FROM THE FIELD TO THE FLOWERBED TO THE LAB: ORNAMENTAL WHITE CLOVER BREEDING AND LEAF TRAIT MAPPING

by

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(Under the Direction of Wayne Parrott)

ABSTRACT

Ornamental white clover (*Trifolium repens* L.) can be developed due to the plant's spreading growth habit and morphological traits that can be combined to create attractive genotypes. Four ornamental genotypes were developed and released after two years of field evaluation. These genotypes were selected due to their highly ornamental phenotypes and comparable or superior performance when compared to currently available ornamental cultivars.

Classic inheritance studies are inconclusive in allotetraploid white clover. Molecular markers can aid locating trait loci at the molecular level. A mapping population containing eight leaf traits was screened for molecular markers and phenotyped. By utilizing published maps, linkage between molecular markers and two different traits were found. The red midrib trait is controlled by dominant genes on two different linkage groups, LG B1 and LG G2. The multifoliolate trait is controlled by two recessive genes on homoeologous groups H1 and H2.

INDEX WORDS: White clover, *Trifolium repens*, ornamental breeding, SSR, trait mapping, red midrib, multifoliolate.

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DEDICATION

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

WHITE CLOVER

White clover is an annual or short-lived perennial found throughout temperate regions of the world (Gibson and Cope, 1985). Originally native to the Mediterranean (Ellison et al., 2006), white clover grows in a wide range of temperatures and in nearly any soil type provided enough moisture and sunlight (Gibson and Cope, 1985). Man has utilized white clover for centuries as forage for livestock due to its high protein value (Van Keuren and Hoveland, 1985). White clover has also been used for many other reasons. The Celtic people of Wales used white clover as a charm to ward off evil spirits (Taylor, 1985) and to this day people search for four-leaf clovers to keep for a good luck charm. A purple-leafed form of white clover was used to treat "the purples (diphtheria) in children and others" during the sixteenth and seventeenth centuries (Parkinson, 1640). More recently, white clover has been cultivated as a nitrogen-fixer in pastures (in addition to its use as a forage crop) and as a living mulch for erosion control (Gibson and Cope, 1985).

White clover is an allotetraploid ($2n = 4x = 32$) that reproduces almost exclusively by outcrossing (Gibson and Cope, 1985). As a result, the plant is highly polymorphic and a wide range of genetic diversity can be found in even a small population of naturalized plants. Wild-type white clover plants have trifoliolate leaves with a white V-mark on the surface of each leaf (Brewbaker, 1955), green stolons, long petiolules branching from the petiole, and white flowers

that give *T. repens* its common name. Variation in white clover traits that can be used for ornamental breeding development includes varying flower color, stolon color, leaflet marks, leaflet number, and petiolule length. The wide range of traits found in the species, in addition to its low, spreading growth habit make white clover ideal for development as an ornamental bedding plant.

GENETICS

The genus *Trifolium* is believed to have originated in the Mediterranean during the Early Miocene era (Ellison et al., 2006). *Trifolium repens* is a member of subgenus *Trifolium,* section *Trifoliastrum* (Ellison et al., 2006). White clover is an allotetraploid (2n = 4x = 32) believed to have resulted from the hybridization of diploids *Trifolium occidentale*, D.E. Coombe and *Trifolium pallescens*, Schreber (Ellison et al., 2006). However, later sequencing efforts by Hand et al. (2008) indicated little homology between *T. repens* and *T. pallescens*. Regardless of ancestral parents, the hybridization event between the two species is believed to have occurred a long time ago (Gibson and Cope, 1985). The disomic inheritance pattern of white clover was confirmed by Weidema (1996) studying the segregation of the isozyme IDH alleles.

White clover has a gametophytic self-incompatibility system (Townsend and Taylor, 1985), which requires pollination from plants that have different self-incompatibility (*S*) alleles than those found in the flower. Self-incompatibility in white clover is controlled by a single gene with many different alleles (Atwood, 1940). Based on data gathered by Atwood in 1942 and 1944, Lawrence (1996) estimated the number of self-incompatibility (*S*) alleles in white clover to be between 74 and 139, including a rare S*f* allele that occurs in less than 1% of a given population (MichaelsonYeates et al., 1997). The self-incompatibility trait in white clover has a

diploid inheritance pattern, which is believed to be due to only one of the original parents containing the self-incompatibility gene during hybridization (Townsend and Taylor, 1985).

ORNAMENTAL TRAITS

Flower Color

White clover gets its common name from its white flowers (Fig.1.1a). However, true white flowers are very rare in the species (Baker, 1987). A light pink pigmentation is often found on the petals of white clover that Brewbaker (1962) stated is environmentally controlled. Conversely, Gillett (1985) noted that the flower color in white clover turns to pink after the flower senesces, perhaps indicating the breakdown of pigment inhibitors in the petals. Brewbaker (1962) studied the light pink (blush) pigmentation (Fig.1.1b) in white clover and determined that the pigmentation is caused by delphinidin, a derivative of cyanidin, and found the trait recessive to white flower color. On two separate occasions, a red-flowered (Fig.1.1c) sport was discovered (Brewbaker, 1962; Pederson and McLaughlin, 1995). Brewbaker's red flower (cyanidin-red) was determined to be controlled by two recessive genes and linked to black seed coat (Brewbaker, 1962). The other red flowering white clover was discovered growing in a lawn in Iowa in 1983 (Pederson and McLaughlin, 1995). Their inheritance study using this trait indicated that the red flower color is recessive to white flower, but the number of genes controlling this trait could not be confirmed (Pederson and McLaughlin, 1995).

Stolon Color

A red stolon color (Fig. 1.2) has been observed in white clover, but no information on the source of the pigmentation or the mode of inheritance is available. In red clover, *Trifolium*

pratense L., Taylor (1982) registered germplasm containing this trait and determined that it is controlled by a single gene, with the red color dominant over green color. The mode of inheritance was later confirmed using isozymes (Xie and Mosjidis, 2001). Red stolon color in *Medicago polymorpha*, an annual legume, was also found to be controlled by one gene, with red color being the dominant trait (De Haan and Barnes, 1998).

Leaflet Marks

Figure 3.1 shows some examples of the different leaf marks found in white clover. The most common leaflet mark found on white clover is a white V-mark. The white V-mark is due to a significantly reduced or complete lack of chloroplasts in the palisade cells on the upper surface of the leaflets (Brewbaker, 1955). For consistency, the gene symbols of Quesenberry et al. (1991) will be used throughout the paper. Both Brewbaker (1955) and Carnahan et al. (1955) studied the inheritance of the white V-mark. The two papers agreed that the trait is controlled by one gene (*V* gene) with multiple alleles, where no white V-mark (*v*) is the recessive phenotype. Carnahan et al. (1955) also described three red-color leaf marks that are controlled by a single gene (*R* gene) - red fleck (*Rf*), red midrib (*Rm*), and red leaf (*Rl*). For this gene, like the *V* gene, lack of a red-color leaf mark (*r*) allele was recessive to the other red leaf mark alleles (Carnahan et al., 1955). These traits were found to have a stronger expression when temperatures were less than 10^{o}C, but were still present during the higher temperatures of summer (Carnahan et al., 1955).

Corkill (1971) studied the inheritance of the different leaflet marks described by Brewbaker and Carnahan, as well as three other red-color leaf marks- diffuse red leaf (*Rld*), controlled by the *R* gene, and red leaflet (*Vrl*) and halo mark (*Vh2*), which are controlled by the

same gene as the white V-mark. Like the traits controlled by the *R* locus, red leaflet expression can be affected by environmental variation (Davies, 1963). All three leaf marks were found to be dominant traits (Corkill, 1971). Hovin and Gibson (1961) studied the inheritance of a redspot mark (*Vr2*) in white clover. The authors stated that this trait is controlled by a novel allele of the *V* gene that is dominant over no-leaf mark (*v*), and co-dominant to the other *V* alleles (Hovin and Gibson, 1961). LeNoble and Papineau (1970) discovered the marginal mark (*Vm*), which is rarely seen in naturalized white clover populations, and found it to be a dominant single gene trait.

Although it is agreed that all leaf marks described above are dominant traits, there is disagreement as to the genetic control of these traits. Carnahan et al. (1955) and Brewbaker (1955) state that the expression of the various leaf marks is controlled by two different genes (*V* and *R*) that each contain multiple alleles. Corkill (1971) hypothesized that the leaf marks are instead controlled by two different loci, each consisting of tightly linked genes, due to a small recombination percentage found in progeny resulting from crosses done using plants with leaf marks from the same gene series. Brewbaker (1955) also found a low rate of recombination among the different white V-marks in his study, but still concluded that the marks were due to a single gene.

Leaf mark traits have been studied in related species of clover, as well. In subterranean clover (*Trifolium subterranium* L.), Tan and Collins (1987) observed 12 different leaf marks, and hypothesized that they are controlled by one gene, with many different alleles that have varying levels of dominance within the gene. In red clover, Smith (1950) determined that the different leaf marks are controlled by four genes- one dominant gene that controls leaf mark expression, another dominant gene that controls the location of the leaf mark, and two dominant genes that

control the expression and location of apical leaf mark (described as marginal mark (*Vm*) in *T. repens*).

Multifoliolate leaves

Most white clover leaves are trifoliolate, but multifoliolate individuals can be found quite often in wild populations (Fig. 3.2). It is believed that ancestral species of *Trifolium* were in fact multifoliolate (Eames, 1961; Jaranowski and Broda, 1978; Zohary and Heller, 1984). The multifoliolate forms of white clover leaves are considered a sign of good luck, especially the four-leaflet form. Variation in leaflet number in white clover ranges from a unifoliate form described by Atwood (1938), up to ten leaflets per leaf (Erith, 1924). The multifoliolate trait was studied in *T. repens* by Ford & Claydon (1996), but they were unable to determine the inheritance of the trait, except to state that it is a recessive trait. A multifoliolate white clover genotype was registered by Baltensperger et al. (1991). No attempt to determine the inheritance of the trait was reported, except to state that there was strong environmental influence (Baltensperger et al., 1991).

Knight (1969) studied multifoliolate leaves in crimson clover (*T. incarnatum* L.) and found that there were two types of multifoliolate leaf traits. One type that was found to be controlled by one recessive gene, had a palmate leaf pattern (Knight, 1969). The other multifoliolate type was strongly influenced by environmental conditions and had a high percentage of pinnate leaves (Knight, 1969). Simon (1962) studied multifoliolate leaves in a small number of red clover testcross populations, and suggested that the trait is controlled by two genes, with at least one of the two genes having to be homozygous recessive for the expression of multifoliolate leaves. Jaranowski and Broda (1978) also studied multifoliolate leaves in red

clover and determined that the trait is controlled by at least three recessive genes. Taylor (1982) also found this trait to be quantitative in red clover. Multifoliolate leaves have also been studied in related genera. In alfalfa (*Medicago sativa* L.), the trait is quantitative and under strong environmental influence (Bingham and Murphy, 1965). In soybean (*Glycine max* (L.) Merr.), the multifoliolate trait is controlled by two different genes- one dominant and one recessive (Palmer and Kilen, 2004). The recessive gene that controls multifoliolate leaf expression in soybean is also under environmental influence (Fehr, 1972).

Extended Petiolules

Wild-type white clover leaflets have long petiolules that extend from the petiole at relatively equal lengths, with the terminal petiolule sometimes slightly longer than the lateral ones (pers. obs.). Variation in petiolule length ranges from very short petiolules to long petiolules and varies by leaflet (Fig.3.2). The long petiolule trait has not yet been researched in white clover. Knight (1969) studied the trait in crimson clover and determined that the trait was controlled by a single recessive gene. In red clover, it was determined that petiolule length is controlled by two genes, where a double recessive genotype at one of the genes is necessary for abnormal petiolule development (Hanson and Hanson, 1952). Broda (1979) also observed elongated petiolules in red clover but found the trait to be single gene recessive.

WHITE CLOVER BREEDING

Conventional Breeding

White clover seed cultivation can be traced back at least to the 1500's in the Netherlands (Leffel and Gibson, 1973). Most white clover breeding effort has focused on improving

agronomic traits in the species. However, there have been a few genotypes released that have ornamental potential. Germplasm containing the cyanidin-red flower color was registered by Pederson and McLaughlin (1995). Baltensperger et al. (1991) registered a multifoliolate germplasm that also had the red leafspot mark (*Vr2*). Two multifoliolate ornamental cultivars ('Crimson Charm' and 'Silver Sprite') were also released from AgResearch Grasslands. One release had red leaves (*Rl*), the other release had green leaves (Caradus and Woodfield, 1997).

The self-incompatibility system makes emasculation of the flowers unnecessary for most genotypes (Cope and Taylor, 1985), which simplifies crosses in white clover breeding programs. Crosses can be done either by hand or by insects such as honeybees (*Apis mellifera*). Younger, newly open flowers were found to have a higher seed set percentage (Cope and Taylor, 1985), so older, more fully open florets are removed to prevent the possibility of pollen contamination. Once the entire flower has been pollinated, the mature seed head is collected about 3-4 weeks later.

Selection of desirable breeding lines in white clover breeding programs is difficult due to the allotetraploid genome and its heterozygosity. The gametophytic self-incompatibility system makes the species impossible to self-pollinate enough generations to fix desirable traits, except in very few lines. Even in self-fertile genotypes, self-pollination is rarely used due to concerns over inbreeding depression. To overcome these obstacles, white clover breeders use recurrent selection over several generations to exert selection pressure on only one trait while maintaining genetic diversity for all other traits in the population (Cope and Taylor, 1985). To avoid inbreeding depression in recurrent selection, a large base population needs to be crossed and maintained (Fehr, 1987), which increases the time required to develop new cultivars when compared to other breeding methods.

In addition to the extended breeding period, there are also replicated field trials that are necessary before the release of any new cultivars. These field trials are done over several years and in different environments. The crossing and testing of new genotypes required in white clover takes a minimum of three growing seasons before a new cultivar can be released. Due to the time and expense involved in the breeding of white clover, researchers are developing molecular methods to make white clover cultivar development more efficient.

Molecular Breeding

The advent of molecular markers in the late 20th century has allowed for their application to be integrated into plant breeding programs in the past decade. Molecular markers have been utilized to identify genes responsible for specific traits, determine the genetic inheritance of traits, map simple and quantitative traits, pedigree analysis, heterogeneity studies, and to assist in breeding programs for both plants and animals (Mohan et al., 1997). By identifying molecular markers associated with traits of interest, breeders are able to identify plants containing those traits during earlier cycles of breeding and selection, thereby increasing the efficiency of the breeding program.

Simple sequence repeats (SSRs), also known as microsatellites, are regions of repeating nucleotide sequences, usually 1-6 bp long, found throughout the genomes of all eukaryotes (O'Hanlon et al., 2000). In 2003, the first SSR and AFLP molecular marker map of white clover was published, allowing for the commencement of gene and QTL mapping in the species (Jones et al., 2003). The map was created using an inbred F_2 mapping population by utilizing the rare *Sf* allele found in the species (Jones et al., 2003) This first map had 18 linkage groups with an average distance of 7cM between the 135 marker loci (Jones et al., 2003). The mapping

population used to create the combined AFLP and SSR map was later used to locate QTLs associated with several agronomic and reproductive traits (Cogan et al., 2006) and SSR markers from the map were used to determine the genetic variability in clover cultivars (George et al., 2006).

In 2004, a molecular map consisting entirely of SSRs in white clover was published that was derived from a mapping population consisting of New Zealand genotypes (Barrett et al., 2004). This map contained 335 polymorphic SSRs and was used to map 493 SSR loci that had an average spacing of 2.3 cM (Barrett et al., 2004). They also mapped the first locus, *R*, which controls red leaf pigmentation, to linkage group B1 (Barrett et al., 2004). This map was later used to map a QTL for resistance to root-knot nematodes (Barrett et al., 2005a) and seed yield QTL (Barrett et al., 2005b).

In 2007, a second white clover SSR map was published that utilized North American genotypes to develop the mapping population (Zhang et al., 2007). This map contained 343 polymorphic SSRs derived from white clover, red clover, *Medicago trunculata* Gaertn., and soybean sources that led to a total of 415 mapped loci with an average spacing of 5 cM (Zhang et al., 2007). The authors of this map used published SSR markers from the two previous maps to develop a very basic consensus map amongst the three maps. As more QTLs and genes are mapped onto molecular maps, it is hoped that white clover breeders will utilize these tools to reduce the time and expense necessary to develop new cultivars.

THESIS OBJECTIVES

White clover has many characteristics that make it amenable to ornamental breeding and cultivar development. As part of the ornamental breeding project, a leaf trait mapping study was

initiated to increase selection of desirable genotypes over a shorter period of time. The goal of this research project is to develop white clover genotypes that can be used for ornamental purposes and to map the leaf traits that are being utilized in the development of ornamental plants.

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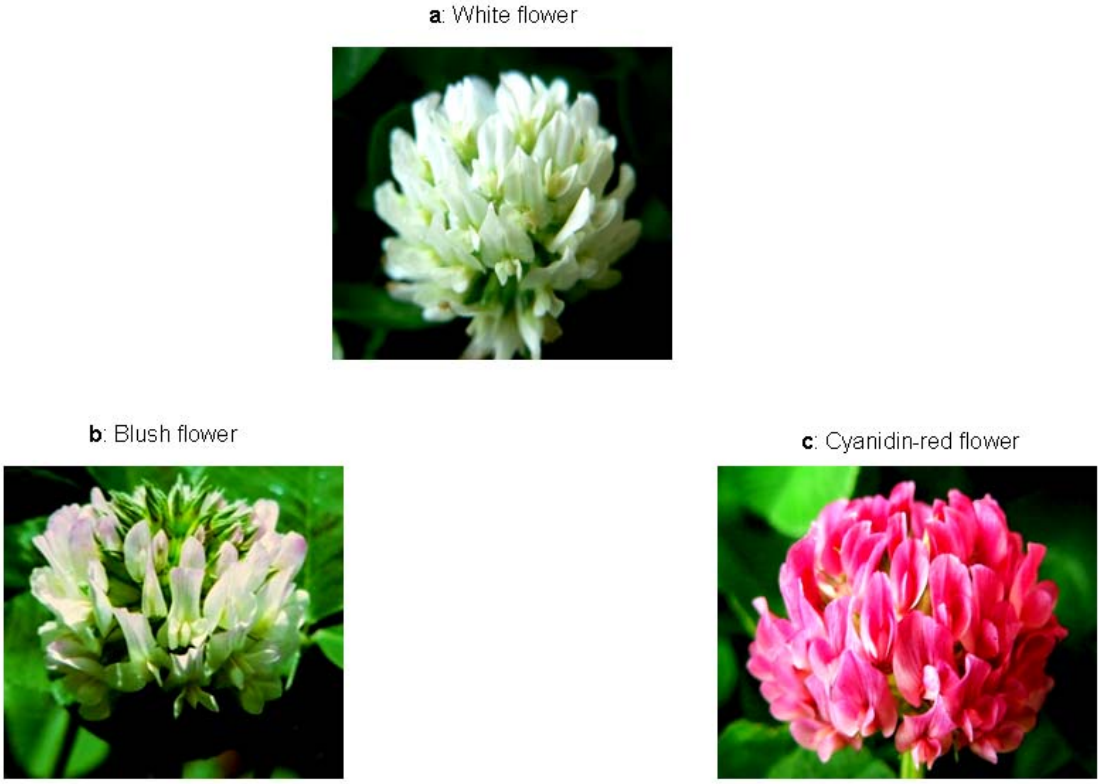

Figure 1.1: Flower colors observed in white clover

a: Green stolon

b: Red stolon

Figure 1.2: Stolon colors observed in white clover

CHAPTER 2

REGISTRATION OF 'FROSTY MORNING', 'PATCHWORK QUILT', 'IRISH MIST' AND 'PISTACHIO ICE CREAM': FOUR WHITE CLOVER CULTIVARS FOR ORNAMENTAL USE[1]

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ABSTRACT

Four white clover [*Trifolium repens* L.] cultivars, ‘Frosty Morning’ (tested as genotype 04-O-2) ‘Patchwork Quilt’ (tested as genotype 04-O-6), ‘Irish Mist’ (tested as genotype 04-O-33) and ‘Pistachio Ice Cream’ (tested as genotype 04-O-53) were developed at the University of Georgia and approved for release in 2008 for use as ornamental garden plants. After two years of evaluations in the field, the four plants were found to be comparable or superior to two commercially available ornamental white clover cultivars in the Southeast region of the United States. These cultivars also have unique phenotypes that are distinct from other commercially available ornamental white clover cultivars that make them suitable for use as ornamental annuals or short lived perennials.

INTRODUCTION

Four white clover [*Trifolium repens* L.] cultivars, ‘Frosty Morning’ (tested as genotype 04-O-2), ‘Patchwork Quilt’ (tested as genotype 04-O-6), ‘Irish Mist’ (tested as genotype 04-O-33), and ‘Pistachio Ice Cream (tested as genotype 04-O-53) were developed at the University of Georgia and approved for release by the University’s Plant Germplasm and Cultivar Release Committee in 2008 for use as ornamental garden plants. These cultivars have unique phenotypes suitable for use as ornamental annuals or short lived perennials. After two years of evaluations in the field, the four plants were found to be comparable or superior to two commercially available ornamental white clover cultivars, ‘Dragon’s Blood’ and ‘Dark Dancer’ in terms of survival and stand quality in the Southeast region of the United States (Figure 2.1).

The four genotypes were developed as part of an ornamental white clover breeding program at the University of Georgia from crossing two commercially available ornamental white clover cultivars ‘Dragon’s Blood’ and ‘Dark Dancer’ with two genotypes developed at the University of Georgia, DS and RD, for ornamental breeding purposes. ‘Dragon’s Blood’ has a combination of red midrib (Carnahan et al., 1955; Corkill, 1971), red fleck (Carnahan et al., 1955) and moon mark (Lenoble and Papineau, 1970) leaf marks, trifoliolate leaves (Gibson and Cope, 1985), and blush flowers (Brewbaker, 1962). ‘Dark Dancer’ has the red leaf (Carnahan et al., 1955; Corkill, 1971) trait, a low level of multifoliolate expression, and blush flowers. This line is believed to be the same variety described by Parkinson (1640) as ‘fower leafed or purple grasse’ (*Quadrifolium fuscum*). DS was selected after two generations of recurrent selection from a cross of MSRLM, a redspot leaf mark white clover germplasm release (Pederson, 1995) and a Dutch-type white clover genotype found in a lawn in Georgia with high multifoliolate expression. It is multifoliolate, has the redspot leaf mark (Hovin and Gibson, 1961), and blush

flowers. RD is an F_1 resulting from the cross of 'Dragon's Blood' x 'Dark Dancer'. It is trifoliolate, has the red leaf trait, and blush flowers. Genotypes 04-O-2 and 04-O-33 are BC_1 plants from the cross of RD ('Dragon's Blood' x 'Dark Dancer') x 'Dragon's Blood'. Genotypes 04-O-6 and 04-O-53 are F_1 plants derived from 'Dragon's Blood' x DS.

MATERIALS AND METHODS

The four genotypes were selected for release after one year of greenhouse evaluation and two years of replicated field trials. In 2004, 22 reciprocal crosses were made with nine different genotypes selected as parents for ornamental traits or stand performance in the Southeast United States. The crosses were done by hand pollination in the greenhouse at the University of Georgia. Seed was harvested by hand three to four weeks later and stored at -20^{o}C to help break dormancy until planted. In 2005, 950 seeds resulting from the crosses were planted in the greenhouse and evaluated for ornamental potential. The seed were germinated by first scarifying between two pieces of 100-C sandpaper, then placing them in 96-cell tray liners containing a potting mix made up of equal parts Fafard #3 potting soil (Conrad Fafard, Inc. Agawam MA), river sand, and farm soil [Cecil sandy clay loam (clayey, kaolinitic, thermic, Typic Kanhapludults)]. The seed was then covered in a fine layer of inoculant (Nitragin, Inc. Milwaukee, WI) containing *Rhizobium meliloti* and *R. luguminosarum* biovar *trifolii*. A thin layer of potting soil was put over the inoculant, and the tray was placed under a misting station for three to four weeks. After removal from the misting station, the plants were placed on a greenhouse bench with 14-hour supplemental lighting. The seedlings were grown in the greenhouse for an additional four to eight weeks, then evaluated for ornamental potential. The seedlings were evaluated twice in the greenhouse – once for phenotype and once for growth

habit. The phenotype evaluation occurred while the plants were in the 96-cell tray liners. Plants that were found to have an ornamental phenotype were potted up into 15-cm pots with the same potting soil and evaluated for growth habit four to six weeks later. Plants that passed the growth habit evaluation were propagated for replicated field trials.

Ninety plants (clonal genotypes) selected for field trials and six of the parental genotypes were planted in two locations in 2006 (Watkinsville, GA and Ardmore, OK) in a randomized complete block experimental design with four clonal reps (blocks) of each genotype at each location. The Georgia field test was established in Mar 2006 in a Cecil sandy clay loam (clayey, kaolinitic, thermic, Typic Kanhapludults) soil with a pH of 6.5. At the Georgia location, the plants were evaluated three times for quality traits – 3 July 2006, 10 Oct 2006, and 6 Apr 2007. The ornamental traits measured were survival, spread, height, canopy density, flower color, flowering amount, leaflet number, and disease symptoms. The experiment was repeated at the Georgia location in 2007. The second-year Georgia field test plot was as described above, except the establishment date was Oct 2007 and soil pH was 6.1. Second-year ornamental quality measurements were taken three times- 20 Nov 2007, 6 Apr 2008, and 14 July 2008, as described above. The Oklahoma field test was planted in a Normangee clay loam (fine, smectitic, thermic Udertic Haplustalfs) soil with a pH of 6.7. At the Oklahoma location, the plants were established in Oct 2005 and were evaluated only once in June 2006. The quality traits measured in Oklahoma were survival, spread, height, flowering amount and seed count.

Spread was measured by taking the average of two perpendicular measurements of the stand diameter. Height was measured at the center of the stand from the soil line to the top of the canopy. Canopy density was measured by a 0-5 rating system based on the amount of soil visible through the stand. A zero value indicated that no leaves were on the plant. A score of

three indicated that 10-15 cm^2 of soil was visible through the canopy. A score of five indicated that the canopy was so dense that no soil was visible. Whenever possible, flower color was defined based on descriptions by Brewbaker (1962) of white clover flower pigmentation, though additional color variations were found within the breeding population at the University of Georgia. Flowering amount was measured by a 0-3 rating system based on the number of flowers, living or dead, present on the stand at the time of evaluation. A zero score indicated that the plant did not flower. A score of one indicated that there were up to 10 flowers on the plant. A score of three indicated that the plant had over 30 flowers. Leaflet number was tabulated as either trifoliolate or multifoliolate. Plants with at least one multifoliolate leaf were scored as multifoliolate plants. Disease incidence was scored on a 0-5 rating system based on the symptoms of the leaves in the stand. A score of zero indicated that no disease symptoms were found on any leaves at the time of evaluation. A score of three indicated that many leaves had necrotic spots, and some leaves were showing symptoms of chlorosis. A score of five indicated that many leaves were dead and all remaining leaves were chlorotic.

The data from each location was combined by year and analyzed to determine which genotype means were statistically different for each ornamental quality trait when compared to the means of two commercialized ornamental white clover cultivars, ‘Dragon’s Blood’ and ‘Dark Dancer’ by Duncan’s Multiple Range Test ($p=0.05$) using SAS 9.1 (SAS Institute, Inc. Cary, NC). Genotypes were selected for cultivar release based on survival, spread, height, canopy density, and lack of disease symptoms.

CHARACTERISTICS

Field measurements

At the GA location, the first year of field evaluations occurred in temperatures that were warmer and drier than the 30-year average. According to the Georgia Automated Environmental Monitoring Network, the average daily maximum temperature from 1 June to 30 September 2006 was 30.82^{o}C, which was 0.8 degrees Celsius higher than the previous year and 0.7 degrees Celsius higher than the 30 year average (1971-2000). During the same period, 31.0 cm of precipitation fell, which was 26.7 cm less than the previous year and 7.6 cm less than the 30 year average (1971-2000). The second year of field evaluations was even warmer and drier than 2006. The average daily maximum temperature from 1 June to 30 September 2007 was 32.02^{o}C, which was 1.2 degrees Celsius higher than the previous year and 1.9 degrees Celsius higher than the 30 year average (1971-2000). During the same period, only 22.2 cm of precipitation fell, which was 8.8 cm less than the previous year and 16.4 cm less than the 30 year average (1971-2000).

'Dragon's Blood' had 17% survival during the first year and 25% survival during the second year of trials at the GA location (Tables 2.1 & 2.2). 'Dark Dancer' had 75% survival during the first year and 58% survival during the second year of trials at the GA location. Genotypes 04-O-2, 04-O-6, and 04-O-33 had 100% survival at both locations, which was statistically higher than the survival rate of both commercial checks over two years at the GA location. Genotype 04-O-53 had 92% survival at the GA location during the first year of field trials, similar to that of 'Dark Dancer', but superior to that of 'Dragon's Blood'. Genotype 04-O-53 had 100% survival at the GA location during the second year of trials, which is better than that of both commercial checks.

The two commercial checks, ‘Dragon’s Blood’ and ‘Dark Dancer’ had 100% survival at the OK location as did all four new genotypes (Table 2.3). In light of its performance during one year in GA (Table 2.1), it is therefore noteworthy that genotype 04-O-53 had 100% survival at this location, which was similar to both commercial checks.

The ‘Dragon’s Blood’ average spread was 57.3 cm at the OK location, 3.1 cm during the first year GA trial, and 1.8 cm during the second year GA trial. The average height of this check was 6.6 cm, 0.4 cm, and 0.3 cm at each environment. The ‘Dark Dancer’ average spread was 50.3 cm at the OK location, 14.4 cm during the first year GA trial and 9.2 cm during the second year GA trial. The average height of the check was 4.9 cm, 1.8 cm, and 2.1 cm at each environment. The four genotypes selected all had spreads similar to ‘Dragon’s Blood’ at the OK location. Genotype 04-O-6 had a spread similar to both ‘Dragon’s Blood’ and ‘Dark Dancer’ at the OK location (Table 2.3). Genotypes 04-O-2 and 04-O-6 had heights similar to ‘Dragon’s Blood’ and were taller than ‘Dark Dancer’ at the OK location. Genotypes 04-O-33 and 04-O-53 were taller than both commercial checks at the OK location (Table 2.3). At the GA location, the four genotypes had spreads superior to both commercial checks and were also taller than both commercial checks for both years of evaluation (Tables 2.1 & 2.2).

The canopy density of ‘Dragon’s Blood’ was 0.4 during the first year GA evaluation and 0.3 during the second year. The canopy density of ‘Dark Dancer’ at the GA location was 2.5 for the first year and 2.3 for the second year. Genotypes 04-O-2, 04-O-6 and 04-O-33 had significantly denser canopies the first year of evaluation at the GA location as compared to the two commercial checks. During the first year GA evaluation, 04-O-53 had a canopy similar to that of the ‘Dark Dancer’ check and denser than ‘Dragon’s Blood’. All genotypes had

significantly denser canopies than both commercial checks during the second year of evaluation at the GA location (Tables 2.1 & 2.2).

The disease incidence of 'Dragon's Blood' was 1.0 for the first year GA evaluation and 4.7 during the second year. The disease incidence of 'Dark Dancer' was 2.0 during both years of evaluation in GA. Three of the genotypes, 04-O-2, 04-O-33, and 04-O-53, had disease incidence measurements similar to those of 'Dragon's Blood' and 'Dark Dancer', while 04-O-6 had disease incidence measurements similar to 'Dark Dancer' for the first year GA evaluation. All four genotypes had disease incidence measurements similar to 'Dark Dancer' and lower than 'Dragon's Blood' for the second year GA evaluation.

Ornamental measurements

The leaves of genotype 04-O-2 are trifoliolate, borne alternately along the stolon, obovate in shape, have serrulate margins, a retuse tip and rounded base that range in width from 2.5 to 3.6 cm when mature. Individual leaflets range in width from 1.2 to 1.9 cm. The leaflets contain the moon and redspot leaf marks. To accurately measure the vegetative and floral color traits of the genotypes, each trait was compared to the *Royal Horticulture Society's Colour Chart* (1995). The moon mark is characterized by a light green crescent mark that corresponds to greyed-green 191-A. The redspot leaf mark is characterized by a greyed-purple 187-A colored V-shape basal to the moon mark that extends to the petiolule as the leaflet matures. The inner leaflet color is green 137-B basal to the red leaf mark. The stolon color corresponds to yellow-green 147-C. The flowers are a deep blush color, which corresponds to red-purple 73-C.

Genotype 04-0-6 is mostly trifoliolate, but also expresses a low level (~12%) of multifoliolate leaves in the summer. The leaves are borne alternately along the stolon, obovate

in shape, have an entire margin, retuse tip and rounded base that range in width from 2.0 to 3.6 cm. Individual leaflets range in width from 0.9 to 1.9 cm. The leaflets contain the moon, red midrib red fleck, and redspot leaf marks. The moon mark is characterized by a light green crescent mark that corresponds to greyed-green 191-A. The red fleck is characterized by greyed-purple 187-A spots of pigmentation appearing randomly along the blade. The redspot leaf mark is characterized by a purple 79-A colored V-shape basal to the moon mark that extends to the petiolule as the leaflet matures. The red midrib is characterized by a greyed-purple 187-A colored herring-bone pattern from the base of the blade to the tip. The stolon color corresponds to yellow-green 147-C. The flower color is pale blush, which corresponds to red-purple 75-C.

The leaves of 04-O-33 are trifoliolate, borne alternately along the stolon, obovate in shape, have a serrulate margin with a retuse tip and rounded base that range in width from 1.7 to 3.0 cm when mature. Individual leaflets range in width from 0.9 to 1.9 cm. The leaflets contain the moon mark and redspot leaf mark traits. The moon mark is characterized by a light green crescent mark that corresponds to greyed-green 189-A. The diffuse red leaf mark is characterized by a greyed-purple 187-A color covering the center of the blade to the petiolule and edge as the leaflet matures. The immature leaflet color is green 138-A basal to the moon mark, which darkens to purple 79-A as the red leaf trait expands. The stolon color corresponds to yellow-green 143-C. The flowers are pale blush colored, which corresponds to red-purple 75-C.

Genotype 04-O-53 is mostly trifoliolate, but also expresses a moderate level (~33%) of multifoliolate leaves in the summer. The leaves are obovate, borne alternately along the stolon, have a serrulate margin, retuse tip and rounded base that range in width from 4.6 cm to 6.7 cm. Individual leaflets range in width from 2.5 to 3.6 cm. The leaflets express the moon and redspot leaf marks. The moon mark is characterized by a light green crescent mark that corresponds to

greyed-green 191-A. The redspot leaf mark is characterized by a greyed-purple 187-A colored V-shape basal to the moon mark that extends to the petiolule as the leaflet matures. The stolon color corresponds to yellow-green 146-D. The flower color is pale blush, which corresponds to red-purple 73-B.

SUMMARY

From nearly one thousand seeds initially planted, four white clover genotypes were selected for ornamental use after two years of field trials and released as the new clonal cultivars, ‘Frosty Morning’, ‘Patchwork Quilt’, ‘Irish Mist’, and ‘Pistachio Ice Cream’. These four highly ornamental genotypes showed superior stand survival, stand performance, and disease resistance in Georgia and superior stand survival and performance in Oklahoma when compared to one of the two currently available ornamental white clover cultivars. The four genotypes showed comparable or superior performance to the second cultivar, ‘Dark Dancer’, which is believed to have been cultivated for over 350 years. In addition, these four genotypes contain novel phenotypes that are not available in any other ornamental white clover cultivars.

AVAILABILITY

Cuttings of 04-O-2, 04-O-6, 04-O-33, and 04-O-53 will be maintained by the University of Georgia, Athens, GA 30602. Plant patents for each genotype are being applied for.

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Table 2.1: Clover genotype performance based on measurements taken during the 2006-2007 growing season at the GA location.

Genotype	Survival (%)	Spread (cm)	Height (cm)	Canopy Density†	Disease Incidence‡
04-O-2	100 a§	40.5 ab	4.2 b	4.1 a	1.8 ab
04-O-6	100 a	47.4 a	5.4 ab	2.8 bc	2.6 a
04-O-33	100 a	35.1 b	4.4 b	3.8 ab	1.7 ab
04-O-53	92 ab	31.9 b	6.8 a	3.3 abc	2.4 ab
'Dragon's Blood'	17 c	3.1 d	0.4 c	0.4 d	1.0 b
'Dark Dancer'	75 b	14.4 c	1.8 c	2.5 c	2.0 ab

† Canopy density: 0 = no canopy to 5 = dense canopy
‡ Disease incidence: 0 = no disease symptoms to 5 = strong disease symptoms
§ Means followed by the same letter are not significantly different at alpha = 0.05 ($P < 0.05$)

Table 2.2: Clover genotype performance based on measurements taken during the 2007-2008 growing season at the GA location.

Genotype	Survival (%)	Spread (cm)	Height (cm)	Canopy Density†	Disease Incidence‡
04-O-2	100 a§	46.4 a	7.5 b	4.7 a	1.4 b
04-O-6	100 a	42.7 a	5.7 b	4.3 a	1.7b
04-O-33	100 a	37.6 a	7.7 b	4.4 a	1.8 b
04-O-53	100 a	46.6 a	10.6 a	4.8 a	1.8 b
'Dragon's Blood'	25 c	1.8 b	0.3 c	0.3 c	4.7 a
'Dark Dancer'	58 b	9.2 b	2.1 c	2.3 b	2.0 b

† Canopy density: 0 = no canopy to 5 = dense canopy
‡ Disease incidence: 0 = no disease symptoms to 5 = strong disease symptoms
§ Means followed by the same letter are not significantly different at alpha = 0.05 ($P < 0.05$)

Table 2.3: Clover genotype performance based on measurements taken at the Ardmore, OK location during 2006.

Genotype	Survival (%)	Spread (cm)	Height (cm)
04-O-2	100 a†	68.0 a	8.8 bc
04-O-6	100 a	60.4 ab	8.0 c
04-O-33	100 a	70.0 a	11.0 b
04-O-53	100 a	63.8 a	13.6 a
'Dragon's Blood'	100 a	57.3 ab	6.6 cd
'Dark Dancer'	100 a	50.3 b	4.9 d

† Means followed by the same letter are not significantly different at alpha = 0.05 ($P < 0.05$)

04-O-2 ‘Frosty Morning’

04-O-6 ‘Patchwork Quilt’

O4-O-33 ‘Irish Mist’

04-O-53 ‘Pistachio Ice Cream’

Figure 2.1: Genotypes selected for cultivar release after field evaluations.

CHAPTER 3

MOLECULAR MAPPING OF THE RED MIDRIB AND MULTIFOLIOLATE LEAF TRAITS IN WHITE CLOVER[1]

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ABSTRACT

Allotetraploid white clover (*Trifolium repens*, L.) is a highly outcrossing heterozygous species in which classic inheritance studies have proven to be inconclusive. With the aid of molecular markers, genes controlling morphological traits in white clover can be determined at the molecular level one gene at a time. A mapping population containing eight morphological traits was developed and phenotyped at two different locations at two different times of the year. By utilizing previously published microsatellite maps, linkage between the molecular markers and two different morphological traits was found in the white clover mapping population. The red midrib trait was found to be controlled by dominant genes on two different linkage groups, LG B1 and LG G2. This finding contradicts previously published inheritance studies on this trait. The multifoliolate trait was found to be controlled by two recessive genes on homoeologous linkage groups H1 and H2 of published white clover microsatellite maps. These findings help to explain the difficulty in fixing a recessive multifoliolate trait in a highly heterogeneous species such as white clover.

INTRODUCTION

White clover, *Trifolium repens* L., is an allotetraploid (2n = 4x = 32) legume that is believed to have resulted from the hybridization of *Trifolium occidentale* (Ellison et al., 2006) and another unknown *Trifolium* species (Hand et al., 2008). Pollination in this species is controlled by a gametophytic self-incompatibility system that was determined by Atwood (1942) to be controlled by a single locus with many different alleles, including a rare *Sf* allele that confers self-compatibility. Due to the tetraploid genome and outcrossing nature of the species, white clover is highly heterozygous.

There are many leaf marks and other morphological traits found within white clover, many of which have been the subject of genetic studies. For consistency, the original genetic nomenclature has been maintained for each trait, but the notation has been modernized to that of Quesenberry et al. (1991). The lack of leaf mark (Fig.1a) is recessive to the presence of all leaf marks (Brewbaker, 1955; Carnahan et al., 1955). The most common leaf mark is the multiallelic white-V mark (Fig.3.1b) on the upper epidermis of each leaflet (Brewbaker, 1955). This trait is highly variable, with the marks ranging from a single V mark to a V mark with a yellow tip (*Vby*, Fig.3.1c). The marginal mark (*Vm*, Fig.3.1d) is rarely seen in naturalized populations (Lenoble and Papineau, 1970). Other leaf marks found in white clover contain anthocyanins. Some examples are the redspot leaf mark (*Vr2*, Fig.1e) (Hovin and Gibson, 1961), red leaflet (*Vrl*, Fig.1f) (Corkill, 1971), red midrib (*Rm*, Fig.1g) mark (Carnahan et al., 1955; Corkill, 1971), and red fleck (*Rf*, Fig.1i) mark (Carnahan et al., 1955).

Although it is agreed that all leaf marks described above are dominant traits, there is disagreement as to the genetic control of these traits. Carnahan et al. (1955) and Brewbaker (1955) concluded that the presence of the various leaf marks is controlled by two different genes

(*V* and *R*) that each contain multiple alleles. In contrast, Corkill (1971) observed low recombination frequencies between the different leaf marks, and concluded accordingly that the *R* and *V* loci each consist of a series of tightly linked genes.

Leaves differ not only in their leaf marks, but also in the number of leaflets. White clover typically has trifoliolate leaves, but multifoliolate genotypes have been observed in naturalized populations. The multifoliolate leaves of white clover are collected as good luck charms, with the four-leaf clover (Fig.3.2a) now recognized as a symbol of good fortune worldwide. Despite the popularity of the four-leaflet trait in clover, it has not been possible to determine its genetic control. Ford and Claydon (1996) determined that the trait was mostly recessive, but were not able to observe any Mendelian segregation in the progeny. Thus, the information available on the genetic control of the multifoliolate trait is limited to that available from other *Trifolium* species. Knight (1969) studied the multifoliolate trait in crimson clover (*Trifolium incarnatum* L.) and found that there were two types of multifoliolate leaves- one that was strongly influenced by environment and one that was not. The environmentally conditioned multifoliolate trait inheritance could not be determined, but the non-environmentally controlled multifoliolate trait was found to be a single gene recessive trait (Knight, 1969). In red clover (*Trifolium pratense* L.), the multifoliolate trait was first studied by Simon (1962) who determined it was conditioned by homozygous recessive alleles at one of two loci, while Jaranowski and Broda (1978) determined that the trait is controlled by homozygous recessive alleles at three loci, and Taylor (1982) determined it was a quantitative recessive trait.

An extended petiolule trait is also found in white clover, which gives the leaf a pinnate shape (Figs. 3.2b and 3.2c). The trait has not yet been studied in white clover, but in red clover, it was found to be controlled by homozygous recessive alleles at a single gene (Broda, 1979) or

homozygous recessive alleles in at least one of two genes (Hanson and Hanson, 1952). In crimson clover, this petiolulate trait was found to be controlled by a single recessive gene (Knight, 1969).

White clover genetics are complicated not only by the allotetraploid genome of the species but also its highly outcrossing nature, such that homozygous lines are not available for inheritance studies. Furthermore, many of the morphological traits under study are highly influenced by environment. For example, many of the traits containing anthocyanin are best observed under temperatures below 10^{o}C (Carnahan et al., 1955). The red leaflet (*Vrl*) trait is conditioned by environment so strongly that it is not visible in the summer (Davies, 1963). In addition, the multifoliolate trait in white clover was found to be environmentally conditioned in a germplasm registered by Baltensperger et al. (1991), which supports Knight's (1969) observations in crimson clover. By looking at these traits at the molecular level, the environmental effects on each trait can be separated from the gene itself. As such, mapping morphological traits found in white clover with molecular markers may be a more effective way to determine the inheritance of these traits, many of which have been studied for nearly a century without satisfactory conclusions.

The development of white clover genetic maps based on molecular markers (Barrett et al., 2004; Jones et al., 2003; Zhang et al., 2007) has allowed some important agronomic traits to be mapped (Barrett et al., 2005a; 2005b; Cogan et al., 2006). In the 2004 white clover microsatellite map, the red fleck mark (*Rf*) mapped onto Linkage Group (LG) B1 (Barrett et al., 2004). The parents used to create the mapping population in that study were forage genotypes, and as such, had limited morphological markers.

The research described here was initiated to map additional leaf morphological traits found in white clover onto the 2007 molecular map (Zhang et al., 2007).

MATERIALS AND METHODS

Plant materials

Two phenotypically distinct white clover genotypes were used as parents to develop a mapping population (Fig. 3.3). One parent, GA02-56 (later named GA43), is an agronomic genotype out of the cultivar 'Durana' (Bouton et al., 2005) that was also used as a parent for the 2007 white clover SSR map (Zhang et al., 2007). This genotype has trifoliolate green leaves, standard petiolules, the intermediate white-V mark (*Vi*), and the red fleck leaf mark (*Rf*). The second parent is 05-O-34, which shows many morphological traits of ornamental value (Tashiro et al., as submitted). This genotype has multifoliolate leaves, an extended petiolule, the marginal mark (*Vm*), red leaflet (*Vrl*), red midrib (*Rm*) and red fleck (*Rf*) leaf marks. This genotype is also self-compatible (*Sf*). A double pseudo-testcross mapping population (Grattapaglia and Sederoff, 1994) was made consisting of 178 F_1s resulting from reciprocal crosses between the two parents. Due to the self-compatibility present in 05-O-34, the F_1 progeny derived from its seed were first tested for hybridity by using 40 SSR marker primers and comparing the individuals to the parents. Only those individuals that had markers derived from both parents were used in the mapping population.

Morphological trait scoring

The 178 individuals in the mapping population were potted up into 12-cm pots using potting mix made up of equal parts Fafard #3 potting soil (Conrad Fafard, Inc. Agawam MA), river sand, and farm soil [Cecil sandy clay loam (clayey, kaolinitic, thermic, Typic Kanhapludults)] and grown in a University of Georgia greenhouse until each individual had filled out the pot. Each individual was scored for the presence/absence of each morphological marker in the greenhouse on 22 Aug 2007 and 29 Mar 2008. 141 individuals that were obtained by selfing the 05-O-34 parent were grown as described above and scored for the presence/absence of each morphological marker in the greenhouse on 12 Aug 2008 and 31 Mar 2009, and used for confirmation of hypothesized genotypes.

Cuttings of both parents and each individual in the mapping population were taken for replicated field plantings. Rooted cuttings of both parents and 140 individuals for which 05-O-34 was the maternal parent were planted at the Plant Sciences Farm (Oconee County, GA) in Cecil sandy clay loam (clayey, kaolinitic, thermic, Typic Kanhapludults) soil with a pH of 5.9. The cloned plants were planted on 75-cm centers in a randomized complete block design with four blocks of each genotype on 6 Dec 2007. Rooted cuttings of both parents and 96 individuals for which GA02-56 was the maternal parent were planted at the Plant Sciences Farm on 18 Apr 2008 with the same experimental design as described above. Each individual in each block was scored for the presence/absence of each morphological trait on 2 July 2008. Individuals in which 05-O-34 was the maternal parent were also scored for the presence/absence of each trait on 26 Mar 2008, and those for which GA02-56 was the maternal parent were scored on 19 Mar 2009. The presence or absence of morphological traits segregating in the 178 individuals used in the mapping population was treated as individual events while attempting to map the traits.

SSR amplification and SSR fragment detection

DNA was extracted from young leaves of each genotype in the mapping population using the Plant DNeasy Mini Kit (Qiagen, Valencia, CA). DNA quantification for each sample was done on a TBS-100 mini-fluorometer (Turner Biosystems, Sunnyvale, CA). After quantification, each sample was diluted to 10 ng μL^{-1} and treated with 0.05 U Longlife RNase (G Biosciences, Maryland Heights, MO). From the original 343 primer pairs used by Zhang et al. (2007) to create their linkage map, 96 were selected based on their even distribution in the different LGs and screened for polymorphism between the two parents used in this mapping population. A total of 78 primer pairs (81%) were polymorphic, which gave a marker spacing of around 20 cM, with between 3 and 6 SSR markers per LG. Fluorescently labeled SSR fragments were amplified as described by Zhang et al. (2007), with the exception of the source of the PCR reagents, which were acquired from Promega (Madison,WI). PCR was performed as described by Zhang et al. (2007) in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA) using either 96- or 384-well PCR plates. After PCR, plates with different fluorescent tags were pooled together for fragment analysis using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) as described by Zhang et al. (2007). SSR fragments were visually scored with GeneMapper 3.7 or 4.0 software (Applied Biosystems) as dominant markers as described by Zhang et al. (2007).

Linkage map development

SSR fragments that segregated in a 1:1 ratio were used to create parental maps using JoinMap 3.0 (Van Ooijen, 2001) as described by Zhang et al. (2007). Rather than pooling the data from the different evaluation dates, the traits segregating in the mapping population were

mapped as dominant markers based on each individual date and place where they were scored (i.e. summer 2008 field or summer 2007 greenhouse) using JoinMap 3.0, with the exception of the multifoliolate trait, which was treated as if conditioned by a recessive allele. Rather than mapping a recessive allele, the multifoliolate trait was instead mapped for its dominant allele, which conditions trifoliolate leaves. Initial attempts to map the multifoliolate trait as a QTL were abandoned, as the data did not have a normal distribution (data not shown). The loci responsible for expression of this trait were found by first linking the trifoliolate trait to molecular markers, based on the trifoliolate parent. Next, the data from the multifoliolate trait were inverted to represent the trifoliolate trait, so that all marker data were dominant, and the trifoliolate trait was mapped based on the multifoliolate parent. Linkage maps were adjusted using the Kosambi mapping function. Those traits that mapped with an LOD score ≥ 4.0 were considered real. The resulting linkage maps were confirmed using the evaluation package of JoinMap 4.0 (Van Ooijen, 2006). After confirmation, the linkage maps were drawn using MapChart 2.2 (Voorrips, 2002).

RESULTS

It was possible to map two of the eight traits studied. The red midrib (*Rm*) and trifoliolate leaf trait each mapped onto two different linkage groups (Figs.3.4 & 3.5) of the 2007 white clover molecular map. One of the genes conditioning the red midrib trait (*Rm,* Fig.3.4) is linked to markers on LG B1, while the second locus is on LG G2. The phenotypic data collected on separate dates and used to map the red midrib trait segregated in a 1:1 ratio (Table 3.1), with each date mapping within 5 cM of each other on both LG B1 and LG G2 (Fig.3.4). Marker

ats084 mapped 15 cM above the red midrib trait on linkage group B1 and marker TRSSRB01B05 mapped within 10 cM of the trait on linkage group G2.

The genes responsible for the trifoliolate leaf trait (Fig.3.5) are linked to molecular markers on linkage groups H1 and H2, which are homoeologous to each other. When the character scored was scored as the trifoliolate trait, the trait mapped to LG H1, while the inverse of the multifoliolate trait mapped onto LG H2. As seen in Figure 3.5, marker TRSSRA02C02 is tightly linked to the genes responsible for winter leaflet number on both LG H1 and H2, and maps to the same location as the winter greenhouse evaluation. As mentioned previously, winter and summer data were mapped separately. The trifoliolate trait always mapped on the same linkage group, but both the data collected in the winter and those collected in the summer are approximately 30 cM apart (Fig.3.5). Within the winter collection date, the data collected in the greenhouse and that collected in the field were 17 cM apart on LG H1 and 18 cM apart on LG H2. Summer data from the greenhouse mapped 24 cM away from that collected in the field for each linkage group.

Of the remaining morphological markers studied, the red fleck (*Rf*) leaf mark segregated in a single gene dominant manner (Table 3.2), with the exception of the summer greenhouse data. This trait is most strongly expressed under low temperatures (Carnahan et al., 1955), so the significant deviation seen in the summer greenhouse data set is most likely due to scoring error while phenotyping the mapping population. The red fleck (*Rf*) leaf mark was not mapped in this study because to map this trait, a bi-parental consensus map needs to be made using co-dominant markers (Zhang et al., 2007). The red leaflet (*Vrl*) trait was only visible during the winter evaluations, but the trait failed to segregate with any of the molecular markers used in this study. The marginal mark (*Vm*) had a 1:1 segregation within the mapping population (Table 3.3), but

likewise, did not segregate with any molecular markers. The intermediate white-V mark (*Vi*) also did not segregate with any of the microsatellite markers screened in this study. The remaining morphological marker studied, extended petiolule, mapped to the same linkage group as the inverse multifoliolate trait (data not shown). This map location is most likely an artifact, since this trait could only be scored with confidence when present with the multifoliolate trait. Thus the data collected for the extended petiolule are incomplete, and the trait was left off the map.

DISCUSSION

The successful mapping of two morphological traits, red midrib and trifoliolate leaves, makes it possible to clarify much of the confusion that has been associated with the genetic control of each of these traits. Adding these markers to the existing molecular maps of white clover also helps saturate the maps available in the public domain. Until now, only one white clover morphological trait had been placed on the molecular map. The *R* locus that maps to LG B1 on the 2004 map (Barrett et al., 2004) is in fact the red fleck (*Rf*) trait. Barrett et al. (2004) followed the Carnahan et al. (1955) hypothesis that red pigmentation on the leaflets of white clover is due to a single gene, with all of the different red-pigmented morphotypes due to different alleles of that gene.

In this study, the red midrib trait was mapped to two different linkage groups (Fig.3.2), with one locus on LG B1 and the other on LG G2. The locus on LG B1 may or may not be the same as the *R* locus mapped by Barrett el al. (2004). Since the red fleck (*Rf*) mark has yet to be mapped in this mapping population, the relationship amongst the two phenotypes believed to be part of the *R* locus has not yet been determined.

Nevertheless, the fact that red midrib is conditioned by loci on two different linkage groups further contradicts the Carnahan et al. (1955) hypothesis that this trait is controlled solely by alleles of the *R* locus. The results also contradict Corkill's (1971) hypothesis that the *R* locus is due to a set of linked genes. The contradictory results between this study and previous studies may be due to the different genetic sources used or differences in phenotype scoring in the study populations. It may be that the *R* allele on LG B1 is necessary for the expression of the red midrib as conditioned by the locus on LG G2. Whenever the red midrib trait is present in the mapping population, the red fleck mark is also present. Conversely, whenever the red fleck mark is expressed in the mapping population, the red midrib mark is not always present. The discovery of two loci which conditions the red midrib trait means the locus on LG B1 should be called *Rm-1*, while the second locus on LG G2 becomes *Rm-2*, in keeping with the nomenclature guidelines of Quesenberry et al. (1991).

The other morphologic marker mapped in this study, the trifoliolate trait, was found to segregate with molecular markers on homoeologous LGs H1 and H2. Direct mapping for the trifoliolate trait mapped the trait to linkage group H1. When the inverse of the multifoliolate trait data was used to map the trifoliolate trait, the morphologic marker mapped to linkage group H2. The winter greenhouse trifoliolate data was tightly linked to marker TRSSRA02C02 on both homoeologues. Since the 2007 map utilized SSR primer pairs published in the two previous molecular maps, this marker is assumed to be the same SSR marker, xtrssra02c02.1, that was mapped to linkage group LG2 of the 2003 molecular map (Jones et al., 2003). This marker was later found to be linked to genes involved with leaf area, leaf width, and leaf length QTLs (Cogan et al., 2006).

Overall, the multifoliolate trait is due to recessive genes at two homoeologous loci. The gene symbols *Tf-1* and *Tf-2*, for trifoliolate, are proposed for the loci located on LG H1 and H2, respectively. The dominant allele suppresses the additional leaflets on each leaf. The trifoliolate trait always mapped to the same linkage groups, although the position varied depending on whether the trait was scored in the winter or the summer. Similar environmental variability in expression of the multifoliolate trait has been described in crimson clover (Knight, 1969) and alfalfa (*Medicago sativa* L.) (Juan et al., 1993). In the case of white clover, the difference in mapping location may simply be an artifact of the population size and the low marker density, or it might mean that different loci control leaflet number in the summer and winter.

Because the basal species of *Trifolium* often have pentafoliolate leaves (Ellison et al., 2006; Zohary and Heller, 1984), it is believed that the genus *Trifolium* originated from multifoliolate ancestors and that the number of leaflets has been reduced during evolution (Eames, 1961; Jaranowski and Broda, 1978; Zohary and Heller, 1984). The presence of loci that inhibit the expression of multifoliolate leaves in white clover supports the premise that the *Leguminoseae* in general, and *Trifolium* in particular, were originally comprised of multifoliolate species (Eames, 1961; Zohary and Heller, 1984). There is another trait which sometimes appears in white clover populations, in which the petiolule of the middle leaflet is elongated. It was noted in this study that whenever the multifoliolate trait and the elongated petiolule were expressed together, the resulting leaves were frequently pinnately compound (Fig.3.2b), rather than palmately compound (Fig.3.2a), thus bearing even greater resemblance of the ancestral legume leaf morphology (Eames, 1961).

The inability to map the red leaflet (*Vrl*), marginal mark (*Vm*), and the intermediate white-V mark (*Vi*) is due to the limited number of microsatellite markers used in this study. The

use of additional microsatellite markers should help further resolve the genetics underlying control of this trait. Mapping of red fleck (*Rf*), should be possible with the development of a bi-parental consensus map. Already, it is evident that the genetic control of many morphological traits in white clover is far more complex than conceived by the early clover geneticists.

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Table 3.1: Segregation of red midrib (*Rm*, Fig.1g) within the mapping and confirmation populations. The presence of two genes controlling the trait has been indicated by designating them as *Rm-1* and *Rm-2*.

Population	Environment	Assumed Genotype†	Red midrib	Green midrib	Expected ratio	χ^2	p value for χ^2
F_1	Summer Greenhouse	*Rm-1 Rm-1 Rm-2 rm-2* x *Rm-1 rm-1rm-1 rm-1*	87	91	1:1	0.06	0.807
F_1	Summer Field	*Rm-1 Rm-1 Rm-2 rm-2* x *Rm-1 rm-1rm-1 rm-1*	89	89	1:1	0.00	1.000
F_1	Winter Greenhouse	*Rm-1 Rm-1 Rm-2 rm-2* x *Rm-1 rm-1rm-1 rm-1*	87	91	1:1	0.06	0.807
F_1	Winter Field‡	*Rm-1 Rm-1 Rm-2 rm-2* x *Rm-1 rm-1rm-1 rm-1*	81	77	1:1	0.06	0.801
S_1§	Summer Greenhouse	*Rm-1 Rm-1 Rm-2 rm-2* ⊗	111	30	3:1	0.77	0.380
S_1	Winter Greenhouse	*Rm-1 Rm-1 Rm-2 rm-2* ⊗	106	35	3:1	0.00	1.00

† Assumed genotype based on segregation pattern within the mapping population.

‡ During the winter field phenotyping, 20 plants had died in all reps in the field, so the chi square values were tested against a population of 158 instead of 178.

§ S_1 population derived from selfing 05-O-34 parent.

Table 3.2: Segregation of red fleck (*Rf*, Fig.1i) within the mapping and confirmation populations.

Population	Environment	Assumed Genotype[†]	Red fleck	No fleck	Expected ratio	χ^2	p value for χ^2
F_1	Summer Greenhouse	*Rfrf* x *Rfrf*	92	86	3:1	68.12	<.0001
F_1	Summer Field	*Rfrf* x *Rfrf*	121	57	3:1	4.72	0.0298
F_1	Winter Greenhouse	*Rfrf* x *Rfrf*	122	56	3:1	4.00	0.0455
F_1	Winter Field‡	*Rfrf* x *Rfrf*	113	45	3:1	0.68	0.4096
S_1[§]	Summer Greenhouse	*Rfrf*⊗	106	35	3:1	0.00	1.000
S_1	Winter Greenhouse	*Rfrf*⊗	110	31	3:1	0.47	0.493

[†] Assumed genotype based on single gene hypothesis of Carnahan et al. (1955).
‡ During the winter field phenotyping, 20 plants had died in all reps in the field, so the chi square values were tested against a population of 158 instead of 178.
[§] S_1 population derived from selfing 05-O-34 parent.

Table 3.3: Segregation of marginal mark (*Vm*, Fig.1d) within the mapping and confirmation populations.

Population	Environment	Assumed Genotype[†]	Marginal mark	No mark	Expected ratio	χ^2	p value for χ^2
F_1	Summer Greenhouse	*Vmvm* x *vmvm*	80	98	1:1	1.62	0.203
F_1	Summer Field	*Vmvm* x *vmvm*	81	97	1:1	1.26	0.262
F_1	Winter Greenhouse	*Vmvm* x *vmvm*	80	98	1:1	1.62	0.203
F_1	Winter Field‡	*Vmvm* x *vmvm*	68	90	1:1	2.80	0.094
S_1[§]	Summer Greenhouse	*Vmvm* ⊗	101	40	3:1	0.77	0.380
S_1	Winter Greenhouse	*Vmvm* ⊗	99	42	3:1	1.61	0.205

[†] Assumed genotype based on single gene hypothesis of LeNoble and Papineau (1970).
‡ During the winter field phenotyping, 20 plants had died in all reps in the field, so the chi square values were tested against a population of 158 instead of 178.
[§] S_1 population derived from selfing 05-O-34 parent.

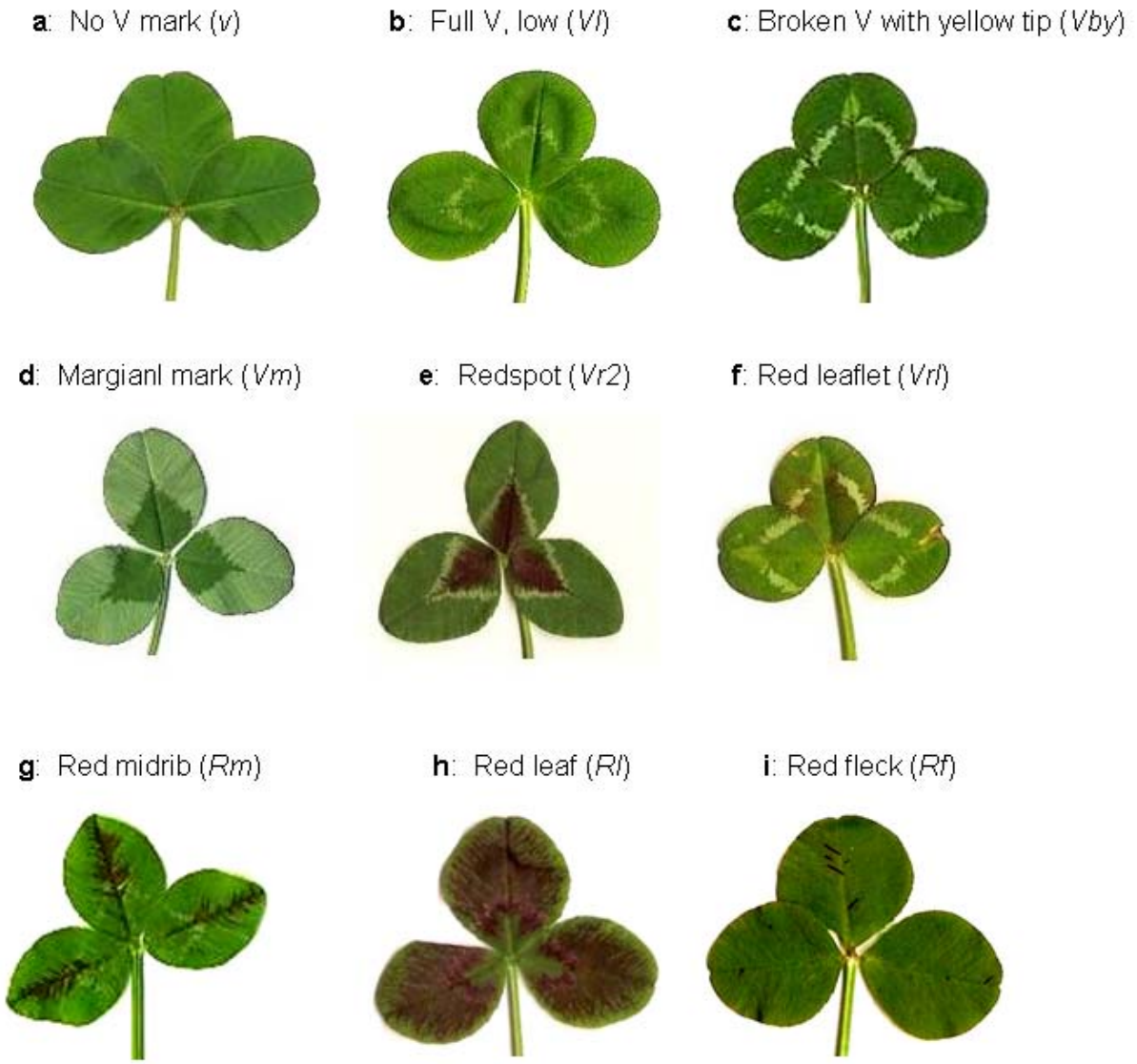

Figure 3.1: Leaf marks found in white clover. The gene symbols are as originally proposed by the authors that described them, but the notation has been modernized as per Quesenberry et al. (1991).

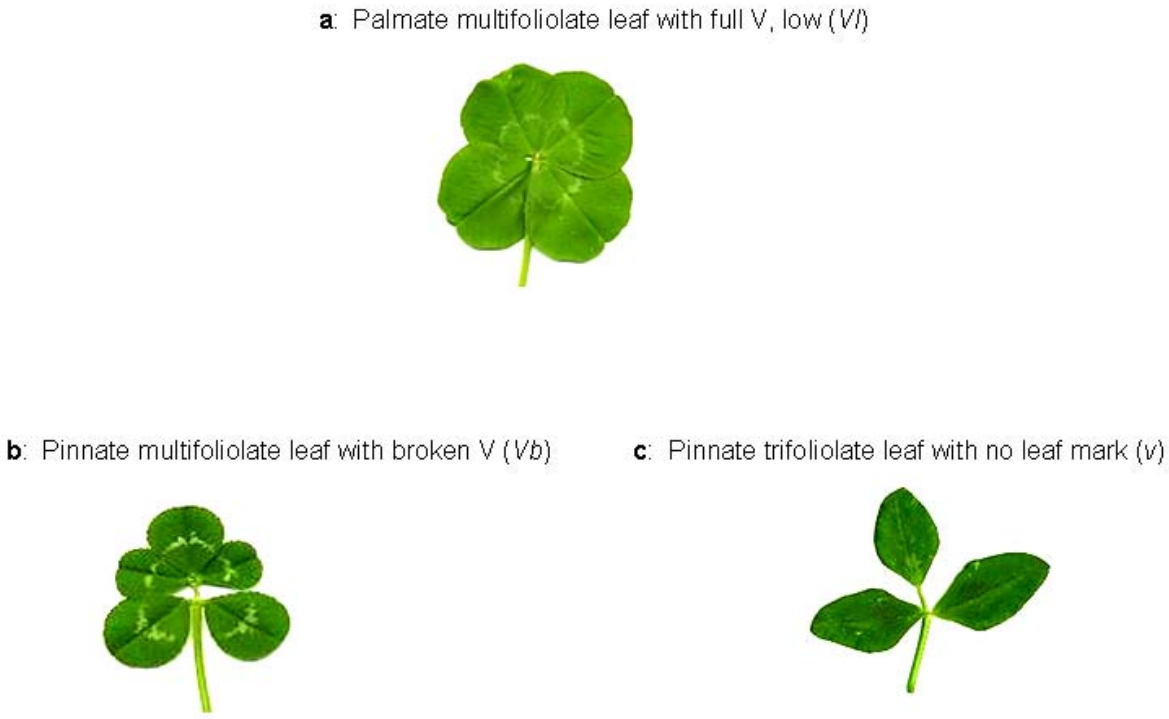

Figure 3.2: Different white clover leaf morphologies found in the species.

Figure 3.3: The parents used to create the mapping population. Left: 05-O-34; Right: GA02-56.

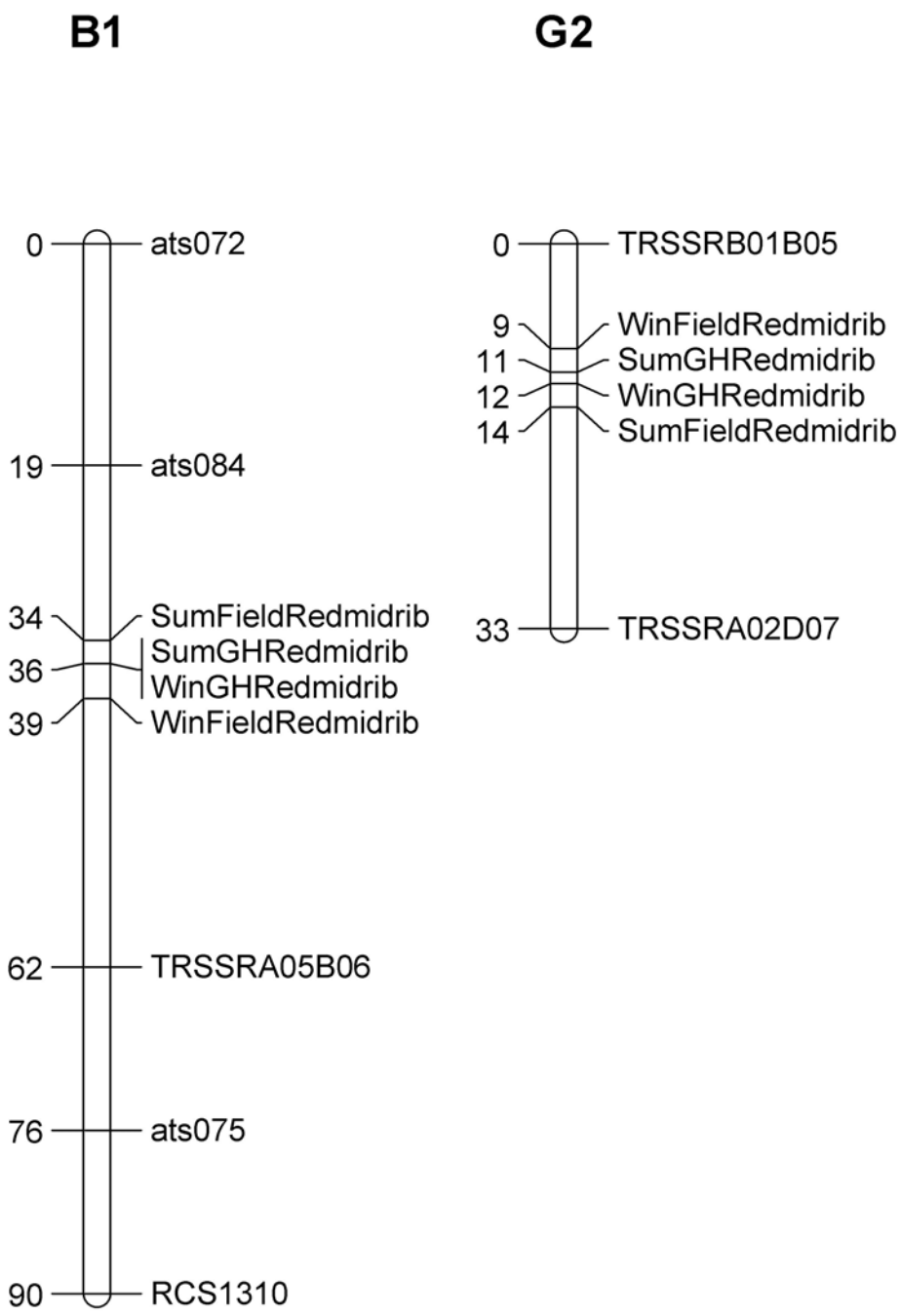

Figure 3.4: Linkage maps showing the location of the loci conditioning for the red midrib trait.

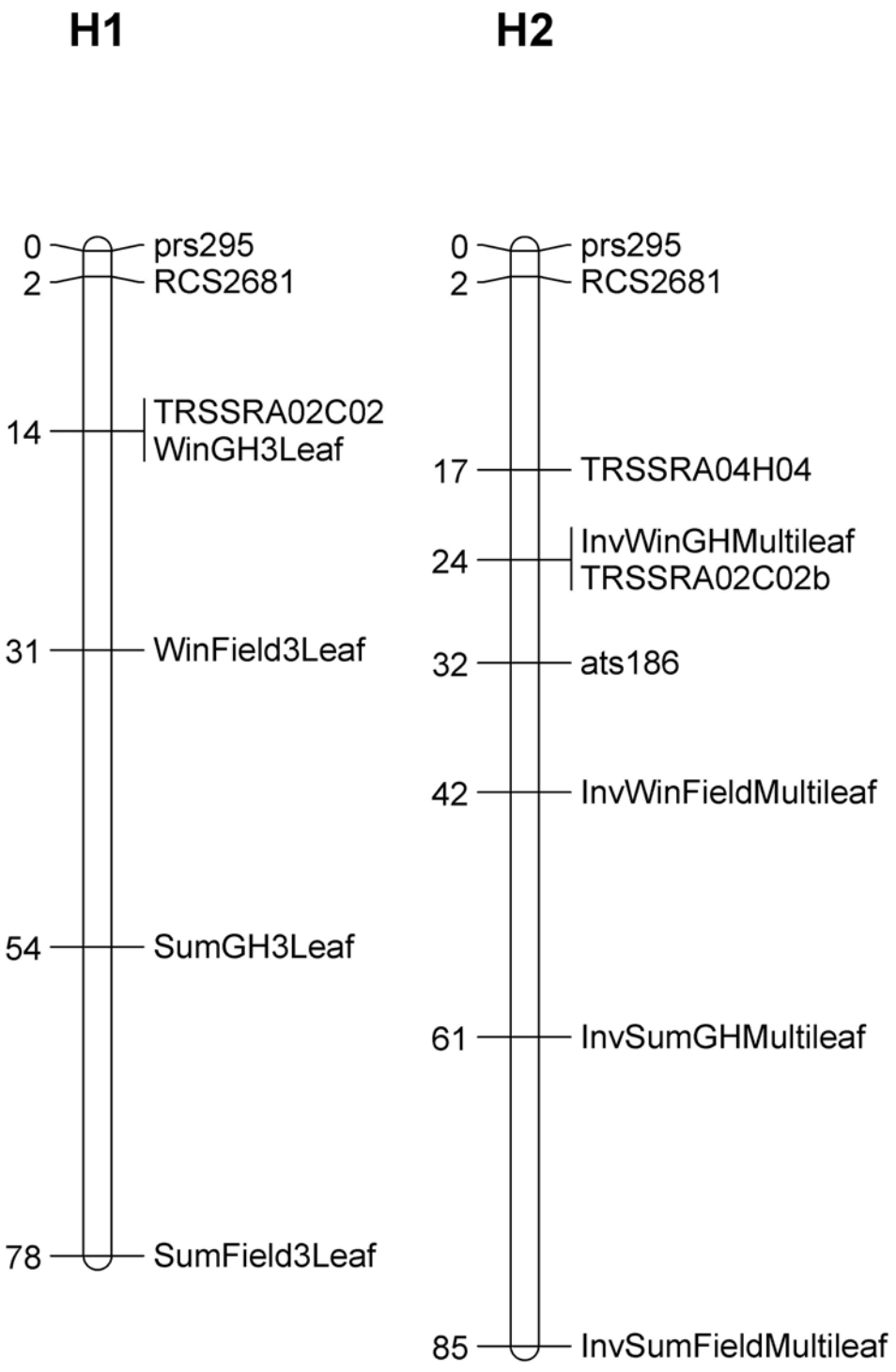

Figure 3.5: Linkage maps showing the location of the loci suppressing multifoliolate leaves.

CHAPTER 4

CONCLUSIONS

Developing white clover for ornamental use is possible due to its growth habit and the number of leaf traits found within the species that can be combined together to create a highly attractive plant. As a result of breeding efforts at the University of Georgia, four white clover genotypes were selected for ornamental use after two years of field trials and released as new cultivars. ‘Frosty Morning’, ‘Patchwork Quilt’, ‘Irish Mist’, and ‘Pistachio Ice Cream’ contain novel phenotypes that are not available in any other ornamental white clover cultivars and showed comparable or superior stand survival, stand performance, and disease resistance in Georgia and comparable stand survival and performance in Oklahoma when compared to the two currently available ornamental white clover cultivars that it was tested against.

Through the course of the ornamental white clover breeding program, it was possible to create genotypes that contained many of the different leaf mark traits found in the species introgressed into one plant. Utilizing one of these ornamental genotypes with many different leaf traits, a mapping population was made in an attempt to map the leaf traits onto an existing white clover microsatellite map. As a result, two leaf traits, the red midrib and trifoliolate leaves, were successfully mapped onto two different linkage groups.

The successful mapping of the red midrib and trifoliolate leaf traits makes it possible to clarify much of the confusion that has been associated with the genetic control of each of these traits. Adding these markers to the existing molecular maps of white clover also helps saturate

the maps available in the public domain. In this study, the red midrib trait mapped to two different linkage groups, with one locus on LG B1 and the other on LG G2 of the 2007 white clover microsatellite map (Zhang et al., 2007). For the locus on LG B1, marker ats084 mapped 15 cM above the red midrib trait. The locus controlling red midrib on LG G2 mapped within 10 cM of marker TRSSRB01B05 of the trait. The fact that red midrib is conditioned by loci on two different linkage groups contradicts the Carnahan et al. (1955) hypothesis that this trait is controlled solely by alleles of the *R* locus. The results also contradict Corkill's (1971) hypothesis that the *R* locus is due to a set of linked genes. In keeping with the standards of Quesenberry et al. (1991), the discovery of a second locus which conditions the red midrib trait means the locus on LG B1 should now be called *Rm-1*, while the second locus on LG G2 becomes *Rm-2*.

The other morphologic marker mapped in this study, the trifoliolate trait, was found to segregate with molecular markers on homoeologous LGs H1 and H2. Direct mapping for the trifoliolate trait mapped the trait to linkage group H1. By inverting the data for the recessive multifoliolate trait (i.e. mapping the trifoliolate data), the trait mapped to linkage group H2. The winter greenhouse trifoliolate data was tightly linked to marker TRSSRA02C02 on both homoeologues. This marker is assumed to be the same SSR marker, xtrssra02c02.1, that was mapped to linkage group LG2 of the 2003 molecular map (Jones et al., 2003), which was later found to be linked to genes involved with several leaf QTLs (Cogan et al., 2006).

Overall, the multifoliolate trait is probably due to recessive alleles at two homoeologous loci. Dominant alleles at these loci suppress additional leaflets on each leaf. The trifoliolate trait always mapped to the same linkage groups regardless of whether mapped for the dominant or recessive trait, although the location varied depending on whether the trait was scored in the

winter or the summer. The difference in mapping location may simply be an artifact of the population size and the low marker density, or it might mean that different loci control leaflet number in the summer and winter. The gene symbols *Tf-1* and *Tf-2*, for trifoliolate, are proposed for the mapped loci located on LG H1 and H2, respectively. Similar environmental variability in expression of the multifoliolate trait was found in other related species (Fehr, 1972; Juan et al., 1993; Knight, 1969).

Because the basal species of *Trifolium* often have pentafoliolate leaves (Ellison et al., 2006; Zohary and Heller, 1984), it is believed that the genus *Trifolium* originated from multifoliolate ancestors, and that the number of leaflets has been reduced during evolution (Eames, 1961; Jaranowski and Broda, 1978; Zohary and Heller, 1984). The presence of loci that inhibit the expression of multifoliolate leaves in white clover supports the premise that the *Leguminoseae* in general, and *Trifolium* in particular, were originally comprised of multifoliolate species (Eames, 1961; Zohary and Heller, 1984), and explains how leaflet number was reduced.

The inability to map the red leaflet (*Vrl*), marginal mark (*Vm*), and the intermediate white-V mark (*Vi*) was due to the limited number of microsatellite markers used in this study. The use of additional microsatellite markers should help further resolve the genetics underlying control of this trait. Mapping of red fleck (*Rf*), should be possible with the development of a bi-parental consensus map. Already, it is evident that the genetic control of many morphological traits in white clover is far more complex than conceived by the early clover geneticists. It is hoped that the successful mapping of these leaf traits will allow for future ornamental breeding efforts in white clover to utilize molecular methods to increase breeding efficiency in the species.

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