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Creating New Germplasm to Improve Aflatoxin Resistance in Maize

Oluwaseun Felix Ogunola

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By

Oluwaseun Felix Ogunola

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By

Oluwaseun Felix Ogunola

Approved:

_____

Marilyn Louise Warburton
(Major Professor)

_____

W. Paul Williams
(Co-Major Professor)

_____

Brian S. Baldwin
(Committee Member)

_____

Teddy P. Wallace
(Committee Member)

_____

Janice DuBien
(Committee Member)

_____

Michael S. Cox
(Graduate Coordinator)

_____

George M. Hopper
Dean
College of Agriculture and Life Sciences

Name: Oluwaseun Felix Ogunola

Date of Degree: December 14, 2018

Institution: Mississippi State University

Major Field: Agronomy

Select Appropriate Title: Dr. Marilyn Louise Warburton

Title of Study: Creating new germplasm to improve aflatoxin resistance in maize

Pages in Study 171

Candidate for Degree of Doctor of Philosophy

Maize (*Zea mays* L), the third largest crop produced in the world is susceptible to pre and post contamination of aflatoxin. Aflatoxin is a secondary carcinogenic metabolite produced by *Aspergillus flavus*, an opportunistic fungus of maize that causes ear-rot and subsequent production of aflatoxin. Host plant resistance is one of the promising long term solution to combat aflatoxin contamination in maize grains. Resistance to aflatoxin is heritable and resistant germplasms that can be used to reduce aflatoxin resistance has been developed. Creating quantitative trait loci (QTL) mapping populations to identify regions within maize chromosomes contributing to aflatoxin resistance help identify markers that can be used for successful introgression of the QTL into desirable backgrounds. To further confirm and validate QTL detected in a mapping population, creating near isogenic lines (NILs) with identified QTL is important. Furthermore, stacking of validated QTL through recurrent selection (RS) will help to improve aflatoxin resistance in maize.

In this study, an $F_{2:3}$ mapping population was derived from crossing CML69, an aflatoxin resistant inbred genotype unrelated to other genotypes used in previous mapping population and Va35, an aflatoxin susceptible inbred germplasm adapted to southeastern U.S. The $F_{2:3}$ families were phenotyped for aflatoxin at Mississippi State, MS and Lubbock

TX in 2016 and 2017 and genotyped using 1331 polymorphic markers. Composite interval mapping (CIM) identified 16 significant QTL in all four environments on chromosomes 1, 2, 3, 4, 5, and 7, and these QTL explains between 4% - 18% of the phenotypic variation observed in the population. Fourteen of the 15 QTL co-locate with previously identified QTL, suggesting that they will be stable in different genetic backgrounds and environments, and one novel QTL will provide additional resistance. Sixteen single nucleotide polymorphism (SNP) markers linked to QTL identified in a previous genome wide association mapping (GWAS) study were also used to create Near Isogenic Lines (NILs) to validate their effect on aflatoxin resistance. Finally, recurrent selection (RS) populations were created to increase the frequency of aflatoxin-resistant alleles from an 8-way cross derived from 8 aflatoxin resistant inbred maize lines.

DEDICATION

I will like to dedicate this dissertation to my parents Pastor and Mrs. Ogunola for their constant prayers, support, and for believing in me even when I doubt myself. Also to my wife, Marquisha Flanagan and my beautiful girls Oluwadarasimi Ogunola and Phiona Ifedayo Ogunola. They are the reasons I kept going till this success was achieved.

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CHAPTER I

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important cereal grains in the world, with a production of approximately 40,861 million bushel in 2017 (USDA FAS, 2018). Maize contains 72% starch, 10% protein and 4% fat, which makes it one of the leading energy supplying food ingredients (365Kcal/100g) in the world (Ranum et al., 2014). It is used for feed, fuel, as a source for fiber, and to extract other industrial components for plastics, paints, glues and pharmaceuticals. Maize is also a model organism used by plant breeders and geneticists, because they can often look for genes identified in maize in other species such as sorghum and wheat (Tenaillon & Charcosset, 2011). The good characteristics of maize are marred by its susceptibility to aflatoxin accumulation produced by *Aspergillus flavus* (Link:Fr), an opportunistic saprophyte and necrophytic fungal pathogen. *A. flavus* is ubiquitously found in soil, water, and air, and grows abundantly on damaged and decaying crops (Klich, 2007). It is known to colonize many economically important oil seed crops such as, legumes, tree nuts, and some cereal grains. Infection can occur pre- or post-harvest under favorable conditions; it is especially common pre-harvest during warm, dry periods and post-harvest when grain is improperly dried before storage or stored in a humid environment (Cotty et al., 1994).

Aflatoxin, a type of mycotoxin, is primarily produced as a toxic secondary metabolite by *A. flavus*, (and to a lesser extent) *A. parasiticus, A. nominus, A. bombycis*,

and *A.pseudotamarii*; Payne & Brown, 1998). Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) are the four major types of aflatoxins produced by *Aspergillus* fungi. The names are derived from the fluorescence they produce under ultraviolet (UV) light (which is blue or green) and the number refers to their relative migration in thin layer chromatography (Klich, 2007). AFB1, is the most potent of all the toxins (Essigmann et al., 1982) and a known naturally occurring carcinogenic, mutagenic, and teratogenic compound. Because of the health hazards it poses to humans, this pathogen has become increasingly important to researchers over the years.

Aflatoxin exposure in humans is a global concern. The action level for the amount of aflatoxin allowed in food stuffs set by the U.S Food and Drug Administration (FDA) is 20 parts per billion (ppb) for grains traded between states and destined for human consumption, to reduce the health problems caused by aflatoxins. The presence of aflatoxin in maize and other starchy cereal grains cause serious health problems in humans and animals that consume contaminated grains especially in developing countries of the world where tolerable levels have not been established or cannot be enforced due to lack of sufficient funds. The first outbreak of aflatoxin was documented in early 1960's when a group of more than 100,000 turkeys (*Meleagris gallopavo*) died after the consumption of a mold contaminated peanut meal (Blount, 1961). Aflatoxins contaminate a variety of staple foods, particularly maize, peanuts or groundnuts and other cereals (Hell & Mutegi, 2011). Consumption of high amounts of aflatoxin is known to cause aflatoxicosis, symptoms of which include hemorrhaging, acute liver damage, edema, problems with nutrient uptake, and possibly death. Chronic exposure to low levels of aflatoxin will in addition cause immunosuppression, cancer, developmental problems with growth, and

other pathological conditions. In 2004 and 2005, consecutive outbreak of acute aflatoxicosis caused over 150 deaths in Kenya (Ochieng et al., 2013).

Over the years, preventive strategies to control aflatoxin contamination have been suggested, including halting the growth of the fungus, controlling environmental factors that influence the growth and development of the fungus, or adoption of pre and post-harvest management strategies. The most effective biological control that has been adopted to date is the use of non-toxigenic strains of *A. flavus*, which competes with the toxigenic strains and can reduce the amount of aflatoxin contamination by up to 90% in some environments (Yin et al., 2008). Pre-harvest control methods such as earlier planting dates, irrigation of fields, and proper fertilization have also been proven to reduce aflatoxin contamination as well (Bruns, 2003; Abbas & Accinelli, 2008). Other cultural practices that can reduce initial infection of *A. flavus* include weed control, low planting densities, and tillage (Jones et al., 1980, Payne & Brown,1998; Bruns, 2003). Though all abiotic factors such as nutrient deficiency, heat, drought etc. that influence *A. flavus* infections cannot be prevented, the practices mentioned here help to reduce its contamination. Some post-harvest practices such as reducing the moisture content of maize to <14% before storage has been proven to reduce fungal growth in storage, which ultimately lead to reduced aflatoxin contamination (Bruns, 2003). Nevertheless, to date, none of these strategies has proven to completely stop the growth of the fungus or its production of aflatoxin in all growing environments (Hell & Mutegi, 2011). Researchers have identified maize lines that are naturally resistant to *A. flavus* and the subsequent production of aflatoxin (Williams & Windham, 2001; 2006). However, transferring resistance from these lines into elite cultivars with many favorable agronomic characteristics such as high yield

and early maturity is difficult due to the quantitative nature of the trait (Clements & White, 2004). Many genes influence quantitative traits. The environment in which maize is grown also has a big influence on the production of aflatoxin, which makes transfer of genes into elite cultivars very hard (Stoloff & Lillehoj, 1981).

Various new strategies involving biotechnology such as identification of quantitative trait loci (QTL) and related markers for marker assisted selection (Warburton et al, 2009), and identification of resistance-associated proteins through proteomics, gene expression studies, and biochemical marker identification (Bhatnagar et al, 2008) may someday be used to enhance host-plant resistance (Warburton & Williams, 2013). Maize lines resistant to aflatoxin accumulation have been bred from tropical sources into US germplasm (Mayfield et al, 2012). These tropical sources are exotic and un-adapted because they originate outside the US Corn Belt dent maize population, which has a temperate growing environment. Although these lines contribute to aflatoxin accumulation resistance in maize, they also contribute undesirable agronomic characteristics such as late maturity, late flowering, poor yield and increased lodging (Brook et al, 2005; Mayfield et al, 2012). To overcome this, exotic germplasm with alleles associated with aflatoxin resistance are used as donor lines to contribute favorable alleles into the US maize germplasm (Goodman, 2005). Through several generation of backcrossing, resistance alleles from exotic germplasm are introgressed into adapted elite cultivars; however, transferring these resistance alleles via phenotypic selection have proven to be difficult due to the highly quantitative nature of the trait (Willcox et al, 2013; Warburton et al, 2013).

Quantitative traits are measurable traits that are influenced by the effect of the cumulative actions of two or more genes coupled with the environment (also known as

polygenic, multifactorial or complex traits) (Collard et al, 2008). Genomic regions containing genes associated with any particular quantitative trait are known as quantitative trait loci (QTL). Identification of causative genes responsible for different traits of interest was not possible until the advent of DNA (molecular) markers in 1980 (Stuber & Edwards, 1986). As a matter of fact, quantitative traits are treated as a cumulative effect of many genes interacting together with the environment using the means and variances of the population (classical biometrical genetics) rather than individual underlying genes in the population each contributing a minor effect to the trait (Stuber & Edwards, 1986). The advent of biochemical and molecular markers coupled with robust statistical analyses has made it possible to estimate the number of genes associated with quantitative traits and the relative genetic location of these genes. A combination of functioning molecular tools, theoretical framework and increased computational ability has given rise to the field now known as molecular quantitative genetics (Mackay, 2001).

Quantitative trait loci are regions within the chromosome of an organism containing one gene, or more genes closely linked together, to influence a trait of interest. Associations between markers and the traits uncover QTL and markers linked to them, but further analysis is required to reveal underlying genes within identified QTL. Linkage or QTL mapping accurately measures the effect of genomic regions contributing to the trait of interest. It aids in detecting causal polymorphisms for a trait of importance between two lines that have contrasting phenotypes for the trait of interest. QTL are easier to identify in linkage mapping although it requires having a population obtained from pure inbred or heterogeneous lines and several approaches have been developed to link QTLs with molecular markers in the populations (Kearsey & Farquhar, 1998). A QTL mapping

population has a balanced proportion of alleles at all polymorphic loci, giving stronger statistical power. However, the QTL are found within larger linkage blocks, due to relatively few generations of meiosis and thus recombination. Populations containing QTL for complex traits (such as aflatoxin accumulation resistance) are grown in replicated field trials in multiple environments to determine the repeatability of the QTL (Kearsey & Farquhar, 1998). Phenotypic and genotypic data generated from QTL mapping studies are analyzed using mapping software to identify potential QTL influencing the trait of interest.

At the end of QTL studies, genetic markers linked to QTL associated with aflatoxin accumulation resistance can be used to develop improved cultivars (Bernardo & Lorenzana, 2009). Past QTL mapping studies have identified various potential QTLs within the maize genome responsible for aflatoxin accumulation resistance (Mayfield et al, 2011). The Corn Host Plant Resistant Research Unit (CHPRRU) of the United States Department of Agriculture (USDA), Agricultural research services (ARS) has previously identified and mapped QTL linked to aflatoxin accumulation resistance in three resistant inbred lines (Mp313E, Mp717, and Mp715) using bi-parental linkage mapping (Brooks et al, 2005; Warburton et al, 2009, 2011; Willcox et al, 2013). Bi-parental linkage mapping utilizes the differential expression of a trait (such as resistance and susceptibility to aflatoxin) between two inbred parents to analyze the inheritance of markers and the phenotypic expression of the trait in the progeny. Although the phenotypic effect of most of the QTL identified in previous mapping studies is small within any single inbred line, through gene pyramiding they may cumulatively create a larger effect. It is expected that when they are introgressed into other backgrounds or measured in other environments, their effects are repeatable, but this must be verified (Warburton et al., 2011).

The unit has also conducted association mapping studies using 300 diverse and publicly available inbred lines, including most of the known aflatoxin accumulation resistant lines adapted to southern US growing conditions (Warburton et al., 2013). Association mapping utilizes the diversity present in the many unrelated lines to identify multiple sequence polymorphisms, determine which of those sequences are related to aflatoxin accumulation resistance and measure the phenotypic effect of the favorable alleles of any associated loci (Warburton et al., 2013). In addition, due to a very large number of historical recombination events in an association panel, resolution can be within hundreds to a few thousand base pairs (Warburton et al., 2013). Results obtained from genotyping 300 lines via genotyping by sequencing (GBS) as described by Elshire et al., (2011) were stored in an in-house maize hapmap database. The database describes where each of the variations identified by GBS occurs within the genome of the 300 lines. Phenotypic values including variation and heritability of aflatoxin levels measured in the association mapping panel as reported in Warburton et al., 2013 shows high level of variation among the test-crossed lines (lines generated by crossing an individual to a phenotypically susceptible individual to determine its zygosity) and also high mean heritability for aflatoxin levels indicating a good data set that will be suitable for genome wide association study (GWAS) analysis. The association mapping panel found various single nucleotide polymorphisms (SNPs) with minor allele frequency (MAS) greater than 1% spanning all 10 chromosomes with an average of 6.3% missing data.

The overarching goal of the first study of this dissertation is to map QTL associated with aflatoxin accumulation resistance in CML69, a resistant maize inbred line unrelated by pedigree to all currently known and mapped resistant donor lines, using Va35 as the

susceptible parent to identify novel QTL associated with aflatoxin resistance. Numerous studies have identified various QTLs using different resistant maize inbred lines such as Mp313E, Mp715 and Mp717 crossed with different susceptible parents. It is hoped that QTL identified from CML69 will be new and will work synergistically when pyramided with QTL from previously mapped resistant lines. Furthermore, information generated from bi-parental mapping studies are specific to the cross being investigated because the map is based off polymorphic markers and QTL identified with such markers.

The aim of the second project in this dissertation is to create near isogenic lines (NILs), also known as introgression lines (ILs) (Bernacchi et al, 1998) or backcross inbred lines (BILs) (Jeuken & Lindhout, 2004). NILs are pairs of plant lines that are identical to each other except at one region or QTL of interest that is being investigated. NILs are important for measuring and validating the effect of a QTL on a trait (Kaeppler, 1997). QTL integration into NILs is important for identification and validation of the QTL in elite cultivars. In the case of aflatoxin accumulation, the QTL of interest being investigated is introgressed into a maize line susceptible to aflatoxin but has other desirable agronomic characteristics with the aim of determining how much effect the introgressed region has on aflatoxin accumulation (i.e., how much aflatoxin is reduced in the new line compared to the original susceptible line). Fine mapping and verification of QTL using NILs have been successful in maize for other traits (Graham et al, 1997). The overall goal of the second study of this dissertation is to have an insight on each of the extracted SNP of interest associated with aflatoxin accumulation resistance per NIL after successful introgression into a susceptible background.

Recurrent selection (RS) is a breeding method involving selecting superior lines with desirable traits from a large population of intermated inbred lines. The selected lines themselves are also intermated, and the process is repeated for several generations. Each generation creates the population for the next cycle of selection, and each generation should show a genetic improvement for the trait of interest over the previous generation (Hallauer et al, 1992). The accumulation of favorable alleles from all lines used in creating the population occurs after every cycle of selection, and the negative alleles are slowly removed (Mock & Eberhart, 1972). Recurrent selection has been applied to develop superior germplasm for some major traits in maize such as cold tolerance (Mock & Eberhart, 1972), four important seed quality traits (protein content, dye binding capacity, tryptophan content, and specific weight, (Pani et al, 1985) and stalk quality (Russells, 1991), among others. Recurrent selection has also been carried out on maize to improve disease resistance traits such northern corn leaf blight (NCLB) caused by Exserohilum turcicum (Ceballos et al., 1991). The goal of the third study of this dissertation is to increase the frequency of alleles associated with aflatoxin accumulation resistance in a population created by crossing eight unrelated resistant inbred lines, resulting in a new population from which to select new inbred lines fixed for the resistance alleles at several loci.

CHAPTER II

REVIEW OF LITERATURE

Maize (*Zea Mays* L)

Maize (*Zea mays* L. ssp. mays) belongs to *Gramineae* family. It is a monoecious plant with the male inflorescence at the top of the stem and the female inflorescence at the base of each branch. The separation of maize's sexual organs makes it an easier plant for breeding in some respects. Archeological and genetic evidence places the time of maize domestication at 9000 BP (Matsuoka et al., 2002) in Mexico from its wild ancestor known as teosinte (*Zea*). Classical taxonomic data possibly suggested that maize probably originated from either *Tripsacum* (gamagrass) or *Zea* (teosinte), (Kellogg, 1997). Maize domestication is undoubtedly one of the best examples of how major morphological changes can occur quickly in response to human selection (Lauter & Doebley, 2002). The major phenotypic differences between teosinte and maize are the multiple long branches with tassels and grains at the tips in teosinte compared to maize, which has a single stalk tipped by the tassel and one or few short branches tipped by ears; the size and shape of the ears in maize vs. teosinte; and the hard covering over individual seeds in teosinte vs. the husk covering the entire ear in maize. Genetic analysis has determined that a single gene, *teosinte branched 1* (*tb1*), largely controls the difference in plant architecture (Doebley & Stec, 1995). Further genetic analysis suggests four more genes: *terminal ear 1, teosinte glume architecture 1* (*tga1*), *single female spikelet (pd1)* and *rind & pith abscission* (*ri1 / ph1*) which control floral stalk, bract and two kernel per cupule are the other genes responsible for the distinct morphological feature of the modern maize (Mangelsdorf et al., 1964). Maize cobs morphologically similar to modern ears have been observed dating back

to 5500 BP from the Tehuacan valley (Long et al., 1989) and 6250 BP from Guila Naquitz (Piperno & Flannery, 2001) in central Mexico. Maize was first recorded in Africa around AD 1500 and spread over to almost all the corners of the continent in less than 500 years (McCann & McCann., 2009).

Maize is one of the leading crops in the world. About 1.07 billion metric tons was produced 2017 with the US leading the production with about 388 million metric tons followed by China, Brazil, The European union and Argentina (USDA FAS., 2017). Approximately 15% of the 388 million metric tons produced in the US is exported to other countries, making US the largest exporter of maize in the world. Maize as a staple food in developing countries contributes to the daily diet of over 200 million people annually with countries like Uganda, Tanzania, Angola, Kenya, Somalia, Malawi, Mozambique, Ethiopia, Zambia and Lesotho using between 70% - 95% of cultivated maize as food or food resources. Other developing countries such as Mexico, Benin, El Salvador, Nicaragua, Zimbabwe and Peru use between 65% - 85% of cultivated maize for food (USDA FAS., 2017). These numbers shows the importance of maize as a staple food source to mostly African and Latin American countries. Maize is also used as a source of fuel (ethanol) and fiber in developed countries and it is used to extract other industrial components for plastic, paints, glues and pharmaceuticals. Due to the importance of maize in food security and because of its easy growing characteristics, maize has been a model plant for geneticists (Tenaillon & Charcosset., 2011).

Aspergillus* and *Aspergillus flavus

Aspergillus is a large genus of fungus, with approximately 250 species classified under the order *Ascomycota* (Scheidegger & Payne, 2003). This genus has been classified into 8 sub-genera and 22 sections with one third of the members producing teleomorphs, a fungal reproductive structure resulting from plasmogamy and nuclear recombination (Geiser et al., 2007). *Aspergillus* was initially classified using morphological, cultural, and biochemical characteristics (Scheidegger & Payne, 2003; Peterson et al., 2008); however, the use of molecular markers, DNA sequencing and other molecular techiques have further helped to have a better understanding of the fungus classification (Wu, 2006; Peterson et al., 2008).

Figure 2.1 Maize kernel infected with *Aspergillus flavus* (Photo by Loeffler and Mideros, 2010).

Species in this genus are abundant and widely distributed in the soil, water, air, and in plants (Klich, 2002). This genus has had significant detrimental impact economically,

ecologically, and medically due to the production of the toxic secondary metabolite, aflatoxin. Only four species of *Aspergillus* produce aflatoxins: *A. flavus, A. parasiticus, A. nomius,* and *A. pseudotamarii* (Richard et al., 2007). Ear-rot caused by *Aspergillus* spp. is considered a minor problem in the U.S., but *A. flavus* infections are taken more seriously because of its ability to produce to carcinogenic aflatoxin (Naidoo et al., 2002). In addition to aflatoxins, *Aspergillus* spp. also produces unrelated mycotoxins known as cyclopiazonic acid (CPA), an indol-tetramic acid that targets the liver, kidneys and gastrointestinal tracts of animals (Table2.1). Maize kernel colonization by *Aspergillus* ssp. occurs at various stages of the plant's life when the spores are brought under the husk or through the silk channel to the kernel surface either by insects or by the wind. Infection of the kernels generally occurs later at the ear development stage (Payne & Brown, 1998). Of the *Aspergillus* species that produce aflatoxins, only *A. flavus* and *A. parasiticus* are of economic importance. Although *A. parasiticus* produces all four major types of aflatoxins while *A. flavus* only produces the AFB1 and AFB2, *A. flavus* is more common on all commodities (Richard et al., 2007).

A. flavus is a ubiquitous, saprophytic and soil-borne fungus which acts as a weak, opportunistic, ear-rot pathogen of maize, especially during periods of heat and drought stress (Payne & Widstrom, 1992; Cotty et al, 1994). The fungus is found most often in regions located between 16 to 35 degrees north or south latitude and optimally grows at a temperature of around 37°C, although the fungus has been shown to grow at temperatures as low as 12°C and as high as 48°C. *A. flavus* is characterized by fast-growing yellow-green colonies (Figure 2.1), usually 65-70 mm in diameter after 7 days growth in the dark at 25°C on Czapek yeast extract (CYA) and it grows well at 37°C (Klich, 2002). *A. flavus*,

as an opportunistic pathogen, has limited direct pathogenic abilities, but specific environmental conditions increase its ability to infect and rot ears, and cause aflatoxin contamination. Although *A. flavus* does not necessarily contribute to economic losses via reduction in yield when it infects the plant, the major concern is when it produces the secondary carcinogenic metabolite known as aflatoxin (Scheidegger & Payne, 2003). *A. flavus* has a broad host range as an opportunistic pathogen/saprobe and infects many economically important crops including corn, cotton, peanut, and many other tree nuts (Sweany et al., 2011), which can then become contaminated with aflatoxin. *A. flavus* undergoes asexual reproduction through conidia produced from conidiophores. It also produces special structures known as sclerotia which survive long periods of time and harsh conditions and can produce conidia and hyphae for further colonization (Horn et al., 2009).

Table 2.2 Aflatoxins and other secondary metabolites produced by different species of *Aspergillus*

Species	Aflatoxins	Other secondary metabolites
Aspergillus avenaceus		Avenaciolide
Aspergillus bombycis	B,G	Kojic acid
Aspergillus caelatus		Kojic acid, aspergillic acid and cyclopiazonic acid
Aspergillus flavus	B,G	kojic acid, nominine, paspaline, paspaliline
Aspergillus lanosus		Griseofluvin, kojic acid, met I
Aspergillus leporis		Antibiotic Y, kojic acid, leporine, pseurotin
Aspergillus nominus	B,G	Aspergillic acid, kojic acid, nominine, pseurotin, tenuazonic acid
Aspergillus oryzae		Cyclopiazonic acid, kojic acid
Aspergillus parasiticus	B,G	Aspergillic acid, kojic acid, parasiticol, parasiticolide A
Aspergillus pseudotamarii	B	Cyclopiazonic acid, kojic acid
Aspergillus sojae		Kojic acid
Aspergillus tamarii		Cyclopiazonic acid, fumigaclavine A, kojic acid
Petromycesalliaceus		nominine, ochratoxin A and B, paspaline.

Reproduced from Scheidegger and Payne, 2005.

Aflatoxin in maize

Under favorable environmental conditions, such as hot and humid environments, *A. flavus* produces a secondary metabolite known as aflatoxin. Aflatoxins are one of the major types of mycotoxins produces by the genus *Aspergillus* (Richard et al., 2009). *Aspergillus* produces four major types of aflatoxin, namely, aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). The names are derived from their color or the fluorescence they produce under ultra-violet light (B = Blue and G = Green). In addition to these 4 groups of aflatoxin, there are also aflatoxin M1 and M2 metabolized from aflatoxin B1 and found in milk of lactating animals and humans following the consumption of contaminated feed (Richard & Payne, 2003). The two most important factors that cause *A. flavus* to produce aflatoxins are drought stress and high temperatures (Payne & Widstrom, 1992). Most oil seed crops such as maize, cotton and tree nuts are susceptible to aflatoxin accumulation because these crops grow in latitudes suitable for the proliferation of *A. flavus* (Klich et al., 2007). Also, the high oil content of the grain and embryos of oil seed are good media for the growth of the fungus.

Discovery of aflatoxin

Aspergillus flavus was first recognized as a maize pathogen in 1920 and was then characterized as an organism that causes "yellow mold" in maize (Taubenhaus, 1920). It drew a lot more attention and became a global serious concern in 1960 with an intense outbreak of an un-known disease in a turkey farm in England (Blount, 1961). This un-known disease killed over 100,000 turkey between May and August 1961. The diseases was named "turkey X disease" pending the time researchers could identify the main cause of the outbreak. The disease was characterized by liver lesions and was said to be

tranmissible from bird to bird (Siller, 1961). Researchers carried out various experiments using commercial feed rations from different affected farms and found similar post-mortem liver lesions from turkey fed similar rations. This led to researchers concluding that commercial feed rations used in the study were the cause of the outbreak, but the main reason why still remained unknown. More light was shed on the outbreak when a second incident occurred north of London in Cheshire County. Researchers found that both mills supplying the ingredients for the feed rations in the London and the Cheshire County outbreaks imported peanut meal from Brazil almost at the same time (Blount, 1961). The peanut meal was used in feed rations to conduct a trial and similar liver lesions observed in previous studies was observed in the new studies. This prompted companies to send representatives to Brazil and they found similar disease problems in livestock fed the contaminated feed rations. This information provoked a whole new study to find what was present in the peanut meal causing the outbreaks (Blount, 1961). Allcroft et al. (1961) sucessfully extracted the "toxic factor" from the Brazilian peanut meal in April 1961. By November 1961, Allcroft had refined his extraction methods and was able to obtain pure "toxic factor" using duckling bioassays.

While these studies were going on in England, there were reports about similar outbreaks in Kenya. These outbreaks was traced back to peanuts imported from Uganda (Asplin & Carnaghan, 1961).There were also some minor outbreaks in England with peanut meal source traced back to India. With reported outbreaks spanning over 5 countires and 3 continents, research intensified. Peanut meals from Nigeria, and some other West African countries, were collected for testing and the "toxic factor" was extraxted using the extraction protocols by Sargeant et al., (1961b). By the end of 1961, it had been confirmed

that peanut meal from three different continents was contaminated with the unknown toxin and was causing massive losses of both poultry and large-animal. A breakthrough article was published by Sargeant et al, (1961), where he observed that contaminated peanut meal from Uganda was heavily infected with an unknown fungus. Fungal cultures was extracted from the Ugandan peanut meal and the cultures possess a characteristic blue fluorescence and produced characteristic liver lesions in the duckling bioassay. The toxin-producing fungus was identified as *A. flavus*. The fungus was then grown on sterilized peanut meal and it contained the blue fluorescent compound and produced liver lesions in the duckling assay. This confirmed that *A. flavus* was the producer of the unknown toxin and therefore, the causative agent of the outbreaks. The toxin was named "aflatoxin" by Sargeant et al., (1961) and was described as the "highly toxic" material produced by *A. flavus*.

Further studies conducted on aflatoxin after its discovery showed that the toxin contains a mixture of related compounds. They are a group of secondary metabolites produced by *A. flavus* and *A. parasiticus* and can be recognized by the yellow-green or gray-green colored growth on corn kernels, respectively. Nesbitt et al., (1962) differentiated aflatoxins B and G based on the blue and green flouresncence. Zijden et al., (1962) and Mateles and Adye, (1964) demostrated the differences in aflatoxins B_1 and B_2 while also demonstrating B_1 to be more toxic than B_2. Chemical structures for all discovered aflatoxin were derived by (Asao et al., 1963), (Figure 2.2) while aflatoxin M_1 present in the milk of lactating animals fed aflatoxin contaminated feed was discovered in 1964 (De Iongh et al., 1964).

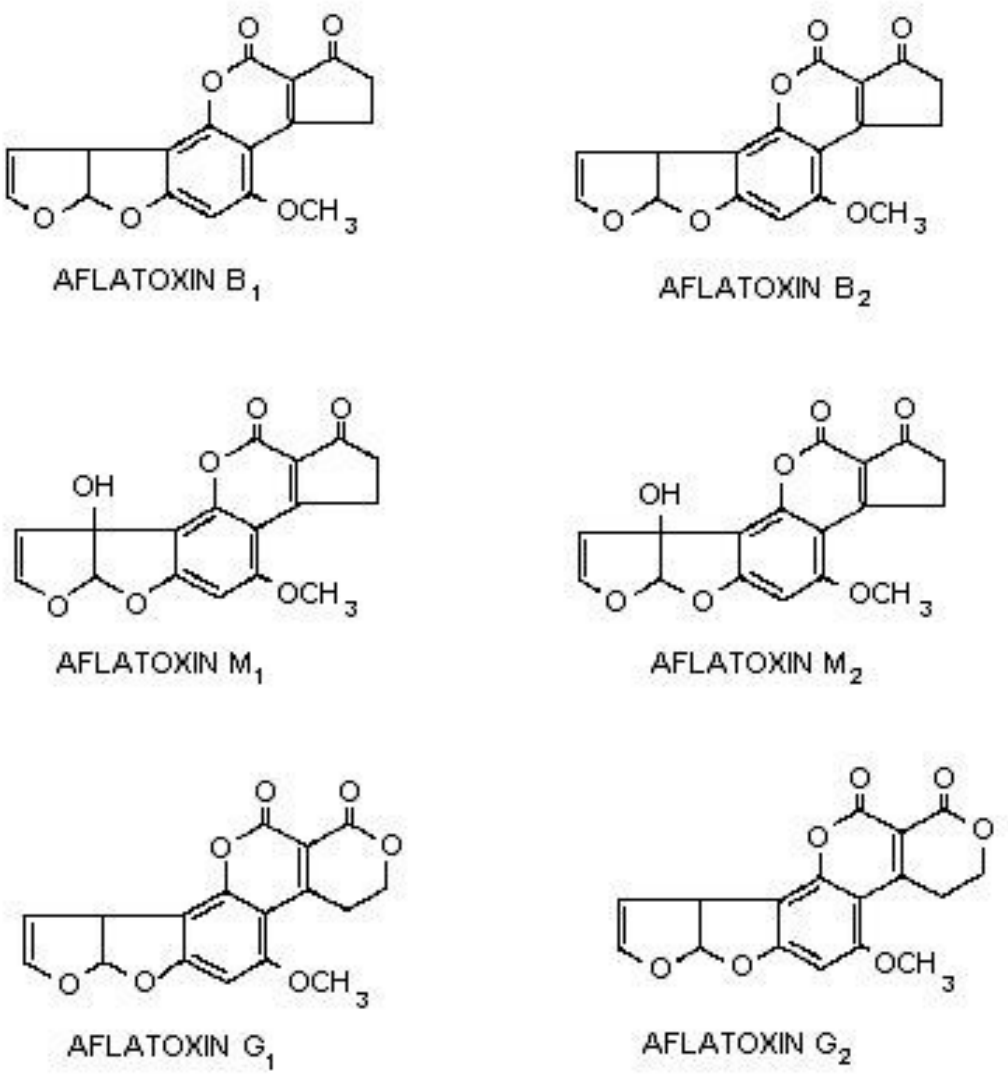

Figure 2.2 The structures of the major aflatoxins. (Asao et al., 1963).

Health implications of aflatoxins

The International Agency for Research on Cancer (IARC) labeled AFB1 as a class 1 human carcinogen in 1988 (Vainio & Wilbourn, 1992). Before that, aflatoxin had been known to be extremely toxic and carcinogenic with AFB1 being the most toxic of the four major types (McCann et al., 1975). Aflatoxins were not a known concern to human health until the identification of aflatoxin as the causative agent of the outbreak of turkey X disease in 1960 (Blount, 1961). Subsequent cases of aflatoxin exposure was documented in dogs (*Canis familiaris*), cattle (*Bos taurus*), and swine (*Sus scrofa*) after its discovery in turkey (Richard & Payne, 2003). Chronic exposure of these animals to aflatoxin causes weight loss, hemorrhages, excessive nasal discharge, discolored body waste and ultimately, death (Miller & Wilson, 1994). The presence of aflatoxin M_1 in milk of dairy animals fed contaminated feed is also a big health concern (Stoloff, 1979). Greater concern for the human population is the possibility of acute and chronic exposures to aflatoxin. Numerous documented cases of aflatoxin related illness and death in Asia and Africa has been recorded, and this is largely due to the lack of proper infrastructures for grain testing to detect aflatoxin contaminations. These areas still have the greatest danger of aflatoxin outbreaks till date (Lewis et al., 2005; Daniel et al., 2011; Yard et al., 2013). The main target organ of aflatoxin is the liver. Usually in severe cases of aflatoxicosis, it causes liver failure and ultimately death of the patient, while chronic exposure to aflatoxin leads to other numerous health issues including cancer (Wild & Gong, 2009).

Bio-activation of AFB1 inhibits cytochrome p53 in liver cell from preventing tumor growth, which drastically increases the chances of individuals exposed to aflatoxin to cancer. The rate of people who develop liver cancer due to aflatoxin exposure increases by

60 times if they have hepatitis B (Wu, 2015). Numerous studies have shown a correlation between exposure to aflatoxin and incidence of liver cancer, especially hepatocellular carcinoma (HCC) (Chuang et al., 2009; Wu, 2015). There is also a proven increased risk of HCC when aflatoxin exposure is combined with hepatitis B (Wild & Gong, 2010). Due to the mutagenic nature of aflatoxin, it is capable of intercalating into DNA to form an AFB1-N7-guanidine adduct once the aflatoxin has been metabolized by the body into the AFB1-8,9-exo-epoxide (Essigmann et al., 1977; Wild & Gong, 2010).

The US and other developed nations have imposed strict action levels on the amount of aflatoxin permitted to be in food and /or feed ingredients due to the health hazards caused by exposure to the toxin. Some countries even further regulated AFB1 specifically (Van Egmond & Jonker, 2004). The Food and Drug Administration (FDA) set the action level of aflatoxin to be 20 parts per billion (ppb) for all food products destined for human consumption (Table 2.2), but the European Union (E.U) has an even more strict action level of 4 ppb (Van Egmond & Jonker, 2004; Klich, 2007) (Table 2.2). The imposed action level helps minimize the level of aflatoxin exposure but, on the other hand, the regulations results in economic losses for farmers and commodity producers, as highly contaminated corn must be destroyed. The estimated cost of direct and indirtect loss the regulations have on farmers in the US totals at least $500 million dollars annually (Robens et al., 2005).

Table 2.2 U.S. Food and Drug Administration action levels for total aflatoxins in food and feed.

Aflatoxin Level	Commodity
20 ppb	For corn, peanut products, cottonseed meal and other animal feeds and feed ingredients intended for dairy animals; for animal species or uses not specified below, or when the intended use is not known.
20 ppb	For corn, peanut products and other animal feeds and feed ingredients, but excluding cottonseed meal, intended for immature animals.
100 ppb	For corn and peanut products intended for breeding beef cattle, breeding swine or mature poultry (e.g. laying hens).
200 ppb	For corn and peanut products intended for finishing swine (100 pounds or more).
300 ppb	For cottonseed meal intended for beef cattle, swine or poultry (regardless of age or breeding status).
300 ppb	For corn and peanut products intended for finishing beef cattle (i.e., feedlot cattle).

Pre- and Post-harvest prevention of aflatoxin contamination in maize

Preventive strategies such as stopping the infection process, control of pre- and post-harvest environmental factors to minimize fungal growth, and crop management strategies to mitigate crop stress can be utilized by maize farmers to minimize the level of aflatoxin contamination in maize. (Hell et al., 2003; Hell & Mutegi, 2011). It is practically impossible to completely avoid aflatoxin contamination in susceptible species. Therefore, numerous studies are being carried out to understand how to reduce initial infection of *A. flavus* colonization or its subsequent production of aflatoxins as much as possible. Pre-harvest practices such as timely harvest, avoidance of kernel breakage from over-drying, and reduction of damage from insects, animals, or mechanical harvest are some of the measures taken by farmer to reduce aflatoxin accumulation (Bruns, 2003; Hell et al., 2003). Delayed harvesting has been shown to increase fungal growth and average aflatoxin titer

(Hell et al., 2003). Biological and environmental factors such as insects, diseases, weeds and drought that can directly cause plant stress also contribute to the process of infection by *A. flavus* and thus have been the subject of much research effort. Insects can also vector fungal spores directly into the kernels through infection sites (Widstrom, 1996). Control of environmental factors through various measures has been practiced to prevent and control fungal penetration, fungal growth and ultimately aflatoxin production and accumulation (Payne & Widstrom, 1992b).

Biological control of aflatoxin has proven to be one of the most effective strategies of preventing aflatoxin accumulation in maize. The most effective biological control of aflatoxin contamination to date is the use of competitive nontoxigenic strains of *A. flavus* and/or *A. parasiticus*. Application of these nontoxigenic strains competitively exclude the natural toxigenic strains and compete with them for substrate, thereby reducing the rate at which they colonize and produce aflatoxin (Yin et al., 2008). Various field experiments have shown between 70% - 90% reduction in aflatoxin contamination by using nontoxigenic *Aspergillus* strains (Dorner, 2004; Yin et al., 2008). Palumbo et al., (2006) also reported several bacterial species such as *Bacillus subtilis*, *Lactobacilli* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp., with ability to inhibit fungal growth and aflatoxin production. Furthermore, saprophytic yeast such as *Candida krusei* and *Pichia anomala* has also being reported to shown considerable promise as biocontrol agents against *A. flavus* (Masoud & Kaltoft, 2006).

Aflatoxin accumulation can increase 10-fold within 3 days when harvested grains are stored in a high moisture environment (Kaaya & Kyamuhangire, 2006). Therefore, drying of maize immediately after harvesting to a moisture content less than 15% is the

most effective post-harvest strategy to reduce aflatoxin accumulation. This practice have being shown to reduce *A. flavus* growth and further aflatoxin contamination (Bruns, 2003; Hell et al., 2003). Other post-harvest practices to reduces aflatoxin contamination is sorting of damaged and contaminated grain, well ventilated storage which allows for proper air flow within the storage units, and complete cleaning of storage units by removal of old grain (Choudhary & Kumari, 2010). Farmers in developong countries such as Nigeria practiced disinfecting methods through smoking to prevent aflatoxin accumulation. Smoking has being shown to reduce the moisture content and also protect maize from fungal infestation. This method was found to correlate with reduced aflatoxin accumulation in the farmers' stores and about 4 to 12% of farmers in different ecological zones in Nigeria use this method to preserve their grain (Udoh et al., 2000; Bankole & Mabekoje, 2004). Nixtamalization, a maize preparation process common in Latin America involving soaking and cooking maize grain in alkaline solution, (usually limewater), has also been proven to effectively reduce aflatoxin contamination by 75-90% (Mendez-Albores, 2004). Cultural practices such as tillage and crop rotation can affect soil inoculum availability (alleviating stress during later plant development) and preventing the inoculum buildup (Jones et al., 1980). Conventional methods of plant disease control, such as fungicide use, can prevent fungal growth, but has proved ineffective in controlling *A. flavus* infection of corn when employed at a concentration that are both cost effective and environmentally safe (Bhatnagar et al., 1993).

There are physical and chemical detoxification methods for reducing the amount of aflatoxin in food and feed products. Extreme high temperatures can decompose aflatoxins; however, cooking and boiling are not effective because the thermal decomposition

temperature of aflatoxin is 267°C (Mohammadi, 2011), well above the temperature of normal cooking. The use of ethanol, acetone, isopropanol, hexane, methanol, water, and acetonitrile have all been used in various combinations to extract aflatoxin from foods and feeds, but many of these solvents are too expensive or toxic in themselves (Campone et al., 2011). Other management practices employed by farmers to reduce aflatoxin accumulation contamination are listed in Table 2.3.

Table 2.3 Managemental practices to manage aflatoxin resistance (Abbas et al., 2009).

Strategy	Method	Rationale
Avoidance	Early planting, supplemental irrigation, short season hybrids	Reduce heat and moisture stress
Fertility management	Provide adequate nutrition	N- deficiency corn more susceptible
Insecticide application	Appropriate timing of application to control insect damage to ears	Insects responsible for enhanced ingress into grains
Bt Hybrids	Hybrids engineered with resistance to ear- damaging insects	Insects responsible for penetration into grains
Natural resistance to insects	Breeding and selecting hybrids for resistance	
Biological control	Use of non-toxigenic isolates of *A. flavus*	Competitive displacement of toxigenic isolates
Fungicides	Control phylosphere fungi	Reduce inoculum density
Soil management	Incorporation of crop residue	Reduce inoculum density

Host Plant Resistance

Of all pre-harvest measures explained earlier, host plant resistant is one of the most effective, efficient and promising ways of reducing *A. flavus* infection and aflatoxin

accumulation in maize (Paul et al., 2003). Host plant resistance is a preventive measure which is an excellent remedy without the need for additional inputs beyond the seeds, and will save farmers money that would have been lost to pre- and post-harvest aflatoxin accumulation (Paul et al., 2003). The first host resistance heritability studies for aflatoxin accumulations was carried out in 1976 (Zuber & Lillehoj, 1979). This came about due to pre-harvest contamination of aflatoxin observed in 1975 in Iowa, thereby making researchers understand pre-harvest aflatoxin accumulation was not just exclusively endemic to the southeastern U.S as earlier believed (Stoloff & Lillehoj, 1981). Maize producers in the Southeast US also experienced a massive aflatoxin outbreak a year after the Iowa incident where over 90% of the maize was contaminated and aflatoxin levels exceeded well above 1000ppb (McMillian et al., 1980). These outbreaks prompted Zuber to examine some selected inbred lines generating 28 single crosses and their reciprocals. Initial studies relied for the most part on natural infection by the fungus and the level of resistance was based solely on kernel infection by the fungus (Scott & Zummo, 1988; Windham & Williams, 2002). The outcome of Zuber's experiment showed a highly significant general combining ability (GCA) but a non-significant specific combining ability (SCA), which indicates that aflatoxin levels are under genetic control. Zuber suggested that to effectively control aflatoxin contamination, identification of lines resistant to aflatoxin should be the major focus of plant breeders.

Given the sporadic and heavily environmentally influenced nature of *A. flavus* infection and subsequent aflatoxin contamination, a more uniform and consistent method of inoculation was needed and developed (Brown et al., 1999; Windham & Williams, 2002). The inbred lines selected by Zuber were artificially inoculated and the progeny

tested for aflatoxin. Zuber et al., (1979) examined the progeny and concluded that aflatoxin levels observed in maize infected with *A. flavus* were under "genetic control" and a cyclic selection program should be effective for developing maize lines resistance to aflatoxin accumulation. Zuber's studies prompted research on different methods to improve host plant resistance for aflatoxin accumulation in maize. Other breeding programs screened open pollinated varieties, hybrids and inbred lines of field maize, popcorn and sweetcorn to find maize lines with resistance to *A. flavus* and aflatoxin accumulation (Lillehoj & Kang, 1978; McMillian et al., 1982; Zuber et al., 1983). Germplasm screening studies were also extensively used to identify a number of maize lines associated with lower grain aflatoxin levels (Windham & William., 1998). This method, however, is not easily implemented largely due to the difficulty in finding elite lines that have high yield, good agronomic performance and resistance to aflatoxin accumulation in multiple environments (Clements & White, 2004).

Over the years, the above mentioned conventional selection methods were used to identify and create maize inbred lines resistant to aflatoxin accumulation, but, due to the highly quantitative nature of the trait, it has been very hard to transfer resistance from these resistant donor lines to elite cultivars (Stoloff & Lillehoj, 1981). This means that a lot of genes are working together to make the plant resistant to aflatoxin accumulation thereby making it hard to transfer all the necessary genes into another germplasm (Williams & Windham, 2008). However, many new strategies are currently being developed and may be used someday to improve host plant resistance to aflatoxin accumulation (Brown et al., 1999; Warburton & Williams, 2014). These new strategies include identification of quantitative trait loci (QTL) and related markers for marker assisted selection (MAS)

(Warburton et al., 2011) which is discussed later and identification of resistance-associated proteins through proteomics and gene expression studies (Bhatnagar et al., 2006). Studies have used comparative proteomic approaches to identify proteins in maize kernels associated with aflatoxin accumulation (Chen et al., 2007). Of the dozens of proteins identified in these studies, a stress related protein *glyoxalase I* known to improve stress tolerance has been characterized (Veenaet al., 1999).

Molecular Mapping

Germplasm lines resistant to aflatoxin accumulation have been released through various breeding programs. These lines are intended to be used as donors of resistance alleles to improve aflatoxin accumulation resistance in elite cultivars or other lines with favorable agronomical characteristics but which accumulate unacceptable levels of aflatoxin. Various mapping studies have been used to identify molecular markers linked to genes or genomic regions known to influence resistance to aflatoxin accumulation (as reviewed in Warburton & Williams, 2014). These molecular markers can be used to improve the introgression of linked genes through MAS. There are three types of markers: morphological (also 'classical' or 'visible') markers which themselves are phenotypic traits or characters; biochemical markers, which include allelic variants of enzymes called isozymes; and DNA (or molecular) markers, which reveal sites of variation in DNA sequences. Molecular markers polymorphic between parents in bi-parental mapping populations have been used to genotype segregating $F_{2:3}$ populations to create linkage maps (Young, 1996). The type of mapping population used to create a linkage map depends on the type of study and the heritability of the trait been investigated. Other mapping populations such as F_2 population, recombinant inbred lines (RIL), backcross (BC)

populations, introgression lines assembled in exotic library, and double haploid lines have been reported to be successful in mapping quantitative traits in plants (Schneider, 2005). Past QTL studies have used different types of molecular markers such as AFLP (amplified fragment length polymorphisms), SSR (simple sequence repeats), RFLP (restriction length fragment polymorphisms), SNP (single nucleotide polymorphism) and other markers for this purpose (Table 2.4).

Restriction fragment length polymorphisms (RFLPs) were the first DNA markers designed for linkage maps (Bernatzky & Tanksley, 1989). RFLPs were ideal for QTL mapping because they allow for the creation of linkage maps with better precision with markers located within or around a QTL. However, they are labor intensive, slow and expensive, making them very hard to work with and less suitable than newer marker types for any genetic work (Bernatzky & Tanksley, 1989). The advent of polymerase chain reaction (PCR) (Saiki et al., 1988) and the development of another type of DNA marker known as simple sequence repeats (SSR) (Tautz, 1989) were a major boost in QTL mapping improvement. SSR development coupled with DNA amplification using PCR created a marker system suitable for easy creation of linkage maps and higher throughput usage for marker assisted selection (MAS). In recent years, another type of DNA marker known as single nucleotide polymorphism (SNP) was developed. This marker represents one nucleotide difference in the genome of an organism.

Table 2.4 Most commonly used markers for QTL analysis with their advantages and disadvantages.

Molecular markers	Codominant or Dominant	Advantages	Disadvantages
Restriction fragment length polymorphism (RFLP)	C	Robust, reliable, transferable across populations	Time consuming, laborious and expensive
Random amplified polymorphic DNA (RAPD)	D	Quick and simple, inexpensive, multiple loci from a single primer possible, small amounts of DNA required	Problems with reproducibility and generally not transferable
Simple sequence repeats (SSR) or "microsatellites"	C	Technically simple, robust and reliable, transferable between population	Large amount of time and labor
Amplified fragment Length polymorphism (AFLP)	D	Multiple loci, high levels of polymorphic generated	Large amount of DNA required and a complicated technology

Adapted from (Collard et al., 2005).

Quantitative Trait Loci

Quantitative traits have been a major focus of genetic studies for over a century because many agriculturally important traits such as yield, quality, abiotic stress resistance, and some forms of disease resistance are controlled by many genes and therefore associated with a quantitative inheritance. Genomic regions within the genome of an organism associated with any specific quantitative trait is referred to as QTL. Until recently, the study of quantitative traits was based only on statistical techniques with limited knowledge as to the location of the genes involved in controlling the trait although, these statistical techniques could estimate approximate gene number, and also gene action (Kearsey &

Farquhar, 1998). Modern QTL mapping takes advantage of molecular marker technology, statistics, and computer science to identify candidate genes associated with quantitative traits. However, despite the large number of publications on QTL mapping studies for different quantitative traits, little has been reported on successful integration of mapped QTL in commercial breeding programs.

The advent of biochemical isozyme markers increased the interest of researchers in mapping underlying genes associated with aflatoxin accumulation resistance. Stuber, (1989) was one of the first researchers to identify the potential of using isozymes to identifying polymorphisms between different individuals and explore their possibilities as genetic markers in maize and other crops. Since isozymes are enzymes with different amino acid sequence but catalyze the same reaction, they can be easily differentiated by starch gel-based electrophoresis. This difference in amino acid structure reveals allelic differences (polymorphism) between individuals that reflect differences in DNA changes, and thus can be mapped on a genetic map (Stuber, 1989). Major breakthrough for characterizing quantitative traits came with the development of DNA markers. These markers reveal polymorphic sites associated with a quantitative trait between two individuals. Mohan et al., (1997) reviewed that "one of the major uses of DNA markers in agricultural research has been in the construction of linkage map for diverse crop species." He further stressed that linkage maps are useful for the identification of regions within the chromosome containing genes (or QTL) associated to a quantitative trait using QTL analysis.

Genetic markers are said to be polymorphic when they reveal differences at the same location among different individuals. This happens by the occurrence of alternative

DNA sequences (alleles) at the same locus. Individuals polymorphic at the same locus, might explains the difference observed in the phenotypes of the individuals with the different sequence at the same locus. The greater the number of polymorphic sites among individuals of the same species, the higher the chances of developing genetic maps and subsequent identification of genes linked to the trait been measured (Liu & Cordes, 2004).

QTL analysis

As researchers continue to develop and optimize QTL detection and mapping using DNA markers, the need arises for better and more adequate statistical methods and tools to analyze data generated from the markers. QTL analysis links two types of information together: the phenotypic data generated from measuring the trait of interest (such as aflatoxin accumulation in this study) and the genotypic mapping data (generated from molecular markers) to determine the genetic basis of the trait (Kearsey & Farquhar, 1998; Lynch & Walsh, 1998). QTL analysis requires two contrasting parental lines in terms of the trait of interest and many molecular markers polymorphic between the parents. A segregating population is generated from the parents using one of a number of different schemes (Darvasi, 1998). The phenotypic and genotypic data generated from the segregating population is scored on all individuals in the mapping population; the date entered into first a linkage mapping program, and then a QTL analysis program.

There are numerous software packages available for mapping quantitative traits and localizing chromosomal regions underlying quantitative traits of interest. Many of the available mapping software programs possess unique characteristics that make them suitable for different purposes depending on the type of population been mapped. Current QTL mapping software includes QTL Cartographer, Mapmaker/QTL, Map Manager QT,

QGene, MapQTL, PLABQTL, MQTL, Multimapper, The QTL Café and Epistat (Manly & Olson, 1999). Statistical analysis of QTL is widely based on three classes: regression (Whittaker & Thompson, 1996); maximum likelihood (Doerge et al., 1997), and Bayesian models (Sillanpää & Corander, 2002). Initially, QTL mapping was carried out using a statistical analysis known as the single-marker analysis. This analysis uses F-tests to determine the level of significance for each segregating marker between the families and tests for significant differences in expression of the quantitative trait between the mean of individuals in each genotypic class (Edwards & Stuber, 1987). The shortcomings of single marker analysis is that it cannot separate the estimates of recombination fraction and QTL effect (Doerge et al., 1997).

An improvement on single marker analysis was made giving rise to interval mapping (IM). IM is used to analyze multiple markers simultaneously compared to just one marker in single-marker analysis (Lander & Botstein, 1989). This concept is based on maximum likelihood parameter estimation and provides a likelihood ratio test for QTL position. IM searches for the presence of QTL using the genetic information of multiple markers in a window of linked chromosome coordinates to estimate the location and effects of QTL. Churchill & George, (1994) further explained the algorithms used to determine the appropriate empirical threshold required to estimate the level of significance of each identified QTL using IM. A setback of IM is that estimates might be biased especially when two or more QTLs are linked (Li et al., 2006). Composite interval mapping (CIM) addresses this issue by combining IM with marker regression analysis. This eliminates the effect of other QTLs that might be linked to the one being tested thereby increasing the precision of QTL detection (Li et al., 2006). Multiple interval mapping (MIM) is an

extension of CIM which tries to incorporate the effect of every QTL identified all at once. The MIM model allows for testing epistatic interactions between all identified QTL (Zeng & Kao, 1999).

The Corn Host Plant Resistance Research Unit (CHPRRU) of the United States Department of Agriculture (USDA), Agricultural Research Service (ARS) has over the years developed mapping populations to identify QTL associated with aflatoxin resistance. The resistant line Mp313E, crossed with the susceptible line Va35, was the first bi-parental QTL mapping population to be phenotyped and scored for aflatoxin accumulation resistance by the unit in 2005 using an $F_{2:3}$ mapping population (Brooks et al., 2005). The study identified an average of 2-5 QTL in 4 locations and they each account for 7 – 18% variation in aflatoxin accumulation depending on the environment (Brooks et al., 2005). Three other bi-parental QTL mapping populations, Mp717 x NC300 (Warburton., 2009), Mp715 x T173 (Warburton et al., 2011), and Mp313E x Va35 (Willcox et al., 2013), have also been phenotyped and scored for aflatoxin accumulation resistance by the unit. These studies have reported multiple QTL, most of which were identified in a single environment with less than 5% of the phenotypic variation observed in the population. However, at least one phenotypically large QTL explaining between 10 – 20% phenotypic variation has been detected in every QTL mapping population (Warburton & Williams, 2014).

Furthermore, availability of phenotypic and genotypic data from the four QTL mapping studies conducted by the CHPRRU with other mapping studies conducted outside the unit has been used to run a meta-analysis for resistance to *A. flavus* infection and aflatoxin accumulation (Mideros et al., 2014). The meta-analysis of QTL consists of a combination of all mapping data (both phenotypic and genotypic data), effect of each

identified QTL, overlapping QTL between different mapping studies. Results generated from the meta-QTL study identified 62 significant meta-QTL twelve of which are located on chromosome 4 (bins 4.07 to 4.09), a maize chromosome region approximately 3% of the maize genome (Warburton & William, 2014). Exploiting this chromosomal region is very promising as it might help maize geneticists identify multiple genes associated to *A. flavus* and other pathogens.

Initially, mapping of QTL associated with a quantitative trait has a precision of around 10-20 cM thereby limiting the resolution of identified QTLs (Paterson et al., 1988). However, in recent years, high-density genotypic data for linkage mapping have allowed for more narrow QTL intervals and a much more better resolutions of the genetic architecture (Fenton et al., 2018). An increase in QTL resolution using high-density linkage mapping with an average interval of 1-2cM between markers have proven to improve the effect of QTL estimates, although this interval still correspond to millions of bases which might contain hundreds of genes. Studies have also shown that it helps to improve the chances of resolving QTL that are closely linked and cannot be separated with low marker density (Stange et al., 2013).

Near Isogenic Lines (NILs)

The success of linkage mapping and the ability to identify genetic locations associated with quantitative traits has made it a little tricky to completely grasp the distinct difference between qualitative and quantitative traits (Tuinstra et al., 1997). Although the difference between both traits is relative depending on how the trait is measured, the type of population used in measuring the traits and the type of markers used to map the location. One way to test the effect of identified QTL in the same genetic background but

independent cross, in new genetic backgrounds, or in new environments is the creation of near isogenic lines (NILs). NILs are not only useful for detecting the effect of QTL, but it is also useful in bridging the gaps in QTL resolution. NILs are defined as homozygous plant lines that are identical to each other except at one genomic region or QTL of interest. NILs are useful for studying phenotypes associated with any specific locus (Dorweiler et al., 1993). They are useful tool for detecting linkages and gene action (epistasis or additive) that exist between quantitative trait loci (QTLs) and the markers used to map the QTLs to their specific locations on the chromosome (Pea et al., 2013). NILs are also good tools for identification of small chromosomal regions consistently associated with a quantitative trait. Integration of a QTL into near isogenic materials is one effective way for the identification, validation, and subsequent incorporation of the QTL into new breeding lines (Kaeppler, 1997). Marker-assisted introgression of favorable alleles from desirable QTL validated with NILs into breeding lines has been reported to be successful in maize elite lines (Bouchez et al., 2002). NILs have been successfully used to verify and fine map QTLs maize (Graham et al.,1997), rice (Yu et al., 1991; Shen et al., 2001) and tomato (Brouwer & Clair, 2004).

NILs that verify markers which reduce aflatoxin levels in maize are created with initial crosses between a line that is resistant to aflatoxin accumulation (donor line) and a line that is susceptible (recurrent line) to get the first progeny (F_1). F_1 progeny genotype is made up of 50% of the resistant parent and 50% of the susceptible parent. Several generations of backcrosses are usually the best method to introgress the region that contains the favorable QTL allele from a donor genome into the recipient genome. The F_1 progeny is backcrossed to the susceptible parent to get the second generation of progeny with 75%

of the susceptible parent and 25% of the resistant parent. Each backcross generation increases percentage of the susceptible parent in the progeny by 50% and reduces the percentage of the donor parent by 50%. The process is continued for 4 to 8 generations making the progeny's genotype approximately between 93% - 98% of the susceptible parents, and 7% - 2% donor parent containing a few genes from the resistant parent. During each generation of backcrossing, there is genetic recombination (or crossover) between homologous pairs of chromosomes thereby creating new phenotypes in the offspring. This gives rise to a segregating population with varying phenotypes therefore making marker assisted selection more important so that favorable alleles are not lost in the population.

In marker assisted backcrossing, marker assisted selection (MAS) is used in each generation after the F_1 to keep track of the progeny that still have the QTL allele from the donor line after each generation of backcrossing. Backcrossing can occur without MAS using phenotypic selection, but within a few generations individual QTL for the trait of interest it may be lost (Tuinstra et al., 1997). Despite the large number of QTLs that have been identified in various experiments and published in the literature, the use of these QTL to develop elite cultivars is very rare (Szalma et al., 2007). The reason behind the lack of usage of these identified QTL is because breeders are unsure whether the QTL and their markers are useful in new genetic backgrounds or expressed in new environments. Using NILs to validate previously identified QTL can help boost the confidence of breeders attempting to introgress the QTL into elite germplasm for the improvement of this germplasm for the trait of interest (Stuber, 1986; 1992).

Recurrent selection

Recurrent selection (RS) is a term that is used for any selection method conducted repeatedly (Hallauer, 1992), but generally refers to selection within the same population. Rezende et al. (2014) defined recurrent selection as breeding programs that involves the incorporation of desired alleles present in distinct group of parents into an individual in order to improve a desired trait. Recurrent selection can be otherwise seen as a process whereby there is an "accumulation of advantages" from different parents into an entire population of different individuals over multiple generations thereby generating a superior population. Accumulation of such favorable alleles in multiple individuals within a population until the alleles are present enough in high frequency increases the chances of finding one individual with all the favorable alleles. Lines can then be selected from the improved population. RS populations are systematically developed to gradually improve traits that are quantitatively inherited. Parents used in RS schemes must have the potential to contribute useful alleles to the population. Many types of populations such as open pollinated cultivars, synthetic cultivars generated by crossing germplasm with desired traits, and F_2 populations derived from intermating two or more inbred lines may all be used for recurrent selection (Hallauer, 1992).

Recurrent selection has been proven to be an important tool for improving maize germplasm, although there are various advantages and disadvantages of different recurrent selection methods (Peng et al., 2007). This is because they all involve mating over several generations, selecting progeny with desired alleles and using them as parents in the next generation to increase the frequency of favorable alleles while maintaining genetic variation (Li et al., 2008). Genomic selection (GS) is a type of selection method similar to

RS. GS predicts the breeding values of lines within a population by analyzing the phenotypes of all individuals with marker scores. The simultaneous incorporation of all marker effect by GS is used to estimate the variation observed due small-effect QTL (Heffner et al., 2009).

For traits that can be phenotypically identified such as yield, plant height and other agronomically desirable traits, phenotypic recurrent section (PRS) is useful because it allows for the evaluation of larger populations when compared to the size of the population required for GS, thereby increasing the chances of selecting desirable plants (Dudley and Lambert., 2004). However, very limited progress can be made using PRS to select desirable plants for quantitatively inherited traits if the traits have low heritability. Such traits are polygenic in nature and highly influenced by genotype x environment interactions, which makes it difficult to measure the true phenotype of a plant. Polygenes are group of genes expressed together to produce a particular trait of interest. Heritability of such genes influencing a particular trait is dependent on the number of genes present with the polygene pool. The proportion of phenotypic variation among individuals within a population that can be attributed to genes present in the genome of an organism is known as heritability (Holland et al., 2003). For example, aflatoxin resistance described as a highly quantitative trait with low heritability (Warburton et al., 2009) because of it's high G X E and also the number of genes involved for the trait to manifest.

Molecular marker-assisted recurrent selection (MARS) is one molecular breeding method used to improve the selection gain of plants with desirable traits if the trait is quantitatively inherited and if markers linked to large QTL have been identified (Beyene et al., 2015). MARS, just like any other recurrent selection scheme, is aimed at

accumulating favorable alleles from multiple parents using molecular markers significantly associated with genes or QTLs influencing a quantitative trait (Bernardo, 2008). A recent publication by Semagn, (2015) reported genetic gains achieved using MARS in 10 tropical bi-parental maize population. The study involved genoyping of 300 $F_{2:3}$ cycle 0 (C_0) progeny with 286 molecular markers. Results obtained from the study showed between 7.9 – 36.5 % higher grain yields than regular hybrids derived using conventional pedigree breeding.

CHAPTER III

HIGH-DENSITY GENETIC MAPPING TO IDENTIFY QTL LINKED TO AFLATOXIN RESISTANCE IN MAIZE

ABSTRACT

Aflatoxin is a secondary carcinogenic metabolite produced by the fungus *Aspergillus flavus* (Link:Fr) under favorable environmental conditions such as the hot and humid environments experienced annually in the southern U.S and many other parts of the world. Pre-harvest contamination of maize (*Zea mays* L.) grain with aflatoxin is a chronic problem causing economic hardship to farmers. It also poses serious health issues in developing countries that lack infrastructure for proper grain testing. Host plant resistance is one of the most efficient methods of reducing aflatoxin accumulation in maize. Identification of aflatoxin accumulation resistant maize germplasm is the first step in breeding for resistance, and resistance sources from tropical growing environments have been found. However, the quantitative nature of the trait makes it hard to transfer resistance from donor lines into elite temperate cultivars. The use of molecular markers linked to quantitative trait loci (QTL) for resistance is one way to hasten this task, and some QTL have been identified to date. To identify additional novel QTL, a bi-parental mapping population consisting of 240 $F_{2:3}$ families was developed by crossing CML69, an aflatoxin resistant inbred line that is unrelated by pedigree to currently known resistant donor lines, with Va35, an aflatoxin susceptible inbred line adapted to southern US. A total of 1331

single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers were successfully mapped. Aflatoxin levels were measured in artificially inoculated field trials in four different locations over 2 years with three replications. Mean aflatoxin levels ranged from 183ng g^{-1} to 1454 ng g^{-1} across all environments. Composite interval mapping (CIM) identified 15 significant QTL on chromosomes 1, 2, 3, 4, 5 and 7 and these QTL explain about 4% - 18% of the phenotypic variation observed in the mapping population. Both parents contributed aflatoxin-resistant alleles to the population. Fourteen of the 15 QTL co-locate with previously identified QTL, suggesting that they will be stable in different genetic backgrounds and environments, and one novel QTL will provide additional resistance. Multiple interval mapping (MIM) analysis also identified 14 significant QTL in 3 of the 4 test environments on chromosomes 1, 2, 3, 4, 5, 7 and 9. Eleven of these QTL corresponds to the ones identified by CIM.

INTRODUCTION

Aspergillus flavus (Link:Fr) is an opportunistic pathogen that colonizes maize (*Zea mays* L.) under favorable environmental conditions, where they can cause ear rot and subsequent production of aflatoxins (Windham & Williams, 2002). Aflatoxins are carcinogenic secondary metabolites that can be lethal or cause serious health concerns when consumed by humans and animals (Miller & Wilson, 1994). Due to the detrimental health effects of aflatoxin, the U.S. food and drug administration (FDA) restricts the interstate action level of maize grain for human consumption under 20 ng g^{-1} (Park & Liang, 1993), potentially causing further economic losses to farmers.

Various methods have been employed to combat aflatoxin accumulation in maize. However, host plant resistance has proven to be one of the most efficient and promising

approaches to date (Moreno & Kang, 2002; Brown et al., 2013). Maize geneticists and breeders have identified resistant maize lines (Scott & Zummo, 1988, Henry et al., 2012). Resistance to aflatoxin accumulation can be achieved either by reducing the amount of fungus present in the grain or by reducing the amount of toxin produced by the fungus after infection (Warburton et al., 2010). Aflatoxin resistant germplasm has been developed, registered and released by the USDA-ARS Corn Host Plant Resistance Research Unit (CHPRRU), including Mp313E, Mp420, Mp715, Mp717, Mp718, and Mp719 (Scott & Zummo, 1992; Williams & Windham 2001, 2006, 2012) and others (Guo et al., 2008; Mayfield et al., 2012; Scully et al., 2016). However, all these lines are ultimately derived from tropical maize germplasm and as such, possess many undesirable agronomical traits such as late maturity and lodging when grown in more temperate environments (Warburton et al., 2009).

Despite the availability of many resistant lines, virtually all commercial hybrid maize lines are still susceptible to aflatoxin accumulation when environmental conditions are favorable for the proliferation of the fungus and production of toxins (Abbas et al., 2002, 2006; Daves et al., 2010). One significant reason that commercial hybrids lack a sufficient amount of resistance is because aflatoxin resistance is a highly quantitative trait with low heritability. The trait is also influenced by different environmental conditions resulting in very high genotype x environment interaction (G X E) (Hamblin & White, 2000). To increase heritability during screening of germplasm for aflatoxin resistance requires artificial inoculation of the fungus in repeated field trials for constant toxin production (Zummo & Scott, 1985). Another main constraint for lack of resistance in commercial hybrid is the fact that none of the QTL identified over six mapping populations

to date explains more than 20% of the phenotypic variation observed and may not be consistent in different locations, (Robertson-Hoyt et al., 2007; Warburton et al., 2009; Paul et al., 2013), making introgression via phenotypic selection inefficient.

Molecular markers developed to identify QTL associated with aflatoxin resistance can also be used to estimate the breeding value of lines derived from the mapped resistant parents. The pyramiding of the best QTL from multiple resistant donors via MAS represents an efficient way to create new highly and stably resistant hybrid maize parental lines. Identification of a few more large effect QTL from resistant genotypes unrelated to known donor lines for improved aflatoxin accumulation will aid in this endeavor. To achieve this purpose, CML69, an aflatoxin resistant genotype released by the International Maize and Wheat Improvement Center, (CIMMYT) and unrelated by pedigree and by molecular marker genetic similarity measurements (Warburton et al., 2013) to all currently known and mapped resistant donor lines (CIMMYT, 2005) was used to create a QTL mapping population. It was crossed to Va35, a southern adapted inbred line as the susceptible parent.

The objectives of this project were to: (i) identify QTL associated with aflatoxin accumulation resistance in CML69 and determine if they are novel compared to previously identified QTL; (ii) to estimate the phenotypic effect of the identified QTL on aflatoxin resistance; (iii) to determine the stability of the identified QTL in multiple environments; and (iv) to identify molecular markers which will help facilitate introgression of identified QTL into new resistant maize lines.

MATERIALS AND METHODS

Germplasm and phenotypic evaluation

A mapping population consisting of 238 $F_{2:3}$ families was created from a cross between CML69, a lowland adapted maize inbred line from CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo (International Maize and Wheat Improvement Centre) Mexico and Va35 a southern U.S. adapted, non-stiff stalk inbred, developed in Virginia by selfing out of the backcross [(C103 x T8) x T8] (Gerdes et al., 1993). Briefly, a cross between both parental lines was used to generate an F1 plant, which was self-pollinated to generate 240 F2 progeny. Each F2 plant was self-pollinated and planted ear to row to create $F_{2:3}$ families, which were sib-mated to generate enough seeds for replicated field trials. Parents, F1, and 240 F2:3 families were grown in a randomized complete block design (RCBD) with three replicates at the R.R. Foil Plant Science Research Center, Mississippi State University in 2016 (MSU2016), twice in 2017 (with one week interval between planting dates to simulate different environments) (MSUA2017 & MSUB2017) and in Lubbock, TX in 2017 (LUBBOCK2017). Each experimental unit consisted of a 5.1 m single plot of each family, with 20 plants per family.

The measurement of aflatoxin accumulation in artificially inoculated plants is the most precise way to measure the level of resistance in maize grains (Windham & Williams, 2002). To determine aflatoxin levels for each family in the mapping population, inoculum was prepared for aflatoxigenic *A. flavus* strain NRRL 3357 (ATCC #200026). This fungus strain has the ability to produce high levels of aflatoxin in maize grain (Windham & Williams, 2002). The top ear of each plant was inoculated at mid-silk (when 50% of plants in the plot has silks), using an Indico-tree-marking gun (Idico Products Co., New York)

fitted with a 35mm 14-gauge hypodermic needle. The needle dispenses 3.4ml suspension containing $3x10^8$ conidia beneath the husk. Inoculated ears were hand harvested approximately 10 – 11 weeks post inoculation, bulked, dried at 38°C to 15% or less moisture content, machine shelled and ground. Aflatoxin concentrations were determined using VICAM AflaTest (VICAM, Watertown, MA) according to manufacturer's directions. VICAM AflaTest uses 50g of ground tissue and can detect all four common aflatoxins (B1, B2, G1, and G2) at a concentration as low as 1 ng g^{-1}.

Genotyping and linkage mapping

$F_{2:3}$ families were genotyped by collecting leaf tissue from plants (20 plants per family) in each family in the first replication of the MSU2016 test. Collected tissue was bulked and frozen in liquid nitrogen, lyophilized and ground using the Tecator Cyclotec-1093 sample mill (FOSS, Inc., Eden Prairie, MN). DNA was extracted from the ground samples using a modified CTAB DNA extraction protocol (Murray & Thompson, 1980). Approximately 4000 markers [single nucleotide polymorphisms (SNP) and simple sequence repeats (SSR)] spanning all ten chromosomes in the maize genome were screened on the parents and F1. Of these, 1331 markers polymorphic between the parents were selected and genotyped on the entire population. Maize SNP genotyping assays from LGC genomics (https://www.lgcgroup.com/maize/#.W9NKe2hKjcs) spanning all 10 chromosomes and polymorphic between the parents of the mapping population were purchased and amplified via polymerase chain reaction (PCR). Polymerase chain reaction products were visualized using the FLUOstar Omega microplate reader (BMG-Labtech, Ortenberg, Germany) and imported into KlusterCaller software (LGC Genomics, Teddington, UK) for allele calls. Furthermore, the 238 families were genotyped using the

illumina maize SNP50 bead chip (https://www.illumina.com/products/by-type/microarray-kits/maize-snp50.html) and tested by Corteva Agriscience (Indianapolis, IN). Additional SSR markers reported by Maize Genetics and Genomics Database (www.maizegdb.org) were selected based on their bin number. Primer pairs for each SSRs were synthesized by Integrated DNA Technologies (IDT Inc., Coralville, IA). Amplified PCR products for SSRs were visualized using 4% agarose gels pre-stained with ethidium bromide.

Genotypic data was input into the Joinmap4 software (version 4) (Van Ooijen, 2006) to generate the summary statistics, estimate the different linkage groups and to calculate distances within groups. Linkage groups were constructed using the maximum likelihood (ML) mapping method by Monte Carlo (Jansen et al., 2001; summarized in Table 3.1). Mapped positions of all markers were confirmed by comparison with known positions in MaizeGDB (Lawrence et al., 2008) and Gramene release 58 http://www.gramene.org/.

Table 3.1 Table 2.4 Total number of polymorphic markers and genetic length (cM) of each chromosome.

Maize chromosome	Polymorphic markers	cM
1	229	404.45
2	140	206.94
3	160	197.74
4	152	208.86
5	132	200.66
6	109	167.87
7	88	160.62
8	137	175.14
9	103	140.31
10	81	98.48
Total	1331	1961.07

Statistical analysis of Phenotypes

Raw aflatoxin level for all 238 $F_{2:3}$ families showed a high level of skewness and kurtosis and were therefore natural log-transformed to normalize the distribution. Descriptive statistics of the natural log-transformed data were obtained using PROC UNIVARIATE function in SAS 9.4 (SAS Institute, 2014, Cary, NC). The means, heritability, and analysis of variance (ANOVA) were calculated using the generalized linear model (GLM) function in SAS 9.4 (SAS Institute, 2014, Cary, NC). Best linear unbiased predictor (BLUPs) for the families across all four environments was calculated using the following model:

$$Y_{ijk} = \mu + fam_i + env_j + \varepsilon_{ijk} \quad (3.1)$$

Where Yijk is the individual observation, μ is the overall mean, fam*i* is the main effect of the *i*th family, env*j* is the main effect of the *j*th environment and ε_{ijk} is the error term.

QTL analysis

Genotypic data from each $F_{2:3}$ family, linkage map data from Joinmap4, and natural log-transformed phenotypic data from the four environments and average over all environments were input into QTL Cartographer version 2.5 (Wang et al., 2012). Composite interval mapping (CIM) (Jansen et al., 2001) was performed using the description by Brooks et al., (2005). The Logarithm of Odds (LOD) threshold was estimated for each environment (regarded as traits in QTL cartographer) as described by Churchill & Doerge, (1994) with 1000 permutations performed for each set of data to maintain a 0.05 level of significance. QTL with peaks above the set 1000-permutation threshold and a minimum of 5-cM distance from any neighboring QTL were declared

significant. Significant QTL results generated from CIM were used as initial model terms for Multiple Interval Mapping (MIM) to further estimate the main effect and the check for epistatic interactions between all the identified QTLs. MIM takes the QTL analysis a step further by searching for new main effect, epistatic interactions between the QTL, test for the significance of the main effects and optimize the position of the final QTL (Jansen et al., 2001).

RESULTS AND DISCUSSION

Phenotypic evaluation

The average raw aflatoxin levels for the 238 $F_{2:3}$ families across all four environments ranged between 177ng g^{-1} in MSUB2017 to 1454 ng g^{-1} in MSU2016, with the highest average aflatoxin level recorded in the MSU2016 test (Table 3.2). In all environments, the susceptible parent Va35 had significantly higher mean aflatoxin levels as expected, while the resistant parent CML69 has the lowest levels of aflatoxin; however, in the LUBBOCK2017 test, all aflatoxin levels were low and the difference between the parents was insignificant. Skewed distribution in aflatoxin levels was observed among the families across all environment in both years and was normalized by natural log-transformation leading to a more normal distribution, a reduced coefficient of variation (CV) and skewness (Table 3.3). The F_1 generation and $F_{2:3}$ families fall somewhere in between both parents. The F_1 means were lower than the $F_{2:3}$ family means, while both generation means were lower than the susceptible parents and higher than the resistant parent in all environment except for the test in LUBBOCK 2017 (Table 3.2).

Table 3.2 Generation means and standard deviations of non-transformed aflatoxin levels for parents, F_1 and 240 $F_{2:3}$ families in 4 environments tested in 2016 and 2017.

Pedigree	MSU2016	MSUA2017	MSUB2017	LUBBOCK 2017
Va35	2711±68	687±11	320±95	120±28
CML69	97±18	55±14	112±95	184±33
F_1	390±14	208±10	65±25	81±25
$F_{2:3}$ Families	1454±48	349±24	177±13	183±8

Table 3.3 Table 3.2Descriptive statistics showing the mean, standard error (SE), standard deviation (SD), coefficient of variation (CV) and skewness of both raw and natural log-transformed [ln(Afl+1)] aflatoxin levels.

Environment	Trait	Mean	SE	SD	CV (%)	Skewness
MSU2016	Aflatoxin	1454.74	47.56	1307.74	112.76	2.30
MSU2016	ln(Afl+1)	5.57	0.07	1.91	34.35	0.16
MSUA2017	Aflatoxin	349.32	16.52	454.20	134.65	2.62
MSUA2017	ln(Afl+1)	4.64	0.07	1.91	41.11	0.16
MSUB2017	Aflatoxin	171.22	10.05	276.33	61.38	3.90
MSUB2017	ln(Afl+1)	3.76	0.07	1.80	47.93	0.16
LUBBOCK2017	Aflatoxin	183.54	8.17	224.57	37.32	4.16
LUBBOCK2017	ln(Afl+1)	4.08	0.06	1.55	38.12	0.16

Mean family heritability was estimated for all families using the natural log-transformed aflatoxin levels within each environment. Family heritability estimates were 0.43 ± 0.022 in MSU2016, 0.24 ± 0.027 in MSUA2017, 0.23 ± 0.036 in MSUB2017 and 0.15 ± 0.032 in LUBBOCK2017. Various studies over the years have presented different heritability estimates in different mapping populations. Estimates from these studies ranges from 0.27 – 0.42 in Mp313E x B73 QTL mapping population (Brooks et al., 2005), 0.22 in Mp717 x NC300 QTL mapping population (Warburton et al., 2009) and 0.28 in Mp715 x T173 QTL mapping population (Warburton et al., 2011). The difference in heritability

estimates can be caused by the type of population being studied, the parents used to create the population, environmental effects, error in measurements, and the type of QTL analysis employed.

Genetic linkage map

A high-density genetic map was constructed using 1331 markers (1303 SNPs and 28 SSRs) scored on both parents, F_1 and 238 $F_{2:3}$ families of the mapping population. Summary statistics of genotypic data including Chi-square test of similarity of family genotypes, similarity of loci (to eliminate redundant markers), and locus genotypic frequencies were calculated using the Joinmap software. Segregation distortion for the families was estimated using Chi-square goodness-of-fit and used to determine deviation of each segregating locus from the expected F_2 Mendelian ratios. Of the 1331 markers, 87 markers showed significant segregation distortion at $p \leq 0.005$ (Supplementary Table 1). Only 37 (~3% of the total number of markers) showed significant segregation distortion at (α=0.05), and these markers were removed from further analysis. The maximum likelihood algorithm (Jansen et al., 2001) and the Haldane mapping function (Haldane, 1919) were used to estimate linkage groups for markers on the same chromosome. Linkage groups were determined using the default independent LOD score for recombination frequency in Joinmap. Ten linkage group corresponding to 10 different maize chromosomes were identified at a LOD of 10.0. The final list of markers and their genetic locations are available on supplementary Table 2.

The final map of this population can be found in Supplementary Figure 1. High-density linkage maps with an average spacing interval of 1cM or less will lead to higher QTL resolutions and an increased chance of identifying all regions influencing aflatoxin

resistance, as well as increased accuracy of effect estimates and improved chances of separating closely linked QTL (Stange et al., 2013; Fenton et al., 2018). This study had a sufficient number of markers to allow for approximately only 1-2 cM between each marker, which is excellent genomic coverage. A total map length of 1961.07 cM and gaps of no greater than 1 to 2cM indicated that most QTL present in this population should be identified.

QTL analysis

Composite Interval Mapping (CIM) analysis detected 15 significant QTL across all four environments and the average of all four environment using the natural log-transformed aflatoxin levels (Table 3.4). Multiple QTL were identified on chromosomes 1, 3 and 4, 2 on chromosome 2, and one each on chromosome 5, 7 and 9. The QTL on chromosome 1 (QTL # 5, 9 and 13) were found in multiple environments in different years and the QTL on chromosome 3 (QTL # 6 and 11) are tightly linked to each other. The 3 QTL identified in LUBBOCK 2017 (QTL # 13, 14 and 15) were not significant at the set threshold, which was unusually high, possibly due to the limited range of variation in the phenotypic data from that environment. The phenotypic variation explained by all the QTL identified by composite interval mapping ranged from 2.1% (QTL #4 in MSU2016) to 19.5% (QTL # 11 in MSUB2017) (Table 3.4).

Of the 15 QTL identified in this study, 14 map to the same location as QTL identified in previous mapping populations, and resistance alleles for these QTL were donated by Mp313E (Brooks et al., 2005) and Mp715 (Warburton et al., 2010) aflatoxin resistant inbred lines. Results generated from this study were also consistent with other previously published reports in which a QTL on chromosome 1 (QTL #1) was identified

in the Mp313 x Va35 bi-parental mapping population, where the resistant allele was donated by the susceptible parent (Va35) (Willcox et al., 2013).

Table 3.4 CIM analysis of natural log-transformed values of aflatoxin levels showing identified QTLs and their relative position on the chromosome.

MSU2016							
QTL	Position	Chr	Bin	LOD	Action	Effect	%
1	129.3	1	1.07	4.2	A	-0.34	4.1
2	66.5	2	2.03	4.6	D	0.063	3.2
3	30.4	3	3.02	4.3	D	0.305	5.5
4	42.7	4	4.02	4.6	D	0.132	2.1
MSUA2017							
QTL	Position	Chr	Bin	LOD	Action	Effect	12.5
5	280.1	1	1.09	4.6	A	0.371	4.4
6	82.8	3	3.06	4.7	D	-0.405	8.9
7	56.4	4	4.06	4	D	-0.294	9.1
8	95.9	7	7.05	4.1	A	-0.421	16.4
MSUB2017							
QTL	Position	Chr	Bin	LOD	Action	Effect	2.3
9	283.1	1	1.09	4.5	A	0.371	3.7
10	161.4	2	2.07	4.6	A	0.246	9.4
11	87.8	3	3.05	4.1	A	-0.47	2.3
12	46	4	4.03	4.8	A	-0.457	2.2
LUBBOCK2017							
QTL	Position	Chr	Bin	LOD	Action	Effect	2.3
13	278.2	1	1.09	4.9	A	0.592	3.6
14	11.4	5	5.01	5	A	-0.417	3.4
15	78.4	9	9.05	5.1	A	0.074	18.5

[a]Reported significant QTLs identified in four tests environments using the natural log-transformed data.
[b]Additive alleles with negative effects indicate the resistant alleles was donated by the resistant parent (CML69) and additive alleles with positive effects indicate resistant allele is donated by the susceptible (Va35).
[c]Dominant alleles with negative effect indicate that the aflatoxin resistant alleles are dominant for aflatoxin reducing alleles and positive effect indicates that the aflatoxin susceptible alleles are dominant for aflatoxin increasing alleles.
[d]Partial R^2 generated from additive and dominance parameters.

Results generated from this study were also compared to a meta-QTL study conducted by Mideros et al. (2014) involving two aflatoxin resistant QTL mapping populations and six ear rot mapping studies for *Aspergillus flavus, Gibberella* (*Fusarium graminearum* (teleomorph: Gibberella zeae) and *Fusarium verticillioides* (teleomorph: *Gibberella moniliformis*). The meta-QTL study identified 62 significant meta-QTL, twelve of which are located on chromosome 4 (bins 4.07 to 4.09), a chromosome region spanning approximately 3% of the maize genome (Mideros et al; 2014). The result of the meta-analysis indicates that this region of the maize genome harbors multiple genes influencing maize response to various pathogens. The current study identified a QTL on chromosome 4 (bin 4.06) that falls within the same confidence interval of one of the QTL identified by the meta-QTL analysis. This QTL very likely contains genes contributing not only to aflatoxin accumulation resistance but also to resistance against other fungal pathogens.

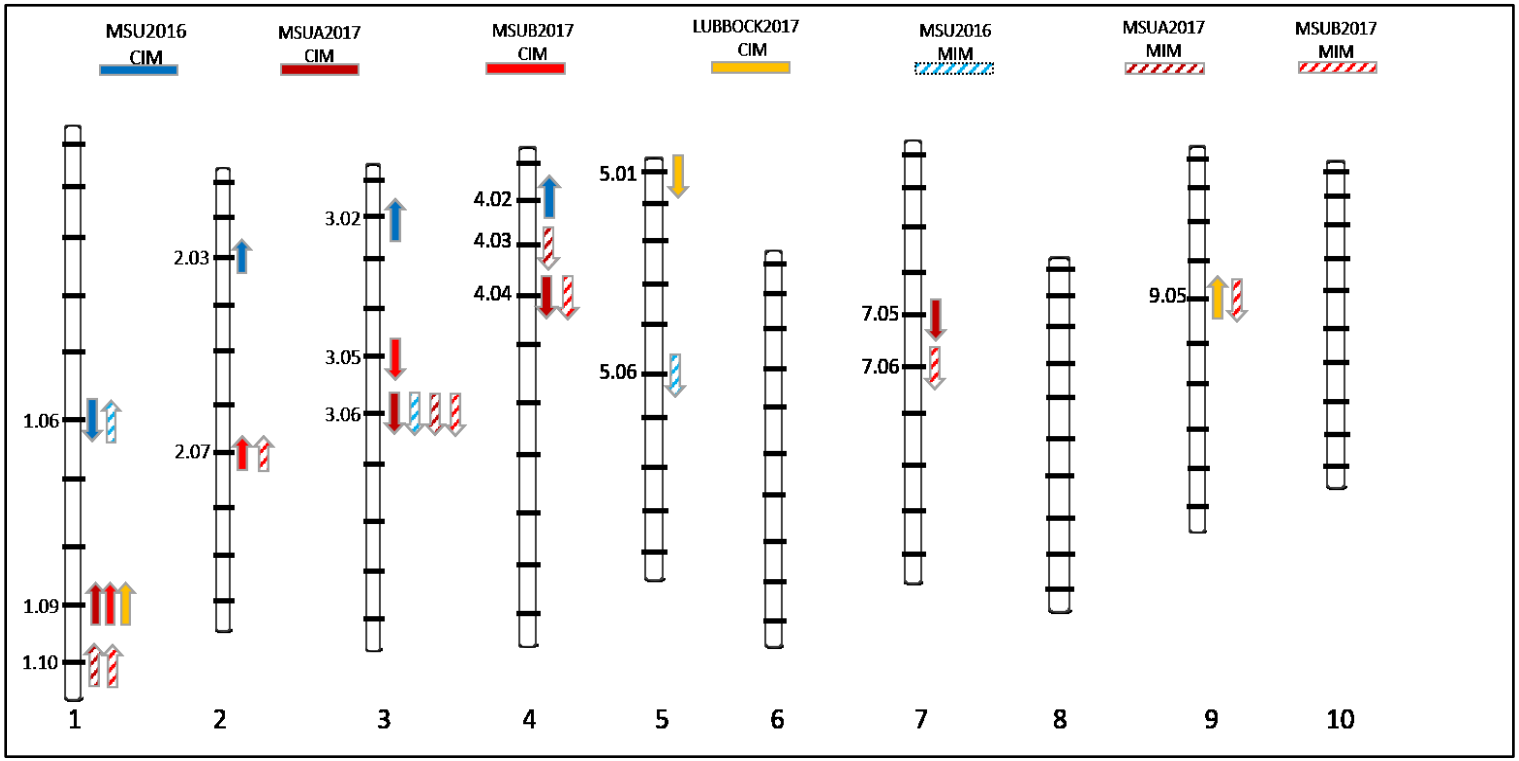

Figure 3.1 Maize chromosomes showing bin locations of significant QTL identified with composite interval mapping (CIM) and multiple interval mapping (MIM) analysis. Arrows pointing up indicates negative additive effect which means that aflatoxin reducing allele is donated CML69 while arrows pointing down indicates a positive additive effect which means that aflatoxin reducing allele is donated by Va35.

Table 3.5 CIM analysis showing markers information for identified QTL using the natural log-transformed aflatoxin levels.

QTLS	Chr	Flanking markers	[a]1-LOD support interval (cM)
MSU 2016			
1	1	PZE-101090535 - PZE101083942	100.0 - 135.5
2	2	PHM1962_33 - PZE-102070469	60.0 - 80.2
3	3	PZE-103017577 - PZE-103048707	28.2 - 55.6
4	4	PZE-104144965 - PZE-104132752	29.9 - 52.6
MSUA2017			
5	1	PZE-101156601 - PZE-101166754	268.5 - 282.2
6	3	PZE-103078297 - PZE-103100160	74.2 - 92.4
7	4	PZE-104136748 - PZE-104099276	50.0 - 75.3
8	7	PZE-107068214 - PZE-107054542	91.2 - 112.0
MSUB2017			
9	1	PZE-101159556 - PZE-101186294	260.5 - 285.4
10	2	PZE-102175032 - PZE-102180077	160.5 - 169.8
11	3	PZE-103092933 - PZE-103110761	80.0 - 100.5
12	4	PZE-104144965 - PZE-104113283	38.2 - 63.0
LUBBOCK 2017			
13	1	PZE-101155680 - PZ3 - 101173330	253.5 - 290.0
14	5	PZE-105163109 - PZE-105146705	0.0 - 21.5
15	9	PZE-109064616 - PZE-109073394	71.5 – 77.3

[a]1-LOD support interval of QTLs refers to the continuous genomic region, which includes the QTL position and all positions to the left, and right with LOD values larger or equal to the LOD value of the identified QTL with a confidence interval of 95%.

Multiple interval mapping (MIM) using the natural log-transformed aflatoxin level obtained from all four environments and the average over all environments was used to refine the QTL models and test for interactions between QTL. Unlike the CIM, where testing of putative QTL is strictly based on one QTL using other markers as covariates and thereby reducing residual variance (Kao et al., 1999), MIM utilizes multiple marker intervals simultaneously to fit multiple putative QTLs directly into the mapping model, thereby increasing the precision and power of the QTL analysis. MIM further readily estimates and analyzes the epistatic interactions between QTL, genotypic values of individuals, and heritability of the quantitative trait under study (Kao et al., 1999; Silva et al., 2012). Multiple interval mapping treats other QTL in the population as covariates when estimating the position and effect of each putative QTL (Silva et al., 2012) allowing for more accurate estimation of detected QTL. The final MIM model provides information about the genetic architecture of the quantitative trait such as the genomic locations, main and epistatic effects and also the proportion of the phenotypic effect of each QTL (Zeng et al., 1999).

Multiple interval mapping identified 4 main effect QTL in MSU2016 on chromosome 1, 3, 4 and 5 (Table 3.6) as opposed to the 4 QTL identified by CIM on chromosomes 1, 2, 3 and 4 (Table 3.4). QTL on chromosome 1, 3 and 4 identified by MIM corresponds to the QTL on the same chromosome detected by CIM based on their 1-LOD support intervals (Table 3.5). An epistatic interaction was detected between the QTL on chromosome 1 and chromosome 4 in MSU2016. In the MSUA2017 test, MIM identified 6 QTL on chromosome 1, 3, 4 (2 QTL), 7 and 9, which correspond to the 4 QTL identified by CIM except for a 2nd QTL on chromosome 4 and one on chromosome 9. . An epistatic

interaction was detected between the QTL on chromosomes 4 and 6. For the MSUB2017 test, MIM detected 4 QTL that corresponds to 4 QTL identified by CIM. Most of the QTL detected by MIM have additive gene action although some of them do have dominant gene action (Table 3.6). MIM did not detect any QTL in LUBBOCK 2017 as opposed to the 3 QTL identified by CIM. This is no surprise because the 3 QTL detected by CIM did not clear the set threshold calculated by the 1000 permutation procedure on the genotypic and phenotypic data of all 238 families.

Table 3.6 MIM analysis of natural log-transformed aflatoxin levels identifying putative QTL detected using multiple marker intervals for MSU2016 test.

MSU2016						
QTL1	QTL2	Type	Chromosome	Position	Effect	LOD
1	X	Additive	1	190.5	0.5254	4.83
	X	Dominant			0.2611	
2	X	Additive	3	82.3	-0.4633	3.45
	X	Dominant			-0.0410	
3	X	Additive	4	75.2	-0.2680	4.13
	X	Dominant			-0.4128	
4	X	Additive	5	86.4	-0.3685	2.61
	X	Dominant			0.3086	
5	1 x 4	D x A			-0.5800	
MSUA2017						
6	X	Additive	1	290.6	0.4254	4.70
	X	Dominant			0.2601	
7	X	Additive	3	92.5	-0.3833	4.00
	X	Dominant			-0.0410	
8	X	Additive	4	65.5	-0.3680	4.08
	X	Dominant			-0.5128	
9	X	Additive	4	90.8	-0.3685	4.01
	X	Dominant			0.3086	
10	X	Additive	7	96	-0.4836	6.41
	X	Dominant			-0.1760	
11	X	Additive	9	84.3	-0.2628	3.87
	X	Dominant			0.1872	
12	7 x 11	D x A			-0.5800	
MSUB2017						
13	X	Additive	1	277.4	0.4820	4.79
	X	Dominant			0.3085	
14	X	Additive	2	169.7	0.2158	3.18
	X	Dominant			-0.5177	
15	X	Additive	3	94.2	-0.4791	4.48
	X	Dominant			0.1249	
16	X	Additive	4	53.4	-0.4350	3.67
	X	Dominant			-0.3394	
17	13 x 14	D x A			0.2904	

[a]QTL 2 refers to a 2nd QTL with an epistatic interaction with QTL 1. QTL with no epistatic interaction is denoted "X".

[b]Negative gene action indicates the resistant alleles were donated by the resistant parent (CML69) and positive effects indicate that the resistant allele is donated by Va35, for both additive and dominant gene action.

Most QTL were identified simultaneously by MIM and CIM (Table 3.4 and 3.6), but MIM analysis revealed some significant QTL that were not identified by CIM. Two QTL were entirely new, on chromosomes 5 and 9 in environments MSU2016 and MSUA2017, respectively (Table 3.4 and 3.6). This might be because the MIM analysis combined all marker intervals to test for putative QTL thereby leading to more markers being analyzed jointly and generating another QTL that could not be detected by CIM using fewer marker intervals. The QTL on chromosome 4 in MSU 2016 may have shifted position with MIM compared to CIM, for similar reasons (of increased numbers of markers in the MIM analysis); this is more likely than that a new, unique QTL was identified in MIM within only a few dozen cM from the one identified in CIM (Table 3.6). Finally, some QTL were found in MIM in common with CIM but in different environments, as happened with QTL on chromosome 4 and 5 (Table 3.4 and 3.6). This tended to happen with QTL that were just below the cutoff in one environment and just above in another; depending on where the significance threshold is set, these will be added or dropped in each analysis. QTL from CIM that were lost in MIM includes one QTL on chromosome 1 in the MSU2016, and all the 3 QTL in LUBBOCK 2017. MIM detected significant epistatic interactions between QTL #1 & QTL #4 in MSU2016 test, QTL # 7 & QTL #11 in MSUA2017 and also between QTL #13 and QTL #14 in MSUB2017 test. There were no significant QTL detected in LUBBOCK2017 therefore, no significant interaction was detected.

Previous studies have identified various QTL associated with aflatoxin resistance, and the QTL with the largest and most stable effect has consistently been identified on chromosome 4 from Mp313E (Brooks et al., 2005). The current study also identified a QTL

on chromosome 4 located in the same region, but with a smaller effect. It may be the same one as the one identified in Mp313E, and a future meta-analysis of mapping data from multiple maps could confirm this.

Other QTL identified in this study were identified in only one environment and indicates they are not entirely stable. The QTL with the largest effect only explained ~12% of the total phenotypic variation observed in this study, which is typical of QTL. Although the QTL identified have very little effect on the phenotypic variation, pyramiding alleles of the largest QTL identified from multiple donors into one single line via marker assisted selection (MAS) might help increase aflatoxin accumulation resistance. However, the QTL identified in the current study only identified one, on chromosome 5, that may aid in this effort as it has not been identified in another donor already.

CONCLUSION

Quantitative trait loci (QTL) identified using bi-parental mapping populations are significant based on the LOD threshold set following CIM, and MIM significance is set before the analysis starts. While CIM uses other polymorphic markers as covariate in determining the effect of a QTL being tested, MIM uses other identified QTL as covariates thereby having a more stringent statistical effect and can measure epistasis between the QTL.

Majority of the QTL identified in this study co-locate with QTL identified in previous mapping populations conducted by CHPRRU in Mp313E and Mp715 mapping population. This study further validates these QTL as stable in different background and in multiple environments. To accurately determine if the co-locating QTL in this study are

the same as the ones detected in other mapping studies, the QTL can either be fine mapped, cloned or be used to create a population for more precise verification. The novel QTL identified on chromosome 5 in this study can be pyramided into other lines or cultivars to improve aflatoxin accumulation resistance.

CHAPTER IV

CONSTURCTING MAIZE NEAR ISOGENIC LINES (NILS) TO TEST GENOMIC REGIONS ASSOCIATED WITH AFLATOXIN RESISTANCE.

INTRODUCTION

Aspergillus flavus is a ubiquitous, saprophytic, pathogenic and opportunistic fungus, which causes ear rot in maize (*Zea mays* L.). In the US maize belt, the fungus rarely causes economic impact or direct damage to maize ears; however, under favorable conditions for growth, it can infect and produce a carcinogenic, immunosuppressive secondary mycotoxin known as aflatoxin, which will accumulate in maize ears (Scheidegger & Payne, 2003). Carcinogenic aflatoxin B1 (AFB1), produced by *A. flavus* is one of the major food safety concerns of maize. AFB1 is of great concern to both human and animal health because of its damaging effect on growth and development, immune system function, and its extreme carcinogenic properties. Thus, most countries have strict regulations to limit the accumulation of aflatoxin in maize and all other agricultural products susceptible to aflatoxin accumulation (Phillips et al., 2008). The U.S Food and Drug Administration (FDA) prohibits interstate commerce of maize grains with an aflatoxin concentration equal to or greater than 20ng/g (Park & Liang 1993).

Majority of the maize lines resistant to aflatoxin accumulation were bred from tropical sources thereby making them un-adapted to the temperate regions (Mayfield et al., 2012). These lines were considered un-adapted because although they contribute aflatoxin reducing alleles, they also contribute major undesirable agronomic characteristics such as high plant and ear height, late maturity, late flowering and high moisture grain content (Mayfield et al., 2012). Although breeding lines combining improved agronomical

characteristics such as early flowering and aflatoxin resistance have been released, these lines were bred for resistance to aflatoxin accumulation and not as parental inbred lines (Williams & Windham, 2012). Released germplasm with increase aflatoxin resistance are used as "donors" of aflatoxin resistant alleles into adapted cultivars. However, transfer of resistant alleles from these donor lines is very difficult because of the highly quantitative nature of the trait (Willcox et al., 2013). Multiple studies have identified Quantitative Trait Loci (QTL) associated with aflatoxin resistance (Widstrom et al., 2003) Researchers use different population structures such as backcross (BC), F_2, double haploids, testcrossed progenies, half-sib and full-sib families, F_2 derived families, recombinant inbred lines (RIL) and diverse inbred population structures to detect and map QTL.

To confirm and validate QTL detected from these mapping studies, it is imperative to create and characterize near isogenic lines (NIL) using QTL identified in the mapping studies. These are generally pairs of lines that differ only in one region, such as a QTL of interest. Pairs of NILs are useful for thorough study of previously detected QTL associated with a trait of interest and can be used for the verification, mapping and incorporation of the desired QTL into an elite cultivar, one with other desirable agronomical characteristics but lacking the trait controlled by the QTL of interest (Eshed & Zamir, 1995; Kaeppler, 1993). If NIL carry more than one QTL at a time, they may be suitable for determining epistatic interactions, genetic linkage and genomic architecture of a trait (Pea et al., 2013). The use of NILs to verify QTL has been successful in maize (Graham et al., 1997), rice (Yu et al., 1991), soybeans (Muehlbaure et al., 1991), tomato (Brouwer & St Clair., 2004) among other species.

In this project, 15 single nucleotide polymorphism (SNP) with the lowest p-value from a genome wide association study (GWAS) conducted by Warburton et al., (2013; 2015) was used to create NILs. The GWAS study where the 15 SNPs were generated focused on identifying new *A. flavus* and aflatoxin accumulation resistant genotypes and resistance genes linked to aflatoxin accumulation reduction. A 300 inbred line panel consisting of maize lines publicly available maize lines known to have a higher level of resistance to aflatoxin contamination, lines developed for resistance to biotic and abiotic stresses and characterized inbred lines from previous studies were used for the GWAS study (Setter et al., 2011). The 300 lines were crossed to Va35, a susceptible, southern adapted inbred line of the non-stiff stalk heterotic pattern. Testcrossed progeny generated from these crosses were phenotyped in College Station, TX, in 2009 and 2010, Lubbock, TX, in 2009 and 2010, Raymond, MS, in 2009, one Starkville, MS, site in 2009, and two sites within Starkville in 2010 (a and b) (Warburton et al., 2013). Primary ears of each testcross progeny were inoculated with a 3.4-mL suspension of 3×10^8 conidia of *A. flavus* isolate strain NRRL 3357 and aflatoxin levels was quantified using the Vicam AflaTest (VICAM). The 300 entries used in the GWAS study were genotyped via genotyping by sequencing (GBS) as described by Elshire et al., (2011). The GBS data called 13,197 SNPs with a minor allele frequency (MAF) greater than 1%. Of these SNPs, 2000 with the lowest missing data and low frequency imbalance covering all 10 chromosomes were used to estimate the genetic diversity and construct structural analysis. TASSEL software 3.0.1 (Bradbury et al., 2007) was used to generate kinship matrix between all entries suing the 2000 SNP subset. PowerMarker 3.25 was used to distance between shared alleles as well as the diversity statistics on the 13,197 SNPs. Fifteen most significant SNPs from the

2000SNP subset with a p-value of ($2.87 \times 10^{-10} < p < 9.78 \times 10^{-5}$) were selected and used in creating NILs.

NILs were constructed with the aim of generating a SNP of interest per NIL so that the effect of each SNP on the trait can be tested separately but unfortunately due to linkage drag and residual homozygosity, this was hard to achieve. Introgression of desired SNP in NILs is achieved by repeatedly backcrossing a donor line (lines with the desirable aflatoxin-resistant alleles in this case) to a recurrent line (aflatoxin-susceptible inbred line that has other desired agronomic traits). After each generation, progeny may be tested with molecular markers (SNPs being the marker of choice) to select individuals that inherited the aflatoxin-resistant alleles, and these individuals are used as parents for the next generation (Martin et al., 1991). Lines generated after several backcrossing generations carry a small segment of the donor line in the recurrent background. A final selfing step for 1 to 2 generations fixes the SNP in homozygous form. After successfully fixing the desirable alleles in the NILs, both the recurrent parent and NILs for each QTL will be grown and phenotyped together by inoculating them with aflatoxigenic *A. flavus* strain NRRL 3357 (ATCC #200026) to determine the effect of the genomic regions identified by the SNP haplotype on aflatoxin accumulation resistance (Kaeppler; 1997).

MATERIALS AND METHODS

Development of plant materials

A total of 7 unrelated maize inbred lines (4 aflatoxin resistant lines and 3 aflatoxin susceptible lines (Table 4.1) were used to start the creation of the NILs. The 4 resistant lines are also referred to as the donor lines, and they carried the favorable aflatoxin-reducing alleles that are being introgressed into the susceptible, or recurrent lines. Briefly,

individual pairs of resistant and susceptible lines were crossed to generate F_1 progenies. Markers were not screened on the F_1 progeny because they are all completely heterozygous for all alleles, including those of interest at the F_1 generation. F_1 progeny obtained from each cross was backcrossed to the recurrent parent to create a segregating BC_1 generation.

Table 4.1 Inbred germplasm used in this study and their pedigree.

Inbred	Pedigree	Trait
CML5	Pob21C5HC133-1-B-##-B	Aflatoxin Resistant
CML348	G26SEQC3-H83-1-1-2-1-B	Aflatoxin Resistant
Mp313E	Old 1133.16-1-1-2-1 from Tuxpan	Aflatoxin Resistant
Mp715	Tuxpan	Aflatoxin Resistant
B73	Iowa stiff stalk synthetic c5	Aflatoxin susceptible
Va35	[(C103 × T8) T8]	Aflatoxin susceptible
Mo17	C.I. 187-2 × C103	Aflatoxin susceptible

Marker-aided recurrent backcrossing started on the segregating BC_1 generation. The progenies were screened with 15 genome-wide association studies (GWAS) SNP markers associated with aflatoxin resistance identified from a study by Warburton et al., (2013; 2015). These 15 SNPs with the lowest p-values ($2.87 \times 10^{-10} < p < 9.78 \times 10^{-5}$; Warburton et al. 2013; 2015), were extracted from an in-house hapmap database for the seven inbred lines used in this study (Table 4.2). KASP assays were designed for all 15 SNPs and screened on every individual from the BC_1 generation to select individuals carried to the next generation. Individuals heterozygous for the aflatoxin-resistant alleles at one or more target loci were selected for further backcrossing in the subsequent generation while individuals homozygous for the susceptible recurrent allele were discarded to eliminate undesirable plants (those that have lost the resistant allele already).

Table 4.2 List of SNPs associated with aflatoxin resistance levels ($p<10^{-4}$) in 7 environments and potentially causing reduction in aflatoxin accumulation. Bin location of all SNPs and association statistics such as the p-value and the R^2 value is shown. Gene name corresponds to the MaizeGDB database, and the SNP locations are given in reference to V2 of the maize reference sequence.

Region	Environment	SNPs	BIN	F	P	R^2	Gene
1	Star10LSM	S1_272220818	1.10	23.12	2.78E-06	0.0994	GRMZM2G 049349
2	Star10LSM	S1_280635931	1.10	25.10	1.14E-06	0.1007	GRMZM2G 009958
3	Star10LSM	S2_153128978	2.06	20.98	8.53E-06	0.1105	GRMZM2G 155437
4	Star09LSM	S2_183190964	2.07	20.84	5.04E-06	0.0963	GRMZM2G 155437
5	Star09LSM	S2_188872911	2.07	21.35	6.08E-06	0.0754	GRMZM2G 026065
6	Star10LSM	S2_205035174	2.08	23.26	2.38E-06	0.0812	GRMZM2G 166337
7	Star10LSM	S3_217359490	3.09	23.73	1.85E-06	0.0797	GRMZM2G 089525
8	Lubb09LSM	S3_217808798	3.09	27.33	3.83E-07	0.1121	GRMZM2G 052991
9	CSta09LSM	S3_217820604	3.09	15.67	9.66E-05	0.0541	GRMZM2G 053047
10	AveLSM	S4_26406913	4.04	16.74	5.63E-05	0.0557	GRMZM2G 003814
11	Star10LSM	S5_206795116	5.07	22.94	3.58E-06	0.1313	GRMZM2G 105874
12	Star10LSM	S8_94752242	8.02	25.30	8.98E-07	0.0890	GRMZM2G 147221
13	AveLSM	S9_107333254	9.04	22.52	3.47E-06	0.0845	GRMZM2G 331766
14	AveLSM	S9_117048731	9.04	43.12	2.87E-10	0.1620	GRMZM2G 108619
15	Star09LSM	S10_91956540	10.04	23.80	2.04E-06	0.0950	GRMZM2G 407650

Linkage Disequilibrium LD was measured by the R2 value. Bin location of all SNPs and association statistics such as the p value and the R2 is shown. Gene names corresponds to the MaizeGDB database.

After each generation of backcrossing to the recurrent parent, each progeny line's genome consists of 50% more of the recurrent parent than the previous generation, eliminating 50% of the donor genome as the individual moves closer to being isogenic with the recurrent parent. The process was repeated for two more generations to reach the BC_3 stage, testing each individuals with the same set of markers to select favorable plants every year. At the BC_3 stage of the NIL creation scheme, 29 individuals with aflatoxin reducing alleles at one or more target loci from the donor parents were selected through MAS for further selfing to fix the alleles (Table 4.2). Each of the 29 individuals make a family with 20 plants per family to generate 580 individual selfed to generate the BC_3S_1 progenies. MAS was also used to select 54 individuals with the aflatoxin reducing alleles from the 580 BC_3S_1 progenies. The 54 individuals were selfed again to generate BC_3S_2 progenies and move more towards homozygosity (Figure 4.1).

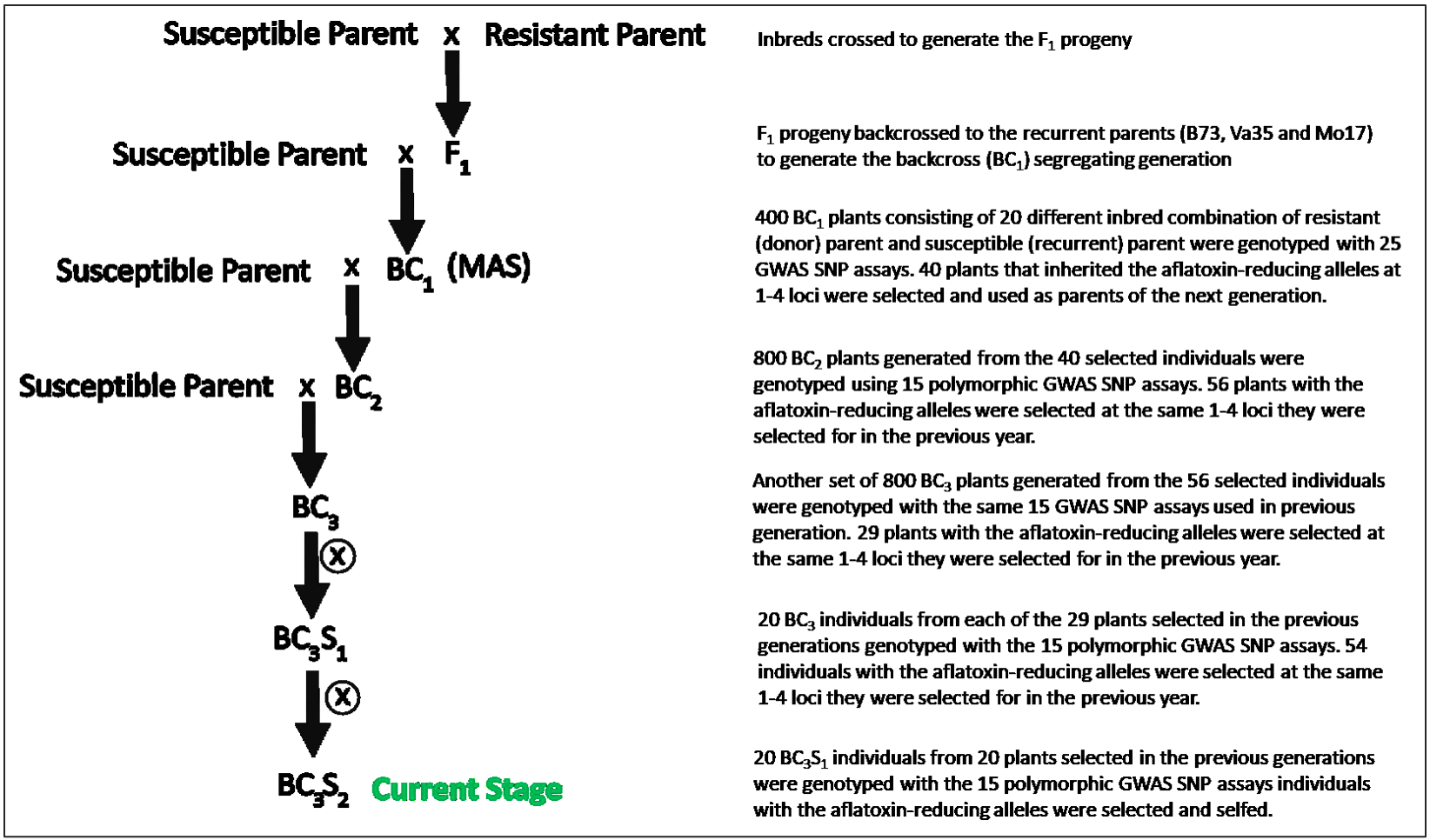

Figure 4.1 A scheme showing how plant materials were created and the number of plant selected in each generation.

SNP markers and KASP assays

Genotype by Sequencing (GBS) data as described by Warburton et al, (2013; 2015) was generated from a panel of 273 diverse maize inbred lines containing aflatoxin accumulation resistant and susceptible genotypes. The GBS data presents the variation in genomic sequence and allelic distribution of each line present in the panel. Polymorphic SNPs among the 7 inbred lines with minor effects were selected and used for creating the NILs.

KASP assays were designed for the SNPs by extracting 100bp of DNA sequence upstream and downstream of the SNP of interest in the B73 V3 reference genome Lawrence et al.; (2005). Twenty five SNPs were initially selected for this project. These 25 SNPs represent genetic locus identified in the GWAS study associated with aflatoxin accumulation resistance. The assays were ordered from LGC genomics (Hurts UK) and tested for amplification and polymorphism on the parental lines. The KASP assays were tested on the progenies from the BC_1 to the BC_3S_2 generation to select the individual progeny with the allele of interest (from the resistant parent) from one generation to the next. Some of the 25 SNPs could not be successfully converted into working assays because they are either too close to other nearby SNPs on the chromosome, which will not allow for proper annealing during polymerase chain reaction (PCR) amplification or they are monomorphic between the parents of the NILs making it impossible to tell the difference between the individuals. In cases like this, SNPs physically close and genetically linked to the initial SNP of interest were chosen since they are likely to be inherited together and KASP assays were designed for these SNPs.

Table 4.3 List of introgressed SNPs and the backgrounds they are being tested in.

Background	Pedigree	Number of selected plants	SNPs selected
B73	B73*CML5	3	S1_272220818, S2_153128978, S3_217820604, S9_117048731
	B73*CML348	4	S1_280635931, S3_217820604, S6_121311207, S9_117048731
	B73*Mp715	2	S3_217820604, S6_121311207, S9_117048731
Va35	VA35*CML5	3	S1_272220818, S4_26406913
	VA35*CML69	5	S2_183190964, S2_205035174, S2_188872911, S5_206795116
	VA35*MP715	2	S2_205035174, S9_117048731
Mo17	MO17*NC388	5	S1_280635931, S2_183190964, S2_188872911, S9_117048731, S10_91956540
	MO17*CML69	4	S2_205035174, S3_217359490, S9_117048731

Genotyping

Leaf tissue samples were collected from 4 inbred lines used as donors and 3 inbred lines used as recurrent parents for creating the NILs. DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method (Murray & Thompson, 1980; Saghai-Maroof et al., 1984). 140 mg of ground tissue was added to 3 ml CTAB extraction buffer (50 mM Tris, pH 7.5/ 0.7 M NaCl / 10 mM EDTA/ 1% hexadecyltrimethylammonium bromide (CTAB)/ 0.1% 2-mercaptoethanol) and incubated for 65°C for 60 min. A 24:1 Chloroform/Octanol mixture (1.4ml) was added and the solution inverted about 50 times and allowed to incubate for 10 minutes at room temperature. Resulting emulsion was centrifuged at 5125 ground force for 10 min at room temperature. After centrifugation, the supernatant was removed and transferred to a new 5ml tube where another 24:1

Chloroform/Octanol mixture (1.4ml) was added and rocked at room temperature for 10 minutes. The mixture was then centrifuged for 10 min at 5125 x g and the supernatant transferred to a new tube where RNAse A (17 µl at 10 mg ml--1 Sigma-Aldrich Co., Saint Louis, MO) was added, mixed by inverting, and allowed to incubate at room temperature for 60 min. 2ml of ice-cold isopropanol was added and the mixture inverted about 50 times. DNA precipitate was hooked out of the mixture using a glass hook and washed in 76% ethanol/ 0.2 M NaOAc and then in 76% ethanol/ 10 mM NH4OAc before being dissolved in 200 µl TE. DNA concentrations was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE).

KASP assays were designed by LGC Genomics (Teddington, UK) for each SNP to determine the allele calls within the parent's genome. Markers were not tested on F1 progenies because they are all theoretically heterozygous at all loci, including the target loci. Marker-aided recurrent backcrossing started at the BC_1 stage. DNA extracted from the samples were genotyped with the 15 SNP KASP assays (described below) and alleles were called using the Klustar-caller software for the OMEGA plate reader by BMG LABTECH GMBH, Orthenberg, Germany. Before genotyping the SNP assays on the progenies, they were individually tested to ensure they mapped to the correct location in the maize genome using the Mp313E x B73 mapping population, one of four previously constructed mapping populations for aflatoxin resistance.

SNP KASP assays work using a 94KDa recombinant thermostable DNA polymerase (KlearTaq). Amplification of DNA at targeted loci using KASP assay involves the use of two specially constructed mixtures, the SNP specific KASP assay mix and the KASP master mix. KASP assays enable bi-allelic scoring of SNPs at specified loci through

competitive allele-specific PCR. The KASP assay mix contains three primers, two allele-specific forward primers that each harbor unique tail sequence connected to a universal FRET (fluorescence resonant energy transfer) sequence, and one common reverse primer. KASP Master mix contains two universal FRET cassettes labeled with FAM or HEX dye (one for each allele), which fluoresce at different wavelengths thereby causing a difference in the genotype call for different alleles when read by a fluorescent-based plate reader (Figure 4.2).

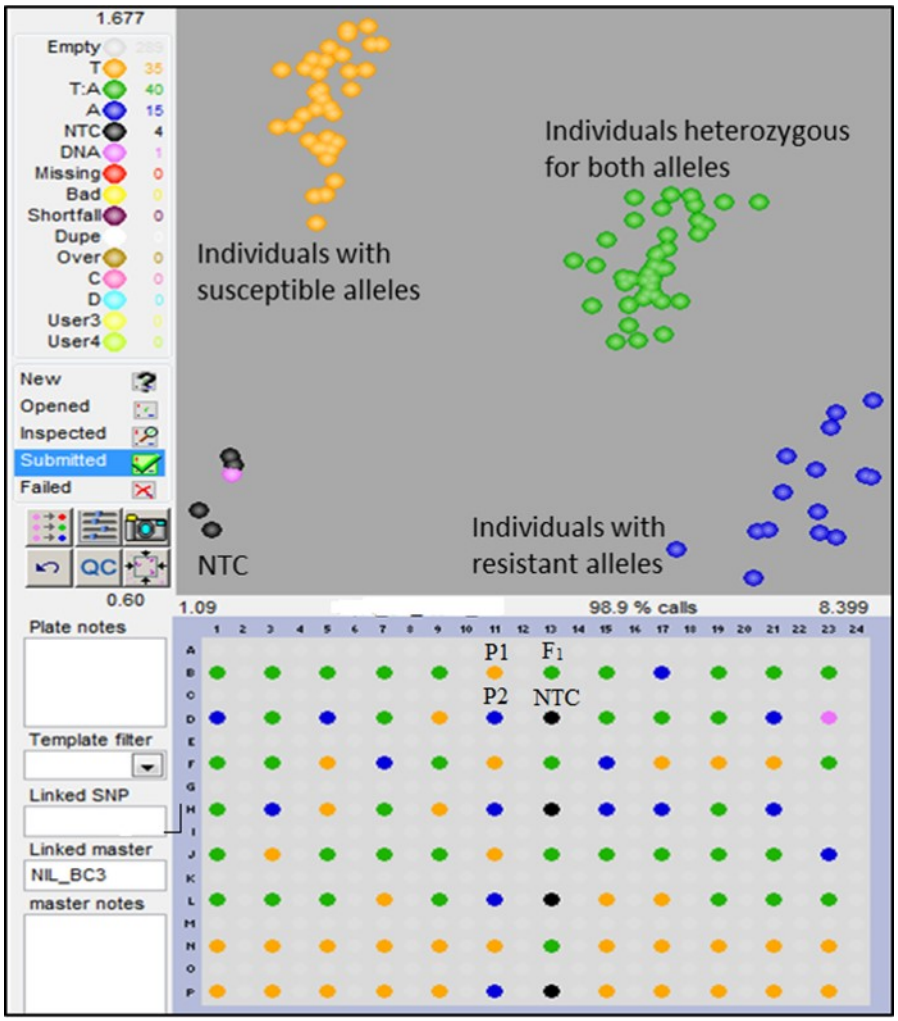

Figure 4.2 A KlustarCaller software output showing the allele calls of 95 individuals (including the parents) and four negative template controls (NTC) for SNP I.D (S3_217359490). Genotypes homozygous for the resistant allele reported by the FAM dye in blue, genotypes homozygous for the susceptible allele reported by the HEX dye in red, and heterozygous genotypes contains both resistant and susceptible alleles in green. The pink spot represents a sample that did not amplify during PCR.

The amplification pattern of two specific alleles (including the two homozygous classes and the heterozygous class) in the KASP assay for a set of 96 individuals in a 96-well micro-titer plate at the end-point fluorescent read after the amplification process is shown in the Klustar-caller plot software in Figure 4.3. One of the two fluorescent signals is generated if the genotype of a given SNP is homozygous and shows up as either yellow

or blue depending on the fluorescent dye attached to the allele while both signals are generated if the genotype is heterozygous for the given SNP showing up as green in the output. The PCR conditions for the KASP assays designed in this study are presented in Table 4.4.

Table 4.4 Thermal cycling conditions using KlearTaq.

Step	Temperature	Time	Number of cycles
1	95°C	15 mins	1 cycle
	95°C	30 sec	
2	61°C	30 sec	34 cycles
	72°C	1 min/kb	
3	72°C	5 mins	1 cycle

Proposed experimental design and field conditions

A set of 28 BC_3S_2 NILs with potentially introgressed alleles from the donor parents alongside the 3 recurrent parents will be evaluated for aflatoxin accumulation in replicated field trials at the R.R. Foil Plant Science Research Center, Mississippi State, Mississippi. Each plot will be a single 5.1 m row with 0.96 m spacing, and 20 plants per plot. Plots will received supplemental furrow irrigation throughout the growing season and standard management of test plots will be practiced. Mid-silk notes (50% of the plants in a plot had emerged silks) will be taken and primary ears will be inoculated 10 days post mid-silk with 3.4 ml suspension containing $3x10^8$ conidia of aflatoxigenic *A. flavus* strain NRRL 3357 (ATCC #200026). Individual inoculated primary ears will be hand harvested, dried at 38°C for 7 days to reduce moisture content to less than 15%, machine shelled and ground. Aflatoxin concentration will be determined using the VICAM AflaTest (VICAM,

Watertown, MA) in compliance with USDA test protocol (USDA, 2002). VICAM Aflatest method requires 50g of ground sample to detect aflatoxin concentration through immunoaffinity chromatography and fluormetric detection, and can quantify concentrations as low as 1.0 ng g^{-1} in grain samples.

Proposed theory

Testing each SNP for phenotypic effect on aflatoxin resistance will be done by observing the differences in aflatoxin accumulation averaged over individuals in which the SNPs has been being successfully introgressed compared to the average aflatoxin accumulation for the recurrent parent. The implementation of this proposed theory starts at the BC_3S_2 stage of this project. At this point, the lines would be tested for each SNP, and those fixed for at least one donor SNP will be chosen. These NILs will be tested for phenotypic effect of each SNP (or SNP combination, if two or more were introgressed). The resistant allele of each SNP will have been completely backcrossed and fixed in the homozygous state in a susceptible background after the series of marker assisted backcrossing steps to the BC_3 stage followed by selfing twice (with MAS) to get the BC_3S_1 and BC_3S_2 generation. The phenotypic effect of each SNP for the fixed NIL carrying it will be calculated as explained by Kaeppler (1997), using the formula for the linear model as follows:

$$Y_{jk} = \mu + \gamma_j + e_{jk} \qquad (3.2)$$

Where Y_{jk} represents the phenotypic value of the *k*th replication of the *j*th line, μ represents the mean of the two lines, γ_j represents the effect of the *j*th line and e_{jk} is the residual error where k =1,2,3 (Kaeppler, 1997). To test the null hypothesis of equality of the means of the created NIL pairs, analysis of variance (ANOVA) will be employed. If

there are significant differences between different NILs, this indicates that the successfully introgressed QTL has a measurable effect in the new background, but if there are no significant differences, the QTL does not have a phenotypic effect in that background, and that the original identification of the QTL was in error; is not repeatable in the new genetic background; or only shows up in specific environments (and not the one measured here).

RESULTS AND DISCUSSION

Creation of NILs requires at least five to six generations of maize backcrosses and subsequent selfing, and must be done with marker assisted selection to determine which of the segregating individuals in each family carry the desired SNP or QTL allele from all the progeny each year of backcrossing. Of the 15 SNP selected for this study, 9 of them were successfully converted into working assays and these were tested on the NILs starting from the backcross generations. The remaining 6 SNPs couldn't be converted into working assays because there are many other SNPs nearby in the assay sequence making it harder to design a working assay. In this case, nearby SNPs linked to the original SNPs were chosen and working assays were designed for these SNPs and screened on the NILs.

At the BC_3 stage, each of the recurrent backgrounds had between 2 and 6 target loci introgressed. B73 had 9 individuals with 6 target loci introgressed from 3 donor parents (CML5, CML348 and Mp715). Va35 on the other hand had 10 individuals with 5 target loci introgressed from 3 donor lines (CML5, CML69 and Mp715). Finally, 9 individuals with 7 target loci from 2 donor lines (CML69 and NC388) were introgressed into a Mo17 background (Table 4.2). Target loci introgressed were from chromosomes 1, 2, 3, 4, 5, 6, 8, 9 and 10.

At the BC_3 generation, it is expected that the genome of all individual NILs will carry ~97% of the recurrent parent and ~3% of the donor parent, including the selected alleles for the favorable SNPs selected by the markers (Figure 4.1). Each NIL is designed to examine one or more specific SNP or chromosomal region and the effect it has on aflatoxin accumulation. NILs are also very important as they can be used in physically observing the phenotypic effect of the introgressed SNP or region, although not all NILs may be physically differentiated visually when grown side by side with the susceptible (recurrent) parent, especially when the effect of the original associated SNP was very small. NILs are also a very useful tool in studying the interactions of two or more SNPs/chromosome regions in the same background and also provide insight on the epistatic interactions between the SNP/loci of interest (Kaeppler, 1997).

Initial results by Williams et al., (unpublished) provide an indication of these interactions, where the phenotypic effect of 2 or 3 QTL identified in QTL mapping studies from the resistant line Mp313E, was introgressed into in a susceptible background (Va35) and the effect of each QTL significantly reduced the level of aflatoxin compared to the un-introgressed Va35. In some cases, two or three QTL together were enough to make the plant as resistant to aflatoxin accumulation as Mp313E (Table 4.4). The QTL measured in the above mentioned study were identified through a QTL mapping population and had larger phenotypic effects on aflatoxin resistance compared to the QTL used in this current study, which were identified from a GWAS study with smaller effect on aflatoxin resistance. It is hoped that the GWAS identified QTL will still have a measurable effect in the NIL compared to the susceptible parents they were generated from. The NILs created by Williams and shown in Table 4.5 can also be very useful for studying high-resolution

mapping if smaller chromosomal regions are introgressed than were mapped in the original QTL mapping populations (since multiple generations of meiosis can provide more recombination, and thus a smaller chromosomal region being tested). Comparisons of successfully introgressed QTL to the recurrent background will be very useful in estimating the effect of the QTL on aflatoxin accumulation. Although as much as 4% of the donor's genome could remain in the NIL after 4-5 generations of backcrosses (Brinkman & Frey, 1977), these regions will be random and vary from NIL to NIL and can be discounted if multiple NIL are created from each donor.

Table 4.5 Validation of QTLs in NILs.

QTLs	Phen. Effect	ppb aflatoxin 2012	ppb aflatoxin 2014
2.05	15%	289	231
3.05	5%	538	401
4.06	10%	303	278
4.09	14%	157	368
3.05, 4.06	5% + 10%	-	242
2.05, 4.09	15% + 14%	-	258
2.05, 3.05	15% + 5%	-	36
3.05, 4.09	5% + 14%	-	28
4.06, 4.09	10% + 14%	-	10
2.05, 3.05, 4.09	15% + 5% + 14%	-	82
2.05, 3.05, 4.06	15% + 5% + 10%	-	12
None	-	690	-
Va35	-	748	411
Mp313E	-	26	1

Table adapted from Williams et al.; (unpublished).

CONCLUSION

Testing the phenotypic effect of potential QTL successfully introgressed into different backgrounds will give an insight on the phenotypic effect of each identified QTL, and potentially the epistatic interaction of each QTL if there are any more than one QTL per NIL. The methodology presented in this study will thus help to validate QTL identified in the GWAS study where the markers linked to the QTL selected for were generated. Due to linkage drag, as much as 4% of the donor parents genome will be present in the NILs even after about 4-5 generations of backcrossing but nonetheless, if the target QTL successfully introgressed, it may help improve aflatoxin resistance making the whole process worthwhile. Although the potential QTL introgressed in this study have not been phenotyped for aflatoxin resistance, the NIL are ready for that test and this is the next step in the process.

CHAPTER V

INCREASING THE FREQUENCY OF ALLELES ASSOCIATED WITH AFLATOXIN RESISTANCE FROM AN 8-WAY CROSS.

INTRODUCTION

Maize (*Zea mays* L.) is a staple food of global importance but is susceptible to aflatoxin contamination. Aflatoxins are a major class of mycotoxins produced *Aspergillus flavus.* At high levels, these hepatocarcinogenic toxins cause acute aflatoxicosis, which ultimately leads to death, and chronic low-level aflatoxicosis results in liver cancer, immune suppression, and other pathological conditions when consumed above permissive levels (Bennett et al., 2007). There are four major types of aflatoxins: AFB1, AFB2, AFG1 and AFG2. AFB1 is the principal member because it is the most common with a higher carcinogenic potency compared to other members of the group (Moreno & Kang, 1998). In the US, the impact of aflatoxin contamination is felt in the southeast, during hot and humid environmental condition suitable for the growth and proliferation of *A. flavus* is experienced annually. In other parts of the US where it is not so hot and humid like southeastern US, aflatoxin contamination is experienced due to drought stress. Aflatoxin contamination causes considerable economic losses to farmers in the US because of strict regulations imposed by the US Food and Drug Administration (FDA) of 20ppb for foods and feed ingredients (Park and Liang, 1993). In developing countries, on the other hand, lack of infrastructures for proper grain testing is the biggest concern, and aflatoxin

contamination causes pose serious health concerns when consumed because of its carcinogenic properties (Gnonlonfin et al., 2013).

Multiple studies have shown host plant resistance to be one of the most effective long-term solutions to combat aflatoxin contamination in maize (Gorman & Kang, 1991; Campbell & White, 1995). Breeding programs have identified and developed germplasm resistant to either *A. flavus* infection or the subsequent production of aflatoxin. Inbred lines such as Mp313E, Mp715, Mp717, Mp719, Tex6, Mo18W, MI82 as well as the population GT-MAS:gk (Scott & Zummo, 1990b, 1992; McMillian et al., 1993; Campbell & White, 1995; Campbell et al., 1997; Williams & Windham, 2001, 2006; Maupin et al., 2003; Clements & White, 2005) have underlying genes and/or quantitative trait loci (QTL) associated with aflatoxin resistance in maize. Since aflatoxin resistance in a highly quantitative trait influenced by multiple QTL, accumulation of these QTL from these inbred lines into one source will help increase resistance to aflatoxin accumulation.

Past studies have successfully mapped QTL for resistance to *A. flavus* infection or aflatoxin suppression. However, majority of the QTL identified in these mapping studies were only identified in one environment, and many explain less than 5% of the phonotypic variation observed in the population (Warburton & Williams, 2014). Another constraint accompanying the low observed phenotypic variation in resistant germplasm is the undesirable agronomic characteristics such as late maturity, high lodging and high husk coverage common to most resistant inbred lines when they are grown in temperate environments, thereby rendering them not suitable for commercial purposes in the US Corn Belt (Betrán et al., 2002).

Recurrent selection (RS) is a plant breeding improvement technique specifically designed to progressively improve quantitatively inherited traits within a population. It is a cyclic process that involves (1) creation of intermated progeny, (2) evaluation of the progenies and (3) intermating for further recombination of selected progenies, to continue the cycle (Weyhrich, 1998). Recurrent Selection can increase the frequency of favorable alleles within a population and still maintain genetic variation within the population (Doerksen, 2003). Hayes & Garber (1919) and East & Jones, (1920), first suggested the effectiveness of concentrating favorable genes for a specific trait by intercrossing selected parents. Hull (1952) further explained RS as a process involving re-selection generation after generation, with intermating among selected progenies within each generation to enhance recombination, thereby increasing the frequency of favorable alleles within the population. Different populations such as open-pollinated varieties, synthetic cultivars, F_2 populations generated from intermating inbred lines and populations created from exotic germplasm have all been used in RS schemes (Hallauer, 1992). Different RS methods such as intrapopulation (selection within one population) or interpopulation (a cross between two populations) and reciprocal recurrent selection have been developed to by to improve maize populations for quantitative traits.

In this study, 8 aflatoxin resistant inbred lines were used to create an 8-way cross. Progenies generated from the 8-way cross were intermated in a cyclic recurrent selection scheme, testing each of the progeny with molecular markers linked to QTL identified in previous QTL mapping and genome-wide association studies (GWAS). Individuals were selected using 15 SNP markers linked to 7 previously mapped QTL by the Corn Host Plant Resistance Research Unit (CHPRRU) in Mp313E and Mp715 bi-parental linkage mapping

(Brooks et al., 2005; Warburton et al., 2009, 2011; Willcox et al., 2013). In addition, 8 SNP markers identified in a GWAS study with the highest association to aflatoxin (Warburton et al., 2013; 2015) were also used to make selections in the RS scheme.

The objectives of this study were to increase the frequency of aflatoxin-reducing alleles in a population generated from an 8-way cross of aflatoxin resistant lines and to test single nucleotide polymorphisms (SNPs) generated from previous QTL and GWAS mapping studies to create 4 different populations to be compared for reduced aflatoxin. An increase in the frequency of favorable alleles increases the overall level of aflatoxin resistance, and genetic variability isstill maintained by intermating selected progenies. In the final population, inbred lines that have successfully combined resistance alleles from multiple parents can be selected. Aflatoxin levels among individuals between each populations will be compared to estimate the cumulative effect of the QTL selected for within each population.

MATERIALS AND METHODS

Plant materials

An 8-way cross population was generated by intermating 8 unrelated aflatoxin resistant inbred lines (CML108, NC334, Tx740, CML348, TZI18, Mp313E, CML311, Mp715; Table 5.1).

Table 5.1 List of the inbred lines used to generate an 8-way cross.

Inbred	Pedigree
CML108	Pob44(STE)C1HC59-3-1-4-4-BB
NC334	SC76^4 x B52
Tx740	Population derived from a mixture of Agricomseeds' (Bolivia) heterotic groups A, C, and E
CML348	G26SEQC3-H83-1-1-2-1-B
TZI18	IITA4001, SeteLagaos (TZSR × 7728) BC (Nigeria)
Mp313E	Tuxpan
CML311	S89500 F2-2-2-2-B*5
Mp715	Tuxpan

A single cross between the pairs of CML108 & NC334, Tx740 & CML 348, TZI18 & Mp313E, and finally CML 311 & Mp715, were used to create F_1 progenies. Double crosses were performed using the F_1 progenies derived from the single crosses to generate a 4-way cross, and the 4-way cross progenies were crossed to generate the 8-way cross (Figure 5.1). Seed from the 8-way cross was used to start the recurrent selection scheme.

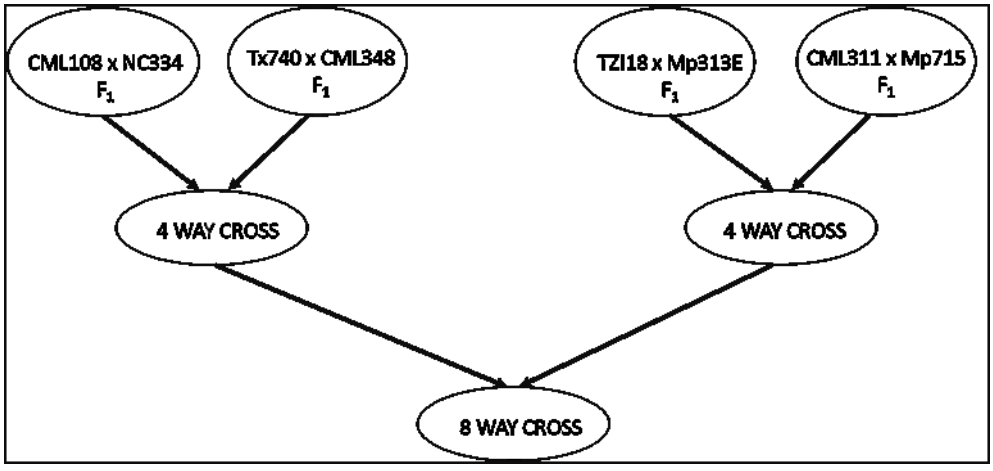

Figure 5.1 A scheme used to generate the 8-way cross.

Genotyping

Leaf tissue samples were collected for the 400 8-way cross individuals in Costar cluster tubes. Tissue samples were frozen in liquid nitrogen and lyophilized using the Freezone Benchtop lyophilizing system, Labconco, Kansas City, MO and ground using the Spex Sample-Prep 2000 Geno Grinder Metuchen, NJ. DNA was extracted from ground tissue samples using a modified CTAB method (Murray and Thompson, 1980; Saghai-Maroof et al., 1984) as explained in the previous chapter. Initial screening of individuals selected for earliness was carried out with a total of 36 markers. Twenty-one of the 36 were markers were linked to 7 QTL identified in previously published QTL mapping studies carried out by the United States Department of Agriculture Corn Host Plant Resistant Research Unit (USDA CHPRRU) (Brooks et al., 2005; Warburton et al., 2009, 2011; Willcox et al., 2013). The other 15 markers were from an association mapping study involving a diverse panel of 300 unrelated lines that included the majority of the aflatoxin accumulation resistant maize breeding lines (Warburton et al., 2013, 2015). Of the 36 markers, only 23 were usefully converted into working assays.

Genotyping of the 400 individuals proceeded using the 23 single nucleotide polymorphism (SNP) markers linked to previously identified QTL. Fifteen of the 23 markers are linked to 7 previously identified QTL from 3 QTL mapping studies conducted by the Corn Host Plant Resistance Research Unit (CHPRRU): Mp313E x Va35 (Brooks et al., 2005); Mp715 x T173 (Warburton et al., 2011); Mp313E x Va35 (Willcox et al., 2013). Each of the 7 QTL is selected using 2 - 3 markers per QTL (preferably 2 flanking markers and one peak marker in the middle of the QTL).

Table 5.2 List of QTL and GWAS markers selected for, their bin and physical locations and the donor parents.

S/N	Marker I.D	Bin Location	Physical location	Donor Parent
1	S1_280635931	1.10	280635931	GWAS
2	PZA02272_3	2.04L	9,963,136	Mp313E
3	S2_168918977	2.06P	168918977	Mp313E
4	S2_195711367	2.06R	195711367	Mp313E
5	S2_153128978	2.06	153128978	GWAS
6	S2_183190964	2.06	183190964	GWAS
7	S2_188872911	2.07	188872911	GWAS
8	S3_2812544	3.03L	2812544	Mp715
9	S3_14229695	3.03R	14229695	Mp715
10	S3_179511489	3.05P	179,511,489	Mp313E
11	PZE03200206675	3.05R	200206675	Mp313E
12	S3_217820604	3.09	217820604	GWAS
13	S4_155804496	4.06L	155804496	Mp313E
14	S4_166924864	4.06P	166924864	Mp313E
15	S4_181338722	4.06R	181338722	Mp313E
16	BNLG_1444_475	4.09L	191767326	Mp313E
17	UMC1574_856	4.09P	235333856	Mp313E
18	PZE0508601096	5.03L	8601096	Mp715
19	S5_19887173	5.03P	19887173	Mp715
20	PZB00765_1	5.07L	198883041	Mp715
21	S8_94752242	8.03	94752242	GWAS
22	S9_107333254	9.04	107333254	GWAS
23	S10_91956540	10.04	91956540	GWAS

An additional 8 SNP markers identified in a genome-wide association study conducted by Warburton et al., (2015) were also used in the selection scheme. Pre-validated SNPs assays were purchased through LGC Genomics (Hurts UK) maize genotyping library. Other markers with no pre-validated SNP assay were custom designed

by sending one hundred base pairs of sequence upstream and downstream of the SNP of interest from the B73 V3 reference genome (Lawrence et al., 2005) to LGC genomics for assay design. Testing of markers started at cycle one (C1) of the recurrent selection scheme.

Assays were designed for each SNP by LGC Genomics (Teddington, UK). Individual assay consists of two competitive allele specific forward primers and one common reverse primer. Each allele specific forward primers contains different fluorescent-labeled component complimentary to two fluorescent resonance energy transfer (FRET) cassettes in the KASP master mix. Allele calls are distinguished from each other due to binding of the FRET cassette to the complementary tail sequence within the allele specific forward primer. Polymerase chain reaction (PCR) was performed using 384 well plates loaded with an epMotion 5073m automated liquid handling system (Eppendorf AG, Hamburg, Germany). Each well consists of 2.5µl template DNA at 10ng μl^{-1}, 2.5µl molecular grade H_20 (Sigma-Aldrich Co., Saint Louis, MO), 2.5µl KASP master-mix and 0.7µl KASP SNP assay (LGC Genomics Limited, Teddington, UK). Plates were sealed using an optical clear seal in a K-Seal heat-based plate sealer (KBioScience, Beverly, MA). PCR was performed using the thermal cycling parameters according to LGC Genomics' KASP thermal cycling conditions manual (Teddington, UK) (Table 5.3).

Table 5.3 Thermal cycling steps for amplification of KASP assays

Step	Temperature	Time (min)	# of cycles
1	95	2	1
2	95	1	34
	61	1	
	72	2	
3	72	5	1

Experimental design and field conditions

Four hundred progenies generated from the 8-way cross (base germplasm) were planted at the R.R Foil Plant Science Research Center, Mississippi State, Mississippi in the summer 2015. Each plot was composed of a single 5.1 m row with 0.96 m spacing. Plots were overplanted and thinned to 20 plants per row at approximately V3-V4 growth stage (Abendroth et al., 2011). Standard management practices was applied to each plot. The earliest maturing plants were selected in the first year using anthesis and silking dates. 200 individuals within the population with the earliest flowering dates based on pollen shed and silk emergence were selected at pollination. Selected individuals were intermated; ears were individually hand-harvested at kernel maturity and dried at 38°C for 7 days to reduce the moisture content to less than 15%. Ears were then machine shelled, and the grain mixed by pouring through a sample splitter twice.

Four hundred early maturing genotypes selected from the base germplasm were planted in summer 2016 at the R.R Foil Plant Science Research Center, Mississippi State,

Mississippi. Leaf tissue were harvested from all 400 individuals and DNA was extracted using the CTAB method explained earlier. All individuals alongside the parents of the 8-way cross were tested with the 23 markers as explained earlier. Four populations (QTL population, GWAS population, QTL + GWAS population and least QTL & GWAS population) were created using the genotypic information generated from the 23 markers. Members of each population were selected based on the number of favorable alleles present in the individual. Individuals were scored according to the zygosity of the inherited alleles from the QTL and the GWAS study. For example, an individual homozygous for the favorable allele in marker S4_181338722 would get a score of 2, an individual heterozygous for the same allele would get a score of 1, while individuals homozygous for the other allele that is not of interest would get a score of 0. The cumulative allele score of each determined their assigned population. Selected individuals within the same population were intermated and individual ears were hand harvested at kernel maturity, dried at 38°C for 7 days, shelled and kernels were saved for the next planting season. The process will be repeated for 5 or more generations with individuals selected generation after generation using molecular marker information generated from the 23 SNP markers.

At cycle six (C_6) progenies generated will be evaluated alongside the resistant inbred lines used to create the 8-way cross for aflatoxin resistance in replicated field trials at the R.R. Foil Plant Science Research Center, Mississippi State, Mississippi. Each plot will consist of a single 5.1 m row with 0.96 m spacing, and 20 plants per plot. Standard managemental practices for test plot will be applied. Silking and tasseling dates will be recorded, and primary ears will be inoculated with 10 days post silking with aflatoxigenic *A. flavus* strain NRRL 3357 (ATCC #200026). Ears will be hand harvested, dried at 38°C

for 7 days, machine shelled and ground. Aflatoxin concentration will be determined using the VICAM AflaTest (VICAM, Watertown, MA) in compliance with the USDA test protocol (USDA, 2002).

RESULTS AND DISCUSSION

The majority of US maize germplasm that are resistant to aflatoxin accumulation originated outside the US Corn Belt and are adapted to tropical regions, thus considered exotic and un-adapted to temperate environments. This causes them to display less desired agronomical characteristics such as late maturity, excessive height, and reduced yield when grown in temperate environments (Brooks et al., 2005; Mayfield et al., 2012). Tasseling and silking dates were recorded for the 8 inbred lines used in this study. The earliest tasseling date was 35 day after planting for (CML108) while the latest tasseling dates recorded was 48 days after planting (CML348). Earliest silking date was 36 days after planting (CML108) while the latest silking date was 55 days after planting (Mp715) Tasseling and silking dates for all inbred lines are presented in Table 5.4.

Table 5.4 Tasseling and Silking dates recorded for the 8 inbred lines used in this study.

Inbred	Days to tasseling	Days to silking
CML108	35	36
NC334	35	37
Tx740	45	47
CML348	48	51
TZI18	38	38
Mp313E	44	46
CML311	38	40
Mp715	52	55

Cycle 1 generated from intermated cycle 0 progenies selected for earliness displayed a shift to earlier maturity, as plants had days to anthesis ranging from 42 – 50 days after planting while the silking dates range from 46 – 53 days (Figure 5.2).

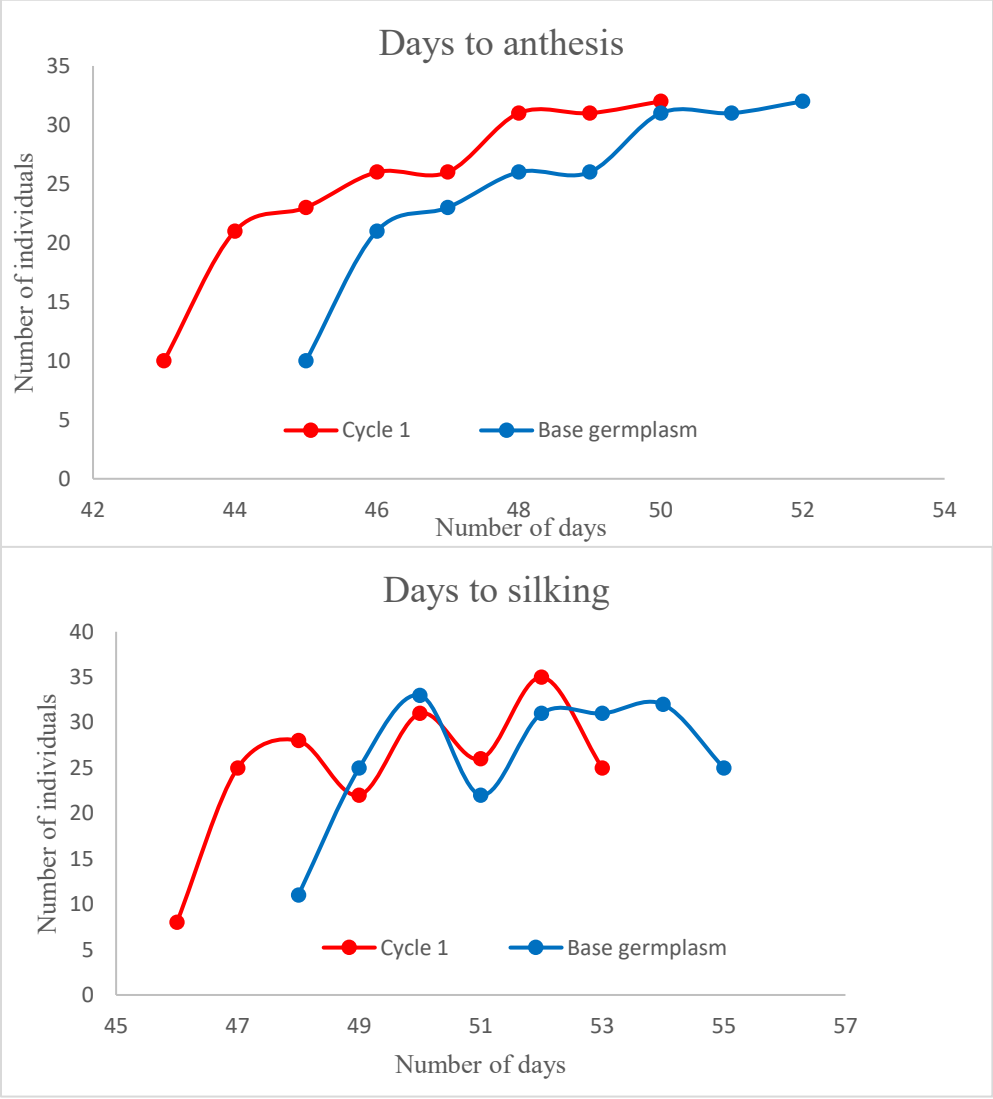

Figure 5.2 Comparison of days to anthesis and days to silking between cycle 0 (base germplasm) and cycle 1.

Following the genotyping of 400 progenies of the 8-way cross, the four populations were created. Population 1 (QTL) consisted of individuals selected for favorable QTL alleles and against the GWAS alleles to avoid overlap of individuals in the population. The cumulative effect of QTL identified via linkage mapping on aflatoxin accumulation will be measured with this population because it consists of individuals selected with more QTL alleles and less GWAS alleles. Population 2 (GWAS) individuals were selected based on the individuals with the greatest number of alleles from the GWAS and the fewest alleles from the QTL. This population will be used to measure the overall effect of GWAS alleles identified via association mapping of 300 maize inbred lines. Population 3 (QTL + GWAS) individuals were selected for positive alleles at both QTL and GWAS alleles to create individuals with more aflatoxin reducing alleles and thus hopefully with increased aflatoxin resistance capabilities. Population 4 (least QTL & GWAS) individuals were selected based on the least number of alleles from both the QTL and GWAS alleles. This population serves as the control population, aimed at showing that accumulation of positive alleles is expected to increase aflatoxin resistance, while accumulating negative alleles decreases resistance when compared to the base C0 population. Table 5.3 shows the breakdown of cumulative scores for each marker and the number of individuals selected for each population.

Table 5.5 Cumulative allele score for each markers used to select individuals within each populations.

Populations	Number of plants selected for each population	QTL markers cumulative score	GWAS markers cumulative score
QTL	34	540	188
GWAS	33	266	282
BOTH	38	572	367
NONE	31	200	129
TOTAL	136	1578	966

FUTURE

Increasing the number of favorable alleles in a population through different RS schemes has being shown to improve the target trait (Hallauer et al., 2010). After every RS cycle, DNA will be extracted from selected individuals within the same population and tested using the same SNP markers they are being selected for. This helps the breeder to keep track of the alleles and make sure they are not lost in the population. Intermating selected individuals every generation increase the frequency of desirable allele combinations in the population ultimately increasing the overall phenotype of aflatoxin resistance among individuals within the population.

Marker-assisted selection and intermating of selected individuals will go for 6 generations to arrive at the cycle 6 progenies. At cycle 6, individuals from all 4 selected populations, and from the C0 population, will be phenotyped by planting them in a replicated field trial and inoculated with aflatoxigenic *A. flavus* strain NRRL 3357 (ATCC #200026). This fungus strain has the ability to produce high levels of aflatoxin in maize grain (Windham & Williams, 2002). VICAM AflaTest (VICAM, Watertown, MA) in compliance with USDA test protocol (USDA, 2002) will be used to determine

aflatoxin concentrations. The VICAM AflaTest method uses immunoaffinity chromatography to isolate aflatoxins (B1, B2, G1, and G2) and fluormetric detection to quantify concentrations as low as 1.0 ng g^{-1} in grain samples.

Phenotyping of individuals within each population starts at the 8^{th} generation of intermating (Cycle 8). Individuals in each population will be planted alongside the parents and 1^{st} generation (cycle 0) progenies to compare their aflatoxin levels. Average aflatoxin concentration will be determined for each individual within the population. Analysis of variance (ANOVA) will be employed to test the mean aflatoxin level between all the individuals within each population and determine how much effect the combination of the alleles has on aflatoxin accumulation. Population 4 (least QTL + GWAS) is expected to have the highest level of aflatoxin, while population 3 (QTL + GWAS) is expected to have the lowest aflatoxin level. Aflatoxin levels for individuals in population 1 (QTL only) and population 2 (GWAS only) is expected to fall between population 3 and population 4.

CHALLENGES

Alleles were miscalled in previous years due to allele coding errors and improper visualization of the alleles. Each of the allele calls traced back to previous generations showed some of the alleles were switched between some of the parents and the F_1 leading to selecting "against" aflatoxin reducing-alleles from donor parent in chosen individuals within the population instead of selecting "for" aflatoxin-reducing alleles in the population. This was observed in all the 4 created selected populations. Defining the alleles and making notes of which alleles is being selected for in which parent is very important. Furthermore, genotyping results were not ready as early as expected in summer 2018 which forced selecting only plants that were still viable and available for pollination. This probably

might have undone the selection for earliness carried out in the first year because only the available individuals with active pollen and silks were selected and intermated.

To combat all these challenges, it is imperative that actual allele calls must be used for selection in subsequent generation or in previous generations to avoid switching of alleles between the parents and selecting wrong individuals. Also, genotyping must be carried out as earliest as possible to increase the chances of selecting all desirable individuals within the population.

CHAPTER VI

CONCLUSION

Aflatoxin is the most potent and most toxic of all mycotoxins. This carcinogenic substance is produced by the fungus *Aspergillus flavus* when the plant comes under any form of stress such as drought stress. Host plant resistance has been proven effective in combating either colonization by the fungus or later production of aflatoxin. The hypothesis of this dissertation tries to answer the question of how to create genetic resources to test previously identified QTL; identify new QTL; and improve aflatoxin accumulation resistance through host plant resistance.

Identification of QTL contributing to aflatoxin resistance in CML69 (an aflatoxin resistant inbred from CIMMTY Mexico) was investigated. A total of 15 QTL were detected in the mapping, and 14 of them co-locate with QTL identified in other mapping populations from Mp313E and Mp715 inbred lines. These 14 QTL are stable in different backgrounds and multiple environments, which was further confirmed by this study. Only one QTL on chromosome 5 was unique and has not being detected in any other mapping population, making CML69 a potential novel source of aflatoxin resistance in maize. Molecular markers within the confidence interval of each identified QTL can be used to introgress the QTL from the resistant donor lines into susceptible background and/or elite cultivars for further verification and determination of the effect of each QTL.

Near isogenic lines (NILs) were created to confirm QTL detected in a previous genome wide association mapping (GWAS) study. Four aflatoxin resistant inbred lines serve as the donor parents. Introgression of single nucleotide polymorphism (SNP) markers linked to 15 QTL with the lowest p-value ($p<10\text{-}4$) from the mapping study was attempted.

In summary, 13 of the 16 QTL were successfully introgressed into different susceptible background, although some of the allele were lost during the backcross progress, and some were lost during the selfing process to fix the desired alleles. This could have been prevented by genotyping the individuals after the first backcross stage (BC_3S_1) to make sure individuals selected still carry the aflatoxin-reducing alleles. Furthermore, a larger population size would have also increased the chances of finding individuals with more combinations of aflatoxin reducing alleles. However, individuals with successfully introgressed alleles will be tested in the future for confirmation and determination of how much influence each QTL provides towards combating aflatoxin accumulation.

Finally, increasing the frequency of aflatoxin-reducing alleles in a population derived from an 8-way cross was performed to test the additive effect of two or aflatoxin-reducing alleles. Four populations were generated from markers associated with 7 QTL (2 flanking markers and 1 peak marker) and 15 GWAS markers. Each population consists of individuals selected with aflatoxin-reducing alleles from the 7 QTL, individuals selected with aflatoxin-reducing alleles from the GWAS studies, individuals selected with aflatoxin-reducing alleles from both the QTL and the GWAS, and individuals selected with the least aflatoxin-reducing alleles from both QTL and GWAS. Intermating selected individual will increase the frequency of desired alleles within the population thereby increasing aflatoxin accumulation resistance. Before this is carried out, individuals should be re-genotyped

In summary, all the projects conducted in this study uses different approach to improve resistance to aflatoxin accumulation. The findings answered some of the questions

posed by the hypothesis and at the same time created new queries that can be exploited further to shed more light on ways to improve aflatoxin accumulation resistance in maize.

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APPENDIX A

COMPOSITE INTERVAL MAPPING (CIM) LOD-PROFILES FOR CML69 x Va35

QTL MAPPING POPULATION

Chromosome 1

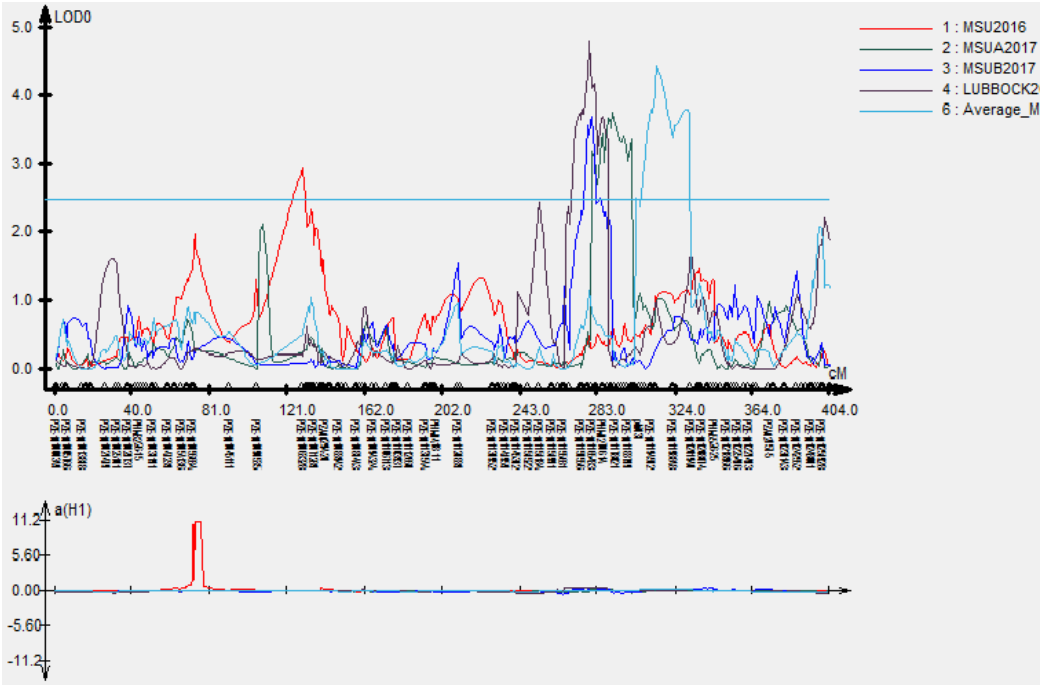
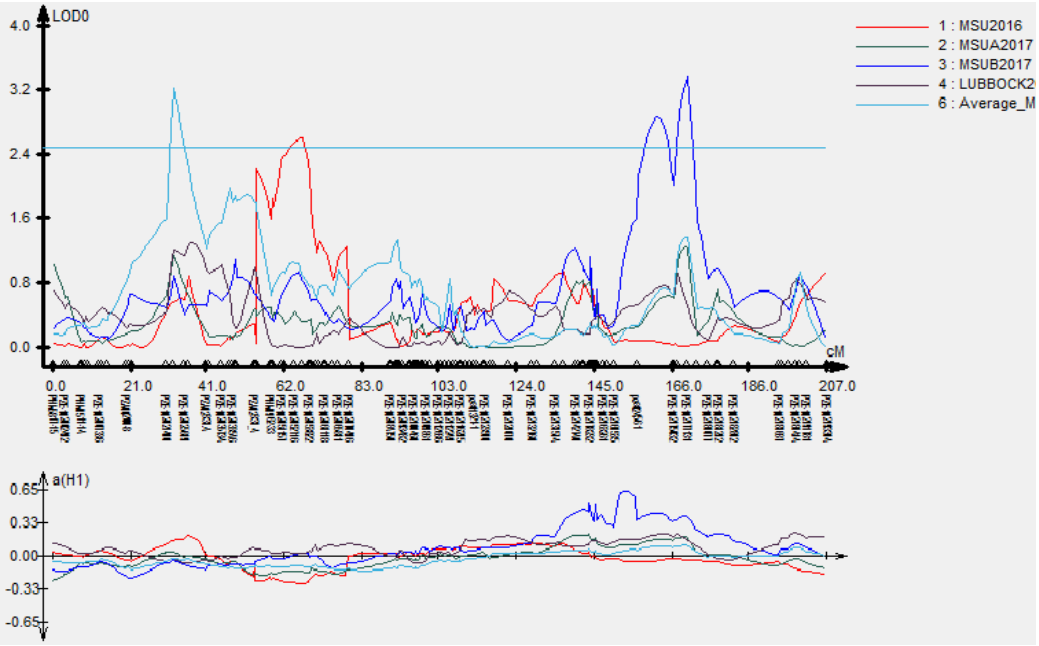


Figure A.1 A graph showing the composite interval mapping results of the full mapping population over 4 years and the average. X-axis represents the genetic length of the chromosomes, with marker positions and labels along the bottom. Y-axis represents the LOD significance. Peaks crossing the LOD threshold of 2.5 (blue horizontal line) were considered significant.

Chromosome 2



Chromosome 3

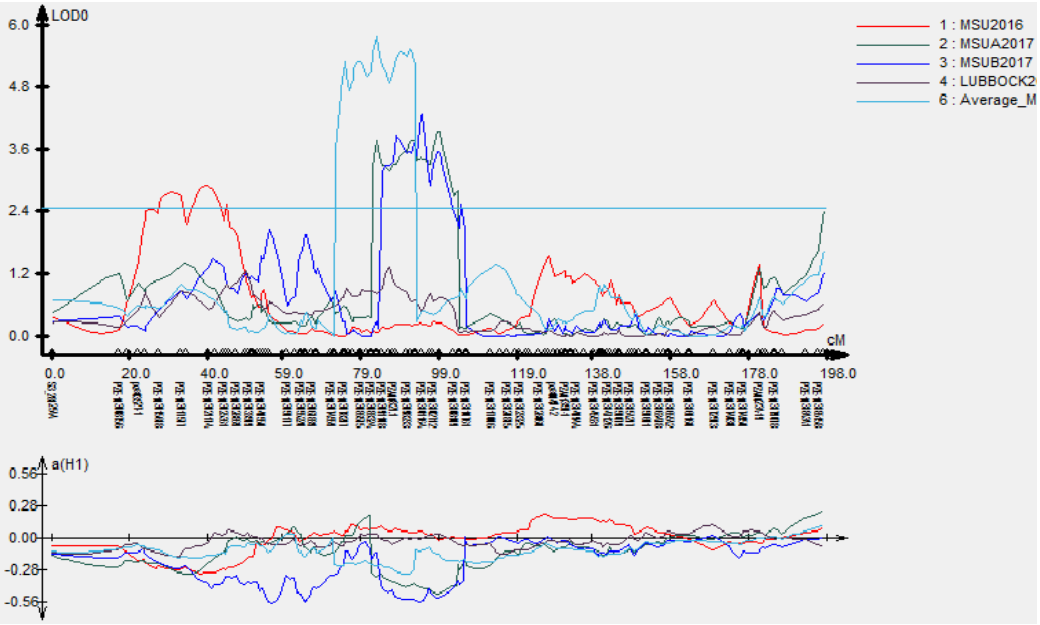
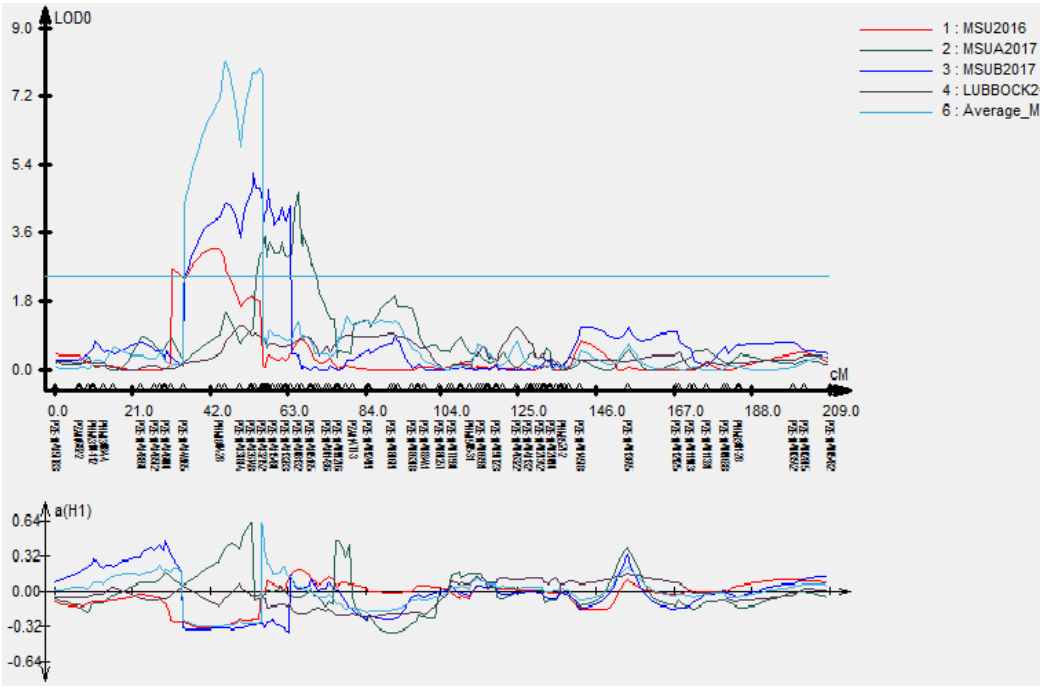


Figure A.1 (continued)

Chromosome 4



Chromosome 5

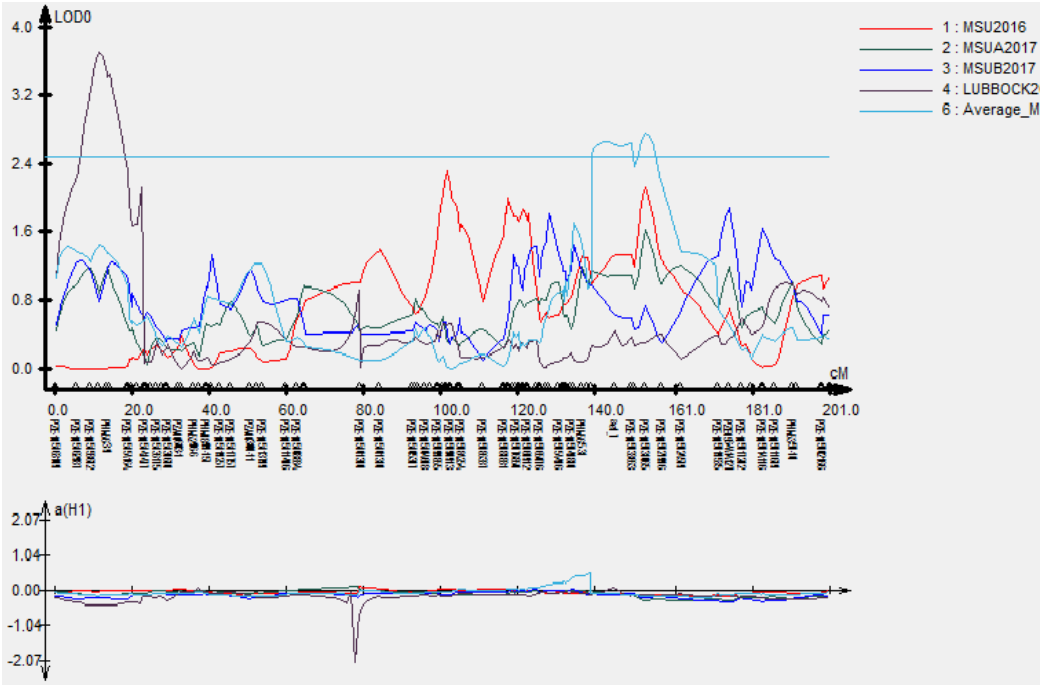
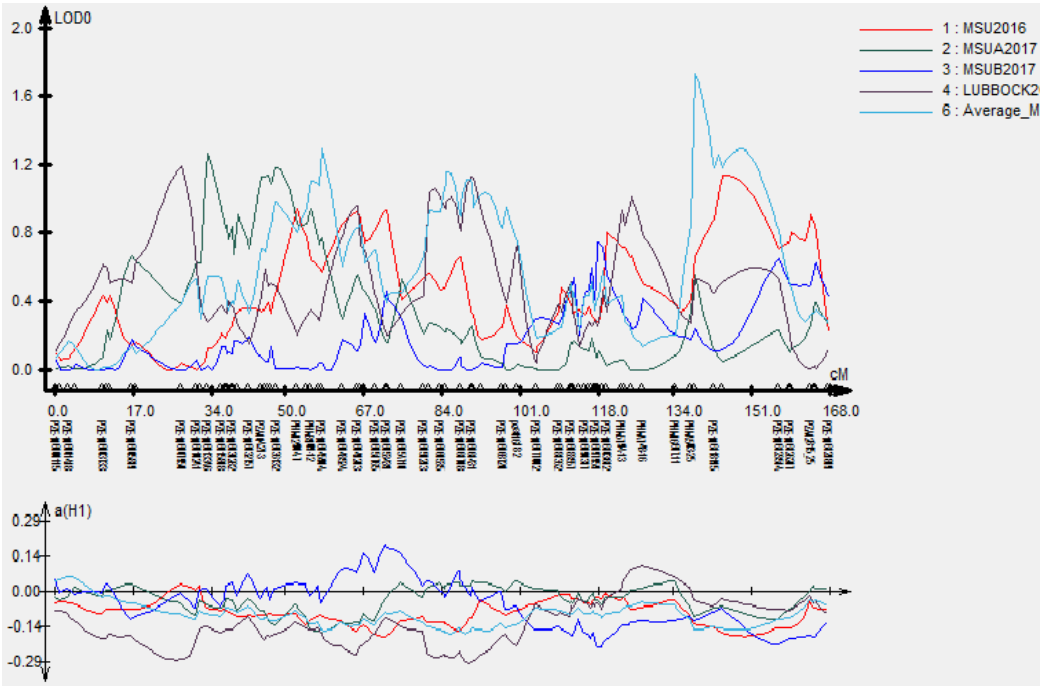


Figure A.1 (continued)

Chromosome 6



Chromosome 7

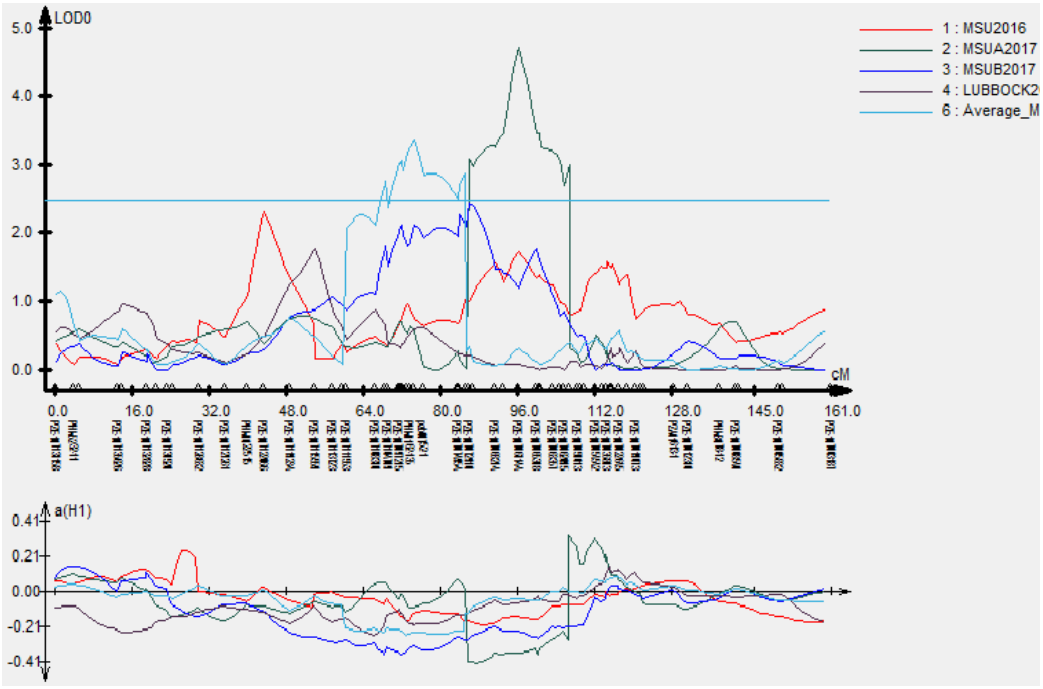
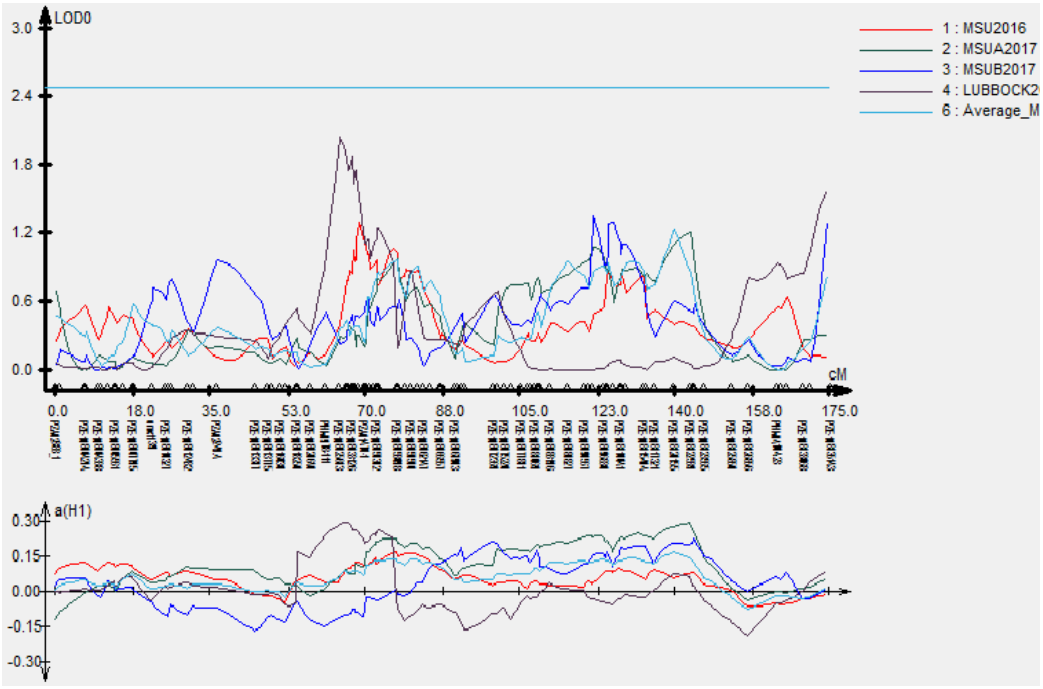


Figure A.1 (continued)

Chromosome 8



Chromosome 9

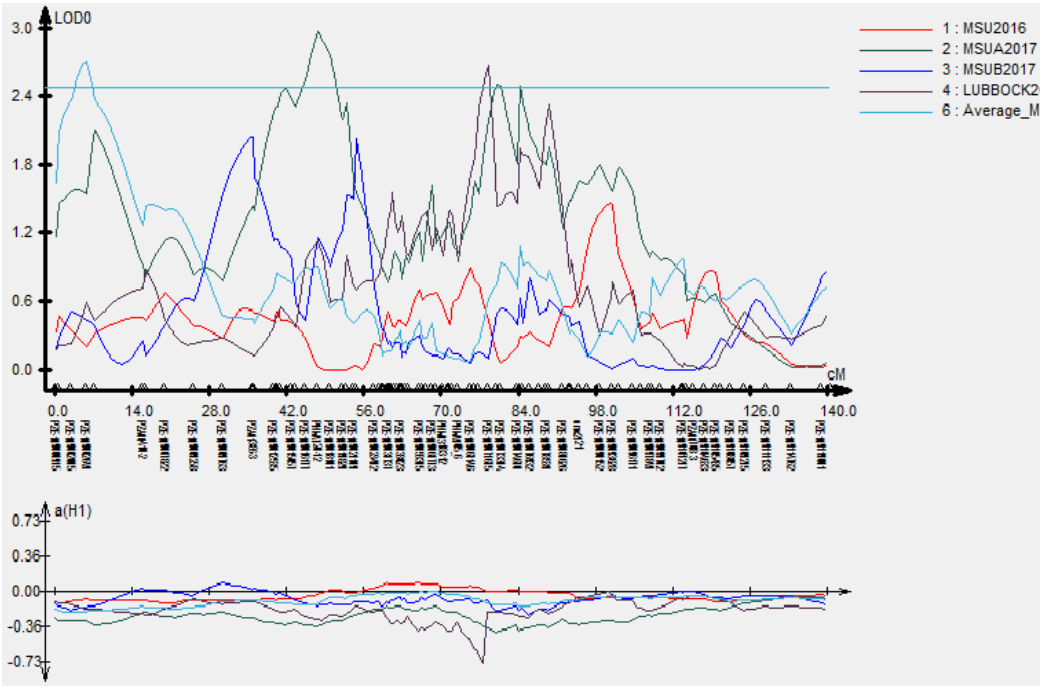


Figure A.1 (continued)

Chromosome 10

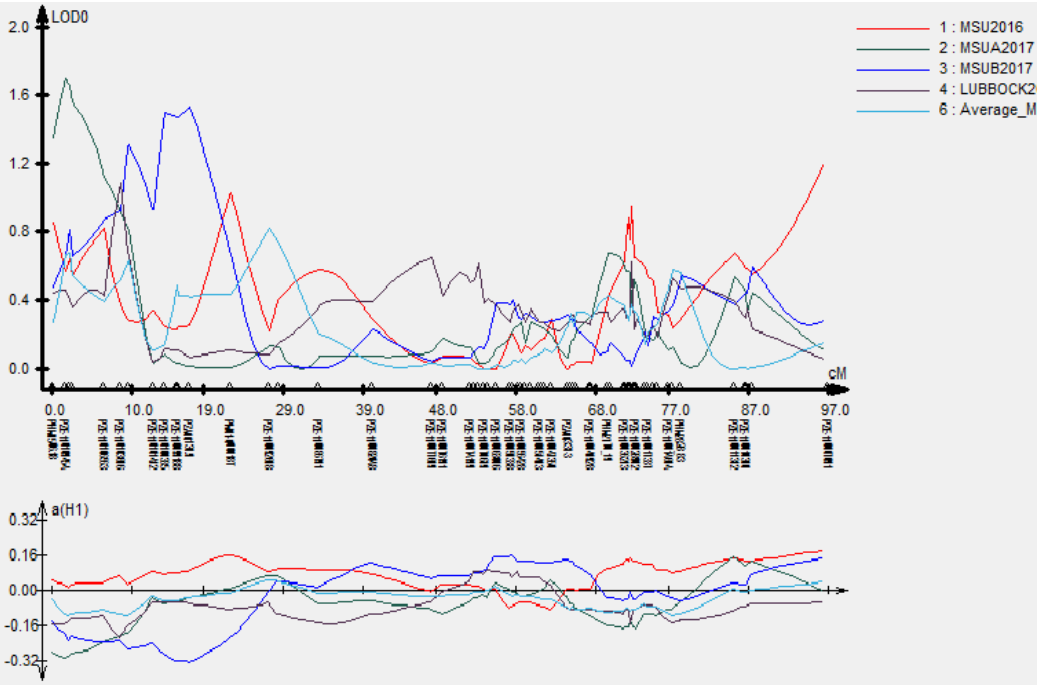


Figure A.1 (continued)

APPENDIX B

CHROMOSOMES AND MARKER POSITIONS (cM) OF 1331 POLYMORPHIC MARKERS USED IN THE STUDY

Table B.1 Chromosomes and marker position (cM) of polymorphic markers used in the CML69 x Va35 QTL mapping population

S/n	Chromosome 1	Position	Chromosome 2	Position	Chromosome 3	Position
1	PZE-101000349	0.0	PHM5817-15	0.0	S3_2812544	0.0
2	PZE-101001044	0.9	PZE-102002492	3.1	PZE-103003116	0.0
3	PZE-101004337	4.1	PZE-102003236	4.0	PZE-103008756	16.9
4	PZE-101005008	5.2	PZE-102003235	4.0	PZE-103010389	19.1
5	PZE-101005766	6.1	PHM1511-14	7.4	PZE-103009843	19.1
6	PZA02869-2	6.3	bnlg108_646	8.1	umc1458_596	19.1
7	PZE-101005765	6.3	PZE-102005684	8.8	pza03527-1	21.9
8	PZE-101013818	14.3	PZE-102006128	9.4	pza00749-1	23.7
9	pza00175-2	16.0	PZE-102007336	12.2	PZE-103013405	23.9
10	PZA00447.6	16.7	PHM5535-8	13.1	PZE-103015618	27.4
11	PZA00731.6	16.9	PZE-102009755	14.4	PZE-103017577	32.7
12	PZE-101016971	18.4	PZE-102009795	14.6	PZE-103017599	32.7
13	PZE-101017463	19.3	PZA00200-8	19.6	PZE-103018049	34.2
14	PZA00566.5	19.3	PZE-102014212	20.6	PZA03527_1	34.2
15	PZE-101021479	26.3	PZA02272_3	21.2	PZE-103021174	39.9
16	PZE-101021926	26.5	PZE-102021400	30.3	PZE-103022202	41.7
17	PZE-101025101	31.7	PZE-102022207	32.1	PZE-103025361	43.8
18	PZE-101027125	33.7	PHM5822-15	32.1	PZE-103024934	44.4
19	PZE-101029737	37.5	PZE-102025619	35.2	PZE-103026250	45.1
20	PZE-101029990	37.8	PZE-102026049	35.2	PZE-103028869	47.1
21	PZE-101031077	38.6	PZE-102028065	36.1	PZE-103032194	49.5
22	PZE-101034091	41.9	PZA02337.4	41.1	PZE-103031323	49.5
23	PHM3226-15	43.6	PZE-102033535	41.8	PZE-103031668	49.5
24	PHM4531-46	44.8	PZE-102035734	45.2	PZE-103033509	50.1
25	PZE-101036318	46.5	PZE-102037264	47.1	PZE-103034530	50.7
26	bnlg1429	47.9	PZE-102037541	47.4	PHM2343.25	51.2
27	PZE-101037191	50.5	PZE-102038546	47.9	PZE-103035524	51.5
28	PZE-101037854	50.7	PZE-102039914	48.9	PZE-103037370	52.4
29	PZE-101039351	51.4	PHM4425-25	49.1	PZE-103040550	53.1
30	PZE-101039440	52.8	PZA02337_4	54.0	PZE-103039677	53.1
31	PZE-101047239	58.7	PZE-102043277	54.2	PZE-103042957	53.4

Table B.1 (continued)

S/n	Chromosome 1	Position	Chromosome 2	Position	Chromosome 3	Position
32	PZE-101048890	59.1	PZE-102043341	54.4	PZE-103044842	53.9
33	PZE-101050072	59.3	PHM6111-5	54.4	PZE-103047477	54.7
34	PHM835-25	59.3	PHM1962-33	58.3	PZE-103048707	55.4
35	PZA00192-6	61.9	PZA02080-1	58.4	PZE-103050177	59.8
36	PHM4597-14	63.0	PZE-102048060	58.8	PZE-103057424	61.8
37	PZE-101054457	63.0	PZE-102047571	59.0	PZE-103055220	63.5
38	PZE-101054136	65.4	PZE-102051157	61.4	PZE-103057464	63.5
39	PZE-101056007	68.8	PZE-102051933	62.0	PZE-103054685	63.5
40	PZE-101055749	69.1	PZE-102052716	64.5	PZE-103061576	63.5
41	PZE-101056684	71.2	PZE-102052725	66.8	PZA02589.1	63.7
42	PHM3726-129	72.1	PZE-102053822	68.5	PZA00627.1	63.7
43	PZE-101059325	72.4	PZE-102055209	68.9	PZE-103065281	64.6
44	PZE-101059816	72.4	PZE-102054468	68.9	PZE-103060872	64.6
45	PZE-101059784	72.4	PZE-102056416	69.4	PZE-103063833	64.7
46	PZE-101060930	72.6	PZE-102058006	70.3	PZE-103063232	64.8
47	PHM11000-21	72.8	bnlg108	70.4	PZE-103065361	64.8
48	PZE-101045111	90.7	PZE-102056984	71.2	PZE-103064200	64.8
49	PZE-101090535	105.0	PZE-102061118	72.7	PZE-103067889	66.1
50	PZE-101063328	129.3	PZE-102061320	73.0	PZE-103069222	67.1
51	PZE-101065758	131.0	PHM10404-8	74.8	PZE-103069689	67.1
52	PZE-101067628	131.0	PZE-102065811	76.4	PZE-103069221	67.1
53	PZE-101069307	132.1	bnlg1175	76.4	PZE-103070266	67.2
54	PZE-101069763	132.4	PZE-102070496	79.1	PZE-103070612	67.8
55	PHM12323-17	133.5	PZE-102084750	90.3	PZE-103074559	71.0
56	PZE-101071162	134.2	PZE-102086050	90.5	PZE-103075872	71.0
57	PZE-101071289	134.4	PZE-102088058	91.5	PZE-103074652	71.0
58	PHM2130-29	134.8	PZE-102087526	92.0	PZE-103076372	71.7
59	PZE-101074049	135.7	PZE-102088558	92.5	PZE-103076996	72.1
60	PZE-101075379	137.2	PZE-102090752	92.9	PZE-103078297	74.3
61	PZE-101077867	138.2	PZE-102091834	92.9	PZE-103078702	74.5
62	PZE-101078026	138.5	PZE-102093482	93.6	PZE-103079483	75.1
63	PZE-101077314	139.0	PZE-102096215	95.3	PZE-103079771	75.5
64	PZE-101077670	139.3	PZE-102096469	95.9	PZE-103083874	76.6
65	PZE-101078713	139.7	PZE-102100460	96.6	PZE-103083575	76.7
66	PZA00378-9	139.7	PZE-102105806	97.0	PZE-103082924	76.8
67	PZA00294-20	140.6	PZE-102101624	97.0	PZE-103086575	78.5
68	PZE-101081568	142.7	PZE-102104318	97.1	PZE-103086917	78.9
69	PZE-101081679	142.7	PM01-000054V	97.2	S3_179511489	78.9
70	PZE-101081882	143.1	PZE-102104129	97.4	PZE-103087007	79.0

Table B.1 (continued)

S/n	Chromosome 1	Position	Chromosome 2	Position	Chromosome 3	Position
71	PZE-101082812	144.0	PZE-102102981	97.8	PZE-103086306	80.1
72	PZE-101083942	147.7	PZE-102104155	97.9	PZE-103088324	81.3
73	PZE-101083968	148.0	PZE-102106394	98.6	PZE-103088477	81.7
74	PHM5481-94	149.9	PZE-102108296	99.2	pza00920-1	81.7
75	PZE-101085771	152.0	PZE-102109182	99.2	PZE-103090601	82.8
76	PZE-101087443	156.6	PZE-102109861	99.7	PZE-103090457	82.8
77	PHM5306.16	157.1	umc1028	99.9	PZE-103090563	83.0
78	PZE-101088395	157.5	PZE-102110466	101.0	PZE-103091108	84.0
79	PZE-101089347	158.8	PZE-102110333	101.0	PZE-103092933	85.9
80	PZE-101090870	160.8	PZE-102112686	102.9	PZA00827.1	86.9
81	PZE-101093639	161.6	umc128_2	104.0	PZE-103097004	87.8
82	PZE-101094324	165.4	PZE-102112805	105.1	PZE-103098233	90.3
83	PZE-101100477	168.0	PHM13648-11	105.1	PZE-103099719	91.5
84	PZE-101106713	172.5	PZE-102112249	105.9	PZE-103100160	92.4
85	PZA02577.1	175.7	PZE-102112161	105.9	PHM9914-11	93.1
86	PZE-101112384	176.2	PZE-102114559	107.1	PZE-103101164	94.1
87	PZE-101112285	176.2	PZA01280.2	108.0	PZE-103102840	96.3
88	PZE-101117251	176.2	PZE-102116295	109.1	PZE-103102712	97.1
89	PZE-101111508	176.4	PZE-102116675	109.3	PZE-103104806	98.7
90	PZE-101108844	177.1	GRMZM2G304548_3	110.5	PZE-103108109	102.4
91	PZE-101105979	177.3	PZE-102117667	110.5	PHM1959-26	103.7
92	PHM3463-18	177.5	PZE-102120321	111.7	PZE-103109587	104.1
93	PZE-101104637	177.9	pza01321-1	112.5	PZE-103110761	105.4
94	PZE-101103230	178.1	PZE-102121608	113.3	pza02402-1	106.0
95	PZA02763.1	178.1	PHM635-23	115.1	PZA00494_2	106.0
96	PZE-101103937	178.4	PZE-102123500	115.5	PZE-103117006	111.8
97	PZE-101112660	184.5	PZE-102126512	117.0	PZE-103119151	114.3
98	PZE-101137544	192.5	PZE-102127730	118.0	PZE-103120875	116.0
99	PZE-101138093	192.5	PZE-102129070	121.8	PHM17210-5	118.3
100	PZE-101135433	193.4	PZE-102132760	127.9	PZE-103123325	119.1
101	PZE-101136154	193.4	PZE-102134204	129.7	PZE-103125004	120.9
102	PZE-101134523	193.6	PZE-102137574	134.4	PZE-103125763	121.5
103	PZE-101133204	194.0	PZE-102140630	136.4	PZE-103128060	124.3
104	PZE-101126587	196.0	PZE-102142740	139.9	PZE-103128464	124.3
105	PZE-101126017	196.0	PZE-102143943	140.6	PZE-103129362	125.8
106	PZE-101123210	196.4	PHM4196-27	141.9	PZA00099-6	126.6
107	PZE-101122861	196.8	PZE-102147076	142.1	pza00494-2	128.1
108	PZE-101122455	197.0	PZE-102178232	143.4	PZE-103133286	129.2
109	PZE-101122614	197.0	PHM499-19	143.6	PZE-103132991	129.2
110	PZE-101122121	197.0	S2_188872782	143.6	PZE-103133859	130.2

Table B.1 (continued)

S/n	Chromosome 1	Position	Chromosome 2	Position	Chromosome 3	Position
111	PZE-101110859	197.7	PZE-102148927	143.9	PZA01359-1	130.7
112	PHM9418_11	199.0	PM01-00005T3	144.2	PZE-103137894	130.9
113	PZE-101120839	210.1	PHM7953.11	144.8	PZE-103139040	131.1
114	PZE-101118063	211.8	PZE-102155387	145.0	PZE-103138646	131.1
115	PZE-101139952	227.8	PZE-102155428	145.0	PZE03200203089	132.4
116	PHM5622_21	229.1	PZA02731-1	145.0	PZE-103139833	132.7
117	PHM3147.18	230.8	PHM14412.4	145.1	PZE-103140944	133.6
118	umc1919	231.9	PZE-102156050	145.5	PZE-103143590	135.9
119	PZA02191.1	233.0	PZE-102159951	145.9	PZE-103145531	138.2
120	PZE-101141959	234.2	PZE-102160834	145.9	PZE-103146956	139.5
121	PZE-101141819	234.2	PZE-102163261	147.2	PZE-103147211	139.9
122	PHM5622-21	236.8	PZE-102165529	148.2	PZA00892-5	140.5
123	PZE-101144216	238.7	PZE-102169535	150.1	mmc0251	140.7
124	PZE-101144895	239.4	pza02456-1	156.4	PHM2919-23	140.7
125	PHM17698-8	239.4	PZE-102175032	165.9	PZE-103147126	141.9
126	PZE-101144838	239.9	pza02418-2	166.4	S3_200247565	142.3
127	PZE-101145302	240.1	PZE-102176300	166.9	PZE-103150197	144.0
128	PZE-101145494	240.3	PZE-102177131	169.7	PZE-103150079	144.4
129	PZE-101145417	240.3	pza02012-7	172.2	PZE-103150095	144.8
130	PZE-101147229	241.0	PZE-102180077	175.2	PZE-103154277	147.2
131	PZE-101151522	246.8	PZA00163-4	177.6	PZE-103154125	147.2
132	PZE-101151184	252.5	PZE-102181292	178.1	PZE-103155594	148.0
133	PZE-101151991	259.0	PZE-102182802	182.0	PZE-103156524	148.9
134	PZE-101152541	259.5	PZE-102187687	194.1	PZE-103157525	149.7
135	PZE-101154881	262.5	PZE-102187906	195.0	PZE-103159691	151.6
136	PZE-101155681	265.0	PZE-102188421	196.7	PZE-103159840	151.8
137	bnlg1057	265.7	PZE-102189414	198.8	PZE-103163478	154.9
138	PZE-101155708	266.4	PZE-102189702	199.7	PZE-103164978	156.0
139	PZE-101156601	268.5	PZE-102191781	201.8	PZE-103165697	157.3
140	PZE-101159556	273.8	PZE-102193394	206.9	PZE-103165542	157.5
141	PZE-101158959	273.8			PZE-103167997	159.7
142	PZE-101159840	273.8			PZE-103169160	162.4
143	PZE-101159230	274.4			PZE-103169263	162.8
144	PZE-101159305	274.9			PZE-103172593	168.8
145	PZE-101161883	275.7			PZE-103174030	172.8
146	PZE-101163518	277.3			PHM3342_31	175.4
147	PZE-101163953	278.2			PZE-103174950	176.1
148	PZE-101165483	280.1			PHM2672-19	176.5
149	PZE-101166754	282.3			PZA00234-19	180.2
150	bnlg1556	282.8			PZE-103177974	181.5

Table B.1 (continued)

S/n	Chromosome 1	Position	Chromosome 2	Position	Chromosome 3	Position
151	PHM12706-14	285.6			PZE-103178597	182.0
152	PZE-101169867	285.6			PZE-103179078	184.1
153	PZE-101169539	285.6			PHM3852-15	184.8
154	PZE-101170476	286.0			PZE-103181174	186.5
155	PZE-101170828	286.2			PZA00143.5	186.5
156	PZE-101173330	288.2			PZE-103184341	192.3
157	PZE-101173927	288.6			PZE-103185158	195.6
158	PHM3690-23	289.8			PZE-103185177	195.6
159	PZE-101175283	290.5			PZE-103186258	196.9
160	PZE-101177112	290.9			PHM13174-18	197.7
161	PZE-101178021	291.8				
162	PHM12693-8	294.2				
163	PZA00403-5	294.2				
164	PZE-101180803	295.5				
165	PZE-101182771	296.8				
166	PZE-101183799	298.6				
167	PZE-101185029	298.8				
168	PZE-101186294	300.4				
169	PZE-101187674	301.0				
170	PZE-101189231	301.9				
171	PZE-101189600	302.8				
172	phi037	304.7				
173	PZE-101190037	305.3				
174	PZE-101190568	306.6				
175	PHM4992-10	307.6				
176	PZE-101194502	310.7				
177	PHM4926-16	310.7				
178	PZE-101195407	311.6				
179	PZE-101196709	313.1				
180	PZE-101196242	313.5				
181	PZE-101198848	322.3				
182	PZE-101199324	322.8				
183	PZE-101201940	331.6				
184	PZE-101205964	335.1				
185	PZE-101205714	335.6				
186	PZE-101207892	336.8				
187	PZE-101208074	337.1				
188	PZE-101210110	340.1				
189	PZE-101210734	340.3				

Table B.1 (continued)

S/n	Chromosome 1	Position	Chromosome 2	Position	Chromosome 3	Position
190	umc1298	341.2				
191	PZE-101210761	342.0				
192	PHM5526-25	343.4				
193	PZE-101213558	343.8				
194	PZE-101215024	345.3				
195	PZE-101215826	347.2				
196	PZE-101218966	350.2				
197	PZE-101218765	350.6				
198	PZE-101221649	351.3				
199	PZE-101220149	351.7				
200	Bin1.08_241404296	353.4				
201	PZE-101222779	354.5				
202	PZE-101222687	354.7				
203	PZE-101225496	356.0				
204	umc1118	359.8				
205	PZA01978.23	359.8				
206	PZE-101227493	361.6				
207	PZE-101229026	364.4				
208	PZE-101230537	365.9				
209	PZA02957-5	372.5				
210	PZE-101236600	374.9				
211	PZE-101236380	375.1				
212	PZE-101239143	380.7				
213	PZE-101239112	380.9				
214	PZE-101242552	386.8				
215	PZE-101245575	389.8				
216	PZE-101246842	391.8				
217	PZE-101248001	393.9				
218	PZE-101249703	395.0				
219	PM01-000019H	395.0				
220	PZA00623-2	395.2				
221	PZE-101249157	395.2				
222	PZE-101250560	396.6				
223	PHM1275-22	398.5				
224	PZE-101251928	399.5				
225	PHM673-33	400.2				
226	pza00856-2	401.4				
227	PZE-101255156	403.8				

Table B.1 (continued)

S/n	Chromosome 1	Position		
228	PHM7616-35	404.2		
229	PHM7616_35	404.5		

Table B.1 (continued)

S/n	Chromosome 4	Position	Chromosome 5	Position	Chromosome 6	Position
1	PZE-104157783	0.0	PZE-105163109	0.0	PZE-106000115	0.0
2	PZA00682-2	6.5	PZE-105162987	5.5	PZE-106000911	1.1
3	bnlg2291_165	6.7	PZE-105158672	9.2	PZE-106000711	1.1
4	PZE-104155312	6.7	PHM4349-3	11.3	PZE-106001418	2.8
5	PZE-104154700	9.0	PHM563-9	13.5	PHM3019-38	2.8
6	PHM4310-112	10.4	S5_217274600	14.0	PZE-106002839	4.4
7	bnlg1444_475	10.6	PZE-105154164	18.5	PZE-106003733	10.4
8	PZE-104153766	10.6	PZE-105154147	19.2	PZE-106004997	11.2
9	PZE-104153828	10.6	PZE-105151649	19.6	PZE-106005121	11.9
10	PHM13084-4	13.0	PZE-105151612	19.9	PZE-106005879	16.4
11	PZE-104152363	15.7	PZE-105146705	21.2	PHM15961-13	17.3
12	PZE-104148860	23.1	PZE-105141477	22.9	PZE-106007950	27.3
13	PZE-104146572	27.0	PZE-105140112	23.5	PZE-106010241	30.7
14	PZE-104146173	28.5	PHM7908_25	23.8	PHM15961_13	30.7
15	PZE-104146082	29.5	PZE-105137112	24.8	PZE-106011532	31.3
16	PZE-104146000	29.9	PHM532.23	24.8	PZE-106013346	32.8
17	PZE-104145773	31.3	PZE-105136175	26.3	PZE-106015808	35.9
18	PZE-104144965	34.7	PHM7908-20	26.3	PZE-106019045	36.7
19	PHM9804-28	44.4	PZE-105132815	27.6	PZE-106024581	36.7
20	PZE-104140188	46.0	PZE-105130799	28.7	PZE-106023350	36.7
21	PZE-104137814	49.7	PZE-105130800	28.7	PZE-106023979	37.1
22	PZE-104136748	53.0	PZE-105130670	29.1	PZE-106022387	37.1
23	PZE-104135927	53.1	PZA00603-1	31.9	PZE-106019350	37.1
24	PZE-104135287	53.1	PZE-105127667	31.9	PZE-106027241	37.6
25	PZA00636-6	53.3	PZE-105126747	32.8	PZE-106030282	38.4
26	PZE-104135978	54.1	PHM5296-6	35.9	PZE-106031629	38.6
27	PZE-104132752	55.8	PZE-105123692	37.1	PZE-106033608	39.5
28	pza00155-1	55.8	PHM1899-157	39.0	PZE-106032757	42.0
29	PZE-104132510	55.8	PZE-105122422	39.5	PZA00427-3	44.6
30	PZE-104131042	56.0	PZE-105120851	40.5	PZE-106034368	45.3
31	PZE-104129724	56.2	PZE-105119257	42.5	PZE-106033954	45.4
32	PZE-104128990	56.2	PZE-105117757	45.5	PZE-106035842	46.0
33	PZA01332.2	56.2	PZA00300-11	50.4	PZE-106035421	46.6
34	PZE-104127500	56.2	PZE-105114143	52.4	PZE-106036132	47.7
35	PZE-104126472	56.4	PZE-105113799	53.6	PHM12904-7	52.4
36	PZE-104125843	56.4	PZE-105111406	59.6	PZE-106041140	52.4
37	PZE-104123813	56.4	PZE-105108884	62.5	PZA00214-1	54.4
38	PZE-104123129	56.4	PZE-105108791	62.5	PHM8909-12	55.4
39	PZE-104122092	56.9	PZE-105107815	64.3	PZE-106044784	57.1

Table B.1 (continued)

S/n	Chromosome 4	Position	Chromosome 5	Position	Chromosome 6	Position
40	PZE-104117455	57.5	PZE-105107675	64.7	PZE-106045904	57.7
41	PZE-104118062	57.5	PZE-105101300	78.8	PZE-106046102	57.7
42	PZE-104116614	57.7	PZE-105101310	84.1	umc1257	57.7
43	PZE-104116480	57.7	PZE-105105397	92.5	PZE-106048524	62.2
44	PZE-104115480	59.0	PZA01796-1	93.3	PZE-106049203	65.3
45	PZE-104114790	60.2	PZE-105106024	93.8	Bin6.03_ss229232045	66.0
46	PZE-104114385	60.9	PZE-105104698	95.5	PZE-106049590	66.0
47	PZE-104113283	62.0	PZE-105103501	95.5	PZE-106050102	67.1
48	PZE-104112702	62.4	PZE-105104504	95.5	PZE-106051765	69.3
49	PZE-104109566	63.5	PZE-105102442	97.0	PZA00942_2	69.3
50	PZE-104108132	65.4	PZE-105099855	98.8	PZE-106053439	71.8
51	PZE-104108744	65.4	PZE-105100373	98.8	PZE-106054182	72.0
52	PZE-104106157	66.5	PZE-105098722	99.4	PZE-106054710	74.8
53	PZE-104106461	66.7	PZE-105098310	100.3	PZE-106059293	79.8
54	PZE-104105165	68.9	PZE-105096406	101.1	PZE-106059969	81.1
55	PZA02194_1	69.1	PZE-105095752	101.5	PZE-106060535	83.3
56	PZE-104104502	70.2	PZE-105090913	102.0	PZE-106057733	84.4
57	PZE-104103747	71.1	PZE-105090726	102.6	PZE-106057620	84.6
58	PZE-104101436	73.2	PZE-105085641	104.3	PZE-106061708	87.7
59	PZE-104101251	73.2	PZE-105088164	104.3	PZE-106061713	88.0
60	PZE-104101517	74.1	PZE-105083270	104.3	PZE-106061431	90.1
61	PZE-104099876	76.0	Zm.67134_148	104.7	PZA00182.4	90.5
62	PZE-104099276	76.4	PZE-105084254	105.0	PZE-106062467	90.5
63	PZE04171144372	76.4	PZE-105086387	110.8	PZE-106064587	92.3
64	PZE-104099380	76.5	PZE-105087881	116.1	PZE-106068720	96.7
65	PZE-104099220	76.6	PZE-105084195	116.3	PZE-106068519	96.7
66	PZE-104097807	78.6	PZE-105087020	116.3	PZA02673_1	97.5
67	PZA01477-3	80.2	PZE-105082207	116.3	PZA00473.5	97.5
68	PZE-104092499	84.5	PZA00881-1	116.3	pza01618-2	100.0
69	PZE04179457445	84.9	PZA00547.6	116.5	PZE-106077002	104.1
70	PZA01289-1	84.9	PZE-105078645	117.0	PZE-106077080	104.4
71	PZE-104089679	90.7	PZE-105077553	117.6	PZE-106081362	109.0
72	PHM9635-30	90.7	PZE-105075120	118.7	PZE-106082237	109.6
73	PZE-104089746	91.4	PZE-105075848	118.7	PZE-106083557	111.5
74	PZE-104088221	92.4	PZE-105076850	119.3	PZE-106085267	112.1
75	PZE-104086308	96.4	PZE-105074128	119.9	PZA02148.1	112.4
76	PZE-104084579	97.9	PZA02818-10	120.4	PZE-106087348	113.4

Table B.1 (continued)

S/n	Chromosome 4	Position	Chromosome 5	Position	Chromosome 6	Position
77	PZE-104084292	98.1	PZE-105073285	120.4	PZE-106090377	114.6
78	PZE-104083441	99.6	PZE-105074008	121.0	PZE-106092279	115.6
79	PZE-104080257	103.6	PZA02862.10	121.4	PZE-106093277	116.0
80	PZE-104080388	103.6	umc1050	121.4	PZE-106096901	116.6
81	PZE-104080267	103.6	PZE-105071303	121.4	PZE-106095393	116.6
82	PZE-104078223	106.3	PZE-105072323	121.5	PZE-106097959	117.1
83	PZE-104077580	107.2	PZE-105069912	122.1	PZE-106098493	117.3
84	PZE-104075457	109.3	PHM5798.39	122.3	PHM15251-5	117.5
85	PZE-104075114	109.7	PZE-105068703	122.3	PHM15251.3	117.5
86	PHM1505-31	111.7	PZE-105065972	124.4	PZE-106101046	118.6
87	PZA00663-5	113.9	PZE-105066316	124.4	PZE-106103612	119.4
88	PZE-104068824	114.1	PZE-105065076	125.2	PZE-106103665	119.4
89	PZE-104069513	114.1	PHM4165-14	125.5	PHM5794-13	122.7
90	PZE-104066589	115.0	PHM3171-5	127.0	PZE-106106978	123.4
91	PZE-104063220	115.6	PZE-105061723	127.2	PZE-106108125	124.8
92	PZE-104062764	116.3	PHM1870.20	127.2	PHM4748-16	127.2
93	PZE-104057462	116.7	PZE-105060871	127.7	PZA00910_1	127.2
94	PZE-104057788	116.8	PZE-105054496	130.2	PHM16607.11	134.2
95	PZE-104058720	116.9	PZE-105053856	130.2	PHM4503-25	137.5
96	PZE-104055631	117.1	PZE-105051986	131.5	PZE-106116156	138.4
97	PZE-104053903	117.1	PZE-105051200	131.7	PZE-106118195	142.6
98	PZE-104055857	117.1	PHM6795-4	131.8	PZE-106118266	142.6
99	PZE-104050723	118.8	PZE-105050668	131.9	PZE-106119113	144.5
100	PZE-104051033	119.1	PZE-105050469	132.1	PZE-106123514	156.7
101	PZE-104049978	119.4	PZE-105049975	132.6	PZE-106126207	159.1
102	PZE-104050087	119.4	PZE-105049870	133.4	PZE-106126667	159.3
103	PZE-104048161	120.7	PZE-105049305	134.3	PZA02815_25	163.7
104	PZE-104047889	120.7	PZE-105048851	134.3	PZE-106128325	163.7
105	PZE-104046322	124.4	PHM565-31	136.3	PHM3466.69	164.5
106	PZE-104041132	127.5	PZE-105037563	137.9	umc1248	164.8
107	PZE-104043510	128.1	PZA02792-16	139.0	PHM5529.4	164.8
108	PZE-104042045	128.4	PZE-105035757	139.0	PZE-106129899	167.3
109	PZE-104041043	128.4	ae1_7	144.8	PZE-106129968	167.9
110	PZE0436718269	129.4	PZE-105033869	144.8		
111	PZE-104034459	129.4	PZE-105033663	149.2		
112	PZE-104032228	130.1	PZE-105033111	149.8		
113	PZE-104032101	130.1	PZE-105031065	152.7		
114	PZE-104027342	130.7	PZE-105027996	156.8		

Table B.1 (continued)

S/n	Chromosome 4	Position	Chromosome 5	Position	Chromosome 6	Position
115	PHM4469-13	130.7	PZE-105027983	156.8		
116	PZE-104028514	130.7	PZE-105024579	161.8		
117	PHM5572-19	130.7	PZE-105019535	171.5		
118	PZA03043-14	130.7	PZE0564414127	174.4		
119	PHM13623-14	130.7	PZE-105018422	174.5		
125	PZE-104022932	131.9	PZE-105011679	186.1		
126	PZA03048_18	132.4	PHM5359-10	190.8		
127	PZE-104021862	133.3	PZE-105006245	191.9		
128	PZE-104021090	133.9	PZB00094_1	191.9		
129	pza00139-4	135.2	PZE-105002166	198.2		
130	PZE-104020214	135.2	PZE-105002105	198.7		
131	PZE-104018939	135.2	PHM662.27	200.4		
132	PZE-104018179	136.5	PZA02462_1	200.7		
133	PHM8527_2	136.5				
134	PZE-104018347	136.5				
135	PHM8527-2	136.7				
136	PZE-104016330	137.6				
137	PZE-104014994	138.6				
138	PZE-104014578	141.7				
139	PZE-104013645	154.4				
140	PZE-104012925	167.4				
141	PZE-104012693	168.3				
142	PZE-104011903	171.1				
143	PZE-104011650	172.4				
144	PZE-104011339	175.5				
145	PZE-104009638	180.5				
146	PZA00436-7	181.2				
147	PHM3301-28	184.2				
148	PZE-104008253	184.2				
149	PZA00436_7	184.4				
150	PZE-104003542	199.0				
151	PZE-104002805	202.2				
152	PZE-104005482	208.9				

B1 (continued)

S/n	Chromosome 7	Position	Chromosome 8	Position	Chromosome 9	Position
1	PZE-107137158	0.0	PZA02388_1	Position	Locus	Position
2	PHM5232-11	3.9	PZE-108001699	0.0	PZE-109000915	0.0
3	PZE-107137797	5.0	PZE-108002184	0.0	PZE-109001327	0.7
4	PZE-107135626	12.9	PZE-108004274	1.1	PZE-109002005	2.9
5	PZE-107134626	13.9	PZE-108004263	6.8	PZE-109002649	5.6
6	PZE-107132828	18.9	PZE-108004388	7.0	PZE-109003256	7.1
7	PZE-107132535	19.1	PZE-108004841	9.7	PZA00410-2	15.8
8	PZE-107131073	21.0	PZE-108004843	10.5	PZE-109006355	15.8
9	PZE-107130520	23.1	PZA01623.3	10.5	PZE-109006730	16.4
10	PZE-107129811	24.3	PHM4512-38	12.1	PZE-109006938	16.4
11	PZE-107128632	29.8	PZE-108005561	12.1	umc1040	16.4
12	PZE-107127261	35.1	PZE-108005788	13.5	PZE-109007822	19.9
13	PHM10225-15	39.7	PZE-108006634	14.0	PZE-109009258	25.1
14	PZE-107122066	43.2	PZE-108007795	15.2	PZE-109009763	30.4
15	PZE-107119234	48.6	PZE-108007877	17.5	PZA01386-3	35.7
16	PZE-107115559	53.9	umc1139	17.8	PZA00466-1	35.9
17	PZE-107113723	57.4	PZE-108009251	21.8	PZE-109012535	39.5
18	PZA03176_4	57.4	PZE-108009325	21.8	PZA03058_22	40.1
19	PZE-107112738	59.6	PZE-108010327	22.0	PZE-109013421	40.2
20	PZE-107112903	59.6	PZE-108010463	25.0	PZA02344.1	40.8
21	PZE-107111553	60.5	PZE-108011044	25.6	PZE-109015057	42.6
22	PZE-107108370	66.2	PZE-108012482	26.4	PZE-109015674	43.6
23	PZE-107105855	68.4	PZE-108012113	30.0	PZE-109016177	45.3
24	PZE-107104709	68.9	PHM9695-8	31.3	PHM4720-12	47.5
25	PZE-107097215	71.2	PZA02249.4	31.3	PZE-109018101	49.8
26	PZE-107095095	71.4	PZE-108013377	36.5	PZE-109019829	52.0
27	PZE-107094078	71.8	PZE-108013775	45.4	PZE-109020361	52.8
28	PZA02722_1	71.9	PZA02955.3	48.1	PZE-109021109	54.0
29	PZE-107091745	71.9	PZE-108016030	49.1	PZE-109021584	54.5
30	PZA02722-1	72.1	PZE-108015703	50.7	PZE-109023492	57.8
31	PZE-107093420	72.1	PZE-108016244	50.8	PZE-109023854	57.8
32	PZE-107090250	72.9	PZE-108016243	50.8	PZE-109025557	58.7
33	PHM9162-135	73.4	PZE-108016906	50.8	PZE-109026940	59.3
34	PZE-107086989	74.4	PZE-108018250	52.1	PHM229.15	59.5
35	pzb00752-1	76.4	PZE-108019866	54.6	PZE-109031737	60.1
36	PZE-107074954	83.4	PZE-108020640	54.8	PZE-109030589	60.5
37	PZE-107074506	83.8	PHM1978-111	57.7	PZE-109030178	60.6
38	PZE-107073339	85.1	PZE-108025613	61.2	PZE-109035156	61.0
39	PZE-107072910	85.8	PZE-108024691	64.2	PZE-109047415	61.5

Table B.1 (continued)

S/n	Chromosome 7	Position	Chromosome 8	Position	Chromosome 9	Position
40	PZA01946.7	85.8	PZE-108027707	64.2	PZE-109039430	62.0
41	PZE-107072122	86.5	PZE-108030174	65.9	PZE-109038023	62.3
42	PZE-107068214	91.2	PZE-108031753	66.1	PZE-109047635	62.7
43	PZE-107067237	93.0	PZE-108033126	66.4	PZE-109046891	62.7
44	PZE-107067144	95.9	PZE-108035435	67.0	pza01791-2	62.7
45	PZE-107065308	99.6	PZE-108036755	67.0	PZE-109047581	62.7
46	PZE-107066119	100.3	PZE-108037876	67.0	PZE-109048180	63.0
47	PZE-107065748	100.5	PZE-108038334	67.4	PZE-109055139	63.3
48	PZE-107063357	103.0	PZE-108038256	67.6	PZE-109054419	63.3
49	PZE-107062980	104.6	PZE-108040317	67.6	PZE-109056967	63.9
50	PZE-107062095	105.5	PZE-108041516	67.8	PZE-109058305	65.8
51	PZE-107061306	106.7	PZE-108042509	68.0	PZE-109060192	66.5
52	PZE-107059013	108.3	PZE-108042588	68.0	PZE-109061001	67.2
53	PZE-107058547	109.0	PZA01257.1	68.0	PZE-109061773	68.1
54	PZE-107057869	109.7	PZE-108043142	68.0	PZE-109061895	68.9
55	PZE-107054542	112.1	PZA01470-1	68.5	PZE-109062229	68.9
56	PZE-107047007	113.3	PZE-108044512	69.8	PHM13183-12	70.3
57	PZE-107046954	113.3	PZE-108044552	69.8	PZE-109064469	71.2
58	PZE-107043973	113.3	PHM5395-34	69.8	PZE-109064251	71.4
59	PZE-107036893	114.1	PZE-108044930	69.8	PZE-109064616	71.9
60	PZA00084.2	114.1	PZE-108046270	70.2	PHM4905_6	72.9
61	PZE-107035269	114.1	PZE-108046705	70.6	PZE-109065712	72.9
62	PHM12830.14	114.1	PZE-108047454	70.6	PZE-109067146	75.2
63	PZE-107032574	114.2	PHM1534-45	71.1	PZE-109067632	76.0
64	PZA03645_1	114.4	PZE-108049414	72.1	PZE-109069697	76.7
65	PZE-107040006	114.4	PZE-108050392	72.1	PZE-109071675	78.4
66	PZE-107033916	114.4	PZE-108050483	72.5	PZE-109072924	79.8
67	PZE-107040240	114.4	PZE-108052389	72.5	PZE-109073394	80.5
68	PZE-107032930	114.4	PZE-108053677	72.7	PZE-109074670	83.6
69	PZE-107030337	115.2	PZE-108053922	72.9	PZE-109075167	84.2
70	PZE-107029322	115.2	PZE-108058098	73.4	PZE-109075943	84.7
71	PZA00132-17	115.5	PZE-108058655	77.2	PZE-109076932	86.0
72	PZE-107022645	116.7	PZE-108059741	77.7	PZE-109077680	87.6
73	PZA00153-3	116.9	PZE-108059500	79.2	PZE-109078539	88.7
74	PZE-107024092	116.9	PZE-108061313	80.3	PZE-109077983	89.3
75	PZE-107020997	118.4	PZE-108062147	81.9	PZE-109080626	91.8
76	PZE-107021672	118.4	PZE-108062040	83.3	PZE-109080822	91.8
77	PZE-107019073	120.2	PZE-108063102	83.3	PZE-109082099	93.1

Table B.1 (continued)

S/n	Chromosome 7	Position	Chromosome 8	Position	Chromosome 9	Position
78	PZE-107018459	121.4	PZE-108066557	84.8	PZE-109083108	93.3
79	PZE-107017393	121.9	PZE-108066339	87.0	umc2121	94.8
80	PZA01613-1	128.4	PZE-108066942	87.0	PZE-109085093	94.8
81	PZE-107012310	131.0	PZE-108067903	87.4	PZE-109087846	96.3
82	PHM3078-12	137.6	PHM3993-16	90.4	PZE-109090152	98.6
83	PZE-107008540	141.1	PHM5805-19	90.9	PZE-109093628	100.8
84	PHM3078_12	141.1	PZE-108069897	92.1	PHM1766-1	101.9
85	PZE-107008696	141.5	PZE-108072369	92.6	PZE-109096171	104.6
86	PZE-107005832	150.0	PZE-108073574	99.1	PZE-109097262	106.0
87	PZE-107005454	150.7	PZE-108075220	100.0	PZE-109097849	107.6
88	PZE-107003187	160.6	PHM5468-25	101.4	PZE-109098623	108.1
89			PZE-108077475	101.4	PZE-109099702	109.5
90			PZE-108077370	103.2	PZE-109101217	113.4
91			PZE-108077871	103.2	PZE-109101698	113.7
92			PZE-108077878	105.3	PZE-109102157	114.3
93			PHM5468_25	105.3	PZA00708_3	115.6
94			PZA00049.12	106.8	PHM816-25	115.6
95			PHM4203-11	106.8	PZE-109104633	117.5
96			PZA03005.19	107.4	PZE-109105485	119.5
97			PZE-108081069	107.4	PHM1766_1	119.5
98			PZE-108081298	108.5	PZE-109106291	120.4
99			PZE-108084409	108.9	PZE-109108057	122.3
100			PZE-108083595	109.5	PZE-109109275	124.8
101			PZE-108084307	109.8	PZE-109111133	128.6
102			PZE-108088196	109.8	PZE-109114762	133.3
103			bnlg666	111.7	PZE-109119001	138.8
104			PZE-108089827	111.7	PZE-109119987	140.3
105			PZE-108090567	115.8		
106			PZE-108091439	119.6		
107			PZE-108092820	120.4		
108			PZE-108096830	121.6		
109			PHM15278-6	124.0		
110			PZE-108100984	124.5		
111			PZA00177-4	124.8		
112			PZE-108104357	124.8		
113			PZE-108107190	125.1		
114			PZE-108110041	126.2		
115			PHM232-30	127.6		
116			PZE-108110593	127.7		
117			Zm.12507	127.8		

Table B.1 (continued)

S/n	Chromosome 7	Position	Chromosome 8	Position	Chromosome 9	Position
118			PZE-108110131	128.0		
119			pza02746-2	128.0		
121			PZE-108116182	133.0		
122			PZE-108117321	133.7		
123			PZE-108120155	135.4		
124			PZE-108122589	139.7		
125			PZE-108123038	143.5		
126			PZA01964_29	144.3		
127			PZE-108123565	144.5		
128			PZE-108125850	146.7		
129			PZE-108126856	153.1		
130			PHM14104-23	156.6		
131			PZE-108130245	163.1		
132			PHM3312-23	163.1		
133			PZE-108131283	164.0		
134			PZE-108133068	165.5		
135			PZE-108133641	169.2		
136			PZE-108133621	170.5		
137			PZE-108135143	170.5		
138			PZE-108135132	175.1		

Table B.1 (continued)

S/n	Chromosome 10	Position
1	PHM1506.18	0.0
2	PHM1506-23	0.0
3	PZA02527_2	0.0
4	PZE-110109454	1.7
5	PHM3736-11	2.1
6	PZE-110108692	2.6
7	PZE-110106563	6.5
8	PZE-110103696	8.5
9	PZE-110103912	8.5
10	PZE-110103108	9.5
11	PZE-110101412	12.6
12	PZE-110100385	14.1
13	PZE-110099188	15.6
14	PZA03603_1	15.8
15	PZE-110099110	15.8
16	PZA00130.9	17.1
17	PM01-000018T	22.2
18	PZE-110092698	27.1
19	PZE-110092504	28.2
20	PZE-110086791	33.3
21	PZE-110082048	39.9
22	PZE-110077699	47.3
23	PZE-110076191	48.8
24	PZE-110074199	52.2
25	PZE-110073500	52.6
26	PZE-110072258	53.3
27	PZE-110070679	53.9
28	PZE-110068690	54.3
29	PZE-110067273	54.3
30	PHM1576-25	54.3
31	PZE-110063006	55.5
32	PZE-110059338	57.1
33	PZE-110058966	57.5
34	PZE-110057767	58.2
35	PZE-110056428	58.5
36	PZE-110056035	59.0
37	PZE-110056000	59.7
38	PZA02993.14	59.7
39	PZE-110051403	60.7

Table B.1 (continued)

40	PZE-110050295	61.0
41	PZE-110049371	61.3
42	PZE-110049023	61.3
43	PZE-110047350	62.2
44	PZA00337-3	64.3
45	PZE-110044488	64.8
46	PZE-110043675	65.0
47	PZE-110043433	65.4
48	PZE-110040928	67.1
49	PZE-110040705	67.3
50	PHM2770_19	69.3
51	PZE-110040068	69.4
52	PZE-110040021	69.7
53	PZE-110036213	71.4
54	PZE-110038474	71.4
55	PZE-110033538	71.6
56	PZE-110026566	71.6
57	PZE-110032930	71.8
58	PZE-110026060	72.0
59	PZE-110032621	72.3
60	PZE-110028687	72.7
61	PZE-110028902	72.7
62	PZE-110029216	72.7
63	PZE-110020091	72.9
64	PZE-110022148	72.9
65	PZE-110018330	74.0
66	PZE-110017901	74.0
67	PZA00310-5	74.0
68	PZE-110017337	74.4
69	PZE-110016288	75.0
70	PZE-110015512	75.4
71	PZE-110016135	75.4
72	PZE-110014694	76.9
73	PZE-110014551	77.4
74	PHM2828_83	78.4
75	PZE-110013211	78.4
76	PZE-110011312	85.2
77	PZE-110010390	86.5
78	PZA01313_2	86.7
79	PZE-110009725	87.3

APPENDIX C

ALLELE CALLS FOR ALL SNPS TESTED ON THE NEAR ISOGENIC LINES.

Family	IND	S1_272 220818	S1_280 635931	S10_91 956540	S2_153 128978	S2_183 190964	S2_188 872911	S2_205 035174	S3_217 359490	S3_217 808798	S3_217 820604	S4_264 06913	S8_947 52242	S9_107 333254	S9_117 048731	BC3S2	S1_272 220818	S1_280 635931	S2_153 128978	S3_217 820604	S9_117 048731
B73 x CML5_2_ 7001	1	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	?	?	B73	C:C	C:C	A:A	T:T	?
	2	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?	CML5	A:A	T:T	?	C:C	C:C
	3	?	C:C	C:C	?	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?	B73 x CML5	C:A	C:C	A:G	?	T:C
	4	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?	2017	C:C	T:T	A:G	T:T	C:C
	5	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?	2016	C:C		A:G	T:T	?
	6	?	T:C	C:C	A:A	C:C	C:C	A:A	A:A	C:C	T:T	?	T:T	A:A	?						
	7	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?			Alleles switched			
	8	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?	B73		T:T			
	9	?	T:T	C:C	G:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?	CML5		C:C			
	10	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	?	?	B73 x CML5		?			
	11	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?			C:C			
	12	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?						
	13	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?						
	14	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?						
	15	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?						
	16	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?						
	17	?	C:C	C:C	A:G	?	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?						
	18	?	T:C	C:C	A:A	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?						
	19	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:T	A:A	?						
	20	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	?	T:T	C:C	T:T	A:A	?						
	B73	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	C:C	C:C	?	T:T	A:A	?						
	CML5	?	T:T	?	G:G	C:C	C:C	A:A	T:A	A:A	T:T	G:G	T:T	A:A	?						
	B73 x CML5	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:C	C:C	T:T	A:A	?						

Figure C.1 Figure showing allele calls for SNPs tested in the NILs. Figure also includes allele comparison in 2016 and 2017.

Red column represents failed SNP assays, green column represents good assays, white column represents assays good assays but alleles doesn't match previous years. Red rows represents the susceptible parents alleles, blue rows represents resistant parent alleles while green rows represents the F_1 alleles.

Family	IND	S1_2722 20818	S1_280 635931	S10_919 56540	S2_153 128978	S2_1831 90964	S2_188 872911	S2_205 035174	S3_217 359490	S3_2178 20604	S4_2640 6913	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S1_27222 0818	S1_28063 5931	S2_15312 8978	S3_21782 0604	S9_11704 8731
B73 x CML5_5_7001	1	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?	B73	C:C	C:C	A:A	T:T	?
	2	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?	CML5	A:A	T:T	?	C:C	C:C
	3	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?	B73 x CML5	C:A	C:C	A:G	?	T:C
	4	?	T:T	C:C	G:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?	2017	A:A	T:T	A:A	T:T	C:C
	5	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?	2016	?	C:C	A:G	T:T	?
	6	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	7	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?						
	8	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	9	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	10	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	11	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	12	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	13	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	14	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	15	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	16	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	18	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	19	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	20	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	B73	?	C:C	C:C	A:A	C:C	C:C	A:A		C:C		T:T	A:A	?						
	CML5	?	T:T	?	?	C:C	C:C	A:A	T:A	T:T	T:G	T:T	A:A	?						
	B73 x CML5	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	T:C	T:T	T:T	A:A	?						

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_280 635931	S10_919 56540	S2_153 128978	S2_1831 90964	S2_188 872911	S2_205 035174	S3_217 359490	S3_2178 20604	S4_2640 6913	S8_947 52242	S9_107 333254	S9_117 048731	BC3S2	S1_27222 0818	S1_28063 5931	S2_1531 28978	S3_217820 604	S9_117048 731
B73*CML5_7_7003	1	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	B73	C:C	C:C	A:A	T:T	?
	2	?	T:T	C:C	G:G	C:C	C:C	A:A	T:A	T:T	C:C	T:T	A:A	?	CML5	A:A	T:T	?	C:C	C:C
	3	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	C:C	T:T	?	?	B73*CML5	C:A	C:C	A:G	?	T:C
	4	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	2017	A:A	T:T	A:A	T:T	C:C
	5	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	2016	?	C:C	A:G	T:T	T:T
	6	?	C:C	C:C	A:A	?	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	7	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	8	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	9	?	?	C:C	A:G	?	?	A:A	T:A	T:T	?	T:T	G:A	?						
	10	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	11	?	?	C:C	?	?	?	?	T:A	T:T	?	?	?	?						
	12	?	?	C:C	G:G	?	?	?	T:A	T:T	?	T:T	G:A	?						
	13	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	14	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	15	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?						
	16	?	T:T	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	17	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	18	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?						
	19	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?						
	20	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?						
	B73	?	C:C	C:C	A:G	C:C	C:C	A:A		C:C		T:T	A:A	?						
	CML5	?	T:T	?	?	C:C	C:C	A:A	A:A	T:T	T:G	T:T	A:A	?						
	B73 x CML	?	T:C	C:C	A:G	C:C	C:C	A:A	C:C	T:C	T:T	T:T	A:A	?						

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_280 635931	S10_919 56540	S2_153 128978	S2_1831 90964	S2_188 872911	S2_205 035174	S3_217 359490	S3_217 820604	S4_264 06913	S8_947 52242	S9_1073 33254	S9_117 048731	BC3S2	S1_272 220818	S1_280 635931	S2_1531 28978	S3_21782 0604	S9_117 048731	S6_12131 1207
B73*CML348_2_7004	1	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?	B73	C:C	T:T	A:A	T:T	C:C	C:C
	2	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	CML348	A:A	T:T	?	C:C	?	C:C
	3	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	B73 x CML348	C:A	T:T	A:G	?	T:C	C:C
	4	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	?	T:T	?	?	2017	A:A	T:T	A:A	T:T	T:C	C:C
	5	?	T:T	C:C	G:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?	2016	?	C:C	A:G	T:T	T:T	T:C
	6	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	7	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?							
	8	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?							
	9	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	10	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	11	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	12	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?							
	13	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	14	?	C:C	?	A:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?							
	15	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	16	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	18	?	T:T	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	19	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	20	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	B73	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	C:C	C:C	T:T	A:A	?							
	CML348	?	T:T	C:C	?	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	B73 x CML348	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	T:C	C:C	T:T	A:A	?							

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_280 635931	S10_919 56540	S2_153 128978	S2_1831 90964	S2_188 872911	S2_205 035174	S3_217 359490	S3_217 820604	S4_264 06913	S8_947 52242	S9_1073 33254	S9_117 048731	BC3S2	S1_272 220818	S1_280 635931	S2_1531 28978	S3_21782 0604	S9_117 048731	S6_12131 1207
B73*CML348_7_7005	1	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	B73	C:C	T:T	A:A	T:T	C:C	C:C
	2	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	CML348	A:A	T:T	?	C:C	?	C:C
	3	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	B73 x CML348	C:A	T:T	A:G	?	T:C	C:C
	4	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	5	?	T:T	C:C	A:A	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	6	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	2017	A:A	T:T	A:A	T:T	T:C	C:C
	7	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?	2016	?	C:C	A:G	T:T	T:T	T:C
	8	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	9	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	10	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	11	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	12	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	13	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	14	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	15	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	16	?	T:C	C:C	G:G	C:C	C:C	A:A	A:A	T:T	?	T:T	A:A	?							
	17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	18	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	19	?	?	T:C	A:G	?	?	A:A	T:A	T:T	?	C:C	?	?							
	20	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	B73	?	C:C	C:C	A:A	C:C	C:C	A:A	A:A	C:C	C:C	T:T	A:A	?							
	CML348	?	T:T	C:C	?	C:C	C:C	A:A	A:A	T:T	C:C	T:T	A:A	?							
	B73 x CML348	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	T:C	C:C	T:T	A:A	?							

Figure C.1 (continued)

Family	IND	S1_27222 0818	S1_280635 931	S10_91956 540	S2_153128 978	S2_183190 964	S2_188872 911	S2_205035 174	S3_217359 490	S3_217808 798	S3_217820 604	S4_26406 913	S5_206795 116	S8_947522 42	S9_107333 254	S9_117048 731	BC3S2	S1_2722208 18	S2_153128 978	S2_1831909 64	S2_188872 911	S3_217820 604	S6_121311 207
B73*MP313E_5_7008	1	?	?	T:C	?	?	T:C	?	T:A	?	?	?	?	C:C	?	?	B73	C:A	G:G	C:C	C:C	T:T	C:C
	2	?	C:C	C:C	G:G	T:T	T:T	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?	MP313E	C:A	G:G	C:C	T:T	C:C	C:C
	3	?	C:C	C:C	G:G	T:T	T:C	A:A	A:A	C:C	T:T	C:C	T:G	T:T	A:A	?	B73 x MP313E	C:A	G:G	C:C	T:C	?	C:C
	4	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	C:C	?	T:G	T:T	A:A	?	2017	C:A	G:G	C:C	T:C	T:C	C:C
	5	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	T:C	?	T:G	C:C	A:A	?	2016	?	A:G	C:C	T:C	T:C	?
	6	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	7	?	C:C	C:C	G:G	T:T	T:C	A:A	A:A	C:C	C:C	?	T:G	T:C	A:A	?							
	8	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	T:C	C:C	T:G	T:T	A:A	?							
	9	?	C:C	C:C	G:G	T:T	T:C	A:A	A:A	C:C	T:T	C:C	T:G	C:C	A:A	?							
	10	?	C:C	C:C	G:G	T:T	T:C	A:A	A:A	C:C	T:T	C:C	T:G	T:T	A:A	?							
	11	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	12	?	C:C	C:C	G:G	T:T	T:T	A:A	A:A	C:C	C:C	?	T:G	T:C	A:A	?							
	13	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	T:T	C:C	T:G	C:C	A:A	?							
	14	?	C:C	C:C	G:G	T:T	T:C	A:A	A:A	C:C	T:C	C:C	T:G	T:T	A:A	?							
	15	?	C:C	C:C	G:G	T:T	T:T	A:A	A:A	C:C	C:C	C:C	T:G	C:C	A:A	?							
	16	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	C:C	?	T:G	T:T	A:A	?							
	17	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	18	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	T:C	C:C	T:G	T:T	A:A	?							
	19	?	?	C:C	G:G	T:T	C:C	A:A	A:A	C:C	C:C	C:C	T:G	?	A:A	?							
	20	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:C	A:A	?							
	B73	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	T:C							
	MP313E	?	C:C	C:C	G:G	C:C	T:T	A:A	A:A	C:C	T:T	C:C	T:G	C:C	A:A	T:C							
	B73 x MP313E	?	C:C	C:C	G:G	?	T:C	A:A	?	C:C	T:C	G:G	T:G	T:C	A:A	T:C							

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_9195 6540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S1_27222 0818	S2_15312 8978	S2_18319 0964	S2_18887 2911	S3_21782 0604	S6_12131 1207
B73*MP313E_14_7009	7009_1	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:G	T:T	A:A	?	B73	C:A	G:G	C:C	C:C	T:T	C:C
	7009_2	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:T	C:C	T:G	T:T	A:A	?	MP313E	C:A	G:G	C:C	T:T	C:C	C:C
	7009_3	?	C:C	C:C	A:G	C:C	T:T	A:A	A:A	C:C	C:C	C:C	T:G	C:C	A:A	?	B73 x MP313E	C:A	G:G	C:C	T:C	?	C:C
	7009_4	?	C:C	C:C	A:G	C:C	T:T	A:A	A:A	C:C	T:T	C:C	T:G	T:T	A:A	?	2017	C:A	G:G	C:C	T:C	T:C	C:C
	7009_5	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:C	C:C	T:G	T:T	A:A	?	2016	?	A:G	C:C	C:C	T:C	?
	7009_6	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:C	A:A	?							
	7009_7	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	7009_8	?	C:C	C:C	A:G	C:C	T:C	A:A	A:A	C:C	T:C	C:C	T:G	C:C	A:A	?							
	7009_9	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	7009_10	?	C:C	C:C	A:G	C:C	T:C	A:A	A:A	C:C	C:C	C:C	T:G	T:C	A:A	?							
	7009_11	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	7009_12	?	C:C	C:C	A:G	C:C	T:C	A:A	A:A	C:C	T:T	C:C	T:G	C:C	A:A	?							
	7009_13	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	7009_14	?	C:C	C:C	A:G	C:C	T:C	A:A	A:A	C:C	T:C	?	T:G	T:C	A:A	?							
	7009_15	?	C:C	C:C	A:G	C:C	?	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	7009_16	?	C:C	C:C	A:G	C:C	T:T	A:A	A:A	C:C	T:T	C:C	T:G	T:T	A:A	?							
	7009_17	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	7009_18	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	7009_19	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:C	C:C	T:G	T:C	A:A	?							
	7009_20	?	C:C	C:C	A:G	C:C	T:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	?							
	B73	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	T:C							
	MP313E	?	C:C	C:C	A:G	C:C	T:T	A:A	A:A	C:C	T:T	C:C	T:G	C:C	A:A	T:C							
	B73 x MP313E	?	C:C	C:C	A:G	C:C	T:C	A:A	?	C:C	T:C	G:G	T:G	T:C	A:A	T:C							
Family	IND	S1_2722 20818	S1_28063 5931	S10_9195 6540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S1_27222 0818	S2_15312 8978	S2_18319 0964	S2_18887 2911	S3_21782 0604	S6_12131 1207

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_9195 6540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S1_27222 0818	S2_15312 8978	S2_18319 0964	S2_18887 2911	S3_21782 0604	S6_12131 1207
B73*MP715_1_7010	7010_1	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	?	T:G	C:C	A:A	?	B73	C:A	G:G	C:C	C:C	T:T	C:C
	7010_2	?	C:C	C:C	A:G	C:C	T:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	G:A	?	MP715	C:A	A:A	T:T	T:T	C:C	T:T
	7010_3	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	?	B73 x MP715	C:A	A:G	T:C	T:C	?	C:C
	7010_4	?	C:C	C:C	A:G	C:C	T:C	A:A	A:A	C:C	C:C	?	T:G	T:T	A:A	?	2017	C:A	G:G	C:C	C:C	T:C	C:C
	7010_5	?	C:C	C:C	A:G	C:C	?	A:A	A:A	A:C	C:C	C:C	T:G	C:C	A:A	?	2016	?	A:G	C:C	C:C	T:C	?
	7010_6	?	T:T	C:C	?	?	T:C	A:A	T:A	C:C	?	?	T:G	C:C	G:G	?							
	7010_7	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	G:A	?							
	7010_8	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	T:C	C:C	T:G	T:T	A:A	?							
	7010_9	?	C:C	C:C	A:G	C:C	T:C	A:A	A:A	C:C	C:C	C:C	T:G	T:C	A:A	?							
	7010_10	?	C:C	C:C	A:G	C:C	T:T	A:A	A:A	C:C	T:C	C:C	T:G	T:T	G:A	?							
	7010_11	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	?							
	7010_12	?	T:T	C:C	A:G	C:C	T:C	A:A	A:A	C:C	T:T	C:C	T:G	T:C	A:A	?							
	7010_13	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	G:G	?							
	7010_14	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:G	T:T	A:A	?							
	7010_15	?	T:C	C:C	?	T:C	T:T	?	T:A	C:C	?	?	T:G	T:T	?	?							
	7010_16	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	?	T:G	T:C	A:A	?							
	7010_17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	G:G	?							
	7010_18	?	T:T	C:C	A:G	C:C	T:T	A:A	A:A	C:C	T:C	?	T:G	T:T	?	?							
	7010_19	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	T:T	C:C	T:G	T:C	G:A	?							
	7010_20	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	T:C	C:C	T:G	C:C	A:A	?							
	B73	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:G	T:T	A:A	T:C							
	MP715	?	T:T	C:C	A:G	T:C	T:T	A:A	C:C	A:A	T:T	C:C	T:G	C:C	G:G	C:C							
	B73 x MP715	?	T:C	?	A:G	?	T:C	A:A	A:A	A:C	C:C	C:C	T:G	T:C	G:A	C:C							

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S2_183190 964	S2_18887 2911	S4_26406 913	S9_11704 8731	S2_18887 2782
Va35*CML69_1_7012	1	?	T:T	C:C	G:G	T:C	T:T	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	T:T	VA35	T:T	T:T	C:C	C:C	T:T
	2	?	T:C	C:C	A:A	C:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	G:G	T:T	CML69	C:C	C:C	G:G	T:T	C:C
	3	?	C:C	C:C	A:G	T:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	C:C	A:A	C:C	VA35*CML69	T:C	T:C	?	T:C	T:C
	4	?	T:C	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	C:C	2017	T:C	T:C	G:C	T:C	T:C
	5	?	C:C	C:C	G:G	T:T	T:C	A:A	A:A	A:A	C:C	G:C	T:G	C:C	G:G	C:C	2016	C:C	T:C	G:C	T:C	T:C
	6	?	T:C	C:C	A:A	C:C	T:C	A:A	A:A	A:A	C:C	G:C	T:G	T:T	G:A	T:T						
	7	?	C:C	C:C	A:G	T:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	8	?	T:C	C:C	A:G	T:C	T:C	?	A:A	A:A	C:C	G:C	T:G	T:T	G:G	C:C						
	9	?	?	C:C	A:A	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	10	?	T:C	C:C	G:G	C:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	C:C	G:A	C:C						
	11	?	C:C	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	G:C	T:G	T:T	G:G	C:C						
	12	?	T:T	C:C	A:G	T:T	T:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	13	?	C:C	C:C	A:G	T:C	T:T	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	T:T						
	14	?	T:C	C:C	G:G	C:C	T:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	G:G	C:C						
	15	?	?	C:C	A:G	C:C	T:C	A:A	A:A	A:A	C:C	G:C	T:G	T:T	A:A	C:C						
	16	?	T:T	C:C	G:G	?	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	17	?	C:C	C:C	A:G	T:T	C:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	G:A	C:C						
	18	?	T:C	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:G	C:C	A:A	C:C						
	19	?	C:C	C:C	A:G	T:T	C:C	A:A	A:A	A:A	C:C	?	T:G	T:T	A:A	C:C						
	20	?	T:T	C:C	A:G	T:T	T:C	A:A	A:A	A:A	C:C	C:C	T:G	C:C	A:A	C:C						
	VA35	?	T:T	C:C	A:A	C:C	T:T	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	T:T						
	CML69	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	G:G	C:C						
	VA35*CML69	?	T:C	C:C	A:G	T:C	T:C	A:A	A:A	A:A	C:C	?	T:G	T:T	G:A	T:C						

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S2_183190 964	S2_18887 2911	S4_26406 913	S9_11704 8731	S2_18887 2782
Va35*CML69_4 7013	1	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	G:C	T:G	T:T	A:A	?	VA35	T:T	T:T	C:C	C:C	T:T
	2	?	C:C	C:C	G:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C	CML69	C:C	C:C	G:G	T:T	C:C
	3	?	T:C	C:C	A:G	T:C	T:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	C:C	VA35*CML69	T:C	T:C	?	T:C	T:C
	4	?	T:T	?	A:G	T:T	T:T	A:A	A:A	A:A	?	G:C	T:G	T:T	A:A	T:T	2017	T:T	T:T	G:C	T:C	T:T
	5	?	C:C	C:C	G:G	C:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	G:G	T:C	2016	T:C	T:C	G:C	T:C	T:C
	6	?	T:T	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	7	?	T:C	C:C	A:G	T:C	T:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	T:C						
	8	?	C:C	C:C	A:G	C:C	T:C	?	A:A	A:A	C:C	G:C	T:G	T:T	G:A	C:C						
	9	?	T:C	C:C	G:G	T:C	T:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	T:C						
	10	?	C:C	C:C	A:G	?	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	11	?	T:C	C:C	A:A	T:C	T:C	A:A	A:A	?	C:C	C:C	T:G	T:T	A:A	T:T						
	12	?	T:T	C:C	A:G	T:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	G:G	T:T						
	13	?	C:C	C:C	A:G	T:T	T:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	C:C						
	14	?	T:T	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	G:A	C:C						
	15	?	T:C	C:C	A:A	T:C	T:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	16	?	T:C	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	G:C	T:G	T:T	G:G	T:T						
	17	?	T:T	C:C	G:G	C:C	T:C	?	A:A	A:A	C:C	C:C	T:G	T:T	G:A	T:C						
	18	?	T:C	C:C	A:A	T:T	T:T	A:A	A:A	A:A	C:C	G:G	T:G	T:T	G:A	C:C						
	19	?	T:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	G:A	T:C						
	20	?	T:C	C:C	A:G	C:C	T:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	T:T						
	VA35	?	T:T	C:C	A:A	C:C	T:T	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	T:T						
	CML69	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	G:G	C:C						
	VA35 x CML69	?	T:C	C:C	A:G	T:C	T:C	A:A	A:A	A:A	C:C	C:G	T:G	T:T	G:A	T:C						

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S2_183190 964	S2_18887 2911	S4_26406 913	S9_11704 8731	S2_18887 2782
Va35*CML69_7 7014	1	?	?	C:C	A:G	T:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	C:C	A:A	C:C	VA35	T:T	T:T	C:C	C:C	T:T
	2	?	?	C:C	G:G	T:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C	CML69	C:C	C:C	G:G	T:T	C:C
	3	?	T:C	C:C	A:G	T:T	T:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	T:T	VA35*CML69	T:C	T:C	?	T:C	T:C
	4	?	?	C:C	A:A	C:C	T:T	A:A	T:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C	2017	T:C	T:C	G:C	T:C	T:C
	5	?	?	C:C	G:G	T:T	T:T	A:A	A:A	A:A	C:C	G:C	T:G	T:T	A:A	C:C	2016	T:C	T:C	G:C	T:C	T:C
	6	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	T:T						
	7	?	?	C:C	A:G	C:C	T:T	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	T:C						
	8	?	?	C:C	G:G	T:T	T:T	A:A	A:A	A:A	C:C	G:C	T:G	C:C	A:A	C:C						
	9	?	?	C:C	A:G	T:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	T:C						
	10	?	T:C	C:C	A:A	T:C	T:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	T:T						
	11	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:G	?	A:A	C:C						
	12	?	C:C	C:C	A:G	T:C	T:C	A:A	A:A	A:C	C:C	G:G	T:G	T:T	A:A	T:C						
	13	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	G:C	T:G	T:T	A:A	?						
	14	?	T:C	C:C	A:G	C:C	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	15	?	T:T	C:C	A:G	T:T	T:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	16	?	?	C:C	G:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:G	C:C	A:A	T:C						
	17	?	?	C:C	A:G	T:C	T:T	A:A	A:A	A:A	C:C	G:C	T:G	C:C	A:A	T:T						
	18	?	T:C	C:C	A:G	T:T	T:T	A:A	A:A	?	C:C	C:C	T:G	T:T	A:A	C:C						
	19	?	?	C:C	A:G	?	T:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	A:A	T:T						
	20	?	?	C:C	A:A	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C						
	VA35	?	T:T	C:C	A:A	C:C	T:T	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	T:T						
	CML69	?	C:C	C:C	G:G	T:T	C:C	A:A	A:A	A:A	C:C	G:G	T:G	T:T	G:G	C:C						
	D22	VA35 x CML69	?	T:C	C:C	A:G	T:C	T:C	A:A	A:A	A:A	C:C	C:G	T:G	T:T	G:A						

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S3_217820 604	S9_117048 731	S6_12131 1207
VA35*MP715_3_7012	1	?	?	C:C	A:G	?	T:T	A:A	?	A:A	T:C	?	T:T	T:T	A:A	?	VA35	C:A	C:C	C:C
	2	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	G:A	?	MP715	C:A	T:T	T:T
	3	?	?	C:C	A:G	T:T	T:T	A:A	?	A:C	T:T	G:C	T:T	T:T	G:A	?	VA35*MP715	C:A	T:C	T:C
	4	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	A:A	?	2017	C:A	T:C	C:C
	5	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:A	?	2016	C:A	T:C	C:C
	6	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	G:C	T:T	T:T	G:G	?				
	7	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	A:A	?				
	8	?	?	C:C	A:G	T:T	T:T	A:A	?	A:C	T:C	C:C	T:T	T:T	A:A	?				
	9	?	?	C:C	A:G	?	T:T	A:A	?	A:A	T:C	C:C	T:T	T:T	G:G	?				
	10	?	?	C:C	A:G	T:T	T:T	A:A	?	A:A	T:T	G:C	T:T	T:T	G:A	?				
	11	?	?	C:C	A:G	?	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	A:A	?				
	12	?	?	C:C	A:G	?	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	?	?				
	13	?	?	C:C	A:G	?	T:T	A:A	A:A	A:C	C:C	G:C	T:T	T:T	A:A	?				
	14	?	?	C:C	A:G	?	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	A:A	?				
	15	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	G:A	?				
	16	?	?	C:C	A:G	?	T:T	A:A	?	A:C	T:C	C:C	T:T	T:T	G:G	?				
	17	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	A:A	?				
	18	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	G:C	T:T	T:T	A:A	?				
	19	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	G:G	?				
	20	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	T:C	C:C	T:T	T:T	G:A	?				
	VA35	?	T:T	?	A:G	?	T:T	A:A	T:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C				
	MP715	?	T:T	C:C	A:G	T:C	T:T	A:A	T:A	C:C	T:T	G:G	T:G	T:T	G:G	C:C				
	VA35*MP715	?	T:T	?	A:G	?	T:T	A:A	A:A	A:C	T:C	C:G	T:T	T:T	G:A	C:C				

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S3_217820 604	S9_117048 731	S6_12131 1207
VA35*MP715_3_7012	1	?	?	C:C	A:G	?	T:T	A:A	?	A:A	T:C	?	T:T	T:T	A:A	?	VA35	C:A	C:C	C:C
	2	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	G:A	?	MP715	C:A	T:T	T:T
	3	?	?	C:C	A:G	T:T	T:T	A:A	?	A:C	T:T	G:C	T:T	T:T	G:A	?	VA35*MP715	C:A	T:C	T:C
	4	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	A:A	?	2017	C:A	T:C	C:C
	5	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:A	?	2016	C:A	T:C	C:C
	6	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	G:C	T:T	T:T	G:G	?				
	7	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	A:A	?				
	8	?	?	C:C	A:G	T:T	T:T	A:A	?	A:C	T:C	C:C	T:T	T:T	A:A	?				
	9	?	?	C:C	A:G	?	T:T	A:A	?	A:A	T:C	C:C	T:T	T:T	G:G	?				
	10	?	?	C:C	A:G	T:T	T:T	A:A	?	A:A	T:T	G:C	T:T	T:T	G:A	?				
	11	?	?	C:C	A:G	?	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	A:A	?				
	12	?	?	C:C	A:G	?	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	?	?				
	13	?	?	C:C	A:G	?	T:T	A:A	A:A	A:C	C:C	G:C	T:T	T:T	A:A	?				
	14	?	?	C:C	A:G	?	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	A:A	?				
	15	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	G:A	?				
	16	?	?	C:C	A:G	?	T:T	A:A	?	A:C	T:C	C:C	T:T	T:T	G:G	?				
	17	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	A:A	?				
	18	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	G:C	T:T	T:T	A:A	?				
	19	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	G:G	?				
	20	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	T:C	C:C	T:T	T:T	G:A	?				
	VA35	?	T:T	?	A:G	?	T:T	A:A	T:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C				
	MP715	?	T:T	C:C	A:G	T:C	T:T	A:A	T:A	C:C	T:T	G:G	T:G	T:T	G:G	C:C				
	VA35*MP715	?	T:T	?	A:G	?	T:T	A:A	A:A	A:C	T:C	C:G	T:T	T:T	G:A	C:C				

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731				
VA35*MP715_9_7018	1	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	G:C	T:T	T:T	A:A	?	BC3S2	S3_217820 604	S9_117048 731	S6_12131 1207
	2	?	?	C:C	A:G	?	T:T	A:A	A:A	A:A	T:T	C:C	T:T	T:T	A:A	?	VA35	C:A	C:C	C:C
	3	?	?	C:C	A:G	?	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	A:A	?	MP715	C:A	T:T	T:T
	4	?	?	C:C	A:G	?	T:T	A:A	?	C:C	T:T	G:G	T:T	T:T	A:A	?	VA35*MP 715	C:A	T:C	T:C
	5	?	?	C:C	A:G	?	T:T	A:A	A:A	A:C	T:C	C:C	T:T	T:T	A:A	?	2017	C:A	T:C	C:C
	6	?	?	C:C	A:G	?	T:T	A:A	A:A	A:C	T:C	C:C	T:T	T:T	G:A	?	2016	C:A	T:C	C:C
	7	?	?	C:C	A:G	?	T:T	A:A	A:A	A:A	C:C	G:G	T:T	T:T	A:A	?				
	8	?	?	C:C	A:G	T:T	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	A:A	?				
	9	?	?	C:C	A:G	?	T:T	A:A	A:A	C:C	C:C	G:C	T:T	T:T	G:G	?				
	10	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	T:T	G:C	T:T	T:T	A:A	?				
	11	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:G	?				
	12	?	?	C:C	A:G	T:C	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:A	?				
	13	?	?	C:C	A:G	?	T:T	A:A	A:A	A:C	T:C	G:G	T:T	T:T	A:A	?				
	14	?	?	C:C	A:G	?	T:T	A:A	?	C:C	T:C	C:C	T:T	T:T	A:A	?				
	15	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	T:C	G:C	T:T	T:T	A:A	?				
	16	?	?	C:C	A:G	?	T:T	A:A	A:A	A:C	T:C	C:C	T:T	T:T	G:A	?				
	17	?	?	C:C	A:G	?	T:T	A:A	?	C:C	T:T	C:C	T:T	T:T	G:G	?				
	18	?	?	C:C	A:G	?	T:T	A:A	A:A	A:C	T:C	G:G	T:T	T:T	G:G	?				
	19	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	T:C	C:C	T:T	T:T	A:A	?				
	20	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	?	G:C	T:T	T:T	G:A	?				
	VA35	?	T:T	?	A:G	?	T:T	A:A	T:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C				
	MP715	?	T:T	C:C	A:G	T:C	T:T	A:A	T:A	C:C	T:T	G:G	T:G	T:T	G:G	C:C				
	VA35*MP715	?	T:T	?	A:G	?	T:T	A:A	A:A	A:C	T:C	C:G	T:T	T:T	G:A	C:C				

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731	BC3S2	S3_217820 604	S9_117048 731	S6_12131 1207
VA35*MP715_11_7019	1	?	?	C:C	A:G	?	T:T	A:A	A:A	A:C	C:C	C:C	T:T	T:T	G:G	?	VA35	C:A	C:C	C:C
	2	?	?	C:C	A:G	?	T:T	A:A	A:A	C:C	T:T	C:C	T:T	T:T	G:G	?	MP715	C:A	T:T	T:T
	3	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	G:G	T:T	T:T	A:A	?	VA35*MP 715	C:A	T:C	T:C
	4	?	?	C:C	A:G	?	T:T	A:A	A:A	A:A	C:C	G:C	T:T	T:T	G:A	?	2017	C:A	C:C	C:C
	5	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	T:C	C:C	T:T	T:T	A:A	?	2016	C:A	C:C	C:C
	6	?	?	?	A:G	T:T	T:T	A:A	A:A	A:A	T:T	G:G	T:T	T:T	G:A	?				
	7	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	C:C	C:C	T:T	T:T	A:A	?				
	8	?	?	C:C	A:G	?	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	A:A	?				
	9	?	?	C:C	?	?	T:C	A:A	?	A:C	T:C	G:G	?	?	?	?				
	10	?	?	?	?	?	?	?	?	A:C	T:C	G:G	?	?	?	?				
	11	?	?	?	?	?	?	?	?	A:C	T:C	?	?	?	?	?				
	12	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:G	?				
	13	?	?	C:C	?	?	?	?	?	A:C	T:C	?	?	?	?	?				
	14	?	?	C:C	?	T:T	T:T	A:A	A:A	A:C	C:C	C:C	T:T	T:T	G:G	?				
	15	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	T:T	G:C	T:T	T:T	A:A	?				
	16	?	?	C:C	A:G	T:T	T:T	A:A	A:A	C:C	T:C	G:C	T:T	T:T	A:A	?				
	17	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	C:C	C:C	T:T	T:T	A:A	?				
	18	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:A	T:C	G:C	T:T	T:T	A:A	?				
	19	?	?	C:C	A:G	T:T	T:T	A:A	A:A	A:C	T:T	C:C	T:T	T:T	A:A	?				
	20	?	?	C:C	A:G	T:T	T:T	A:A	A:A	C:C	C:C	G:G	T:T	T:T	G:G	?				
	VA35	?	T:T	?	A:G	?	T:T	A:A	T:A	A:A	C:C	C:C	T:G	T:T	A:A	C:C				
	MP715	?	T:T	C:C	A:G	T:C	T:T	A:A	T:A	C:C	T:T	G:G	T:G	T:T	G:G	C:C				
	VA35*MP715	?	T:T	?	A:G	?	T:T	A:A	A:A	A:C	T:C	C:G	T:T	T:T	G:A	C:C				

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731		S3_217808 798	S9_107333 254	S9_11704 8731
MO17*CML69_1_2020	1	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:A	T:T	MO17	A:A	A:A	C:C
	2	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	G:C	T:T	T:T	A:A	T:C	CML69	C:C	G:G	T:T
	3	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	G:G	T:T	MO17*CML69	A:C	G:A	T:C
	4	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:T	T:T	G:A	T:T	2017	A:C	G:A	T:C
	5	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:G	C:C	2016	A:C	G:A	T:C
	6	?	C:C	C:C	A:G	C:C	C:C	?	A:A	C:C	C:C	C:C	T:T	T:T	A:A	T:T				
	7	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:A	T:C				
	8	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	A:A	C:C				
	9	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:C	T:T	T:T	G:G	T:T				
	10	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:A	T:T				
	11	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:G	T:C				
	12	?	C:C	C:C	A:G	C:C	C:C	A:A	?	A:C	C:C	G:C	T:T	T:T	A:A	T:T				
	13	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	A:A	C:C				
	14	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:G	T:T				
	15	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:A	T:C				
	16	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:G	C:C				
	17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	A:A	T:T				
	18	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	G:C	T:T	T:T	G:A	T:C				
	19	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:G	T:T				
	20	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:T	T:T	A:A	C:C				
	MO17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:G	T:T	A:A	T:T				
	CML69	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	G:G	C:C				
	MO17*CML69	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	?	T:T	T:T	G:A	T:C				

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731		S3_217808 798	S9_107333 254	S9_11704 8731
MO17*CML69_6_2020	1	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	G:C	T:T	T:T	G:A	T:T	MO17	A:A	A:A	C:C
	2	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:A	C:C	CML69	C:C	G:G	T:T
	3	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:T	T:T	A:A	T:C	MO17*CML69	A:C	G:A	T:C
	4	?	T:C	C:C	A:G	C:C	?	A:A	?	A:C	?	?	T:T	?	G:A	T:T	2017	A:C	G:A	T:C
	5	?	C:C	?	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:G	C:C	2016	A:C	G:A	T:C
	6	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:T	T:T	G:A	T:C				
	7	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	G:G	T:T	T:T	A:A	T:C				
	8	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:C	T:T	T:T	G:G	T:T				
	9	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:T	T:T	A:A	T:C				
	10	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:T	T:T	A:A	T:T				
	11	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	G:G	T:C				
	12	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:T	T:T	A:A	T:C				
	13	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	G:C	T:T	T:T	G:A	T:C				
	14	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	A:A	C:C				
	15	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	G:G	T:T	T:T	G:G	T:T				
	16	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:C	T:T	T:T	A:A	T:C				
	17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:G	T:T				
	18	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:A	T:C				
	19	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	G:G	T:T	T:T	G:G	C:C				
	20	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	A:A	T:C				
	MO17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	T:T				
	CML69	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	G:G	T:C				
	MO17*CML69	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	?	T:T	T:T	G:A	T:T				

Figure C.1 (continued)

Family	IND	S1_2722 20818	S1_28063 5931	S10_91956 540	S2_1531 28978	S2_18319 0964	S2_18887 2911	S2_20503 5174	S3_21735 9490	S3_21780 8798	S3_21782 0604	S4_2640 6913	S5_2067 95116	S8_94752 242	S9_10733 3254	S9_1170 48731		S3_217808 798	S9_107333 254	S9_11704 8731
MO17*CML69_12_2020	1	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	A:A	C:C	MO17	A:A	A:A	C:C
	2	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:A	T:C	CML69	C:C	G:G	T:T
	3	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	G:G	T:C	MO17*CML69	A:C	G:A	T:C
	4	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	G:G	T:T	T:T	G:G	C:C	2017	A:C	G:A	C:C
	5	?	C:C	?	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:A	T:T	2016	A:C	G:A	C:C
	6	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	A:A	T:C				
	7	?	C:C	C:C	?	C:C	C:C	A:A	A:A	A:C	C:C	G:G	T:T	T:T	G:A	T:T				
	8	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	A:A	T:C				
	9	?	C:C	C:C	A:G	C:C	C:C	?	A:A	A:C	C:C	C:C	T:T	T:T	G:G	T:T				
	10	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:C	T:T	T:T	A:A	T:T				
	11	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	G:C	T:T	T:T	A:A	T:C				
	12	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:C	T:T	T:T	G:G	C:C				
	13	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:G	T:T				
	14	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	G:G	T:T	T:T	A:A	T:C				
	15	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:C	C:C	C:C	T:T	T:T	A:A	C:C				
	16	?	C:C	C:C	A:G	C:C	C:C	?	A:A	C:C	C:C	G:C	T:T	T:T	G:G	T:C				
	17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	G:A	T:T				
	18	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	G:G	T:T				
	19	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	A:A	C:C	C:C	T:T	T:T	A:A	T:C				
	20	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:T	T:T	G:G	T:T				
	MO17	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	C:C	T:G	T:T	A:A	T:T				
	CML69	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	G:G	T:T	T:T	G:G	T:C				
	MO17*CML69	?	C:C	C:C	A:G	C:C	C:C	A:A	A:A	C:C	C:C	?	T:T	T:T	G:A	T:T				

Figure C.1 (continued).