Analysis of the Outcrossing Ratio of German Chamomile (*Matricaria recutita* L.) using SSR-markers

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By

Maureen Woytowich

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ABSTRACT

German chamomile (*Matricaria recutita* L.) is one of the most ancient and widely used medicinal plant species. Plant breeding efforts for desired agronomic traits have existed for over sixty years; however, the breeding programs for chamomile are small in comparison to other crop plant species. The objective of this thesis was to examine the variability of chamomile's outcrossing ratio based upon multiple environmental factors in order to provide a basic understanding of pollination biology to inform the breeding of chamomile. Two studies were conducted to achieve this objective. The first study evaluated the outcrossing ratio at multiple geographical locations throughout central Germany. The second study evaluated greenhouse compared to field conditions, as well as, potential temperature effects on the outcrossing ratio. Chamomile exhibited a wide range of outcrossing frequencies in the crosses regardless of both location and ploidy. The results did not show a statistical difference between geographical locations; however, in all crosses there appeared to be an effect associated with crossing direction. In many crosses, the frequency of outcrossed progeny was considerably higher in one cross direction than the other. The greenhouse and field comparison could not be made due to progeny germination difficulties. The greenhouse crosses could have been negatively affected by poor pollination, seed dormancy, and unfavorable temperature conditions during germination. Although this study had technical issues, and the outcrossing ratio could not be analyzed as expected, valuable knowledge was gained for the direction of future research in chamomile.

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TABLE OF CONTENTS

PERMISSION TO USE i
ABSTRACT ii
ACKNOWLEDGEMENTS iii
TABLE OF CONTENTS iv
LIST OF TABLES vi
LIST OF FIGURES vii
LIST OF ABBREVIATIONS ix
CHAPTER 1 1
1.0 Introduction 1
CHAPTER 2 4
2.0 Literature Review 4
2.1 Plant Reproduction 4
2.1.1 Sexual Fertilization: Outcrossing and Selfing 4
2.2 Marker-Assisted Selection (MAS) 5
2.3 Molecular Markers 5
2.3.1 Simple Sequence Repeat (SSR) Markers 6
2.3.2 Practical Applications of SSR Markers 7
2.4 German Chamomile 8
2.4.1 Cultivation 8
2.4.2 Medicinal Importance 10
2.4.3 Reproduction 11
2.4.4 Breeding 11
2.5 Polyploidy 11
2.5.1 Polyploidy as a Breeding Tool 12
2.5.1.1. Seedless Watermelon 14
2.5.1.2. Chamomile Polyploidy 14
CHAPTER 3 16
ABSTRACT 16
3.0 Evaluating Geographical Location Effect on the Outcrossing Ratio 16
3.1 Introduction 16
3.2 Materials and Methods 17
3.2.1 Plant Material and Description of Growing Locations 17
3.2.2 Experimental Procedures 24
3.2.2.1 DNA Extraction 24
3.2.2.2 Polymerase Chain Reaction (PCR) 25
3.2.2.2.1 Primers and Microsatellites 26
3.2.2.3 Microsatellite Fragment Preparation 29
3.2.2.4 Capillary Electrophoresis 29
3.2.2.5 Fragment Analysis 30

3.2.2.6 Statistical Analysis 34
3.3 Results 34
3.3.1 Diploid Results 36
3.3.2 Tetraploid Results 39
3.4 Discussion 43
3.4.1 Location Effect 43
3.4.2 Crossing Direction and Parent of Origin Effects 43
3.4.3 Ploidy 44
3.5 Conclusion 44
CHAPTER 4 46
4.0 Evaluating the Effects of Field versus Greenhouse Environments on the Outcrossing Ratio 46
4.1 Introduction 46
4.2 Materials and Methods 47
4.2.1 Plant Material and Description of Growing Locations 47
4.2.2 Experimental Procedures 49
4.2.2.1. Fragment Analysis 50
4.2.2.2 Flow Cytometry 51
4.3 Results 52
4.4 Discussion 56
4.4.1 Pollination 56
4.4.2 Climatic Conditions 57
4.4.3 Seed Dormancy 59
4.5 Conclusion 60
CHAPTER 5 61
5.0 General Discussion 61
REFERENCES 63
APPENDICES 70

LIST OF TABLES

Table 3.1. Diploid plant material used for assessment of outcrossing ratio at different geographical locations.

Table 3.2. Tetraploid plant material used for assessment of outcrossing ratio at different geographical locations.

Table 3.3. Latitude and longitude coordinates of the towns used for diploid plot locations in central Germany.

Table 3.4. Latitude and longitude coordinates of the towns used for tetraploid plot locations in central Germany.

Table 3.5. SSR markers that exhibited polymorphism selected for analysis.

Table 3.6. Original contig sequences of each primer used for the development of the microsatellite analysis. Bold and underlined sections representing the forward primer and reverse primer.

Table 3.7. Peak (loci) locations on electropherogram for diploid parents of *Matricaria recutita* used in crossing trials.

Table 3.8. Peak (loci) locations on electropherogram for tetraploid parents of *Matricaria recutita* used in crossing trials.

Table 3.9. CEQ™ peak scoring criteria used to standardize amplicon size between plates by comparison of the CEQ™ internal size standards. Variation in overall signal strength between plates caused different thresholds to be used to assign peak presence or absence.

Table 3.10. Results of diploid parents 20-2 and 13-2 reciprocal cross.

Table 3.11. Results of diploid parents 12-5 and 20-04 reciprocal cross.

Table 3.12. Results of diploid parents 04-2 and 18-2 reciprocal cross.

Table 3.13. Results of tetraploid parents 24-01 and 29-04 reciprocal cross.

Table 3.14. Results of tetraploid parents 23-04 and 29-01 reciprocal cross.

Table 3.15. Results of tetraploid parents 22-06 and 16-08 reciprocal cross.

Table 4.1. Diploid plant material used for evaluating the effects of field versus greenhouse environments.

Table 4.2. Tetraploid plant material used for evaluating the effects of field versus greenhouse environments.

Table 4.3. Crosses peak (loci) locations on electropherogram.

Table 4.4. CEQ™ peak scoring criteria used to standardize amplicon size between plates by comparison of the CEQ™ internal size standards. Variation in overall signal strength between plates caused different thresholds to be used to assign peak presence or absence.

Table 4.5. Crossing results of successful trials in 2017.

LIST OF FIGURES

Figure 3.1. Map of Germany. Red indicates the area used for diploid crosses, green indicates the area used for tetraploid crosses.

Figure 3.2. Locations of diploid crosses completed throughout central Germany.

Figure 3.3. Locations of tetraploid crosses completed throughout central Germany.

Figure 3.4. High and low temperature recordings of Erfurt, Bretleben, Borxleben, Hettstedt, Freyburg, and Schönfeld, Germany in July 2014.

Figure 3.5. High and low temperature recordings of Erfurt, Ritteburg, Tiefthal, and Töttelstädt, Germany from August 28 to September 15, 2014.

Figure 3.6. High and low wind speed recordings of Erfurt, Bretleben, Borxleben, Hettstedt, Freyburg, and Schönfeld, Germany in July 2014.

Figure 3.7. High and low wind speed recordings of Erfurt, Ritteburg, Tiefthal, and Töttelstädt, Germany from August 28 to September 15, 2014.

Figure 3.8. Plant material. (A) Cross layout. Two parental lines of the same ploidy were transplanted into a plot and allowed to intercross. Each genotype, A and B, had between three and five clonal plants per plot with 15 cm spacing between plants. (B) Mature chamomile flower head.

Figure 3.9. 1% agarose gel image of DNA extraction used to determine approximate concentrations for DNA dilutions. The molecular weight marker standard is 1 kb Bioline HyperLadder™ with fragment sizes of 10037 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500/1517 bp, 1000 bp, 800 bp, 600 bp, 400 bp, and 200 bp.

Figure 3.10. 1% agarose gel image of PCR amplified products from Plate 25 using primer KamSSR-36. The molecular weight marker standard is 50 bp Bioline HyperLadder™ with fragment sizes of 2000 bp, 1800 bp, 1600 bp, 1400 bp, 1200 bp, 1000 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 200 bp, 100 bp, and 50 bp. Progeny were compared to controls of parent 1 (P1) in duplicate, parent 2 (P2) in duplicate, and a negative control (H_2O) in duplicate. The loci analyzed were 142 bp and 224 bp (Table 3.8).

Figure 3.11. Electropherogram from CEQ™ 8000 of four samples using primer KamSSR-36 on PCR plate 24. The Y axis is the relative fluorescence (dye signal), and the X axis is fragment length in nucleotides. Red peaks are size standards. The top two panels show the presence of peak 142 (blue) in parent 23-04 (in duplicate) and the bottom two panels absence of peak 142 in parent 29-01 (in duplicate).

Figure 3.12. Histograms of log e-values from a plant EST tBLASTx of next generation sequencing (NGS) contig sequences (Table 3.6) from which primers were developed for the analysis here. Primer 85 had no significant similarity found and therefore was excluded from this graph.

Figure 3.13. Histograms of log e-values from a plant EST BLASTn of next generation sequencing (NGS) contig sequences (Table 3.6) from which primers were developed for the analysis here.

Figure 3.14. Boxplots showing the comparison of diploid and tetraploid crosses. The first six crosses (white) are diploid and the last six crosses (gray) are tetraploid.

Figure 4.1. Plot images. (A) Field plot showing thermometer in waterproof bag. (B) Field plot showing isolation cage. (C) Greenhouse plot.

Figure 4.2. Field map of chamomile crosses completed on the IPK Gatersleben campus in June 2017. Yellow stars indicate plot locations, pink stars indicate plot locations with an isolation cage, and red arrow indicate distance between nearby plots.

Figure 4.3. Profile of diploid parent 16-080-22 to the left (labeled RN1, FL1 position 50) and internal *Vicia faba* standard to the right (labeled RN2, FL1 position 220).

Figure 4.4. Profile of tetraploid parent 16-03-01 to the left (labeled RN1, FL1 position 100) and internal *Vicia faba* standard to the right (labeled RN2, FL1 position 220).

Figure 4.5. Bar graph representing the frequency outcrossed for all successful field crosses involving diploid parents.

Figure 4.6. Greenhouse temperature readings from date of isolation (13:10:00 on June 6, 2017) to date of harvest (23:55:00 on June 26, 2017). Temperature readings were taken at 15-minute intervals, totaling 1965 observations throughout the isolation period. The x-axis represents the observation number out of the 1965 readings (0= June 6 at 13:10:00, 200= June 8 at 14:55:00, 400= June 10 at 16:55:00, 600= June 12 at 18:55:00, 800= June 14 at 20:55:00, 1000= June 16 at 22:55:00, 1200= June 19 at 00:55:00, 1400= June 21 at 2:55:00, 1600= June 23 at 4:55:00, 1800= June 25 at 6:55:00).

Figure 4.7. Field temperature readings from date of isolation (13:00:00 on June 6, 2017) to date of harvest (23:45:00 on June 26, 2017). Temperature readings were taken at 15-minute intervals, totaling 1965 observations throughout the isolation period. The x-axis represents the observation number out of the 1965 readings (0= June 6 at 13:00:00, 200= June 8 at 14:45:00, 400= June 10 at 16:45:00, 600= June 12 at 18:45:00, 800= June 14 at 20:45:00, 1000= June 16 at 22:45:00, 1200= June 19 at 00:45:00, 1400= June 21 at 2:45:00, 1600= June 23 at 4:45:00, 1800= June 25 at 6:45:00).

Figure 4.8. Temperature readings from Greenhouse 5 that was used for selfing parental genotypes from 13:10:00 on June 6, 2017 to 23:55:00 on June 26, 2017. Temperature readings were taken at 15-minute intervals, totaling 1965 observations throughout the isolation period. The x-axis represents the observation number out of the 1965 readings (0=June 6 at 13:10:00, 200= June 8 at 14:55:00 , 400= June 10 at 16:55:00 , 600= June 12 at 18:55:00, 800= June 14 at 20:55:00, 1000= June 16 at 22:55:00, 1200= June 19 at 00:55:00, 1400= June 21 at 2:55:00, 1600= June 23 at 4:55:00, 1800= June 25 at 6:55:00).

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AFLP	Amplified Fragment Length Polymorphism
GBS	Genotyping By Sequencing
GIFS	Global Institute for Food Security
GWAS	Genome Wide Association Study
IPK Gatersleben	Leibniz Institute of Plant Genetics and Crop Plant Research
ISSRs	Inter Simple Sequence Repeats
MAS	Marker-Assisted Selection
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SNPs	Single-Nucleotide Polymorphism
SSRs	Simple Sequence Repeats
TKW	Thousand Kernel Weight
WHO	World Health Organization

CHAPTER 1

1.0 Introduction

The relationship between plants and human health has been documented for thousands of years. Early humans acquired the knowledge to utilize plants for healing benefits through observation and the experience of trial and error over many centuries. Records of medicinal plant use date back approximately 4000 years where clay tablets were used to record plant remedies (Karunamoorthi *et al*., 2013), including oils of *Cedrus* species (Cedar) and *Cupressus sempervirens* (Cypress), *Glycyrrhiza glabra* (Licorice), *Commiphora* species (Myrrh) and *Papaver somniferum* (Poppy juice) for various illnesses (Gurib-Fakim, 2006). Medicinal plant use in Europe is believed to have originated with Hippocrates (460–377 BC) and Aristotle (384–322 BC) (Gurib-Fakim, 2006). In the first century AD, the Greek physician Dioscorides wrote the famous book “De Materia Medica” that became one of the most powerful influences for medicinal plant use. This book is commonly accepted as the first European herbal book and was considered the standard book of reference for over 1000 years (Gurib-Fakim, 2006). Medicinal plants were cultivated as early as 800 AD at monasteries in Central Europe (Gurib-Fakim, 2006). In 300 BC, philosopher and natural scientist Theophratus noted the ability of medicinal qualities of plants to change characteristics through cultivation (Gurib-Fakim, 2006). Many of the medicinal plants used as remedies for ailments, ranging from coughs to parasitic infections in ancient times, are still used today.

Plants have formed the foundation of sophisticated traditional medicinal systems that have been in existence for thousands of years, and they continue to provide humankind with new remedies. These plant-based systems continue to play an essential role in health care worldwide for various reasons, including the rising human population, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs, and development of resistance, together which have led to increased emphasis on the use of plant materials as a source of medicine (Karunamoorthi *et al*., 2013). In 2011, the World Health Organization (WHO) estimated that 70 to 90% of people in developing countries rely primarily on traditional medicine systems for their health care remedies (Robinson and Zhang, 2011). Although modern medicine is available in many developing countries, many people still rely heavily on medicinal plants to meet primary health care needs due to historical and cultural reasons (Gurib-Fakim, 2006). Herbal remedies have become increasingly popular in North America in recent years; however, they are often considered as nutritional supplements rather than medicines (Gurib-Fakim, 2006). In many countries of Europe, herbal medicine is a part of everyday life, where the use of medicinal plants is considered a sophisticated and rational method to treat aliments as a supportive means rather than curative. As an alternative to synthetically derived medicinal products, the use of herbal tea and

phytomedicines are common in Europe (Gurib-Fakim, 2006). Herbal medicines may have remained popular over time due to the tendency of medicinal plants to work slowly, and typically have minimal toxic side effects (Srivastava *et al*., 2010).

There are over 300,000 plant species that exist on earth; however, it is estimated that only 15% of plants have been evaluated to determine their medicinal value (Palhares *et al*., 2015). A medicinal plant is defined as a plant that, in one or more of its organs, contains a substance that can be used for therapeutic purposes (Karunamoorthi *et al*., 2013). Medicinal plants contain many different chemical compounds that can act independently or in concert to improve human health. For example, one single plant can have phenolic compounds that act as antioxidants, tannins that act as natural antibiotics, and alkaloids that improve mental well being (Gurib-Fakim, 2006). Herbal medicines are made from the processed products of medicinal plant species. Certain steps have been established in Pharmacopoeias, official publications that contain a list of medicinal drugs with their effects and directions for their use, in order to guarantee the quality of herbal medicines. These steps include correct identification of the plant species, analysis of purity, and confirmation of the minimum concentration of the active ingredients (Palhares *et al*., 2015).

One of the plant families that is important for medicinal purposes is the Asteraceae. This family consists of approximately 25,000 species in 1400 genera that are distributed in most ecosystems across all continents with the exception of Antarctica (Gurib-Fakim, 2006). Chamomile is an annual plant belonging to the Asteraceae, which originates from southeastern Europe and western Asia (Franke and Schilcher, 2005). Today, chamomile is cultivated worldwide due to its ability to grow over a wide range of climate and soil types (Ahmad *et al*., 2011). There are two plants commonly known as chamomile, German chamomile (*Matricaria recutita* L., syn. *Matricaria chamomilla* L, syn. *Chamomilla recutita* L.) and Roman or English chamomile (*Chamaemelum nobile* L.) (Kong *et al*., 2017). Whereas both types of chamomile may look similar and are often mistaken for one another, they belong to different species and have a few defining characteristics. German chamomile is an annual species, whereas Roman chamomile is a perennial species. Botanically, there are floral morphological differences between German and Roman chamomile (Singh *et al*., 2011). Additionally, the essential oils and chemical constituents of German and Roman chamomile are significantly different (Mann and Staba, 1986). Although belonging to different genera, both types of chamomile have been used as medicinal plants for thousands of years.

This study is focused on the more widely cultivated German chamomile (*Matricaria recutita* L.) and therefore, this species will be referred to as chamomile throughout this document. The objective of this thesis was to examine the variability of chamomile's outcrossing ratio relative to multiple environmental factors in order to provide a basic understanding of fertilization for future breeding methodology. The first study evaluated the outcrossing ratio between synthetic crosses of plants of the

same ploidy, either diploid or tetraploid, at multiple geographical locations throughout central Germany. The second study evaluated outcrossing among synthetic crosses grown under both field and greenhouse conditions, as well as potential temperature effects on the outcrossing ratio.

CHAPTER 2

2.0 Literature Review

2.1 Plant Reproduction

Flowering plants, or angiosperms, possess exceptionally versatile reproductive and mating strategies. There are three phases of reproduction in angiosperms: flowering and pollination, fruiting and seed dispersal, and seed germination and seedling establishment (Barrett and Eckert, 1990). Two general reproductive strategies exist in plants, sexual and asexual reproduction. Sexual reproduction requires the fusion of the male gamete transmitted by pollen, with the female gamete (egg) in the ovule (Richards, 1997). In contrast, asexual reproduction allows offspring to arise from a single parent (Richards, 1997). Although there are advantages to both means of reproduction, sexual reproduction is observed far more frequently in multicellular eukaryotes, including plants (Neiman *et al*., 2014).

The most successful reproductive strategy found in vascular plants is the formation of seeds, either sexually (i.e. amphimixis) or asexually (i.e. apomixis). Seed-forming plants represent the most species-rich lineage within vascular plants (Wang and Köhler, 2017), and seed is one of the key factors of crop productivity in modern agriculture (Barcaccia and Albertini, 2013). Plants that reproduce sexually through seed have three mating systems: obligate outcrossing, predominant selfing (autogamy), and simultaneous outcrossing and selfing, otherwise known as mixed mating (Barrett *et al.*, 1996). Approximately one-third of the species that have had their mating system quantified are considered to be mixed mating, and thus exhibit a broad mixture of selfing and outcrossing (Barrett *et al.*, 1996). Reproductive success depends on a variety of ecological factors, including growing conditions, presence of pollinators, dispersal agents, as well as pest and disease pressure (Barrett and Eckert, 1990).

2.1.1 Sexual Fertilization: Outcrossing and Selfing

It is essential to understand variation in fertilization processes and crossing barriers in order to assess plant breeding methods (Faehnrich *et al*., 2015). For example, there are advantages and disadvantages to both means of fertilization, outcrossing and selfing. Self-fertilization is beneficial when the presence of pollinators or potential mates is limited (Herlihy and Eckert, 2002). However, large reductions in genetic diversity can be observed in selfing plant species (Barrett, 2003). Self-fertilization becomes unfavorable when the progeny suffer reduced viability because of inbreeding depression (Herlihy and Eckert, 2002). Significant inbreeding depression can be observed in the fitness of selfed progeny due to the expression of recessive deleterious mutations in the homozygotic condition (Lande and Schemske, 1985). Inbreeding depression is a major factor that selects for the maintenance of cross

fertilization in many plant species (Charlesworth and Charlesworth, 1987). There is evidence that suggests many plant species shift from outcrossing to selfing as a result of unfavorable environmental conditions (Barrett and Eckert, 1990).

There are many mechanisms in plants that have evolved to prevent self-fertilization, and therefore promote outcrossing including self-incompatibility, heterostyly, and dichogamy (Lande and Schemske, 1985). Plants in the Asteraceae family often have either dichogamous protandry, where anthers mature before the stigma becomes receptive, or sporophytic self-incompatibility as a mechanism to prevent selfing (Huang and Kao, 2013). Self-incompatibility can be a desired property in plant breeding in order to control and establish particular crossings because it eliminates the need for male-sterile mother lines (Faehnrich *et al*., 2015).

2.2 Marker-Assisted Selection (MAS)

Plant breeding can be enhanced and made more efficient by utilizing marker-assisted selection (MAS). MAS is an indirect selection method that allows a phenotypic trait to be selected using molecular markers genetically linked to a genetic factor influencing the trait (Ntsomboh Ntsefong *et al.*, 2015; Lidder and Sonnino, 2012). The method of using molecular markers is based on naturally occurring DNA polymorphisms (Ntsomboh Ntsefong *et al.*, 2015). Conventional breeding systems require numerous breeding cycles in order to combine target traits in a particular genotype (Devi *et al.*, 2017). However, MAS can significantly decrease the time and resources in breeding programs by enhancing the accuracy of selection (Lidder and Sonnino, 2012). MAS is especially valuable when phenotyping is difficult or the evaluation of specific traits is time consuming in certain species, such as tree crops that have long juvenile periods (Kalwade and Devarumath, 2014). Successful MAS requires a high correlation (i.e. linkage) between the gene of interest and the molecular marker (Devi *et al.*, 2017).

2.3 Molecular Markers

A molecular marker is a DNA sequence which can be readily detected and whose inheritance can be monitored, thus providing a way to differentiate plants (Kumar *et al*., 2009). They are used for many purposes in plant science, including understanding genome relationships, germplasm identification, gene mapping, and marker assisted breeding (i.e. MAS) (Swapna *et al.*, 2011). There are many different molecular marker systems available for monitoring genetic diversity (Baraket *et al.*, 2011); the best molecular marker system depends on the study and the species being examined. Random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSRs), single-nucleotide polymorphism

(SNPs), and simple sequence repeats (SSRs) are commonly used molecular markers because they are easier, cheaper, and faster compared to other molecular markers such as restriction fragment length polymorphism (RFLP), and amplified fragment length polymorphism (AFLP) markers (Mall and Chawla, 2014). In many crop species, including chamomile, next generation sequencing (NGS) approaches have been used for the examination and exploitation of genetic resources in plant breeding (Otto *et al*., 2017). NGS led to the establishment of genotyping by sequencing (GBS), which allows thousands of molecular markers to be examined genome wide (Deschamps *et al*., 2012). In chamomile, GBS has been used to evaluate the genetic structure of cultivated varieties and to perform genome-wide association studies (GWAS) that focused on genes affecting flowering time and the content of alpha-bisabolol, a medicinal component found in the essential oil (Otto *et al*., 2017).

2.3.1 Simple Sequence Repeat (SSR) Markers

Microsatellites, alternatively known as simple sequence repeats (SSRs), are the molecular marker system selected for this study. SSRs are composed of locus-specific DNA regions which flank short tandem repetitive simple DNA motifs of 1 to 6 base pairs in length (Li *et al*., 2014). SSRs are abundant across eukaryotic genomes in both coding and non-coding regions (Xiao *et al.*, 2015). SSRs comprise a large fraction of noncoding DNA and are relatively rare in protein-coding regions (Li *et al.*, 2002).

The foundation behind the development of SSRs was based on human and mammalian biology (Powell *et al.*, 1996), and since then SSRs have been found in numerous eukaryotes including mammals, birds, fish, insects, yeast, and plants (McCouch *et al*., 1997). The isolation and cloning of the first plant microsatellites was performed with tropical tree species (Condit and Hubbell, 1991) and subsequently studies to identify microsatellites have been undertaken in many important crop species including rice, barley, wheat, maize, soybean, tomato, grapevine, forest trees, sunflower, and *Brassica* species (McCouch *et al.*, 1997).

SSRs can serve as highly informative genetic markers, and in conjunction with the use of polymerase chain reaction (PCR) technology, enable the detection of length variation, which is treated as allelic states (Powell *et al.*, 1996). Natural sources of variation in SSR markers which provide population-level variation can occur due to replication slippage of DNA polymerase, in addition to the influence of other factors (Michael *et al*., 2007). SSRs are often preferred molecular markers due to many desirable attributes, including high level of polymorphisms, their multi-allelic nature, co-dominant inheritance, wide genomic distribution, reproducibility, and ease of analysis by PCR (Kalwade and Devarumath,

2014; McCouch *et al.*, 1997; Powell *et al.*, 1996; Singh *et al.*, 2010; Swapna *et al.*, 2011; Xiao *et al.*, 2015).

SSRs show high levels of polymorphism based on the differences in the number of DNA repeat units (i.e. DNA motifs) at a given locus, which can vary in an allelic fashion to reveal co-dominant inheritance (Swapna *et al.*, 2011). Polymorphisms are revealed by PCR amplification from genomic DNA using locus-specific distinctive primers that flank and consequently define the microsatellite locus (Powell *et al.*, 1996). Amplification products obtained from different individuals can be resolved on high resolution agarose gels to reveal the polymorphism (Powell *et al.*, 1996); however, capillary-based fragment analysis methods provide greater resolution for separating and sizing microsatellite alleles than gel-based methods (Swapna *et al.*, 2011).

2.3.2 Practical Applications of SSR Markers

SSRs can be used in a wide range of studies, and practical applications of SSRs in plant science include: genetic analysis, molecular assisted breeding (MAS), genome mapping, gene tagging, genetic diversity detection, variety identification, and germplasm fingerprinting (Kalwade and Devarumath, 2014; Mall and Chawla, 2014; Xiao *et al.*, 2015). The first applications of microsatellites in plants included cultivar identification by genotyping species such as soybean (Rongwen *et al.*, 1995) and grapevine (Thomas and Scott, 1993). SSRs have been the markers of choice for various applications in many crop species such as sugarcane (Swapna *et al.*, 2011), apricot (Campoy *et al*., 2011) and rice (Singh *et al.* 2010) due to their abundance, codominant inheritance, and high levels of polymorphism. SSRs have also been significant in long-term germplasm conservation to ensure their proper maintenance, validate accessions or cultivars, determine the degree of relatedness among individuals or groups of accessions, clarify genetic structure, and to help determine the presence of a specific gene or gene complex in particular accessions (McCouch *et al*., 1997).

Paternity analysis can be accomplished by any genetic marker system that is sufficiently polymorphic between parental lines (Gerber *et al*., 2000), as has been studied in many tree species (Gerber *et al*., 2000; Sampson, 1998; Ziegenhagen *et al.,* 1998). It is advantageous to use SSRs for determining gene flow through reconstruction of relationships between parental and offspring generations because the multiallelic, codominant nature of SSRs allows single plants to be individually genotyped (Gerber *et al*., 2000). SSRs represent single loci and therefore the problems associated with multiple banding patterns obtained with other marker systems can be avoided (Powell *et al*., 1996).

2.4 German Chamomile

Chamomile is an annual, herbaceous plant belonging to the Asteraceae. It has an upright stem that grows between 10 and 80 cm tall (Singh *et al*., 2011). The feathery foliage is due to leaves that are alternate, long and narrow, and bi- to tri-pinnate (Singh *et al*., 2011). Chamomile has many inflorescences per plant. Each inflorescence is a paniculate flower head (capitula), that consist of a ring of white male-sterile outer ray florets and yellow inner hermaphroditic disc florets (Otto *et al*., 2015), causing the inflorescence to resemble a single flower. Flower heads are placed separately and have a diameter of 10 to 30 mm (Singh *et al*., 2011). A distinctive characteristic of *M. recutita* in comparison to *C. nobile* is the elongated cone-shaped and hollow receptacle (Sharafzadeh and Alizadeh, 2011). The fruit of chamomile is a small and dry achene, which will be referred to as a seed. Chamomile has very small seeds with a thousand kernel weight (TKW) of 0.02 to 0.06 g for diploid forms and 0.04 to 0.12 g for tetraploid forms (Franke and Schilcher, 2005).

2.4.1 Cultivation

Recommendations for chamomile cultivation are typically company-specific and are adapted to geographic location and genetic variety (Franke and Schilcher, 2005). Chamomile is extremely tolerant of a variable growing environment; the plant is able to withstand cold temperatures as low as 2°C although the ideal growing temperature is 7 to 26°C (Franke and Schilcher, 2005). It grows equally in all soil types; however, wild growing locations are often in sandy to loamy soils (Franke and Schilcher, 2005). In Hungary, chamomile is grown extensively on clay soils with lime, which are considered too poor for any other crop (Singh *et al*., 2011). Furthermore, chamomile has been successfully grown on alkaline soils with a pH as high as 9 in India (Singh *et al*., 2011). In this light, examining the effects of sowing date, plant density, fertilization, weed control, and harvest date with respect to the soil and climate conditions could be used to improve yield and quality of chamomile (Honermeier *et al*., 2013).

Chamomile originates from southeastern Europe and western Asia (Franke and Schilcher, 2005) but today chamomile is cultivated in temperate regions worldwide due to its ability to grow over a range of climate and soil types (Ahmad *et al*., 2011). The key countries responsible for current production are Argentina, Bulgaria, Czech Republic, Egypt, Germany, Hungary, and Poland (Otto, personal communication). In Germany, chamomile is cultivated on approximately 1000 ha (Honermeier *et al*., 2013). There are two cultivation timings used in chamomile, sowing in autumn and sowing in spring. Autumn-sown crops overwinter at the 6 to 8 leaf stage and are harvested after the beginning of flowering during summer of the second year (Honermeier *et al*., 2013). For spring cultivation, chamomile needs to be sown as early as possible, typically March, to ensure adequate growth and formation of side branches

for high flower yield (Rafieiolhossaini *et al*., 2010). When sown in March or April, harvest occurs in July (Honermeier *et al*., 2013). An increased pressure of disease and insects in the summer months is a negative aspect of spring-sown crops in comparison to autumn-sown crops (Franke and Schilcher, 2005). Plant diseases that are known to cause damage to chamomile include powdery mildew (*Erysiphe cichoracearum*), downy mildew (*Peronospora leptosperma*, *Peronospora radii*), white rust (*Albugo tragopogonis*), rust (*Puccinia matricaiae*), leaf spot disease (*Stemphylium botryosum*), and wilt and stem rot (*Fusarium culmorum*) (Franke and Schilcher, 2005; Singh *et al*., 2011). Thrips (*Thrips tabaci* and *Thrips physapus*) are known to attack chamomile flower heads, causing them to disintegrate (Hrudová *et al*., 2006). Aphids (*Aphis fabae*, *Brachycaudus helichrysi*, *B. cardui*) damage chamomile plants by sucking on the leaves and stems (Hrudová *et al*., 2006). In addition to damage in the field, fungi and insects can cause extensive damage to the dry flowers during storage and reduce the quality of the dried raw product (Singh *et al*., 2011).

In Germany, chamomile crops are cultivated at a seeding rate of 2.0 to 2.5 kg/ha with a row spacing most commonly being 0.25 metres, but can range between 0.16 to 0.40 metres (Otto, personal communication). Chamomile is an obligate light-dependent germinator and therefore is sown on a flat soil surface (Franke and Schilcher, 2005). For germination and continuous development, it is critical for the soil to have good moisture; otherwise, a patchy and poor germination can occur (Singh *et al*., 2011). A second method of chamomile field cultivation is transplanting. This method can significantly reduce the mortality of seedlings if transplanted within 4 to 5 weeks of germination (Singh *et al*., 2011).

Fields of chamomile are harvested during full bloom. Chamomile flowers over a period of 50 to 65 days with the need for multiple harvests due to the continuous production of new flowers (Franke and Schilcher, 2005). The way chamomile is harvested substantially influences the quality of raw flower material (Ehlert *et al*., 2011). Hand picking allows for a very high flower quality; however, the quantity of material harvested per hour is very low (3-5 kg h^{-1}) (Ehlert *et al*., 2011). This method of harvest is common in low-wage countries such as India (Singh *et al*., 2011). It is common in several countries for chamomile flower heads to be harvested manually with small picking combs (Honermeier *et al*., 2013). A handheld comb can increase productivity of hand picking up to 18 kg h^{-1} (Ehlert *et al*., 2011). Productivity can be further increased by the addition of comb shovels and picking carts (Franke and Schilcher, 2005). In Germany, the harvest of chamomile is completed by machine-based combs combined with a rotating drum in special picking machines (Honermeier *et al*., 2013). Other picking principles like rotating virtual combs and cutting bars can be used. Due to the strong influence on picking quality and flower head losses in chamomile, the comb parameters have to be optimized (Honermeier *et al*., 2013). During harvest, the comb moves through the upper plant stand of a chamomile field, which causes the stems with side shoots to be arranged between the teeth of the comb and glide down in the gaps up to the

inflorescences. The flower head diameter is larger than the comb gap width and therefore the gliding stops and the breaking point is reached. The stem tears within a few millimetres of the flower head, resulting in high quality flower yield (Ehlert *et al*., 2011). Flower head drug yield, the amount and quality of essential oil extracted, is the most important yield component of chamomile production. If the breaking point of the stem is too far below the flower head, the quality of the raw material and associated profit decreases.

2.4.2 Medicinal Importance

Chamomile is one of the most ancient and widely used medicinal plant species with numerous applications. The government authorities of twenty-six countries recognize chamomile as an official drug (Baghalian *et al*., 2011). The medicinal properties of chamomile are attributed to the flower heads and the essential oils extracted from them (Baghalian *et al*., 2011). Although the flowers contain the highest amount of essential oil, it is actually present in all organs of a chamomile plant (Franke and Schilcher, 2005). Dry flowers of chamomile contain between 0.2 to 1.9% essential oil (Singh *et al*., 2011), which has many terpenoids and flavonoids that contribute to its medicinal properties (Srivastava *et al*., 2010). There are approximately 120 secondary metabolites in chamomile, including 28 terpenoids and 36 flavonoids (Srivastava *et al*., 2010). The active constituents of the essential oil of particular interest in pharmaceutical application include (-)-α-bisabolol (levomenol), a monocyclic sesquiterpene alcohol, matricin, and flavone derivates (Franke and Schilcher, 2005). Medicinal ingredients are extracted from the dry flower heads by using water, ethanol or methanol as solvents, and the corresponding extracts are known as aqueous, alcoholic and/or methanolic extracts (Srivastava *et al*., 2010). Optimum chamomile extracts contain approximately 50% alcohol (Srivastava *et al*., 2010). Many different preparations for the use of chamomile have been developed, including dry powder, herbal beers, lotion, essential oils, and the most popular, herbal tea (Srivastava *et al*., 2010). Chamomile tea is one of the world's most desired herbal teas, having approximately one million cups consumed every day (Srivastava *et al*., 2010). Chamomile has been used for a wide range of healing applications. WHO recommends internal, external, and inhalation as methods for medicinal use of chamomile (World Health Organization, 1999). Internal uses are suggested for symptomatic treatment of digestive ailments, restlessness, and insomnia due to nervous disorders (World Health Organization, 1999). External uses are recommended for inflammation and irritations of the skin and mucosa, hemorrhoids, and infections of the mouth and gums (World Health Organization, 1999). Inhalation is advised to relieve anxiety, general depression (Srivastava *et al*., 2010) and symptomatic relief for irritations of the respiratory tract caused by the common cold (World Health

Organization, 1999). In addition to medicinal use, the essential oils of chamomile are used extensively in cosmetics and aromatherapy (Srivastava *et al*., 2010).

2.4.3 Reproduction

Chamomile is considered to be mainly outcrossing (Faehnrich *et al*., 2016), with a self-incompatibility mechanism that is reported to be highly variable (Faehnrich *et al*., 2015). Wild populations of chamomile are diploid (2n =18). However, cultivars can be either diploid or colchicine-induced tetraploid (4n =36). Diploid cultivars have higher outcrossing rates than tetraploid cultivars due to stronger self-incompatibility (Faehnrich *et al.*, 2013). Little is known regarding variability in outcrossing ratio and how this is correlated with environmental conditions and genetic background of breeding lines or cultivars of chamomile.

2.4.4 Breeding

Due to the significant medicinal properties and a vast number of uses, chamomile has great economic value and demand (Singh *et al*., 2011). Farmed chamomile originated from wild-collections, and breeding efforts for desired agronomic traits have existed for over sixty years; however, the breeding programs for chamomile are small in comparison to other plant species (Faehnrich *et al*., 2016; Otto *et al*., 2015). Centers of breeding efforts are Czech Republic, Germany, Hungary, and Poland (Otto, personal communication). In Europe, several diploid and tetraploid chamomile cultivars are available for production. These cultivars have different quality parameters and are therefore classified into four different chemotypes: high matricin content, low matricin content, high matricin and bisabolol content, and high matricin and bisabolol oxide content (Honermeier *et al*., 2013). The characteristics important for a chamomile crop include larger flower heads, disease resistance, the chemical compound profile, quantity of oil, and a synchronization of flowering time for mechanical harvest (Albrecht *et al*., 2016). The primary goals of a chamomile breeding program are to increase the yield of the valuable medicinal components and to improve the suitability of the plant for mechanical harvest (Beier and Ehlert, 2014).

2.5 Polyploidy

Polyploidy is a condition where an organism possesses more than two complete sets of chromosomes (Woodhouse *et al*., 2009). For example, organisms with three sets of chromosomes (2x + x =3x) are termed triploids, and organisms with four sets of chromosomes (2x + 2x =4x) are called

tetraploids. Polyploids can arise within a species, known as autopolyploids, or due to the hybridization of two distinct species, known as allopolyploids (Woodhouse *et al*., 2009).

Polyploidy can be observed in both plants and animals with different species exhibiting different levels of tolerance for polyploidy. Although polyploidy is less abundant in animals in comparison to plants (Paterson, 2005), it can occur in some species of invertebrates, amphibians, and fish (Dheilly *et al*., 2014). Triploids have larger body size and faster growth in comparison to diploids. However, increased size is at the expense of reproductive development because in most species, triploids are sterile or infertile (Dheilly *et al*., 2014). An example of animal triploidy is seen in oysters. Since the 1980s, oyster aquaculture has used the differences in growth and reproductive capacity observed between diploids and triploids to increase production (Dheilly *et al*., 2014). Triploid oysters present a benefit due to faster growth, strong reduction of gonad development, and better survival rate (Dheilly *et al*., 2014). Although triploids demonstrate more rapid growth, there is more variation in growth observed in triploids than in diploids (Nell, 2002). The faster growth becomes more pronounced after the first year, when diploids become more sexually active (Nell, 2002). Oysters grow faster at least partly because of energy reallocation from gametogenesis to growth, although this does not explain why triploids grow faster than diploids before sexual maturation (Nell, 2002). A further advantage of triploid oysters is that they can be marketed year-round while diploids are not consumable during summer while spawning (Nell, 2002; Payton *et al*., 2017). In the past, physical stress such as heat shock and hydrostatic pressure had been used to induce triploidy in oysters (Nell, 2002). Currently two main methods are used to produce triploid oysters, inhibiting polar body formation after fertilization or by crossing diploid and tetraploid brookstock (Dheilly *et al*., 2014).

In the plant kingdom, polyploidy is a major force in the evolution of both wild and cultivated plants (Ma *et al*., 2004; Sattler *et al*., 2016). At least 50% of angiosperms are estimated to have experienced polyploidy at least once in their evolutionary history (Ma *et al*., 2004; Wendel, 2000) A large number of plant species, including several important crops, have polyploid genomes. Natural allopolyploid crops include bread wheat (Haider, 2013), durum wheat (Haider, 2013), coffee (Clarindo *et al*., 2012), oat (Ansari and Thomas, 1983), and strawberry (Nosrati, 2015). Natural autopolyploid crops include alfalfa (Havananda *et al*., 2011), and potato (Sattler *et al*., 2016).

2.5.1 Polyploidy as a Breeding Tool

Plant breeders have used natural polyploidy and/or induced polyploidy in many ways to develop

increasingly productive and adapted cultivars (Sattler *et al*., 2016). Polyploid organisms often outperform their diploid counterparts in several aspects (Sattler *et al*., 2016). Consequences of polyploidy most important for plant breeding include buffering of deleterious mutations, increased heterozygosity, heterosis, reduced fertility, and increase in the size of plant organs known as the "giga" effect (Sattler *et al*., 2016). Polyploids may exhibit larger organs such as roots, leaves, fruits, flowers, and seeds, but polyploidy plants have lower growth rates (Sattler *et al*., 2016). Consequently, polyploids tend to flower later or over a longer period of time, which is desirable for ornamental plants (Singh, 1979). Furthermore, polyploids can be used as a bridge for genetic transfer between species when crossing is not possible due to differences in ploidy level (Sattler *et al*., 2016).

Sexual polyploidization or somatic doubling can be used to induce polyploids in plants (Sattler *et al*., 2016). Sexual polyploidization was commonly used for creating polyploids before the discovery of colchicine by Blakeslee and Avery in 1937, which allowed a breakthrough in somatic polyploidization research (Blakeslee and Avery, 1937; Ramanna and Jacobsen, 2003). Colchicine is an alkaloid which is extracted from meadow saffron (Sattler *et al*., 2016). Colchicine is now the most commonly used antimiotic substance for inducing ploidy in plants (Sattler *et al*., 2016). The chromosome number is doubled as a result of colchicine binding to α- and β-tubulin dimers, inhibition of microtubule polymerization during the cell cycle, and prevention of chromosome/chromatid migration during anaphase; thus, chromosomes do not pull apart as they normally do (Sattler *et al*., 2016). Before the work of Blakeslee and Avery (1937), other methods such as exposure to high or low temperature were attempted to induce somatic polyploidy (Blakeslee and Avery, 1937).

After the induction of chromosome doubling, it is essential to confirm the ploidy of the plants. Polyploids can be identified through direct and indirect methods (Sattler *et al*., 2016). The examination of physiological and morphological traits are examples of indirect methods. The stomata of plants are commonly examined as polyploid plants typically have larger stomata in lower density with higher number of chloroplasts per guard cell in comparison to diploids (Sattler *et al*., 2016). Indirect methods are quick and simple but are often inaccurate; therefore, direct methods are the best means of ploidy detection (Sattler *et al*., 2016). Chromosome counting is considered the most accurate way to detect polyploidy; however, it is highly specific to each species and time consuming. As an alternative, flow cytometry is a quick, reliable, and simple method to confirm the success of polyploidy production (Otto *et al*., 2015). With flow cytometry analysis, the ploidy level is determined by its correlation with relative or absolute DNA content (Sattler *et al*., 2016). Cultivated allopolyploidy species that have been successfully generated synthetically include triploid marigold (Sattler *et al*., 2016), and triticale (Ma *et al*., 2004). Synthetic autopolyploid crops include tetraploid rye (Wiśniewska *et al*., 2013), triploid sugar beet

(Ellerton and Hendriksen, 1959), and triploid watermelon (Grange *et al*., 2003). Although polyploids have been achieved in a large number of crop species, the polyploids do not always exhibit higher quality or yield than their diploid relatives, or the improvement occurs in plant organs that are not of interest for commerce (Sattler *et al*., 2016).

2.5.1.1. Seedless Watermelon

One of the most acknowledged and successful cultivated polyploidy crop is triploid seedless watermelon (*Citrullus vulgaris* Schard.). Reduced fertility as a result of abnormal meiotic pairing is considered a beneficial result of autotriploidy, as low number of seeds is a desirable characteristic in watermelon production (Sattler *et al*., 2016). Consumer preference of seedless watermelon is increasing; however, growing triploids is risky compared to the natural diploid forms. The process of creating triploid seed is slow and labour intensive (Grange *et al*., 2003). A diploid ($2x = 22$) watermelon seedling is treated with colchicine at the apical meristem to obtain a tetraploid ($4x = 44$) form (Grange *et al*., 2003; Sattler *et al*., 2016). The tetraploid watermelon is then selfed until a stable line is developed (Grange *et al*., 2003). The pollen of the diploid form is used to pollinate the stigma of the induced tetraploid to produce triploid progeny ($3x = 33$) (Sattler *et al*., 2016). Triploid cultivars require special production due to their need for pollination by diploid cultivars as the triploids produce sterile pollen (Núñez *et al*., 2008). It is critical for the cultivars of different ploidies to be easily identifiable to prevent seedless and seeded fruit mixing during harvest (Núñez *et al*., 2008). It costs approximately four times more to produce triploid seedless varieties of watermelon in comparison to diploids, therefore increasing the market price (Sattler *et al*., 2016). High production costs of triploid watermelon are due to the high labour cost of producing the seed (Grange *et al*., 2003); therefore, it is important to obtain high yields in order for the crop to be profitable for the farmer (Núñez *et al*., 2008).

2.5.1.2. Chamomile Polyploidy

Polyploidy is important for the production of chamomile. Approximately one quarter of chamomile varieties currently cultivated worldwide are autotetraploids that have been generated artificially by colchicine (Otto *et al*., 2015; Sattler *et al*., 2016). Tetraploid forms for chamomile outperform diploid forms in biochemical and morphological traits, including quantity of essential oils, taller standing height, larger flower heads, and a higher seed weight (Sattler *et al*., 2016). The flowering time in chamomile depends on genetics as well as ploidy level, with tetraploids flowering later than

diploids (Otto *et al*., 2017). Furthermore, tetraploid varieties are hypothesized to protect against unwanted hybridization of cultivated chamomile with wild diploids (Otto *et al*., 2015). This is important as the contamination of cultivated chamomile with wild chamomile can decrease yields in subsequent generations and potentially not fulfill the requirements for medicinal use (Otto *et al*., 2015). In 1962, the first tetraploid (4x = 36) variety called Bodegold was developed as a cultivar for production in Germany (Sattler *et al*., 2016). Subsequently, tetraploid varieties were released by Slovakia, Poland, Romania, and Bulgaria (Sattler *et al*., 2016).

This thesis is part of a larger study with the goal to exploit naturally-occurring genetic and phenotypic variation to breed triploid chamomile varieties (Otto *et al*., 2015). Triploid chamomile is desirable because of the production of sterile seeds, and advantages for the flower heads. Crossing diploids and tetraploids produces triploid plants. In order to produce triploid chamomile efficiently, a mechanism to prevent self-fertilization of the mother plants is necessary (Otto *et al*., 2015). Through the distorting effects of an odd chromosome number on meiosis, triploidy leads to non-viable gametes and sterility of seeds (Otto and Whitton, 2000). Chamomile is fairly resistant to herbicides and is able to lay dormant for 10 to 15 years before germination (Otto *et al*., 2015). Therefore, sterile seeds are desirable in chamomile production because this would prevent or minimize the germination of chamomile seeds as a weed in the same field in subsequent years (Otto *et al*., 2015). Furthermore, triploid chamomile plants are advantageous as they are expected to have a longer flowering period, thus lengthening the harvesting period due to delayed senescence (Faehnrich *et al.*, 2013). As seen in oysters, sterility in triploid chamomile could also be associated with increased energy allocation to the growth of the economically important flower heads rather than sexual reproduction (Otto *et al*., 2015). It is critical to understand the fertilization and crossing barriers of chamomile, as well as how the environment affects fertilization, in order to conduct a successful plant-breeding program.

CHAPTER 3

ABSTRACT

The environment in which a plant grows directly affects variation in crop performance. Understanding the fertilization process and potential crossing barriers of chamomile is a critical first step in improving the breeding and commercial production of chamomile. The objective of this study was to examine the outcrossing ratio of multiple field crosses completed at various locations within Germany in 2014. Six synthetic crosses, where the parental lines of the same ploidy level were grown in a plot and allowed to intercross, were performed. Each cross was completed between plants of the same ploidy replicated at two or three different field sites within the region of chamomile production in Germany. There was a wide range of outcrossing frequencies exhibited in the crosses tested. The overall percentage of outcrossed diploid progeny ranged from 18.75% to 89.29%, with an overall average of 57.56%. The overall percentage of outcrossed tetraploid progeny ranged from 5.41% to 97.92%, with an overall average of 58.23%. The results did not show a statistical difference between geographical locations; however, in all crosses there appeared to be an effect associated with crossing direction. In many crosses the frequency outcrossed was considerably higher in one cross direction than the other. Future research should focus on parent-of-origin effects in chamomile and how they affect the fertilization process.

3.0 Evaluating Geographical Location Effect on the Outcrossing Ratio

3.1 Introduction

The environment in which a plant grows directly affects variation in crop performance (Elias *et al*., 2016). A major challenge faced by plant breeders is the differential response of genotypes from one environment to another, a phenomenon known as genotype by environment (GxE) interactions. Environmental influences on the mating systems of plants are considered to be important (Cruzan *et al*., 1994). As a result, breeding programs aim to develop new varieties based on adaptation to specific environmental conditions (Elias *et al*., 2016). In order to release a new variety and provide cultivation recommendations, regional crop variety trials conducted at multiple sites within a targeted production region should be completed.

Understanding the fertilization process and potential crossing barriers of chamomile is a critical first step in improving the breeding and commercial production of chamomile. Accordingly, it was of interest to industry partners to evaluate how the outcrossing frequency would be affected according to location throughout the key commercial growing locations in central Germany. The objective of this study was to examine the outcrossing ratio of multiple field crosses completed in various locations within Germany in

2014. Two hypotheses were constructed. Firstly, it was hypothesized that there would be no significant difference in the outcrossing ratios between the different geographical locations tested, as all locations were in the commercial growing region of Germany. Secondly, it was hypothesized that diploid crosses would exhibit higher outcrossing rates than tetraploid crosses due to strong self-incompatibility.

3.2 Materials and Methods

3.2.1 Plant Material and Description of Growing Locations

Prior to beginning the research for this Master thesis, six synthetic crosses, whereby parental lines of the same ploidy level were grown in a plot and allowed to intercross, were completed throughout central Germany during 2014. Permission to use this plant material as part of this thesis was formally given by Dr. Lars Otto of the Leibniz Institute of Plant Genetics and Crop Plant Research (Appendix 1), who has worked with Dr. Sharbel on chamomile research for approximately 10 years. Diploid and tetraploid parental genotypes were selected in order to capture a wide range of genetic diversity in addition to the availability of elite breeding material and registered varieties at the time of study (Tables 3.1, 3.2).

Table 3.1. Diploid plant material used for assessment of outcrossing ratio at different geographical locations.

Parent	Variety/Population	Source	Country of Origin
20-2	Promyk	Pharmaplant GmbH	Poland
13-2	Argenmilla	Pharmaplant GmbH	Argentina
12-5	Germania	N.L. Chrestensen GmbH	Egypt
20-04	Promyk	Pharmaplant GmbH	Poland
04-2	MAT19	Genebank, Gatersleben	Germany
18-2	Population	Martin Bauer GmbH	Croatia

Table 3.2. Tetraploid plant material used for assessment of outcrossing ratio at different geographical locations.

Parent	Variety/Population	Source	Country of Origin
24-01	Elite Material	Pharmaplant GmbH	Germany
29-04	Lutea	Farm Dilshofen	Germany
23-04	Elite Material	Pharmaplant GmbH	Germany
29-01	Lutea	Farm Dilshofen	Germany
22-06	Elite Material	Pharmaplant GmbH	Germany
16-08	Trade Material	Company Agbina	Russia

The parental genotypes were diploid and tetraploid elite breeding material produced by *in vitro* culture propagation by Pharmaplant GmbH, Germany. Parental plants were raised in a greenhouse and clonally propagated by cuttings prior to geographical isolation. Each cross was completed between plants of the same ploidy replicated at two or three different field sites (Figures. 3.1, 3.2, 3.3) within the chamomile production region of Germany. All locations were within a relatively small area of the country; latitude and longitude of each location were recorded in Tables 3.3 and 3.4. These field sites were private gardens carefully selected by the industry partner Pharmaplant GmbH, to ensure no other chamomile plants were in the area, thus preventing cross contamination of pollen. No specific information on the pollinators present in each growing location was available. Temperature and wind historical data from the time of planting was collected from an online source and summarized in Figures 3.4, 3.5, 3.6, and 3.7 (timeanddate.com). Parents 12-5, 20-04, 29-04, 24-01 were transplanted to the field August 28 and flower heads were harvested September 15. Parents 20-2, 13-2, 04-2, 18-2, 29-01, 23-04, 22-06, and 16-08 were transplanted to the field and flower heads were harvested at unknown dates in the month of July. Therefore, growing locations used in July included Erfurt, Bretleben, Borxleben, Hettstedt, Freyburg, and Schönfeld. Erfurt, Bretleben and Borxleben are located relatively close to one another (Figures 3.2, 3.3) and thus considered under the same weather data, as are Hettstedt and Freyburg. The growing locations used from August 28 to September 15 include Erfurt, Ritteburg, Tiefthal, and Töttelstädt. As all of these locations are close together, they were considered under the same weather data.

Figure 3.1. Map of Germany. Red indicates the area used for diploid crosses, green indicates the area used for tetraploid crosses.

Figure 3.2. Locations of diploid crosses completed throughout central Germany.

Table 3.3. Latitude and longitude coordinates of the towns used for diploid plot locations in central Germany.

Town	Latitude	Longitude
Hettstedt	51.633330 N	11.500000 E
Borxleben	51.396670 N	11.228330 E
Bretleben	51.336435 N	11.232659 E
Töttelstädt	51.008100 N	10.883600 E
Erfurt	50.978060 N	11.029170 E

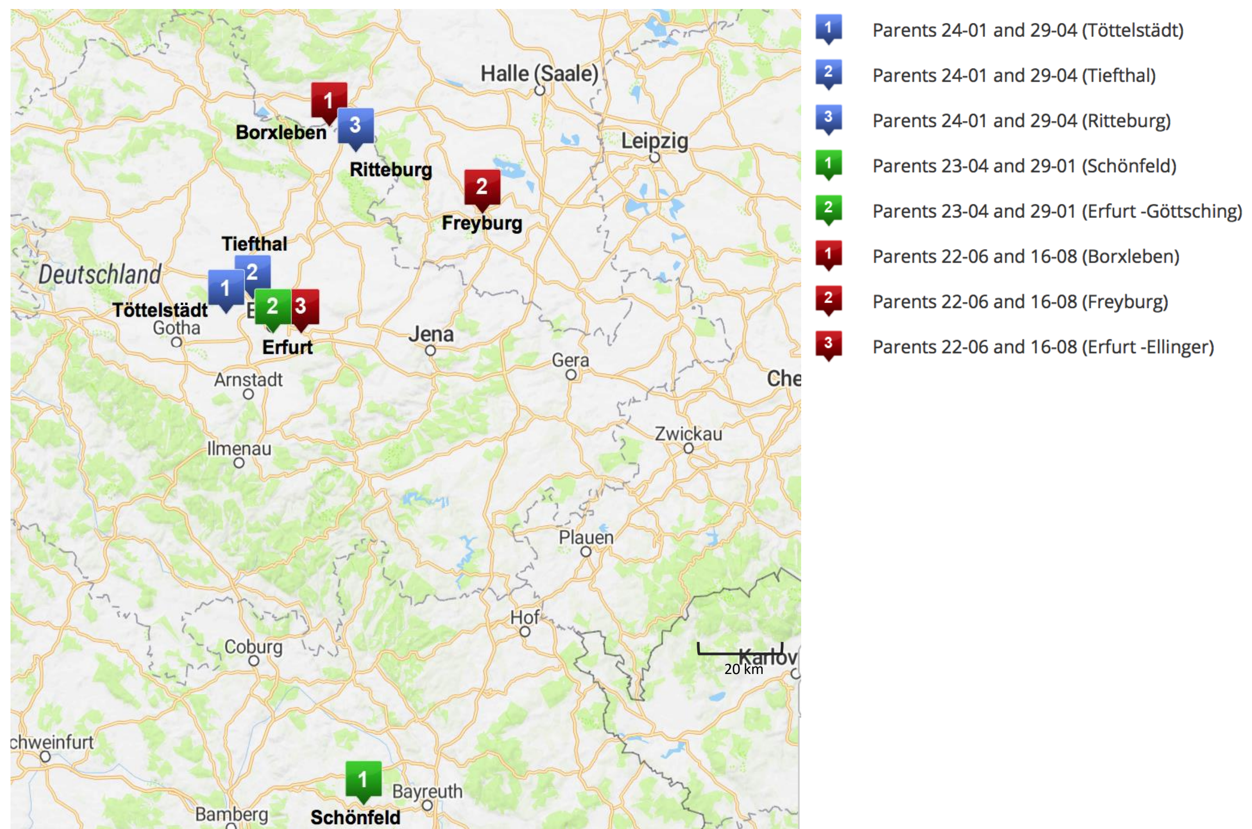

Figure 3.3. Locations of tetraploid crosses completed throughout central Germany.

Table 3.4. Latitude and longitude coordinates of the towns used for tetraploid plot locations in central Germany.

Town	Latitude	Longitude
Ritteburg	51.344754 N	11.326311 E
Freyburg	51.212780 N	11.769720 E
Borxleben	51.396670 N	11.228330 E
Töttelstädt	51.008100 N	10.883600 E
Tiefthal	51.022200 N	10.948900 E
Erfurt	50.978060 N	11.029170 E
Schönfeld	49.934891 N	11.352323 E

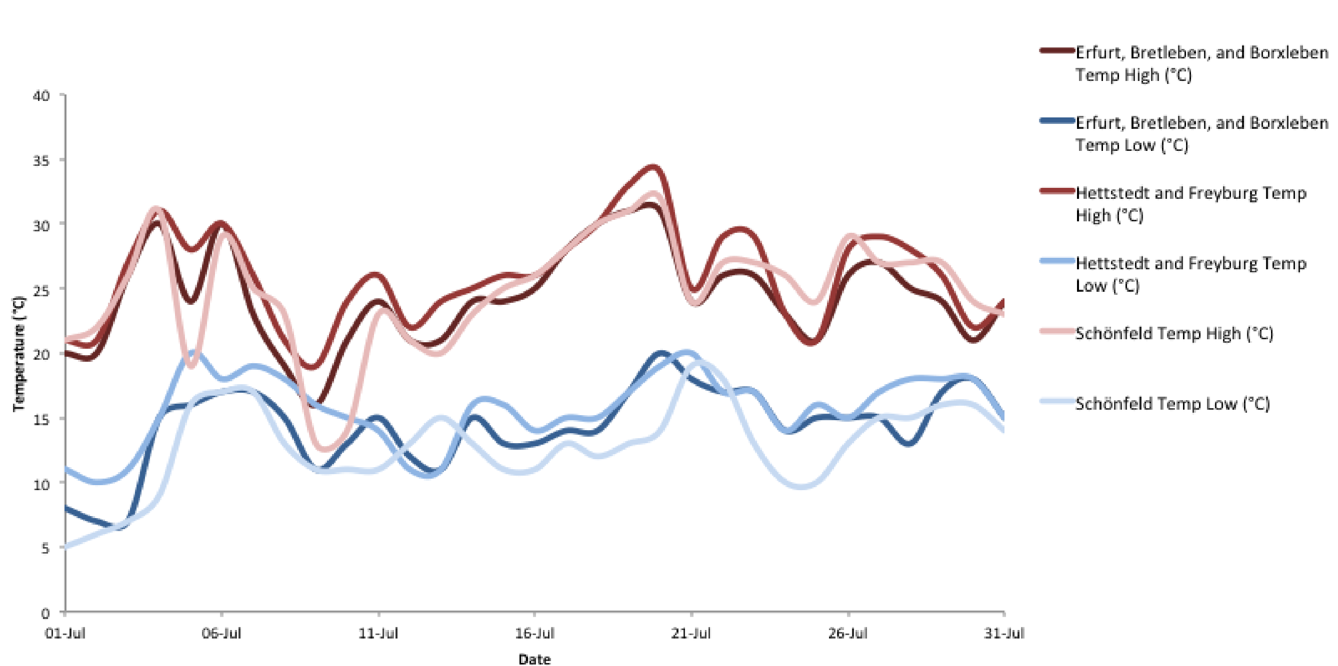

Figure 3.4. High and low temperature recordings of Erfurt, Bretleben, Borxleben, Hettstedt, Freyburg, and Schönfeld, Germany in July 2014.

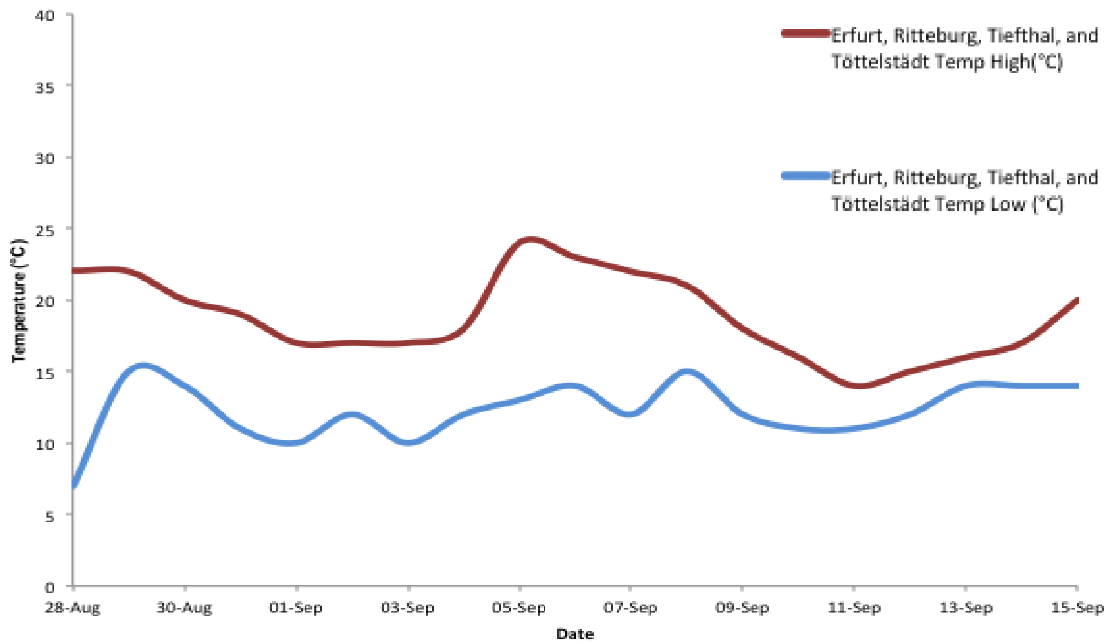

Figure 3.5. High and low temperature recordings of Erfurt, Ritteburg, Tiefthal, and Töttelstädt, Germany from August 28 to September 15, 2014.

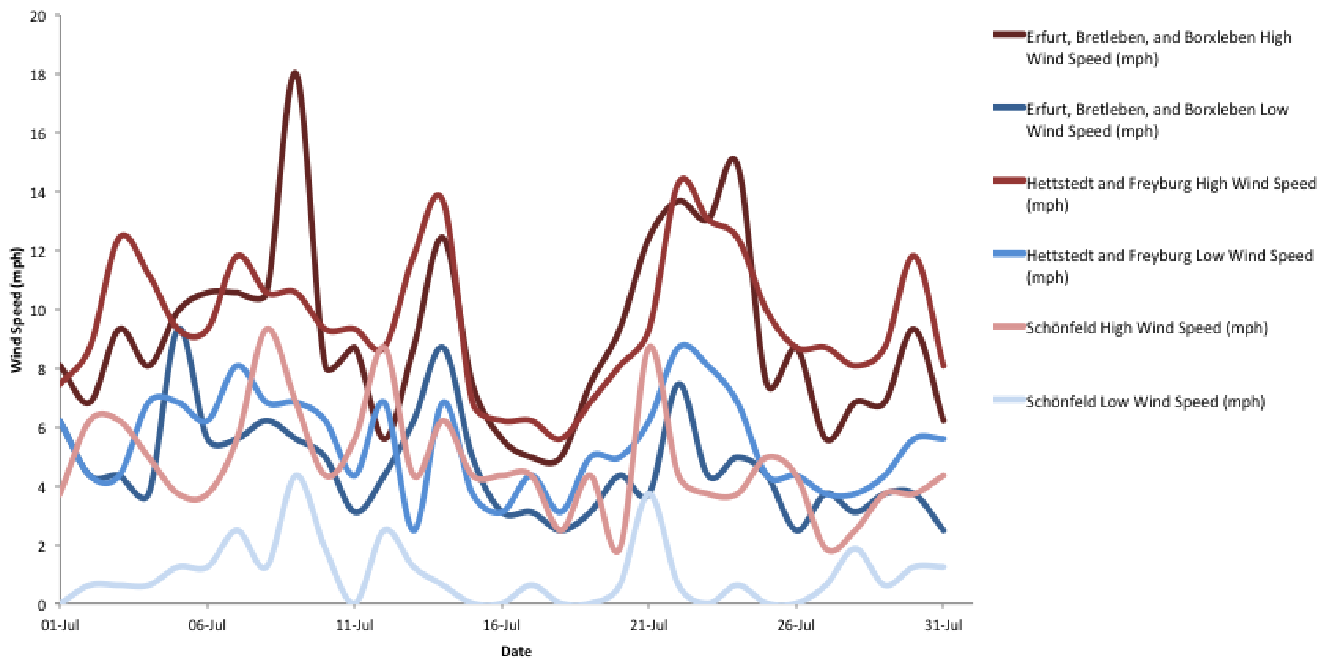

Figure 3.6. High and low wind speed recordings of Erfurt, Bretleben, Borxleben, Hettstedt, Freyburg, and Schönfeld, Germany in July 2014.

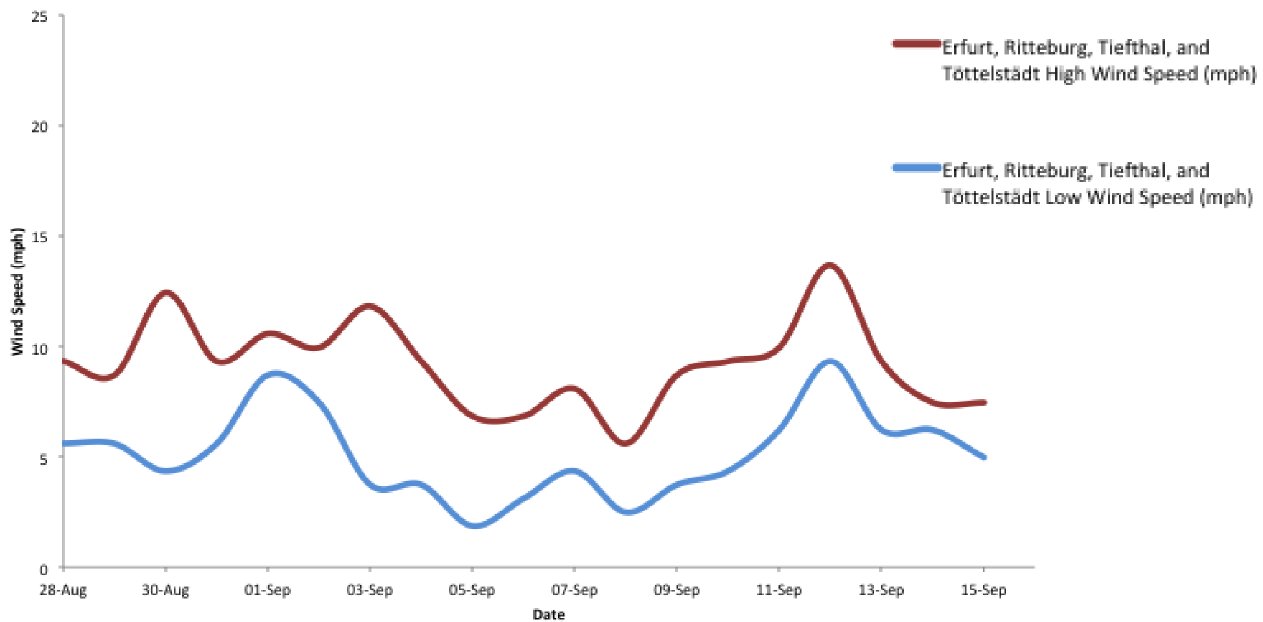

Figure 3.7. High and low wind speed recordings of Erfurt, Ritteburg, Tiefthal, and Töttelstädt, Germany from August 28 to September 15, 2014.

For all crosses, between three and five clonal plants of each genotype were transplanted prior to flowering to a rectangular plot with 15 cm spacing between plants (Figure 3.8A). This allowed each genotype to act as both a mother and father plant. Flower heads were hand-harvested on multiple days when the individual flower heads were mature. Maturity was determined when the white ray florets were parallel to the stem (Figure 3.8B). Progeny (seeds) were sown in the greenhouse approximately 8 weeks after harvest for further analysis. For each cross, approximately 40 mg of fresh leaf tissue from F1 plants was collected into separate wells of a 96-well deep-well plate containing two grinding balls on ice. The plate was then thermo-sealed for storage at -80°C for approximately two years until further analysis, beginning with DNA extraction as part of this thesis. Fourteen plates with 96 progeny DNA samples per plate were prepared, totaling 1344 individual DNA samples. Completed crosses from 2014 are summarized in supplementary tables in Appendix 2.

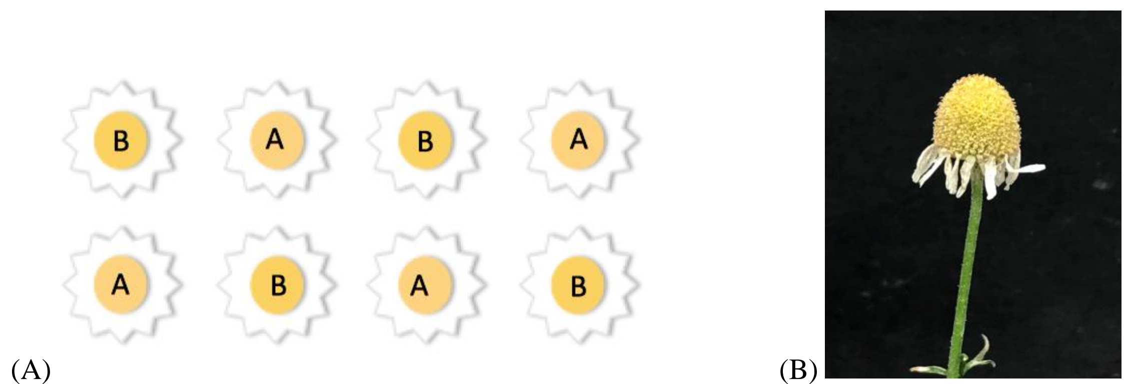

Figure 3.8. Plant material. (A) Cross layout. Two parental lines of the same ploidy were transplanted into a plot and allowed to intercross. Each genotype, A and B, had between three and five clonal plants per plot with 15 cm spacing between plants. (B) Mature chamomile flower head.

3.2.2 Experimental Procedures

3.2.2.1 DNA Extraction

The progeny DNA was extracted from the leaf tissue using the Beckman Coulter Agencourt Choloropure kit. A plate with F1 DNA was taken out of -80°C storage and placed into liquid nitrogen for 3 minutes and then ground twice at 1700 rpm for 20 seconds using a Geno/Grinder (SPEX SamplePrep) to homogenize the samples. The plate was then centrifuged briefly for approximately 15 seconds to bring the ground plant material to the bottom of the well. Afterwards, 300 µL of Lysis Master Mix (300 µL of Lysis Buffer and 2 µL of RNase per sample) was added to each well. The plate was then thermo-sealed and placed into a 65°C water bath for 10 minutes with periodic shaking to the plate for mixing purposes. After incubation, the plate was centrifuged at 5000 rcf for 10 minutes. Following this step, 150 µL of clear supernatant was aspirated into a new 96-well special plate and 150 µL of Bind Buffer mix (6 µL Bind Buffer and 150 µL 100% isopropanol per sample) was added to each well and mixed well. The supernatant and Bind Buffer mixture was incubated for 5 minutes at room temperature and then placed onto a magnetic separation stand for 5 minutes. Once the magnetic particles had migrated to the wall, the supernatant was removed. The plate was then removed from the magnetic separation stand and 300 µL of wash buffer was added to each well to re-suspend the pellet. After two minutes of incubation at room temperature, the plate was once again placed onto the magnetic separation stand until the magnetic particles had migrated to the wall, this step taking approximately 5 minutes. The supernatant was removed, the plate was removed from the magnetic separation stand, and the pellet was the washed twice with 300 µL of freshly prepared 70% ethanol. After two minutes of room temperature incubation, the plate was placed on the magnetic separation stand and the supernatant was removed after the magnetic particles had migrated to the wall. After the supernatant was removed, the plate was placed in a vacuum

concentrator for 5 minutes at 45°C to dry the magnetic particles. Once dried, 80 µL of TE buffer was added and the DNA re-suspended. The plate was sealed and placed in a water bath for incubation at 65°C for 2 to 4 minutes before being placed onto the magnetic separation stand for a final 5 minutes. Once the magnetic particles had migrated to the wall, 50 µL of the supernatant was transferred into a new 96-well plate. Following the DNA isolation procedure, the DNA concentration of the samples was checked on a 1% agarose gel using gel-electrophoresis (Figure 3.9). This gel was used as guide to determine approximate DNA concentration in order to prepare the DNA dilutions prior to running the PCR. The very bright bands were diluted 49:1 H_2O to DNA, mid-brightness bands were diluted 9:1 H_2O to DNA, and faint bands were not diluted.

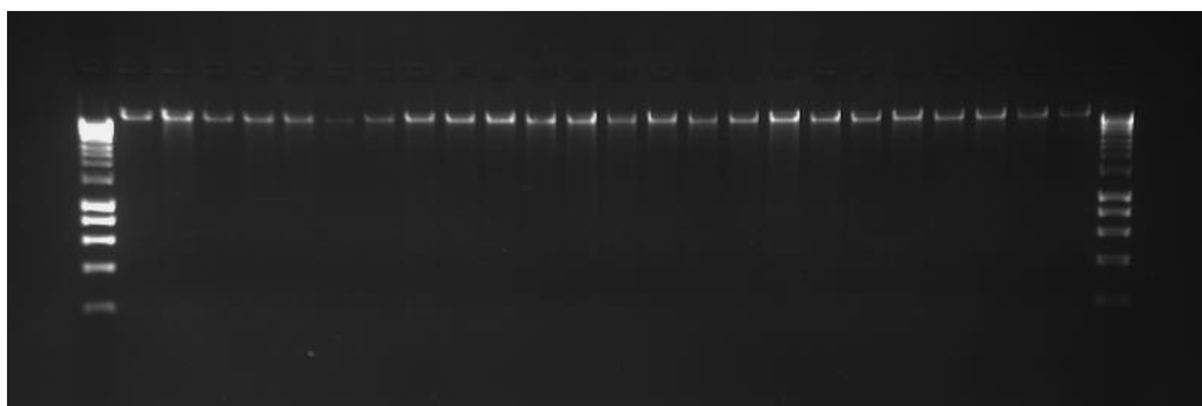

Figure 3.9. 1% agarose gel image of DNA extraction used to determine approximate concentrations for DNA dilutions. The molecular weight marker standard is 1 kb Bioline HyperLadder™ with fragment sizes of 10037 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500/1517 bp, 1000 bp, 800 bp, 600 bp, 400 bp, and 200 bp.

3.2.2.2 Polymerase Chain Reaction (PCR)

PCR was performed on 90 samples from each plate of DNA with three controls: maternal DNA, paternal DNA, and a negative control (total 96 reactions per plate). Each control was run in duplicate to ensure there was no contamination. Each PCR reaction contained 0.5 µL of 10 µM forward labelled and reverse primers, 4.0 µL nuclease free water, 1.0 µL DNA template, and 6.0 µL of 2x Taq FroggaMix. PCR was run with the following protocol: one cycle of 95°C for 5 minutes, followed by 34 cycles of 94°C for 50 seconds, 55°C for 45 seconds, 72°C for 2 minutes, then a final extension of 72°C for 5 minutes and a 12°C hold. PCR reactions were carried out using an Eppendorf Mastercycler pro S PCR system. Following PCR, the product was run on a 1% agarose gel to confirm the presence of amplified products (Figure 3.10).

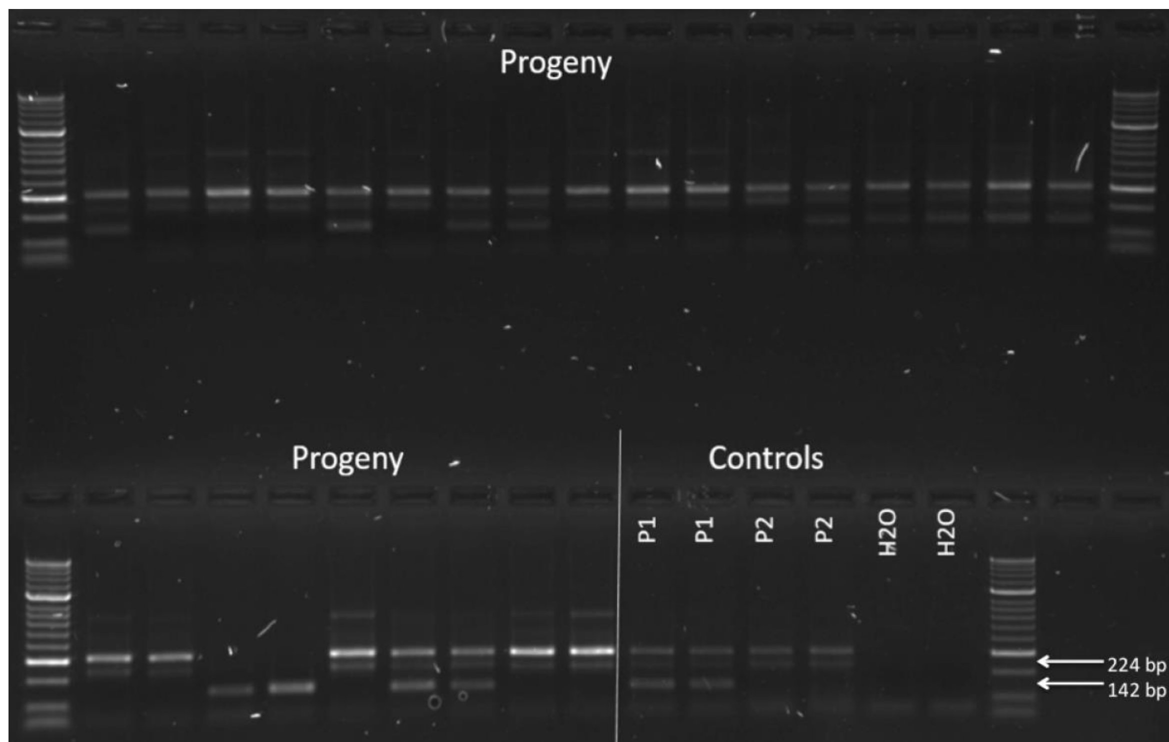

Figure 3.10. 1% agarose gel image of PCR amplified products from Plate 25 using primer KamSSR-36. The molecular weight marker standard is 50 bp Bioline HyperLadder™ with fragment sizes of 2000 bp, 1800 bp, 1600 bp, 1400 bp, 1200 bp, 1000 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 200 bp, 100 bp, and 50 bp. Progeny were compared to controls of parent 1 (P1) in duplicate, parent 2 (P2) in duplicate, and a negative control (H_2O) in duplicate. The loci analyzed were 142 bp and 224 bp (Table 3.8).

3.2.2.2.1 Primers and Microsatellites

Previously, 17,751 candidate simple sequence repeat (SSR) markers were generated from RNAseq reads in an inflorescence-specific transcriptome data set using the SciRoKo program (Kofler *et al*., 2007; Sharbel and Otto, unpublished data). PCR primers for 100 preselected microsatellite loci were developed and tested for polymorphisms in four genotypes from different origins that were expected to be reasonably diverse. Six SSR markers that exhibited polymorphism were selected to be used for analysis in this M.Sc. thesis (Table 3.5). Original contig sequences of each primer used for the development of the microsatellite analysis are summarized in Table 3.6. It was important to develop SSR markers that showed polymorphism with different alleles between the crossing parents in order to analyze the paternity of the progeny plants.

Table 3.5. SSR markers that exhibited polymorphism selected for analysis.

Primer Name	Forward Sequence (5’-3’)	Reverse Sequence (5’-3’)	Microsatellite Motif$_{(length)}$
KamSSR-8	CAGCCTAAATCCTGGGGTACT	GTTCTTTCATGAATTCCCGTTC	$(TAAC)_4$
KamSSR-23	AATACGAACCAAACGGACTGA	CAAAATTGACAGGTCGAAACC	$(AACCG)_3$
KamSSR-36	GTCACAGAGAGTCGAGCTTGG	GATGTTGTTTCGGGTTCAAAG	$(GTGA)_4$
KamSSR-65	AGACTGGATGGGTGAAGTGTG	GCTGTTGGAGCAAAATGAGAG	$(TCAC)_3$
KamSSR-81	CTACCCCACCACAACAACAAC	CACCAACACCATCCTCAGAGT	$(AAC)_6$
KamSSR-85	GGCATGCATAGAGTCCACAAC	GCTGCAGTTTCAGATCAGGAG	$(AC)_9$

Table 3.6. Original contig sequences of each primer used for the development of the microsatellite analysis. Bold and underlined sections representing the forward primer and reverse primer.

Primer Name	Original Contig Sequences used for the Development of the Microsatellite analysis
KamSSR-8	TGATCTTCAGCAACGCCGGAATGCGTGGATTAGTTAGGGAAAATTCACATCGTTCTTCAATTCAACAATA ACAATATCATCAAAAACGCAAAACCAGTTTTGGATTAAGAAAAAACCGTAAGAATTAAATCACAAAGGC ATATCG**CAGCCTAAATCCTGGGGTACT**CTTATAAGATATATAAACATCAGCACATTTTATGAACCTATG ATCTTATGTTTTCCTTAATATACATTGTTAACTAACTAACTAACTATGTGAATTTGGGCCAAAATCCTAAA ACAATCAATAGGCATATAAGCAATTTCAAAGAGCTATCCAAACACACTATAA**GAACGGGAATTCATGAA** **AGAAC**ATTAACATATACAAACAAAGACACAATTAGCACATAAGCACTCAAATAAGCATATAGCTACGCA CAGAATTCACAAAAAGAACATATCATTCGCCTTCTTTTTAAGGATATGAGTTCCATTAAAGACTTCACTC
KamSSR-23	TATATATCCTTCTGGCTTCGGTTTGAAATGGTTCGGTTTTGTTCAAGCCAGCACTGACAAAAAAACACATT TAAAACGTAT**AATACGAACCAAACGGACTGA**CATAAAACCGAACCGAACCGAGTCACTTCAGTTTGATT CCCAGTTCTTGTATTTCATAATCGATTTTTCGATTTTTGGCTTGGTTCGGTTTGGTCGGGCCGGGTC**GGTTT** **CGACCTGTCAATTTTG**ACCCATTTACTAAAA
KamSSR-36	CAGAGAGTGGACGTA**GTCACAGAGAGTCGAGCTTGG**CTTTTAGCTCAGAGTGTCGCCATGGTGAGTGA GTGAGTGAGTTCAACTTCAAACATCTGTCTGATGAATTCTCTGATGTTCAATAACTCAGCCGAAATT**CTTT** **GAACCCGAAACAACATC**TGATGATATGACCAGAGATTCTTTGGCATAGAGACTGTGGTCAGCA
KamSSR-65	CAAGAAAGAGTGTTACTGTAATAAACAATAAGAATGTGTTCACTTTATTACAAGGTTCAGCATATAAAAA GGGCAATAACTATTTTACAAATGTGACAGATATGCAGGAAATTGGTACTCAATAGACTAACTAAGATAAC ATAAGATGTGACATCTTTGACCCATTTTTAATAACCGTCGCCTTGACGTGTGTTTCATCTCAGTAGCTTTG AACGAGTTAAGCTAACATTAAGTAAATAGGAAACATGTAAAGCAGGTTAAAAACATACCAAGCGTATTC AAATGCATCAAGCATCTGAAATCCCTGTTATATATTTTTGCAGTAAGATTACTTTTTTTGTATGAAGGAA ACCCCACCGAGCCACCTTTGAGAAACTTTAGGCTTCACAGACCACCCCGCAAGACCCAAGGAGTATGGTG AATCATGGTCAATAATAAGACAAGGGCGGGTGGACCCAAGGAGTTTGCAGTTAGCGGAATTCTAACTTTG AGACCTCATATTTGGGACTCTCAAACCCTTACCAGTAGGCTGCCCCCTTGGGGTTTAATAACATAACTTCT GTAATCATGTTTAGCACACATTATATTTCTTAAATTCAAAT**AGACTGGATGGGTGAAGTGTG**TGTTGGTC ACTCACTCACTCAACATGTGTAACTACATAAATAGCCCTCTAAGTTCAACAAACTAGTCAGTCAGATTAC ACTCACAATTGTAACTTAACTACATCTACTAAAAGGC**CTCTCATTTTGCTCCAACAGC**TCAGTTAGCTTG TACTGTGTGACTGAAACTAATATTTGAGCATGACTGAATTCCACACACCAGTCAATACATTCATAATATA GCTATTAGCACTACATAACCTAGCCGTGATTTATATCAGACTTTAAGCCCTGCCCAAATATGTCAGAACTC TCATAAGCATGCAAAATAACATAATCGCGTGAGTGGAATTTCAACCAAGGAGAGGGTTCTTTCAAAACTA AAACAGACATGTATATGTGATTTAGCAAGACTCGTGTGATTTAGCAACTATCATACGAGATTTAGCAAGC AAAGTATAATTTGTGAGGTATAGAGTTTGTGCAGGCAAACATGAGTTTCTCCAAAGCTGTGTGCATGAAC ACAGCTAAGGGGCTGTTTACTTTTTGCTTAATGGCCCATCTGGTAACAATATCCTTCTCAAACAGAATTTT TTGTATTTTCTTTGTTACTTATTGGCAAGAGGAATCATTATTGTGGAAGAGTGTAAAAACCCAACTCATTC CTATCCAGGATATCCTTAAATATTTCTTTTATGTATTCACTCATCATCAAAGTTAAATATACCCAATTCATT CCTCCATCACCACCGATCACCCTTCCAATCCTCCATCACCACAATCACCCTTGTTCATACTGACCCATTCC CACCGAGCTGCTCACTCCGACACCTCCTTCAACGTCATCAGTTTTGCATTTCTTCATATCCCTTGTTTGCTT ACGTGGATATCGTTTCCCTCAATCGTTTCGCTTCCTTTCATTTATTGGACTAAAAGATTCAACAA
KamSSR-81	CAACCTTCGTCCTAAAATGTTTCCACTCACCATACTCTTCACAACACTCGTCGTCACATCCATCTCCAACG ACGCCTACCCATCACC**CTACCCCACCACAACAACAAC**CTGCGACCTTAATAACAACAACAACAACAACC CAACACCAGTTCGTAAAGAAACATACGACAATGGTCAGATAATAGACATAAGTCACAGGTACCATCCTG ACATGCCATCATGGG**ACTCTGAGGATGGTGTTGGTG**AGATTATAAGTTTGCCTAGGAGTATGAAGAAT GGGTCACTTG
KamSSR-85	TCTCTGAGGCACGGTGAATCTCTGAAAACACACAGCATATGAATAACTGATGGTGCCACACAGACACCCG TCATAAAACCAAAGAATGAACTGTAAACAGGACAAAAGGTATGATACTTTTTATCGACTAATAGTCATGT AA**GGCATGCATAGAGTCCACAAC**TACTACCTGAGGCAAATCATGCTTTTAAACTAAGTTATACATGGAC CATTGTACACACACACACACACACTCATAATTCCTAACGAGTTGGCCTACGAAGAT**CTCCTGATCTGAA** **ACTGCAGC**TTGCGATGTCTCAAACGTCGGATGTGCAGGATATATACTGATTGTTGGAAGGAATTATTTAG TGTATCGCATTACCTATATTACAAATATGGCAGGACTAAACAACACTACAACAGCAGCCCTATGTGTACC TTCATATTTTATTGACTCCTTCAACTTTATTACCTGGTTTATAGAACGGTTACCATTCAATATGTTGTAGAA TCTTAATTTTAGATTTACCACAACTAAGAAAAGATTAGTAAGTCAGGTTAGATCAAGTACCTAGAGGCAT TCCGAAAG

3.2.2.3 Microsatellite Fragment Preparation

An ethanol precipitation was conducted to prepare the samples for the CEQ™ 8000 Genetic Analysis System from Beckman Coulter. PCR products were diluted according to the label color: D2 (black) 1.2 µL, D3 (green) 2 µL, D4 (blue) 1.0 µL, with nuclease free water up to 5 µL for each PCR product. Multiple PCR products of the same DNA sample were combined into one sample with a maximum of one PCR product of each colour per sample. Then 60 µL of ice-cold 100% ethanol was added to each sample and the plate underwent centrifugation (4750 rpm, 40 minutes, 4°C) before discarding the supernatant. Each sample was washed with 200 µL of ice-cold 70% ethanol and the plate once again underwent centrifugation (4750 rpm, 40 minutes, 4°C) before discarding the supernatant. The plate was vacuum dried in a concentrator for 30 minutes at 45°C. Once dried, 25 µL of sample loading solution and size standard master mix (24.8 µL sample loading solution, 0.3 µL CEQ™ size standard) was added to each sample and mixed well, ensuring no bubbles had formed. If bubbles were present, the plate was centrifuged for approximately 20 seconds. The plate was incubated in the dark at room temperature for 30 minutes. Following incubation, the samples were transferred to a special 96 well plate for the capillary fragment analyzer (Beckman Coulter CEQ™ 8000 Genetic Analysis System), covered with one drop of mineral oil to prevent evaporation, and then denatured for 5 minutes to 95°C. The plate was then immediately put on ice and covered with tin foil to prevent light penetration during transport between machines before loading into the CEQ™ 8000 analyzer.

3.2.2.4 Capillary Electrophoresis

Amplified fragments were separated with the Beckman Coulter CEQ™ 8000 Genetic Analysis System. This machine is a fluorescence-based system that uses capillary electrophoresis to measure PCR amplicon size (Beckman Coulter, 2004). The machine analysis parameters and size standard were decided depending on the size of the fragments observed on the agarose gel. The Frag-3 method standard settings (capillary temperature 50°C for 45 minutes separation time per sample) and size standard 400 were used for samples 400 base pairs or less. The Frag-4 method standard settings (capillary temperature 50°C for 60 minutes separation time per sample) and size standard 600 were used for samples 600 base pairs or less.

3.2.2.5 Fragment Analysis

The CEQ™ 8000 produced an electropherogram in which the peaks represent alleles. These peaks were manually scored and recorded in Microsoft Excel. In the Beckman Coulter software, GenomeLab GeXP Genetic Analysis System, the parental genotypes were observed for peaks present in one parent and absent in the other (Figure 3.11). These identified peaks (Tables 3.7, 3.8) were then scored in the progeny as absent, present, or unable to score using conservative scoring criteria. Identical peaks that were present in both parents of a cross were not considered. Amplicon size was standardized between plates by comparison of the CEQ™ internal size standards. As a result of variation in overall signal strength between plates, different thresholds were used to assign peak presence and absence scoring criteria (Table 3.9).

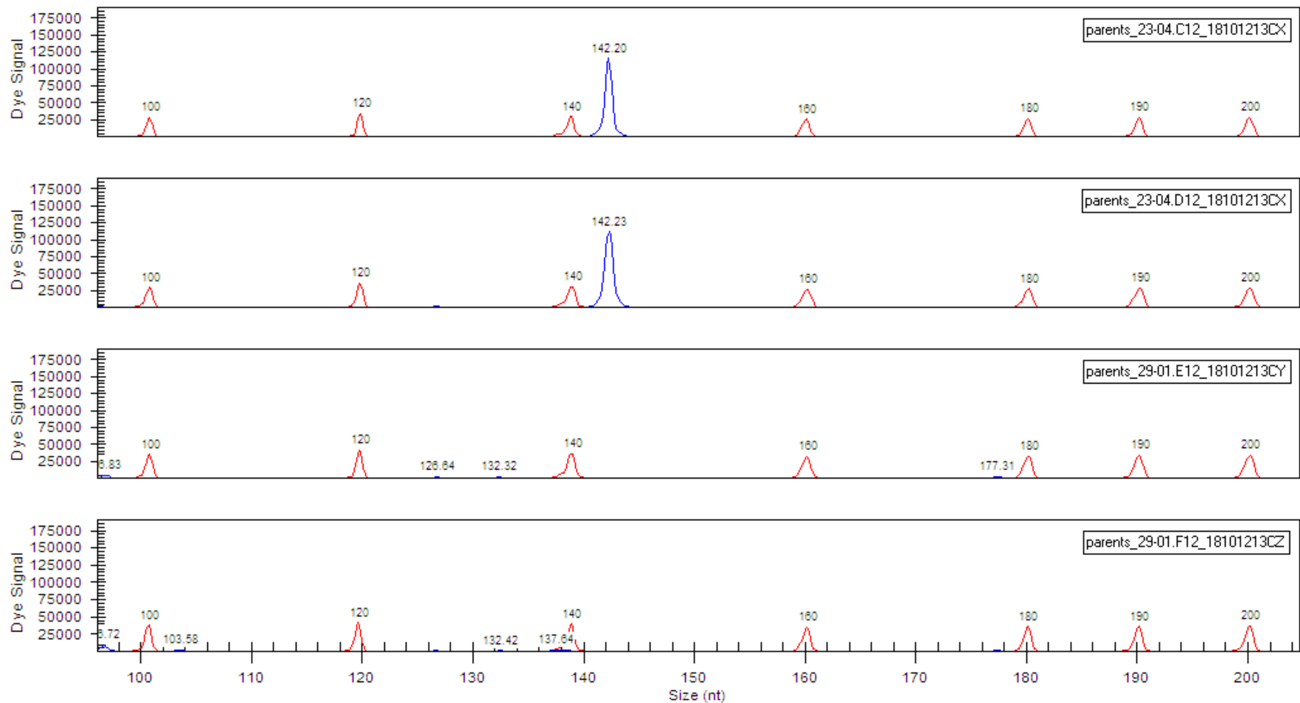

Figure 3.11. Electropherogram from CEQ™ 8000 of four samples using primer KamSSR-36 on PCR plate 24. The Y axis is the relative fluorescence (dye signal), and the X axis is fragment length in nucleotides. Red peaks are size standards. The top two panels show the presence of peak 142 (blue) in parent 23-04 (in duplicate) and the bottom two panels absence of peak 142 in parent 29-01 (in duplicate).

Table 3.7. Peak (loci) locations on electropherogram for diploid parents of *Matricaria recutita* used in crossing trials.

Mother	Father	Ploidy	Location	PCR Plate Number	Primer Used	Peak Locations (bp)
20-2	13-2	2x	Bretleben	18	KamSSR-36	224
						241
			Erfurt	18	KamSSR-36	224
						241
			Borxleben	19	KamSSR-36	224
						241
13-2	20-2	2x	Bretleben	18	KamSSR-65	111
			Erfurt	19	KamSSR-65	111
			Borxleben	19	KamSSR-65	111
12-5	20-04	2x	Töttelstädt	20	KamSSR-36	322
						482
			Erfurt -Hübner	20	KamSSR-36	322
						482
			Erfurt -Beier	21	KamSSR-36	322
						482
20-04	12-5	2x	Töttelstädt	20	KamSSR-65	366
					KamSSR-36	237
						318
			Erfurt -Hübner	21	KamSSR-65	366
					KamSSR-36	237
						318
			Erfurt -Beier	21	KamSSR-65	366
					KamSSR-36	237
						318
04-2	18-2	2x	Erfurt -Grimmer	26	-	-
			Hettstedt	27	KamSSR-36	282
						381
			Erfurt -Trautvetter	28	-	-
18-2	04-2	2x	Erfurt -Grimmer	26	-	-
			Hettstedt	27	-	-
			Erfurt -Trautvetter	28	KamSSR-36	210

Table 3.8. Peak (loci) locations on electropherogram for tetraploid parents of *Matricaria recutita* used in crossing trials.

Mother	Father	Ploidy	Location	PCR Plate Number	Primer Used	Peak Locations (bp)
24-01	29-04	4x	Töttelstädt	22	KamSSR-36	312
						481
			Tiefthal	22	KamSSR-36	312
						481
			Ritteburg	23	KamSSR-36	-
29-04	24-01	4x	Töttelstädt	22	KamSSR-36	238
			Tiefthal	23	KamSSR-36	238
			Ritteburg	23	KamSSR-36	238
23-04	29-01	4x	Schönfeld	24	KamSSR-8	213
			Erfurt	25	KamSSR-8	213
29-01	23-04	4x	Schönfeld	24	KamSSR-36	142
						224
			Erfurt	25	KamSSR-36	142
						224
22-06	16-08	4x	Borxleben	29	KamSSR-36	240
					KamSSR-65	364
			Freyburg	30	KamSSR-36	240
					KamSSR-65	364
			Erfurt	31	KamSSR-36	240
					KamSSR-65	364
16-08	22-06	4x	Borxleben	29	KamSSR-36	237
			Freyburg	30	KamSSR-36	237
			Erfurt	31	KamSSR-36	237

Table 3.9. CEQ™ peak scoring criteria used to standardize amplicon size between plates by comparison of the CEQ™ internal size standards. Variation in overall signal strength between plates caused different thresholds to be used to assign peak presence or absence.

Plate	Primer	Peak	Criteria (dye signal)
18	KamSSR-65	111	absent <1000, present >5000
	KamSSR-36	224	absent <20,000, present >50,000 (obvious size difference between parents)
		241	absent <1000, present >5000
19	KamSSR-65	111	absent <1000, present >2000
	KamSSR-36	224	absent <5000, present >40,000 (obvious size difference between parents)
		241	absent <1000, present >5000
20	KamSSR-65	366	absent <1000, present >2000
	KamSSR-36	237	absent <10,000, present >50,000
		318	absent <2000, present >5000
		322	absent <2000, present >10,000
		482	absent <5000, present >10,000
21	KamSSR-65	366	absent <1000, present >2000
	KamSSR-36	237	absent <10,000, present >50,000
		318	absent <5000, present >10,000
		322	absent <2000, present >10,000
		482	absent <1000, present >10,000
22	KamSSR-36	238	absent <2000, present >10,000
		312	absent <5000, present >50,000
		481	absent <8000, present >50,000
23	KamSSR-36	238	absent <2000, present >3000
24	KamSSR-8	213	absent <1000, present >10,000
	KamSSR-36	142	absent <4000, present >30,000
		224	absent <500, present >1000
25	KamSSR-8	213	absent <1000, present >5000
	KamSSR-36	142	absent <4000, present >14,000
		224	absent <1000, present >1500
26	-	-	-
27	KamSSR-36	282	absent <500, present >700
		381	absent <500, present >1000
28	KamSSR-36	210	absent <400, present >800
29	KamSSR-65	364	absent <2000, present >3000
	KamSSR-36	237	absent <2000, present >3000
		240	absent <2000, present >10,000
30	KamSSR-65	364	absent <1000, present >2000
	KamSSR-36	237	absent <1000, present >3000
		240	absent <5000, present >10,000
31	KamSSR-65	364	absent <100, present >300
	KamSSR-36	237	absent <200, present >3000
		240	absent <1000, present >7000

Analysis of raw scoring results was completed by finding peaks present in one parent and absent in the other parent. To differentiate between outcrossing and selfing, the parent that had a peak present was considered the father. Therefore, the progeny that had the same peak must have been outcrossed as the mother plant did not have that specific allele at the locus. For example, in PCR plate 24 (Figure 3.11), parent 23-04 was considered the father and parent 29-01 was considered the mother. Therefore, any progeny with peak 142 present must have been outcrossed. If more than one paternal marker (locus) was scored for a single progeny and there were conflicting results with at least one peak displaying outcrossing, that progeny sample was considered outcrossed. Several markers were used per cross because there was no evidence for homozygosity at any marker (Otto, unpublished data). Once raw scoring results were completed, the number of progeny identified as outcrossed at each crossing location was divided by the total number of scored progeny for each crossing location and then converted to a percentage to determine the outcrossing ratio (frequency outcrossed).

3.2.2.6 Statistical Analysis

To determine if there was a significant difference in the outcrossing ratio (outcrossed:selfed) based upon location, the outcrossing frequencies from the diploid and tetraploid crosses were analyzed separately. For each ploidy, the reciprocal crosses were grouped by location and a one-way ANOVA was carried out. The locations were then divided into two categories, north and south growing regions, and another one-way ANOVA was performed.

To analyze if there was a significant difference in the outcrossing ratio based upon ploidy, a one-way ANOVA was completed with the data which was pooled and divided into two groups, diploid and tetraploid.

Finally, a BLASTn and tBLASTx was performed on the original genomic sequences from which primer sequences were developed, in order to possibly assess genomic positions of the markers used. All analyses were done using the NCBI website, and both plant EST and genomic databases were used.

3.3 Results

Results of the BLASTn and tBLASTx on the microsatellite-containing sequences show that the contig of primer KamSSR-81 had many hits, some of which were highly significant (e.g. E-values of -30 to -40; Figures 3.12, 3.13). No other microsatellite-containing sequences were characterized by significant matches in the different databases. Primers KamSSR-8, KamSSR-23, KamSSR-36, KamSSR-65, and

KamSSR-85 only had log E-values between -3 and +3 for both the EST tBLASTx and the EST nBLAST. However, primer KamSSR-36 showed the best polymorphism on the agarose gel and electropherogram.

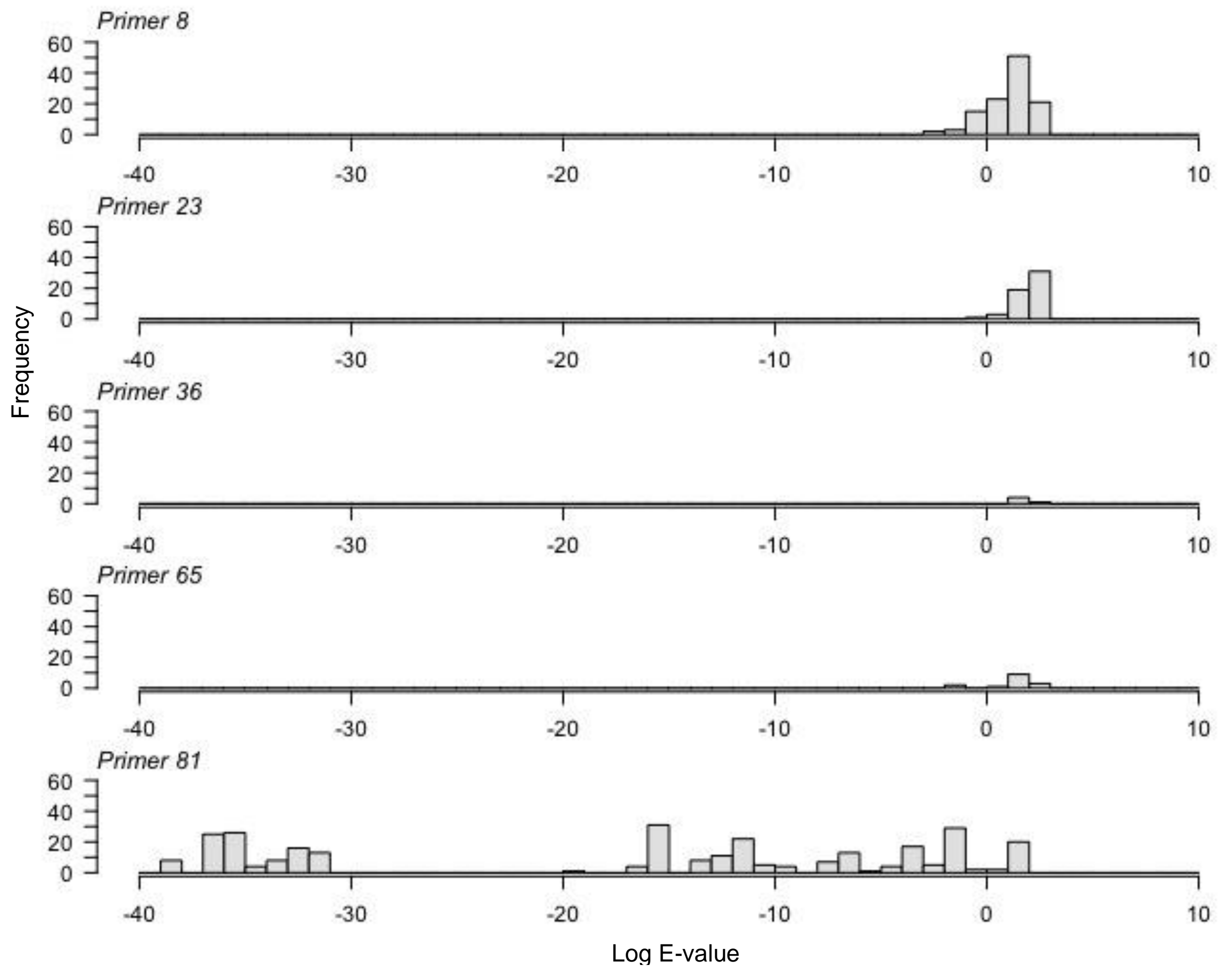

Figure 3.12. Histograms of log e-values from a plant EST tBLASTx of next generation sequencing (NGS) contig sequences (Table 3.6) from which primers were developed for the analysis here. Primer 85 had no significant similarity found and therefore was excluded from this graph.

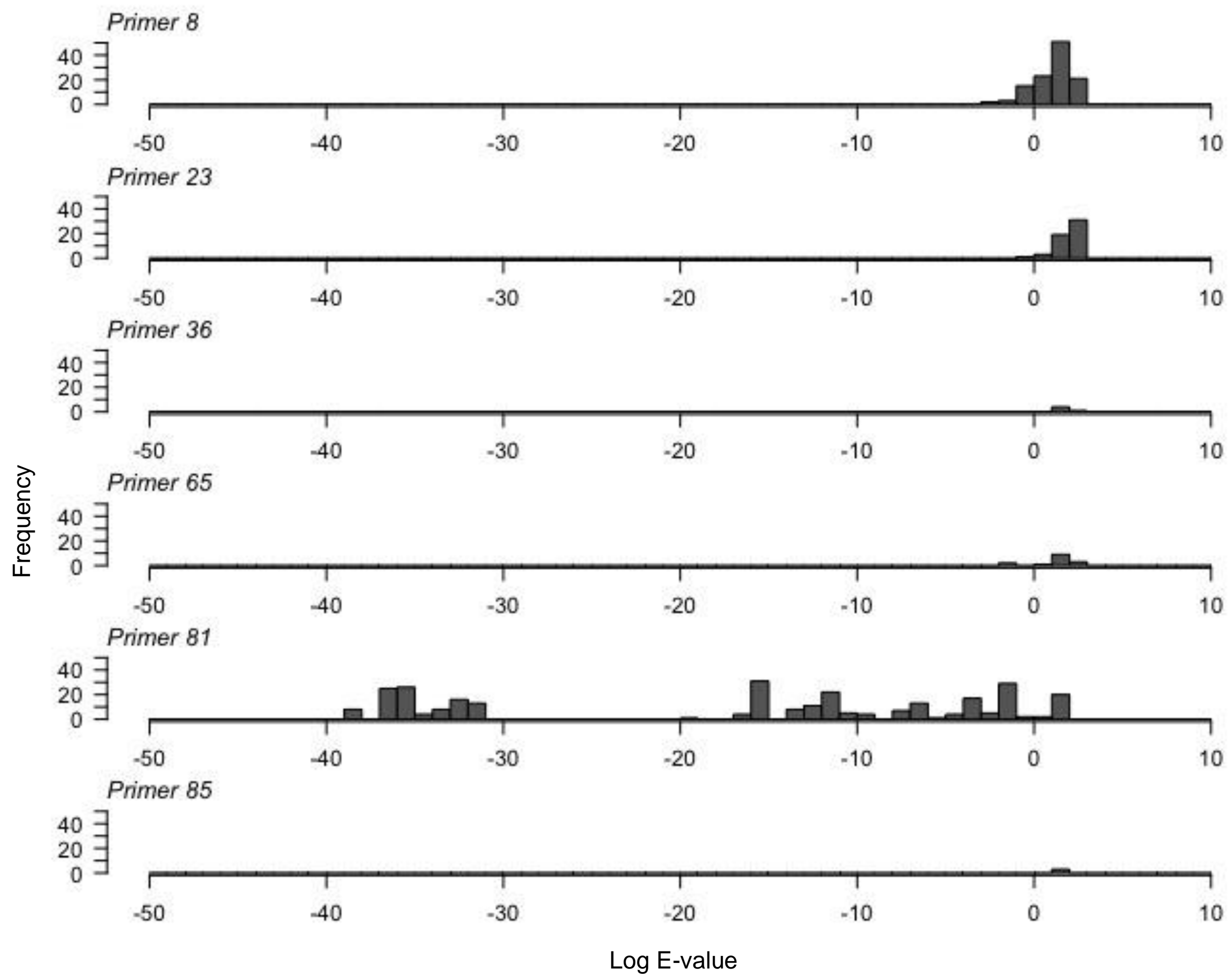

Figure 3.13. Histograms of log e-values from a plant EST BLASTn of next generation sequencing (NGS) contig sequences (Table 3.6) from which primers were developed for the analysis here.

3.3.1 Diploid Results

The first reciprocal crosses with diploid parents 20-2 and 13-2 were completed in Bretleben, Erfurt, and Borxleben, Germany (Table 3.10). With 20-2 as the mother, two loci (224 bp and 241 bp) on the electropherogram were scored for each location. However, when 13-2 was the mother, only one locus (111 bp) on the electropherogram was scored at each location. Samples that failed during the capillary electrophoresis protocol were labeled as dropouts and not considered in further analysis. A dropout could be due to biological or technical reasons, for example if the primer did not work with that genotype due to sequence divergence. Biological and technical errors could not be differentiated and therefore samples that could not clearly be scored were not included in subsequent analyses.

After the dropouts were identified, the number of samples analyzed from Bretleben, Erfurt, and Borxleben for the cross 20-2 X 13-2 were 28, 26, and 32, respectively; and for cross 13-2 X 20-2 were 28, 29, and 25, respectively. Subsequently, the frequency of outcrossed progeny for cross 20-2 X 13-2 were calculated as 89.29% at Bretleben, 57.69% at Erfurt, and 87.50% at Borxleben. For cross 13-2 X 20-2 the frequency of outcrossed progeny was calculated as 42.86% at Bretleben, 51.72% at Erfurt, and 28.00% at Borxleben.

Table 3.10. Results of diploid parents 20-2 and 13-2 reciprocal cross.

Cross (Mother X Father)	**Plate Number**	**Location**	**Total Number of Loci Scored**	**Dropout Frequency** (%)	**Proportion of Father Fertilized Offspring** (plants)	**Frequency of Outcrossed Progeny** (%)
20-2 X 13-2	18	Bretleben	2	12.50	25/28	89.29
	18	Erfurt	2	0	15/26	57.69
	19	Borxleben	2	0	28/32	87.50
13-2 X 20-2	18	Bretleben	1	12.50	12/28	42.86
	19	Erfurt	1	9.38	15/29	51.72
	19	Borxleben	1	3.85	7/25	28.00

The second reciprocal cross between different diploid parents, 12-5 and 20-04, was completed at Töttelstädt and two locations in Erfurt: the gardens of Hübner and Beier (Table 3.11). In the electropherogram for the cross 12-5 X 20-04, there were two loci (322 bp and 482 bp) scored, and for the cross 20-04 X 12-5, three loci (237 bp, 318 bp, and 366 bp) were scored. Once dropouts were removed, the number of samples analyzed from Töttelstädt, Erfurt-Hübner, and Erfurt-Beier for cross 12-5 X 20-04 were 30, 26, and 30, respectively; and for cross 20-04 X 12-5 were 32, 32, and 26, respectively. The frequency of outcrossed progeny for cross 12-5 X 20-04 was calculated to be 73.33% at Töttelstädt, 88.46% at Erfurt-Hübner, and 53.33% at Erfurt-Beier. For the cross 20-04 X 12-5, the frequency of outcrossed progeny was calculated to be 37.50% at Töttelstädt, 65.63% at Erfurt-Hübner, and 57.69% at Erfurt-Beier.

Table 3.11. Results of diploid parents 12-5 and 20-04 reciprocal cross.

Cross (Mother X Father)	**Plate Number**	**Location**	**Total Number of Loci Scored**	**Dropout Frequency** (%)	**Proportion of Father Fertilized Offspring** (plants)	**Frequency of Outcrossed Progeny** (%)
12-5 X 20-04	20	Töttelstädt	2	6.25	22/30	73.33
	20	Erfurt -Hübner	2	0	23/26	88.46
	21	Erfurt -Beier	2	6.25	16/30	53.33
20-04 X 12-5	20	Töttelstädt	3	0	12/32	37.50
	21	Erfurt -Hübner	3	0	21/32	65.63
	21	Erfurt -Beier	3	0	15/26	57.69

The third reciprocal cross used parents 04-2 and 18-2 in Hettstedt, and two Erfurt locations, the gardens of Grimmer and Trautvetter (Table 3.12). Cross 04-2 X 18-2 was not able to be scored on plates 26 and 28 which contained progeny samples from both Erfurt sites, due to PCR failures. However, at the Hettstedt site there were two loci (282 bp and 381 bp) that could be scored. Similarly, cross 18-2 X 04-2 was not able to be scored on plates 26 and 27 due to PCR failures.

For the cross 18-2 X 04-2, one locus (210 bp) could be scored for the Erfurt-Trautvetter site only due to failed PCR of the other sites. There were 48 plant samples scored for 04-2 X 18-2 at Hettstedt and 37 plant samples scored for 18-2 X 04-2 at Erfurt-Trautvetter, with the frequency of outcrossed progeny being 18.75% and 54.05%, respectively.

Table 3.12. Results of diploid parents 04-2 and 18-2 reciprocal cross.

Cross (Mother X Father)	**Plate Number**	**Location**	**Total Number of Loci Scored**	**Dropout Frequency** (%)	**Proportion of Father Fertilized Offspring** (plants)	**Frequency of Outcrossed Progeny** (%)
04-2 X 18-2	26	Erfurt -Grimmer	0	-	-	-
	27	Hettstedt	2	0	9/48	18.75
	28	Erfurt -Trautvetter	0	-	-	-
18-2 X 04-2	26	Erfurt -Grimmer	0	-	-	-
	27	Hettstedt	0	-	-	-
	28	Erfurt -Trautvetter	1	11.90	20/37	54.05

When comparing the first reciprocal cross, 20-2 X 13-2 and 13-2 X 20-2, it is evident that with 20-2 as the mother plant, the frequency outcrossed was greater at all locations. The average frequency outcrossed was 78.16% with 20-2 as the mother and 40.86% with 13-2 as the mother. The second diploid reciprocal cross exhibits similar patterns to that of the first reciprocal cross, whereby the frequency appeared to differ with respect to crossing direction. The average frequency outcrossed for 12-5 X 20-04 was 71.71% and for 20-04 X 12-5 was 53.61%. The frequency outcrossed was higher in cross 12-5 X 20-04 at Töttelstädt and Erfurt -Hübner; however, for Erfurt -Beier the higher outcrossing rate was observed with cross 20-04 X 12-5. The third reciprocal cross, 04-2 X 18-2 and 18-2 X 04-2, only had one location per crossing direction, whereby Hettstedt and Erfurt -Trautvetter could be successfully analyzed.

The overall percentage of outcrossed diploid progeny ranged from 18.75% to 89.29%, with an overall average of 57.56%. The outcrossing frequencies for the reciprocal crosses were grouped by location and one-way ANOVA was performed. There was no significant difference of the outcrossing frequency between individual geographical locations (P-value =0.769054). The locations were then divided into north and south growing regions (north including Hettstedt, Borxleben, and Bretleben, and south including Töttelstädt, Erfurt, Erfurt –Hübner, Erfurt –Beier, and Erfurt –Trautvetter), and another one-way ANOVA was completed. There was no significant difference of the outcrossing frequency between the north and south growing regions in this experiment (P-value =0.606188).

A one way ANOVA was used to compare the difference in crossing direction of the diploid reciprocal crosses. There was a significant difference (P-value = 0.039268) in the crossing direction of crosses 20-2 X 13-2 and 13-2 X 20-2. There was no significant difference (P-value = 0.241495) in the crossing direction of crosses 12-5 X 20-04 and 20-04 X 12-5. The last reciprocal cross, parents 04-2 and 18-2, could not be compared due to poor results.

3.3.2 Tetraploid Results

The first crosses were between tetraploid parents 24-01 and 29-04 at Töttelstädt, Tiefthal, and Ritteburg (Table 3.13). For the cross 24-01 X 29-04, there were two loci (312 bp and 481 bp) scored for Töttelstädt and Tiefthal. However, due to failed PCR no loci could be scored for Ritteburg. The final number of samples analyzed for Töttelstädt and Tiefthal were 30 and 24, respectively. The calculated frequency of outcrossed progeny was 90% at Töttelstädt, and 75% at Tiefthal. For the cross 29-04 X 24-01, there was one locus (238 bp) scored. There were 29, 31, and 26 plants analyzed from Töttelstädt, Tiefthal, and Ritteburg, respectively. The calculated frequency of outcrossed progeny was 65.52% at Töttelstädt, 32.26% at Tiefthal, and 28.00% at Ritteburg.

Table 3.13. Results of tetraploid parents 24-01 and 29-04 reciprocal cross.

Cross (Mother X Father)	**Plate Number**	**Location**	**Total Number of Loci Scored**	**Dropout Frequency** (%)	**Proportion of Father Fertilized Offspring** (plants)	**Frequency of Outcrossed Progeny** (%)
24-01 X 29-04	22	Töttelstädt	2	6.25	27/30	90.00
	22	Tiefthal	2	7.69	18/24	75.00
	23	Ritteburg	0	-	-	-
29-04 X 24-01	22	Töttelstädt	1	9.38	19/29	65.52
	23	Tiefthal	1	3.13	10/31	32.26
	23	Ritteburg	1	3.70	7/26	28.00

The second set of tetraploid reciprocal crosses were between parents 23-04 and 29-01 at Schönfeld and Erfurt (Table 3.14). For the cross 23-04 X 29-01, there was one locus (213 bp) scored and the number of offspring samples analyzed totaled 43 plants from Schönfeld and 45 plants from Erfurt. The frequency of outcrossed progeny was 95.35% at Schönfeld and 71.11% at Erfurt.

For the cross 29-01 X 23-04, there were two loci (142 bp and 224 bp) scored. The final number of offspring plant samples analyzed was 42 from Schönfeld and 41 from Erfurt, with the frequency of outcrossed progeny calculated to be 45.24% and 53.66%, respectively.

Table 3.14. Results of tetraploid parents 23-04 and 29-01 reciprocal cross.

Cross (Mother X Father)	**Plate Number**	**Location**	**Total Number of Loci Scored**	**Dropout Frequency** (%)	**Proportion of Father Fertilized Offspring** (plants)	**Frequency of Outcrossed Progeny** (%)
23-04 X 29-01	24	Schönfeld	1	10.42	41/43	95.35
	25	Erfurt - Göttsching	1	6.25	32/45	71.11
29-01 X 23-04	24	Schönfeld	2	0	19/42	45.24
	25	Erfurt-Göttsching	2	2.38	22/41	53.66

The third set of tetraploid reciprocal crosses were completed between parents 22-06 and 16-08 at Borxleben, Freyburg, and Erfurt (Table 3.15). Cross 22-06 X 16-08 had two loci (240 bp and 364 bp) scored. This resulted in 46, 47, and 48 offspring plants being analyzed from Borxleben, Freyburg, and Erfurt, respectively. The frequency of outcrossed progeny was calculated to be 89.13% at Borxleben, 68.09% at Freyburg, and 97.92% at Erfurt.

One locus (237 bp) was scored for 16-08 X 22-06. The total number of offspring samples evaluated was 41 plants from Borxleben, 40 plants from Freyburg, and 37 plants from Erfurt. As a result, the frequency of outcrossed progeny was calculated as 26.83% at Borxleben, 30.00% at Freyburg, and 5.41% at Erfurt.

Table 3.15. Results of tetraploid parents 22-06 and 16-08 reciprocal cross.

Cross (Mother X Father)	**Plate Number**	**Location**	**Total Number of Loci Scored**	**Dropout Frequency** (%)	**Proportion of Father Pollinated Offspring** (plants)	**Frequency of Outcrossed Progeny** (%)
22-06 X 16-08	29	Borxleben	2	4.17	41/46	89.13
	30	Freyburg	2	2.08	32/47	68.09
	31	Erfurt -Ellinger	2	0	47/48	97.92
16-08 X 22-06	29	Borxleben	1	2.38	11/41	26.83
	30	Freyburg	1	4.76	12/40	30.00
	31	Erfurt -Ellinger	1	11.90	2/37	5.41

The first reciprocal cross, 24-01 X 29-04 and 29-04 X 24-01, had a higher outcrossing value at all locations when 24-01 was acting as the female. The average outcrossing frequency for 24-01 X 29-04 was 82.50% and 41.93% for 29-04 X 24-01. The second tetraploid reciprocal cross with parents 23-04 and 29-01 showed a higher outcrossing value at all locations when 23-04 was acting as the female with an average of 83.23%, compared to when 23-04 acts as the male parent with an average outcrossing value of 49.45%. The third tetraploid reciprocal cross, 22-06 X 16-08, displayed the same pattern of one crossing direction being considerably higher in outcrossing value than in the other direction. With 22-06 as the mother, the average frequency outcrossed was 85.05% and with 16-08 as the mother, the average frequency outcrossed was 20.75%.

The overall percentage of outcrossed tetraploid progeny ranged from 5.41% to 97.92%, with an overall average of 58.23%. The outcrossing ratios for the reciprocal crosses were grouped by location for a one-way ANOVA. There was no significant difference in the outcrossing frequency between individual geographical locations (P-value =0.960199). The locations were then divided into two categories, north and south, and another one-way ANOVA was completed. The north locations were Ritteburg, Freyburg and Borxleben, and the south locations were Töttelstädt, Tiefthal, Erfurt -Göttsching, Erfurt -Ellinger, and Schönfeld. There was no significant difference of the outcrossing frequency between the north and south growing regions in this experiment (P-value =0.374801).

A one way ANOVA was used to compare the difference in crossing direction of the tetraploid reciprocal crosses. There was no significant difference in the crossing direction of crosses 24-01 X 29-04 and 29-04 X 24-01 (P-value = 0.088556), 23-04 X 29-01 and 29-01 X 23-04 (P-value = 0.119047). There was a significant difference in the crossing direction of crosses 22-06 X 16-08 and 16-08 and 22-06 (P-value = 0.005419).

A comparison of the diploid and tetraploid crosses is illustrated in Figure 3.14. Both ploidy levels exhibited large variation in outcrossing frequency. When all diploid crosses were considered, the maximum, minimum, and average frequency outcrossed were 89.29%, 18.75%, and 57.56%, respectively. Similarly, when all tetraploid crosses were considered, the maximum, minimum, and average frequency outcrossed were 97.92%, 5.41%, and 58.23%, respectively. The data was pooled and divided into two groups, diploid and tetraploid crosses, and a one-way ANOVA was completed. There was no significant difference of the outcrossing frequency between the two groups (P-value = 0.944381).

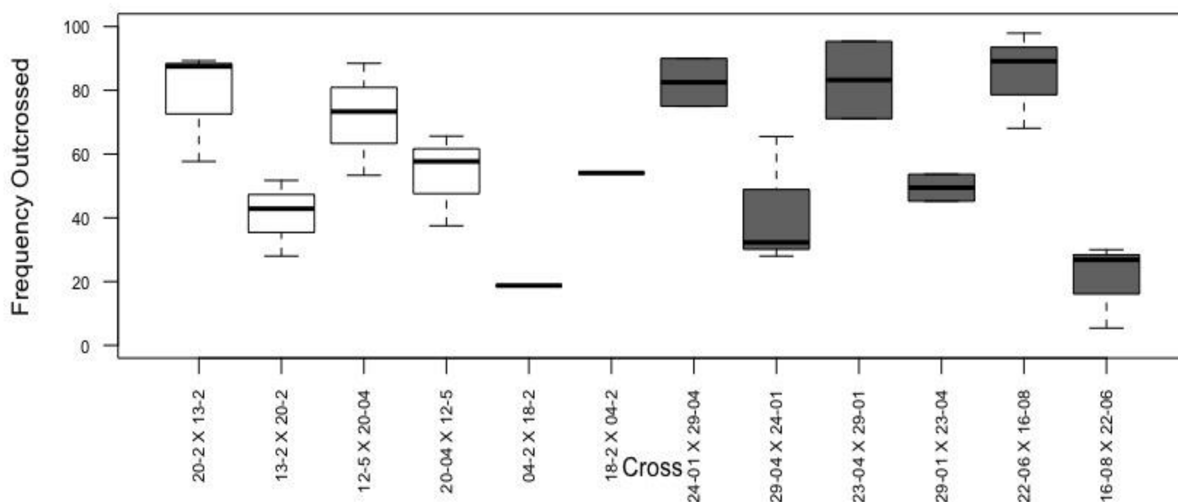

Figure 3.14. Boxplots showing the comparison of diploid and tetraploid crosses. The first six crosses (white) are diploid and the last six crosses (gray) are tetraploid.

3.4 Discussion

3.4.1 Location Effect

Chamomile exhibited a wide range of outcrossing frequencies in the crosses tested. Comparable to this study, a wide range of outcrossing frequencies has been previously observed in a field study of *Capsicum annuum* L, where outcrossing ranged from 2% to 90% depending on location, environment, and space between plants (Pickersgill, 1997).

The hypothesis specific to Experiment 1, was that there would be no significant difference in the outcrossing rate between the different locations, considering the high outcrossing levels of this species. Therefore, it was thought that genetic factors would have a greater effect on the outcrossing rate than environmental factors at the specific locations. Diploid and tetraploid crosses were analyzed separately; initially, each location was considered individually and then with the locations categorized in north and south growing regions. As the hypothesis predicted, there was no significant difference in the outcrossing rate found between the individual locations tested or in the north and south categories for the diploid or tetraploid crosses. In contrast to this experiment, a lentil experiment also completed in central Germany successfully detected a difference in outcrossing frequency based upon location (Horneburg, 2006). However, the locations tested with lentils were vastly different in altitude and in the presence of natural flowering plants, which could affect the presence of pollinators (Horneburg, 2006).

There has been a geographical pattern observed in the reproductive system of other Asteraceae; for example, there were differences in self-compatibility and outcrossing frequencies observed in populations of *Hypochaeris salzmanniana* between western and eastern geographical locations in Spain (Arista *et al*., 2017). The amount of pollinators present in the different locations affected the outcrossing rates in *H. salzmanniana* as selfing allowed reproductive insurance when the pollinator attendance was low (Arista *et al*., 2017). Although the study of *H. salzmanniana* examined the evolution of a mixed-mating system of plants in different localities through time, the effect of pollinator presence on the mating system could be applicable to investigations of genotypes that have been planted in novel locations. Therefore it could be plausible that in this study there was a large range of outcrossing frequencies observed due to each location having varying pollinator presence.

3.4.2 Crossing Direction and Parent of Origin Effects

The results of outcrossing did not show a statistical difference between geographical locations; however, in all crosses there appeared to be an effect associated with crossing direction. In many crosses, the frequency outcrossed was considerably higher in one cross direction than the other. The most obvious

difference was observed with parents 22-06 and 16-08, where the outcrossing frequencies were higher with 22-06 as the mother and between the cross directions there was a difference of 62.30% at Borxleben, 38.09% at Freyburg, and 92.51% at Erfurt. These observations were statistically significant between reciprocal crosses with parents 20-2 and 13-2, as well as parents 22-06 and 16-08. Therefore, being a male or female in these crosses matters. Maternal and paternal parents have different genetic and epigenetic contributions to seed development in angiosperms (Bai *et al*., 2016). When the phenotypic expression, and consequently, the effect of an allele depends on if it is inherited from the mother or father, it is known as the parent-of-origin effect (Lawson *et al*., 2013). As a result of genomic imprinting, two alleles at a single locus can be functionally non-equivalent. This is considered to be the primary epigenetic phenomenon that leads to the display of parent-of-origin effects (Lawson *et al*., 2013). Parent-of-origin effects can be exhibited by loci required for gametophyte development or by imprinted genes needed for seed development (Bai *et al*., 2016). For example, parent-of-origin effects on kernel mutants in maize have been identified by phenotyping reciprocal crosses with inbred lines (Bai *et al*., 2016).

There was also a difference observed in the dropout rate depending on cross direction. For example, in the crosses 20-2 X 13-2 and 13-2 X 20-2, when 20-2 was the mother, both Erfurt and Borxleben had a dropout rate of 0%. Conversely, when 13-2 was the mother, Erfurt and Borxleben had dropout rates of 9.38% and 3.85%, respectively. It would be advantageous to conduct further experiments to determine if a high outcrossing mother or father retains this behavior in crosses with other genotypes.

3.4.3 Ploidy

It was hypothesized that all diploid crosses would exhibit higher outcrossing rates than tetraploid crosses due to stronger self-incompatibility (Faehnrich *et al.*, 2013). A boxplot comparison between ploidy types (Figure 3.14) shows that there was a wide variation in the outcrossing frequency and that the upper and lower levels were similar in both ploidy types suggesting that there was no significant difference in the outcrossing rate between diploid and tetraploid crosses.

3.5 Conclusion

In conclusion, there was no significant difference in the outcrossing ratio observed based on the geographical location of the cross. There was a large variation in the frequency outcrossed regardless of both location and ploidy. The most prospective reason for the large range of outcrossing frequencies is varying pollinator presence; however, pollinator data was not collected as part of this study. A noteworthy observation that came about from this study was that the crosses appeared to be affected according to the

direction in which the cross occurred. Future research should focus on parent-of-origin effects in chamomile and how they affect the fertilization process.

CHAPTER 4

ABSTRACT

A field growing environment is vastly different than a greenhouse growing environment as it is uncontrolled. The objective of this study was to examine how the outcrossing ratio of chamomile might be affected in a controlled greenhouse environment compared to a field environment. Seven crossing pairs were chosen based on maturity, height, presence of disease, and ploidy. Genotypes of the sample ploidy were selected as crossing pairs, diploid with diploid, and tetraploid with tetraploid. All crosses were completed in parallel in both greenhouse and field locations on the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben) campus. The outcrossing ratio between the two growing conditions could not be compared because the greenhouse progeny seeds did not germinate for any of the crosses. There are three stages of plant development that could have been negatively affected: gamete formation, fertilization, and germination. Three probable explanations to why the greenhouse crosses failed are poor pollination, unfavorable climate conditions, and seed dormancy. The addition of flies into greenhouses growing chamomile could increase pollination efficiency. Future research should be focused on germination requirements and methods to break dormancy in chamomile.

4.0 Evaluating the Effects of Field versus Greenhouse Environments on the Outcrossing Ratio

4.1 Introduction

A crucial step in plant breeding is taking the prospective cultivars from the lab and greenhouse into field-testing. A field environment is vastly different than a greenhouse environment as it is uncontrolled. In a greenhouse, the temperature, irrigation, air humidity, and light can all be efficiently managed to achieve the best plant growth potential. Many factors including climate, disease, pests, pollinators, and soil can all affect how a plant performs in the field. Therefore, it was of interest to study how the outcrossing ratio of chamomile might be affected in a controlled greenhouse environment compared to a field environment.

Three hypotheses were formulated for this study. Firstly, it was hypothesized that higher outcrossing rates would be observed in field crosses in comparison to greenhouse crosses. This was expected because in the field there are pollinators that transfer pollen between plants and thus aid in outcrossing, whereas no pollinators were introduced to the greenhouse. The second hypothesis was that increased ambient temperature would be correlated to lower levels of outcrossed offspring due to stress on the plant at higher temperatures. The third hypothesis was repeated from the first study in chapter 3,

that diploid crosses would exhibit higher outcrossing rates than tetraploid crosses due to stronger self-incompatibility.

4.2 Materials and Methods

4.2.1 Plant Material and Description of Growing Locations

Multiple clones, genetically identical plants produced by cuttings, of 14 parental genotypes (Tables 4.1, 4.2) were grown in the greenhouses of the IPK Gatersleben (Germany). Seven crossing pairs were chosen based on maturity, height, presence of disease, and ploidy. Genotypes of the same ploidy were selected as crossing pairs, diploid with diploid, and tetraploid with tetraploid.

Table 4.1. Diploid plant material used for evaluating the effects of field versus greenhouse environments.

Parent	Variety/Population	Source	Country of Origin
16-721-10	Wild Collection	Priors Halton	England
16-080-22	Kirschkamille	Pharmaplant GmbH	Germany
16-064-6	MAT 2	Genebank, Gatersleben	Germany
16-067-24	MAT 16	Genebank, Gatersleben	Germany
16-004-14	Camoflora	Martin Bauer GmbH	Germany
16-020-11	Wild Collection	Dover	England
16-066-6	Pohorelicky Velkokverty, MAT 15	Genebank, Gatersleben	Czech Republic
16-066-21	Pohorelicky Velkokverty, MAT 15	Genebank, Gatersleben	Czech Republic

Table 4.2. Tetraploid plant material used for evaluating the effects of field versus greenhouse environments.

Parent	Variety/Population	Source	Country of Origin
16-03-01	Bodegold	Pharmasaat GmbH	Germany
16-03-101	Bodegold	Pharmasaat GmbH	Germany
16-007-22	Goral	Pharmasaat GmbH	Slovakia
16-006-17	Zloty Lan	Pharmasaat GmbH	Poland
16-006-25	Zloty Lan	Pharmasaat GmbH	Poland
16-005-20	Lutea	Farm Dilshofen	Germany

All crosses were completed in parallel in both greenhouse and field locations. Parental plants were just beginning to flower prior to isolation; therefore, before the plants were placed in their respective locations for crossing, any existing flower heads were clipped to ensure no pollen cross contamination. Each genotype had four to five clones in both locations, with each cross having the same number of plants from each genotype (Figure 3.8A). Plants were placed in close proximity to one another, with approximately 15 cm spacing between individuals. The plants for the greenhouse crosses were kept in the original pots and each cross was placed into separate large cabinets isolated with a door (Figure 4.1C). All field locations were on the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben) campus (Figure 4.2). These plants were transplanted into the soil as a rectangular plot with alternating genotypes and approximately 15 cm spacing between plants. If field locations were less than 150 m apart, isolation cages were used to prevent pollen cross contamination (Figure 4.1B). Thermometers were placed at every greenhouse location and in most field locations. Each thermometer was placed in the corresponding location from the beginning of isolation to the end of harvest, recording 24 hours a day at 15-minute intervals. Field thermometers were placed in a waterproof bag (Figure 4.1A). Plants were hand-harvested once flower heads were mature, roughly 20 days after isolation. Flower heads were determined to be mature when the white ray florets the became parallel to the stem (Figure 3.8B). F1 seeds were sown in the greenhouse and young leaf samples were collected for further analysis.

(A) (B) (C)

Figure 4.1. Plot images. (A) Field plot showing thermometer in waterproof bag. (B) Field plot showing isolation cage. (C) Greenhouse plot.

One clone of each parental genotype was selfed to investigate homozygosity. The selfing plants were placed into one greenhouse cabinet with a bag covering each plant to ensure proper isolation and self-fertilization. Due to the high temperatures in the greenhouse and the bag method of isolation, the selfed plants did not survive long enough to flower and produce seed.

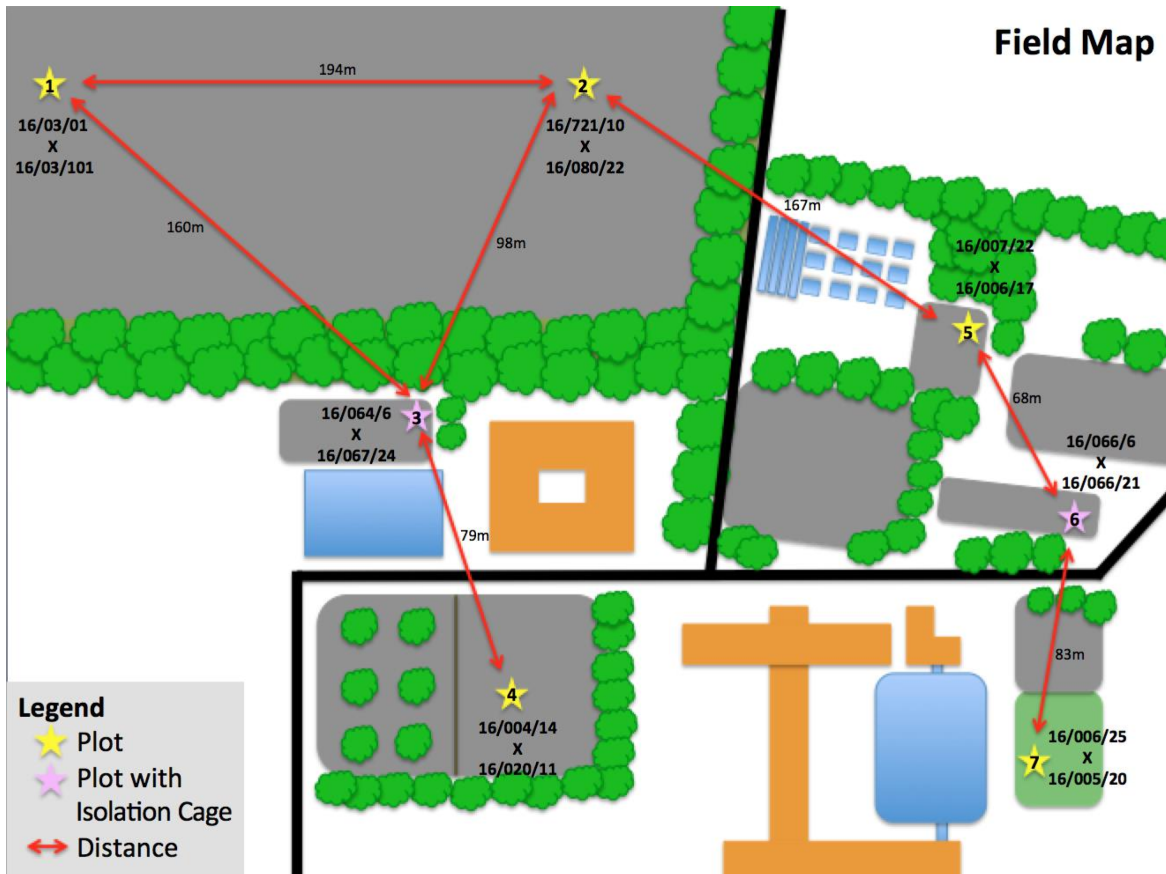

Figure 4.2. Field map of chamomile crosses completed on the IPK Gatersleben campus in June 2017. Yellow stars indicate plot locations, pink stars indicate plot locations with an isolation cage, and red arrow indicate distance between nearby plots.

4.2.2 Experimental Procedures

The following procedures as explained for Experiment 1 in chapter 3.2.2 were repeated: DNA extraction, PCR, primers and microsatellites, ethanol precipitation, capillary electrophoresis, and fragment analysis.

4.2.2.1. Fragment Analysis

As in chapter 3.2.2, the CEQ™ 8000 produced an electropherogram in which the peaks represent alleles. These peaks were manually scored and recorded in Microsoft Excel. In the Beckman Coulter software, GenomeLab GeXP Genetic Analysis System, the parental genotypes were observed for peaks present in one parent and absent in the other (Figure 3.11). These identified peaks (Table 4.3) were then scored in the progeny as absent, present, or unable to score using conservative scoring criteria. Identical peaks that were present in both parents of a cross were not considered. Amplicon size was standardized between plates by comparison of the CEQ™ internal size standards. As a result of variation in overall signal strength between plates, different thresholds were used to assign peak presence and absence scoring criteria (Table 4.4). Analysis of raw scoring results and frequency outcrossed calculation was completed as in chapter 3.2.2.5.

Table 4.3. Crosses peak (loci) locations on electropherogram.

Mother	Father	Ploidy	Location	PCR Plate Number	Primer Used	Peak Locations (bp)
16-721-10	16-080-22	2x	Field	PP11	KamSSR-36	210
16-080-22	16-721-10	2x	Field	PP11	KamSSR-36	231 312 381 503
16-064-6	16-067-24	2x	Field	PP4	KamSSR-85	143

Table 4.4. CEQ™ peak scoring criteria used to standardize amplicon size between plates by comparison of the CEQ™ internal size standards. Variation in overall signal strength between plates caused different thresholds to be used to assign peak presence or absence.

Plate	Primer	Peak	Criteria (dye signal)
PP11	KamSSR-36	210	absent <7500, present >15,000
PP11	KamSSR-36	231	absent <25,000, present >75,000 (obvious size difference between parents)
		312	absent <5000, present >75,000
		381	absent <1000, present >7500
		503	absent <1000, present >5000
PP4	KamSSR-85	143	absent <1000, present >5000

4.2.2.2 Flow Cytometry

Flow cytometry was used to determine the ploidy of parent plants before crossing (Otto *et al*., 2015). A *Vicia faba* leaf sample was added to every *M. recutita* leaf sample as an internal standard for flow cytometry analysis. Plants were classified as diploid or tetraploid based on a clearly defined single peak (Figures 4.3, 4.4). The peak of each species was recorded and the ratio was calculated. Samples with a *Matricaria recutita* to *Vicia faba* ratio of 0.44 to 0.48 were classified as tetraploids and plants with a ratio of 0.23 to 0.26 were classified as diploids. The control species tested were *Hordeum vulgare*, *Vicia faba*, and *Hordeum vulgare* plus *Vicia faba*.

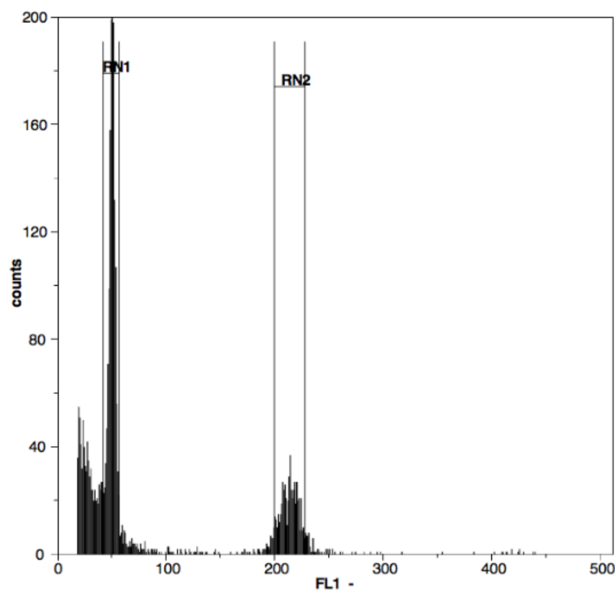

Figure 4.3. Profile of diploid parent 16-080-22 to the left (labeled RN1, FL1 position 50) and internal *Vicia faba* standard to the right (labeled RN2, FL1 position 220).

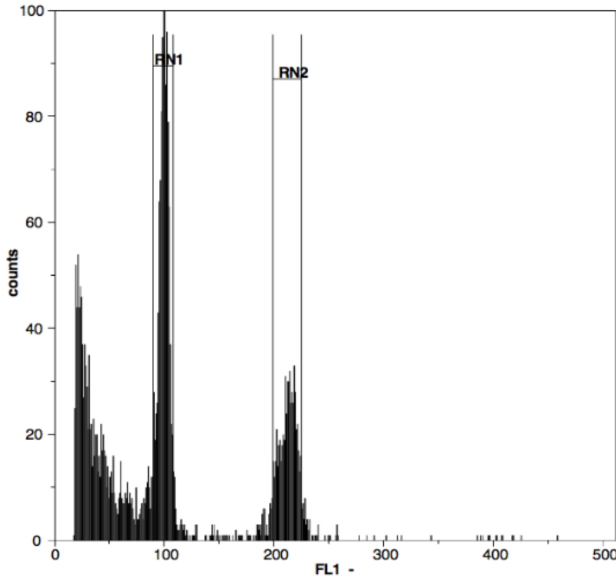

Figure 4.4. Profile of tetraploid parent 16-03-01 to the left (labeled RN1, FL1 position 100) and internal *Vicia faba* standard to the right (labeled RN2, FL1 position 220).

4.3 Results

Seven crosses were prepared in parallel in both field and greenhouse locations at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK Gatersleben) campus. Parental genotypes were selected based on capturing a wide range of genetic diversity and the availability of elite breeding material and registered varieties at the time of study. However, the only field reciprocal cross that could be analyzed in both directions was 16-721-10 X 16-080-22 and 16-080-22 X 16-721-10. The field cross 16-064-6 X 16-067-24 could be analyzed; however, the reciprocal cross did not produce viable seeds (Table 4.5). Although there were viable seeds analyzed from reciprocal field crosses of parents 16-006-25 and 16-005-20, no father specific markers were found in the progeny. The remaining reciprocal field crosses as well as all the greenhouse crosses did not have viable seeds to analyze.

The cross 16-721-10 X 16-080-22 had one locus scored (210 bp), and 39 out of the 63 analyzed plants with a father-specific marker; thus, the frequency of outcrossed progeny was 61.90%. When the parents were reversed (16-080-22 X 16-721-10) there were four loci scored (231 bp, 312 bp, 381 bp, and 503 bp). Four father-specific markers were observed in 69 out of the 86 analyzed plants, and so the outcrossed progeny was calculated as 80.23%.

Table 4.5. Crossing results of successful trials in 2017.

Cross (Mother X Father)	**Location**	**Total Number of Loci Scored**	**Number of Dropouts** (%)	**Proportion of Father Fertilized Offspring** (plants)	**Frequency of Outcrossed Progeny** (%)
16-721-10 X 16-080-22	Field	1	25.00	39/63	61.90
16-080-22 X 16-721-10	Field	4	2.27	69/86	80.23
16-064-6 X 16-067-24	Field	1	0.00	4/45	8.89

Cross 16-064-6 X 16-067-24 had one locus scored (143 bp). Only 4 out of 45 plants analyzed had a father-specific marker; therefore, this cross had an outcrossing frequency of 8.89%. Results for the successful field crosses are shown in Figure 4.5. The outcrossed frequency is considerably different between cross 16-064-6 X 16-067-24 and the reciprocal cross with parents 16-721-10 and 16-080-22.

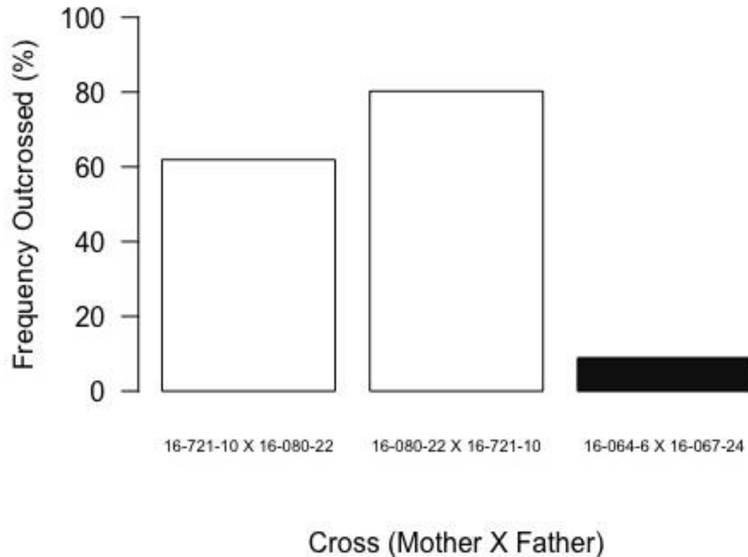

Figure 4.5. Bar graph representing the frequency outcrossed for all successful field crosses involving diploid parents.

Thermometers were placed at every greenhouse location and in field locations 1, 3, 4, 6, and 7. Temperatures were recorded 24 hours a day at 15-minute intervals from the beginning of the isolation period to the end of harvest to monitor if there was variation in temperature and how that variation affected the outcrossing ratio. Greenhouse temperature readings are summarized in Figure 4.6. When comparing all greenhouses, a notable difference in temperature was exhibited in Greenhouse 7, which contained parents 16-080-22 and 16-721-10. Greenhouse 7 had the lowest minimum, maximum, and average temperature at 12.2°C, 25.3°C, and 16.9°C, respectively. The remaining greenhouses had similar temperature readings to one another with a minimum temperature between 14.1°C and 18.2°C, a maximum temperature between 32.0°C and 38.9°C, and an average temperature between 21.8°C and 25.6°C.

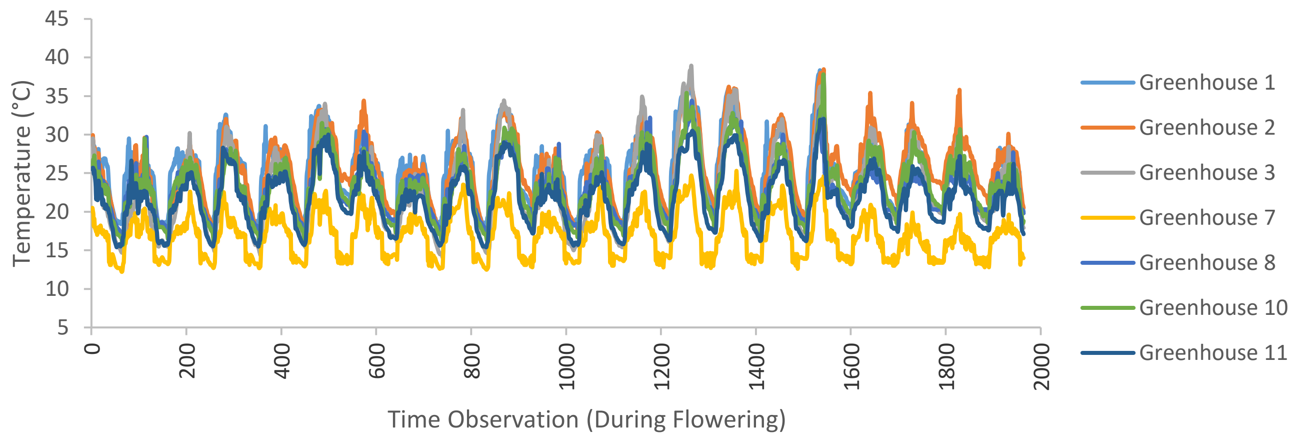

Figure 4.6. Greenhouse temperature readings from date of isolation (13:10:00 on June 6, 2017) to date of harvest (23:55:00 on June 26, 2017). Temperature readings were taken at 15-minute intervals, totaling 1965 observations throughout the isolation period. The x-axis represents the observation number out of the 1965 readings (0= June 6 at 13:10:00, 200= June 8 at 14:55:00, 400= June 10 at 16:55:00, 600= June 12 at 18:55:00, 800= June 14 at 20:55:00, 1000= June 16 at 22:55:00, 1200= June 19 at 00:55:00, 1400= June 21 at 2:55:00, 1600= June 23 at 4:55:00, 1800= June 25 at 6:55:00).

When comparing the field locations, there were no obvious differences in temperature (Figure 4.7). All locations had a minimum temperature between 7.9°C and 9.6°C, a maximum temperature between 38.2°C and 43.0°C, and an average temperature between 20.18°C and 21.57°C.

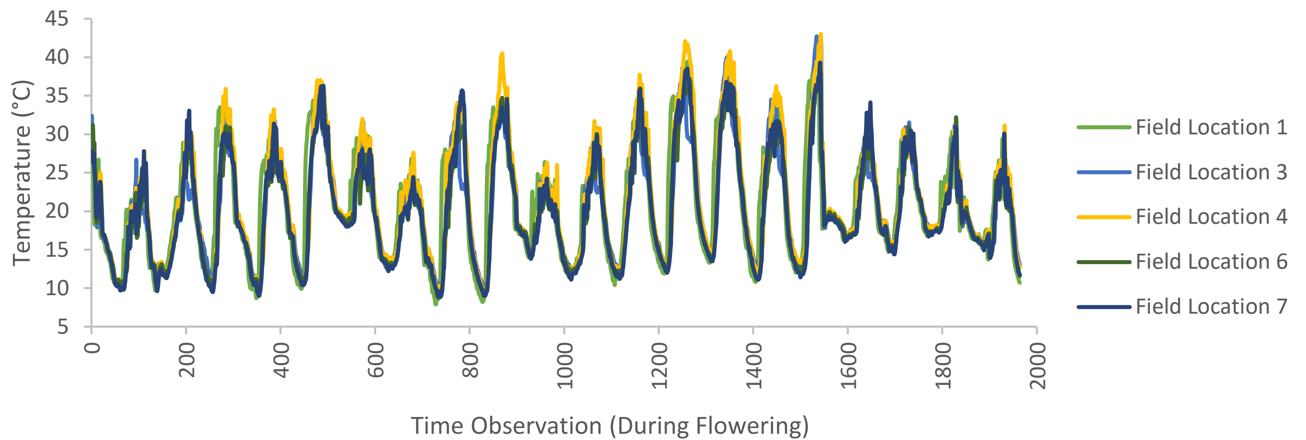

Figure 4.7. Field temperature readings from date of isolation (13:00:00 on June 6, 2017) to date of harvest (23:45:00 on June 26, 2017). Temperature readings were taken at 15-minute intervals, totaling 1965 observations throughout the isolation period. The x-axis represents the observation number out of the 1965 readings (0= June 6 at 13:00:00, 200= June 8 at 14:45:00, 400= June 10 at 16:45:00, 600= June 12 at 18:45:00, 800= June 14 at 20:45:00, 1000= June 16 at 22:45:00, 1200= June 19 at 00:45:00, 1400= June 21 at 2:45:00, 1600= June 23 at 4:45:00, 1800= June 25 at 6:45:00).

Growing conditions between greenhouse and field growing locations could not be compared because the greenhouse progeny seeds did not germinate. The temperature in the greenhouse used for germination and seedling growth of F1 plants (Greenhouse 5) was not measured, as this was not expected to affect the experiment. However, Greenhouse 5 was used for selfing parental genotypes and had temperatures recorded June 6 to June 26 (Figure 4.8).

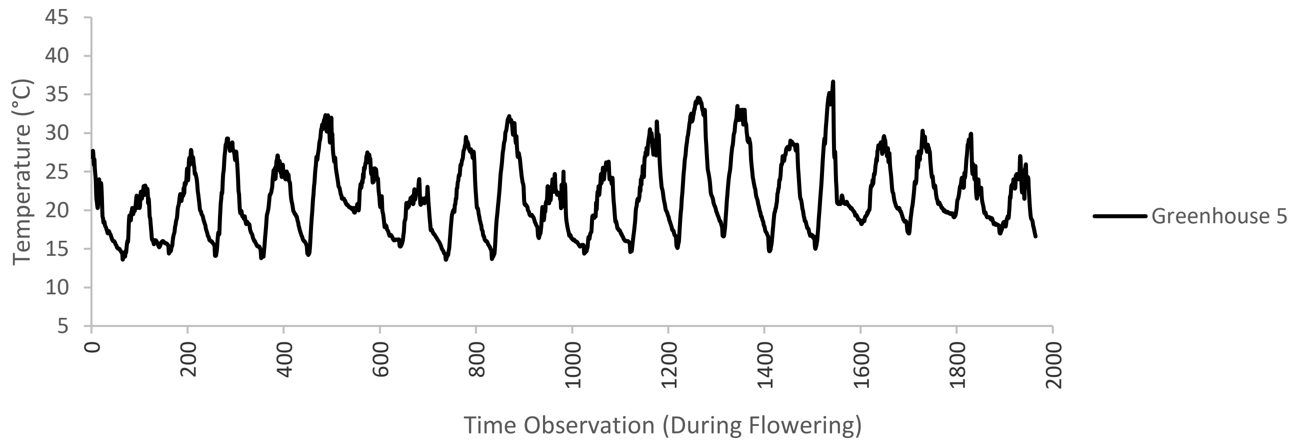

Figure 4.8. Temperature readings from Greenhouse 5 that was used for selfing parental genotypes from 13:10:00 on June 6, 2017 to 23:55:00 on June 26, 2017. Temperature readings were taken at 15-minute intervals, totaling 1965 observations throughout the isolation period. The x-axis represents the observation number out of the 1965 readings (0=June 6 at 13:10:00, 200= June 8 at 14:55:00 , 400= June 10 at 16:55:00 , 600= June 12 at 18:55:00, 800= June 14 at 20:55:00, 1000= June 16 at 22:55:00, 1200= June 19 at 00:55:00, 1400= June 21 at 2:55:00, 1600= June 23 at 4:55:00, 1800= June 25 at 6:55:00).

4.4 Discussion

The main objective specific to this experiment was to determine the effect of greenhouse conditions compared to field conditions on the outcrossing ratio of chamomile. However, the hypothesis that field crosses would exhibit higher outcrossing rates due to enhanced pollination from wind and insects could not be properly analyzed, as none of the greenhouse crosses produced viable seeds. Furthermore, the hypothesis that all diploid crosses will exhibit higher outcrossing rates than tetraploid crosses due to stronger self-incompatibility could not be analyzed in this experiment because the three crosses that produced viable seeds were all diploid. There are three stages of plant development that could have been negatively affected: gamete formation, fertilization, and germination. Three probable explanations to why the greenhouse crosses failed are poor pollination, unfavorable climate conditions, and seed dormancy.

4.4.1 Pollination

Greenhouses are a valuable growing system because they allow for a more efficient use of water, fertilizer, pesticides, and labour, although these systems also pose major constraints for pollination (James and Pitts-Singer, 2008). Pollination, the act of transferring pollen grains from the stamens to a stigma in order to facilitate fertilization, is accomplished by both biotic pollinators (e.g. insects) and abiotic factors

(e.g. wind) which are not present in greenhouses. Thus it is possible that greenhouse crosses in this experiment were not successful due to a lack or decrease in pollination success due to insect composition and/or numbers. Studies have shown that the introduction of insect pollinators into greenhouse production has improved production of many crop species including strawberry (Malagodi-Braga and Kleinert, 2004), sweet pepper (*Capsicum annuum* L.) (Shipp *et al*., 1994), watermelon (Bomfim *et al*., 2014), zucchini (Roldán-Serrano and Guerra-Sanz, 2005), cucumber, eggplant, tomato, and green bean (James and Pitts-Singer, 2008). Many horticulture crops produced in a greenhouse depend on efficient pollination, as approximately 45% of greenhouse crop production value is attributed to this process (James and Pitts-Singer, 2008). Honeybees and bumble bees are the most frequently used pollinators in greenhouses; however, the foraging activity of bumble bees is affected by high temperatures of about 30°C (James and Pitts-Singer, 2008). As bees are limited in production by temperature and this experiment experienced high temperatures, introduction of bees likely would not have helped pollination.

Additionally, bees are complicated to propagate which leads to a higher cost to the researcher. Conversely, flies are inexpensive to propagate and economical to obtain from pet shops in large quantities. Although bees might be superior pollinators, larger amounts of flies can be used to compensate the pollinating abilities. Therefore, flies are the preferred pollinator to introduce to greenhouses being used for chamomile research in Germany (Otto, personal communication). Flies are known to be pollinators of many species in the Asteraceae family including *Chrysanthemum* sp., *Helianthus* sp., *Spilanthes acmella* L., *Tagetes patula* L., and *Taraxacum officinale* (Mitra and Banerjee, 2007). In future greenhouse experiments, flies should be introduced to help improve pollination of chamomile plants.

4.4.2 Climatic Conditions

The development of chamomile plants depends on environmental conditions and should be tested under different climatic conditions (Rafieiolhossaini *et al*., 2010). The effect of environmental conditions on the production of chamomile has been documented by observing morphological changes, differences of flower yield, and differences of essential oil yield (Honermeier *et al*., 2013). When comparing chamomile production in both temperate and subtropical zones, Karami *et al*. (2009) found that the different growing conditions had an effect on the essential oil content and compositions. The highest amount of oil content was obtained from temperate zones (Karami *et al*., 2009). Chamomile field experiments conducted in Belgium found that the planting date had a more pronounced effect on growth, yield, essential oil content and main essential oil compounds, as compared to the age of seedling at transplanting (Rafieiolhossaini *et al*., 2010). With early planting, and thus reduced temperature stress, chamomile has optimum growth and development opportunities (Rafieiolhossaini *et al*., 2010). Hence, it

is possible that the conditions during flowering within this study could have been improved with a lower temperature in the greenhouse and an earlier transplanting date in the field.

The chamomile plants used for the greenhouse crosses were isolated into greenhouse chambers already occupied by other experiments at the IPK Gatersleben, and therefore the climate controls could not be altered. As chamomile is native to southeastern Europe and western Asia, it requires cool, temperate growing conditions with temperatures ranging from 7°C to 26°C (Franke and Schilcher, 2005). It was hypothesized that increased ambient temperature would be correlated with lower levels of outcrossed offspring, as environmental stresses, such as heat, are known to promote self-pollination in other species (Holtsford and Ellstrand, 1992; Shivrain *et al*., 2009). As indicated in Figure 5, Greenhouses 1, 2, 3, 8, 10, and 11 had maximum temperatures between 32.0°C and 38.9°C, temperatures above the optimal growing conditions for the species and potentially causing heat stress during flowering. However, seeds were still produced but did not germinate. Greenhouse 7 was within the ideal growing conditions (maximum temperature of 25.3°C), and plants from this greenhouse produced seeds that did not germinate.

Global mean surface temperature is predicted to rise by 1.8°C to 4.0°C by the year 2100 (Schmidhuber and Tubiello, 2007). Overcoming the effects of high temperature will be essential for all crops and for food security in the future. Pollen grains becoming exposed to the environment is a critical step in the reproductive cycle of plants. The release of pollen into the environment is controlled by an equilibrium between the physiological state of the sporophyte and atmospheric conditions (Begcy and Dresselhaus, 2018). When a mother plant experiences stress, all stages of male gametophyte development, as well as the timing of pollen dispersal, are adversely affected (Pacini and Dolferus, 2019). Pollen sterility induced by abiotic stress, including high temperature stress, is a problem affecting many crop species (Pacini and Dolferus, 2019). High temperature stress is defined as the rise in temperature beyond a critical threshold for a period of time sufficient to cause irreversible damage to plant growth and development (Rang *et al*., 2011), and has been studied in many species. For example, high temperature stress one day prior to anthesis in rice showed an effect on the normal functioning of the anther dehiscence and pollen viability (Rang *et al*., 2011). Furthermore, temperatures of 35°C and greater lasting for more than one hour at anthesis can lead to high sterility in rice (Jagadish *et al*., 2007). Since seeds were produced at all tested temperatures in this experiment, including beyond the optimum growing temperature, gamete formation and fertilization was likely not a problem. Therefore, temperature effects during germination were examined as an alternative influence.

All seeds, both field and greenhouse grown, were placed in Greenhouse 5 for germination and seedling growth from July 9 to August 8. The optimum temperature for good seed germination in

chamomile is between 10°C and 20°C (Singh *et al*., 2011). The temperature of this greenhouse was not measured after the crossing plants were removed, as it was not expected to affect the experiment. However, from the temperature conditions present in Greenhouse 5 from June 6 to June 26 (Figure 7), it can be predicted that similar temperature conditions in this greenhouse would have been present three weeks later during germination. These temperature conditions are considerably higher than the optimum germination temperature, although the field reciprocal crosses with parents 16-721-10 and 16-080-22 were able to germinate under these conditions with a high outcrossing frequency similar to genotypes tested in Experiment 1. This finding could mean that these specific genotypes are tolerant to high temperatures and are potential candidates for breeding chamomile varieties capable of adapting to abiotic stresses. Heat stress could also explain why the field cross 16-064-6 X 16-067-24 germinated but had a very low outcrossing frequency, as this cross could have a low tolerance to high temperatures during germination. Iloh *et al*. (2014) demonstrated that increased temperature had a negative effect on the germination of maize, rice, and sorghum seeds. Hence, it is possible that the conditions during the germination of seeds in this study could have been improved with a lower temperature in the greenhouse.

4.4.3 Seed Dormancy

Germination is defined as the process that begins with water uptake by the seed and ends with the emergence of the embryonic axis through the structures surrounding it (Bewley *et al*., 2013). When a seed absorbs water, metabolic processes are activated that consequently lead to the expansion of the embryo and the penetration of the radicle through the surrounding tissues (Bewley *et al*., 2013). When in a mature and dry state, seeds are resting organs with low moisture content and little to no metabolic activity (Gallardo *et al*., 2001). Induction of dormancy in seeds is genetically programmed and the release of dormancy only occurs under certain environmental conditions (Pacini and Dolferus, 2019). In order for germination to occur, seeds need to uptake water under ideal conditions, such as at a suitable temperature and in the presence of oxygen. Some seeds require an additional action in order to break dormancy, for example, light or scarification (Taiz *et al*., 2015). After one year of field cultivation, chamomile is known to have seeds that can stay dormant for 10 to 15 years before germinating as a weed species in a new crop (Otto *et al*., 2015). It is possible that in this experiment, the majority of F1 seeds planted remained dormant due to unfavourable temperature conditions in the greenhouse during germination. Chamomile seeds are known to require high humidity for germination and for rapid seedling development (Franke and Schilcher, 2005). Pharmaplant has undertaken using Giberellic acid to break dormancy in chamomile seeds, with inconsistent results (Otto, personal communication).

4.5 Conclusion

In conclusion, the outcrossing ratio from field and greenhouse crosses could not be compared as the greenhouse crosses did not produced viable seeds. The greenhouse crosses could have been negatively affected by poor pollination, seed dormancy, and unfavorable temperature conditions during germination. As such, it would be valuable to add flies into greenhouses growing chamomile to increase pollination efficiency. Future research should be focused on seed germination requirements and methods to break dormancy in chamomile.

The hypothesis, that increased ambient temperature would be correlated to lower levels of outcrossed offspring due to stress on the plant at higher temperatures, could not be tested as the temperature differences were observed only in the greenhouse. The crosses in which progeny germinated had no difference in temperature, as they were completed in field locations within 100m of each other. Interestingly, these seeds were produced when the temperature during flowering and germination was much higher than the optimal growing temperatures of chamomile. This is an important observation as these specific genotypes are tolerant to high temperatures and are potential candidates for breeding chamomile varieties capable of adapting to abiotic stresses.

As demonstrated in the previous study (Chapter 3), diploid crosses exhibited higher outcrossing rates than tetraploid crosses due (at least in part) to stronger self-incompatibility. In this study, the three crosses that produced viable seeds were all diploid.

CHAPTER 5

5.0 General Discussion

The analysis of the outcrossing ratio described in this thesis can be used as a foundation to help design future breeding methodologies in chamomile. Although no precise outcrossing frequency was determined, important observations were made throughout this research. It was hypothesized that there would be no significant difference in the outcrossing frequency based on the location of the cross in central Germany. The results of this study confirmed this hypothesis; the outcrossing ratio was wide ranging with no significant difference based on specific location or based on northern or southern growing region within central Germany. Both diploid and tetraploid crosses were tested and analyzed separately as it was hypothesized that diploid crosses would exhibit higher outcrossing rates due to stronger self-incompatibility. No significant difference in the outcrossing frequency was observed based on ploidy. Although diploid cultivars tend to show stronger self-incompatibility (Faehnrich *et al*., 2013), plants grown in temperatures above 30°C have overcome the self-incompatibility mechanism (Otto, unpublished data). According to the temperature observed during the field isolation of these crosses (Figures 3.4 and 3.5), temperature above 30°C cannot be the reason diploids did not have a stronger self-incompatibility mechanism in this study. It is possible that the outcrossing ratio varied greatly according to the pollinator presence as seen in the natural populations of another mixed-mating Asteraceae species (Arista *et al*., 2017). This first experiment would have been improved if the same genotypes were used at all locations throughout central Germany, as genotype-specific effects were not able to be determined. Furthermore, specific data that could help explain the differences in outcrossing ratio was not recorded, including the amount and types of pollinators present, as well as detailed information on potential wind barriers.

The second study evaluating the effects of field versus greenhouse environments on the outcrossing ratio was technically challenged. A major requirement for the analysis of the outcrossing ratio using the methods described in this thesis was the production of viable seeds in order to grow the F1 generation. Without these seedlings, there was no progeny DNA available to determine the method of fertilization. Initially it was believed that high temperature in the greenhouse during flowering was the cause of non-viable seeds. However, a limited number of field crosses were able to produce viable seeds at temperatures above the optimum growing temperatures for chamomile. Therefore, it is more plausible that the crosses did not produce viable seeds due to issues with pollination or seed dormancy. A sample of seeds from each cross was observed under a microscope to examine the physical features of the seed and it was determined there was nothing physically wrong with the seed, such as abortion due to malformed endosperm, to stop germination. Due to the technical issues, the hypotheses formulated could not be

tested. A recommendation is made to utilize pollinators (such as flies) to help with pollination in future greenhouse experiments involving chamomile.

The most valuable information obtained from this thesis study came from observations that indicated the need of future research in chamomile. Primarily, another field study should take place with a focus on how the presence of pollinators affects the outcrossing ratio using the same genotypes at multiple locations. It would also be beneficial to know the chemical profiles of all genotypes tested to determine if the insects are attracted to specific chemical attractants. An unanticipated finding was that all crosses in the first experiment (Chapter 3) appeared to have an effect on the outcrossing ratio associated with the crossing direction. Parent-of-origin effects in the fertilization method of chamomile should be further researched to determine the impact of maternal and paternal parent among different crosses. The second experiment's (Chapter 4) technical difficulties highlighted the need to introduce flies to help improve pollination of chamomile plants in greenhouses. As well as the need for future work focused on germination requirements and methods to break dormancy in chamomile.

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APPENDICES

Appendix 1. Plant material transfer agreement between Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, and Global Institute for Food Secuirty (GIFS), Saskatoon, Canada.

MATERIAL TRANSFER AGREEMENT

Between

Leibniz-Institute of Plant Genetics and Crop Plant Research
Correnssstrasse 3, OT Gatersleben 06466 Seeland, Germany

- hereinafter collectively referred to as PROVIDER -

and

Global Institute for Food Security (GIFS)
110 Gymnasium Place
University of Saskatchewan
Saskatoon, SK S7N 4J8 Canada

- hereinafter referred to as RECIPIENT -

Art. 1 Subject Matter of this Agreement

(1) IPK owned PLANT MATERIAL of *Matricaria recutita* L. plants (syn. *Chamomilla recutita*; German chamomile).

(2) This Agreement governs RECIPIENT's access to and use of the PROVIDER`s PLANT MATERIAL and related information transferred with the PLANT MATERIAL for the PURPOSE to perform further DNA analysis based on the PLANT MATERIAL provided by IPK in the frame of the Master-thesis "Analysis of the outcrossing ratio of German chamomile (*Matricaria recutita* L.) using SSR-markers" of Ms Maureen Woytowich (hereinafter referred to as PURPOSE).

Art. 2 Definitions

(1) PLANT MATERIAL shall mean leaf material (to isolate the DNA) from *Matricaria recutita* L. plants (syn. *Chamomilla recutita*; German chamomile. PLANT MATERIAL means also any progeny, derivatives and modifications including but not limited to their inherent components (e.g. DNA molecules).

Art. 3 Acknowledgement of PROVIDER's Property

(1) RECIPIENT acknowledges PROVIDER's sole property of all PLANT MATERIAL delivered by PROVIDER in accordance with this Agreement and RECIPIENT refrains from all actions which may affect PROVIDER's rights relating to the PLANT MATERIAL.

(2) Nothing contained in this Agreement shall be deemed to constitute by implication or otherwise the granting of any licence, property right, right of use and enjoyment by PROVIDER to RECIPIENT, other than explicitly stated in this contract.

Art. 4 Confidentiality Obligation

(1) RECIPIENT shall keep all received PLANT MATERIAL strictly confidential and shall not pass it to any third party. Unless explicitly stated otherwise in this Agreement, any disclosure of PLANT MATERIAL to third parties requires prior written consent of PROVIDER.

(2) RECIPIENT shall disclose PLANT MATERIAL only to those of its employees who need the information for the PURPOSE granted in this Agreement directly and who have been obligated to keep confidential all of PROVIDER's knowledge and information they learn about directly or indirectly under this agreement and not to disclose such knowledge and information to third parties, except as required by law.

(3) This confidentiality obligation shall not apply to PLANT MATERIAL, for which RECIPIENT is able to produce evidence that it:
   a) was known to RECIPIENT prior to the date it was received under the terms of this Agreement, or
   b) is now in, or hereafter comes into, the public domain without breach of this confidentiality obligation, or

c) was disclosed lawfully to RECIPIENT at any time from a third party being entitled to disclose the same without obligation of secrecy and without breach of any confidentiality obligation, or
d) has been developed by RECIPIENT independently, or
e) has to be disclosed due to mandatory legal requirements by RECIPIENT to authorities, provided that RECIPIENT indicates the authorities to the confidential character of the PLANT MATERIAL.

(4) Detailed confidential information shall not be excepted from this confidentiality obligation when included in more general information, for which the exception may apply. The same shall be in effect for combinations of single information, when only parts of the information are excepted from this confidentiality obligation but not the combination itself.

(5) RECIPIENT shall give not less than one (1) month prior written notice of its intention to plead an exception from the confidentiality obligation. Documentary material is to be furnished.

(6) This confidentiality obligation is applicable beyond the termination of this contract without any temporal limitation.

Art. 5 Conditions for the use of MATERIAL

(1) RECIPIENT may use the PLANT MATERIAL under the conditions here stipulated for the non-commercial PURPOSE only. RECIPIENT shall not use any PLANT MATERIAL for any personal or other purpose or commissioned research whatsoever. The right to use excludes explicitly the use of the PLANT MATERIAL for services and the commercialisation of new plant varieties.

(2) RECIPIENT especially agrees to handle PLANT MATERIAL in compliance with all national and international laws, regulations and guidelines which are applicable at the time of use and for the PURPOSE. PROVIDER is not responsible for the compliance with all national and international laws, regulations and guidelines which are applicable at the time of use and for the PURPOSE. PLANT MATERIAL shall be handled only by those with sufficient skill, knowledge, experience and ability to use the PLANT MATERIAL.

(3) RECIPIENT will keep PROVIDER informed in writing about the progress related to the preparation of the respective application of the field trial and if required provide PROVIDER with a final written report on their evaluation of the PLANT MATERIAL.

(4) MATERIAL as well as lines resulting from the PLANT MATERIAL and DNA or RNA extracted from the PLANT MATERIAL, may be used by RECIPIENT for the PURPOSE only.

(5) RECIPIENT is not entitled to give PLANT MATERIAL as such, as well as lines resulting from the PLANT MATERIAL and DNA and RNA extracted from the PLANT MATERIAL, to, or make available to or transfer in any other way to any third parties nor to use PLANT MATERIAL outside its facilities (on the address specified in the MTA).

(6) For the purposes of allowing PROVIDER to protect patentable subject matter and to prevent disclosure of confidential PROVIDER information, RECIPIENT agrees to give IPK an opportunity to review any proposed publication or presentation containing confidential PROVIDER information relating to the PLANT MATERIAL by sending a full copy of any such proposed publication or presentation at least sixty (60) days prior to the intended date of publication or it being first submitted for publication or presentation (whichever occurs first), or the intended date of presentation. IPK shall have 30 days, after receipt of said copies, to approve such proposed presentation or publication (the "Response Period").

(7) If IPK object in writing to the proposed publication or presentation within the Response Period, then RECIPIENT shall refrain from making such presentation or publication (i) until the parties have agreed to a version which protects the confidential information from public disclosure, or (ii) until IPK have filed patent application(s) directed to the patentable subject matter or otherwise sought protection for the information contained in the proposed presentation or publication.

(8) The result of any research based upon the PLANT MATERIAL thereof and other biological material or information or results obtained or discovered from other sources but which could not have been obtained or discovered without the disclosure of the PLANT MATERIAL shall neither be included in any patent application, business secret or any title deed nor commercialised or put to any other than strictly non-commercial use without a prior written Agreement between PROVIDER and RECIPIENT concerning ownership and commercial

exploitation of the results. In any event, IPK shall have - each to full extent - the irrevocable right to use such results and biological material free of charge for any non-commercial research purposes.

(9) RECIPIENT shall ensure that its employees and/or co-workers engaged in use under this Agreement proceed in the same way as set out in this Agreement.

Art. 6 Warranty, Liability, Indemnification

(1) PROVIDER provides the PLANT MATERIAL with no warranty or representations as to the purity, activity, safety, reliability, quality, commercial utility or usability or fitness for a particular purpose or any other warranty, express or implied. In particular, PROVIDER does not warrant that the exercise of this Agreement nor the use of the PLANT MATERIAL does not interfere with or infringes upon any third party patents, copyrights, trademarks or other rights.

(2) RECIPIENT recognizes that the PLANT MATERIAL is of an experimental nature. PROVIDER disclaims any and all liability for any damage (including, but not limited to, damage derived or later originating from an initial damage, and claims to consequential losses) that may arise from use, storage or disposal of the PLANT MATERIAL and that is not the result of PROVIDER´s wilful misconduct or gross negligence. PROVIDER will not be liable to the RECIPIENT for any loss, claim or demand made by the RECIPIENT, or made against the RECIPIENT by any other party, due to or arising from the use of the PLANT MATERIAL by the RECIPIENT, except to the extent permitted by law when caused by the gross negligence or wilful misconduct of PROVIDER.

(3) RECIPIENT will indemnify, defend and hold harmless PROVIDER, and its officers, agents and employees against any claims, costs, product or other liabilities, of whatsever nature, which may arise from or in connection with this Agreement or as a result of RECIPIENT's use, handling, storage, release (including inter alia distribution to a third party) and disposal of the PLANT MATERIAL, DATA and/or any result of the RECIPIENT'S research related to the PLANT MATERIAL and DATA.

(4) It is further agreed that the furnishing of PLANT MATERIAL to RECIPIENT shall not constitute any grant or license to RECIPIENT under any legal rights now or hereinafter held by PROVIDER. The obligations of RECIPIENT under the terms of this Agreement shall remain in effect until the material becomes generally/publically available or until two years from date of signature. Upon termination of this Agreement, RECIPIENT will discontinue use of the PLANT MATERIAL. Upon the termination of this agreement all PLANT MATERIAL provided and supplied herein by IPK to RECIPIENT including all copies made or parts separated by RECIPIENT shall at IPK's election without delay either be destroyed or sent back to IPK. In the case of destruction, RECIPIENT shall supply IPK with certification of destruction. PLANT MATERIAL may not further be used by the RECIPIENT.

(5) IPK can terminate this Agreement on written notice to RECIPIENT, upon the occurrence of any breach of this agreement by RECIPIENT. This termination does not affect any of IPK's claims due neglect of duty by RECIPIENT.

(6) The provisions of clauses 1- 6, and 7 shall survive termination of this Agreement.

Art. 7 Term and Termination

(1) This Agreement shall commence with the execution by both RECIPIENT and PROVIDER. This Agreement shall remain in effect without any temporal limitation unless terminated by one of the parties as stated herein.

(2) This Agreement will terminate on the earliest of the following dates:
(a) when the PLANT MATERIAL becomes generally available from third parties, for example, through reagent catalogs or public depositories, or

(b) on completion of the RECIPIENT's current research for the PURPOSE with the PLANT MATERIAL, or

(c) on thirty (3) months written notice by either party to the other, provided that:

(i) if termination should occur under 8 (a), the RECIPIENT shall be bound to the PROVIDER by the least restrictive terms applicable to the PLANT MATERIAL obtained from the then-available sources; or

(ii) if termination should occur under 8 (b), the RECIPIENT will discontinue its use of the PLANT MATERIAL and will, upon direction of the IPK, return or destroy any remaining PLANT MATERIAL. In the case of destruction, RECIPIENT shall supply IPK with certification of destruction. PLANT MATERIAL may not further be used by the RECIPIENT and

(iii) in the event the IPK terminates this Agreement under 8 (c), other than for breach of this Agreement or for cause such as an imminent health risk or patent infringement, the IPK will defer the effective date of termination for a period of up to one year, upon request from the RECIPIENT, to permit completion of research in progress. Upon the effective date of termination, or if requested, the deferred effective date of termination, RECIPIENT will discontinue its use of the PLANT MATERIAL and will, upon direction of the IPK, return or destroy any remaining PLANT MATERIAL and supply IPK with certification of destruction and (iv) provided that termination shall not relieve the RECIPIENT of any obligation or liability accrued hereunder prior to the effective date of such termination.

(3) PROVIDER can also terminate this Agreement on written notice to RECIPIENT, upon the occurrence of any breach of this Agreement by RECIPIENT. This termination does not affect any of PROVIDER's claims due neglect of duty by RECIPIENT. Upon the termination of this Agreement all PLANT MATERIAL provided and supplied herein by PROVIDER to RECIPIENT including all copies made or parts separated by RECIPIENT shall at PROVIDER's election without delay either be destroyed or sent back to PROVIDER. In the case of destruction, RECIPIENT shall supply PROVIDER with certification of destruction. PLANT MATERIAL may not further be used by the RECIPIENT.

(4) The provisions of clauses 1, 3, 4, 5, 6 and 8 shall survive termination of this Agreement.

Art. 8 Miscellaneous

(1) This Agreement may not, in its entirety or in parts, be transferred or assigned by RECIPIENT in any manner whatsoever to any third party without previous assent by PROVIDER.

(2) This Agreement is the only authoritative instrument. There are no oral collateral agreements. Any alteration, modification, amendment and completion of this agreement may be done in a written form only. The same applies to any waiver of the written form requirement.

(3) If any of the provisions of this Agreement or a part thereof should be or become nugatory, then the validity of the remaining Agreement shall not be affected thereby. The same applies in the event that any provision or parts thereof should prove unenforceable. In the case that the unenforceability or invalidity of a clause rests on its material, spatial or temporal extent the clause shall be valid in its largest and widest enforceable extent possible.

(4) PROVIDER and RECIPIENT agree to replace an unenforceable or nugatory clause or part of this Agreement by a new clause to be negotiated in good faith which covers the economic content of the invalid clause as far as possible. This may also be applicable in the case of a supplementary interpretation of the agreement.

(5) This Agreement shall be governed and construed in accordance with German Law and the parties hereto submit to the jurisdiction of the German Court which is locally competent in Gatersleben.

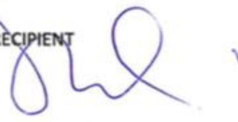
(6) This Agreement may have been supplied to RECIPIENT as an electronic data file. RECIPIENT hereby represents that it has not altered the text of the Agreement. This Agreement shall be null and void if its text differs in any way from the text of the electronic data file that was provided to RECIPIENT.

Gatersleben, 25.01.2018

For PROVIDER

Prof. A. Graner
Managing Director

For RECIPIENT



Prof. Dr. T. Sharbel
~~Administrative Director~~
Chair, Seed Development
Global Institute for Food Security

Appendix 2. Supplementary tables on crosses completed in 2014.

Parents	Ploidy of Both Parents	Grower	Location	Number of Progeny DNA for Analysis	Plate Number
20/2 X 13/2	2x	Hoffmann	Bretleben	32	18
13/2 X 20/2				32	18
20/2 X 13/2	2x	Großmann	Erfurt	32	18
13/2 X 20/2				32	19
20/2 X 13/2	2x	Schaller Eltern	Borxleben	32	19
13/2 X 20/2				32	19

Parents	Ploidy of Both Parents	Grower	Location	Number of Progeny DNA for Analysis	Plate Number
12/5 X 20/04	2x	Steinmetz	Töttelstädt	32	20
20/04 X 12/5				32	20
12/5 X 20/04	2x	Hübner	Erfurt	32	20
20/04 X 12/5				32	21
12/5 X 20/04	2x	Beier	Erfurt	32	21
20/04 X 12/5				32	21

Parents	Ploidy of Both Parents	Grower	Location	Number of Progeny DNA for Analysis	Plate Number
24/1 X 29/4	4x	Steinmetz	Töttelstädt	32	22
29/4 X 24/1				32	22
24/1 X 29/4	4x	Reh	Tiefthal	32	22
29/4 X 24/1				32	23
24/1 X 29/4	4x	Freist Mutter	Ritteburg	32	23
29/4 X 24/1				32	23

Parents	Ploidy of Both Parents	Grower	Location	Number of Progeny DNA for Analysis	Plate Number
23/4 X 29/1	4x	Neumann	Schönfeld, Hof	48	24
29/1 X 23/4				48	24
23/4 X 29/1	4x	Göttsching	Erfurt	48	25
29/1 X 23/4				48	25

Parents	Ploidy of Both Parents	Grower	Location	Number of Progeny DNA for Analysis	Plate Number
04/2 X 18/2	2x	Grimmer	Erfurt	48	26
18/2 X 04/2				48	26
04/2 X 18/2	2x	Ullrich	Hettstedt	48	27
18/2 X 04/2				48	27
04/2 X 18/2	2x	Trautvetter	Erfurt	48	28
18/2 X 04/2				48	28

Parents	Ploidy of Both Parents	Grower	Location	Number of Progeny DNA for Analysis	Plate Number
22/6 X 16/8	4x	Schaller	Borxleben	48	29
16/8 X 22/6				48	29
22/6 X 16/8	4x	Eulau	Freyburg	48	30
16/8 X 22/6				48	30
22/6 X 16/8	4x	Ellinger	Erfurt	48	31
16/8 X 22/6				48	31