary, NC).



A B C

Figure 2.4 Pollen grains observed under the microscope. (A) Big, round and black stained pollen are considered as male fertile lines. (B) Small, wrinkled and lightly stained pollen are considered as male sterile lines. (C) Combination of stained and lightly stained pollen grains are considered as partially fertile/sterile lines

2.3 Results and Discussion

2.3.1 Percent Pollen Fertility and Seed Set

Highly significant differences (p value <0.01) were found for percent pollen fertility and seed set using ANOVA when Variety was used as main effect for both short-day and long-day conditions. No significant differences were observed for daylength treatment as main effect or its interaction with variety on pollen fertility and seed set (Table 2.1).

Table 2.1. Analysis of Variance of pollen fertility and seed set under culture room conditions for male sterile lines 2008S, 2009S and fertile lines Catahoula, CL131, Cypress and 10HHB020.

Trait	Daylength[1] p-value	Variety p-value	Replicate p-value	Treatment x Variety interaction
Mean (%) pollen fertility	0.40 ns	<0.01**	0.93 ns	0.66 ns
Mean (%) seed set	0.21 ns	<0.01**	0.59 ns	0.17 ns

[1]Daylength treatment = long day (14 hrs.) short day (10 hrs.)
**highly significant at p-value ≤0.01
ns = not significant

Although there were significant differences in pollen fertility under greenhouse conditions, Seed set did not show significant differences among lines/varieties indicating that the two male sterile lines can have seed setting similar to male fertile lines at low temperature and short daylength conditions (Table 2.2).

Table 2.2. Analysis of Variance of pollen fertility and seed set on "Variety" as treatment under greenhouse conditions for male sterile lines 2008S, 2009S and fertile lines Catahoula, CL131, Cypress and 10HHB020.

Trait	Replicate p-value	Treatment p-value
Mean percent (%) pollen fertility	0.22 ns	0.01**
Mean (%) seed set	0.98 ns	0.20 ns

**highly significant at p-value ≤0.01.
ns = not significant.

When grouped using Fisher's least significant difference (LSD), pollen fertility of male sterile lines 2009S and 2008S belonged to the same group A; Catahoula in group B; Cypress, CL131 and 10HHB020 in group C for both long day (14 hrs.) and short day (12 hrs.) conditions. (Figures 2.5, A and 2.5, B).

When grown under greenhouse conditions, pollen fertility of 2008S and 2009S were still significantly different from the fertile lines Catahoula, 10HHB020, Cypress, and CL131 using Fisher's least significant difference (LSD). Sterile lines 2009S and 2008S belonged to group A with 16% and 42% pollen fertility respectively while fertile lines Catahoula, 10HHB020, CL131 and Cypress belonged to group B with 76%, 89%, 91%, and 86% pollen fertility respectively (Figure 2.5, C).

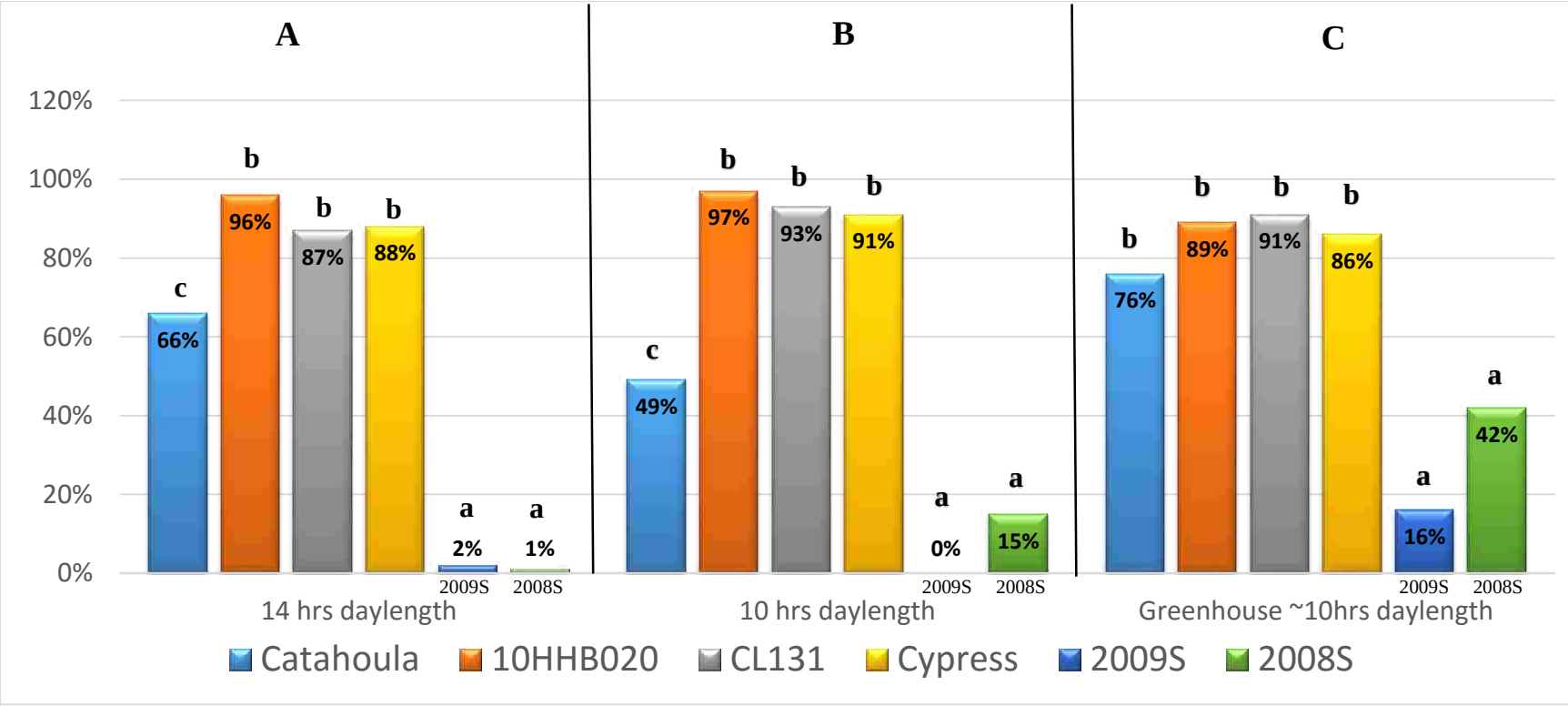


Figure 2.5 Mean percent (%) pollen fertility data for lines treated in the culture room under (A) long daylength (14 hrs.); (B) short daylength (10 hrs.) under constant high temperature conditions (28-30°C); (C) Greenhouse at short daylength (~10 hrs.) at high temperature (28°C) during the day and low temperature (22°C) during the night. Means of the same letter are not significantly different among varieties using Fisher's least significant difference (LSD).

An increase from 1% to 4% seed fertility from long to short daylength exposure was observed for male sterile line 2008S (Figures 2.6, A and 2.6, B). When grouped using Fisher's LSD for seed setting, 2009S, 2008S and Catahoula were grouped as A with 0%, 1% and 5% seed set respectively. Fertile lines 10HHB020 and CL131 were grouped as B with 41% and 39% seed set respectively. Cypress belonged to both A and B under 14 hours daylength with 23% seed set.

Under ten hours of daylength, both 2009S and 2008S belonged in group A with 0% and 4% seed set respectively. Catahoula and CL131 belonged in group A and B with 30% and 15% seed set respectively. Cypress belonged in group B and C (41% seed set) and 10HHB020 in C (66% seed set).

When varieties and lines were grown in the greenhouse with high temperature during the day (~28^0C) and low temperature at night (~22^0C) at a 12- hour photoperiod, an increase of 26% and 34% seed set were observed in 2009S and 2008S respectively. When grouped using Fisher's least significant difference (LSD), Catahoula is in group A with 14% seed set and 2009S is in group A and B with 26% seed set. 10HHB020, CL131, Cypress and 2008S are in grouped B with 60%, 39%, 30% and 34% seed set respectively.

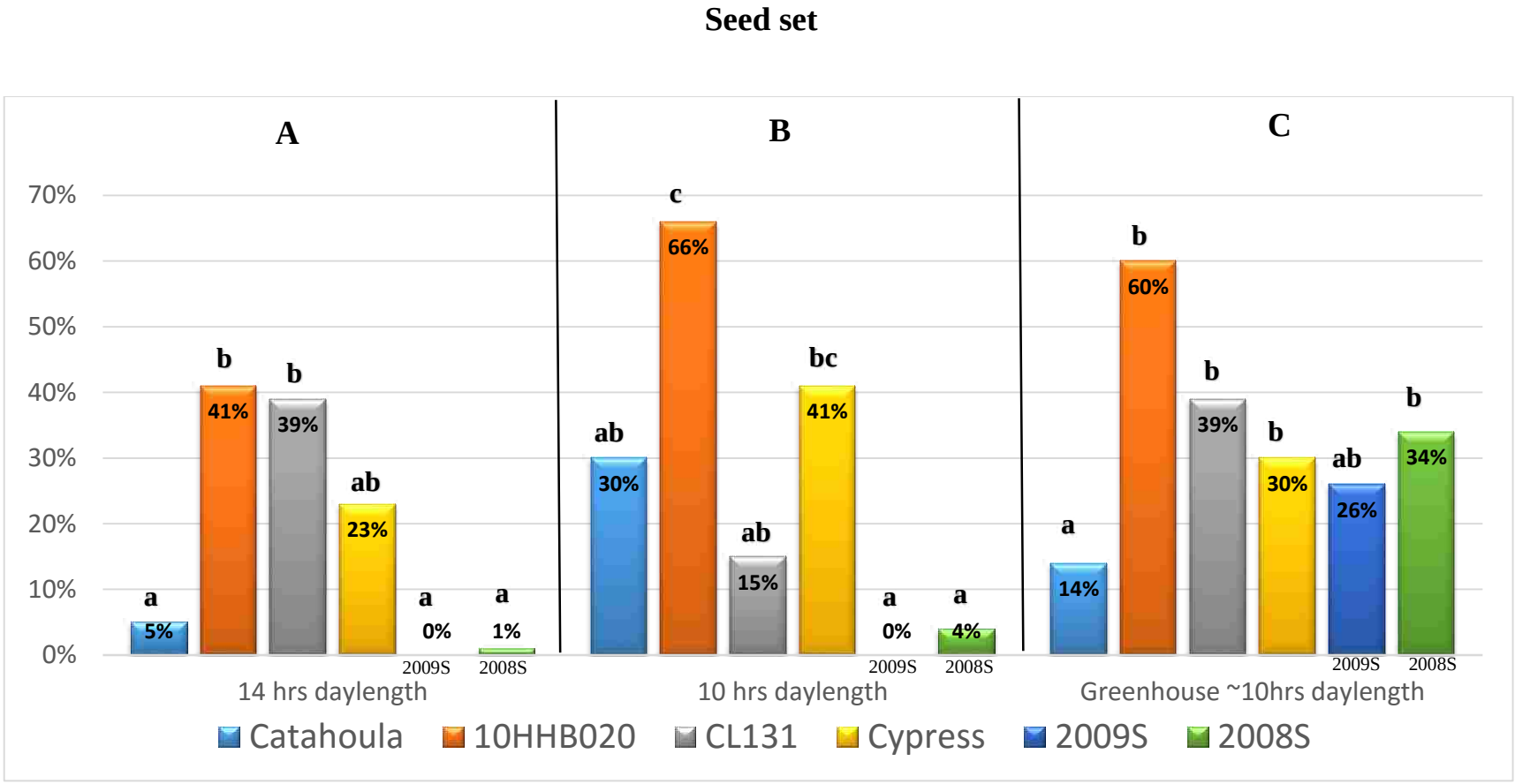


Figure 2.6 Mean percent (%) seed set data for lines treated in culture room under (A) long daylength (14 hrs.); (B) short daylength (10 hrs.) under constant high temperature conditions (28-30°C); (C) Greenhouse at short daylength (~10 hrs.) at high temperature day (28°C) low temperature night (22°C). Means of the same letter are not significantly different among varieties using Fisher's least significant difference (LSD)

2.3.2 Other Agronomic Characteristics

There was a significant daylength x variety interactions for mean days to heading when analyzed using SAS Ver. 9.4 PROC GLM. No significant height differences were detected for daylength treatment. However, between variety mean height showed significant differences for short day and long day conditions. There was a significant difference among varieties for panicle length, and an interaction occurred between daylength and variety. Significant differences were observed for the number of spikelets between short and long daylength exposure (Table 2.3).

Table 2.3. Analysis of variance of agronomic traits on Daylength treatment under culture room conditions of male sterile lines 2008S, 2009S and fertile lines Catahoula, CL131, Cypress and 10HHB020.

Trait	Daylength[1] p-value	Variety p-value	Replicate p-value	Daylength x Variety interaction
Mean days to heading[2]	<0.01 **	<0.01**	0.21 ns	0.02*
Mean height (cm)	0.81 ns	<0.01**	0.48 ns	0.10 ns
Mean length of panicle (cm)	0.72 ns	<0.01**	0.53 ns	0.01**
Mean number of spikelets[2]	<0.01 **	0.12 ns	0.81 ns	0.62 ns

[1]Daylength treatment = long day (14 hrs.) short day (10 hrs.)
[2]Values were log-transformed for normality
*highly significant at $\alpha \leq 0.01$
*significant at $\alpha \leq 0.05$
ns = not significant

Under greenhouse conditions, significant differences were detected among varieties for mean days to heading, mean height, panicle length, and mean number of spikelets (Table 2.4).

Table 2.4. Analysis of Variance for agronomic traits on "Variety" as treatment under greenhouse conditions of male sterile lines 2008S, 2009S and fertile lines Catahoula, CL131, Cypress and 10HHB020.

Trait	Replicate p-value	Treatment p-value
Mean days to heading	0.97 ns	<0.01**
Mean height (cm)	0.67 ns	<0.01**
Mean length of panicle (cm)	0.89 ns	<0.01**
Mean number of spikelets	0.66 ns	0.04*

*highly significant at $\alpha \leq 0.01$.
*significant at $\alpha \leq 0.05$.
ns = not significant.

Using Fisher's LSD among varieties in each treatment (Table 2.5) showed male sterile lines with the shortest panicle length at short day, although it was not significantly different from 10HHB020 under long day conditions. Also under short day conditions, the two male sterile lines showed late heading which was not significantly different from 10HHB020.

Table 2.5. Fisher's least significant difference test (LSD) among varieties under three different treatment conditions.

Treatment	Variety	Mean days to heading	Mean plant height (cm)	Mean length of panicle (cm)	Mean number of spikelets
Long Day[1]	Catahoula	99 c	65.00 ab	13 b	81 ns
	10HHB020	100 bc	33.50 d	8 c	66 ns
	CL131	113 abc	50.00 c	11 b	48 ns
	Cypress	98 c	72.00 a	18 a	73 ns
	2009S	119 ab	48.00 c	6 c	50 ns
	2008S	121 a	57.50 bc	8.0 c	93 ns
Short Day[2]	Catahoula	114 b	67.50 a	12 a	45 ns
	10HHB020	136 a	43.70 b	13 a	44 ns
	CL131	112 b	61.00 a	12 a	43 ns
	Cypress	100 c	62.00 a	14 a	47 ns
	2009S	132 a	41.00 b	6 b	27 ns
	2008S	133 a	54.50 ab	7 b	40 ns
Greenhouse[3]	Catahoula	120 a	70.50 a	18 ac	108 ab
	10HHB020	128 b	61.00 b	18 ac	69 b
	CL131	113 c	58.00 bd	14 ad	86 ab
	Cypress	97 d	63.30 b	18 bc	68 bc
	2009S	114 c	52.80 cd	11 bd	56 d
	2008S	132 e	62.70 b	13 bd	125 ab

Means with the same letter are not significantly different using Fisher's LSD at p-value ≤ 0.05.
[1]Long day (14 hrs.) and constant high temperature (28-30^{0}C) conditions.
[2] Short day (10 hrs.) and constant high temperature (28-30^{0}C) conditions.
[3]Daylength: ~10 hrs. Set temperature: 28^{0}C day, 22^{0}C night at 12 hr. period.

2.3.3 Stage and Duration for Pollen Fertility Reversion of 2009S

The results showed that there was no significant difference for duration of treatment on partially exposed and fully exposed flag leaves, indicating that additional five days of exposure to short daylength and low temperature conditions did not affect the pollen fertility of 2009S (Table 2.6).

Table 2.6. Effect of duration and stage of exposure at short day, low temperature on pollen fertility of 2009S.

Treatment	DF	F-value	p-value
Duration (10 and 15 days)	1	0.86	0.35 ns
Stage (partially and fully exposed)	1	5.97	0.02*
Duration x Stage	1	0.52	0.49 ns
Replicate	11	0.53	0.87 ns

*significant using F test at p-value ≤ 0.05
ns = not significant

Significant differences were observed between partially exposed and fully exposed panicles. The mean percent pollen fertility was higher for both ten days and fifteen days of treatment in partially exposed panicles with 69% and 47%, respectively compared to the fully exposed panicle with 28% and 24%, respectively (Figure 2.7).

Studies conducted by Liang et al. (2008) on male sterile lines showed that the bract primordium stage of panicle initiation was the most responsive stage for fertility reversion and diminishes at pollen mother cell formation. In addition, Zhou et al. (2014) treated male sterile lines in different photoperiod and temperature conditions when the panicles reached ~1 cm (before the stage of premeiosis), but were exposed for two weeks or until flowering and found

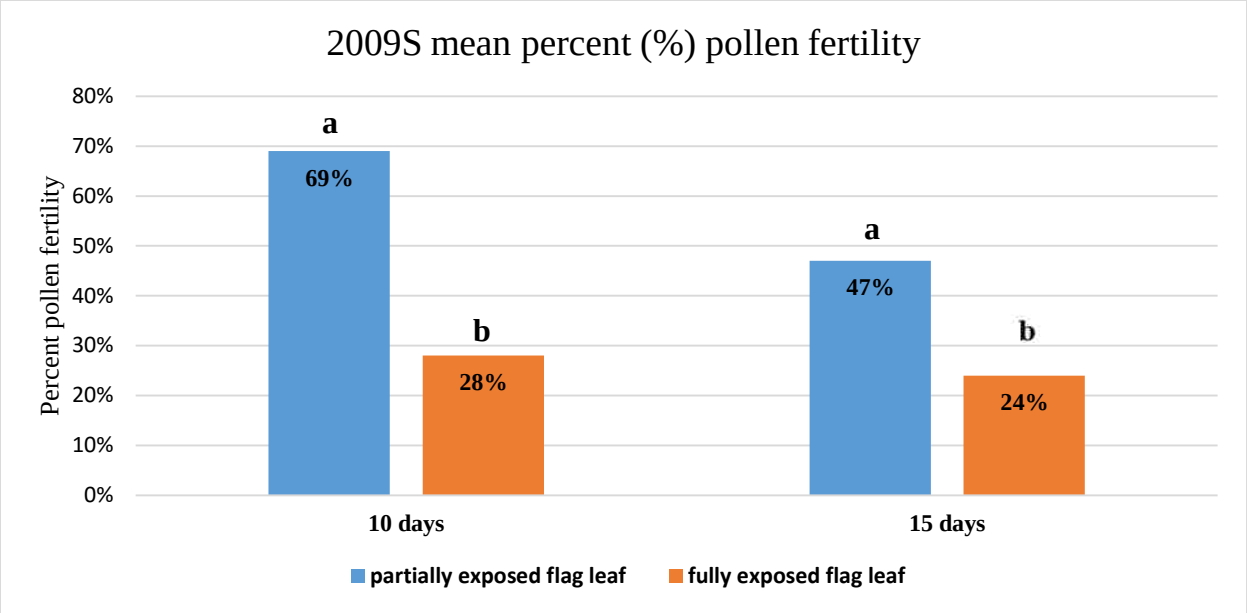


Figure 2.7 Mean percent (%) pollen fertility of partially exposed and fully exposed flag leaf of 2009S treated to 10 and 15 days of low temperature and short day treatment.
Means of the same letter are not significantly different at $\alpha = 0.05$.

fertility reversion under low temperature conditions. Chen et al. (2010) recommended transferring male sterile lines to low temperature conditions at the fourth stage of young panicle initiation for five to seven days and when distance between flag leaf (collar) and the second leaf was ~2 cm to restore fertility. These studies showed that the response to fertility reversion ranges from early panicle initiation stage to the meiotic stage (booting). Sanchez and Virmani (2005) reported that different temperature sensitive genetic male sterile lines (TGMS) displayed different critical stages for sterility ranging from panicle differentiation to meiotic division. Moldenhauer et al. (2013) classify the panicle differentiation (R1) stage as the stage between panicle differentiation to collar formation. Panicles with a fully exposed flag leaf collar (or termed as "early boot" measuring ~2 inches) was seen as the period for the start of meiotic division (Dunand and Saichuck 2015). This study found that when lines were subjected to low temperature and short daylength during panicle differentiation stage (R1) (which can be visually identified as partially exposed flag leaf), high pollen fertility was observed in contrast to low

fertility at the early booting stage. The result of this study provides valuable information for breeders in deciding the developmental stage to treat male sterile lines to maximize pollen fertility.

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CHAPTER 3. INHERITANCE OF PHOTOPERIOD AND THERMOSENSITIVE GENETIC MALE STERILITY IN F_2 AND BC_1F_2 POPULATIONS AND DEVELOPMENT OF MOLECULAR MARKERS

3.1 Introduction

Hybrid rice is a proven technology that over the last 20 years has been shown to increase yield up to 30% over inbred varieties under irrigated conditions (Virmani et al. 2003). Hybrid rice requires the use of a male sterility system consisting of either a three-line method that uses cytoplasmic male sterility (CMS) or a two-line approach that uses photoperiod and/or thermosensitive genetic male sterility (PTGMS) (Xu et al. 2011). A spontaneous *japonica* mutant variety Nongken 58S discovered in 1973 (Shi 1985) that showed certain fertility characteristics provided the opportunity to replace the CMS system in hybrid seed production (Yuan 1990). The two-line system can be used as a pollen sterile female for hybrid seed production under long day and high temperature conditions and as a fertile maintainer under short day and low temperature environments. Furthermore, numerous hybrid combinations can be generated because normal fertile male parents can function as restorers (Xu et al. 2011).

3.1.1 Inheritance of Photoperiod and Thermosensitive Genetic Male Sterile Rice Lines

Studies on crossing conventional male *indica* and *japonica* lines to Nongken 58S, which was the original *japonica* photoperiod male-sterile spontaneous mutant, showed all F_1's to be male fertile (Virmani et al. 2003). Studies using F_2 plants from crosses to Nongken 58S and three other fertile lines, Yang et al. (1992) reported that inheritance of pollen sterility was governed by a single recessive gene under long-day conditions. Furthermore, a cross between Nongken 58S and Nongken 58 (fertile) showed a typical single-gene recessive segregation (Mei et al. 1999a; Mei et al. 1999b). Many studies have been conducted on male sterile lines grown under natural field conditions of long daylength and high temperature that showed F_2 segregation fitting a 3:1 fertile: sterile single locus model (Lopez et al. 2003; Peng et al. 2010; Xu et al. 2011; Qi et al.

2014). The most comprehensive and recent work conducted by Zhou et al. (2014) reported thermosensitive genetic male sterile (TGMS) lines AnS-1 and Zhu1S were controlled by the recessive gene *RNase ZS1* responsible for pollen sterility under high temperature conditions.

In contrasting research, Zhang et al. (1994) report that genetic male sterile lines derived from Nongken 58S crossed to fertile lines showed a two-locus genetic model that was non-allelic to the original Nongken 58S. Several studies that used various *indica* male sterile lines showed 15:1 fertile: sterile pollen segregation ratios (Subudhi et al. 1997; Dong et al. 2000; Zhou et al. 2011) under natural long daylength and high temperature. Other studies detected segregation that followed either a bimodal or continuous distribution in each population (Mei et al. 1990; Alcochete et al. 2005).

3.1.2 Candidate Genes for Photoperiod and Thermosensitive Genetic Male Sterility

Numerous candidate genes associated with photoperiod and thermosensitive genetic male sterility (PTGMS) have been reported. Zhou et al. (2011) evaluated the *indica* PTGMS line Peiai64S and identified candidate gene *pms1(t)* by bulked segregant analysis (BSA) using simple sequence repeat (SSR) markers. Fourteen markers were mapped to the *pms1(t)* locus LOC_Os7g12130 that encoded a MYB-like protein containing a DNA-binding domain (Zhou et al. 2011). RT-PCR analysis revealed that mRNA levels were altered in different photoperiod conditions and treatments (Zhou et al. 2011). A long, non-coding RNA (lncRNA) was discovered that was reported to regulate PTGMS in an F_2 population from a cross of the PTGMS Nongken 58S *japonica* male sterile line with a non-PTGMS doubled haploid line (Ding et al. 2012a). The lncRNA was 1236 bp in length and described as a long-day specific, male-fertility-associated RNA (LDMAR) located at LOC_Os12g36030 on chromosome 12. A spontaneous mutation causing a single nucleotide polymorphism (SNP) between the wild-type and mutant

was thought to modify the structure of LDMAR (Ding et al. 2012a). This mutation was associated with increased methylation in the promoter region and reduced transcription of LDMAR under long-day conditions, presumably resulting in pollen sterility (Ding et al. 2012a). Genetic analysis by Xu et al. (2011) used bulked segregant analysis (BSA) and fine mapping to identify the *ptgms2-1* locus containing LOC_Os02g12290 on chromosome 2 that encoded a ribonuclease Z in the PTGMS line Guangzhang63S. A SNP in LOC_Os02g12290 that created a premature stop codon in the Guangzhang 63S allele, was identified as a candidate marker for sterility (Xu et al. 2011).

3.1.3 Molecular Markers for Photoperiod and Thermosensitive Genetic Male Sterility

Transfer of male sterility to different genetic backgrounds in hybrid rice breeding can be facilitated using molecular markers tightly linked to the trait. Tanee et al. (2014) used simple sequence repeat markers (SSR) located on chromosome 2 near the *tmsX* gene (Peng et al. 2010) in backcrossing thermo-sensitive male sterility to Thai cultivars. Lopez et al. (2003) used four SSR markers RM2, RM10, RM11 and RM214 on chromosome 7 near the *tms2* locus with 97% accuracy in selection of male sterile plants in an F_2 population. A SCAR (sequence characterized amplified region) marker for the male sterile locus *tms6* located on chromosome 3 was developed from mapping a TGMS mutant using an F_2 segregating population (Wang et al. 2004).

3.1.4 Genotyping Using CEL 1 Nuclease

Targeting induced local lesions in genomes (TILLING) is a reverse genetics approach that takes advantage of the mismatch-specific nuclease to detect mutations in a target region (Barkley and Wang 2008). The TILLING method has been used to detect mutations that existed in mutagenized or natural populations using CEL1 nuclease (Oleykowski et al. 1998) or mung bean nucleases (Colbert et al. 2001; Comai et al. 2004; Till et al. 2004). However, the

complexity of the method prevented its use for routine detection of SNP or InDels (Kadaru et al. 2006). Integrated DNA Technologies (IDT Coralville, IA) offers a product that uses mismatch-specific nucleases derived from celery (Surveyor ^{tm} Mutation detection kit) to visualize small DNA fragments with standard agarose gel electrophoresis or reverse-phase HPLC using the WAVE and WAVE HS systems (http://www.idtdna.com). Kadaru et al. (2006) developed an alternative Ecotilling protocol involving simple gel systems and unlabeled primers. With the optimized conditions, he successfully identified four new SNPs in the rice *alk* gene that were verified by Sanger sequencing. Jiang et al. (2013) reported a simplified, label-free CEL 1 based protocol with agarose gels or capillary systems for detection of mutations, marker assisted breeding and genome-wide association studies.

3.1.5 QTL Mapping by Selective Genotyping

Whole population QTL mapping is often laborious and costly (Zhang et al. 1994). Bulk segregant analysis (BSA) (Michelmore et al. 1991; Quarrie et al. 1999) and selective genotyping (SG) are two methods for mapping QTLs that can reduce the time and cost of genotyping. BSA can provide information on both the polymorphism of the parents and possible linkage between markers and the targeted QTL (Sanabria 2015). The method has allowed the identification of several loci for photoperiod and thermosensitive genetic male sterility in rice (Zhang et al. 1994; Wang et al. 1995; Subudhi et al. 1997; Dong et al. 2000; Reddy et al. 2000; Peng et al. 2010; Xu et al. 2011; Zhou et al. 2011; Qi et al. 2014). BSA involves less genotyping given that the DNA of extreme samples are pooled. However, it can create false positives because the allele frequency estimates relies on band intensities (Quarrie et al. 1999; Sanabria 2015). A potentially more precise strategy is to use selective genotyping (SG) that is also compatible with PCR-based markers run in agarose gels. SG detects significant QTLs by genotyping each selected individual

line with contrasting phenotypes (Sun et al. 2010). Navabi et al. (2009) reported detection of QTLs by genotyping no more than 20 extreme individuals. A comparative analysis between whole population mapping, BSA, and SG approach showed that SG can effectively detect major QTLs for rice grain yield (Vikram et al. 2012). It was also used to detect QTLs for root length (Toorchi et al. 2002), drought resistance (Subashri et al. 2009), salt tolerance (Rana et al. 2009), resistance to kernel fissure (Pinson et al. 2013) and marker discovery for sheath blight resistance in rice (Sanabria 2015).

The primary goal of this study was to determine inheritance of pollen sterility/fertility in the two photoperiod and thermosensitive male sterile lines 2008S and 2009S using F_2 and BC_1F_2 segregating populations. SNP markers were developed for specific alleles within LOC_Os12g36030 and LOC_Os07g12130 to evaluate marker-trait associations in a segregating F_2 population derived from 2008S. QTL mapping using the selective genotyping approach was also carried out to discover additional markers for increased precision of selecting male sterile lines in the 2008S background. In addition, F_2 and BC_1F_2 populations derived from 2009S were genotyped using CEL 1 nuclease assay to detect the presence of SNP in LOC_Os02g12290 associated with male sterility.

3.2 Materials and Methods

3.2.1 Plant Populations

A total of 1,038 randomly sampled F_2 plants, derived from a cross of *indica* male sterile 2008S to tropical *japonica* lines CL131 (U.S. Pat. No. 7,786,360) (Linscombe 2013), Cypress (Reg. no. CV-91, PI561734) (Linscombe et al. 1992) and 9502008-A, were evaluated from March to May of 2012, 2013, and 2014 at the H. Rouse Caffey Rice Research Station (Crowley, LA.) to determine inheritance of pollen male sterility. To validate the inheritance, F_2 male sterile

line 82 from the 2008S/Cypress cross and F_2 line 91 from the 2008S/CL131 population were backcrossed to their original fertile parents to produce the BC_1F_1 (18 and 6 plants, respectively) and were selfed to generate the BC_1F_2 (184 and 177 plants, respectively). In cooperation with Dr. Greg Berger, 658 randomly-sampled F_2 plants from 2008S/CL131 and 2008S/Cypress, were evaluated for seed fertility in 2013 and 2014 at the Rice Research and Extension Center, Stuttgart, AR.

For the 2009S inheritance study, 301 F_2 plants from the cross 2009S/10HHB020 and 308 F_2 plants from 2009/07-1643 were planted at Crowley in March 2013. To confirm the inheritance in a backcross population, one F_2 plant with sterile pollen grains and a second F_2 plant with no detectable pollen grains from each population were planted in the greenhouse in 2013 and backcrossed to male fertile parents 10HHB020 and 07-1643. A total of 41 BC_1F_1 plants from all crosses were produced and selfed to generate four BC_1F_2 populations ranging in size from 131 to 301 individuals.

3.2.2 Collection of Data for Pollen and Seed Fertility

Data on pollen sterility were obtained by collecting five to ten florets just before anther dehiscence/extrusion from one or more panicles of each plant with subsequent storage in 70% ethanol. Anthers from three florets of each plant were placed in tubes of an 8 x 12 PCR microtiter plate (Biorad, Hercules, CA) with 150 µL of 1% I_2KI. Anthers were broken up with a wooden toothpick to release pollen grains. Approximately 20 µL of the sample solution with broken anthers were removed and placed on a haemocytometer. The number of fertile and sterile pollen for each plant was determined by manual counting up to 200 stained (fertile) and unstained (sterile) pollen grains using a light microscope at 40x magnification. Large, dark round pollen grains were considered fertile (Figure 3.1, A). Small, shriveled, unstained or lightly-

stained pollen grains were scored as sterile (Figure 3.1, B). Partial fertile/sterile has the presence of both fertile and sterile pollen (Figure 3.1, C). Percent pollen sterility was calculated by dividing sterile pollen to total number of pollen. ≥90-100% sterile pollen were scored as sterile and <90% sterile pollen were scored as fertile. Samples with no visibly stained or unstained pollen were scored as "no pollen" types with 100% sterility (Figure 3.1, D). Mean seed set data of 2008S/CL131 and 2008S/Cypress from Arkansas in 2013 and 2014 were calculated by the average number of filled grains over the total number of spikelets from three panicles of each plant.

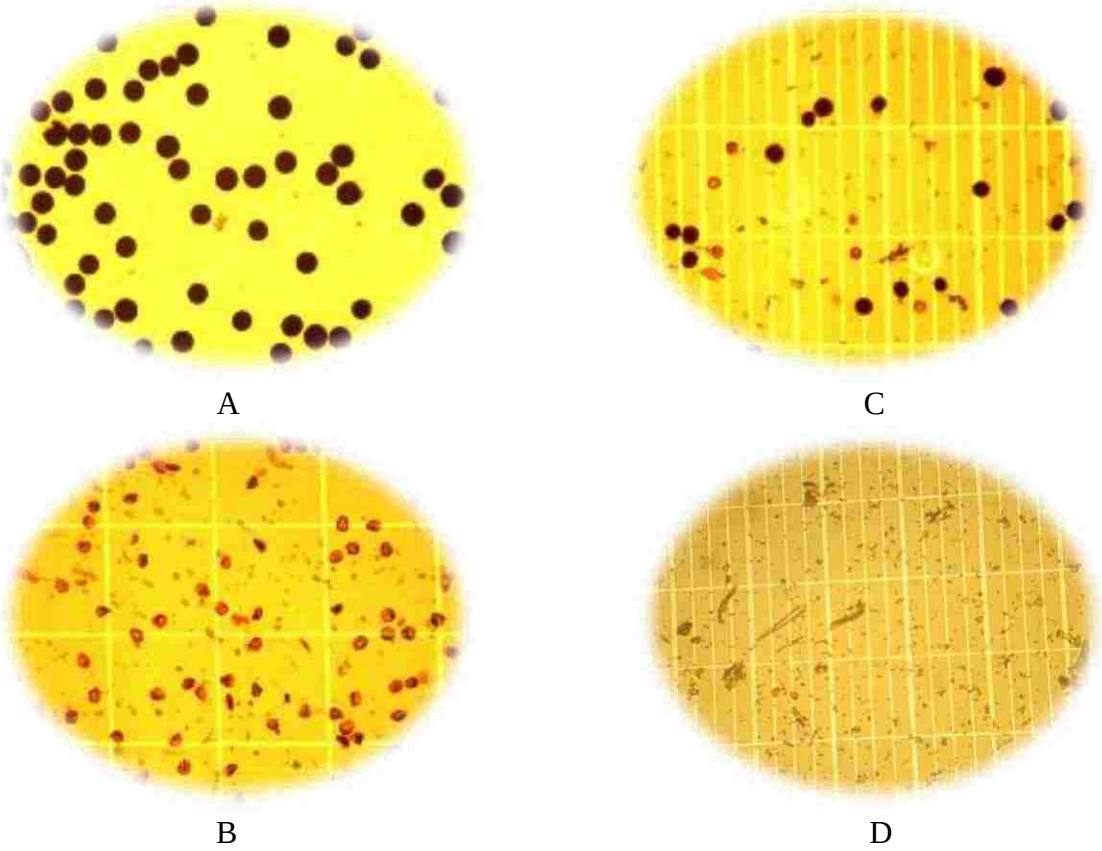


Figure 3.1 Pollen grains observed under the microscope (40X magnification). (A) Big, round, and black stained pollen are considered as male fertile lines. (B) Small, wrinkled and lightly stained pollen are considered as male sterile lines. (C) Combination of fertile and sterile pollen grains are considered as partially fertile/sterile lines. (D) "No pollen" type showed no visible stained or unstained pollen (D).

3.2.3 Design of SNP Markers for Genotyping Male Sterile Lines

Sequence data from the Nipponbare reference genome (http://www.gramene.org) was used to design SNP primers using the SNAP program (http://ausubellab.mgh.harvard.edu/). SNPs were identified by aligning DNA sequences of Nipponbare with the published sequences of Zhou et al. (2011) for LOC_Os07g12130 and Ding et al. (2012a) for LOC_Os12g36030 using Clustal X ver. 2 (Larkin et al. 2007). Forward primers for each SNP were designed to contain a mismatch within three nucleotides of the 3' end of the putative fertile allele, and a 3' mismatch with the sterile allele (Drenkard et al., 2000). Reverse primers were designed by matching all nucleotides adjusted for the target PCR length. All primers were designed to amplify ~ 350 bp fragment for visual scoring of markers on a 1.5% agarose gel (Figure 3.2). Data were analyzed by single marker analysis and two-way ANOVA using PROC GLM in SAS ver. 9.4 (SAS Institute, Cary NC).

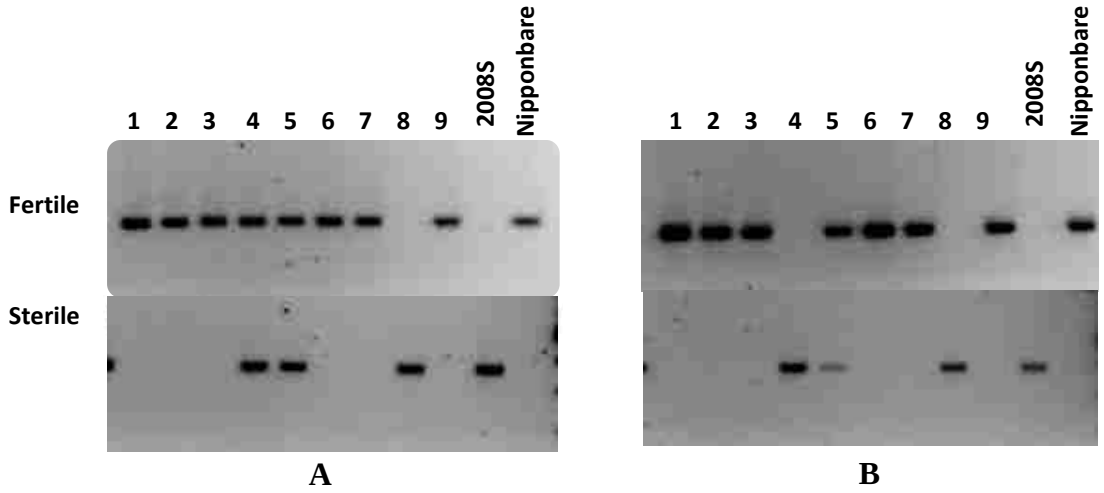


Figure 3.2 SNP genotypes at (A) LOC_Os07g12130 and (B) LOC_Os12g36030. Bands present in fertile genotype and absent on sterile genotype are male fertile (ex. Nipponbare). Bands present in sterile genotype but absent on fertile genotype are male sterile (ex. 2008S). Bands showing in both fertile and sterile genotypes are heterozygote and scored as fertile.

For the LOC_Os02g12290 sequence (Xu et al. 2011), primers were designed using primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) to amplify 498 bp of the target region containing the SNP in 2009S (Table 3.1). The CEL 1 nuclease assay was then used to genotype fertile and sterile lines. For amplification of the target region, 10 µL PCR reactions were prepared with a mixture of the following: 1 µL of 20 ng/ µL DNA template, 1 µL of 10X PCR buffer (containing 1mM $MgCl_2$), 0.8 µL of 10mM dNTPs mix, 0.2 µL each of 20 µM forward and reverse primers, 0.08 µL of 5U/ µL Takara La Taq with high GC Buffer II (Clonetech, Mountain View, CA) and 6.8 µL of distilled water. The PCR profile included the following steps: 95°C, 3 min, 95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec, repeat 30X previous three steps, 72°C, 5 min with final holding temperature at 4°C.

Table 3.1 Primer sequences used to genotype SNPs in LOC_ Os02g12290, LOC_Os07g12130 and LOC_Os12g36030.

Primer Name	Marker Type	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')	Size (bp)
LOC_Os02g12290 CEL1	SNP (C/A)	CGACCGAGTAG ACATTGACTTG	CTGAGCTCGGA CTGGTCCAT	498
LOC_Os07g12130 REF SNP 10	SNP (C)	TGCACAGAGAA AGAACTAGGAT CCCTTAGAGC	ACGCAAGCTGC AGGTAGCAACG A	373
LOC_Os07g12130 ALT SNP 10	SNP (A)	AAATTGCACAG AGAAAGAACTA GGATCCCTTAC ATA	CCCAGGGTTGG TCAAAGGAGGG T	351
LOC_Os12g36030 REF	SNP (C)	GTGTTGATAAA AATTTTACTCTT GATGGATGGGA G	CAAATCCTTTAG CATTTTTAACCC GAAAACTTG	356
LOC_Os12g36030 ALT	SNP (G)	CGTGTTGATAA AAATTTTACTCT TGATGGATGGT TC	CAAATCCTTTAG CATTTTTAACCC GAAAACTTG	357

3.2.4 SNP Genotyping Using CEL 1 Nuclease Assay

Presumably due to high GC content (72%) of the first exon in LOC_Os02g12290, standard PCR protocol using SNP markers did not amplify any visible bands from 2009S (unpublished results). Therefore, a commercial CEL 1 nuclease (Integrated DNA Technologies Inc., Coralville, IA) was used to assay the genotype of F_2 and BC_1F_2 populations. Initial PCR was performed using primers for LOC_Os02g12290. Approximately 50 ng/µL of PCR products (5 µL) from individual F_2 plants were mixed with ~50 ng/µL (5 µL) of the PCR product from the fertile parent 10HHB020 or 07-1643. Formation of heteroduplex DNA molecules in the mixture was accomplished by running in a PCR using 96 well iCycler (BIORAD, Hercules, CA) as per the Surveyor ™ Mutation Discovery kit protocol (IDT, Coralville, IA). The 1:1 PCR product mix was then made to a final 13 µL reaction volume by adding 1 µL of 0.5 M $MgCl_2$, 1 µL of enhancer solution, and 1 µL of nuclease S (CEL 1 nuclease). The reactions were incubated at 42°C for one hour and then terminated by adding 1 µL of stop solution. All samples were mixed with 1 µL 6x blue loading dye and immediately loaded onto a 1.5% agarose gel stained with ethidium bromide and electrophoresed at 170 volts for 60 minutes (Figure 3.3).

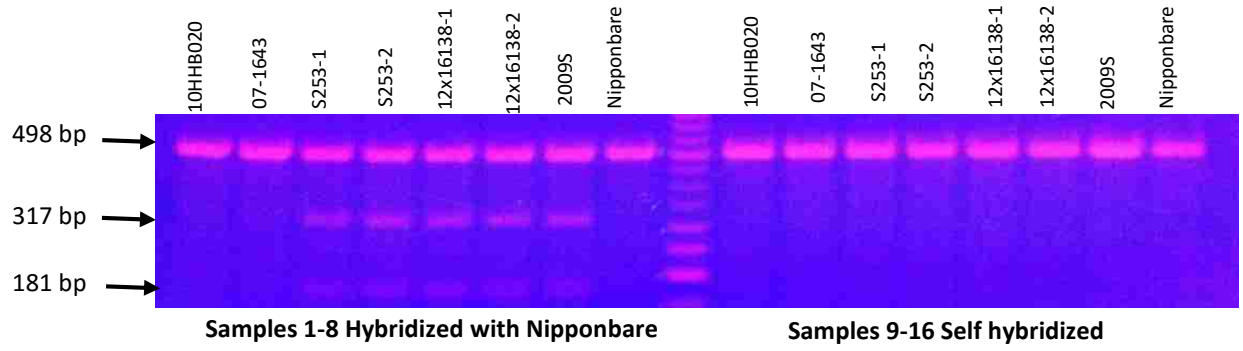


Figure 3.3 Genotyping using CEL 1 nuclease assay. Samples genotyped were fertile 10HHB020, 07-1643, Nipponbare; sterile S253-1, S253-2, 12x16138-1, 12x16138-2 and 2009S. Sample DNA were mixed with Nipponbare (hybridized) to form heteroduplex where CEL 1 cleaved at the SNP mismatch showing three bands for male sterile lines. Self-hybridization (no DNA mixing) showed single band indicating that all lines were homozygous.

3.2.5 Confirmation of SNPs in LOC_Os12g36030, LOC_Os07g12130 and LOC_Os02g12290

To confirm the presence of the nucleotide variant between fertile varieties (CL161, Nipponbare) and sterile lines (2008S, 2009S), the regions in LOC_Os12g36030, LOC_Os07g12130 in 2008S and LOC_Os02g12290 in 2009S were sequenced using Sanger sequencing. DNA samples were taken with three replicates in each plant to be sequenced. Primer design was carried out using primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and selected to have a product length of 500-600 bp. A 20 µL PCR reaction was prepared from 3 µL 20 ng/ µL DNA template, 2 µL 10X PCR buffer (containing 1mM $MgCl_2$), 1.6 µL of 10 mM dNTPs mix, 0.4 µL each of 20 µM forward and reverse primers, 0.16 µL of 5U/ µL of Invitrogen Platinum Taq DNA polymerase high fidelity (Thermo Fisher Scientific, Waltham, MA), and 12.44 µL of distilled water. PCR reactions run on a BioRad ICycler (BIORAD, Hercules, CA) consisted of the following steps: 95°C, 3 min, 95°C, 30 sec, 68°C, 30 sec, 72°C, 30 sec, repeat 35X previous three steps, and 72°C, 5 min. PCR products were visualized on a 2% agarose gel run at 170 volts for 60 minutes and stained with ethidium bromide. Bands were removed from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced at the Genomics Core Facility at Pennington Biomedical Research Center (Baton Rouge, LA.) using an Applied Biosystems 3130XL sequencer. The sequences were read and edited using Sequence Scanner Software v1.0 (Applied Biosystems, Foster City, CA). The Clustal X ver. 2 software (Larkin et al. 2007) was used to align the sequences and identify SNPs between fertile and sterile lines.

3.2.6 QTL Mapping Using Selective Genotyping

A total of 86 polymorphic SNP markers from *indica* Jasmine 85 x tropical *japonica* Lemont whole genome sequence (sequences available in Oard lab, unpublished) and 36 SSR

markers from the universal core genetic map (Orjuela et al. 2010) covering all twelve chromosomes were used to identify QTLs in 2008S linked to PTGMS. The strategy of selective genotyping was employed using 22 pollen sterile (70-100% sterile) and 22 pollen fertile (100% fertile) individuals from 2008S/CL131 F_2 segregating population planted in 2013 and 2014. Marker trait association and linkage analysis were calculated using SAS ver. 9.4 (SAS Institute, Cary NC) and ICIM QTL mapping software Ver. 4.0.6.0. (http://www.isbreeding.net)

3.3 Results and Discussion

3.3.1 Pollen and Seed Data Analysis Across Years and Locations

Pollen sterility data (Table 3.2) for 2008S/9502008-A, 2008S/CL131 and 2008S/Cypress F_2 segregating populations planted from 2012 to 2014 showed no significant differences to 15:1 pollen fertile: sterile ratio using chi square goodness of fit test. The ratio confirmed that the inheritance of male sterility was controlled by two recessive genes.

Table 3.2 Chi square analyses and ratios of pollen sterility in 2008S F_2 and BC_1F_2 populations planted in 2012 to 2014 at H. Rouse Caffey Rice Research Station, Crowley LA.

Year	Generation	Population	Total plants	Fertile pollen	Sterile Pollen	Pr>Chisq 15:1 ratio	Pollen ratio fertile: sterile
2012	F_2	2008s / 9502008-A	223	206	17	0.94 ns	15:1
2012	F_2	2008s / CL131	217	197	20	0.82 ns	15:1
2013	F_2	2008s / Cypress	152	145	7	0.25 ns	15:1
2013	F_2	2008s / CL131	117	111	6	0.39 ns	15:1
2014	F_2	2008s / Cypress	134	128	6	0.31 ns	15:1
2014	F_2	2008s / CL131	207	200	7	0.06 ns	15:1
2014	BC_1F_2	2008s / CPRS-82//CPRS	184	177	7	0.17 ns	15:1
2014	BC_1F_2	2008s / CL131-91 //CL131	177	167	10	0.74 ns	15:1

ns = not significant at p-value ≤0.05

Frequency distribution of pollen sterility displayed positive skewness showing higher frequency of pollen male fertile plants with a small increase in frequency at 90-100% sterility displayed in all segregating F_2 populations from 2012 to 2014 (Figures 3.4 - 3.9).

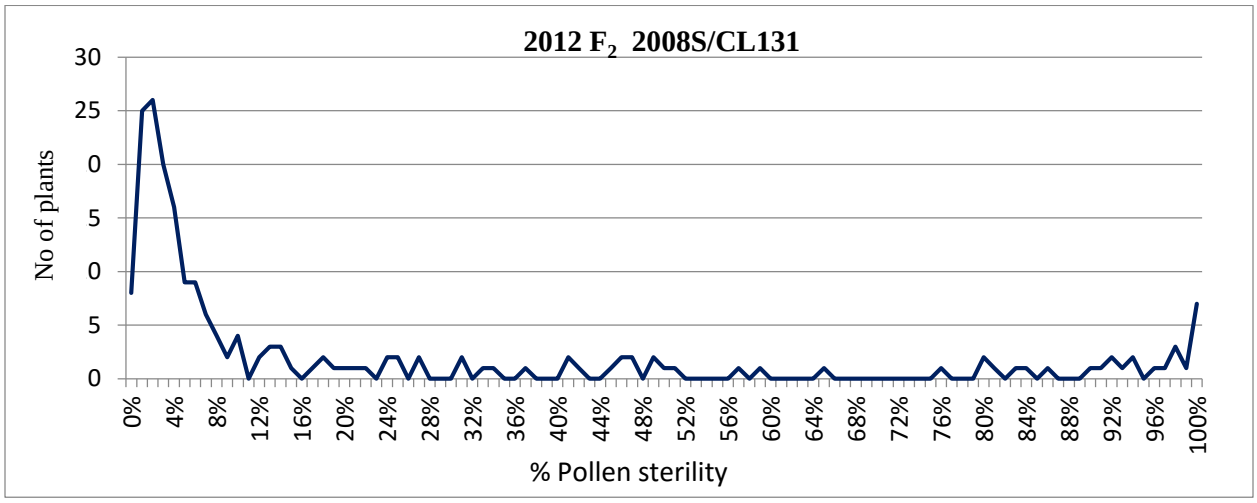


Figure 3.4 Frequency distribution of percent pollen sterility of 2008S/CL131 F_2 population planted in 2012.

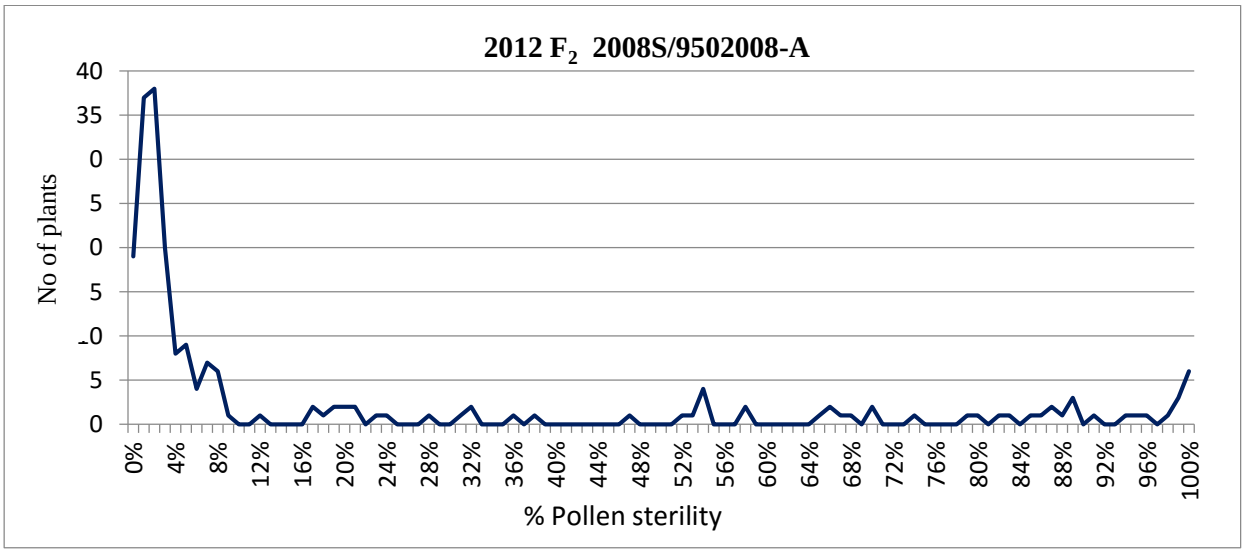


Figure 3.5 Frequency distribution of percent pollen sterility of 2008S/9502008-A F_2 population planted in 2012.

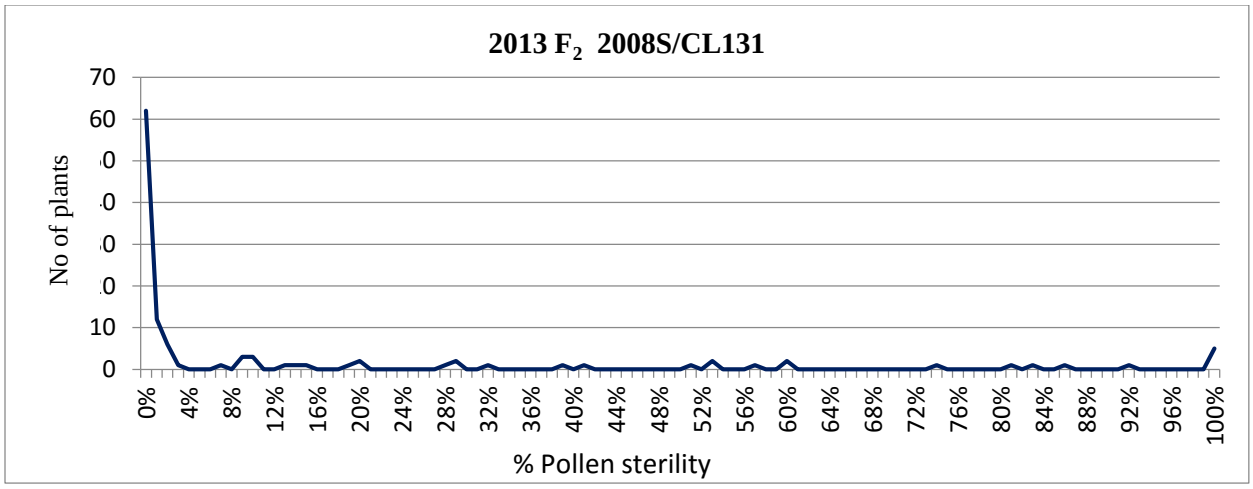


Figure 3.6 Frequency distribution of percent pollen sterility of 2008S/CL131 F_2 population planted in 2013.

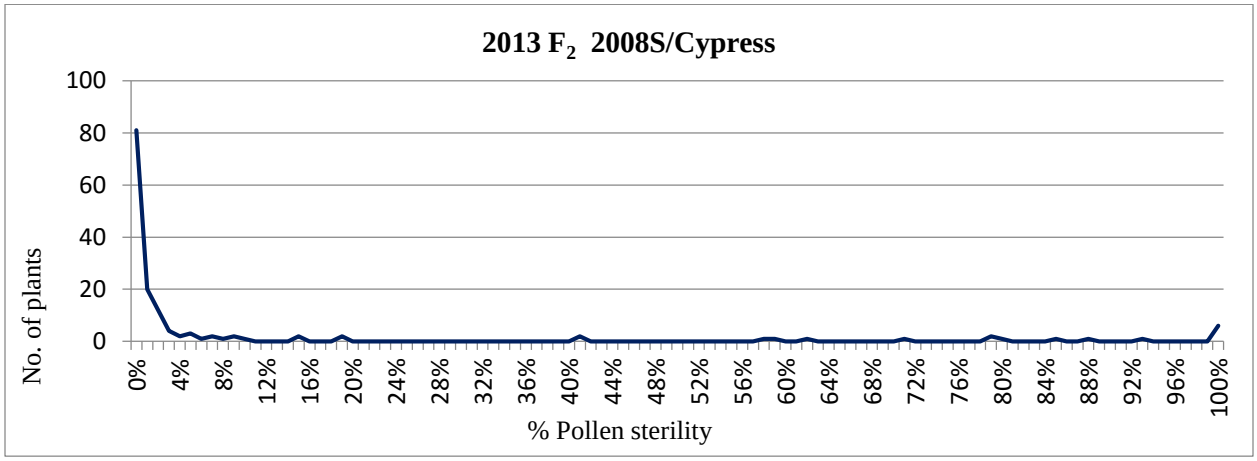


Figure 3.7 Frequency distribution of percent pollen sterility of 2008S/Cypress F_2 population planted in 2013.

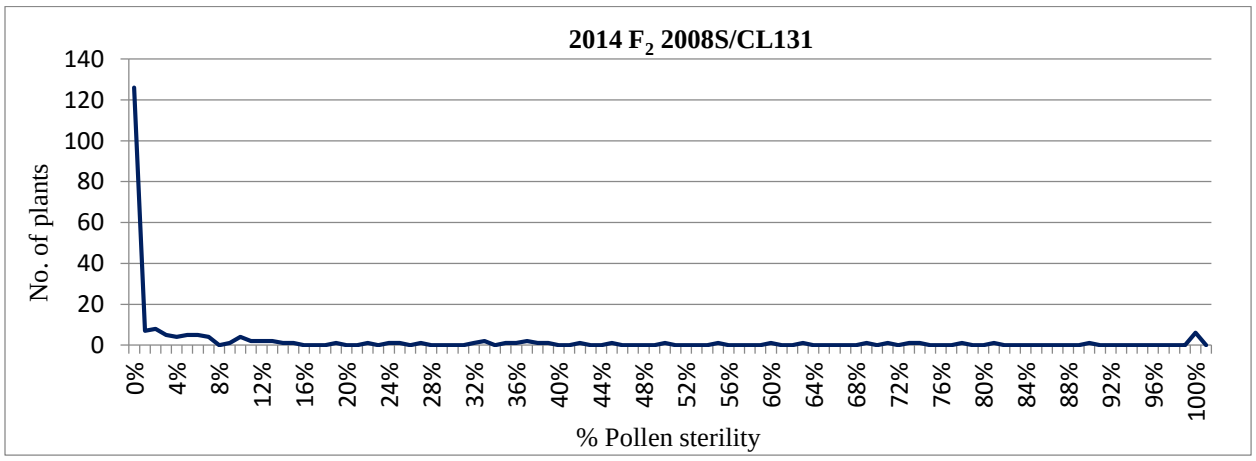


Figure 3.8 Frequency distribution of percent pollen sterility of 2008S/CL131 F_2 population planted in 2014.

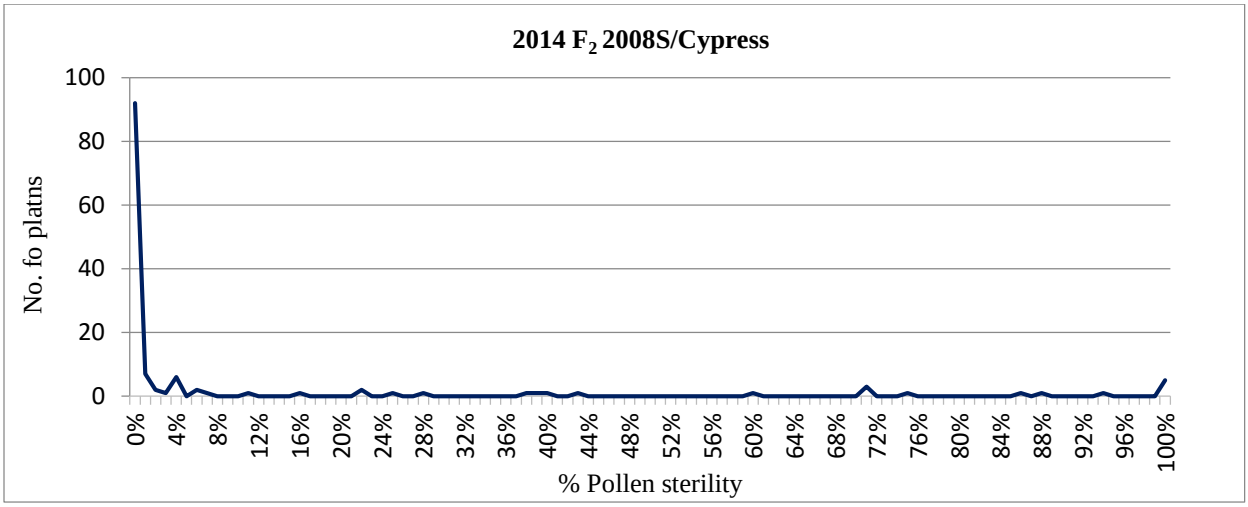


Figure 3.9 Frequency distribution of percent pollen sterility of 2008S/Cypress F_2 population planted in 2014.

BC_1F_2 populations of 2008S/Cypress-82//Cypress and 2008S/CL131-91//CL131 showed similar 15:1 ratio of pollen fertile: sterile demonstrating a two gene recessive segregation using chi square analysis (Table 3.2).

As shown in Figures 3.10 and 3.11, the frequency distribution of pollen sterility in the backcross populations was similar to the F_2 segregating material. Overall, the data indicate that the male sterile trait from the original parent 2008S was present in both backcross populations.

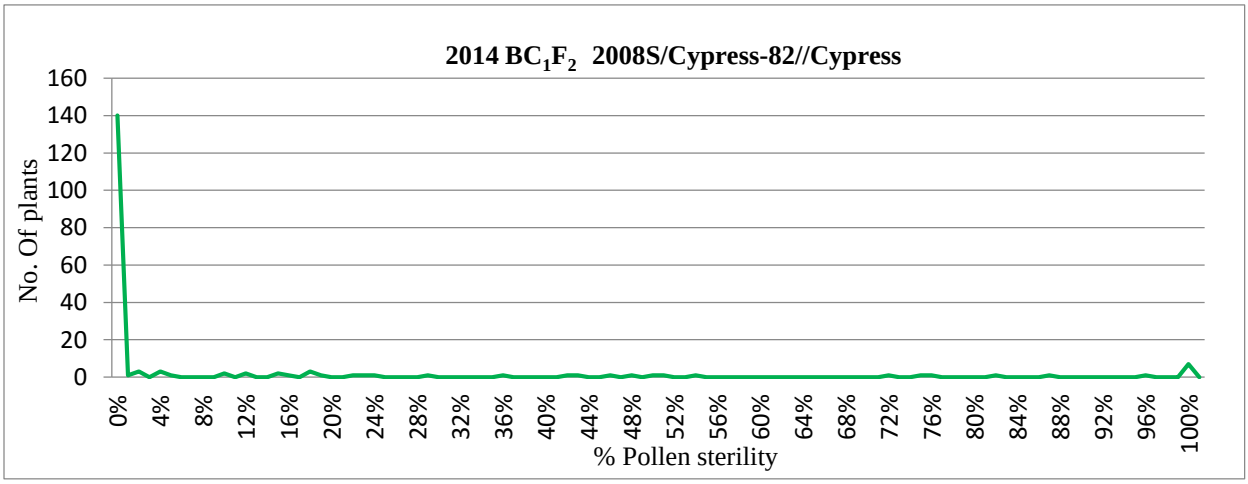


Figure 3.10 Frequency distribution of percent pollen sterility of 2008S/Cypress-82//Cypress BC_1F_2 population planted in 2014.

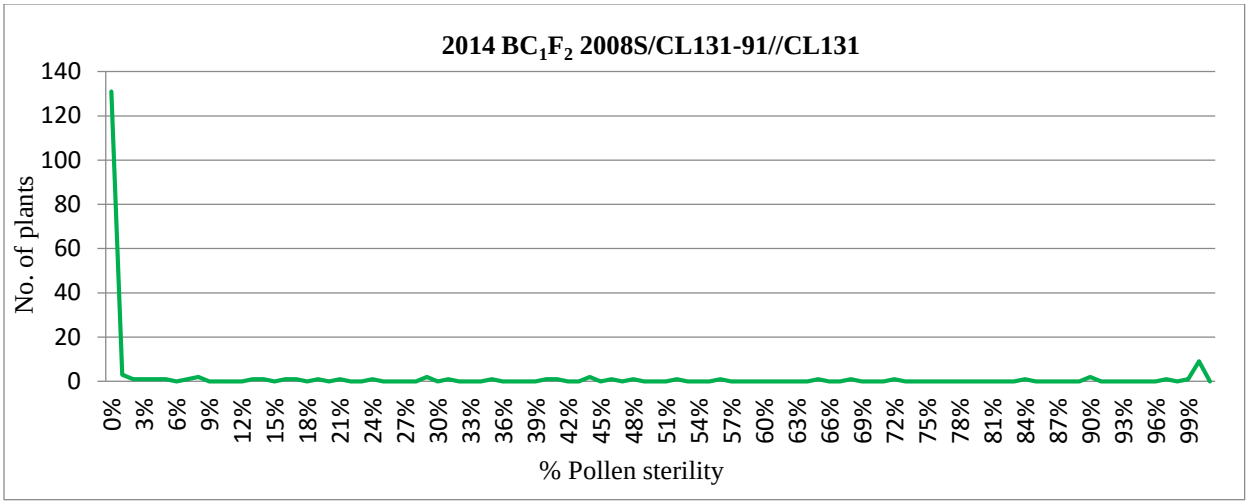


Figure 3.11 Frequency distribution of percent pollen sterility of 2008S/CL131-91//Cypress BC_1F_2 population planted in 2014.

Data from Arkansas for seed sterility (unfilled grains) of 2008S/CL131 and 2008S/Cypress F_2 segregating populations in a two-year study (2013 to 2014) showed positive skewness with high frequency of plants with low seed sterility (Figures 3.12 to 3.15).

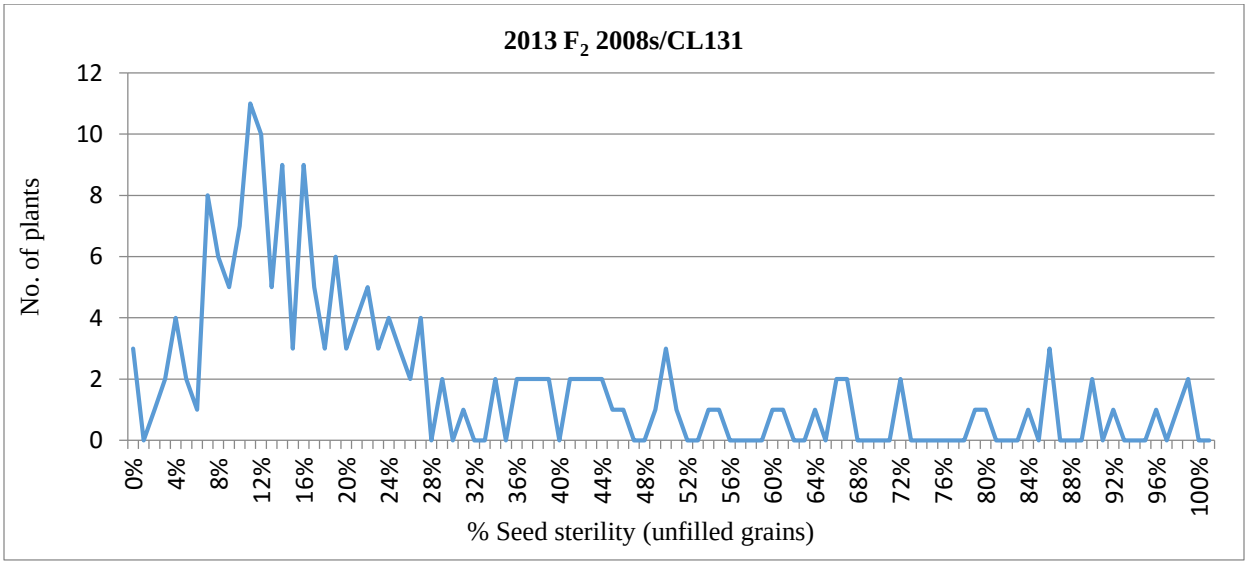


Figure 3.12 Frequency distribution of percent seed sterility (unfilled grains) of 2008S/CL131 F_2 population planted in 2013.

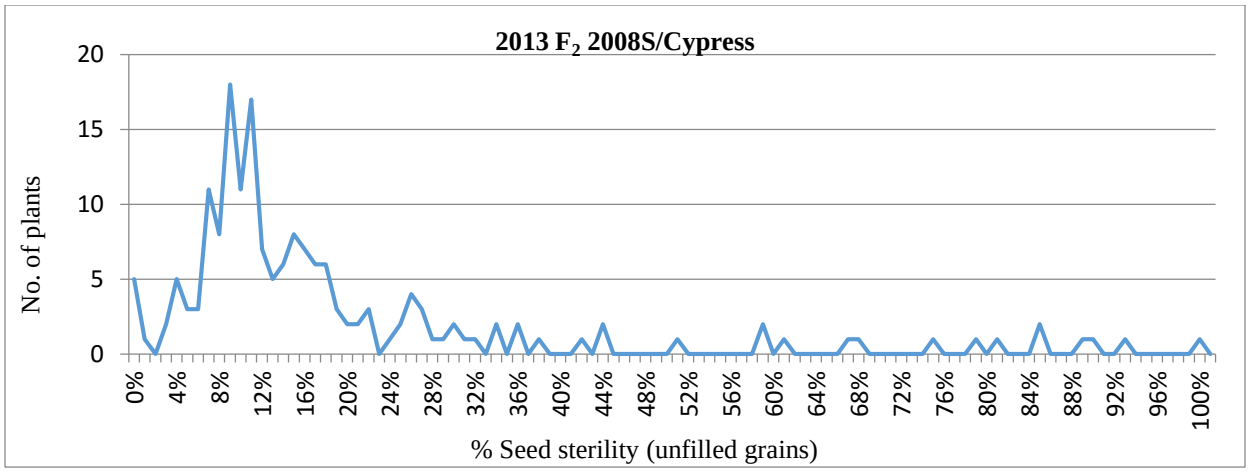


Figure 3.13 Frequency distribution of percent seed sterility (unfilled grains) of 2008S/Cypress F_2 population planted in 2013.

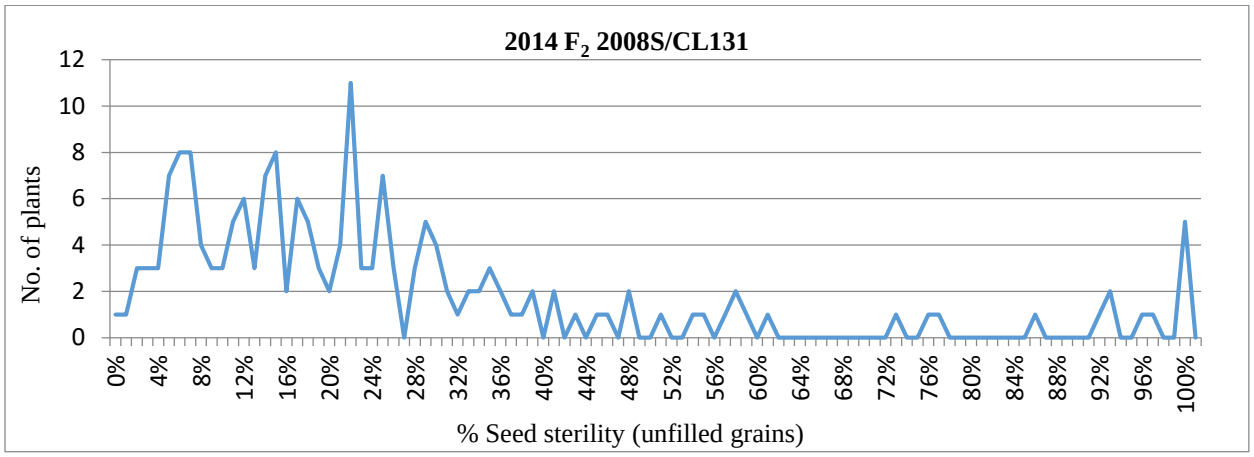


Figure 3.14 Frequency distribution of percent seed sterility (unfilled grains) of 2008S/CL131 F_2 population planted in 2014.

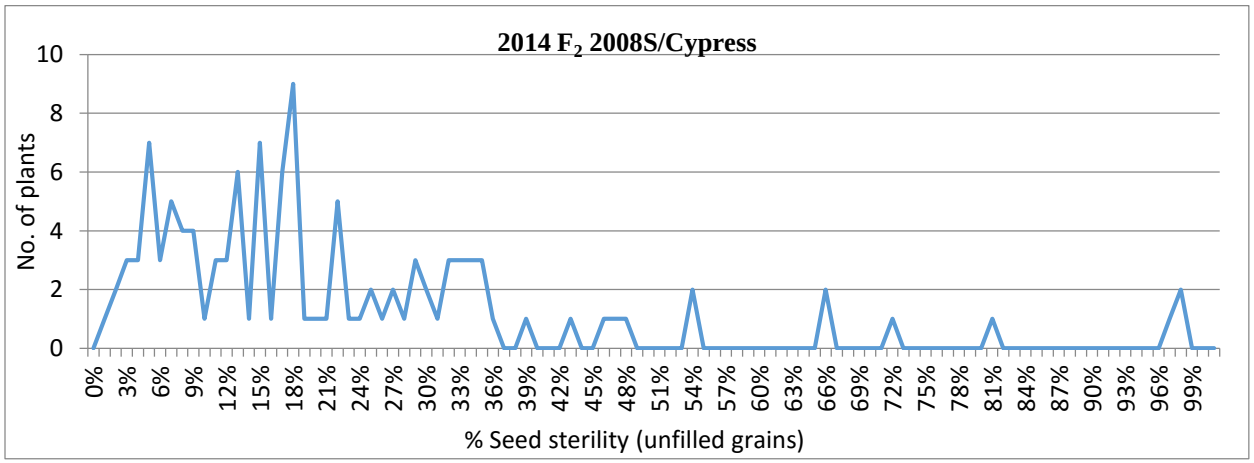


Figure 3.15 Frequency distribution of percent seed sterility (unfilled grains) of 2008S/Cypress F_2 population planted in 2014.

Plants were classified as seed sterile when 80 to 100% of spikelets in a sample panicle produced unfilled grains while plants were classified as fertile when spikelets were more than 20% filled grains as described by (Zhang et al. 1994). The seed sterility segregation ratio fit 15 fertile: 1 sterile using chi square goodness of fit test (Table 3.3). Partially fertile plants were also observed that may be the result of higher field outcrossing for pollen sterile plants due to exposed panicles during heading and other conditions affecting seed setting rates for pollen fertile plants.

Table 3.3 Chi square goodness of fit tests for the ratio 15 fertile: 1 sterile in 2008S/CL131 and 2008S/Cypress F_2 population planted in 2013 and 2014 at Arkansas Rice Research and Extension Center, Stuttgart AR.

Year	Generation	Population	Total plants	Fertile plants	Sterile plants	Pr>Chisq 15:1 ratio	Seed ratio fertile:sterile
2013	F_2	2008S / Cypress	178	171	7	0.20 ns	15:1
2013	F_2	2008S / CL131	181	169	12	0.83 ns	15:1
2014	F_2	2008S / Cypress	119	115	4	0.19 ns	15:1
2014	F_2	2008S / CL131	180	169	11	0.94 ns	15:1

ns = not significant

Two F_2 populations (2009S/10HHB020 and 2009S/07-1643) planted in 2013 showed pollen sterility segregating 3 fertile: 1 sterile using chi-square goodness of fit test. Four BC_1F_2 population also displayed typical single gene recessive locus models. Segregation revealed that male sterility in 2009S was controlled by a single recessive gene (Table 3.4).

Table 3.4 Chi-square test for single recessive gene inheritance in 2009S x 10HB020 and 2009S x 11-12134 F_2 population planted from 2013 to 2014 at H. Rouse Caffey Rice Research Station, Crowley LA.

Year	Generation	Population	Total plants	Plants with fertile pollen	Plants with sterile pollen	Pr> Chi square (3:1)	Ratio fertile: sterile
2013	F_2	2009S/10HB020	301	228	73	0.76 ns	3:1
2013	F_2	2009S/07-1643	308	245	63	0.06 ns	3:1
2014	BC_1F_2	2009S/10HHB020-114//10HHB020	131	102	29	0.45 ns	3:1
2014	BC_1F_2	2009S/10HHB020-283//10HHB020	231	178	53	0.47 ns	3:1
2014	BC_1F_2	2009S/07-1643-205//07-1643	208	162	46	0.34 ns	3:1
2014	BC_1F_2	2009S/07-1643-225//07-1643	225	174	51	0.42 ns	3:1

ns = Not significant at p-value >0.05

Frequency distribution of pollen sterility in F_2 and BC_1F_2 populations showed high frequency of male fertile and low frequency for male sterile lines (100%) with very few partially fertile/sterile individuals (Figures 3.16 to 3.21).

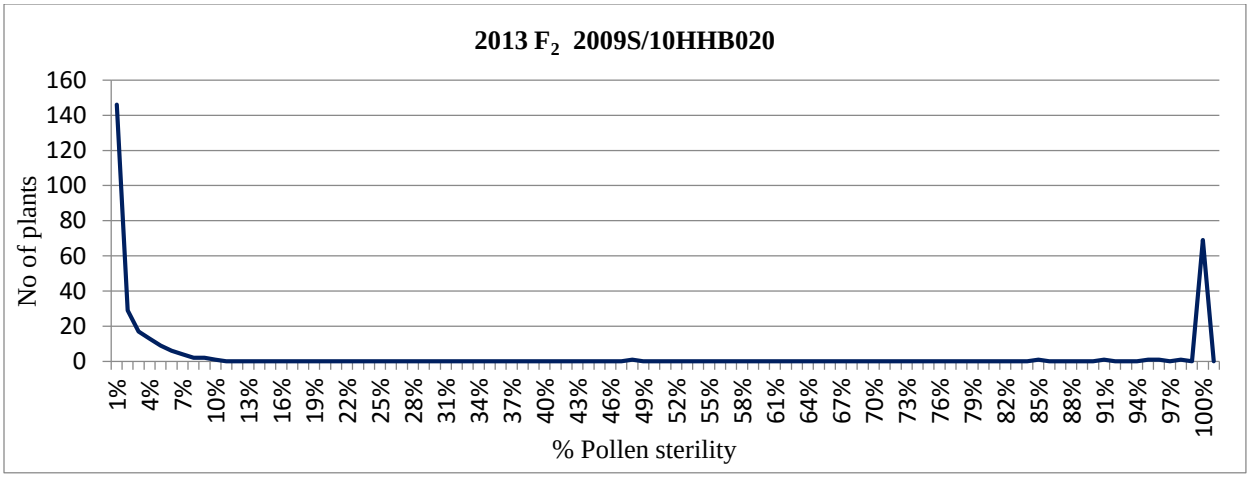


Figure 3.16 Frequency distribution of percent pollen sterility of 2009S/10HHB020 F_2 population planted in 2013.

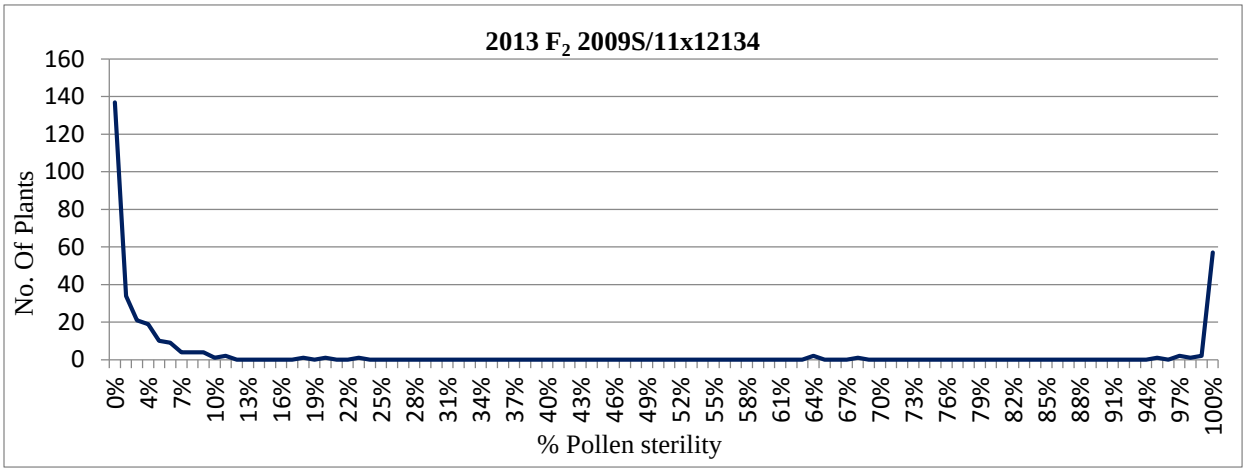


Figure 3.17 Frequency distribution of percent pollen sterility of 2009S/11x12134 F_2 population planted in 2013.

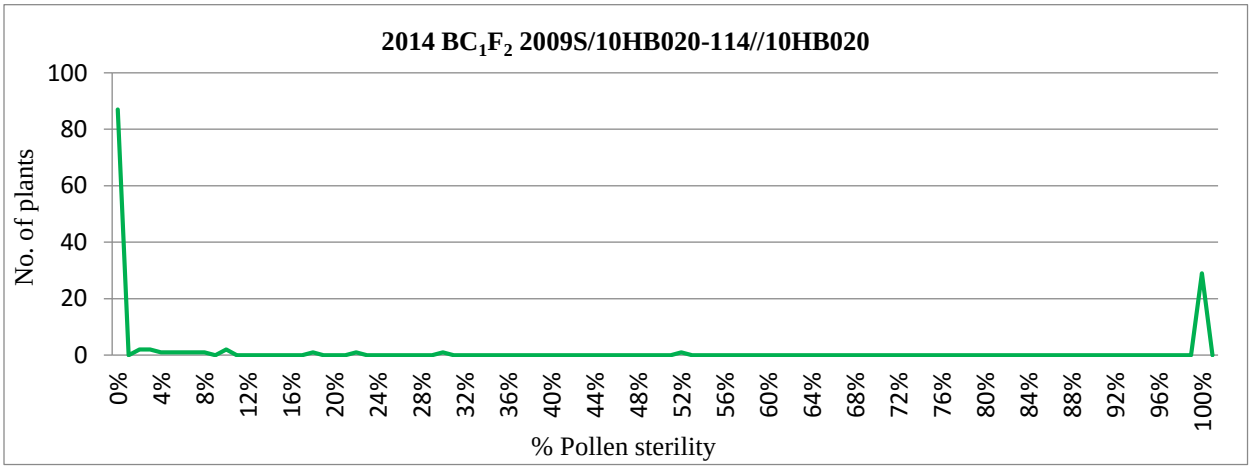


Figure 3.18 Frequency distribution of percent pollen sterility of 2009S/10HB020-114//10HB020 BC_1F_2 population planted in 2014.

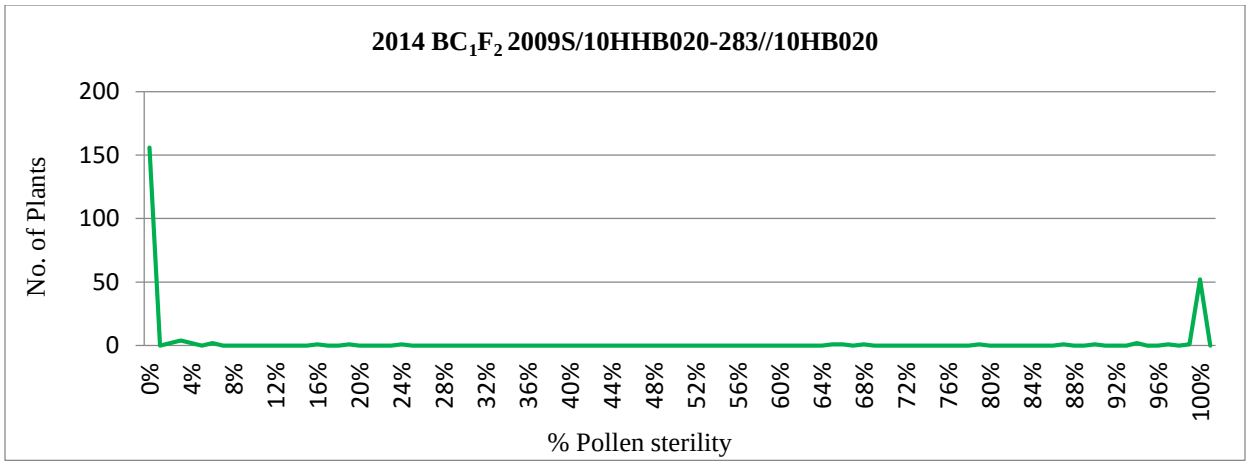


Figure 3.19 Frequency distribution of percent pollen sterility of 2009S/10HHB020-283//10HHB020 BC_1F_2 population planted in 2014.

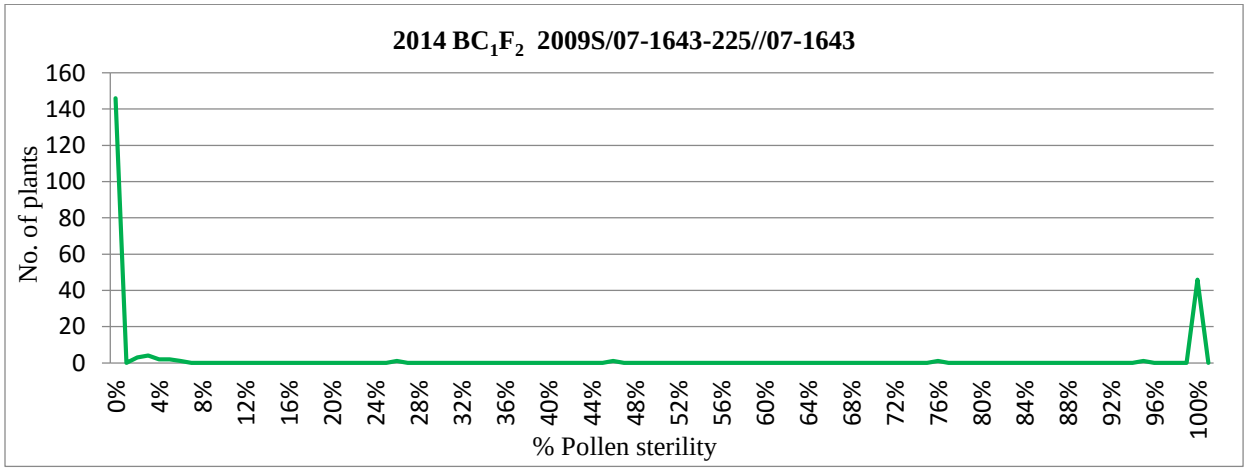


Figure 3.20 Frequency distribution of percent pollen sterility of 2009S/07-1643-225//07-1643 BC_1F_2 population planted in 2014.

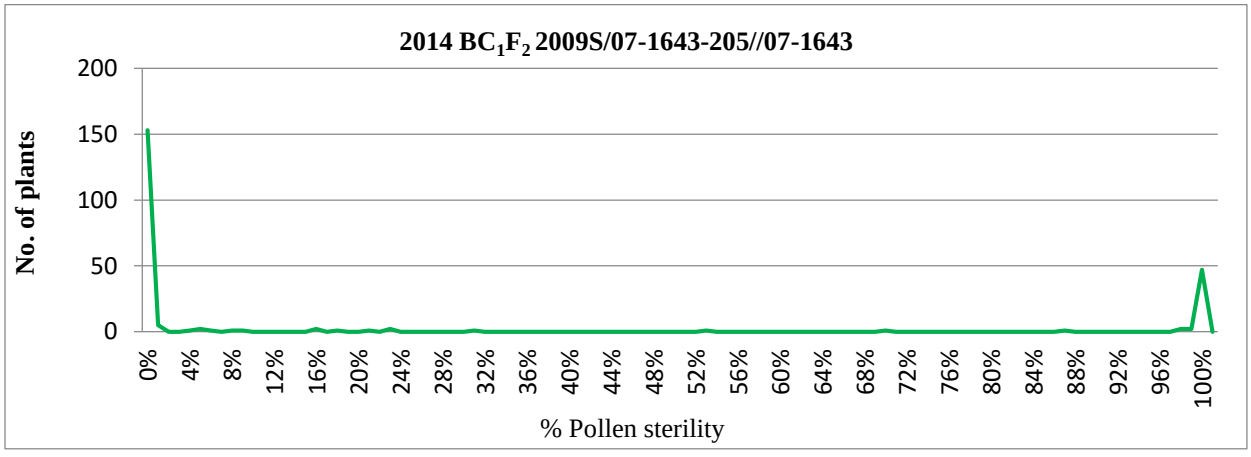


Figure 3.21 Frequency distribution of percent pollen sterility of 2009S/07-1643-205//07-1643 BC_1F_2 population planted in 2014.

The presence of sterile plants with no pollen were observed in both 2009S/10HB020 and 2009S/07-1643 F_2 populations. From the 63 sterile plants in 2009S/10HB020 F_2 population, 33 were classified as no-pollen type and 36 exhibit typical sterile pollen grains while 2009S/07-1643 produced 29 no-pollen types and 28 typical male sterile pollen plants. Segregation of the pollen and no-pollen type were examined in each of the BC_1F_2 populations. Results showed that the pollen type BC_1F_2 population 2009S/07-1643-205//07-1643 had 17 pollen and 29 no-pollen type male sterile plants, while 2009S/10HHB020-114//10HHB020 had two pollen and 27 no-pollen types male sterile plants. Examination of no-pollen type BC_1F_2 population 2009S/10HHB020-283//10HHB020 revealed 29 pollen type and 24 no-pollen type male sterile lines, while 2009S/07-1643-225//07-1643 revealed 46 pollen types and five no-pollen type male sterile lines (Table 3.5).

Table 3.5 Number of pollen and no-pollen type sterile plants from BC_1F_2 populations derived from pollen and no-pollen type sterile F_2 plants.

Type of pollen male sterility in F_2	BC_1F_2 Population	Plants with sterile pollen	Sterile pollen classification	
			Pollen type	No-Pollen type
Pollen type	2009S/10HHB020-114//10HHB020	29	2	27
No-pollen type	2009S/10HHB020-283//10HHB020	53	29	24
Pollen type	2009S/07-1643-205//07-1643	46	17	29
No-pollen type	2009S/07-1643-225//07-1643	51	46	5

Overall, the observed segregation of pollen and no-pollen types male sterility was not consistent with known genetic ratios such as 9:3:4 and 12:3:1. for simple Mendelian trait. Previous reports showed that there were male sterile lines classified as no-pollen type as reported by Peng et al. (2010). Tanee et al. (2014) showed that male sterile lines changed to no-pollen type when temperature reached 30°C whereas typical sterile pollen grains were observed at 25°C. Similar findings were described by Zhou et al. (2014) where no-pollen type was observed when lines were grown at 28°C to 30°C and showed pollen type male sterility when subjected at 26°C depending on the male sterile background.

3.3.2 Single Marker Analysis and Genotype x Year (environment) Interactions

Single marker analysis using ANOVA for SNP markers LOC_12g36030 and LOC_07g12130 showed highly significant differences (p-value <0.01) in 2008S/9502008-A, 2008S/CL13, and 2008S/Cypress F_2 populations planted from 2012 to 2013 (Tables 3.6 to 3.9). Single marker analysis for all F_2 segregating populations showed the SNP marker in LOC_Os12g36030 produced higher variance than LOC_07g12130 with an R^2 value ranging from 11% to 38%.

Table 3.6 Single Marker Analysis using ANOVA PROC GLM for LOC_Os07g12130 and LOC_Os12g36030 in 2008S/9502008-A F_2 population planted in 2012.

Source	DF	Type III SS	Mean Square	F Value	Pr > F	R-square
LOC_Os07g12130	2	1.7848	0.8924	9.02	<0.01**	0.08
LOC_Os12g36030	2	2.5839	1.2919	13.56	<0.01**	0.11

** highly significant at p-value ≤0.01

Table 3.7 Single Marker Analysis using ANOVA PROC GLM for LOC_Os07g12130 and LOC_Os12g36030 in 2008S/CL131 F_2 population planted in 2012.

Source	DF	Type III SS	Mean Square	F Value	Pr > F	R-square
LOC_Os07g12130	2	1.1378	0.5689	6.08	<0.01**	0.06
LOC_Os12g36030	2	2.1648	1.0824	12.21	<0.01**	0.11

** highly significant at p value ≤0.01

Table 3.8 Single Marker Analysis using ANOVA PROC GLM for LOC_Os07g12130 and LOC_Os12g36030 in 2008S/Cypress F_2 population planted in 2013.

Source	DF	Type III SS	Mean Square	F Value	Pr > F	R-square
LOC_Os07g12130	2	1.2974	0.6487	10.50	<0.01**	0.12
LOC_Os12g36030	2	3.6352	1.8176	39.64	<0.01**	0.35

** highly significant at p value ≤0.01

Table 3.9 Single Marker Analysis using ANOVA PROC GLM for LOC_Os07g12130 and LOC_Os12g36030 in 2008S/CL131 F_2 population planted in 2013.

Source	DF	Type III SS	Mean Square	F Value	Pr > F	R-square
LOC_Os07g12130	2	1.6574	0.8287	13.04	<0.01**	0.19
LOC_Os12g36030	2	3.3662	1.6831	34.84	<0.01**	0.38

** highly significant at p value ≤0.01

Test of interactions for both markers using two-way analysis of variance (ANOVA) showed that 2008S/9502008-A planted in 2012 was not significant with a p-value of 0.1205 (Table 3.10). Similarly, 2008S/CL131 F_2 planted in the same year (2012) showed no significant differences in genotype interactions exhibiting a p-value of 0.0618 (Table 3.11). The variance explained by the model (R^2) was 21% for 2008S/9502008-A and 18% for 2008S/CL131.

Table 3.10 Two Way ANOVA using PROC GLM for LOC_Os07g12130 and LOC_Os12g36030 in 2008S/9502008-A F_2 population planted in 2012.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOC_Os07g12130	2	1.5066	0.7533	8.71	<0.01**
LOC_Os12g36030	2	1.8650	0.9325	10.78	<0.01**
Interaction	4	0.6403	0.1600	1.85	0.1205ns

R-square = 0.21
** highly significant at p value ≤0.01
ns = not significant

Table 3.11 Two Way ANOVA using PROC GLM for LOC_Os07g12130 and LOC_Os12g36030 in 2008S/CL131 F_2 population planted in 2012.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOC_Os07g12130	2	0.5811	0.2905	3.49	0.03*
LOC_Os12g36030	2	2.2454	1.1227	13.49	<0.01**
Interaction	4	0.7600	0.1900	2.28	0.0618ns

R-square = 0.18
** highly significant at p value ≤0.01
ns = not significant

However, when the F_2 population 2008S/CL131 and 2008S/Cypress were planted in 2013 and genotyped using both SNP markers, two-way ANOVA showed that interaction effects were highly significant with p-value less than 0.01 (Tables 3.12 and 3.13). The variances explained (R^2) by the model were and 65% in 2008S/Cypress and 54% for 2008S/CL131 F_2 populations which were higher compared to 2008S/9502008-A (21%) and 2008S/CL131 (18%) F_2 populations planted in 2012.

Table 3.12 Two Way ANOVA using PROC GLM for LOC_Os07g12130 and LOC_Os12g36030 in 2008S/Cypress F_2 population planted in 2013.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOC_Os07g12130	2	1.4409	0.7204	28.46	<0.01**
LOC_Os12g36030	2	3.4405	1.7202	67.96	<0.01**
Interaction	4	2.1094	0.5273	20.83	<0.01**

R-square = 0.65
** highly significant at p value ≤0.01

Table 3.13 Two Way ANOVA using PROC GLM for LOC_Os07g12130 and LOC_Os12g36030 in 2008S/CL131 F_2 population planted in 2013.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOC_Os07g12130	2	0.8633	0.4316	11.40	<0.01**
LOC_Os12g36030	2	1.5223	0.7611	20.09	<0.01**
Interaction	4	0.5319	0.1329	3.51	0.01**

R-square = 0.54
** highly significant at p value ≤0.01

Effects of genotype x year (environment) interactions were tested using SAS PROC GLM in 2008S/CL131 F_2 population planted in 2012 and 2013 (Table 3.14). The "Year" source of variation and all interactions of "Year" with genotype LOC_Os12g36030 and LOC_Os07g12130 were not significant suggesting that years planted (2012 and 2013) did not influence expression of pollen male sterility with stable expression across both years.

Table 3.14 Test of environment effect "Year" by marker interaction in 2008SxCL131 F_2 for 2012 to 2013 using markers LOC_Os07g12130 and LOC_Os12g36030.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
LOC_Os07g12130	2	1.4096	0.7048	10.44	<0.01**
LOC_Os12g36030	2	3.3590	1.6795	24.87	<0.01**
Year	1	0.1340	0.1340	1.99	0.16ns
LOC_Os07g12130*Year	2	0.1784	0.0892	1.32	0.27ns
LOC_Os12g36030*Year	2	0.0943	0.0471	0.70	0.50ns
LOC_Os07g12130* LOC_Os12g36030	4	1.1655	0.2913	4.32	<0.01**
LOC_Os07g12130* LOC_Os12g36030* Year	4	0.1261	0.0315	0.47	0.76ns

R-square = 0.30
** highly significant at p value ≤0.01
ns = not significant

To validate the utility of SNP markers that were developed in this study, all pollen male sterile lines in 2008S/CL131 BC_1F_2 were genotyped using the SNP marker in LOC_Os12g36030 and LOC_Os07g12130. Seven out of 10 plants (70%) in the BC_1F_2 population produced sterile genotypes at both loci while 19 out of 20 (95%) showed genotypes of either heterozygote or homozygote fertile at one or both loci (Table 3.15).

Table 3.15 Genotypes of pollen sterile plants and randomly sampled pollen fertile plants from 2008S/CL131 BC_1F_2 populations planted in 2014 using both LOC_Os12g36030 and LOC_Os07g12130 markers.

2008S/CL131 BC_1F_2 population	Number of plants	LOC_Os12g36030 and LOC_Os07g12130 Genotype	% plants with correct genotype for both markers
Pollen Sterile	10	7[a]	70
Pollen Fertile	20	19[b]	95

Genotype: [a] Homozygous sterile; [b] Homozygous or heterozygous fertile in one or both markers

3.3.3 Genotypic Analysis of 2009S Population

To test the SNP in LOC_Os02g12290 for association with pollen male sterility, all pollen male sterile lines from 2009s/10HHB020 and 2009S/07-1643 F_2 populations were genotyped using the CEL 1 nuclease assay. For the 2009s/10HHB020 population, a majority (71/73) of the male sterile lines carried the male sterile allele in LOC_Os02g12290. A random sample of highly fertile lines (100% fertile) from the same population showed 61 out of 63 (97%) carried either the homozygous fertile or heterozygote genotype. Similarly, the 2009S/07-1643 F_2 population produced 20 out of 20 (100%) pollen sterile plant that possessed the locus for male sterility while 20 out of 21 (95.2%) randomly-selected pollen fertile lines showed genotypes of either heterozygote or homozygote fertile for LOC_Os02g12290 (Table 3.16).

Table 3.16. LOC_Os02g12290 marker genotypes and percent (%) plants with correct genotypes from randomly-sampled male pollen-sterile and male pollen-fertile plants from 2009S/10HB020 F_2 and 2009S/11x12134 F_2 populations planted in 2013.

F_2 Population	Phenotype	Number of plants	Genotype	% plants with correct marker genotype
2009S/10HHB020	Sterile	73	71[a]	97
2009S/10HHB020	Fertile	63	61[b]	97
2009S/07-1643	Sterile	20	20[a]	100
2009S/07-1643	Fertile	21	20[b]	95

Genotype: [a] Homozygous sterile; [b] Homozygous or heterozygous fertile

To validate the SNP markers in the backcross population, 2009S/07-1643 BC_1F_2 pollen male sterile lines were genotyped. A majority (35/39; 89.7%) exhibited the genotype of sterile LOC_Os02g12290. Randomly-sampled pollen male fertile lines showed 20 out of 20 (100%) plants produced the genotype of either homozygote fertile or heterozygote at LOC_Os02g12290 locus (Table 3.17).

Table 3.17 LOC_Os02g12290 marker genotypes and percent (%) plants with correct genotype from randomly-sampled male pollen-sterile and male pollen-fertile plants from 2009S/07-1643-225//07-1643 BC_1F2 populations planted in 2013.

BC_1F_2 population	Phenotype	Number of plants	SNP Genotype[1]	% plants with correct marker genotype
2009S/07-1643-225//07-1643	Sterile	39	35[a]	90
2009S/07-1643-225//07-1643	Fertile	20	20[b]	100

[1]Genotype: [a] Homozygous sterile; [b] Homozygous or heterozygous fertile

3.3.4 SNP Validation by Sequencing

A substitution of nucleotide C to A in the first exon of LOC_Os02g12290 was reported to be a causal variant responsible for male sterility in thermosensitive male sterile rice (Xu et al. 2011; Zhou et al. 2014). Sequencing of the first exon from position 6,397,342 bp to 6,397,560 bp in LOC_Os02g12290 showed two SNPs after alignment of 2009S with fertile Nipponbare and CL161 (Fig. 3.22) using Clustal X ver. 2 sequence alignment tool (Larkin et al. 2007). The exact position of the SNP was determined by running the basic local search alignment tool (BLAST) in the Gramene (www.gramene.org). Comparison of the sequence of Nipponbare reference genome, sequence of 2009S and published sequences of Guangzhang 63S (Xu et al. 2011) and Zhu1S (Zhou et al. 2014) showed that the two SNPs were located specifically at 6,397,411 bp and 6,397,412 bp position in the first exon of LOC_Os02g12290 where the latter position was the nucleotide substitution from C to A (www.gramene.org, rice.plantbiology.msu.edu). These findings showed that the SNP (C/A) found in male sterile line Guangzhang 63S (Xu et al. 2011) and Zhu1S (Zhou et al. 2014) was identical to the SNP found in 2009S at LOC_ Os02g12290.

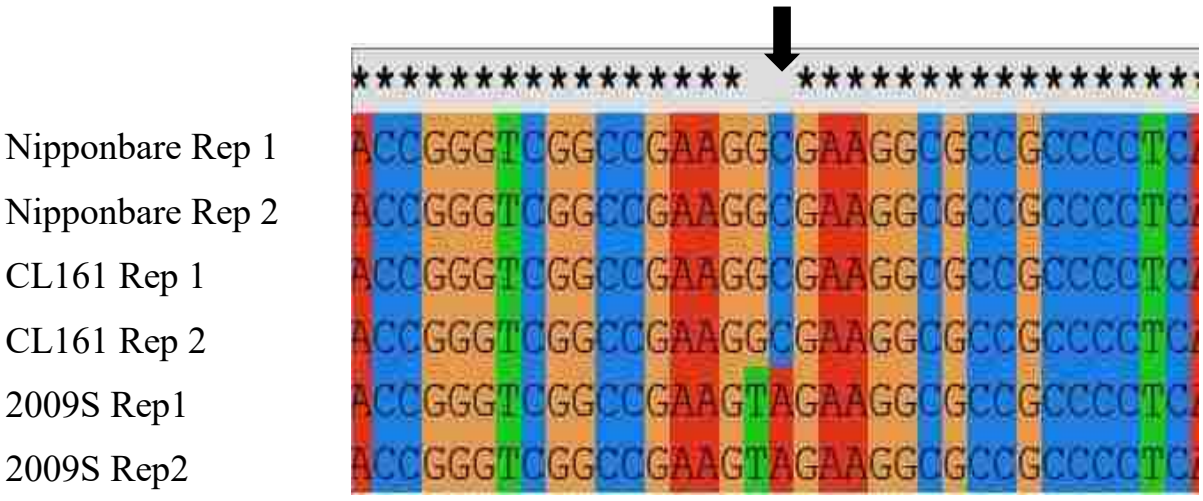


Figure 3.22 DNA Sequence alignment comparison using Clustal X between fertile CL161 and sterile 2009S at locus LOC_Os02g12290 showing a SNP (C to A) marked with black arrow.

A G to C nucleotide substitution at LOC_Os12g36030 was reported to be responsible for male sterility in Nongken 58S (Ding et al. 2012a). 2008S and CL161 Sanger sequencing at position 22,054,461 bp to 22,054,982 showed a nucleotide variant from G to C (Figure 3.23). The sequence was searched in BLAST (http://www.gramene.org/) and the Rice Genome Annotation Project website (http://rice.plantbiology.msu.edu/). Comparison of the sequence of Nipponbare reference genome, 2008S and the published sequence of Nongken 58S (Ding et al. 2012a) showed the SNP found in Nongken 58S was identical to SNP found in 2008S. The SNP was specifically located at 22,054,886 bp in chromosome 12.

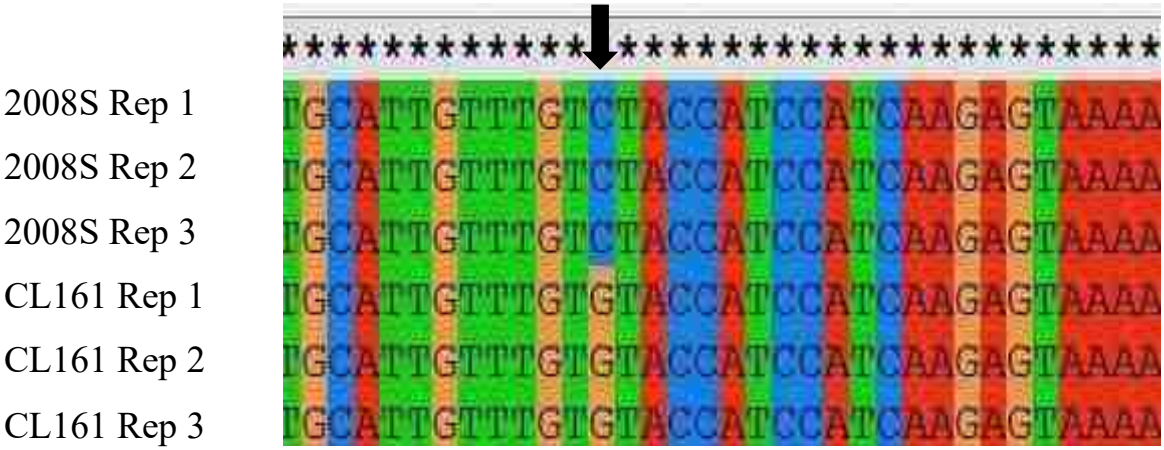


Figure 3.23 DNA Sequence alignment comparison using Clustal X of fertile CL161 and sterile 2008S at locus LOC_Os12g36030 showing a SNP (G to C) marked with black arrow.

LOC_Os07g12130 was reported to be associated with male sterility, and this gene was found to have 25 SNP variants between fertile Nipponbare and male sterile line Pei'ai 64S (Zhou et al. 2011). To verify the presence of SNP in LOC_Os07g12130 as reported by Zhou et al. (2011), the first exon was sequenced at position 6,781,839 bp to 6781320 bp of chromosome 7. A SNP from nucleotide C to G were found between 2008S and CL161 (Fig 3.24). Nipponbare reference genome, 2008S, CL161 and the published sequence of Pei'ai 64S at LOC_

Os07g12130 were compared. The results confirmed that the *indica* male sterile line 2008S carried identical SNP at the first exon identified by Zhou et al. (2011) from mapping *indica* male sterile line Pei'ai64S.

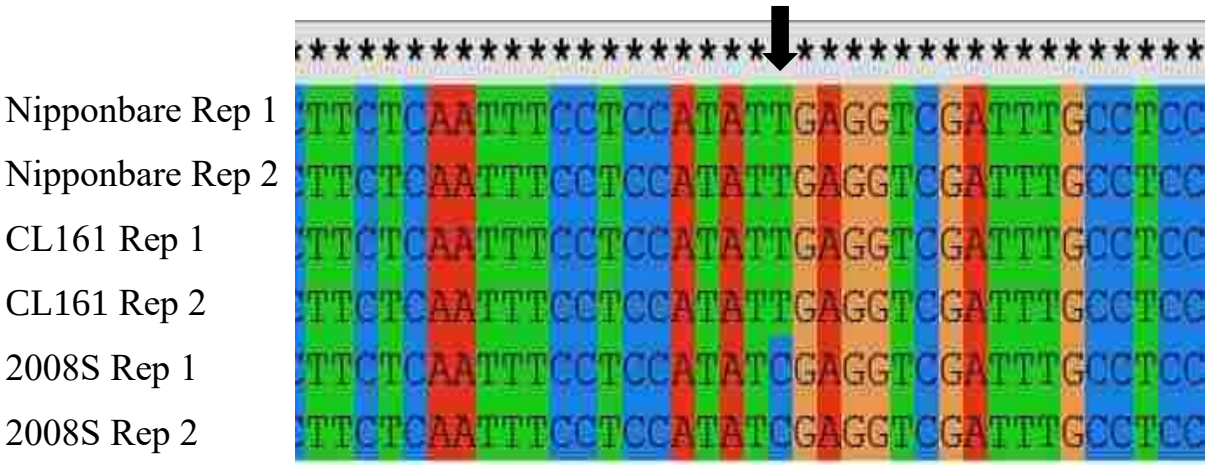


Figure 3.24 DNA Sequence alignment comparison using Clustal X of fertile Nipponbare CL161 and sterile 2008S at locus LOC_Os07g12130 showing a SNP (T to C) marked with black arrow.

3.3.5 QTL Mapping of Pollen Male Sterility in 2008S

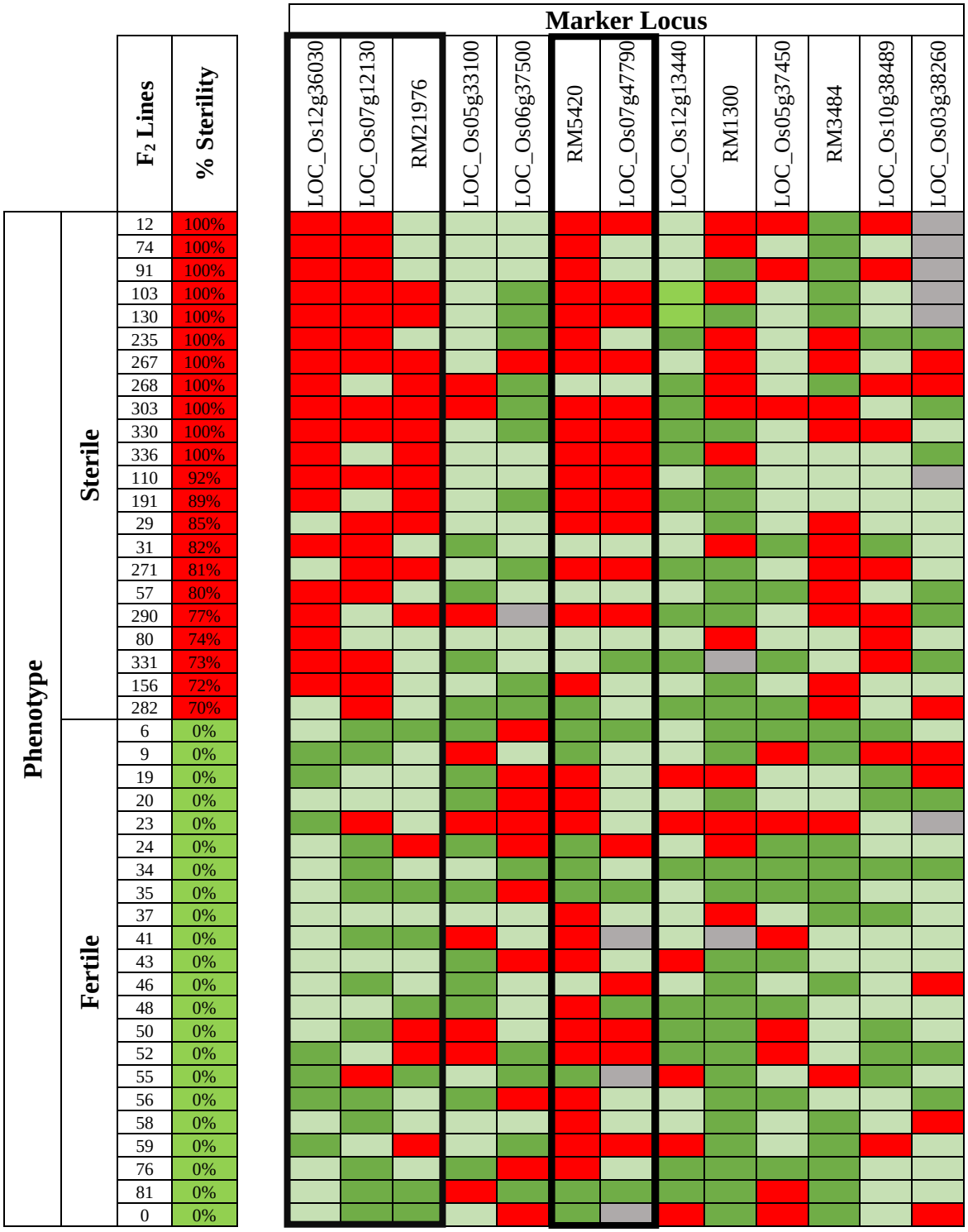
Redundant markers were removed and genotype data from 110 polymorphic markers were used for mapping QTLs on all 12 chromosomes (Appendix B). QTL mapping by single marker analysis was used to generate p-values of QTL based on raw ANOVA, Hochberg, step-down Bonferroni and false discovery rate in SAS (SAS codes in Appendix C). The p-values were ranked lowest to the highest. Based on raw p-values (ANOVA), thirteen markers showed significant differences with p-values less than 0.05 (Table 3.18). Two QTLs were detected at LOC_Os12g36030 and LOC_Os07g12130 that mapped within previously reported QTL regions (Zhou et al. 2011; Ding et al. 2012a). The variance explained by the top three QTLs ranked by p-value was 81% for LOC_Os12g36030, 53% for LOC_Os07g12130 and 27% for RM21976. Additional QTLs on chromosomes 3, 5, 6, 7, 10, and 12 produced R^2 values ranging from 5% to 21% (Table 3.18).

Table 3.18 ANOVA single marker analysis by selective genotyping twenty-two pollen sterile and twenty-two pollen fertile F_2 plants from 2008S/CL131 population.

Rank	Locus	F	Raw_P	Hochberg p-value	Stepdown Bonferroni p-value	False Discovery rate p-value	R^2 value
1	LOC_Os12g36030	84.7625	0	0	0.0000	0	0.81
2	LOC_Os07g12130	22.822	0	0.00002	0.0000	0.00001	0.53
3	RM21976	7.6182	0.00154	0.17062	0.1706	0.0579	0.27
4	LOC_Os05g33100	5.3407	0.00868	0.9552	0.9552	0.21627	0.21
5	LOC_Os06g37500	5.234	0.00957	0.9995	1.0000	0.21627	0.21
6	RM5420	4.6623	0.01498	0.9995	1.0000	0.26064	0.19
7	LOC_Os07g47790	4.1382	0.02307	0.9995	1.0000	0.26064	0.19
8	LOC_Os12g13440	4.2723	0.02064	0.9995	1.0000	0.26064	0.17
9	RM1300	5.7278	0.02148	0.9995	1.0000	0.26064	0.13
10	LOC_Os05g37450	3.5188	0.03887	0.9995	1.0000	0.29285	0.15
11	RM3484	3.8432	0.02953	0.9995	1.0000	0.29285	0.16
12	LOC_Os10g38489	3.6008	0.03625	0.9995	1.0000	0.29285	0.15
13	LOC_Os03g38260	2.9385	0.04471	0.9995	1.0000	0.31579	0.05

LOC_Os05g33100 and LOC_Os06g37500 showed highly significant differences with p-values less than 0.01. This finding is unexpected because these markers are heterozygous or have fertile genotypes that were consistently showing on male sterile lines. Hence, additional studies are needed to explain this observation. LOC_Os12g13440, RM1300, LOC_Os05g37450, RM3484, LOC_Os10g38489 and LOC_Os03g38260 are minor effect QTL that can be studied further because of the low frequency of sterile genotype for sterile plants and high occurrence of heterozygous genotype (Table 3.19). Markers RM5420 and LOC_Os07g47790 both with R^2 value of 19% showed high occurrence of sterile genotypes (16 and 12, respectively, out of 22) for male sterile lines and therefore may be a useful marker for marker assisted selection (Table 3.19).

Table 3.19 Genotype data from 13 significant (Raw_p-values) QTLs by single marker analysis using SAS ver. 9.4. Black box highlights markers and genotypes that have the most number of sterile genotype for sterile F_2 lines. Homozygous sterile genotype (red), homozygous fertile genotype (green), heterozygous (light green), missing data (gray).



The ANOVA single-marker analysis in SAS described above was based on p-values. An alternative for single markers based on LOD scores was carried out using Inclusive Composite Interval Mapping software ver. 4.0.6.0 (https://www.integratedbreeding.net/). The top QTL identified with LOD score of 15.62 was LOC_Os12g36030 that showed 78% variance explained (PVE). The QTL at LOC_Os07g12130 produced a LOD score of 7.13 and PVE at 51.5% while SSR marker RM21976 showed a LOD score of 2.2 and PVE of 20.2% (Table 3.20). Additive and dominance effects were also estimated where the additive effects were 0.45, 0.39 and 0.35 and dominance effects were -0.32, 0.02, 0.05 for LOC_Os12g36030 LOC_Os07g12130 and RM21976, respectively.

Table 3.20. QTLs detected in 2008S/CL131 F_2 population using Single Marker Analysis option (LOD scores) with Inclusive Composite Interval Mapping (ICIM) ver. 4.0.6.0.

Chr	Position (cM)	Marker Locus	LOD	PVE(%)	Add	Dom
12	90.95	LOC_Os12g36030	15.6192	78.6958	0.4530	-0.3234
7	23.31	LOC_Os07g12130	7.1276	51.4822	0.3945	0.0165
7	95.61	RM21976	3.0074	26.4779	0.3475	0.0536
5	104.66	LOC_Os05g33100	2.1907	20.1978	0.0445	0.3962

Inclusive Composite Interval Mapping was also used to identify marker intervals for significant association with pollen male sterility. A QTL with relatively large effect was detected at the interval between LOC_Os12g36030 and RM1300 showing a LOD score of 16.3 and percent variance explained of 38.9%. A second QTL was detected between marker RM3484 and LOC_Os07g12130 with LOD score of 10 and 19 % percent variance explained (PVE). Minor effect QTLs were also detected in marker intervals between LOC_Os12g13440 and

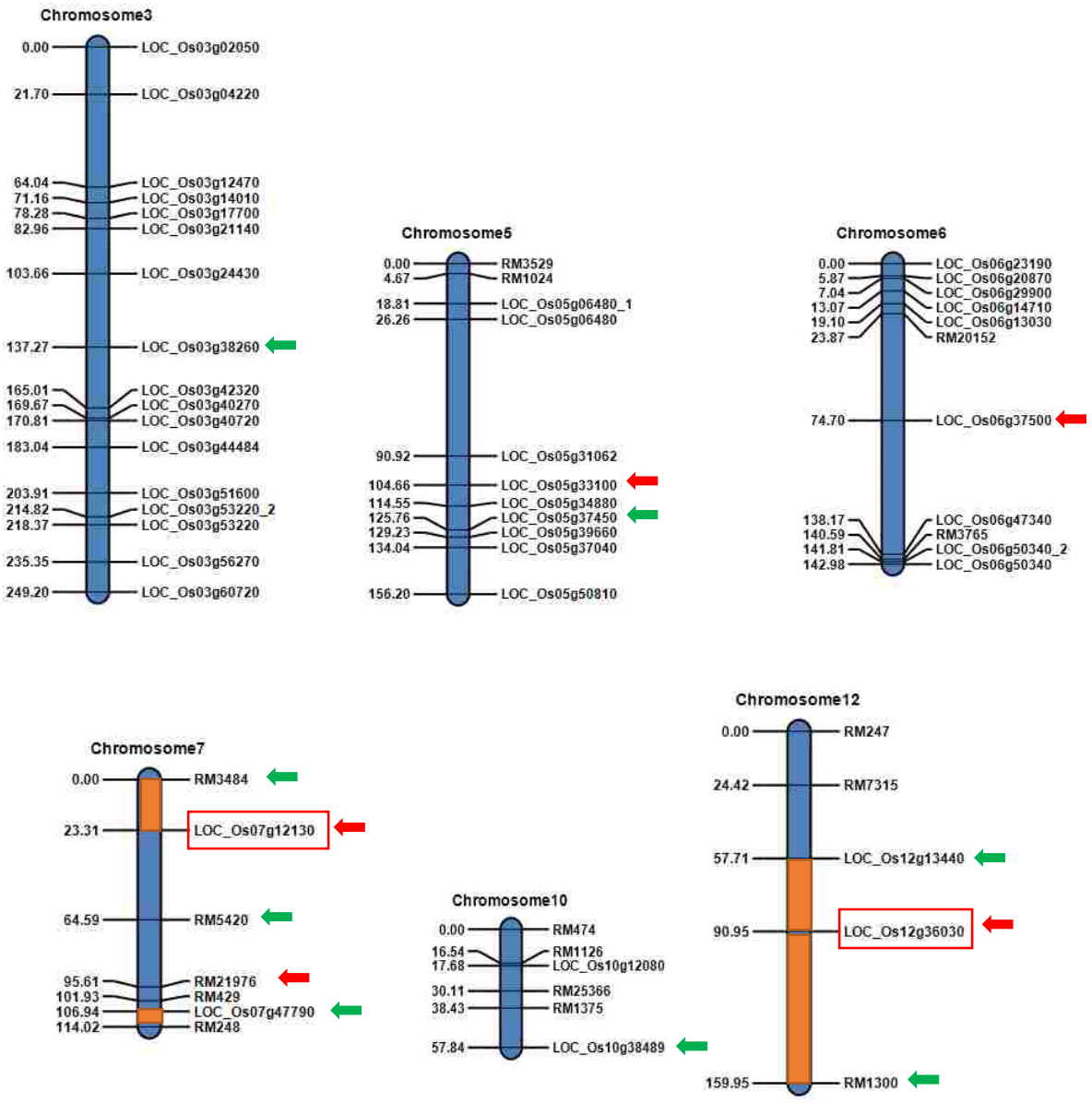
LOC_Os12g36030 with LOD score of 3.5 and 14% variance explained. The interval between LOC_Os07g47990 and RM249 generated a LOD score of 4.19 and variance explained of 11.9% (Table 3.21).

Table 3.21. QTLs detected in 2008S/ CL131 F_2 population using Inclusive Composite Interval Mapping (ICIM) ver. 4.0.6.0

Chr	Interval (cM)	LeftMarker	Right Marker	LOD	PVE(%)	Add	Dom
7	23.31	RM3484	LOC_Os07g12130	10.42	19.23	0.241	0.012
7	7.08	LOC_Os07g47790	RM248	4.20	11.93	0.241	-0.026
12	33.24	LOC_Os12g13440	LOC_Os12g36030	3.56	14.80	-0.273	0.182
12	69	LOC_Os12g36030	RM1300	16.27	38.90	0.369	-0.101

A linkage map was constructed for all 12 chromosomes (Figure 3.25) that showed the major and minor QTLs identified by single marker analysis approach and inclusive composite interval mapping. Based on the map, the common QTL region identified between single marker analysis and interval mapping are the following; LOC_Os12g36030, LOC_Os12g13440, and RM1300 in chromosome 12; LOC_Os07g12130, RM3484, LOC_Os07g47790 and RM248 in chromosome 7.

The study identified 12 new QTLs, these are: LOC_Os03g38260, RM21976, LOC_Os05g33100, LOC_Os05g37450, LOC_Os06g37500, RM5420, LOC_Os07g47790, LOC_Os10g38489, LOC_Os12g13440, RM1300, RM3484 from single marker analysis and RM248 from interval mapping (Figure 3.25).



Major QTL (R^2 value ≥20%)
Minor QTL (R^2 value <20%)
QTL by inclusive composite interval mapping (LOD score greater than 2.0)
Red Box = Previously reported QTL/gene

Figure. 3.25 Linkage map of chromosomes 3, 5, 6, 7, 10, and 12 showing major (red arrows) and minor (green arrows) QTLs detected using SAS ANOVA single marker analysis. QTLs using inclusive composite interval mapping were also identified (orange covered).

3.4 Summary

A three-year study (2012, 2013 and 2014) of inheritance of pollen male sterility in F_2 and BC_1F_2 populations derived from 2008S revealed a 15 fertile: 1 sterile two-locus segregation model. Seed fertility data of 2008S F_2 population also showed a 15 fertile: 1 sterile ratio when evaluated at the Arkansas Rice Research and Extension Center in 2013 and 2014. Taken together, all pollen and seed data from this study indicate that environmentally sensitive male sterility in 2008S is governed by two recessive genes. For the 2009S male sterile evaluated in this study, segregating populations for the F_2 population planted in 2013 and the BC_1F_2 planted in 2014 showed a 3 fertile: 1 sterile single-locus segregation model. All data demonstrated that male sterility in 2009S is controlled by a single recessive gene that should facilitate use of this material for Louisiana hybrid development.

Analysis using ANOVA for SNP markers in LOC_Os07g12130 and LOC_Os12g36030 showed significant effects on all populations tested in 2012 and 2013 while interaction (two-way ANOVA) effects were significant only in 2013. ANOVA using marker genotype LOC_Os07g12130, LOC_Os12g36030 and Years planted (2012 and 2013) as main effects and its interactions were examined and the result revealed that "Year" as main effect or with interaction with the marker genotypes LOC_Os07g12130 and LOC_Os12g36030 were not significant and that the trait expression was stable across years.

All male sterile lines and 20 randomly sampled fertile lines in F_2 and BC_1F_2 population derived from 2009s were genotyped using CEL 1 nuclease assay targeting the SNP in LOC_Os02g12290. Overall results showed that genotyping correctly identified 90-100% of pollen male sterile lines containing homozygous sterile genotypes and 95 to 100% of pollen fertile lines having heterozygous or homozygous fertile genotypes.

Sequence comparison of Nipponbare reference genome, Sanger sequence data of CL161 and 2009S and published sequences of Guangzhang 63S, (Xu et al. 2011) and Zhu1S (Zhou et al. 2014) showed a nucleotide substitution of C to A in the first exon of LOC_Os02g12290. The investigation showed that male sterile lines 2009S, Guangzhang 63S and Zhu1S had identical SNP (A) that was located in position 6,397,412 bp of chromosome 2 which was reportedly associated with male sterility in rice.

Comparison of Nipponbare reference genome, Sanger sequencing from 2009S, CL161 and published sequence of male sterile line Nongken 58S at LOC_Os12g36030 (Ding et al. 2012a) showed Nongken 58S and 2008S having identical SNP (C) at position 22,054,886 bp while fertile lines Nipponbare and CL161 had the fertile SNP (G) in chromosome 12. The nucleotide substitution of G to C at LOC_Os12g36030 in chromosome 12 was reported to be responsible for male sterility in Nongken 58S (Ding et al. 2012a).

Sanger sequencing of the first exon of LOC_Os07g12130 in 2008S showed identical sterile SNP (A) found in male sterile line Pei'ai 64S (Zhou et al. 2011) at 6,781,581 bp position while fertile Nipponbare and CL161 had the fertile SNP (T) in chromosome 7.

All Sanger sequencing results identified and confirmed the presence of SNPs in 2009S and 2008S that corresponds to SNPs previously reported in LOC_Os02g12290 (Xu et al. 2011; Zhou et al. 2014), LOC_Os12g36030 (Ding et al. 2012a) and LOC_Os07g12130 (Zhou et al. 2011) associated with photoperiod and thermosensitive genetic male sterility in rice. The molecular markers based from these SNPs will greatly enhance selection in developing male sterile lines adapted under Louisiana conditions.

QTLs for male sterility were detected by selective genotyping strategy using twenty-two 100% pollen fertile and twenty-two 70-100% pollen sterile plants in the F_2 population of

2008S/CL131. Based on p-values from single marker analysis (ANOVA), previously identified QTLs/genes LOC_Os12g36030 and LOC_Os07g12130 were significantly associated with the trait. New QTLs RM21976 in chromosome 7 and LOC_Os05g33100 on chromosome 5 were also detected. However, many heterozygous genotypes in LOC_Os05g33100 were showing on male sterile lines which needs further studies to explain the significant p-values. Eight minor QTLs were also discovered on chromosomes 3, 5, 6, 7, 10, and 12. When analyzed by LOD scores using single marker analysis option in ICIM software, four QTLs with LOD scores greater than 2.0 were identified, these were: LOC_Os12g36030, LOC_Os07g12130, RM21976 and LOC_Os05g33100.

Inclusive composite interval mapping (ICIM) were used to compare results with single marker analysis. The method revealed four QTLs for male sterility. One QTL was identified at LOC_Os07g12130 and RM3484; two QTLs in the intervals of LOC_Os12g36030 and LOC_Os12g13440; LOC_Os12g36030 and RM1300; the fourth QTL was detected in the interval of LOC_Os07g47790 and RM248 on chromosome 7.

Overall results for QTL mapping revealed 12 new QTLs identified from single marker analysis and interval mapping. This new markers can complement previously identified QTL/gene in chromosome 7 (Zhou et al. 2011) and 12 (Zhou et al. 2012; Ding et al. 2012a) to increase prediction in identifying male sterile lines. Furthermore, the information herein can be used for future studies in fine mapping genes responsible for male sterility under the 2008S genetic background and develop new markers to facilitate the development of male sterile lines for the two line hybrid breeding program in Louisiana.

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CHAPTER 4. EVALUATION OF ALLELES AT S5n WIDE COMPATIBILITY LOCUS AND THEIR EFFECTS ON SPIKELET FERTILITY OF *INDICA* X *JAPONICA* HYBRIDS.

4.1 Introduction

Hybrid rice is proven to be a very effective technology in increasing yield not only in China, but also in many other countries such as the Philippines, Bangladesh, Indonesia, Pakistan, Ecuador, Guineas and the U.S. (Yuan 1990). Hybrid rice technology currently uses inter-sub specific crosses of *indica/japonica* to exploit maximum heterosis compared to intra-sub specific hybrids *indica/indica* and *japonica/japonica* counterparts that have almost reached their yield ceiling (Virmani et al. 2003).

Indica/japonica rice hybrids exhibit heterosis for different traits, but most of these inter-sub specific combinations cannot be used directly in rice production due to low spikelet fertility (Ji et al. 2010). The *S5* locus (LOC_Os06g11010) was recently reported to regulate spikelet fertility of an *indica/japonica* rice hybrid (Ji et al. 2012). There are three known alleles at the *S5* locus: an *indica* allele (*S5i*), a *japonica* allele (*S5j*), and a wide compatibility allele (*S5n*). The *S5* locus is not essential for embryo sac development, although interactions between *S5i* and *S5j* have deleterious effects on embryo sac formation resulting in the reduction of spikelet fertility (Ji et al. 2012). However, the *S5* locus was reported to be associated with fertility of *indica/japonica* hybrids with the *S5n* allele from the so-called wide-compatibility varieties (WCV), capable of overcoming the inter-sub specific fertility barrier (Ji et al. 2010). A yeast two-hybrid assay showed that the *S5i* and *S5j* aspartyl protease (Asp) formed homodimers and heterodimers, while the *S5n* was incapable of dimerization. A 136 base-pair deletion resulted in a nonfunctional allele in the Asp N-terminus while the heterozygous A/C site at position 1834 bp relative to the gene LOC_Os06g11010 in the Nipponbare reference genome was proposed as the main cause of

partial sterility in the *indica/japonica* hybrids (Ji et al. 2010). Site-directed mutagenesis proved that the single amino acid difference between *S5i* and *S5j* encoding aspartyl proteases was the main cause of embryo sac abortion (Ji et al. 2012).

4.1.1 Molecular Markers for S5n, S5i and S5j Alleles

In developing near isogenic lines (NIL) with the wide compatibility gene *S5n*, Wang *et al*. (2005) used SSR markers RM253 and RM276 that flanked the *S5* locus. Functional molecular markers S5-t1 and S5-t2 were also used, based on the 136 bp deletion in the sequence of *S5* locus, to genotype 646 rice lines. Some 28 genotypes including 16 cultivated, nine *Oryza rufipogon* and three *Oryza nivara* accessions were found to carry the *S5n* allele (Yang et al. 2012). A survey of 197 varieties from Chinese micro-core rice collections found a small number of accessions (10) with the *S5n* wide compatibility allele (Yang et al. 2009b). A PCR-based multiplex marker system was successfully developed targeting the deletion and the SNPs for determining the allelic status at the *S5* locus (Sundaram et al. 2010). The 136 base-pair deletion primer pair was generated based on sequences reported for *S5n* (Chen et al. 2008), while a SNP marker was designed by targeting the A/C SNP at the *S5* locus for *japonica* and *indica* alleles. The simultaneous amplification of both SNP and InDels using a multiplex polymerase chain reaction (PCR) has allowed the successful genotyping of 584 rice lines where 116 were shown to carry the *S5n* allele (Sundaram et al., 2010).

4.1.2 Integrating S5n with Other Wide Compatibility Alleles into Rice Breeding

Improved spikelet fertility in *indica/japonica* hybrids by 33.6-46.7% was recently accomplished by stacking *f5n* (Wang et al. 2006) which is a wide compatibility allele for pollen fertility and *S5n* through integrated backcrossing (Mi et al. 2015). Similarly Wang et al. (2005) was able to increase pollen and spikelet fertility through introgression of three neutral alleles *f5*-

Du, f6-Du and *S5* by successive backcrossing. These results further showed that molecular marker-assisted strategy can improve spikelet fertility using *S5n.* It is also compatible in integrating other loci such as *f5n* (Wang et al. 2006) that rescue pollen fertility and *f6-Du* (Wang et al. 2005) for embryo sac fertility to gain maximum heterosis in *indica / japonica* rice hybrids.

One of the objectives of the research herein was to test the effect of the *S5n* gene on spikelet fertility, yield and other agronomic traits using the *indica*/*japonica* RiceCAP SB5 RIL mapping population. The other objective was to test the presence of *S5n* in the current Louisiana varieties and male sterile lines to identify potential parents carrying homozygous *S5n* alleles for use in the LSU hybrid rice breeding program.

4.2 Materials and Methods

4.2.1 Design of SNP and InDel Markers

The SNAP program (http://ausubellab.mgh.harvard.edu/) was used to design SNP primers targeting the S5 locus using the sequence of the Nipponbare reference genome (http://www.gramene.org). Forward primers for each SNP were designed to contain a mismatch within three nucleotides of the 3' end of the reference allele (A), and a 3' mismatch with the alternative allele (C) (Drenkard et al. 2000). Standard procedure using the SNAP program that align all nucleotides in the sequence were used to design the reverse primers adjusted for the target PCR product length. Reference (*S5j*) and alternate (*S5i* or *S5n*) primers were designed to generate a 326 base-pair fragment for visualization on a 1.5% agarose gel (Figure 4.1). InDel marker S5-t1 was obtained from the published primer sequences for the *S5* locus (Yang et al. 2009b). The PCR fragment size for *S5n* was shorter at 381 bp while *S5j* and *S5i* generated a longer fragment size of 517 bp (Figure 4.2).

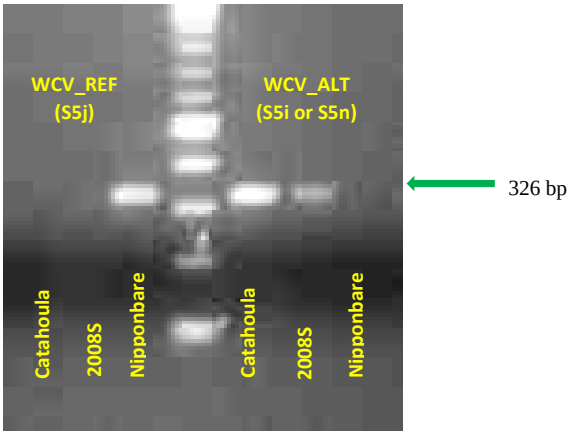

Figure 4.1 SNP-based marker WCV_REF and WCV_ALT. Varieties Catahoula and 2008S contain the same target "C" SNP for *S5i* and *S5n*, respectively, while Nipponbare shows the presence of "A" SNP for *S5j*.

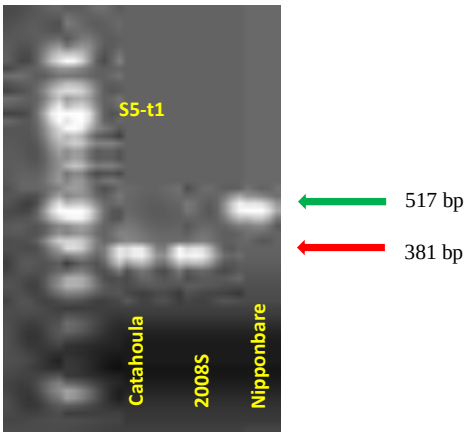

Figure 4.2 InDel marker S5-t1. Catahoula and 2008s have a deletion in the *S5* locus (LOC_Os06g11010) that shows a 381 bp fragment size while Nipponbare showed no deletion producing a 517 bp fragment size.

4.2.2 Genotyping RILs of SB5 Mapping Population and Hybrids with Nipponbare

Genotyping recombinant inbred lines (RILs) allowed classification of lines that are homozygous for *S5i*, *S5n* and *S5j* (Ji et al. 2012). Classification was done by random selection of 97 recombinant inbred lines (F8) from the RiceCAP SB5 mapping population (Jasmine 85 x Lemont cross) and genotyping using InDel and SNP markers for the *S5* locus (Table 4.1). The primer sequences of S5-t1 were used to amplify the target region of LOC_Os06g11010 starting at 301 bp to 818 bp covering the first exon while the SNP markers targeted the A/C SNP at

position 1834 bp relative to the LOC_Os06g11010 sequence of the Nipponbare reference genome.

Table 4.1 InDel and SNP primer sequences used to genotype the S5 locus LOC_Os06g11010.

Primer Name	Marker Type	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
S5-t1	InDel	CGTCTTGCTTCTTCATTCCC	GTAGGTAAACACAGGCAGAG
WCV_REF	SNP (A)	AGGGTACCCTGATATTCTGAGTTACAAGGCAATA	TTGATGTCCGGTGATACCCAATCGA
WCV_ALT	SNP (C)	GGGTACCCTGATATTCTGAGTTACAAGGCTTTC	TTGATGTCCGGTGATACCCAATCGA

10 µL PCR reaction mixture was prepared by combining the following: 1 µL of 20 ng/µL DNA template, 1 µL of 10X PCR buffer (containing 1mM $MgCl_2$), 0.8 µL of 10mM dNTPs mix, 0.2 µL each of 20 µM forward and reverse primers, 0.08 µL of 5U/ µL Taq polymerase and 6.8 µL of distilled H_2O. The PCR profile included the following steps: 95°C, 3 min, 95°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec, repeat 30X previous three steps, 72°C, 5 min with final holding temperature at 4°C. Amplified PCR products were visualized on 1.5% agarose gel stained with ethidium bromide.

Ten RILs that do not have the 136 bp deletion were selected as representative samples homozygous for *S5i S5i* and another ten RILs that have the 136 bp deletion were selected as homozygous for *S5n S5n*. All twenty RILs have a "C" SNP when genotyped with the SNP based markers (Table 4.2). Nipponbare was considered homozygous for *S5j S5j* due to the presence of an "A" SNP and with no deletion at the LOC_Os06g11010.

Table 4.2 Genotypes using InDel and SNP markers at the S5 locus for 20 RILs from SB5 mapping population and corresponding hybrids.

Variety/ RIL	136 bp Deletion	SNP (A/C)	Variety/RIL Genotype	Hybrid (F1) Genotype[1]
Jasmine 85	No	C	*S5i S5i*	-
Lemont	Yes	C	*S5n S5n*	-
Nipponbare	No	A	*S5j S5j*	-
108	No	C	*S5i S5i*	*S5i S5j*
125	No	C	*S5i S5i*	*S5i S5j*
150	No	C	*S5i S5i*	*S5i S5j*
252	No	C	*S5i S5i*	*S5i S5j*
274	No	C	*S5i S5i*	*S5i S5j*
296	No	C	*S5i S5i*	*S5i S5j*
312	No	C	*S5i S5i*	*S5i S5j*
324	No	C	*S5i S5i*	*S5i S5j*
385	No	C	*S5i S5i*	*S5i S5j*
419	No	C	*S5i S5i*	*S5i S5j*
157	Yes	C	*S5n S5n*	*S5n S5j*
173	Yes	C	*S5n S5n*	*S5n S5j*
409	Yes	C	*S5n S5n*	*S5n S5j*
500	Yes	C	*S5n S5n*	*S5n S5j*
502	Yes	C	*S5n S5n*	*S5n S5j*
568	Yes	C	*S5n S5n*	*S5n S5j*
596	Yes	C	*S5n S5n*	*S5n S5j*
602	Yes	C	*S5n S5n*	*S5n S5j*
623	Yes	C	*S5n S5n*	*S5n S5j*
656	Yes	C	*S5n S5n*	*S5n S5j*

[1] Individual RILs were crossed to Nipponbare to produce hybrids with *S5i S5j* or *S5n S5j* genotype.

4.2.3 Generation and Field Evaluation of Hybrids using Nipponbare (S5j) as Parent

All twenty RILs that are homozygous for *S5i S5i* and *S5n S5n* were crossed to Nipponbare (*S5j S5j*) creating two groups of hybrid genotypes: *S5i S5j* and *S5n S5j* (Table 4.2). A total of twenty seeds from each cross were placed in petri dishes for 10 days with subsequent seedling transfer to the greenhouse. Plants were transplanted after ~ 30 days in a randomized complete block design with 2 replicates at the H. Rouse Caffey Rice Research Station at Crowley, LA. in May 2014. Each replicate consisted of a single, two-meter row with 10 plants. Standard management practices for fertilization, pest management, and weed control were followed.

4.2.4 Phenotypic Data Collections

Phenotypic data were collected as follows: number of days from planting to heading were recorded when panicles first emerged from the boot for 50% of the plants in each row, mean height (cm) was measured from ground to tip of the longest panicle, mean grain yield/plant (g) was calculated based on total weight of grains harvested across all plants in row at ~12% moisture divided by total number of plants, mean number of panicles were counted and averaged from three randomly-sampled plants within each row, mean yield per panicle (g) was calculated by dividing the mean grain yield/plant (g) to the mean number of panicles, mean percent (%) spikelet fertility was determined from three randomly-sampled panicles within each row by dividing the filled grains over total number of spikelets multiplied by 100, and mean number of seeds per panicle was computed by dividing total number of fertile seeds over the number of panicles from three randomly sampled plants within each row. Data were analyzed using SAS PROC ANOVA (SAS Institute, Cary, NC).

4.3 Results

4.3.1 Analysis of Spikelet Fertility and Other Agronomic Traits

Each trait was analyzed using F-test in PROC ANOVA (SAS Institute, Cary, NC) comparing group means of *S5i S5j* and *S5n S5j*. Mean (%) percent spikelet fertility showed highly significant differences between *S5i S5j* and *S5n S5j* genotypes at p-value ≤0.01. Mean yield per plant between two genotypes likewise showed significant differences with *S5n S5j* hybrids having a higher mean yield of 55.38 g compared to 42.83 g for the *S5i S5j* counterpart. The analysis also revealed significant differences for days to heading, mean height (cm), mean yield per panicle (g), and mean number of seeds per panicle. There was no significant difference in panicles per plant. Earlier heading plants were shorter than late heading plants. Under field conditions, plants with low spikelet fertility were visually identified by erect panicles while panicles with high spikelet fertility bend due to the weight of the grains (Figure 4.3).

A **B**

Figure 4.3 Panicles of Hybrid (A) 274 x Nipponbare-*S5i S5j* and (B) 602 x Nipponbare-*S5n S5j* (B) on August 2014 at the H. Rouse Caffey Rice Research Station, Crowley, LA.

The spikelet fertilities of wide compatibility hybrids (with *S5n*) developed by Yang et al. (2012) averaged 60.69% in contrast to *S5i S5j* (7.75%) control hybrids. Similar high spikelet fertilities were also observed by Kumar and Virmani (1995) on crossing wide compatible varieties (*S5n*) to *indica, aus and japonica* cultivars.

The *S5n S5j* genotypes produced an average percent spikelet fertility of 59% (Table 4.3). Our results are comparable to wide compatible hybrid seed setting rate of 61% reported by Yang et al. (2012) but we obtained spikelet fertilities in S5i S5j that are higher (48%) than in their *indica/japonica* control hybrids (7.75%). A probable explanation is that the hybrids developed from our study came from RILs of the same population which are closely related while Yang et al. (2012) developed hybrids from parents that were diverse and unrelated. In a study of closely related populations, hybrid combinations from *S5n* near isogenic lines (NIL) developed by Ji et al. (2010) exhibited higher spikelet fertilities that ranged from 55.07% to 88.36% while *S5i S5j* hybrids had 41.07% to 64.64%.

Table 4.3 Analysis of variance using PROC ANOVA on trait means for *S5n S5j* and *S5i S5j* genotypes.

Trait	Num DF	F Value	P-value	Mean *S5n S5j*	Mean *S5i S5j*
Mean yield per plant (g)*	1	5.4	0.02	55.38	42.83
Days to heading**	1	6.72	0.01	124	132
Mean height (cm)**	1	14.5	<0.01	126.00	140.00
Mean number of panicles/plant	1	0.01	0.92	28	28
Mean yield per panicle (g)**	1	9.19	<0.01	1.97	1.53
Mean % spikelet fertility**	1	8.51	<0.01	59	48
Mean number of seeds per panicle**	1	16.69	<0.01	116	78

** Highly significant using PROC ANOVA at p-value ≤ 0.01
*Significant at p value ≤ 0.05

4.3.2 Correlation of Spikelet Fertilities to Other Agronomic Traits

Percent spikelet fertility (PSF) is positively correlated with mean yield per plant (YPL), mean yield per panicle (YPA) and number of seed/panicle (NSP) which is expected as more grains are produced due to successful fertilization and normal embryo development (Table 4.4). PSF as a function of grain yield is also known to have positive significant association with the number of effective tillers/hill, panicle/m^2, and thousand grain weight, (Hasan et al. 2011), (Mulugeta et al. 2012). PSF showed significant negative correlation with mean days to heading (DH) and plant height (HT). As a consequence, other yield related traits such as YPL and NP were also negatively correlated to DH, while YPL was negatively correlated to HT (Table 4.4). A study by Rahman et al. (2007) also found significant negative correlation of spikelet fertility to that of days to heading, days to maturity and number of panicles in identifying QTL's for agronomic traits in rice using an introgression line from *Oryza minuta.*

Table 4.4 Descriptive statistics and Pearson's correlation matrix for seven traits measured in S5i S5j and S5n S5j genotypes.

TRAIT	YPL	DH	HT	NP	YPA	PSF	NSP
YLP	1						
DH	-0.300	1					
HEI	-0.116	0.617*	1				
NP	0.567*	-0.385*	0.079	1			
YPA	0.855*	-0.152	-0.187	0.086	1		
SPF	0.444*	-0.601*	-0.572*	0.125	0.494*	1	
NSE	0.675	-0.416	-0.396	0.230	0.698	0.548*	1

*Significant correlation at p-value ≤0.05

Notes: YPL: Mean yield per plant (g); DH: Days to heading; HT: Mean height (cm); NP: Mean number of panicles/plant; YPA: Mean yield per panicle (g); PSF: Mean % spikelet fertility; NSP: Mean number of seeds per panicle.

4.3.3 Genotyping Varieties and Potential Breeding Lines from the LSU Hybrid Breeding Program.

We surveyed a total of 91 existing varieties and male sterile lines from the LSU rice hybrid breeding program for the presence of 136 bp deletion and the A/C SNP to identify potential parent carrying the *S5n* allele. Based on our results, 29 plants carried the homozygous *S5n* allele, while 56 were homozygous for *S5i* and four were heterozygous for the 136 bp deletion. Nipponbare (*japonica*) and Bengal (*tropical japonica*) were the only varieties that were homozygous for *S5j* (Appendix D). The majority of the plants that were homozygous for *S5i* came from early generation lines derived from *indica* male sterile parent 2009S while S5n-carrying plants typically came from U.S. *tropical japonica* varieties and from the 2008S (*indica*)/CL161 (*tropical japonica*) population of male sterile lines.

4.4 References

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CHAPTER 5. SUMMARY AND CONCLUSIONS

5.1 Evaluation of Environmentally-Sensitive Male Sterile Lines Under Controlled Conditions

A study was conducted to determine if male sterile lines 2009S and 2008S respond to differences in daylength conditions under high temperatures. The results indicated that at high temperature (28-30°C) for both short (10 hrs.) and long (14 hrs.) daylengths, 2009S exhibited 98-100% pollen and seed sterility. This study showed that 2009S exhibited a thermosensitive response as shown by consistent high pollen and seed sterility under both short and long daylengths. In contrast, 2008S was 100% sterile under high temperature and long daylength, but showed partial pollen and seed fertility when exposed to short daylengths. However, the investigation resulted in no statistical difference in pollen and seed sterility between the 2008S and 2009S. While 2008S responded to one or both stimuli, further research will be needed to determine the developmental stage, critical temperature, and photoperiod for male sterility. A study conducted in the greenhouse under short day and low temperature conditions showed that the 2008S and 2009S produced fertile pollen and seeds. These investigations illustrated that the male sterility in 2008S and 2009S is compatible with the two- line hybrid breeding system and will be very useful as a female parent for hybrid breeding purposes.

The stage and duration of treatment for reversion to fertile pollen in 2009S were investigated. Results showed that the male sterile line exhibited higher pollen fertility when treated at the panicle differentiation stage or when the flag leaf has not completely emerged from the boot compared to when the flag leaf was completely exerted. Comparison between 15-day and 10-day exposure to low temperature and short daylength also did not result in significant difference in percent pollen fertility. The identification of the duration of temperature and daylength treatments will be useful for controlled environments such as chambers or greenhouses

to study and manipulate male sterile lines for hybrid development. Finally, use of morphological markers by visually identifying the proper developmental stage will maximize the pollen and seed yield potential of treated male sterile line 2009S. This information will be invaluable when other environmentally-sensitive male sterile lines are discovered and characterized for applied plant breeding efforts.

5.2 Single and Two-Gene Recessive Inheritance of Male Sterile Lines

Results from a three-year study of F_2 and BC_1F_2 populations derived from 2008S demonstrated two-gene recessive inheritance for pollen male sterility. Moreover, seed fertility of the two populations planted from 2013 to 2014 in Arkansas showed a similar pattern of 15 fertile: 1 sterile for a two-locus segregation model. For the F_2 and BC_1F_2 populations derived from 2009S, male sterility was governed by a single recessive gene as demonstrated by the 3 fertile: 1 sterile segregation ratios. This study indicates that both 2008S and 2009S have good potential as sources of male sterility that would be useful in the development of hybrids for the Louisiana industry.

5.3 Development and Use of SNP Markers and Association with Genetic Male Sterility

SNP markers developed for LOC_Os07g12130 and LOC_Os12g36030 targeting alleles in the 2008S background were evaluated in this study. The results showed significant marker effects using single marker analysis on all populations in 2012 and 2013 while interaction effects were significant in 2013 alone. Analysis of variance using SNP markers LOC_Os07g12130, LOC_Os12g36030 and Years planted (2012 and 2013) as main effects and its interactions were analyzed and the result revealed that “Year” as main effect or with interaction with the SNP markers LOC_Os07g12130 and LOC_Os12g36030 were not significant and that the trait expression was stable across years. The selection of both LOC_Os07g12130 and

LOC_Os12g36030 together increased R^2 values compared to the use of single markers. The highest percent variance explained (65.6%) using both markers was found in the 2008S/Cypress F_2 population planted in 2013. However, there was substantial variation not explained by using the two markers, so additional markers are needed to increase the prediction in identifying male sterile lines through QTL mapping. The SNP markers developed in LOC_Os12g36030 and LOC_Os07g12130 can be used effectively for marker assisted selection in identifying male sterile lines under the 2008S genetic background.

Selected F_2 and BC_1F_2 population from 2009s were genotyped using the CEL 1 nuclease assay targeting the SNP in LOC_Os02g12290. Overall results showed the assay correctly identified 90 to 100% of pollen male sterile lines containing homozygous sterile genotypes and 95 to 100% of pollen fertile lines having heterozygous or homozygous fertile genotypes. Due to the high prediction for male sterility of this marker, the method is currently used to increase efficiency in selection and verify presence of the alleles in male sterile breeding lines of the LSU AgCenter hybrid rice breeding program.

5.4 QTL Mapping of Male Sterility in 2008S

QTL mapping for male sterility by selective genotyping was performed using extreme phenotypes in the F_2 population of 2008S/CL131. Based on the ranking of p-values from single marker analysis (ANOVA), QTLs in LOC_Os12g36030 and LOC_Os07g12130 were significantly associated with the trait. Three additional major QTLs were detected at LOC_Os05g33100, LOC_Os06g37500, and RM21976. Eight minor QTLs were also detected on chromosome 3,5,7, 10, and 12. When analyzed using single marker analysis option in inclusive composite interval mapping (ICIM) software by LOD scores, four QTLs with a LOD score greater than 2.0 were detected. This includes LOC_Os12g36030, LOC_Os07g12130, RM21976

and LOC_Os05g33100. QTLs found in LOC_Os5g33100 and LOC_Os06g37500 needs further studies because many heterozygous genotypes were present in male sterile lines. Interval mapping using ICIM revealed four QTLs. The first QTL is in the interval of LOC_Os12g36030 and RM1300 while the second QTL is in the interval of LOC_Os07g12130 and RM3484. The third QTL is in the interval of LOC_Os07g47790 and RM248. The fourth QTL is in LOC_Os12g36030 and LOC_ Os12g13440. Additional markers are needed to shorten QTL intervals that will increase precision of QTL detection for the male sterile trait. This study detected the presence of previously identified QTLs/genes in LOC_Os12g36030 (Zhou et al. 2012) and LOC_Os07g12130 (Zhou et al. 2011) and twelve new QTLs associated with male sterility. QTLs discovered in this study will provide additional markers that can be used for marker assisted selection under the genetic background of 2008S. Furthermore, the results of the study can be used in the future to fine map QTLs and discover genes that are associated with rice photoperiod and thermosensitive male sterility.

5.5 Wide Compatibility Gene S5n in Hybrids and its Effect on Spikelet Fertility

Most *indica* x *japonica* hybrids have low spikelet fertilities due to the presence of incompatible heterozygous *S5i S5j* genotypes. Previous research (Ji et al. 2012) has reported that the presence of wide compatibility gene *S5n* removes the fertility barrier and increases spikelet fertility in *indica* x *japonica* hybrids. The effect of wide compatibility gene *S5n* in spikelet fertility was tested by generating hybrids heterozygous for *S5i S5j* and *S5n S5j* by crossing ten SB5 RILs homozygous for *S5i* and ten SB5 RILs homozygous for *S5n* to Nipponbare homozygous to *S5j*. The presence of the *S5* locus was validated by utilizing a SNP and insertion-deletion markers to classify genotypes with *S5i S5j* and *S5n S5j* hybrids. The overall result of the replicated yield trial revealed that mean spikelet fertility was significantly higher in hybrids

having the *S5n S5j* than *S5i S5j*. In addition, there were positive correlation with the increase of spikelet fertility to number of grains per panicle and increase in yield per plant of those hybrids carrying the *S5n*. A total of 91 existing male sterile lines from the LSU hybrid rice breeding program was surveyed and it was found that 29 carried the *S5n* allele, 56 carries the *S5i*, four were heterozygous and two contained the *S5j* allele. The results of the experiment and the markers developed in this study will be an important tool to identify potential parents carrying homozygous *S5n* alleles for use in the LSU hybrid rice breeding program.

APPENDIX A. QUANTITATIVE TRAIT LOCUS MAPPING USING SINGLE MARKER ANALYSIS AND INCLUSIVE COMPOSITE INTERVAL MAPPING (ICIM).

Table A1. Rank of markers based on p-values using single marker analysis in SAS ver. 9.4

Rank	Code	Marker Locus	F value	Raw_P	Hochberg p-value	Stepdown Bonferroni	False Discovery Rate p-value
1	X111	LOC_Os12g36030	84.7625	0	0	0	0
2	X69	LOC_Os07g12130	22.822	0	0.00002	0	0.00001
3	X71	RM21976	7.6182	0.00154	0.17062	0.1706	0.0579
4	X50	LOC_Os05g33100	5.3407	0.00868	0.9552	0.9552	0.21627
5	X63	LOC_Os06g37500	5.234	0.00957	0.9995	1	0.21627
6	X70	RM5420	4.6623	0.01498	0.9995	1	0.26064
7	X73	LOC_Os07g47790	4.1382	0.02307	0.9995	1	0.26064
8	X109	LOC_Os12g13440	4.2723	0.02064	0.9995	1	0.26064
9	X112	RM1300	5.7278	0.02148	0.9995	1	0.26064
10	X53	LOC_Os05g37450	3.5188	0.03887	0.9995	1	0.29285
11	X68	RM3484	3.8432	0.02953	0.9995	1	0.29285
12	X100	LOC_Os10g38489	3.6008	0.03625	0.9995	1	0.29285
13	X28	LOC_Os03g38260	2.9385	0.04471	0.9995	1	0.31579
14	X22	LOC_Os03g04220	3.1775	0.05213	0.9995	1	0.3465
15	X55	LOC_Os05g39660	2.8769	0.06774	0.9995	1	0.40286
16	X89	RM6839	2.9301	0.06465	0.9995	1	0.40286
17	X1	RM1247	2.562	0.08944	0.9995	1	0.42264
18	X7	RM12276	2.6658	0.08188	0.9995	1	0.42264
19	X13	LOC_Os02g44980	2.558	0.08976	0.9995	1	0.42264
20	X35	LOC_Os03g53220_2	2.7461	0.07599	0.9995	1	0.42264
21	X72	RM429	2.571	0.0897	0.9995	1	0.42264
22	X6	RM11635	2.7059	0.10947	0.9995	1	0.45482
23	X14	LOC_Os02g48130	2.4268	0.1009	0.9995	1	0.45482
24	X43	LOC_Os04g58920	2.3035	0.1127	0.9995	1	0.45482
25	X93	LOC_Os09g36420	2.3595	0.10717	0.9995	1	0.45482
26	X17	LOC_Os02g52130	1.8931	0.16385	0.9995	1	0.54455
27	X21	LOC_Os03g02050	1.8985	0.16273	0.9995	1	0.54455
28	X44	RM3531	1.9251	0.15914	0.9995	1	0.54455
29	X49	LOC_Os05g31062	1.9277	0.15844	0.9995	1	0.54455
30	X74	RM248	1.964	0.15328	0.9995	1	0.54455
31	X108	RM247	1.9496	0.15747	0.9995	1	0.54455
32	X94	LOC_Os09g38640	1.758	0.18514	0.9995	1	0.58114

Table A1 Continued

Rank	Code	Marker Locus	F value	Raw_P	Hochberg p-value	Stepdown Bonferroni	False Discovery Rate p-value
33	X113	RM7315	1.7787	0.18165	0.9995	1	0.58114
34	X37	LOC_Os03g60720	1.6388	0.20668	0.9995	1	0.63122
35	X2	RM1196	1.567	0.22092	0.9995	1	0.65695
36	X105	LOC_Os11g47550	1.499	0.23534	0.9995	1	0.68187
37	X51	LOC_Os05g34880	1.4051	0.25718	0.9995	1	0.72654
38	X57	LOC_Os06g13030	1.3449	0.27208	0.9995	1	0.7287
39	X67	LOC_Os06g50340_2	1.3247	0.27729	0.9995	1	0.7287
40	X97	RM1126	1.3293	0.27583	0.9995	1	0.7287
41	X66	LOC_Os06g50340	1.2837	0.28792	0.9995	1	0.73942
42	X36	LOC_Os03g56270	1.2601	0.29465	0.9995	1	0.7399
43	X99	RM1375	1.1703	0.32041	0.9995	1	0.7871
44	X12	LOC_Os02g44120	1.0604	0.35561	0.9995	1	0.84019
45	X20	LOC_Os02g56320	1.0566	0.35689	0.9995	1	0.84019
46	X11	LOC_Os02g42870	0.9123	0.40961	0.9995	1	0.84466
47	X15	LOC_Os02g49986	0.9826	0.38318	0.9995	1	0.84466
48	X33	LOC_Os03g51600	1.0055	0.37472	0.9995	1	0.84466
49	X34	LOC_Os03g53220	0.9536	0.39393	0.9995	1	0.84466
50	X39	LOC_Os04g40150	0.9084	0.41112	0.9995	1	0.84466
51	X76	LOC_Os08g07290	0.9352	0.40074	0.9995	1	0.84466
52	X96	LOC_Os10g12080	0.9251	0.40463	0.9995	1	0.84466
53	X60	LOC_Os06g23190	0.8531	0.43351	0.9995	1	0.86124
54	X95	RM474	0.8509	0.43443	0.9995	1	0.86124
55	X18	LOC_Os02g52210	0.7928	0.4594	0.9995	1	0.87986
56	X83	RM210	0.8042	0.45533	0.9995	1	0.87986
57	X9	LOC_Os02g14900	0.7603	0.47401	0.9995	1	0.88836
58	X40	LOC_Os04g47420	0.7482	0.47956	0.9995	1	0.88836
59	X16	LOC_Os02g51070	0.7153	0.49504	0.9995	1	0.90215
60	X41	RM17377	0.6989	0.50297	0.9995	1	0.90215
61	X87	RM5779	0.6824	0.51136	0.9995	1	0.90287
62	X62	RM20152	0.6289	0.53824	0.9995	1	0.90778
63	X91	LOC_Os09g31430	0.6316	0.53684	0.9995	1	0.90778
64	X92	LOC_Os09g32910	0.6364	0.53436	0.9995	1	0.90778
65	X58	LOC_Os06g14710	0.584	0.56225	0.9995	1	0.92079
66	X64	RM3765	0.5897	0.55939	0.9995	1	0.92079

Table A1 Continued

Rank	Code	Marker Locus	F value	Raw_P	Hochberg p-value	Stepdown Bonferroni	False Discovery Rate p-value
67	X56	LOC_Os05g50810	0.5513	0.58054	0.9995	1	0.92395
68	X59	LOC_Os06g20870	0.5582	0.57652	0.9995	1	0.92395
69	X42	LOC_Os04g56070	0.5265	0.59461	0.9995	1	0.93321
70	X23	LOC_Os03g12470	0.4988	0.61091	0.9995	1	0.94565
71	X52	LOC_Os05g37040	0.4522	0.64063	0.9995	1	0.96774
72	X107	RM202	0.4478	0.64231	0.9995	1	0.96774
73	X19	LOC_Os02g54890	0.4056	0.66926	0.9995	1	0.96957
74	X27	LOC_Os03g24430	0.4091	0.66693	0.9995	1	0.96957
75	X79	RM22694	0.4287	0.65425	0.9995	1	0.96957
76	X3	LOC_Os01g18290	0.2053	0.81524	0.9995	1	0.97388
77	X10	LOC_Os02g19750	0.1909	0.82697	0.9995	1	0.97388
78	X32	LOC_Os03g44484	0.2027	0.81737	0.9995	1	0.97388
79	X45	RM3529	0.1823	0.83401	0.9995	1	0.97388
80	X47	LOC_Os05g06480	0.2313	0.79455	0.9995	1	0.97388
81	X48	LOC_Os05g06480_1	0.3169	0.73024	0.9995	1	0.97388
82	X61	LOC_Os06g29900	0.2234	0.80077	0.9995	1	0.97388
83	X65	LOC_Os06g47340	0.2492	0.78058	0.9995	1	0.97388
84	X75	LOC_Os08g03620	0.1667	0.84706	0.9995	1	0.97388
85	X77	RM22529	0.2263	0.79849	0.9995	1	0.97388
86	X78	LOC_Os08g14210	0.2273	0.79767	0.9995	1	0.97388
87	X80	RM22839	0.1594	0.85322	0.9995	1	0.97388
88	X81	LOC_Os08g24350	0.1664	0.84729	0.9995	1	0.97388
89	X82	LOC_Os08g30820	0.3838	0.68368	0.9995	1	0.97388
90	X84	RM3845	0.2448	0.78402	0.9995	1	0.97388
91	X86	LOC_Os08g45000	0.3405	0.71338	0.9995	1	0.97388
92	X88	RM5526	0.3685	0.69408	0.9995	1	0.97388
93	X90	RM257	0.1753	0.83984	0.9995	1	0.97388
94	X98	RM25366	0.3339	0.71805	0.9995	1	0.97388
95	X102	LOC_Os11g12810	0.1707	0.84371	0.9995	1	0.97388
96	X106	RM6327	0.1639	0.8494	0.9995	1	0.97388
97	X25	LOC_Os03g17700	0.1396	0.8701	0.9995	1	0.9756
98	X103	LOC_Os11g25990	0.1374	0.87199	0.9995	1	0.9756
99	X4	LOC_Os01g41340	0.1225	0.88505	0.9995	1	0.98043
100	X29	LOC_Os03g40270	0.1106	0.89554	0.9995	1	0.98043

Table A1 Continued

Rank	Code	Marker Locus	F value	Raw_P	Hochberg p-value	Stepdown Bonferroni	False Discovery Rate p-value
101	X31	LOC_Os03g42320	0.103	0.90234	0.9995	1	0.98043
102	X24	LOC_Os03g14010	0.0674	0.9349	0.9995	1	0.99663
103	X85	RM3480	0.0728	0.92986	0.9995	1	0.99663
104	X5	RM11307	0.0005	0.9995	0.9995	1	0.9995
105	X8	LOC_Os01g55040	0.0082	0.99182	0.9995	1	0.9995
106	X26	LOC_Os03g21140	0.0406	0.96021	0.9995	1	0.9995
107	X30	LOC_Os03g40720	0.0183	0.98191	0.9995	1	0.9995
108	X38	RM551	0.0308	0.96968	0.9995	1	0.9995
109	X46	RM1024	0.0386	0.96213	0.9995	1	0.9995
110	X104	LOC_Os11g29230	0.0245	0.97583	0.9995	1	0.9995

Table A2. Rank of markers using single marker analysis option (LOD score) in ICIM ver. 4.0.6.0

Rank	Chromosome	Marker Locus	LOD score	PVE(%)	Add	Dom
1	12	LOC_Os12g36030	15.6192	78.6958	0.4530	-0.3234
2	7	LOC_Os07g12130	7.1276	51.4822	0.3945	0.0165
3	7	RM21976	3.0074	26.4779	0.3475	0.0536
4	5	LOC_Os05g33100	2.1907	20.1978	0.0445	0.3962
5	7	RM5420	1.9473	18.108	0.2168	0.3808
6	12	LOC_Os12g13440	1.7974	16.8543	-0.2948	0.1586
7	6	LOC_Os06g37500	1.7646	16.6451	-0.2278	0.1419
8	7	LOC_Os07g47790	1.7413	16.4141	0.2669	-0.0206
9	7	RM3484	1.6224	15.4283	0.1955	-0.2
10	10	LOC_Os10g38489	1.529	14.6016	0.2586	0.0102
11	5	LOC_Os05g37450	1.4926	14.3171	0.0227	0.3419
12	3	LOC_Os03g04220	1.3546	13.1146	0.0084	-0.3339
13	9	RM6839	1.2587	12.2215	0.1658	0.2796
14	5	LOC_Os05g39660	1.2343	12.0269	0.0381	0.3105
15	12	RM1300	1.2275	12.0222	0.1707	-0.1438
16	3	LOC_Os03g53220_2	1.1805	11.545	-0.1403	0.2318
17	1	RM1247	1.1074	10.8572	0.2207	-0.1515
18	2	LOC_Os02g44980	1.1025	10.842	0.0564	0.2845
19	7	RM429	1.062	10.4511	0.1825	-0.0899
20	2	LOC_Os02g48130	1.0477	10.3446	0.0758	0.2761
21	1	RM12276	1.0431	10.2708	0.1890	-0.2183
22	9	LOC_Os09g36420	1.0205	10.0872	-0.1142	-0.209
23	4	RM3531	1.0081	9.9731	-0.1265	0.2133
24	4	LOC_Os04g58920	0.9978	9.8718	-0.1252	0.2076
25	12	RM247	0.8967	8.8997	0.2193	0.0383
26	7	RM248	0.8548	8.5438	0.1592	-0.0891
27	5	LOC_Os05g31062	0.8376	8.3998	0.0344	0.2548
28	1	RM11635	0.8254	8.194	0.0183	0.4584
29	3	LOC_Os03g02050	0.8243	8.2831	-0.0250	-0.265
30	12	RM7315	0.7745	7.8021	0.1481	-0.1259
31	9	LOC_Os09g38640	0.7686	7.7187	-0.1872	-0.0541
32	2	LOC_Os02g52130	0.7677	7.7379	0.0864	0.231
33	3	LOC_Os03g60720	0.7138	7.2344	0.0347	0.2438
34	1	RM1196	0.684	6.9397	0.0868	-0.2118
35	5	LOC_Os05g34880	0.6835	6.9352	-0.0200	0.238
36	6	LOC_Os06g50340_2	0.6637	6.7593	0.0897	-0.1984
37	11	LOC_Os11g47550	0.6562	6.6594	-0.1789	-0.0424
38	8	RM210	0.5888	6.036	-0.1356	0.099

Table A2 Continued

Rank	Chromosome	Marker Locus	LOD score	PVE(%)	Add	Dom
39	10	RM1126	0.5809	5.951	-0.0240	0.2263
40	6	LOC_Os06g50340	0.5587	5.7586	0.0390	-0.2102
41	10	RM1375	0.5093	5.2781	0.1120	0.0977
42	5	LOC_Os05g37040	0.4929	5.1189	0.0134	0.2053
43	6	LOC_Os06g13030	0.4685	4.9054	-0.0547	0.1691
44	3	LOC_Os03g56270	0.4622	4.813	-0.1160	0.0995
45	2	LOC_Os02g44120	0.461	4.8069	0.0210	0.197
46	3	LOC_Os03g53220	0.46	4.7943	-0.1099	0.1175
47	2	LOC_Os02g56320	0.4596	4.7903	-0.0417	0.1988
48	9	RM5779	0.4426	4.6208	-0.0511	-0.1809
49	3	LOC_Os03g51600	0.4372	4.5693	-0.1168	0.1154
50	6	RM3765	0.4346	4.5607	0.034	-0.1889
51	2	LOC_Os02g49986	0.4247	4.4524	0.0556	0.1714
52	8	LOC_Os08g07290	0.4056	4.2638	-0.0574	-0.1893
53	10	LOC_Os10g12080	0.4023	4.2193	-0.0385	0.1889
54	2	LOC_Os02g42870	0.3958	4.1637	0.1119	0.035
55	4	LOC_Os04g40150	0.3925	4.1468	-0.1263	0.1101
56	10	RM474	0.3677	3.8946	0.0363	0.1697
57	6	LOC_Os06g23190	0.3673	3.9043	-0.1090	0.0696
58	2	LOC_Os02g52210	0.3416	3.6388	0.0186	0.1797
59	2	LOC_Os02g14900	0.3271	3.4949	0.0836	0.1195
60	4	LOC_Os04g47420	0.3215	3.4412	-0.1156	0.0496
61	2	LOC_Os02g51070	0.3067	3.2951	0.0155	0.1714
62	11	RM202	0.3013	3.2571	0.0647	-0.1232
63	5	LOC_Os05g50810	0.2993	3.2235	0.0121	0.1647
64	4	RM17377	0.2992	3.2218	-0.082	0.125
65	9	LOC_Os09g32910	0.2724	2.9422	-0.1089	-0.0546
66	9	LOC_Os09g31430	0.2698	2.9209	-0.1113	0.0612
67	6	RM20152	0.2668	2.9088	-0.0305	0.1422
68	6	LOC_Os06g14710	0.2414	2.7068	-0.0750	0.082
69	6	LOC_Os06g20870	0.2349	2.5906	-0.1045	-0.0615
70	2	LOC_Os02g54890	0.2342	2.5717	0.0239	0.1319
71	4	LOC_Os04g56070	0.221	2.447	-0.0894	0.0546
72	3	LOC_Os03g12470	0.2102	2.3214	-0.0412	0.1302
73	3	LOC_Os03g38260	0.1894	2.1356	-0.0764	-0.0781
74	9	RM5526	0.1838	2.0556	-0.0803	-0.0535
75	8	RM22694	0.1762	2.0019	-0.0083	-0.1361
76	8	RM22529	0.1596	1.8285	0.0705	0.0549
77	3	LOC_Os03g24430	0.1581	1.9122	0.0939	-0.1518

Table A2 continued

Rank	Chromosome	Marker Locus	LOD score	PVE(%)	Add	Dom
78	8	LOC_Os08g30820	0.1567	1.7961	0.0514	0.1168
79	8	LOC_Os08g45000	0.1368	1.5968	-0.0619	-0.0664
80	10	RM25366	0.1338	1.5665	0.0838	-0.0145
81	5	LOC_Os05g06480	0.117	1.406	-0.0627	-0.0384
82	5	LOC_Os05g06480_1	0.1	1.2255	0.0381	-0.0782
83	6	LOC_Os06g47340	0.094	1.1738	0.0551	-0.0563
84	8	RM3845	0.093	1.1531	-0.009	-0.0984
85	8	LOC_Os08g14210	0.084	1.0718	0.0235	-0.0817
86	1	LOC_Os01g18290	0.0736	0.9688	0.0614	0.0118
87	3	LOC_Os03g44484	0.073	0.9566	-0.0554	-0.0243
88	6	LOC_Os06g29900	0.0682	0.9209	-0.0657	-0.0311
89	2	LOC_Os02g19750	0.0647	0.9016	0.0596	-0.0419
90	5	RM3529	0.0633	0.8614	0.0031	0.084
91	11	LOC_Os11g12810	0.0577	0.8068	0.0104	-0.0792
92	11	RM6327	0.0572	0.8179	-0.0683	-0.0544
93	8	LOC_Os08g03620	0.0565	0.7881	0.0014	-0.081
94	8	LOC_Os08g24350	0.0558	0.7869	0.0225	-0.0671
95	8	RM22839	0.0525	0.7539	-0.0208	-0.0832
96	3	LOC_Os03g17700	0.0437	0.6612	-0.0291	-0.058
97	11	LOC_Os11g25990	0.042	0.6507	-0.0531	-0.044
98	1	LOC_Os01g41340	0.035	0.5803	0.0495	0.0086
99	3	LOC_Os03g40270	0.0292	0.5244	0.0099	0.0628
100	3	LOC_Os03g42320	0.0257	0.4886	0.0066	0.0617
101	9	RM257	0.0255	0.4741	-0.0469	0.0035
102	8	RM3480	0.0132	0.3461	0.0298	-0.0286
103	3	LOC_Os03g14010	0.0105	0.3205	-0.0357	0.021
104	1	RM11307	0	0.0662	0.0029	-0.0232
105	2	LOC_Os01g55040	0	0.0392	-0.0135	0.0079
106	3	LOC_Os03g40720	0	0.087	-0.0071	0.0265
107	3	LOC_Os03g21140	0	0.1934	-0.0284	0.0127
108	4	RM551	0	0.1468	0.0290	-0.0058
109	5	RM1024	0	0.1838	-0.0243	0.0241
110	11	LOC_Os11g29230	0	0.1165	-0.0178	-0.0291

APPENDIX B. SSR AND SNP PRIMER SEQUENCES USED IN QTL MAPPING OF 2008S/CL131

Table B1. SSR markers from the Universal Core Genetic Map.

No.	SSR Markers	Chr	Position start	Position end	Forward	Reverse
1	RM1247	1	1655880	1656025	TTCTCAGCTGCTTGTGCATC	CCTCCAAGGTAAAGGGGTTC
2	RM1196	1	22230958	22231393	AGCTGCCGTGAGCCTCAAG	TCCAAAACGCTCTCTTCGTC
3	RM11307	1	24257472	24257619	AAAGCTCTGCAATCTTCTCTCC	GAATACGACATCAGAACAGTGC
4	RM11635	1	30878124	30878417	TTGTCACCCTTACTAGGATCAGC	GTGTGACTCTTGATGTAACTCAGC
5	RM12276	1	43371168	43371356	GTCGACGGCTTCCTCAAGATTGG	TGAGACCTCTGTGAAGGCACTCG
6	RM3529	4	91445	91850	CGCGCCACCTCGATATATAC	GCTCAGGTTAACCAAGGTGG
7	RM551	4	168674	168859	AGCCCAGACTAGCATGATTG	GAAGGCGAGAAGGATCACAG
8	RM1024	4	1212762	1213054	GCATATACCATGGGGATTGG	GGGATTGGGATAATGGTGTG
9	RM17377	4	28931144	28931312	ATATTACTTCGACGCTGGATCAGG	GTCAGTTCGTCAGGCACAACG
10	RM3531	4	35177538	35177688	TATCGCCACTCGTCCATTCTCC	CAGCTACCAACACCTCCATACCC
11	RM20152	6	19542217	19542406	GGGTGGGCGTATTATAGATTCAAGG	TTAGGCCCAATAGTGGAGGAAGG
12	RM3765	6	29722485	29722669	AGGTCTTAGGCCTCCCTGAG	TGGATGCTTCCAATCCTCTC
13	RM3484	7	4418197	4418381	TCCGGTCGTCCTCATCGTATCC	GCCCTCTTGCTCCCACATCG
14	RM5420	7	19356355	19356477	CCTGATCTCAACACACACGC	GAAGTCTTGTTGCGCGTATG
15	RM21976	7	25589679	25589873	CTTCCTCCTACCTTCCTCCATCC	GCACCATCACCTCCATCTCTAGC
16	RM429	7	26805357	26805467	TCCCTCCAGCAATGTCTTTC	CCTTCATCTTGCTTTCCACC
17	RM248	7	29339144	29339414	TCCTTGTGAAATCTGGTCCC	GTAGCCTAGCATGGTGCATG
18	RM22529	8	5182161	5182326	TGCGAGTATTTAACTCACCCATCC	CTTGCCTCACAAGATCCAAACC
19	RM22694	8	8963832	8963986	TTAGCTGTATTTAGCCCGACATAGCC	CGCCGGTTCTTCTCCTCTTAGG
20	RM22839	8	12402657	12402916	ACGGTACGGATTATAGCGATGC	ACGCTCATCAACCGAGAAAGG
21	RM210	8	22341749	22341902	TCACATTCGGTGGCATTG	CGAGGATGGTTGTTCACTTG
22	RM3845	8	24629788	24630035	AGCTCGATCTCCTCTCTAGACC	GCTTCAGCCTTCAGGTCAAC
23	RM3480	8	27260326	27260513	GTGCCAAGGAGATTGGATTG	ATGGTCTGCAACTCTGCATG

Table B1 continued

No.	SSR Markers	Chr	Position start	Position end	Forward	Reverse
24	RM5779	9	3802710	3802855	ATCGAACCATCCAGGATGAC	TTGCACAAGAGGCAACACTC
25	RM5526	9	7313032	7313202	TCAGCCTGGCCTCTCTTATC	ATGATCCTCCACCCACTAGC
26	RM6839	9	14566026	14566255	GAACAGAGGAGGAGATCGAGAGG	CTTCTTGGGAGATGCAGAAATGG
27	RM257	9	17719681	17719852	CAGTTCCGAGCAAGAGTACTC	GGATCGGACGTGGCATATG
28	RM474	10	1804009	1804200	AAGATGTACGGGTGGCATTC	TATGAGCTGGTGAGCAATGG
29	RM1126	10	9439397	9439643	AGAAAAGGCTGCATCAGTGC	TCCAACGACAGACTGTACGG
30	RM25366	10	13212303	13212407	TCGGTCTCTGTGCCGTGATTAGG	CACCAGCGCAGCAACTAACATCC
31	RM1375	10	16386736	16387024	CTACACGCGCAAACTCTGTC	ATGAAGGTCTAGGCTGCACC
32	RM6327	11	364087	364425	CAGCCTAGGGCGTCATAGAC	GATTGGGTGATGGATAGCAC
33	RM202	11	8984931	8985221	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA
34	RM7315	12	2181917	2182069	CACAAAGGCGTGTGGGTTAG	GAGTCACGGGATGTTGCC
35	RM247	12	3185384	3185581	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG
36	RM1300	12	25965402	25965555	CAGCCATGAATGTTGGCTAC	GCCATGTCCATTTATGGTGC

Table B2. SNP marker primer sequences used for QTL mapping of 2008S/CL131 F_2.

No.	Locus	Chr	SNP	Position	Forward	Reverse
1	LOC_Os01g18290	1	Ref	10271979	CCGTTAACCTGCACCACGAGATGTG	TTTTATCATGAGAATCAGCGAAGGTATAGTCAGCT
			Alt		GCCGTTAACCTGCACCACGAGATGTA	ATTGTGAACCTCAGCTGTGCGGCT
2	LOC_Os01g41340	1	Ref	23392669	ATCTGGAGGCCGATGTTAGTGATGAGTGT	CCAGCCTACATACCACATGCAGTAAGCC
			Alt		CATCTGGAGGCCGATGTTAGTGATGAGTAC	AGGACAAAAGCCTATTCTCTTGCTCTTTTGATC
3	LOC_Os01g55040	1	Ref	31644508	AGGATGCCATAAAATGCCCAGATGAGA	GACGAAGCTGGTCCTTGTTGTACAGAACAT
			Alt		GGATGCCATAAAATGCCCAGATGATG	GCGCTCTAAAAAGTTTTTCCAATCATCTTCATAA
4	LOC_Os02g14900	2	Ref	8316395	GGTTGAGCTCAAAGCACCAGCTCG	CAAAACTTGAACGATCTGCACCAGCA
			Alt		CGGTTGAGCTCAAAGCACCAGCTAA	GTGAACCCATTCCGTAGTCGTAAGCATG
5	LOC_Os02g19750	2	Ref	11553574	CTAGCCAGATCAGCAGGCTGCCA	TCCTCGCTGGAGTTGAGAATGGTGA
			Alt		CCATTTCTAGCCAGATCAGCAGGCTGTAG	GTCTTGAGGCGGAGGAGGAAGTTTAGC
6	LOC_Os02g42870	2	Ref	25773316	CACACTGTGCATGGCTCCTCGCT	TTAATACTTCGCGTAGGTGTTCAAACGTAAAATGT
			Alt		CACTGTGCATGGCTCCTCGCG	AATTAATCTAGCATCACATTGTTAAATCATGGAGCA
7	LOC_Os02g44120	2	Ref	26663691	GCTGTGCAACACGCGACGAAGA	CAGGTGCGGCCTTGACGAGG
			Alt		TCGTGATGCTCTCTTCTTCTTCGTCGA	AGTCTCGTTGCGGCTGCTCTCG
8	LOC_Os02g44980	2	Ref	27237273	CATGTTTTCTATTTGCCGATCCATCACAC	AGGCACCAAGGATGCGCAAAATTTA
			Alt		CATGTTTTCTATTTGCCGATCCATCACAT	CGCTGATGCTTTACAAGACGAGCTCAG
9	LOC_Os02g48130	2	Ref	29460692	CCTTGACCGGCTAAAGAAAGTGGACAC	AATCATACACTACCACATCAAATTCCTCTTGTGAAC

Table B2 continued

No.	Locus	Chr	SNP	Position	Forward	Reverse
			Alt		CCTTGACCGGCTAAAGAAAGTGGACAT	ACCTGTCTTTTGTGTAGCACCAGCATTAAGTTT
10	LOC_Os02g49986	2	Ref	30534494	CCTGAAGCAGCAGCAGCAGCAGTAT	AGCCAGCCCAGTCAATGTCAAGAATG
			Alt		CCTGAAGCAGCAGCAGCAGCAGTAG	TGATCAAAGCCAACGTCAGAGCATTG
11	LOC_Os02g51070	2	Ref	31231826	ATGGCGTCGCGAAGGCGAATA	ATCAAGCAACTGTAGTTCTAGCAACATGGCTC
			Alt		ATGGCGTCGCGAAGGCGAAAG	AGCGAGGTAACATTTCCGAATAACAAATGG
12	LOC_Os02g52130	2	Ref	31905607	CGTGTACCTAAGCTTGCAGTTGCAGTCAC	ATCTCTCCATAAAACACTCCCAACCAACAGAT
			Alt		GTGTACCTAAGCTTGCAGTTGCAGTCGT	TCTTGTCCAATAGATGAGGCAGCAGATTG
13	LOC_Os02g52210	2	Ref	31961689	CAAGGTCCAAATCCAACTGGCACG	CAGCGTGCGGAGTCGTACTAAATAGTAAAGG
			Alt		CCAAGGTCCAAATCCAACTGGCAAA	GTGCGCGTGCATCCTAGCTCG
14	LOC_Os02g54890	2	Ref	33610607	TGGGAGCAGTGGGGCAACAAGA	ATGGATGGCAAGTGGATTCCTTTAACAGA
			Alt		TGGGAGCAGTGGGGCAACAATG	GTTGCTATTTCAAATGGATTGGATCGGTTT
15	LOC_Os02g56320	2	Ref	34470208	GCATTGTAAGTTCACAATCTCTAGGACACTACTGAG	TGCATCATTCACAGCTTCCTAATAACGGA
			Alt		GCATTGTAAGTTCACAATCTCTAGGACACTACTTTC	AAACAACTGGACTTACAGGATTGTTTATGGGC
16	LOC_Os03g02050	3	Ref	649831	TTGCCAGCTCTGATCCTATATCGATCTCC	ACATTACTTTCAACACCCTGCGTCCTTTTT
			Alt		GCCAGCTCTGATCCTATATCGATCCCG	GCTGGCCCCAAATAGCGTGACA
17	LOC_Os03g04220	3	Ref	1938015	GGAACGGGAACGGCAACGGTT	GGGAGGTGGGACAGGTCGGC

Table B2 continued

No.	Locus	Chr	SNP	Position	Forward	Reverse
			Alt		GAACGGGAACGGCAACGGGA	GTACTCCCACGACTCGCGGCTG
18	LOC_Os03g12470	3	Ref	6592478	CGACAGGATCGGGAAAGGCACC	TTAGCGGTCCCCCAAGACTTGTCC
			Alt		CGAGCTTCACCTCGTTCATCACGAA	TTAACCGCAATTGTAATTGGATTGCATGAT
19	LOC_Os03g14010	3	Ref	7599183	AACGAGAAGATGACGAAGCTTGAACC	TAACTGAGCATGATACATTCGGACATAACCAAG
			Alt		AACGAGAAGATGACGAAGCTTGCAGT	CCTGAAAATGCCATGTTAGGCCTGTGTAATTT
20	LOC_Os03g17700	3	Ref	9848183	CTTTTTAATAGCTGCCTTCGCCTCTCGTAC	CTGGTACTTGTTCGTCACCTCGAACTTGTT
			Alt		TTTAATAGCTGCCTTCGCCTCTCGTGT	CAAGAAATCTAGGGCAGGCAGGGAAA
21	LOC_Os03g21140	3	Ref	12047872	GCACTCCAATAGCACAAACGAGATGACG	CAGGCAGACCGACCGAAGTAACACAT
			Alt		AGCACTCCAATAGCACAAACGAGATGAAA	CGACCAAGAAATTACAAGGATCAGTAGTGTCG
22	LOC_Os03g24430	3	Ref	13913667	GATGGAGAAATTGTCTCGGCACTGAAG	GAAGTCTGAACTACATGCACACACTGAAATTAGTTG
			Alt		TGGAGAAATTGTCTCGGCACTGCTC	CAGCTTAGCGTGTCATTAGTGTGCTATTTGG
23	LOC_Os03g38260	3	Ref	21237842	GAGAGGAAGATTGCCAGGCGTGTC	CCAGGCAAATATGCTTTACCATGGAACAT
			Alt		GAGAGGAAGATTGCCAGGCGTCAG	TCTTGGGCCCTGTTTGTCATCATAAAAGT
24	LOC_Os03g40270	3	Ref	22380154.5	CAAGTAGATTGATCCTGGGAGCTTGTCTG	ATAACACCATTTAGCAACCAAAGCACGACTATC
			Alt		AAGTAGATTGATCCTGGGAGCTTGTGCC	AATGAGGGCCTTGACCATTCGTCC
25	LOC_Os03g40720	3	Ref	22642198	GCCGGAGGTGGCCAACTACTGGTA	CCGGAGGTGGCCAACTACTGGAG

Table B2 continued

No.	Locus	Chr	SNP	Position	Forward	Reverse
			Alt		TGGTCCCAGTCGAACTTGTTCATCACTAG	TCGTAGGCGTCCCACGACACG
26	LOC_Os03g42320	3	Ref	23544941	CCAATCTCTTCACTGAGGCCCCG	TTGGCAAGCTGGGCCTCAATTTC
			Alt		CCAATCTCTTCACTGAGGCCCCC	GTCAATTCTGGGAGCGAGTTCACAGC
27	LOC_Os03g44484	3	Ref	25030823	GGCATCGCCTAGATGCAGGTTCC	TCTTTGACCGCAATTGGCTGAATATGA
			Alt		GGCATCGCCTAGATGCAGGTCGT	ACTTTAATAGCACCTAATTTATCTCCGTTCCCGTTA
28	LOC_Os03g51600	3	Ref	29521388	GCCTCATGTACCGCGGCGCT	CCTCCATGCCCTCACCCACGTA
			Alt		GCCTCATGTACCGCGGCCAC	CTCCTCGTAGTCCTTCTCCAACGCG
29	LOC_Os03g53220	3	Ref	30523344	TTCTCCTTCCTGCTCATAGATTGCTCGA	CGACGATGTCCTGCAGCTGGG
			Alt		GAGATTCTCCTTCCTGCTCATAGATTGCTCTT	TCAAGAACTTGGAAAGCCATGTCGATG
30	LOC_Os03g53220_2	3	Ref	30523344	GATACACATGGAGAGGGAATAATACTAACTGACCAA	CTGTTTCCATAATATCTGCTGGTAAACCGTACAAC
			Alt		GATACACATGGAGAGGGAATAATACTAACTGATCAC	CCTAAGAGAGCATAGTGGGATTGTGGAAATG
31	LOC_Os03g56270	3	Ref	32076293	ACATCGGCAACGAGACGAAACAGAA	TTTGCAGATACAGATAGAGAAACAGAAGCACCTC
			Alt		ACATCGGCAACGAGACGAAACAGAG	CAAATTCTTGGGGAACCTTTCTGTTCTTTG
32	LOC_Os03g60720	3	Ref	34502302	CACACAGCCTCCTCACGCGTAGTAAAGT	CCTTTTTCGCTTTCCGCTGTGTTGT
			Alt		CACAGCCTCCTCACGCGTAGTAAAGC	GAGTCAAGCGTGTAGTTGGGAGGAGTAGTAGAAT
33	LOC_Os04g40150	4	Ref	23705609	CGGGCTTTGTGTCGAAGGGC	GCTGCTCCGTATATGCACTGAATAATCTGG
			Alt		TGCGGGCTTTGTGTCGAAGACA	ACTTGGGCCCTCCATTTGGCC

Table B2 continued

No.	Locus	Chr	SNP	Position	Forward	Reverse
34	LOC_Os04g47420	4	Ref	27954240	GTCCTGCAACACACACACAGCGAA	TGGGTGAGGTCGGCCAAATGAC
			Alt		GTCCTGCAACACACACACAGCGAG	GGGATGGGGACGCCGAACAT
35	LOC_Os04g56070	4	Ref	33207408	CTTGCCAGATCTACAAATTGCTGCTACAACTAG	TATGAATGGTACACAGTGCTCACAGGAATCA
			Alt		CTTGCCAGATCTACAAATTGCTGCTACAACTTA	TGGAAATCATCAGGATTTGTCAGTGCATACT
36	LOC_Os04g58920	4	Ref	34867120	ACTAAGGCGGGTCTGTCGATGTGGTAAT	GCCAAACCGCCATTGTTCTCGA
			Alt		AAGGCGGGTCTGTCGATGTGGTAAC	GGCTCGTGACCTTCTAGTAGAAATCCTGCTTA
37	LOC_Os05g06480	5	Ref	3335474	TGAGCATTAGGGTTCCTTAACCAGTTGAATC	TAGTTTAGAAAAGCGTGCACGCGAA
			Alt		GAGCATTAGGGTTCCTTAACCAGTTGCAAT	ATTAAGAGGCTGTGTTTGGGGGAGAGG
38	LOC_Os05g06480_1	5	Ref	3335496	TGCATTATCCCGAGCTACAATTATTACTCTTATGGA	TAGTTTAGAAAAGCGTGCACGCGAA
			Alt		TGCATTATCCCGAGCTACAATTATTACTCTTATCTA	AAGAAGTACATCGAGGCCGGGGC
39	LOC_Os05g31062	5	Ref	17995322	CCATGGTGCCCACCATTTGTTCTCTAT	ATTGGTTGATGTCCCACTCCCAAACA
			Alt		CCATGGTGCCCACCACCATTTGTTCTCTAC	ATAGGGAGCCTGACATTTATATCCTTCATGTTAGC
40	LOC_Os05g33100	5	Ref	19349759	GATCAGCAAAGGTGGTGGTAGAGTTAGCG	ACTTACCCCTGACTTTGAAGAAAGAACCTGC
			Alt		ATGGATCAGCAAAGGTGGTGGTAGAGTTAGAA	TGACAGTTTGGCACTATGGTAAATCTTGAGAACT
41	LOC_Os05g34880	5	Ref	20661893	GGAGTTGTCCTCAATGTGCTCTAACCTTTG	AATACTCCAATATGTTATACCTCCAATGGCTAATCC
			Alt		GGGAGTTGTCCTCAATGTGCTCTAACCTTTA	TTGAACATGAAATTGTCAAAGGATCTAAACACTAGG

Table B2 continued

No.	Locus	Chr	SNP	Position	Forward	Reverse
42	LOC_Os05g37040	5	Ref	21585027	TCGAGAGTGCACTCGTGGCATTGT	CGTGGATCGGATATGTACATGTGCTCC
			Alt		CGAGAGTGCACTCGTGGCATTGC	AAACAGTTCATCGATAATAGCAAGGGAAAATGAC
43	LOC_Os05g37450	5	Ref	21853461	GCGGTCATGCAAAATGAATCTGGTG	AAAAGGATCACATTTCAATCAGCGAGCATA
			Alt		AATCTAGCGGTCATGCAAAATGAATCTAGCT	CAGTCACTACTTTGCATCATGACTCCTCTAAGCTA
44	LOC_Os05g39660	5	Ref	23220648	GGAGAGGACGCTCGCTCTGACATC	AGAGGGAGTGGAGAAAATTTAGATCATTGGTATAGG
			Alt		GGAGAGGACGCTCGCTCTGACACT	TTAAATGCAAGGAAAAACCTCAGAGAGAGAGG
45	LOC_Os05g50810	5	Ref	29079709	GATGGAGAAATTGTCTCGGCACTGAAG	CATTAGATTCGTCTCACAAAATAGCCTAGGGG
			Alt		CTTGGCACTAAAATTTCGGAATCAGAATTGAT	TAGCCCTCCGGAATCTTGCTATTTAGGAGTA
46	LOC_Os06g13030	6	Ref	7133249	AAAGACACCTGAAAGCAGGGGAACG	GATCTGTGATTTGTTATCAGCAGTCACATTGG
			Alt		CAAAGACACCTGAAAGCAGGGGAAAA	GTATCAGCTTGCGATTTATTTGCAGTAAGTGGTA
47	LOC_Os06g14710	6	Ref	8282369	CGTCGAGGCGCAGGCAGAAGA	GGAGGTGCGTCGTCCGCATC
			Alt		GTCGAGGCGCAGGCAGACGC	GGTTCATTTCATTCATCCATACCCACGAT
48	LOC_Os06g20870	6	Ref	12055335	TGCAGAAGGGCTTTGATGGAACCAATA	TCGACTGCAGGCTGATAAAACACTAACAAAA
			Alt		AATGGCATCGTTGTAACAATATCATATGCTTCAT	TGCAGAAGGGCTTTGATGGAACCATAG
49	LOC_Os06g23190	6	Ref	13542620	ATGGCCGTTGCTATTGCAGTTGACA	CCATCTTATGCAGATCCAACACCTTGATG

Table B2 continued

No.	Locus	Chr	SNP	Position	Forward	Reverse
			Alt		GCCGTTGCTATTGCAGTTGCCC	ATAAACCAATGCCCTGAACTCATTGCCT
50	LOC_Os06g29900	6	Ref	17221736	GACGTACCGCAGCAGCGGGC	ATTCACTGATTCTCCCTCCACACCCATAA
			Alt		GCCGTTGCTATTGCAGTTGCCC	ATAAACCAATGCCCTGAACTCATTGCCT
50	LOC_Os06g29900	6	Ref	17221736	GACGTACCGCAGCAGCGGGC	ATTCACTGATTCTCCCTCCACACCCATAA
			Alt		TGACGTACCGCAGCAGCACGA	TGCAGAGCGGCAGCTGACACATA
51	LOC_Os06g37500	6	Ref	22193906	TCTCATCGTCACTAGCTTCCTCTCTACTGTGC	TGAAGGGTGTCGGCTGAGAGAGTGATA
			Alt		CATTTCTCATCGTCACTAGCTTCCTCTCTACTATCA	CATCCTATCAACCGACACGTCGATACG
52	LOC_Os06g47340	6	Ref	28694837	CACCAACGCCACCGCGTTCC	TCCATCTTCCTTTCCCGGATCACC
			Alt		CACCAACGCCACCGCGTACA	CTTCACTAAGTCGTGGCTGGTCTGCTG
53	LOC_Os06g50340	6	Ref	30472113	GTGGAGCTCACCGACAACCTGCTAAC	GTCTGGAATGGCGCCGGTGA
			Alt		GTGGAGCTCACCGACAACCTGCTAAT	CGCCGGTGAGCCTGTTCCTG
54	LOC_Os06g50340_2	6	Ref	30473110	GCTCACCGACAACCTGCGCAC	GTCTGGAATGGCGCCGGTGA
			Alt		GGAGCTCACCGACAACCTGCTGAT	CGCCGGTGAGCCTGTTCCTG
55	LOC_Os07g12130	7	Ref	6781538	TGCACAGAGAAAGAACTAGGATCCCTTAGAGC	ACGCAAGCTGCAGGTAGCAACGA
			Alt		AAATTGCACAGAGAAAGAACTAGGATCCCTTACATA	CCCAGGGTTGGTCAAAGGAGGGT
56	LOC_Os07g47790	7	Ref	28540477	CAGTAATAACTAGCTACCATGCATGCTATTGCCAT	TGGGGAAGTTGACCTTGGCCTTG
			Alt		CAGTAATAACTAGCTACCATGCATGCTATTGCTTC	GGGTCGTCGAGTGGCGGGTC
57	LOC_Os08g03620	8	Ref	1710029	AGCAGACTAGAGCACTGTGGCTTACCATACA	AATCCACCTGACTCCAGTGTGTAGACAACC

Table B2 continued

No.	Locus	Chr	SNP	Position	Forward	Reverse
			Alt		CAGCAGACTAGAGCACTGTGGCTTACCATACT	CAGCATCACCTCGGCCCCTG
58	LOC_Os08g07290	8	Ref	4070113	GCATGCAAAAACCAGAGAAACAATTCTACGT	GCTTCTTCCAGAAGACCAGACGCAATAATT
			Alt		GCATGCAAAAACCAGAGAAACAATTCTATCC	GCAACTGGATGCATATGAACCATAAGTCAAGTA
59	LOC_Os08g14210	8	Ref	8494086	TGGGCGTATCAAAACCGACTGGTGT	AATACTTCTACTGTGACCCGCACTCTGGG
			Alt		GTGGGCGTATCAAAACCGACTGGTAC	GGATCAATATATGGGTGCGAAGGGGTT
60	LOC_Os08g24350	8	Ref	14695765	GACTGGAGAAGAAACCTGCAAGAGTACTCCAT	GCGTGTTCTCGTGCACCTTGGTC
			Alt		GACTGGAGAAGAAACCTGCAAGAGTACTCAGA	CCTACCATCTCCCACGCGATATTGG
61	LOC_Os08g30820	8	Ref	19029711	TTATCGGTAGCTGGACATCAGGATGAGG	GGATGAACCGGAGTGTATGTCAAGCTCTT
			Alt		AAGTTATCGGTAGCTGGACATCAGGATGAGA	CAAATTGGCGAGACACCGGCA
62	LOC_Os08g45000	8	Ref	28242684	GCTTCTTCATGATGACCCTCTTCATGCTTA	GGCTCTGCGCCAGGTACAGGAAC
			Alt		TCTTCATGATGACCCTCTTCATGCTGG	ACATCTCCTCCAGCGACTTCCCCTT
63	LOC_Os09g31430	9	Ref	18905063	GGACCCTATCGTGCATGGTGACTACCTA	CACGTACGCACCTGTGGACCGAT
			Alt		CCCTATCGTGCATGGTGACTACCGG	AGTTGAAGAAGATCGGTGTAAATTCCTAGACATGA
64	LOC_Os09g32910	9	Ref	19616203	GTTGGCTTCAGATTGCTGTTACACCGTT	TAAGTTCCAACCGCTCCAAAGCATAAGAA
			Alt		CAGTTGGCTTCAGATTGCTGTTACACCTTC	AGATTTGGAGCACTATTCTCTATGACTTGCAGC
65	LOC_Os09g36420	9	Ref	20572452	CAAGGCCATCCTGTTTGTACCCAAGTG	ACGCATTTCTTCACAAGGTTCTTGCG
			Alt		TCAAGGCCATCCTGTTTGTACCCAAGTA	AACTCATCACCACTCTTGGTGGAGTGGTAC

Table B2 continued

No.	Locus	Chr	SNP	Position	Forward	Reverse
66	LOC_Os09g38640	9	Ref	22222793	CGAGGAGTCGAAGAAGAAGATGGACGAT	GCACGTGATCCTACGAGCTGTCGTC
			Alt		AGGAGTCGAAGAAGAAGATGGAGGCG	TGTTGGCCCAACCAATGGATCAA
67	LOC_Os10g12080	10	Ref	6731557	TCGTCGACGCCCTGTTCACCTT	TGGGTGCAGCCGGAACGACT
			Alt		GTCGACGCCCTGTTCACCGA	GCTACTCGAGCGGGTTGTCCCAG
68	LOC_Os10g38489	10	Ref	20489019	ACCCGTACCGTACGTTAGCTAGTCCTGACA	AGACAATAAGCTTCAGCCCGGTTTATATATTTTTGTAT
			Alt		CCCGTACCGTACGTACGTAGCTAGTCCTGTGG	ATTGTACACTAATTTTCGTAGACGGACTATCCGC
69	LOC_Os11g12810	11	Ref	7254467	CGCCAACCTGGTACGTGATATGACAAG	GGTCTGCTTGTGGTGCTTGGGGTA
			Alt		CGCCAACCTGGTACGTGATATGACAAA	TTTGCAACGCTGCCATTGCTACAG
70	LOC_Os11g25990	11	Ref	14385352	CCAGAGTGGCGCGAAGGCGT	CGAGGCAGTGGCGTATCTCCTCC
			Alt		CCAGAGTGGCGCGAAGCCTC	ACCACTCCACCACCATCCCGC
71	LOC_Os11g29230	11	Ref	16479181	CATGGCGAAGACGGGCGCTA	GCTTCCCCAAATCGACATCGCA
			Alt		CATGGCGAAGACGGGCGCTG	CCTCCAGAACGCCCTCAACGC
72	LOC_Os11g47550	11	Ref	28213265	ATGAAGAAGTCGATGCCGTCGAACG	CTACAACACCGTCATCATCTCCTTCCTCA
			Alt		CGGCCATTCGGCAACGCTGT	TACCTGTCCCAGATCATCTTGCCACC
73	LOC_Os12g13440	12	Ref	7522959	GGATGGGGTCGATGATGGCCTAA	ATGATATCAATGACAGCATTTCATCTATAGGAG
			Alt		GGATGGGGTCGATGATGGCCTCT	ACCCACTGTTCTTTGCTCCATCGATTC
74	LOC_Os12g36030	12	Ref	22054886	GTGTTGATAAAAATTTTACTCTTGATGGATGGGAG	CAAATCCTTTAGCATTTTTAACCCGAAAACTTG

APPENDIX C. SAS CODES FOR SINGLE MARKER ANALYSIS GENERATING P-VALUES RANKED ACCORDING TO THE HIGHEST SIGNIFICANCE.

```
libname Mylib "C:\Users\cdeguzman\Desktop\sterile" ;   run ;

%macro TTT (SNP) ;

proc glm  data=MYLIB.sterile  outstat=&SNP  noprint ;
     class &SNP ;
     model sterile = &SNP / ss3 ;
     quit ;
RUN ;
data &SNP ;   set &SNP ;
     if (_type_ = 'SS3') ;
     Locus = _source_ ;
     keep Locus F Prob ;


%mend TTT ;
%TTT   (X1)
%TTT   (X2)
%TTT   (X3)
%TTT   (X4)
%TTT   (X5)
.
.
.
%TTT   (X122)
data SNPsRanking ;   set
X1
X2
X3
X4
X5
.
.
.
X122;
ttt+1 ;
Test = compress ("test" || ttt) ;
Raw_P = Prob ;
drop ttt Prob ;

proc multtest  pdata=SNPsRanking  holm  hoc  fdr  out=Mylib.SNPsRanking
noprint ;

proc sort  data=Mylib.SNPsRanking ;   by fdr_p ;

ODS  html  file='jsGLMforSNPs.htm'  style=Journal ;
proc print  data=Mylib.SNPsRanking  label ;
     var Locus F Raw_P hoc_p stpbon_p fdr_p ;

Run;
ODS  html  close  ;
RUN ;
```

APPENDIX D. GENOTYPE OF VARIETIES AND POTENTIAL MALE STERILE BREEDING LINES FROM LSU RICE RESEARCH HYBRID BREEDING PROGRAM.

No	Variety/Line	Pedigree	136 bp deletion	SNP A/C	Genotype
1	Nipponbare	-	no	A	S5j S5j
2	Catahoula	-	yes	C	S5n S5n
3	2008S	-	yes	C	S5n S5n
4	Jasmine 85	-	no	C	S5i S5i
5	Cypress	-	yes	C	S5n S5n
6	LaGrue	-	yes	C	S5n S5n
7	Tequing	-	no	C	S5i S5i
8	Lemont	-	yes	C	S5n S5n
9	Mermentau	-	yes	C	S5n S5n
10	Lemont	-	yes	C	S5n S5n
11	MY2048	-	yes	C	S5n S5n
12	CL 114	-	no	C	S5i S5i
13	CL 151	-	yes	C	S5n S5n
14	CL 161	-	yes	C	S5n S5n
15	Cocodrie	-	yes	C	S5n S5n
16	Wells	-	yes	C	S5n S5n
17	Bengal	-	no	A	S5j S5j
18	CL131	-	yes	C	S5n S5n
19	Shufeng	-	no	C	S5i S5i
20	Trenesse	-	yes	C	S5n S5n
21	CIAT6	-	no	C	S5i S5i
22	CIAT2	-	no	C	S5i S5i
23	MCR	-	no	C	S5i S5i
24	O8A	-	no	C	S5i S5i
25	793s	-	no	C	S5i S5i
26	805s	-	no	C	S5i S5i
27	1-14-2005	2009S-2-5F5	yes	C	S5n S5n
28	1-15-210	2009S-2-5F5	yes	C	S5n S5n
29	1-16-2010	2009S-5-1F5	no	C	S5i S5i
30	1-16-2002	2009S-5-1F5	no	C	S5i S5i
31	1-18-2002	2009S-5-1F5	no	C	S5i S5i
32	1-19-2013	2009S-5-1F5	no	C	S5i S5i
33	1-23-1930	2009S-5-1F5	no	C	S5i S5i
34	1-26-1943	2009S-5-1F5	no	C	S5i S5i
35	1-29-1954	2009S-5-1F5	no	C	S5i S5i
36	1-31-1963	2009S-5-1F5	no	C	S5i S5i
37	1-34-73	2009S-5-1F5	no	C	S5i S5i
38	1-34-75	2009S-5-1F5	no	C	S5i S5i
39	1-35-79	2009S-5-1F5	no	C	S5i S5i

APPENDIX D continued

No	Variety/Line	Pedigree	136 bp deletion	SNP A/C	Genotype
40	1-35-80	2009S-5-1F5	no	C	S5i S5i
41	1-40-99	2009S-5-1F5	no	C	S5i S5i
42	1-41-101	2009S-5-1F5	no	C	S5i S5i
43	1-41-102	2009S-5-1F5	no	C	S5i S5i
44	1-54-156	2009S-5-1F5	no	C	S5i S5i
45	1-62-27	2009S-5-1F5	no	C	S5i S5i
46	1-63-28	2009S-5-1F5	yes/no	C	Heterozygous
47	1-63-29	2009S-5-1F5	yes/no	C	Heterozygous
48	1-75-14	2009S-6-2F5	no	C	S5i S5i
49	1-75-16	2009S-6-2F5	no	C	S5i S5i
50	1-76-20	2009S-6-2F5	no	C	S5i S5i
51	2-1-2003	2009S-6-1F5	no	C	S5i S5i
52	2-1-2029	2009S-6-1F5	no	C	S5i S5i
53	2-1-1932	2009S-6-1F5	no	C	S5i S5i
54	2-4-1941	2009S-6-1F5	no	C	S5i S5i
55	2-4-1942	2009S-6-1F5	no	C	S5i S5i
56	2-7-1954	2009S-6-1F5	no	C	S5i S5i
57	2-8-1959	2009S-6-1F5	no	C	S5i S5i
58	2-11-2004	2009S-6-1F5	no	C	S5i S5i
59	2-11-1971	2009S-6-1F5	no	C	S5i S5i
60	2-13-1977	2009S-6-1F5	no	C	S5i S5i
61	2-14-1980	2009S-6-1F5	no	C	S5i S5i
62	2-15-2001	2009S-5-2F5	no	C	S5i S5i
63	2-15-2002	2009S-5-2F5	no	C	S5i S5i
64	2-16-2005	2009S-5-2F5	no	C	S5i S5i
65	2-16-2006	2009S-5-2F5	no	C	S5i S5i
66	2-16-2007	2009S-5-2F5	no	C	S5i S5i
67	2-17-2009	2009S-5-2F5	no	C	S5i S5i
68	2-18-2013	2009S-5-2F5	no	C	S5i S5i
69	2-18-2015	2009S-5-2F5	no	C	S5i S5i
70	2-18-2016	2009S-5-2F5	no	C	S5i S5i
71	2-19-2017	2009S-5-2F5	no	C	S5i S5i
72	2-21-2027	2009S-5-2F5	no	C	S5i S5i
73	2-22-2029	2009S-5-2F5	no	C	S5i S5i
74	2-22-1931	2009S-5-2F5	no	C	S5i S5i
75	2-23-1934	2009S-5-2F5	no	C	S5i S5i
76	2-32-37	2008S/CL161	yes/no	C	Heterozygous
77	2-24-2001	2008S/CL161	yes	C	S5n S5n
78	2-24-2,3	2008S/CL161	yes	C	S5n S5n
79	2-24-2004	2008S/CL161	yes	C	S5n S5n

APPENDIX D continued

No	Variety/Line	Pedigree	136 bp deletion	SNP A/C	Genotype
80	2-26-2009	2008S/CL161	yes	C	S5n S5n
81	2-26-2012	2008S/CL161	yes	C	S5n S5n
82	2-27-2013	2008S/CL161	yes	C	S5n S5n
83	2-28-2019	2008S/CL161	yes	C	S5n S5n
84	2-29-23	2008S/CL161	yes	C	S5n S5n
85	2-30-25	2008S/CL161	yes	C	S5n S5n
86	2-31-29	2008S/CL161	yes	C	S5n S5n
87	2-34-2	2008S/CL161	yes	C	S5n S5n
88	2-35-7	2008S/CL161	yes/no	C	Heterozygous
89	2-37-12	2008S/CL161	yes	C	S5n S5n
90	2-37-14	2008S/CL161	yes	C	S5n S5n
91	2-43-1	2008S/CL161	no	C	S5i S5i

VITA

Christian “Stan” Torres De Guzman was born in Caloocan City and grew up in Navotas Metro Manila, Philippines. He is the third child among four siblings. He attended San Jose Academy from kindergarten to elementary then went to Navotas Municipal High School for his secondary education. He graduated top 3 of his class and carried the role of the Citizens Army Training (CAT) Cadet Corps Commander. He was awarded with full scholarship from the Department of Science and Technology (DOST scholar) and entered the University of the Philippines Los Baños for his undergraduate education. He earned a degree in Bachelor of Science in Agriculture, Major in Horticulture in 2001.

Following his education, he was a technical assistant in poultry breeding. A year after, he joined East West Seed Company in the Philippines starting as a Research Farm Foreman then became Research Farm Supervisor. After five years, he then took the position of Junior Plant Breeder in the same company from 2007 to 2010, with concentration on breeding of hybrid sweet and waxy corn for the Southeast Asian market.

He came to LSU Agcenter in 2010 as a visiting research associate. After a year, he was accepted in the doctoral program at Louisiana State University and was awarded an assistantship through the School of Plant, Environmental and Soil Sciences through the mentorship of Dr. James H. Oard. After 5 years, he is graduating in the summer of 2016.

He is married to Jennifer and blessed with wonderful twins – Dathan and Nasya (2 years old).