12-2020

Characterization of Genetic Sources Associated with Restorability and Seed Dimension in Arkansas Restorer Rice Lines

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Characterization of Genetic Sources Associated with Restorability and Seed Dimension in Arkansas Restorer Rice Lines

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Crop, Soil, and Environmental Sciences

by

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Bachelor of Science in Agriculture, 2009

December 2020
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This thesis is approved for recommendation to the Graduate Council.

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ABSTRACT

Hybrid rice (*Oryza sativa L*.) breeding offers a significant opportunity to enhance rice production, and the cultivation of a male sterile line is the most important factor in the success of cross-breeding. One of the key elements of hybrid rice production is to develop a restorer line that is assigned as the male parent. The restorer lines provide viable pollen for fertilization of the male sterile plant due to the presence of a restorer gene (*Rf*) in their genomes. Any superior restorer line applied to hybrid rice production must contain genes/quantitative trait loci (QTL) associated with the desirable agronomic traits in its genome. The objectives in this study were to 1) identify the genetic source of restorability in two Arkansas-developed restorer lines, 367R and 396R, and 2) identify QTLs associated with seed dimensions in two restorer lines. The study was performed at the University of Arkansas System Division of Agriculture, Rice Research and Extension Center, Stuttgart, Arkansas. Three populations resulting from crosses between 367R and Arkansas advanced lines RU1501139, and 396R crossed with Tropical *Japonica* cultivar "Newbonnet (PI474580) or Arkansas advanced line RU1501047 were developed. Five F_3 plants from each $F_{2:3}$ line were selected for testcross with an Arkansas developed cytoplasmic male sterile (CMS) line 873A. Five testcross F_1 plants resulting from each selected pollen donor plant were grown in the greenhouse. Pollen fertility was tested via a pollen stain procedure (Virmani et al, 1997). The results showed that 367R contains one restorer gene and 396R possesses two restorer genes within their genomes. The genotypic analysis showed that there are two major QTLs, in chromosome ("chr" hereafter) 10, which is co-localized with two previously reported QTLs where *Rf4* and *Rf5* genes were mapped. For the second part of this study, the parental lines were evaluated for grain length, width, thickness, 100-seed weight, and heading date. The population 367R × RU1501139 ("Population-A", hereafter) was selected for evaluation of grain

length, thickness, and length/width ratio. A total of 300 F_3 seeds from F_2 plants grown in greenhouse conditions were harvested, cleaned and evaluated using the WinSEEDLE ^{TM} image analyzer (Regent Instruments Inc., Canada). A total of 17 QTLs for grain dimensions were identified. Two QTLs in chr. 3 and one each QTL in chr. 7 and 11 were associated with grain length, while two QTLs located in chrs. 3 and 7 were associated with grain length/width ratio. Three QTLs located in chrs. 5, 6, and 8 were associated with grain thickness, while nine grain weight QTLs were identified that included four QTLs in chr. 12, two QTLs in chrs. 1 and 10, and one QTL in chr. 3. These results can be used for developing superior restorer lines and applied to hybrid rice production via marker-assisted selection.

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Rice (*Oryza sativa* L.) is the principal staple food around the world, supplying almost 25% of the global nutrition source for humans. Around 50% of the world's population relies on rice for food and income (Feng et al., 2014). In *Oryza* genus, there were two major common cultivated species and twenty-one wild species. Today's rice varieties originated from the perennial wild rice *Oryza rufipogon* (Londo et al., 2006). The common cultivated rice species *O. sativa* is originally from Asia and was important in agriculture during ancient times, while *O. glaberrima* derives from western Africa (Ansari et al., 2015).

An increase in the world's human population will require more rice production to feed people in the near future. Additionally, environmental degradation and urban expansion will cause a decrease in arable lands. To meet the growing need for rice, world rice production should be increased 40% by 2030; therefore, rice varieties with much improved yield potential need to be developed (Zhou et al., 2016). In 2018, approximately 782 million metric tons of rice were harvested from 167 million ha around the world (FAO, 2018).

Rice Production in Arkansas

In 1902, Lonoke County had the first commercial rice production field in Arkansas, U.S.A. (Hardke, 2018). In the following years, Arkansas continued expanding rice acreage and currently is the largest rice producing state in the U.S. Arkansas produces around 48% of the total U.S. rice production, followed by California, Louisiana, Texas, Mississippi, and Missouri. The Eastern side of Arkansas is the main rice production area; additionally, the Arkansas River Valley and the Red River Valley, located between northern Texas and southwest Arkansas, are

two other important rice growing regions in the state (Hardke, 2018). Arkansas rice production had a peak of 0.722 million ha (1.785 million acres) of harvested area in 2010. Nine years later, Arkansas harvested almost 4.2 million tons (84.0 million hundredweight) of rice from 0.577 million ha (1.427million acres) (USDA, 2019).

Rice cultivation utilizes three tillage methods. The main method is the conventional tillage method, which includes fall and spring tillage followed by the preparation of the seedbed prior to plant. This method was used for just over 50% of the rice field in 2018. The second most popular method was stale seedbed planting at around 43% of the rice acreage. No-till rice production was used in some limited areas (Hardke, 2018).

Fertilization practices relative to soil types and crop rotations are the most important and costly practices in rice production. Approximately 50% of Arkansas' rice fields are classified as silt loams soils, 24% as clay, and 23% as clay loam soil (Roberts et al., 2018).

Generally, rice varieties in US are classified based on their kernel size in combination of their physicochemical characteristics into three groups, long-grain, medium-grain, and short-grain. In the long-grain rice varieties, the ratio of kernel length to its width is more than 3.0. In Arkansas, long-grain varieties have cooking qualities defined as typical Southern US long-grain rice: the cooked rice appears fluffy, non-aromatic, non-sticky with intermediate amylose content (20-24%), and a medium gelatinization temperature between 70 ºC and 74 ºC (Juliano, 1992; Suwannaporn et al., 2007). Long-grain cultivars are generally used in the parboiled, canned, frozen or similar fabricated products (Webb et al., 1985).

For medium-grain, the ratio of kernel length to width is between 2 to 3. The medium-grain varieties consist of a sticky and moist structure due to their low amylose content (10 to 20%) as well as low gelatinization temperatures (Juliano 1992; Suwannaporn et al., 2007; Biselli

et al., 2014). Medium-grain and short grain rice varieties are mainly used in the production of ready-to-eat dry foods such as cereals, baby foods and beverages (Webb et al., 1985; Hardke et al., 2018).

Long-grain rice and medium-grain rice have the biggest share at around 75% and 25%, respectively, followed by around 1.5 % for short-grain rice in United States' rice production. Ninety percent of Arkansas rice production is from long grain cultivars (Mcbride et al., 2018).

Hybrid Rice Production

Hybrid rice is defined as commercially grown filial 1 (F_1) seeds resulting from a cross between two genetically diverse parents. Hybrid rice demonstrates greater yield potential (10-15%) as well as durable resistance/tolerance to biotic and abiotic stresses compared to conventional cultivars (FAO, 2004). Such superb performance by hybrid rice is due to a phenomenon known as heterosis. Heterosis can have positive effects such as increasing yield, or negative effects, such as reducing maturity days (Virmani et al, 1997).

Greater seed yield is the foremost goal of hybrid rice production. Several studies showed that heterosis effectively influences several yield components such as the panicle and spikelet numbers (Anandakumar and Sreehangasamy, 1984; Chang et al., 1971, 1973; Amrithadevarathinam, 1984). A study conducted in China showed that hybrid rice cultivars produced 18 - 41% higher grain yield than conventional cultivars, and the yield advantage was due to the higher number of panicles per m^2 produced in the hybrid rice cultivars (Huang et al., 2013). Production of hybrid rice in Arkansas has been growing rapidly in the last decade due to hybrid rice's net revenue advantage over inbred lines (Lyman and Nalley, 2013). Currently, over 40% of Arkansas' rice acreage are planted to hybrid rice (Hardke, 2018). Since 2010, the

University of Arkansas at Fayetteville, as one of the major crop variety developers in the state, has aimed to release hybrid rice cultivars with increased yields and enhanced grain quality.

Before hybrid rice technology, semi-dwarf rice varieties increased rice yield around 1.5 tonnes per hectare (ha) between the 1960s and 1975. Rice is a self-pollinated plant; thus, to improve hybrid seed production, developing a male sterile line assigned as a female parent is required. Shinjyo and Tamura (1966) identified cytoplasmic male sterility in a B1F1 generation of a population originated from Chinsurah Boro II and Taichung 65(Shinjyo, 1969). In 1964, Dr. Yuan Longping observed some male sterile rice plants on *Indica* rice genotypes. In 1970, natural male sterility called a wild abortive system (WA) was discovered in wild rice plants and called a wild abortive system (WA). This discovery enabled the use of hybrid systems for large-scale production by developing commercial rice hybrids (Zebing and Yingguo, 1988; Li et al., 2009).

Hybrid Rice Systems

There are three main systems for hybrid rice production: Sterility induced by chemical, two-line hybrid rice system, and three-line hybrid system. Sterility in the chemically-induced male sterility method is achieved with chemical hybridizing agents (CHAs) such as Ethrel®, monosodium methyl arsenate and sodium methyl arsenate. This method can shift those lines to become partially sterile to completely sterile, if proper chemical application is used. Disadvantages of using such a method include: 1) the CHAs may not be completely effective to convert a fertile plant to complete sterility, 2) some chemical agents such as methyl arsenate or sodium methyl arsenate can cause health problems like cancer, and 3) it can be a costly method for hybrid rice production (Virmani et al., 2003).

The two-line hybrid rice production system requires a rice genotype as a pollen donor and an environment-sensitive genetic male sterile line (EGMS) as the female parent. In EGMS several genes lead to sterility, but the expression of these genes is regulated by specific environmental conditions, such as temperature (TGMS), day length (PGMS), or both (PTGMS). Sterility can be induced by temperature in TGMS (Temperature-sensitive genic male sterile) lines. For example, sterility can be conferred, when the temperature is over 30°C at the daytime and a minimum of 24°C at the night. Daylight can induce sterility in PGMS (Photoperiod-sensitive genetic male sterility) lines. For example, daylight of 13.75 hours or greater is required for sterility of some PGMS lines. Photo-thermosensitive genic male sterility (PTGMS) respond to both day length and temperature: a 14-hour day length and approximately 12 hours of 30^0C temperature keeps the lines sterile. Since the system requires one sterile line and one pollen donor, it is easier and more profitable than the three-line system. Also, cultivated varieties can be used as the pollen donor. However, the challenge is that any changes in environmental conditions can turn the lines fertile (Virmani at al., 2003).

The three-line system is another method for hybrid rice production. It involves the use of three different lines: cytoplasmic male sterility (CMS), maintainer, and restorer (R) lines. A CMS line contains a sterile cytoplasm and recessive restorer (*rf*) gene in its nucleus. A maintainer line is an isogenic line of the CMS line, but it has a normal cytoplasm. Maintainer lines are utilized for propagation of the CMS line. Restorer lines are used as a pollen donor for hybrid rice production (Virmani et al., 1997). To produce fertile F_1 seeds in the three-line system, the CMS line must be crossed with a restorer plant, which carries a dominant restorer *Rf* gene in its genome (Xu, 2003).

The sterility in the CMS lines result from specific nuclear and mitochondrial interactions. Several sources of cytosterility have been identified including Wild-abortive (WA), Chinsurah boro II (BT), Honglian (HL), Dissi type (DI), Dwarf wild rice abortive pollen (DA), Indonesian paddy (IP), and Chinese wild rice (CW). Wild-abortive and BT are the most common types of cytosterility in hybrid rice production. The three-line cytoplasmic male sterility system is not affected by external factors; thus, the CMS method is considered the most reliable method (Li and Zhu 1988; Lin and Yuan 1980; Virmani et al., 1997; Shinjyo, C., 1969; Shinjyo, 1975; Huang., 2000).

Seventeen *Rf* genes have been identified so far including *Rf1* in the chr. 10 of the BT-type maintainer line Taichung-65B. *Rf2* was identified in chr. 2 in the LD-type in a japonica cultivar called Fukuyama (Shinjyo, 1975). *Rf3* was identified in chr. 1 in the WA-type in an indica cultivar IR24. *Rf4* is located in chr. 10 in the WA-type in the IR24 (Zhang et al., 1997). *Rf5* was detected in chr. 10 in the HL-type in indica line Miyang-23. *Rf6* was identified in chr. 8 in the HL-type in indica line 93-11 (Huang., 2000 & Liu et al., 2004). *Rf7* was found in chr. 12 in a *japonica* variety (Akebono) (Yabuno T., 1977). *Rf9* was identified in chr. 10 in the BT-type in an indica line (H-103). *Rf10*, *Rf11*, *Rf12*, *Rf13*, *Rf14*, and *Rf15* were detected in chr. 10 in the BT-type in indica lines H-103, H-406 and I-130 (Maekawa M., 1982 & Kato et al., 2007). *Rf17* was identified in chr. 4 in CW-type in a japonica cultivar Taichung-65 (Fuji, S., & Toriyama, K., 2005). *RFWA2 (Rf8)* was identified in chr. 10 in the WA-type in an indica IR24 (Tan et al., 1998). However, four restorer genes of *Rf1*, *Rf2*, *Rf3*, and *Rf4* have been widely used for developing hybrid rice (Zhang et al., 2017).

The induction of fertility of the restoration *Rf*1 gene was identified on chr. 10 in the BT-type maintainer line Taichung-65B (Shinjyo, 1975). The initial cross of Taichung-65B with a

CMS line resulted in ~8% partial fertility and increased the fertility ratio in further generations (Sano et al. 1990). Further studies revealed that *Ifr1* gene restores fertility by reducing the level of B-atp6-orf79 RNA in the mitochondria, which then restores fertility to ~8-12% (Ohta et al., 2010). The *Rf1* gene is commonly used to restore pollen fertility in the BT type CMS line. The gene is located on chr. 10 and has been cloned (Komori et al., 2004). Six *Rf1* alleles (*Rf1A* to *Rf1F*) have been identified (Kato et al., 2007). Additionally, the gene was identified in Sunflower (*Heliantus annuus L.*) as an important pollen restorer gene and was cloned via a simple sequence repeat (SSR) marker ORS511 (Yue et al., 2009). The *Rf2* gene is located on chr. 2 and is only effective for the LD type of CMS. The fertility restoration is gametophytically-determined (Itabashi et al., 2011). The *Rf2* gene is also located on chr. 2 in *Sorghum.* Two SSR makers were introduced for marker-assisted selection, which works in sorghum (Madugula et al., 2018). *Rf3* is positioned on chr. 1 and restores pollen fertility in the WA CMS type. A study by Pranathi et al. (2016), validated a candidate gene *SF2* as the restorer *RF3* and developed the marker RMS-SF21-5 for identification of the presence of the gene in the genome. *Rf4*, located on chr. 10, is widely used for hybrid rice production due to the *Rf* gene's large restorability compared to that of other *R* genes and is used in the WA CMS type. Moreover, one of the latest investigations of over 300 rice cultivars showed that 90 lines have *Rf3*, 65 lines have *Rf4* and 45 lines have both the *Rf3* and *Rf4* genes with about 97% restorability (Namaky et al., 2016). By developing the SSR marker RMS-PPR9-1 it was determined that PPR9-782-(M, I) is indeed the candidate gene for *Rf4* (Pranathi et al., 2011). The *Rf5* gene was identified on chr. 10 for honglian (HL) CMS type (Huang et al., 2000; Liu et al., 2004). The *Rf5* is a major restorer gene with around 50-94% restorability (Hu et al., 2012; Huang et al., 2015). Interestingly, two major QTLs for BT-type CMS lines, *qSF8-1* and *qSF10-1* (*Rf1a* allele) are located on chr. 10 at the same region as the *Rf5*

gene. These results indicated that *Rf1a* allele was the same with *Rf5* gene (Zhang et al., 2017). The *Rf6* gene was identified on chr. 8 for Honglian (HL) CMS type (Huang et al., 2000; Liu et al., 2004; Zhang et al., 2017). The *Rf6* is a major restorer gene with around 50-94% restorability (Hu et al., 2012; Huang et al., 2015). The *Rf5* and *Rf6* genes were also mapped and cloned on chrs. 10 and 8, respectively (Hu et al., 2012; Huang et al., 2015). The *Rf7* gene was detected in a study that utilized the cross from a *Japonica* variety 'Akebono', also called pollen fertility restoration-ak (Yabuno T., 1977). Subsequent studies indicated that the *Rf7* gene was located on chr. 12 and identified as a restorer gene for WA CMS line (Bazrkar et al., 2008: Yarahmadi et al., 2017). The *Rf7* gene restores fertility to as much as 80% (Nematzadeh A. and Kiani G., 2010). Small GTP-Binding Protein-1 (*RfWA2, Rf8* and Rf(u)) acts as a restorer gene for WA CMS type (www.gramene.org). Bharaj et al (1995) found two restorer genes *RfWA-1* and *RfWA-2* located on chr. 7 and 10, respectively. The genes restored the fertility in CMS lines between 40-80%. *RfWA-2* was a weaker restorer gene, which restores around 10% fertility in a recessive (*rf*) genotype and almost 72% fertility in a dominant (*Rf*) genotype (Tan et al., 1998). The pollen fertility restoration-9 gene (*Rf9*) was identified in the *Rf-1* locus in chr. 10, which primarily restores BT-type CMS lines. The *Rf-a* gene was reported as a synonym of *Rf9* gene that restores fertility to ~70 percent (Maekawa M., 1982; Wang et al., 2006). The *Rf-1* locus also included two adjacent restorer genes (*Rf-1a* and *Rf-1b*), where the *Rf-1b* gene had lower restorability than the *Rf-1a* gene (Komori et al., 2004; Kato et al., 2007). Several pollen fertility restorer genes were identified on crosses involving BT-type CMS lines and a *Japonica* line Taichung-65. Six crosses were developed which constituted a combination of either normal or sterile cytoplasm having three restorer genotypes *RfRf, Rfrf,* and recessive *rfrf* genes.

In 1982, M. Maekawa, conducted a study involving a BT-type CMS line crossed with *Indica* or *Japonica* lines that carried a *Rf-1* locus. The lines that successfully restored pollen fertility were classified into four subgroups: *Rf-a, Rf1-b, Rf1-c,* and *Rf1-d*. Another study regarding the fertility restoration of the *Rf-1* locus in the BT-type CMS line revealed six distinct *Rf* genes: *Rf-a, Rf1-b, Rf1-c, Rf-d, Rf1-e,* and *Rf1-f* , which were also known as *Rf10, Rf-11, Rf12, Rf13, Rf14,* and *Rf15* (Kato et al., 2007; www.gramene.org). The pollen fertility restoration-17 gene (*Rfcw*) was derived from a *Japonica* cultivar Taichung-65 as a restorer gene on chr. 4, which restores the CW-type CMS line (Fuji, S., & Toriyama, K., 2005). The *Rf17* gene was identified as a synonym of the *Rfcw* gene that restores fertility to ~75% (Fuji, S., & Toriyama, K., 2009; Toriyama et al., 2019).

Seed Dimension

Since rice is one of the most important food crops, plant scientists have always aimed to increase the productivity of rice (Xue et al., 2008). Both genetic and environmental factors are effective in increasing rice yield potential (Weng et al., 2008). The number of panicles, grains and weight per panicle can increase grain yield. Grain size such as grain length, width and thickness are components of grain weight, which is one of the traits of interest when breeding (Fan et al., 2006). Grain shape measured by its length, width and the length/width ratio is becoming valuable factors for grain quality and consumer preference. The USA and the majority of Asia prefer long and slender grains while South Korea, Japan and Sri Lanka prefer short and thick grain varieties (Shao et al., 2010). Rice preferences vary by countries and are affected by culture, traditions, and industrial usage. In the US and most Asian countries, the preference is mainly for long grain rice because of its color, price, chalkiness, non-sticky texture, and better

cooking quality criteria for the ready to eat food production industry. In the Philippines, people prefer rice varieties with low amylose content, while Indonesians consider higher amylose content for non-sticky structure and better milling quality (Webb et al., 1985; Shao et al., 2010). Medium-grain and short-grain varieties are favored in Australia, USA, Japan, South Korea, Taiwan and about 40 % of China and in the cooler parts of these countries. These two types are primarily used in the production of ready-to-eat dry foods such as cereals, baby foods and beverages because of the lower amylose content but in some Asian countries the preference is due to the longer storability without electricity (Webb et al., 1985; Hardke et al., 2018).

Current Research in QTL Mapping

Seed dimensions such as length and width are quantitative traits controlled by several genes. All 12 rice chrs. have grain shape related QTLs; however, there are limited studies on seed size QTL. Several QTLs associated with grain length have been identified. Fan (et al., 2006) detected *GS3* located near the centromere on chr. 3 in a population obtained from the cross between two indica lines Minnhui-63 and Chuan-7, and it explains over 55% of the phenotypic variation. Wan et al. (2006) identified QTL, *gl-3*, with an 87.5 kb size close to the centromere on chr. 3 in a population from the cross between a japonica line 'Asominori' and an indica 'IR24' that explained about 33 % of the phenotypic variation. A QTL *qGL7-2* was detected on chr. 7 in a population resulting from the cross between a *javanica* line 'D50' and an indica 'HB277' that explained about 20 % of the phenotypic variation (Shao et al., 2010). Quantitative trait loci for grain width were identified in several chrs. *GW2* is located on chr. 2, *qSW5* (equal to *GW5*) is located on chr. 5 in a population resulting from the cross between a japonica line 'Asominori' and an indica 'IR24' that explained around 39 % of the phenotypic variation (Shomura et al.,

2008., Weng et al., 2008). Another QTL *Qss7*, which is related to increased grain length and decreased grain width, was detected with 23 kb on the long arm of chr. 7 from the population resulting from the cross between an indica 'Zhenshan97' and a japonica 'Cypress' and explained around 16% of the phenotypic variation (Qiu et al., 2012).

The University of Arkansas can play a pivotal role in hybrid rice production by developing novel hybrid rice varieties that would contribute to Arkansas keeping its status as the major rice producer in the country with almost 50% of USA total rice production. Since large scale hybrid production and yield are desired, introducing new restorer lines is critical to the success of the hybrid system. While there are limited restorer lines, QTL studies have been conducted to find new restorer genes. Grain weight is associated with grain size such as grain length, width, and thickness. Genetic background is highly connected with grain dimensions; however, studies over grain dimensions are still scarce. Identifying new QTLs on restorer lines can promote higher grain yield expectations. Restorer lines 367R and 396R are being used for several traits associated with agronomic traits. Detected QTLs associated with grain sizes could be used for developing superior restorer lines and marker-assisted selection can play a significant role in the production of high-quality hybrid rice cultivars.

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Chapter 2

INHERITANCE AND ALLELIC RELATIONSHIP OF RESTORABILITY IN ARKANSAS RESTORER LINES

ABSTRACT

Rice (*Oryza sativa L.*) production has increased considerably after the introduction of hybrid rice technology. The process of hybrid breeding relies on developing hybrid parental lines that include male sterile lines as the female parent and fertility restorer lines that are assigned as the male parent. A restorer line that carries restorer (*Rf*) genes in its nucleus is an essential part of hybrid rice breeding. The University of Arkansas (UA, hereafter) hybrid rice program has developed two restorer lines (367R and 396R). However, there is no information about the genetic sources of restorability in these two lines. The objectives in this study were to identify the inheritance and allelic relationships of restorability in these two lines. An experiment was conducted at the University of Arkansas, System, Division of Agriculture, Rice Research and Extension Center, Stuttgart (RREC). Three bi-parental populations were developed: one resulting from a cross between "367R" and a UA advanced line of "RU1501139" and two crosses between "396R" as the female parent and a UA advanced line "RU1501047" and cultivar "Newbonnet" as the male parent. F_2 leaves from the population of 367R x RU1501139 and 396R x RU1501047 were collected and used for genotypic analysis. The $F_{2:3}$ lines from each population were test-crossed using a UA developed CMS line 873A to determine the restorability status in each line via test cross procedure. The results showed that 367R and 396R restorer lines each contain two restorer genes in their genomes. Genotypic analysis on the population of 367R x RU1501139 detected two major QTLs on the chromosome (chr. hereafter) 10 that were co-localized with formerly reported QTLs of the *Rf4* and *Rf5* genes. The results of this study can be used for

developing markers for identification of restorer lines/plants within populations via marker assisted selection.

INTRODUCTION

Hybrid rice definition

Hybrid rice (*Oryza sativa*) is a commercially grown filial 1 (F_1) seed resulting from a cross between two genetically distinct parents. Hybrid varieties yield more seeds (10% to 15%) and demonstrate greater tolerance to biotic and abiotic stresses compared to the conventional rice varieties (Virmani et al., 1997). Rice is a strictly self-pollinated plant, which makes hybrid rice production difficult; therefore, developing a male sterile line designated as a female parent is essential for hybrid rice production. Male sterile florets not only have a functional stigma, but also sterile pollen that prevent any seed production via self-pollination (Li, 1977; Virmani et al., 2003). However, cytoplasmic male sterility can be restored via one or more dominant restorer genes (*Rf*) from a restorer male line (Li et al., 2009).

Generally, male sterility can be produced via three ways: environment-sensitive genetic male sterility that is used for two-line hybrid rice production, cytoplasm male sterility (CMS) system that is used for three-line hybrid rice production, and chemically induced male sterility method based on chemical usage (Yuan, 1994; Virmani et al.., 1997). In this study we focus on the three-line hybrid rice production.

The first hybrid rice cultivar was developed in China in 1964 via the three-line system (Yuan, 1966). The resulting wild-abortive (WA) CMS line was introduced in 1970 (Li, 1977). The three-line system includes a cytoplasmic male sterile (CMS, A) line, a maintainer (B) line and restorer (R) line. Sterility of CMS is a result of the interaction between the nucleus and genetic factors in the cytoplasm (Virmani et al., 2003). The advantages of the three-line system include but are not limited to: sterility not influenced by environmental conditions and *Rf* is a single dominant gene controlling restorability that can be transferred from one generation to the

next more easily (Virmani et al., 1997). The disadvantages of the three-line system include, but are not limited to: 1) this system requires developing three-lines of CMS, B, and R lines, therefore it is more challenging compared to the two-line system, 2) since male parents have to carry a dominant *Rf* gene, more male parent varieties should be developed, 3) the sterility condition can be broken by some diseases such as blast, 4) CMS lines rarely bring reverse outcomes to the yield and quality traits in the hybrid seeds (Virmani et al., 1997) and 5) a number of pollen donor cultivars carry restorer genes. Sterility in a CMS line results from the incompatibility between the sterile mitochondrial cytoplasm in a plant cell and a homozygous recessive nuclear gene (*rf*). In such conditions, a protein from a mitochondrial gene causes dysfunction in the process of pollen development in the florets. The process of this protein can be regulated by a specific restorer gene in the cell's nucleus and, as a result, the plant turns fertile. There are several types of CMS lines including wild-abortive (WA), Chinsurah boro II (BT), Hong-Lian (HL), Dissi type (DI), Dwarf wild rice abortive pollen (DA), Indonesian paddy (IP), and Chinese wild rice (CW). Hybrid rice production in China is primarily based upon WA, BT and, to some extent, HL systems. The WA system is primarily used outside of China (Guo and Liu, 2009; Sattari et al., 2008).

A maintainer is an isogenic line to its correspondent CMS line, but, due to its normal cytoplasm, maintains its fertility. The B lines are used for propagation of the CMS line by crossing the CMS line (female parent) with the B line as a pollen donor (Virmani et al., 1997).

A restorer line is required as a male parent in hybrid rice seed production. In hybrid rice production, the female parent is a CMS line; thus, in order to produce seeds, the CMS line should be crossed with a restorer male parent. Restorer lines carry at least one restorer gene (*Rf*) with a normal or sterile cytoplasm (Virmani et al., 2003). The interaction of a specific gene (*Rf*) with

the mitochondria makes a CMS line fertile. In this interaction, the majority of the *Rf* genes code for pentatricopeptide repeat (PPR) proteins. The PPRs play a role in mRNA synthesis by editing, splicing, cleaving, and stabilizing the RNA strain by binding the 3' ends of RNA (Barkan and Small., 2014; Tang et al., 2017). So far, 17 *Rf* genes have been reported to restore CMS lines. Of these 17 *Rf* genes, six genes (*Rf1, Rf2, Rf3, Rf4, Rf5 and Rf6*) are commonly used for hybrid rice production. The three most common CMS types are WA, BT, and HL (Tang et al., 2017).

The *Rf3* and *Rf4* genes that restore the WA-type CMS line were detected on chr.s 1 and 10, respectively (Zhang et al., 1997; Tang et al., 2014). In the WA-type CMS line, sterility comes from the accumulation of the *WA352* gene that interacts with a mitochondrial protein, COX11, and causes early death of pollen cells in the anther tapetum (Luo et al., 2013). The role of the *Rf4* gene is to regulate the quantity of *WA352* PPR repeats to ~25%; thus, preventing the death of pollen cells (Luo et al., 2013; Tang et al., 2014; Barkan and Small, 2014). The *Rf3* gene has a different mechanism and a weaker effect than the *Rf4* gene for fertility restoration (Suresh et al., 2012). The amount of *WA352* PPR repeats does not change the presence of the *Rf3* gene. Thus, the *Rf3* gene's function is not clear, but the *Rf3* gene could have an effect after the translational process (Luo et al., 2013; Katara et al., 2017).

The *Rf1a* and *Rf1b* genes were identified as restorers of the BT-type CMS line identified between 7.5 cM to XNpb291 and 3.7cM to OSRRf markers on chr. 10 (Wang et al., 2006; Komori et al., 2004). BT-type CMS lines are restored by preventing the accumulation of a cytotoxic *B-atp6* protein coded by the open reading frame (*orf79*) gene fragment. The *Rf1a* gene is responsible for the cutting of the *B-atp6-orf79* mRNA fragment, thus preventing the synthesis of cytotoxic *orf79* mRNA, while the *Rf1b* gene mediates the degradation of *B-atp6-orf79* mRNA (Komori et al., 2004; Wang et al., 2006).

The *Rf5* and *Rf6* genes, which were identified as restorers of HL-type CMS lines, are located on chrs. 10 and 8, respectively (Huang et al., 2000; Hu et al., 2012). The restorability was a result of energy deficiency (ATP/ADP; energy-carrying molecules) in the mitochondria. The *B-atp6-orfH79* gene fragment corresponded to mitochondrial activity and reduced the energy that caused the sterility of pollen (Hu et al., 2012). The *Rf5* gene cleaves the *B-atp6-orfH79* gene fragment by interconnecting with gene fragments that help with the separation of the *B-atp6-orfH79* gene fragment (Hu et al., 2012; Huang et al., 2012). The *Rf6* gene restorer effect is similar to *Rf5*. The *Rf6* gene breaks the *B-atp6-orfH79* gene fragment by interacting with a different protein (OsHXK6) fragment and prevents the synthesis of *B-atp6-orfH79* that finally results in fertility restoration (Hu et al., 2012; Huang et al., 2012; Tang et al., 2017).

Yan et al. (2012) developed 13 restorer lines for production of hybrid rice at the University of Arkansas, System, Division of Agriculture, Rice Research and Extension Center (RREC), Stuttgart. Two R lines, 367R and 396R, showed good potential for developing hybrid rice cultivars. WA-CMS is the most common hybrid rice production system for three-line systems (Huang et al., 2014). However, the number of WA-CMS lines is limited. The majority of *indica* lines have been determined to be restorer lines, including IR24 and IR64, which are two popular cultivated *indica* varieties (Toriyama and Kazama, 2016). The development of WA CMS lines as both CMS and maintainer lines will broaden the development for *indica* hybrids. In order to do this, Toriyama and Kazama (2016) successively backcrossed IR24 and IR64 with both Taichung 65 CMS and CMR lines. As a result, CMS and restorer lines were identified for IR24 and IR64 elite restorer lines.

In a subsequent study with CW-type CMS lines, several elite *Indica* varieties were used to develop restorer and CMS lines by applying *Rf17* fertility restoration. Two elite *Indica*

varieties, IR 24 and IR 64, were the restorers of fertility to CW-types (Toriyama and Kazama, 2016). The IR 64 had the CW-type cytoplasm and *Rf17* nuclear gene resulting from crosses using CWR-IR 64 lines. The CWR-IR 64 lines were crossed with several elite *Indica* varieties and F1 seeds were harvested. Then, the F1 generation was backcrossed with elite *Indica* varieties. After two backcrossing, the seeds with dominant *Rf* gene(s) were selected as candidates for R-lines by using single nucleotide polymorphism (SNP) markers. After two generations of self-pollination, several restorer lines were developed with around 80% fertility restoration. The seeds with recessive *rf* genes were selected for the CMS lines. These CMS lines were then backcrossed with elite *Indica* varieties and, after four backcrosses, CMS lines were developed (Toriyama et al., 2019).

In another research project, 148 exotic rice resources were screened to identify CMS, maintainer, and restorer (*Rf*) lines. All 148 lines were evaluated by checking their pollen fertility. Of the 148 lines, 16 were completely sterile and 16 were completely fertile. To identify maintainers for the completely sterile lines, the 16 sterile lines were crossed with stable maintainer lines: GAN 46B, BRRI 1B, IR 58025B, IR 62820B, and IR 68888B. This facilitated the identification of the corresponding maintainer line for each sterile line. On the other hand, the 16 fertile lines, which showed > 80% pollen fertility, were classified as restorer lines. To confirm their restorer capability, the 16 fertile lines were crossed with five standard CMS lines. The resulting F_1s were evaluated for pollen and spikelet fertility and those F_1s that showed 80% or more of fertile offspring were considered as new restorer lines (Islam et al., 2015).

In 2016, another study showed that about 97% restorability was observed on 65 lines that carried the *Rf4* gene (Namaky et al., 2016). By developing the simple sequence repeat (SSR) markers for the candidate genes PPR9-782-(M, I) (Tang et al., 2014) and PPR762 (Suresh et al.,

2012), the *Rf4* gene was identified as a major restorer gene located between $1.92x10^7$ and $1.94x10^7$ base pairs (bp) (Pranathi et al., 2011). Several studies identified that the *Rf4* gene was located on the long arm of chr. 10 (Zhang et al., 1997; Tan et al., 1998; Ahmadikhah and Karlov, 2006; Tang et al., 2014).

The restorer *Rf5* gene originated from the BT-type CMS line with a 94% restorability (Hu et al., 2012). Previous studies identified two major candidate QTLs: *qSF8-1* and *qSF10-1* (*Rf1a allele*) on chr. 10 (Akagi et al., 1996; Komori et al., 2004; Wang et al., 2006). A study by Zhang et al. showed that QTLs *qSF8-1* and *qSF10-1* (*Rf1a allele*) were the same with the *Rf5* gene. Additionally, the *Rf5* gene was mapped as a major restorer gene between SNP locations $1.69x10^7$ and $1.84x10^7$ bp (Zhang et al., 2017).

Another conducted study detected a QTL associated with fertility between $1.45x10^7$ and $2.0x10^7$ bp in chr. 10 with a ~3.2 logarithm of the odds (LOD – a statistical evaluation of gene location on chr.) score (Zhang et al., 2019). Previous studies reported that the restorer gene *Rf5* was in the same location as the Honglian type CMS line (Huang et al., 2000; Liu et al., 2004). Hu et al. (2012) mapped and cloned the *Rf5* gene and found that the *Rf5* gene restored the sterility to ~94%. The BT-type CMS lines have two major QTLs, *qSF8-1*, and *qSF10-1* (*Rf1a allele*) on chr. 10. Research identified that one of BT-type restorer QTLs *qSF8-1* and *qSF10-1* (*Rf1a allele*) was the same with the *Rf5* gene.

Objectives

The University of Arkansas hybrid rice program developed several restorer lines. Among these lines, 367R and 396R showed the largest yield potential for hybrid rice cultivation. However, genetic resources (*Rf* genes) and their positions on the chromosomes were unknown.

Therefore, the objectives of this study were to identify the inheritance (number of *Rf* genes in the genomes) and allelic relationship (identification of the position of *Rf* genes in the genome) of these restorer lines.

MATERIALS AND METHODS

Plant Materials

The experiments were conducted at the University of Arkansas System, Division of Agriculture, Rice Research and Extension Center (RREC) in Stuttgart, Arkansas from 2016 to 2019. Six rice genotypes were used for this study, including two restorer lines (367R and 396R), and three non-restorer lines (RU1501139, RU1501047 and Newbonnet) and a CMS line (873A) developed at the UA hybrid program. The restorer line 367R [Katy/IR30//IR140(PI 458443)/Jasmine 85(PI 595927)] is a medium-grain variety and has high yield potential for hybrid rice production. Other restorer line 396R [Francis/4/ IR 1586-2(PI-400793)/3/Bengal//L202/Lemont] is a long-grain variety and has greater yield potential than other developed restorer lines for hybrid rice production. Both restorer lines were developed by the hybrid rice program at RREC in 2012. Non-restorer genotypes RU1501139 (LBNT/9902/3/DAWN/9695//STBN/4/ LGRU/5/WLLS/6/RU9201179/7/IRGA409/RXMT/5/LGRU//LMNT/RA73/3/LGRU/4/LGRU) and RU1501047 (IR-TGRT 30 RADS) are two long-grain, advanced lines developed by the RREC long-grain program. Newbonnet is a mid-season, long-grain, dwarf cultivar developed by crossing “Dawn” and “Bonnet 73” in 1983. The WA CMS line, 873A (Iaca Claro(PI 392687,Guinea-Bissau)//II-32/Jin-23) had a non-aromatic background. The restorer lines, CMS line 873A, and Newbonnet were obtained from RREC hybrid rice breeding lines seed collection

and RU1501139 and RU1501047 were provided by Dr. Karen Moldenhauer of the University of Arkansas, RREC long-grain rice breeding program.

Phenotypic Studies

A- Developing Bi-parental Populations

In Summer 2016, three bi-parental populations were developed, which resulted from crosses of 367R with RU1501139; 396R with RU1501047 and Newbonnet, respectively. In 2017, the F_1 plants were grown in greenhouse and tested by means of genotypic markers to make sure the resulting plants were true hybrids. The F_2 seeds were collected from each single F_1 plant. The F_2 seeds were planted in 3.78-L plastic pots filled with 3.78-L Baccto® premium potting soil in greenhouse during fall 2017. Twelve pots were placed in a plastic tub immersed in 10-15 cm of water (Fig. 1). Fertilizer, Osmocode® (15N-9P-12K), was applied to the top of pots by adding 1/2 scoopful per 3.78-L pot, and pesticides were applied according to the standard recommendations in Arkansas. The greenhouse lighting system was set to 12 hours of day light, which was ideal for rice growth (Harrington, 2010). The $F_{2:3}$ seeds from each F_2 plant were harvested for the field study.

Six separate soil samples were collected from 0 to 15cm depth in RREC field and sent for testing at the Soil Testing and Research Laboratory in Marianna, AR, during Summer 2018. The results of soil testing showed that the soil texture was silt loam and silty clay loam with a 5.5-5.8 pH level and soil organic matter was 2% in Summer 2018. The $F_{2:3}$ lines were planted in the field. 30 seeds from each line were planted in a row of 2.1 m long spaced 0.4 m apart on three planting dates: May 22nd, May 30th, June 6th of 2018. Germination started on the 5th, 12th and 19th of June, respectively (Fig. 2). After each planting, the bays were flushed to improve germination.

Meanwhile, the UA CMS line 873A was planted for test crossing in six planting dates of 10th, 22nd, and 29th of May and 12th, 18th, and 27th of June 2018 into 3.78-L plastic pots containing potting soil under greenhouse conditions. The greenhouse was programmed for 30ºC during the day and 23ºC at night with 75% humidity. Seed germination occurred 5-6 days after planting. Urea was applied as a source of nitrogen at a rate of 56 kg/ha before flooding the bays on the 5th and 12th of July at the V5 stage. The bays were flooded on the same day of fertilization. Weeds were controlled by pulling them manually from the field, no diseases were observed, and no chemicals were used for disease control.

B- Test Cross Procedure

At the heading stage, five panicles from five randomly selected plants from each row were carefully collected and used for test crossing with the 873A CMS line in the sterile room of the greenhouse (Fig. 3).

The F_1 (test cross) seeds were harvested, and 10 seeds for each F_1 plant were planted into 3.78-L plastic pots (3 seeds/each) containing Baccto® premium potting soil in a greenhouse. Twelve pots were placed in a plastic tub to keep the water around 15 cm deep. Maintenance for watering and fertilization of urea (46-0-0) in the greenhouse followed the standard rice growth recommendations for Arkansas (Roberts et al., 2019). At panicle exertion (R3-R4 growth stages), when one or more florets reached anthesis, 15-20 spikelet were collected between 7-10:00 am for pollen staining from five randomly selected plants. A total of 25 crosses were tested for pollen staining from each line. The pollen staining procedure is described in Table 1. In 1997, Virmani et al., 1997 classified pollen viability based on appearance and a pollen sterility/fertility ratio. Sterile pollen can appear to be translucent either in an unstained, withered or spherical shape,

while fertile pollen is stained and round (completely dark) (Fig. 4). There are six classifications based on the sterility/fertility ratio: completely sterile (100% pollen sterility), sterile (91-99%), partially sterile (71-79%), partial fertile (31-70%), fertile (21-30%), and fully fertile (0-20% sterility). Since the purpose of this study was to identify R lines for the hybrid rice breeding program, the pollen variability from the samples were classified into two classes of sterile (>91% sterility) and fertile (<91% sterility)(Table 2).

DNA Extraction and Genotyping:

The tissue samples from each F_2 plant from the populations of 367R x RU1501139 were collected at the V5 growth stage, labeled, and freeze-dried for genotyping via Single Nucleotide Polymorphism (SNP) markers. The samples were sent to an Illumina sequencing company, located in River Falls, Wisconsin, to be genotyped using an Infinium Rice 7K Chip (Morales et al., 2020). The Infinium SNP chip is a silicon-based bead chip that has microscopic beads on the surface and is attached to a specific oligonucleotide fragment. Each oligo fragment represents a specific region within the plant genome. The DNA samples run over the beads and, as a result, the DNA fragments complimentary to the oligo fragments bond to each other and are then extended. The hybridized fragments were stained with different color dyes and detected with a laser (Illumina SNP Genotyping, 2017). In this study, the F_2 plants were genotyped using 7,000 SNP Infinium markers. Then, the $F_{2:3}$ seeds from each single plant were harvested.

Statistical Analyses:

Determination of how many restorer gene(s) were in the restorer lines 367R and 396R was evaluated by using a Chi-square test. Chi-square tests were used to evaluate the goodness-

of-fit of the observed data (the results of the test crossed according to S, all F and segregating from the $F_{2:3}$ lines from each population) to expected ratio by using Excel®. The Chi-square was calculated via the formula below:

$$\chi 2 = \sum \frac{(Observed\ frequency - Expected\ frequency)^2}{Expected\ frequency}$$

For example, the phenotypic ratio of fertility restoring of 3R:1S was expected for one restorer gene and 15R:1S was expected for two restorer genes in the restorer line. JMP Pro was used to observe association between detected QTL and *Rf* genes.

QTL Mapping:

The linkage map was constructed with inclusive composite interval mapping (ICI) software with the genotypic and phenotypic data collected from the F_2 and $F_{2:3}$ populations to identify QTL associated with the restorability (Meng et al., 2015). The Kosambi function was used for the linkage map and the markers were ordered into the linkage map based on SNP markers. For identification of any QTL and its power, an Inclusive Composite Interval Mapping was performed using the additive and dominant QTL function with a 2.5 LOD for threshold. Only QTL with a P-value $\leq 10^{-3}$ (LOD score of ≥ 3.0) was declared as a major QTL. The detected QTL associated with fertility were compared to the previously reported QTLs regions using the Gramene database (https://www.gramene.org/).

RESULTS

Inheritance Analysis

As shown in Table 1, the majority of $F_{2:3}$ lines from both populations of 367R x RU1501139 and 396R x RU1501047 were segregating for fertility. The Chi-square test for 367R

population (x^2=0.7504, p-value=0.3863) and 396R (x^2=0.3604, p-value=0.5483) fit into the 15F:1S ratio (Fig.5). Therefore, 367R and 396R each possesses two restorer genes in its genome.

QTL Analysis for Allelic Relationship

To detect the position of the R genes in the 367R and 396R genomes, the populations derived by 367R and 396R were genotyped using 7K SNP genotypic markers. Among 300 F_2 plants from populations derived from 396R, only 723 polymorphic SNP markers were identified, thus the detection of major QTLs in this population was not possible because of low LOD values. However, among 295 F_2 plants from 367R x RU1501139 population, 2595 polymorphic markers were identified. The QTL analysis on the population using QTL ICIMapping software detected one region with a LOD>3.0 on chr. 10. Two adjacent QTLs associated with fertility were detected on chr. 10. The first QTL was positioned between $1.45x10^7$ and $1.46x10^7$ bp, which was co-localized with the previously reported restorer gene *Rf5*. Several SNP markers, such as SNP-10557866 and SNP-10562661, with 17-18 % phenotypic variations explained (PVE) were located at the same places. The second QTL, was located in $1.93x10^7$ and $1.98x10^7$ bp that was co-localized with the previously reported gene *Rf4*. Several markers with significant p-value (p-value<0.01). markers including SNP-10.18986400, SNP-10.18995837, SNP-10735601 and SNP-10.20184542 were located at the same region with around 2-3% PVE values (Table 3).

The results showed there is a strong association between *Rf5*, detected QTL, and two SNP markers SNP10557866 located in (14,503,250 bp) and SNP10562661 located in (14,664,0458 bp) positioned on left and right side of the gene. There was a minor linkage association between detected QTL and *Rf4*, and the SNP marker to this gene was SNP-

10.19278971, located in (19,350,417 bp) and 10734306 located in (19,860,755 bp) positioned in right side of the gene (Fig.6).

One of the two common restorer genes *Rf4* was located on chr. 10 (Gramene database, 2020). The *Rf4* gene was identified as a restorer gene via several studies on the long arm of chr. 10 (Zhang et al., 1997; Tan et al., 1998; Ahmadikhah and Karlov, 2006; Tang et al., 2014).

DISCUSSION

Hybrid rice breeding enables a significant increase (10-15%) in rice production (FAO, 2004). Crosses between genetically distinct parents can increase yield by taking advantage of the heterosis effect. In this project, our aim was to identify the number and the position of *Rf* genes in the genomes of two restorer lines developed in Arkansas (367R and 396R).

In this study, a chi-square test confirmed that restorer lines 367R and 396R have two restorer (*Rf*) genes. Quantitative trait loci analysis detected one major QTL for the restorer line 367R located between SNP: 10557866 and SNP: 10760864 ($1.45x10^7$….$2.0x\ 10^7$) in chr. 10 with a ~3.2 LOD score and this QTL was co-localized with previously reported restorer *Rf4* and *Rf5* genes.

The genetic mapping analysis on 367R detected a QTL associated with fertility in chr. 10 that colocalized with *Rf4* (Zhang et al., 1997; Tan et al., 1998; Ahmadikhah and Karlov, 2006; Tang et al., 2014). Other studies published the position of the *Rf4* gene by using candidate genes PPR9-782-(M, I) (Tang et al., 2014) and PPR762 (Suresh et al., 2012) as a major restorer gene between $1.92x10^7$ and $1.94x10^7$ base pairs (Pranathi et al., 2011).

The 7K SNP platform did not have enough resolution. Of the 7000 SNP markers, only 735, and 2345 polymorphic SNP markers were detected. Moreover, the polymorphic SNP

markers were not evenly distributed throughout the genomes. This assumption was supported by Rice et al. (2019).

Although *Rf4* is a major fertility gene, there is a low linkage associated with the detected QTL. Pranathi et al. (2016) reported that when the two major genes of *Rf3* and *Rf4* presents in a genome, one displays as a major, while the other exhibits as a minor gene. Therefore, it can be assumed that, in 367R, *Rf5* has a major gene influence, while *Rf4* is minor.

We inferred the origin of the gene of interest by analyzing the history of the crosses that led to the creation of the 367R and 396R restorer lines. The 367R was the result of crosses between the lines [Katy/IR30//IR140(PI458443)/Jasmine-85(PI595927)]. A previous study showed that IR262, one of the parental lines of cultivar Jasmine-85, possesses *Rf4* in its genome (Bharaj et al., 1995). It has been reported that Tetep and IR262 which are the parental lines of Katy and Jasmine-85, respectively, possess *Rf5* in their genomes (Bharaj et al., 1995; Seshu and Zang, 1989). Therefore, it can be assumed that *Rf4* and *Rf5* originated from Katy and/or Jasmine-85 and Jasmine-85, respectively.

Likewise, searching of the 396R parental lines [Francis//// IR 1586-2(PI400793)///Bengal//L202/Lemont] showed that Black Gora, which is the ancestral line of L202, has a restorer gene (Ntanos and Koutroubas, 2002), so it can be assumed that one of the restorer genes is derived from L202.

CONCLUSION

Restorer genes are a crucial part of hybrid rice production. However, lack of the restorer lines limits the genotypic diversity and causes biotic vulnerability (Virmani et al., 1997). To improve genetic diversity and efficiency of the three-line system, novel restorer lines are

introduced (Virmani et al., 1997; Kazama and Toriyama, 2014). Several restorer lines were developed at the RREC in Stuttgart, Arkansas (Yan et al., 2012). These lines were restorer, but the resource and number of *Rf* genes were unknown. In this study, we detected a major QTL, which included several SNP markers: SNP-10.18986400, SNP-10.18995837 and 10735601 that were adjacent with the *Rf4* gene and 10557866 and 10562661 that were adjacent with the *Rf5* gene. These markers can be used in marker-assisted selection and can improve the test cross process.

TABLES AND FIGURES

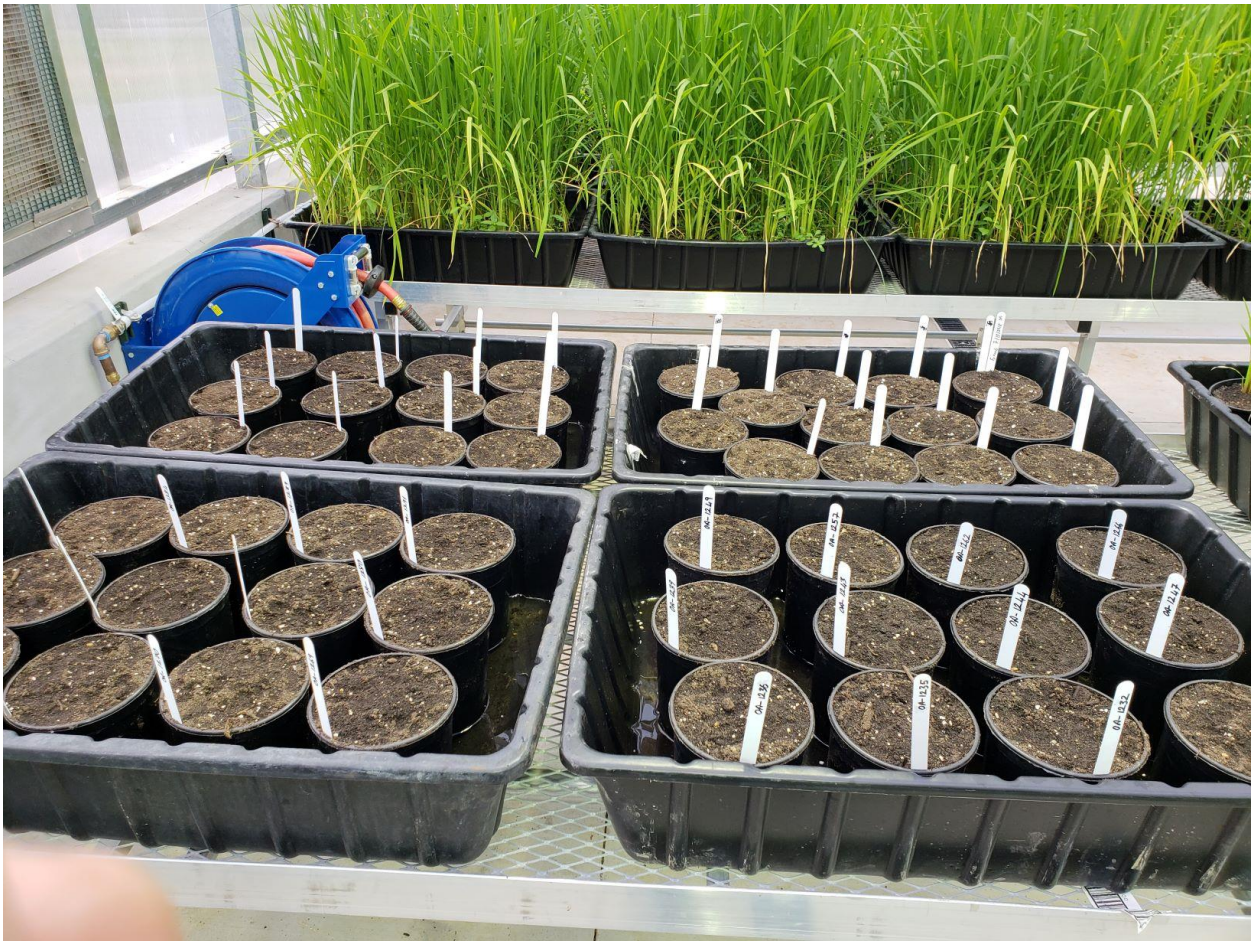


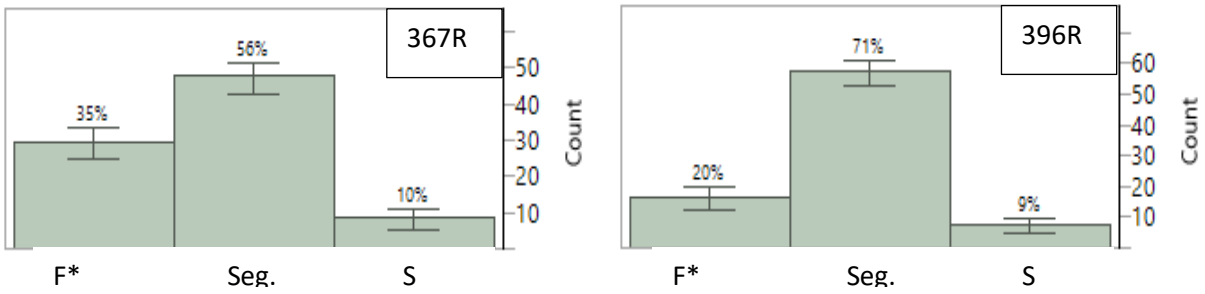
Figure 1: Plants grown in the greenhouse. Photographed by Ozgur Azapoglu.

Figure 2: 22 May planting in the field. Photo by Ozgur Azapoglu.

Figure 3: Test crossing at the sterile room in the greenhouse. Photo by Ozgur Azapoglu.

Category	Appearance	Classification
Unstained withered sterile (UWS)		Sterile
Unstained spherical sterile (USS)		Sterile
Stained round (light) sterile (SRS)		Sterile
Stained round fertile (SRF)		Fertile

Figure 4: Pollen staining scale (Virmani et al., 1997).



*Classification of $F_{2:3}$ lines F, all fertile; Seg., partial fertile; S, all sterile

Figure 5: Fertility frequency of 367R and 396 (a)

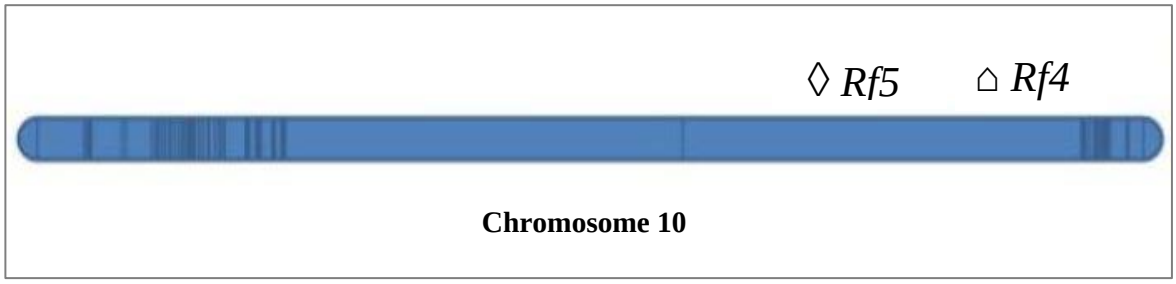


Figure 6: Linkage Map and QTL position for Restorer Gene

Table 1: Pollen-stain protocol (Guzman et al., 2011)

STEP	PROCESS
1	Stock solution prepared with 100 ml distilled water, 1 gr iodine crystals and 3 gr potassium iodide.
2	Dilute the stock solution in a rate, one-unit stock solution and four-units distilled water.
3	Collect several young spiclets at the flowering phase.
4	Anthers are removed manually by separating palea and lemma.
5	Place the anthers onto a proper slide and treat with I2K solution for 5 minutes.
6	Check the anthers with a microscope using 10x or 20x lens.
7	Fertile pollens have a dark-black color, sterile pollens will have translucent color (Fig. 4).
8	Visually estimate the pollens to determine the sterility level.

Table 2: Chi-square test from the phenotypic ratio.

Restorer Line	Chi-square ($\chi 2$)	P-value	P<0.01	P<0.05
367R	0.7504	0.3863	0.5636	0.5483
396R	0.3604	0.5483	0.7640	0.5636

Table 3: List of parental detected quantitative trait loci.

QTL	Parental origin of positive allele	Chromosome	LeftMarker	RightMarker	Base Pair Position (bp)	Logarithm of the odds (LOD)
qTL-1	367R	10	10557866	10760864	14503250	3.5476
qTL-2	367R	10	10735601	SNP-10.20184542	20743450	0.6819

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Chapter 3

EVALUATION OF TRAITS ASSOCIATED WITH SEED CHARACTERISTICS IN ARKANSAS RESTORER LINES

ABSTRACT

The primary objective of most rice (*Oryza sativa* L.) breeding programs is to enhance grain yield. Grain shape is one of several important factors to increase yield capacity (Huang et al., 2013). Grain shape is measured by its length, width, thickness, and the ratio of length-width. Since the importance of these agronomic traits were realized, researchers have taken further interest in grain shapes. In this context, an experiment was conducted during fall 2017 to 2020 identify seed dimension quantitative trait loci (QTL) on both 367R and 396R bi-parental populations in Stuttgart, Arkansas. Five seed dimension traits including seed length, seed width, seed thickness, seed length-width ratio and 100-seeds weight were obtained for QTL detection. The study detected a total of 17 QTL. Four QTL were associated with seed length. Of these QTL, two were identified in chr. 3, one in chr. 7 and one in chr. 11. Two QTL related to seed length-width ratio were identified in chrs 3 and 7. Whereas a total of three QTL were identified for seed thickness, one each in chrs. 5, 6 and 8. Eight QTL were associated with seed weight, four in chr. 12, two each in chrs. 1 and 10, and one in chr. 3 for the population of 367RxRU1501139. Since the yield and seed dimensions could be controlled by multiple genes, the detected QTL can play a role in introducing superior parental lines for hybrid rice production.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the major crops for food and income resources for almost half of the world's population. With the rapid increase in the world's population, rice production must continuously increase as well. To satisfy the demand in rice, an increase of 30% rice production by 2050 is necessary (World Bank, 2013; Feng et al., 2014). In order to speed-up the improvement of rice yields, yield components must be improved. In particular, the number of grains per panicle, panicle number and seed weight (SW hereafter) should be further studied (Weng et al., 2008 & Wang et al., 2015). Of these aforementioned components, SWT, which is controlled by multiple genes and several identified quantitative trait loci (QTLs), has the greatest chance in improving yield (Weng et al., 2008; Huang et al., 2013 & Wang et al., 2015). Additionally, increasing grain dimensions are key breeding factors for more yield. Seed dimensions that affect the yield potential are seed length (SL hereafter), seed width (SWT hereafter), seed thickness (ST hereafter) and seed length width ratio(SLWR hereafter) (Huang et al., 2013; Qiu et al., 2012 & Wang et al., 2015). While rice is classified according to grain forms as rough, brown, and milled rice, SL is the primary factor in rice classification. Based on SLWR, rice is classified into three subgroups: long-grain, medium-grain and short-grain (Hardke et al., 2018 & Qiu et al., 2017). Seed length and SWT, and their ratio determine the kernel size where the ratio is between 3.0 to 1 or greater in long-grain rice and 2.0 to 1 in medium-grain and short-grain rice).

In regard to the classification of rice, cooking characteristics are affected by the chemical structure such as fluffy, aroma, sticky and amylose content (Hardke et al., 2018). In the United States, long- (~75%) and medium-grain (~25%) varieties are primarily cultivated (Mcbride et al., 2018). In the long-grain varieties, moderate amylose (20-24%) content brings fluffy and non-

sticky structure when cooked. The non-sticky structure, quicker cooking times and partial boiling are advantages that makes long-grain rice a favorite for ready food production, such as quick cooking rice, canned rice, canned-dry soups, and frozen foods. On the other hand, medium- and short-grain varieties are sticky and have a moist structure when cooked because of low amylose content (10-20%). Ready dry foods, such as cereals, baby foods and beverages, are produced from the medium-grain varieties (Webb et al., 1985; Hardke et al., 2018; Hardke, 2018).

Researchers have reported several QTLs related to yield and grain sizes, but the knowledge of other seed dimension traits QTLs are limited. Thus, studies focusing on QTLs that could be related to grain dimensions are essential (Fan et al., 2006 & Wang et al., 2015). Che et al (2015) conducted a QTL study on an F_2 population created by crossing two *indica* rice lines (RW11 x BobaiB) that were significantly distinct (about 37 %) from each other in terms of their SLs. Che et al (2015) developed two backcross populations between an F_2 population and RW11- BobaiB, separately. The QTL was identified on chr. 2 and identified as GL2 from the backcross with RW11. Then, RW11 crossed with Nipponbare (*Japonica* variety). The GL2 improved the grain dimensions around 24% for SL, 16% for SWT, and about 27% more in 1000 grain weight.

Qiu et al (2020) conducted a two-year (2015-2016) genetic mapping study to clarify the QTLs associated with grain dimensions. Qiu et al (2020) used 1016 accessions in five populations: *indica*, *japonica*, *aus*, *basmati*, and *admixture* from the 3K Rice Genome Project (accessions collected from China, India, Philippines, Bangladesh, Japan, and other Asian countries). Seventy QTL were identified for seed dimensions (SL, SWT, SLWR) on all 12 chromosomes. Twenty-four QTLs were identified on chrs. 1-7, 9-11 for SLR, and the phenotypic effect was between 1-30%. Twenty-one QTLs were identified on all chromosomes excluding

chrs. 10 and 12 for SWT and the phenotypic variation changed between 1 and 42%. They detected 25 QTLs for SLWR on chrs. 1-8, 11, and 12 with between about 1 and 28% phenotypic variation (Qiu et al., 2020).

Eizenga et al., (2018) identified a total of 27 QTLs for yield-related traits. A RIL population developed by using two *tropical japonica* lines 'Estrela' and 'NSFTV199.' F_1 seeds were advanced to F_7, producing a final population size of 256 RILs. Grain' dimension traits studied include SL, SWT, SLWR, and 100-Seed weight (SW hereafter). The research detected seven QTLs including a major QTL '*qHULGRLG3'* explaining around 40 % of the phenotypic variation on chr.3. Six QTL for SW were identified with the major QTL '*qHULGRWD5*' explaining 38% of the phenotypic variation. Eight QTL were identified for SLWR, which were at the same locations as QTL '*qHULGRLG3* and *qHULGRWD5*' with 32.6% and 38.9% phenotypic variations. Six QTL were identified for SW. The objective of this study was to identify QTL associated with seed characteristics including SL, SWT, ST,SLWR, and SW. Results of this study could contribute to the improvement of the genetic background of yield-related QTLs through introduction of each QTL themselves for the improvement of rice's yield potential.

MATERIALS AND METHODS

Plant Materials

A bi-parental population resulting from a cross between the restorer line '367R' and a non-restorer line 'RU1501139' were developed for this study. Restorer line 367R is a medium-grain rice and was developed at the University of Arkansas's Rice Research and Extension Center (RREC), Stuttgart by Yan et al. (2012). Restorer line 367R is derived from

Katy/IR30//IR140(PI-458443)/Jasmine-85(PI-595927) crosses. Non-restorer line RU1501139 is a long-grain, advanced line developed by the RREC long-grain program. The population development and the methods of plant management from the start to the F_2 plants production were discussed in Chapter 2. A total of 300 F_2 plants from this population were grown in three replications in a greenhouse using a completely randomized design (CRD) to evaluate traits associated with seed characteristics. The $F_{2:3}$ seeds were harvested and used for phenotypic evaluation.

Preliminary Study

Two genotypes of 367R and RU1501139 were grown in three replications in the greenhouse using a randomized complete block (RCB) to evaluate traits associated with seed characteristics (seed SLR, thickness, width, SLR and SW). Each replication consisted of three plants. The panicles for each parent were randomly collected in the greenhouse. The panicles were dried (15% moisture) and threshed in Stuttgart, Arkansas. In order to analyze the seed dimensions, 30 seeds from each line were randomly selected, cleaned and evaluated via Mettler Toledo® balance and Winseedle® Pro (Fig. A) to measure the grain dimensions' significance level. According to the JMP Pro 14 software (SAS Institute Inc., Cary, NC), an ANOVA analysis followed by Student's T-test had significant results regarding SL, SWT, SLWR, ST and SW. The results indicated that SL and SLR had a significant effect, but seed thickness, SW and SLWR had no effect.

Phenotyping

Based on the preliminary study of parental lines, the population 367R x RU1501139 was used in this study. The $F_{2:3}$ seeds harvested in the greenhouse were measured for SLR, SWT, ST, SLWR, and SW in the Spring of 2018 at RREC in Stuttgart, Arkansas. To evaluate seed dimensions and SW, 100 seeds from each $F_{2:3}$ lines were measured via Winseedle® Pro and the Mettler Toledo® balance, respectively. Then, the three replications of all 100-seeds had an average value for every 300 lines that were calculated in an Excel® file. One-way ANOVA analysis followed by Student's T-test significance results of the seed dimensions (SL, SWT, SLR, ST, and SW) (Table 1). Multivariate analysis was run to understand the correlations between traits by using JMP Pro 14 software (Fig. 1).

Genotyping

The tissue samples were collected from both parental lines; 367R, 396R, Newbonnet, RU1501139, RU1501047 and each F_2 plant from the populations of 367R x RU1501139 at the V5 growth stage for genotyping via single nucleotide polymorphism (SNP) markers. The parental line samples and the F_2 plant population samples were sent to an Illumina sequencing company, located in River Falls, Wisconsin, to be genotyped with an Infinium Rice 7K Chip (Morales et al., 2020). In this study, the F_1 plants for parental lines and F_2 plants for the population 367R x RU1501139 were genotyped using 7,000 SNP Infinium markers. Then, the $F_{2:3}$ seeds from each single plant were harvested in three separate replications. The linkage map was created via inclusive composite interval mapping (ICI) software by using genotypic data from F_2 and phenotypic data from $F_{2:3}$ seeds while creating QTLs related to seed dimensions. The ICI Mapping was used with the Kosambi function for linkage mapping and SNP markers were

ordered for linkage mapping. The identification and detection of the QTLs, 2.5 LOD score was considered as a threshold level for a major QTL. The Oryzabase database was used to detect any co-localized QTLs. The distribution of the seed dimensions and detected QTLs were analyzed using JMP Pro 14 software (Fig. 2). Oryzabase a comprehensive rice data source, was used to identify candidate genes.

RESULTS

Preliminary Study

The ANOVA study showed that there are significant differences between 367R and RU1501139 on SL, SLWR (p-value< 0.001), and SW, SWT(p-value< 0.05). There was no difference for ST between these two lines (Table 1).

Parental Significance Analysis of $F_{2:3}$ Population

The ANOVA analysis for the Population-A was used to find significance levels of seed dimensions between parents within a linkage map. The results indicated that seed length and length-width ratios had a significant effect, but seed thickness, 100-seed weight and seed-width had no effect.

Seed Length: The distribution of $F_{2:3}$ for SL followed a normal distribution (Fig. 2). SL had a mean of 9.7 mm with a range from 8.2 to 11 mm. The trait had a standard deviation (SD) of 0.48 and a standard error (SE) of 0.03. These two values explained the significance of seed length with a p-value < 0.001 for the population (Table 1).

Seed Width: The distribution of $F_{2:3}$ for SWT followed a normal distribution (Fig. 2). There was no difference in seed width with a mean of 2.5mm and range from 2.3 to 2.7mm. Seed width had a 0.15 SD and SE of 0.06. While the trait was not significant at p-value of 0.001, it had significance with a p-value < 0.05 (Table 1).

Seed Length-Width Ratio: The distribution of $F_{2:3}$ for SLWR followed a normal distribution (Fig. 2). There was no difference for seed width; however, the seed length-width ratio had a significant difference with a mean of 3.74 mm and a range from 3 to 4.5 mm and a SD of 0.29 and SE of 0.018. Significant difference between parents 367R and RU1501139 expressed with a p-value < 0.001 for the population (Table 1).

Seed Thickness: The distribution of $F_{2:3}$ for ST followed a normal distribution (Fig.2). For ST, the mean number of thickness in the population was 2.11 mm and range from 1.7 to 2.11 mm. The SD for thickness was 0.09 and SE was 0.012. The difference between parents 367R and RU1501139 was not significant with a value p-value > 0.05 (Table 1).

100-seed weight: The distribution showed majority of the $F_{2:3}$ lines ranged between 1.25 to 1.5gr (Fig. 2). For SW, the mean was 2.5gr, ranging from 2.3 to 2.7gr. The trait had a 0.15 SD and SE of 0.06 in the population. The difference between parents in the population expressed a p-value < 0.05(Table 1).

Multivariate analysis showed that a positive significant correlation (p-value>0.001) between SL and SLWR (r=0.44) and ST (r=0.23), and SWT (r= 0.166, p-value>0.01). The results revealed that SLWR has a strong negative correlation with SWT(r=0.622, p-value>0.001)

but positive with SW(0.213, p-value>0.01). The analysis showed that ST has a positive correlation with SW (0.48, p-value>0.001) (Table. 1).

Genotypic study

A total of 17 major QTL were identified in the bi-parental population of 367R x RU1501139. For SL, four QTL were identified including two QTL, *qSL3-1* and *qSL3-2* on chr. 3, and one QTL *qSL7-1 qSL11-1* each on chrs. 7 and 11, respectively (Fig. 3). The detected QTL were linked to RU1501139 and infer increasing seed yield and explained 5.1 to 8.4% of phenotypic variation (PVE) on the population (Table 2).

No major QTL for SWT were detected; however, 12 minor QTL were identified including 8 minor QTL with (2<LOD<3): two QTL on chr. 2, and three QTL each on chrs. 7 and 10, respectively.

Two major QTL, *qSLWR3-1*, *qSLWR7-1* were detected on chrs. 3 and 7 for SLWR. These two QTL were co-localized with the QTL, *qSL-2* and *qSL7-1*, which identified SLs. The detected QTLs were linked to RU1501139 and explained 5.5 to 11.1% of phenotypic variation (PVE) on the population. The *qSLWR3-1* and *qSLWR7-1* were co-localized with other detected QTL, *qSL3-2* and *qSl7-1*, respectively (Table 3) .

Eight QTL were identified for SW including two QTL on each chr. of 1, 2, 10 and 12. Seven of these QTLs were co-localized with previously reported QTLs, AQEI043, AQBA011, AQAP004, AQCI003, AQCS003, AQAE008 and AQF014, respectively (Table 3). Furthermore, all eight QTL originated from 367R .

Three QTLs were identified on chrs. 5, 6 and 8 associated with ST. The QTL *qST5-1* on chr. 5 co-localized with a previously reported QTL AQFU013 (Table 3) for seed thickness. The detected QTLs linked to the 367R had a range of 4.6 to 7.5% phenotypic variation.

Detection of Candidate Genes for Major QTL

A total of five candidate genes were identified via rice genomic annotation using the online rice database of Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/), including four for SL and two candidate genes for SW (Table 3).

Two candidate genes of the *GL-7* and *OsGASR9* are identified within a detected QTL *qSL7-1* ($2.3x10^6..2.3x10^6$) associated with SL. *GL-7* is a previously reported gene that regulates seed length by increasing the length and starch structure in endosperm (Wang et al., 2015). *OsGASR9* is a transcript gene for plant growth and development. The *OsGASR9* increases grain length and weight by increasing the efficiency of gibberellic acid (Li et al., 2019). It is worth noting that *qSL7-1* is co-localized with another detected QTL *qSLWR7-1* associated with SLWR.

Two candidate genes were identified on the detected QTL *qSL11-1* ($16.28 x10^6.. 17.69 x10^6$) associated with SL on chr. 11 including Rice Big Grain-1 (*RBG1*) and Flower and Leaf Color Aberrant (*FLA*). The *RBG1* gene is responsible for grain development, abiotic stress tolerance and the gene improves root development by enhancing the plant's auxin level (Lo et al., 2020). The *RBG1* is 948 bp and its four allelic genes are located near the *RBG1*gene, 5 kb to M37341, ~27 kb to M37342 and M82594l, 46 kb to M44256 (Lo et al., 2020). The *FLA* gene is a ubiquitously expressed gene and a key factor for flower and chloroplast development. The *FLA* improves grain length and rice yield. The *FLA* is located between the marker *M11-3* and *S6* with 56 kb on the long arm of chr. 11 (Ma et al., 2019).

One gene (*HAP5L*) is located within a detected QTL, *qSW10*-1(6.64×10^6 ..9.26×10^6) associated with SW. The *HAP5L* is an endosperm-specific gene that regulates starch accumulation and protein concentration (Xiong et al., 2019). The accumulation of starch increases the width, but any decrease in *HAP5L* causes sharp decreases to grain weight (Xiong et al., 2019).

DISCUSSION

In this study, we aimed to identify the genetic sources associated with seed characteristics in rice. The preliminary study on two genotypes (367R and RU1501139) determined significant differences between the two genotypes for four seed characteristics of SL, SWT, SLWR, and SW. restorer line 367R is a medium-grain rice that is shorter (< 3mm) than typical long-grain rice. Seed length-width ratio is an important measurement for classification of rice cultivars. The results showed a positive correlation between SLWR and SL, but a negative correlation with SWT. The data showed a positive correlation between SW with SL. Although there was no significant correlation between SW and SWT, the data showed a weak negative correlation between these two trait. Furthermore, results revealed that there was a positive correlation between SWT and ST. Therefore, it can be assumed that longer and thicker seeds are heavier than shorter and wider seeds.

Enhancing grain yield, milling, and eating quality of rice can be achieved through development of superior cultivars by incorporating a number of agronomic traits, such seed dimension and seed weight. The majority of these traits are classified as quantitative traits and are controlled by several QTL located in different parts of the rice genome. Each QTL has different impact on the phenotypic variation. In a breeding program, a breeder considers only

those QTL that have the greater impact on the phenotypic variations. In this study, we identified 17 major QTL and several minor QTL associated with seed characteristics. Annotation analysis revealed that five detected QTL contain genes associated with seed characteristics and 11 were co-localized with previously reported QTLs (Huang et al., 1997; Redona et al., 1998; Xing et al., 2001; Jiang 2004; Alam et al., 1998; Zhu et al., 2000; Xu et al., 2002; Mei et al., 2003; Wissuwa et al., 1998; Sato et al., 2003; Cui et al., 2002; Zuang et al., 2001; Aluka et al., 2004). It can be concluded that 1) the annotation analysis of the QTL validates our finding via previously reported genes/QTLs associated with traits, and 2) these QTLs can be incorporated into the genomes of new superior genotypes.

For example, one important detected QTL is *qSL7-1* on chr. 7 associated with SL. The QTL is co-localized with *qSLWR7-1* associated with SLWR. Further investigation identified two candidate genes, GL7 and *OsGASR9*, in this genomic region. One important detected QTL *qSL3-2* on chr. 3 associated with SL is co-localized with *qSLWR3-1* and is associated with SLWR.

On chr. 11, one QTL *qSL11-1* was detected for SL. Two candidate genes, *RBG1* and *FLA* were identified on chr. 11 for SL. The *RBG1* gene is associated with grain, root development and stress tolerance by enhancing cell division and auxin levels; thus, it helps to improve root development and stress tolerance, which are important factors for having a greater yield. (Lo et al., 2020). The second candidate gene, *FLA*, is a cell membrane protein that belongs to the Ubiquitin-specific proteases. The *FLA* is a common amino acid for eukaryotic cells. The *FLA* improves grain length and yield by regulating chloroplast and flower development (Ma et al., 2019). We can summarize that the QTLs *qSL7-1* and *qSL11-1* contain several candidate genes associated with seed length and have major impact on the phenotypic variations, thus these two QTL can be integrated in a new generation of long-grain rice cultivars.

Although the ANOVA analysis showed significance of SWT in this population, no major QTL were identified on chrs. However, a total of 12 minor QTL were detected with an LOD of 8 QTL range from 2 to 3 LOD score. It can be assumed that SWT is controlled by several minor QTLs that, overall, significantly enhance SWT.

The ANOVA analysis showed there was no difference between 367R and RU1501139 for the ST trait, but the genotypic analysis identified three major QTLs associated with the ST trait. Genotypic analysis showed that the two QTL of *qST5-1* and *qST6-1* originated from 367R, while *qST8-1* originated from RU1501139. Therefore, despite no statistical significance, there is a biological significance between these two genotypes due to these detected QTLs.

CONCLUSIONS

In rice breeding, the ultimate goal is to increase grain yield. Grain yield is affected by several components such as SL, SWT, SLWR, ST and SW. In this research, 17 QTL associated with seed characteristics were identified. Further studies are needed to identify major genes associated with these characteristics and developing molecular markers that can be used for marker assisted selection in breeding programs.

TABLES AND FIGURES

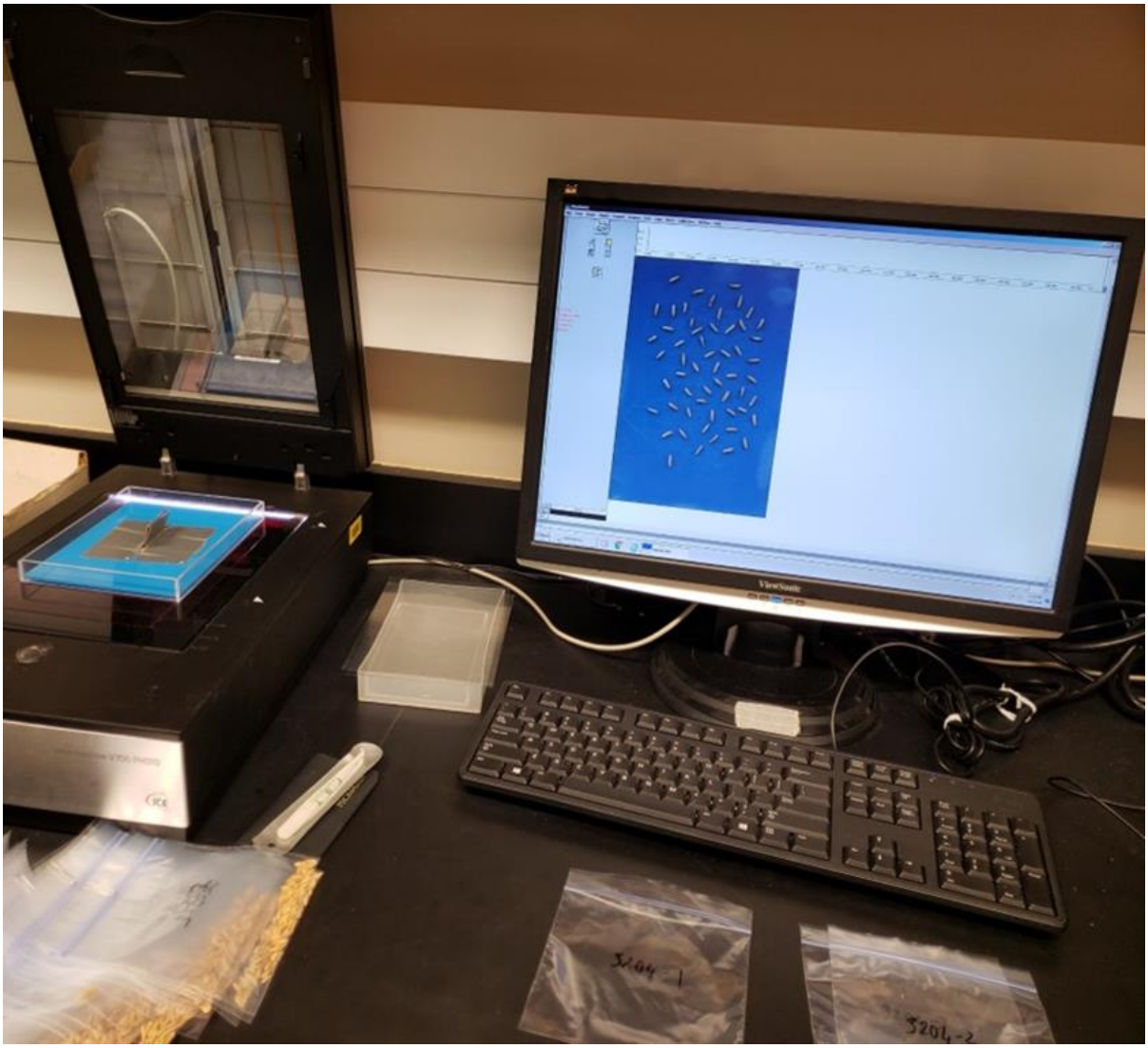


Figure A: Winseedle® Pro Grain dimension measurement.

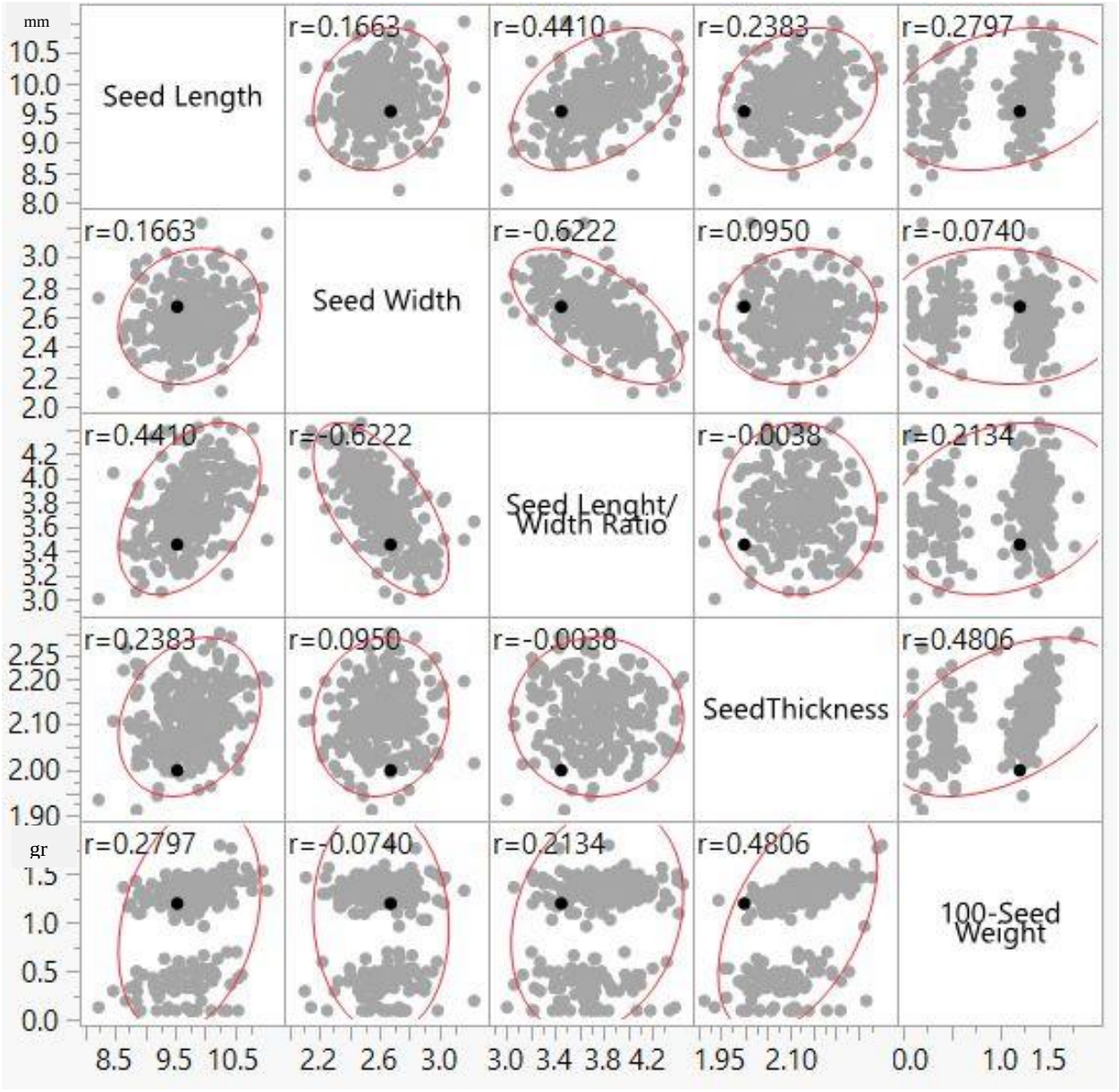
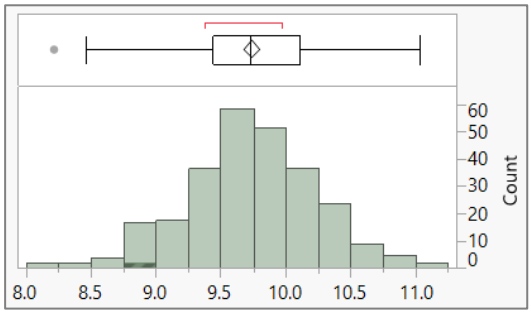
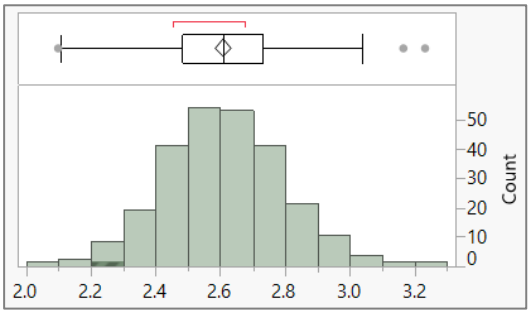


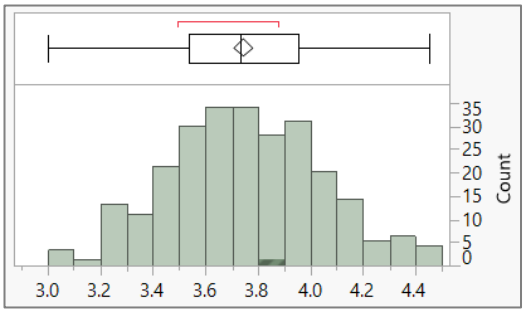
Figure 1: Multivariate correlation analysis of Seed Dimensions in F_2



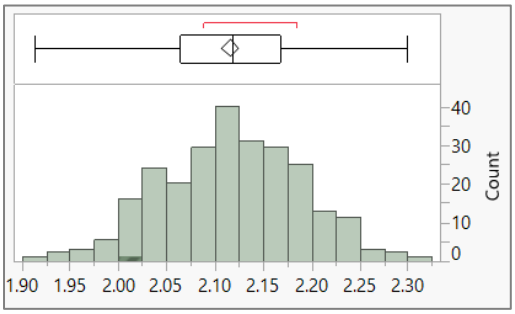
A: Distribution of Seed Length



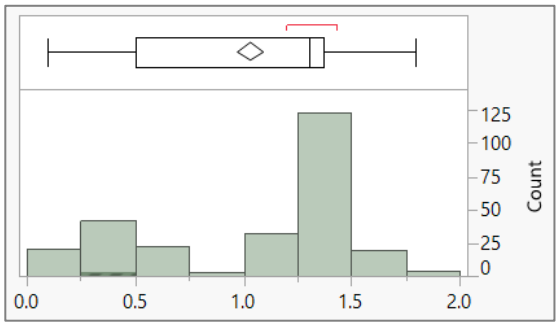
B: Distribution of Seed Width



C: Distribution of Seed Length/Width



D: Distribution of Seed Thickness



E: Distribution of 100-Seed Weight

Figure 2: Distribution of Seed Dimensions

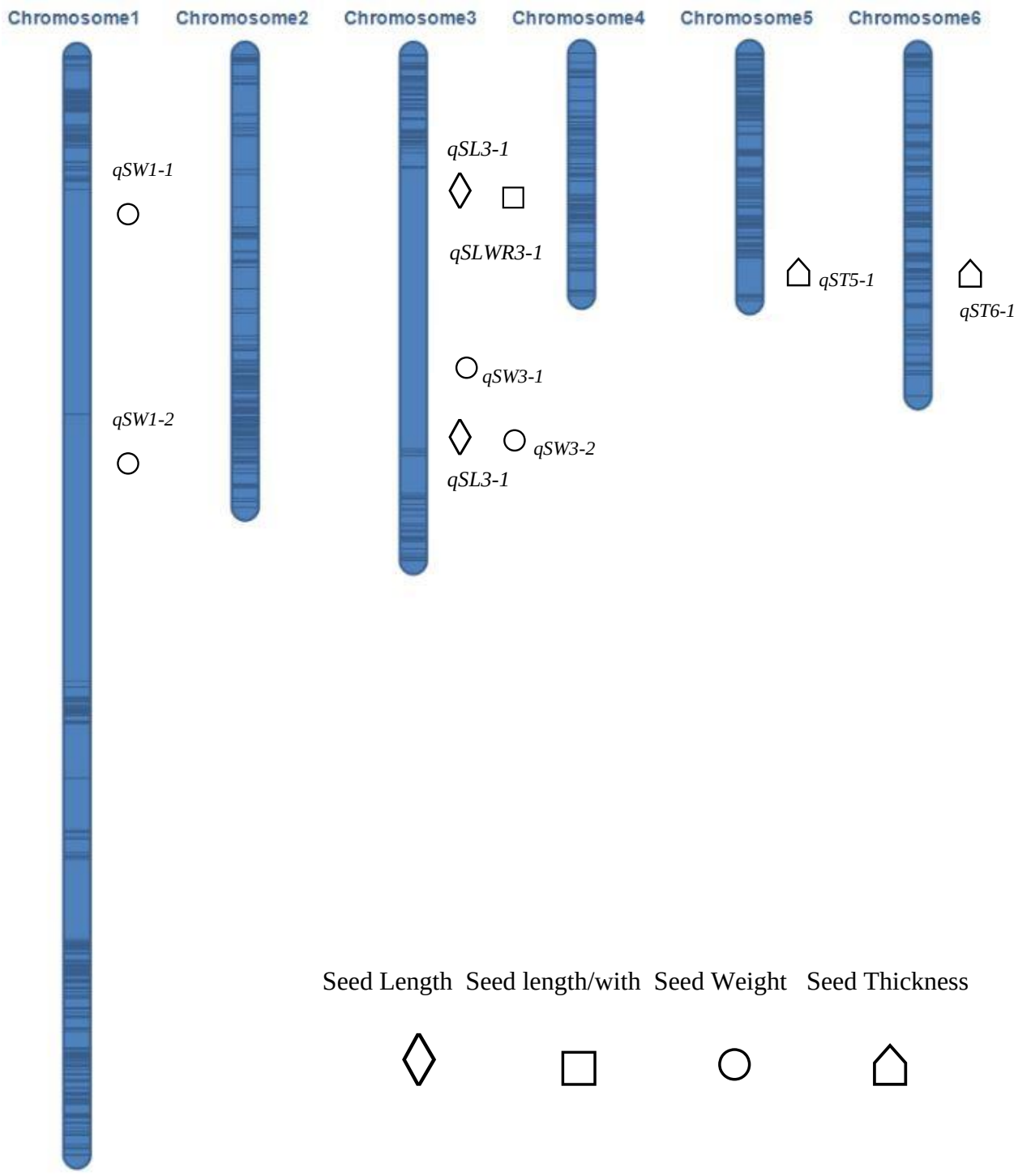


Figure 3: Linkage Map and QTL position for Seed Dimensions

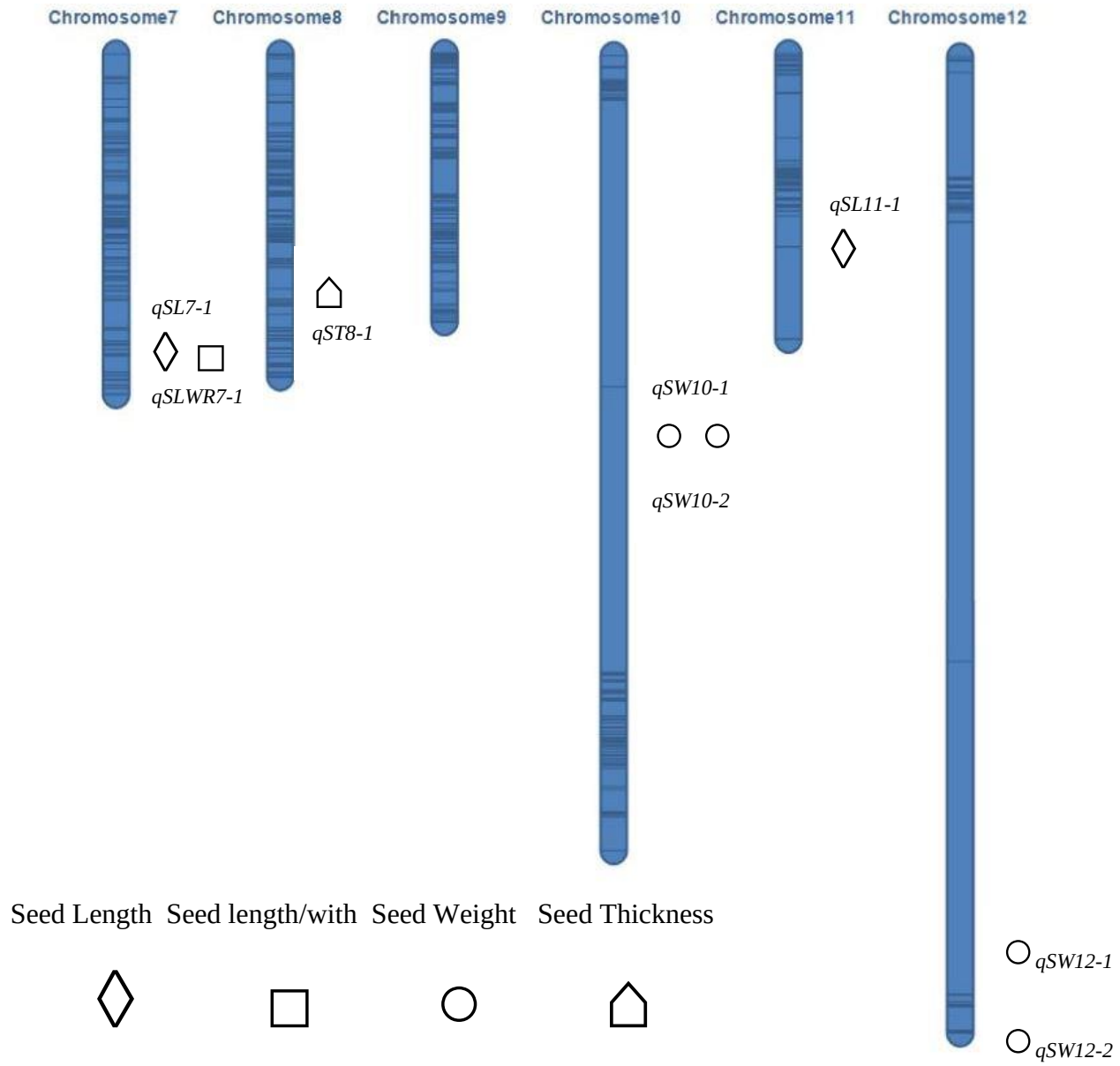


Figure 3: Cont.

Table 1 : ANOVA analysis of Seed Dimensions in $F_{2:3}$ population

Trait	µ367R	µRU1501139	µPopulation-A	Range	Standard Deviation	Standard Error Mean	F
SL	10.24	9.06	9.73	8.2-11.03	0.48	0.03	118.0720**
SWT	2.56	2.5	2.61	2.1-3.23	0.185	0.01	3.1865*
SLWR	4	3.62	3.75	3.0-4.45	2.89	0.018	42.0621**
ST	1.96	1.93	2.12	1.91-2.3	0.07	0.004	0.8935
SW	2.5	2.36	1.03	0.1-1.8	0.478	0.02	32.000*

Effects with P-values < 0.01 given *
Effects with P-values < 0.001 given **

Table 2: List of QTL detected and parental origin of positive allele for major QTL

QTL	Parental origin of positive allele	LeftMarker	RightMarker	BP Position	LOD	PVE(%)	Add.	Dom.
qSL3-1	*367R*	2624847	2641058	$6.22x10^6$-$6.99x10^6$	3.4262	5.2782	0.0334	0.2151
qSL3-2	*367R*	id3014217	3417192	$30.12\ x10^6$-$30.45\ x10^6$	3.8808	5.9111	0.1625	0.0063
qSL7-1	*RU1501139*	7818489	7869914	$23.68\ x10^6$-$25.52\ x10^6$	4.8256	8.3937	-0.1989	0.0649
qSL11-1	*RU1501139*	11465340	c11p17119245	$17.23\ x10^6$-$17.11\ x10^6$	3.4371	5.1480	0.1511	0.0813
qSLWR3-1	*367R*	id3014217	3417192	$30.12\ x10^6$-$30.45\ x10^6$	3.8508	5.4423	0.0958	0.0525
qSLWR7-1	*RU1501139*	id7004041	SNP-7.23491886.	$23.08\ x10^6$-$23.49\ x10^6$	7.4073	11.0463	-0.1451	0.0299
qSW1-1	*367R*	255699	312212	$8.29\ x10^6$-$10.34\ x10^6$	14.2588	2.4703	0.0571	0.8289
qSW1-2	*367R*	312212	id1007778	$10.34\ x10^6$-$10.80\ x10^6$	19.0820	2.5001	0.4698	0.3372
qSW3-1	*367R*	2650075	3399945	$7.3\ x10^6$-$29.75\ x10^6$	18.4045	2.4567	-0.4345	0.4044
qSW3-2	*367R*	id3014217	3417192	$30.12\ x10^6$-$30.45\ x10^6$	4.0224	0.2401	0.1734	-0.0022
qSW10-1	*367R*	SNP-10.8934622.	10348161	$9.00\ x10^6$-$9.2\ x10^6$	14.5954	2.4698	-0.0112	-0.8350
qSW10-2	*367R*	10348161	SNP-10.9220148.	$9.2\ x10^6$-$9.3\ x10^6$	14.6125	2.4710	-0.0148	-0.8353
qSW12-1	*367R*	12661368	SNP-12.20165789.	$15.9\ x10^6$-$20.19\ x10^6$-	13.8334	2.4302	0.0080	-0.8212
qSW12-2	*367R*	SNP-12.20165789.	SNP-12.21730645.	$20.19\ x10^6$-$21.76\ x10^6$	21.5835	2.5226	-0.4770	0.3241
qST5-1	*367R*	5604007	5612073	$22.29\ x10^6$-$22.59\ x10^6$	4.3585	7.5423	0.0254	-0.0093
qST6-1	*367R*	6642523	6684382	$21.53\ x10^6$-$22.48\ x10^6$	2.7006	4.6149	0.0220	-0.0011
qST8-1	*RU1501139*	8757429	8764880	$18.74\ x10^6$-$18.91\ x10^6$	3.4779	5.8963	-0.0203	-0.0155

Table 3: List of Previously reported co-localized QTL

QTL	Candidate Genes	Synonyms	Previously Reported QTL	Reference
qSL3-2		*GL11*	AQDH002	(Huang et al., 1997)
qSL7-1	*GL7 - OsGASR9*	-	AQEO012	(Redona et al., 1998)
qSL11-1	*RBG1 - FLA*	*GL11*	AQCA006	(Xing et al., 2001)
qSW1-1		-	AQEI043	(Jiang 2004)
qSW3-1		*Pdw3-1*	AQBA011, AQBX006	(Alam et al., 1998; Zhu et al., 2000)
qSW3-2		*QBphr3*	AQAP004, AQCU183	(Xu et al., 2002; Mei et al., 2003)
qSW10-1	*HAP5L*	-	AQCI003	(Wissuwa et al., 1998)
qSW10-2		-	AQCS003	(Sato et al., 2003)
qSW12-1		*qLS12-1*	AQAE008	(Cui et al., 2002)
qSW12-2		-	AQCF014	(Zuang et al., 2001)
qST5-1		*MR5*	AQFU013	(Aluka et al., 2004)

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GENERAL CONCLUSION

Rice (*Oryza sativa L.*) is one of the most crucial crops around the world. Hybrid rice breeding promises to increase rice yield by using male sterile lines in cross-breeding. The hybrid rice system requires restorer lines that provide viable pollen for fertilization of the male sterile plant as a result of the presence of a restorer gene (*Rf*) in their genomes. All restorer lines should contain genes/QTL associated with restorability in its genome. Two of the developed several restorer lines, 367R and 396R in Stuttgart, Arkansas showed higher yield capacity. In this study, the number of *Rf* genes and resources of the *Rf* genes were identified. A chi-square test on phenotypic data proved the presence of two *Rf* genes for both restorer lines. Then, a major QTL was identified between SNP: 10557866 and SNP: 10760864 ($1.45x10^7$….$2.0x\ 10^7$) in chr. 10 with a ~3.2 LOD score for the 367R. This QTL included SNP markers: SNP-10.18986400, SNP-10.18995837 and 10735601 that were adjacent with the *Rf4* gene and 10557866 and 10562661 that were adjacent with *Rf5* gene. These markers can be used in marker assisted selection and can improve the test-cross process.

Since the main objective of breeding is to increase grain yield, the second study involved, parental lines that were evaluated for several traits associated with agronomic traits, such as seed length, seed width, seed thickness and 100-seed weight for the 367R × RU1501139 population. Seventeen QTL were identified for seed dimensions. Four QTL were associated with seed length in chrs. 3, 7 and 11. Eight QTL were associated with seed weight in chrs. 1, 3, 10 and 12. Two QTL located in chrs. 3 and 7 were associated with seed length-width ratio. Three QTLs located in chrs. 5, 6 and 8 were associated with seed thickness.