COLOR AND CAROTENOID CONTENT IN SQUASH (*Cucurbita* spp.)

By

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To my grandparents, Edwin and Marie Paulone, who were unable to pursue advanced degrees and sacrificed in countless ways to give future generations of their family the opportunity

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LIST OF ABBREVIATIONS

a*	CIE L*a*b* numeric color space value measuring color direction in red (a* > 0) or green (a* < 0).
b*	CIE L*a*b* numeric color space value measuring color direction in yellow (b* > 0) or blue (b* < 0).
BB	U.S. winter squash butternut cultivar ‘Butterbush’ (*Cucurbita moschata*.)
C	CIE L*a*b* numeric color space value measuring chroma (saturation or vividness). As chromaticity increases a color becomes more vivid; as it decreases a color becomes more dull.
H	CIE L*a*b* numeric color space value measuring (tint of color). This is an angular measurement where 0° = red, 90° = yellow, 180° = green, and 270° = blue.
H	Broad-sense heritability measuring the ratio of genotypic variation in relation to the phenotypic variation present among individuals.
h^2	Narrow-sense heritability measuring the ratio of additive variation in relation to the phenotypic variation present among individuals.
HPLC	High Performance Liquid Chromatography.
HRM	High Resolution Melting.
L*	CIE L*a*b* numeric color space value measuring lightness or darkness. The measurement ranges from black (0) to white (100).
PI	Plant Introduction.
TGA	U.S. winter squash acorn cultivar ‘Table Gold Acorn’ (*Cucurbita pepo*.)
TKB	U.S. winter squash acorn cultivar ‘Table King Bush’ (*Cucurbita pepo*.)
RAPD Marker	Random Amplified Polymorphic DNA Marker.
SDub	French winter squash butternut heirloom variety ‘Sucrine DuBerry’ (*Cucurbita moschata*.)
SNP Marker	Single Nucleotide Polymorphic Marker.
SSR Marker	Simple Sequence Repeat Marker

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Carotenoids serve as a cancer preventative and as antioxidants that protect against degenerative health problems such as cardiovascular disease, cataract formation, and macular degeneration in older adults. Carotenoids with high importance in human health are those that serve as precursors to vitamin A (pro-vitamin A carotenoids), and function in normal vision, bone growth, cell division and differentiation, and reproduction. Squash (*Cucurbita* spp.) is an excellent dietary source of carotenoids. The diversity and range of carotenoid types and concentrations within squash provide a means to increase the nutritional value through plant breeding.

The objectives of this research were to: 1) determine if the carotenoids of squash could be correlated with colorimetric analysis using the CIE L*a*b* color value system, 2) determine heritability and gene action of flesh color in nine *Cucurbita* populations derived from three crosses, 3) create a genetic linkage map in *C. moschata* (2n=2x=40) with sufficient marker density for quantitative trait loci (QTL) studies to map genomic regions associated with color, and 4) design a protocol to recover monomorphic simple sequence repeat (SSR) markers using high resolution melting (HRM) curve analysis.

Strong correlations were found between color value a* and total carotenoids (r = 0.91) and color value b* and chroma with lutein (r = 0.87). Broad-sense heritabilities ranged from 0.19 to 0.82 for L*, 0.12 to 0.32 for a*, 0.40 to 0.93 for b*, 0.36 to 0.92 for chroma, and 0.14 to 0.15 for hue across all three crosses. Significant combinations of additive and dominance gene effects were identified for color space values L* and hue ($P \leq 0.05$). A linkage map was constructed using random amplified polymorphic DNA (RAPD) and SSR markers. QTL associations were examined using composite interval mapping in MapQTL 5.0. Twenty-one linkage groups were obtained with QTLs detected on LG7 and 18 for L*, LG3, LG10, LG18 for a*, LG 5 and LG17 for b* and chroma, and LG 3, LG13, LG14, LG15, and LG19 for hue. Four of the ten monomorphic markers tested, M042, M120, M009, P098 were able to be recovered using HRM for addition to the linkage map data set.

CHAPTER 1
INTRODUCTION

The Cucurbitaceae Family

Cucurbitaceae, the gourd family, is a large plant family comprised of 825 species within 118 genera (Wang et al., 2007). Of the 118 genera, *Citrullus, Cucumis,* and *Cucurbita* are the most economically important, with the *Cucurbita* genus containing pumpkins, squash and gourds (Lebeda et al., 2007). Members of the Cucurbitaceae family, often referred to as 'cucurbits' in short, have subtropical and tropical distribution (Wang et al., 2007), with origins in India (cucumber, *Cucumis sativus*), Africa (*Cucumis melo*, melon and *Citrullus lanatus*, watermelon), and Central and South America (*Cucurbita* spp., squash and pumpkin) (Wehner and Maynard, 2003). Yet, cucurbit crops can be adapted to grow in many agricultural environments, such as monocultures in developed countries and in small garden settings with low input levels in non-developed countries (Lebeda et al., 2007).

Members of this family have been used by humans for both cultural and nutritional value for over 12,000 years (Brothwell and Brothwell, 1969) and are associated with the origins of present-day agriculture and the development of human civilizations. Additionally, although an Old World plant, cucurbit crops were one of the first to be domesticated in the New World (Bisognin, 2002). Cucurbits are also noted for a variety of shapes, sizes and colors of plants and fruit (Bates, Robinson, and Jeffrey, 1990). In addition to food usage, fruit from Cucurbitaceae have been used as storage containers and as a fiber source for sponges and shoe production (Moravec, Lebeda, and Kristkova, 2004).

Taxonomy of the Cucurbitaceae. The Cucurbitaceae family is in the phylum Magnoliophyta, class Magnoliopsida, subclass Dileniidae, order Violales (Walters and Keil, 1996). Additionally, all species commonly cultivated in the Cucurbitaceae family belong to the Cucurbitoideae subfamily (Wehner and Maynard, 2003). Plants of this subfamily are trailing herbs with climbing tendrils (Walters and Keil, 1996) that are sensitive to frost and complete their lifecycle within one year (Wehner and Maynard, 2003). Leaves are palmately veined and alternate, and can be deep to simple lobed and estipulate. Cucurbits are monoecious, with separate male (staminate) and female (pistillate) flowers both borne along the vine at the base of the leaf petiole (Wehner and Maynard, 2003) and rarely are perfect (Walters and Keil, 1996). Flowers can have five distinct sepals, or sepals can be varying degrees of connate to absent entirely, with an inferior ovary. Fruit is characterized by two, five-connate carpels and one locule (Walters and Keil, 1996). Flowers are insect-pollinated by bees since the pollen is too large and sticky to travel by wind (Wehner and Maynard, 2003). Additionally, there are several parietal ovules that contain up to five locules with an additional axile ovule present. Fruit of this family has been classified as a berry, pepo, capsule or achene (Walters and Keil, 1996).

Economic Importance of Pumpkins, Squash and Gourds

In 2007, the top five producers of pumpkins, squash and gourds were China, India, Russia, the U.S. and Egypt and in 2009, 43,900 acres of both fresh market and processing squash were harvested in the U.S. totaling a production value of $203,464,000 (FAOSTAT, 2010). In 2009, Florida harvested 8,800 acres totaling $51,480,000 gross revenue and currently ranks 1st in U.S. followed by California

($32,160,000), Georgia ($28,892,000), New York ($23,004,000), and Michigan ($11,739,000).

The average price for U.S. grown squash was $28.20 / hundred weight (cwt) in 2009. The price for Florida squash sold in 2009 was $45.00 / cwt., which decreased from $53.00 in 2008 and $52.80 in 2007. In 2009, Florida accounted for 20% of total U.S. harvested squash acreage, and 25% of total U.S. cash receipts (USDA-NASS, 2010). In the most recent census of agriculture (2007), Miami-Dade County was the leading county for squash production in Florida, accounting for 4,689 of Florida's 7,349 harvested acres, produced on only 18 farms. Hillsborough County was second in the state for acreage with 623 harvested acres and 33 farms, followed by Hardee County with 133 harvested acres and 9 farms.

Of the 188 farms producing 7,349 acres of squash in 2007, only 10 farms were larger than 100 acres, yet they accounted for 5,915 (80%) of the total squash acreage in Florida (USDA-NASS, 2009). Florida produces a large amount of the U.S. summer squash (*C.pepo*) crop, but also produces winter squash (*C. moschata* and *C. pepo*), calabaza (*C. moschata*), banana squash (*C. maxima*), gourds (*Lagenaria* spp. and *Luffa* spp.) and tropical squash (*Cucurbita* spp.) (Swaider and Ware, 2002). The Florida squash crop is produced almost entirely for fresh market sale, and is among a limited number of crops that is shipped from Florida 12 months of the year (Mossler and Nesheim 2003) with shipments at their peak from November to April (Pollack, 1996).

Nutrition of Squash

An important component of the nutritional value of squash, *Cucurbita* spp., is its carotenoid content which serves as one source of pro-vitamin A in plant tissues (Gross, 1991). Winter squash (*Cucurbita* spp.) is also noted as a good source of dietary iron

(Garrison and Somer, 1995) Developing countries produce roughly four times the total number of *Cucurbita* spp., pumpkins, squashes, and gourds, as do developed countries. The number of *Cucurbita* species that are produced in developing countries as compared to that of developed countries is reflective of *Cucurbita* spp. being a staple food crop in the developing world (Lebeda et al., 2007). In light of this, an increase in the nutritional content of squash would benefit both developed and developing nations; however it may have a greater benefit on the diets of people in developing nations.

Importance of Carotenoids and Vitamin A in Human Health

Roughly 60 different carotenoids occur naturally in plant tissues (Seroczynska et al., 2006) and serve as a cancer preventative as well as antioxidants that protect the body against degenerative diseases (Murkovic et al., 2002) such as cardiovascular disease, the formation of cataracts, and macular degeneration in older adults (Tang, 2010). More specifically, carotenoids of high importance in human health are pro-vitamin A carotenoids, which function to promote normal vision, bone growth, cell division and differentiation, and reproduction (ODS/NIH, 2006).

Although plant sources do not provide vitamin A directly as animal sources do, their precursors are referred to as ‘pro-vitamin A’ sources and are converted into the bioavaliable form of retinol in the human body. Over 40 carotenoids have been identified as vitamin A precursors in plants (Santos and Simon, 2006). Carotenoids from plants that are common sources of pro-vitamin A include beta-carotene, alpha-carotene, and beta-cryptoxanthin; beta-carotene is made into retinol twice as efficiently as the latter two (ODS/NIH, 2006; Olson, 1996; Pavia and Russell, 1999). Further, since plants supply the precursors to vitamin A, the amount of pro-vitamin A carotenoids within plant tissues is converted into retinol activity units, RAE. RAEs can then be used

to compare vitamin A biological activity among the differing forms of vitamin A (Trumbo et al., 2001).

The active form of vitamin A is retinol, which can be made into both retinal and retinoic acid (Trumbo et al., 2001). Retinal is used as a pigment which is linked to a protein, opsin, to compose the chromoprotein rhodopsin. This protein is found in light sensitive rod cells in the eye and aids the eye in adaptation to dim light. In bright light, rhodopsin breaks down and in low light the process is reversed (Alpern, 1971; Zile, 2001). Retinoic acid functions to aid in vertebrate embryonic development (Zile, 2001), as well as maintaining epithelial tissues such as skin, hair and mucous membranes, reproductive health in sperm formation, and immune system functioning by aiding some white blood cells in efficiently fighting infection (ODS/NIH, 2006).

Both the pre-formed animal sources, vitamin A, such as liver, and pro-vitamin A carotenoid sources, such as red, orange and yellow pigmented fruit, are absorbed in the intestine, transported in the blood as vitamin A esters, and stored in the liver when the amount ingested is <1mg (ODS/NIH, 2006; Olson, 1999; Pavia and Russell, 1999). With larger amounts, the absorbed carotenoids are stored in the blood plasma and other tissue (IVACG, 1999).

Recommended Dietary Allowance (RDA) for Carotenoids and Vitamin A

To date, no official RDAs have been issued for carotenoid levels (Murkovic et al., 2002). However, there are established RDA levels for Vitamin A released by the Institute of Medicine (Trumbo et al., 2001). Currently, for infants less than one year of age, the RDA range is from 400 – 500 µg RAE. RDAs for children are 300, 400 and 600 µg RAE for age ranges of 1-3, 4-8 and 14-18 years, respectively. Adult males require 900 µg RAE and adult females 700 µg RAE. Pregnancy alters these levels by

increasing the RDA by 70 µg RAE. Additionally, lactating women have nearly double the requirement of non-lactating women with 1,300 µg RAE.

Until 2001, it was commonly accepted that 6 µg β-carotene had the same vitamin A activity as 1 µg retinol when converting values into RAEs. In 2001, the U.S. Institute of Medicine (IOM) set the new RAE standard as 12 µg β-carotene to 1 µg retinol. The conversion rate of pro-vitamin A carotenoids was reevaluated after observing individuals who had adequate intake of plant sources of pro-vitamin A, but blood serum levels still showed deficiency. Questions then arose regarding the bioavailability of pro-vitamin A in plant sources. Further studies also suggest that the conversion rate may be closer to 1 µg retinol : 21 µg β-carotene. Under the previous conversion rate of 1 µg retinol : 6 µg β-carotene, all populations have to ability to meet vitamin A daily requirements from existing food sources. However, the new ratios make it increasingly difficult for those who obtain their primary source of vitamin A from pro-vitamin A sources in vegetable crops to consume recommended levels of vitamin A, thus increasing the probability of vitamin A deficiency (West et al., 2002). This new standard shows an increased need to select for and increase carotenoid content in plants, such as squash, through plant breeding.

Increasing Pro-Vitamin A Rather than Pre-formed Vitamin A Content

As reported by the World Health Organization (WHO) in 2001, 118 countries have noted vitamin A deficiencies as a common health problem, primarily in Africa and South-East Asia. There are an estimated 100 to 140 million Vitamin A deficient children, resulting in 250,000 to 500,000 cases of childhood blindness annually. Unfortunately, half of these children die within 12 months of lost sight. Child-birth related deaths in

women are estimated at 600,000 annually, with the majority being caused by Vitamin A deficiency (Santos and Simon, 2006).

Pre-formed vitamin A sources are found in animal products such as meat, liver and eggs, although these products are typically more expensive than plant pro-vitamin A sources. In developing countries, it is often easier to obtain sources rich in pro-vitamin A carotenoids instead of pre-formed vitamin A (Ong and Tee, 1992). Paradoxically, although many developing nations have ample vegetables rich in pro-vitamin A carotenoids, vitamin A deficiencies still occur. The cause of this is likely due to diets which are low in fat and protein, both of which aid in absorption of vitamin A precursors (Ameny and Wilson, 1997). However, vitamin A deficiency may also present itself if the individual has a lower capacity to convert pro-vitamin A forms into usable vitamin A within the body or if he or she has a high body mass index (Tang, 2010). These results suggest that increasing pro-vitamin A forms may be more beneficial in terms of lower cost for the consumer, and increases in pro-vitamin A carotenoid levels in plants could beneficially impact both people in the developed and developing world.

Analysis of Squash Fruit

Carotenoid Analysis

The predominant carotenoids found within squash (*Cucurbita* spp.) include lutein, β -carotene, and zeaxanthin (Gross, 1991). The most accurate way to analyze carotenoid content in plant tissues is through high performance liquid chromatography (HPLC). Although highly precise and repeatable, this extraction method requires specialized lab training. This method also forces the researcher to examine the costs and benefits to using this method by considering the number of samples to be tested against the operation cost of running the equipment and hazardous solvents used

(Rodreiguez-Amaya and Kimura, 2004). In addition, carotenoid analysis is complicated by the variable composition of vegetables throughout the year, differences in what is actually eaten and what is sampled, and different cooking methods that influence carotenoid bioavailability (Heinonen *et al.*, 1989). To decrease time and increase efficiency in plant breeding selection, an alternative to chemical carotenoid analysis would be useful.

Color Analysis

Since carotenoids are a class of plant pigments, their levels have a direct impact on the color of plant tissues. For example, tomato carotenoid biosynthesis has been studied thoroughly as it pertains to fruit ripening. As the fruit matures, color development proceeds through stages of green, orange, pink and red; this is due to accumulation of the carotenoid lycopene within the chromoplasts of the fruit (Hirschberg, 2001). Within squash, a range of white, cream, yellow, and orange colors in the mesocarp of squash are also conditioned by carotenoids (Gross, 1991). A way to indirectly and objectively measure the carotenoid content in squash by its color would be beneficial for manipulating squash color in a breeding program.

The human eye is able to discern different colors and intensities. In the human eye, there are three types of cones used to detect the primary colors of light - red, green and blue - and their interaction allows the perception of color. However, due to subjectivity of the human eye from factors such as lighting on the object, background color behind the object and age of the eye, an objective method of color observation is desirable. The tristimulus color measurement system has three color sensors which have spectral sensitivity curves similar to that of the human eye and are referred to as color-matching functions. These three functions are detected as x, y, and z coordinates,

and can be converted into a numerical value system (Konica Minolta Sensing, Inc., 1998). These coordinates can then be converted into one of many color systems. The most preferred systems to measure color of fruit and vegetables are the Hunter tristimulus system, and the CIE L*a*b* system (Seroczynska *et al.*, 2006).

Many studies have been conducted that correlate the measurement of color with pigment concentration in other agricultural products such as paprika (Ramakrishnan and Francis, 1973), leaves of parsley (Berset and Caniaux, 1983), blueberry (Francis, 1985), salmon (Ando *et al.,* 1992), red pepper (Reeves, 1987), grapes (Watada and Abbott, 1975), peaches (Morrison, 1990), sweet potato (Ameny and Wilson, 1997; Takahata *et al.,* 1993; Simonne *et al.*, 1993), carrots (Ling *et al.,* 1996), Swiss chard (Ihl, 1994), squash (Seroczynska *et al.*, 2006; Francis, 1962), cranberries and wines (Francis, 1969).

Due to the correlation of color intensity with nutrients such as β -carotene in sweet potato (Simonne *et al.*, 1993; Ameny and Wilson, 1997), color intensity may be considered for use as a reliable indication of nutrient value, such as vitamin A. Currently, visual observations of pumpkin varieties (*C.pepo, C. maxima,* and a cross between *C. maxima* and *C. moschata*) by Murkovic *et al.* (2002) indicated that flesh with a higher carotene level had an orange appearance while flesh with higher lutein content and lower carotene content had bright yellow coloration.

The benefit to correlation of plant color with pigment concentration is both the rapidity of the method as well as the quantitative and descriptive evaluation of color in a single determination (Ameny and Wilson, 1997). Pigment concentration correlated with color values would also enable the plant breeder to indirectly select for increased

nutrient content with a faster turn-around in the selection process. Additionally, it would open the opportunity to breed for changing squash color to fit specialty markets.

Gene Number and Inheritance of Color in Cucurbitaceae

Flesh color in squash spans a wide range of color and intensity, indicating that expression of flesh color is likely a quantitative trait, controlled by many genes and influenced by environmental factors, rather than a qualitative trait controlled by few genes. Knowledge of the gene number and heritability of flesh color would allow plant breeders to estimate the population size and time needed to manipulate flesh color in squash.

Factors affecting rind and flesh color in various plants in Cucurbitaceae have been studied. In *C. pepo*, thirteen loci have been identified that control rind color (Paris et al., 2003). However, in *C. maxima* only three loci determining light and dark green rind color were identified (Lopez et al., 2003). In watermelon (*Citrullus lanatus*) canary yellow to scarlet red flesh color was found to be controlled by a single, dominant gene, while salmon yellow, white and red fleshed cultivars deviated from the ratio of a one gene model. Deviated ratios in salmon yellow, red and white flesh were suggestive of quantitative traits controlling flesh color (Gusmini and Wehner, 2006). Flesh color in melon (*Cucumis melo)*, has been found to be controlled by both major and minor genes (Cuevas *et al.*, 2010). In melon, flesh color in both the F_2 and BC_1 populations fit an epistatic two-gene model. Heritabilities of melon flesh color, as a reflection of β-carotene content were moderate, 0.55 in the F_1, F_2, and BC populations and 0.68 in the F_3 populations.

To date, gene number and the inheritance of flesh color have not been studied in *Cucurbita moschata* or *Cucurbita pepo*.

Molecular Tools for Increasing Color Content of Squash

Marker assisted selection (MAS) is a tool used by plant breeders to make selection decisions based on a plant's genotype rather than phenotype. When traits are found to have significant associations with markers, MAS can be used to more effectively and efficiently select for desired traits in a population with a faster turn-around time than by examination of phenotypic information alone. Marker types that have been used in the cucurbits include protein, morphological, and nuclear and plastid markers (Lebeda et al., 2007). However, as a whole, little genomic information is available for most cucurbit species. At present, the largest amount of information in the *Cucurbitaceae* family exists in three principle economic crops: cucumber, watermelon, and melon (Wang et al., 2007).

Molecular Markers Used in Cucurbitaceae

Morphological markers are typically used for cucurbit germplasm characterization aimed at screening for important traits (Lebeda et al., 2007). However, the use of these phenotypic markers has been proven insufficient in mapping genomes within Cucurbitaceae due to the limited number available (Wang et al., 2007).

Variations in isozyme profiles have assayed diversity in *Cucumis, Citrullus, Momordica*, and *Cucurbita*. The most developed and comprehensive information is available for cucumber (Knerr et al., 1989; Meglic et al., 1996). However, isozyme markers used in *Cucurbita* have produced varied, and at times, conflicting results in defining taxonomic relationships (Puchalski and Robinson, 1978; Decker-Walters et al., 1990). Protein markers have also been used to evaluate enzymes involved in seed storage in both melon, and cucumber (Bretting and Widrlechner, 1995). The most thorough isozyme marker analyses have been examining relationships between wild

and domesticated individuals in *C. pepo* (Ignart and Weeden, 1984; Decker and Wilson, 1987; and Decker-Walters et al., 1993).

Plastid markers, both chloroplast and mitochondrial, have been useful in determining phylogenetic relatedness due to their high level of conservation (Decker-Walters, et al., 2004a). In *Cucurbita*, mitochondrial sequences have been used to evaluate different accessions, suggesting a minimum of six independent domestication events (Sanjur, et al., 2002). Relationships between domesticated *Cucurbita* and its wild relatives have also been examined using chloroplast DNA (Wilson et al., 1992).

Nuclear markers provide higher levels of variation and are more useful in determining relationships within and below the level of plant species than morphological, enzyme, or plastid markers (Lebeda et al., 2007). Random amplified polymorphic DNA (RAPD) markers are a dominant marker system with primers composed of randomly assembled 10 base pair sequences. This marker system is commonly used as a starting point in crops that do not have a high level of advancement in molecular marker systems, such as members of the Cucurbitaceae family. RAPD and simple sequence repeat (SSR) markers have been most widely used across cucurbit crops, including *Lagenaria, Citrullus, Cucumis* and *Cucurbit*a. RAPDs have been used at the intraspecific level for cucurbits (Lebeda et al., 2007). However, due low variation in and low repeatability of RAPD markers, cucurbit breeders have sought other more stable and codominant marker systems where heterozygotes can unambiguously be determined (Dijkhuizen et al., 1996; Garcia-Mas et al., 2000).

Restriction fragment length polymorphisms (RFLPs) were among the first types of codominant marker systems used in cucurbit crops. With the establishment and

availability of SSR markers, SSRs quickly became the most used codominant marker system in cucurbit crops. PCR-based molecular marker systems that have been utilized less frequently in cucurbit crops include amplified fragment length polymorphisms (AFLPs), inter simple sequence repeats (ISSRs), cleaved amplified polymorphic sequences (CAPS), restriction satellites, and sequence-based amplified polymorphisms (SBAPs) (Lebeda et al., 2007).

Genetic Linkage Maps Available in the *Cucurbita* genus

Molecular markers can also be used to map the chromosomes in the haploid genome for use in studying regions of the genome associated with complex phenotypic traits controlled by many genes in quantitative trait loci (QTL) studies. Knowledge of both desirable and undesirable QTL alleles in relation to the breeder's trait of interest is equally useful. Knowing the location of genes affecting different phenotypic traits in relation to one another provides information on genetic linkage and the likelihood of recombination during meiosis. This information will aid the plant breeder in decisions such as best crosses to make, population size, and number of selfing generations needed until homozygosity is attained.

Linkage map development within *Cucurbita* is limited, and only five genetic maps for the *Curcubita* now exist. The first two maps constructed were of interspecific crosses between *C. moschata* and *C. pepo*, and were composed solely of RAPD markers. The first, published by Lee et al. (1995), was a RAPD map of an F_2 population. The second map, created from a BC_1 population, was published by Brown and Myers (2002) and was composed of both RAPD and morphological markers. A third map in *Cucurbita* was created in *C. pepo* using an F_2 population with RAPD, SSR, and, morphological, markers (Zraidi and Lelley, 2004).

This third map was recently expanded and compared to a second *C.pepo* map. Zraidi et al. (2007) used two F_2 populations, created from two intraspecific crosses, to create the first consensus map for *C. pepo* using RAPD, AFLP, SSR, and SCAR markers. Because of the limited number of SSRs available for *Cucurbita*, Gong et al. (2008a), developed over 500 SSR primers which were polymorphic in *C. pepo*, *C. moschata*, and *C. ecuadorensis*. The researchers used these SSRs to update their first *C. pepo* map (Zraidi et al., 2007) and constructed a fourth published map in *Cucurbita* and the first SSR-based map in *C. pepo* in 2008. The fifth and most recent *Cucurbita* map was also published in 2008. Gong et al. (2008b) constructed the first SSR-based map of *C. moschata* using an F_2 population, and examined its synteny with their most recent *C. pepo* map.

The limited availability of genetic maps within *Cucurbita* indicates a need for genotypic information to be acquired to aid in breeding for desirable traits in squash, such as increasing color and carotenoid content.

Quantitative Trait Loci (QTL) Detection in Cucurbitaceae

Within Cucurbitaceae, most of QTL identification has been with Cucumis sativus and Cucumis melo. Major areas of QTL identification in Cucumis sativus, include disease resistance, fruit quality traits and components of yield (sex expression, earliness, and lateral branching) (Staub et al., 2008). In Cucumis melo, QTL identification similarly includes fruit quality and disease resistance, in addition to floral biology QTLs. Furthermore, Cucumis melo, melon, is considered the model plant within Cucurbitaceae; most genomic work has been done in this species. Within melon, QTL regions and identified genes are sufficient in number to produce a synthetic genetic map (Pitrat, 2008).

Unlike cucumber and melon, no QTLs have been identified for squash (Cucurbita spp.) or within Cucurbita.

Research Rationale

Knowledge of color and carotenoid content in squash, *Cucurbita* spp., is currently limited. A better understanding of the carotenoid content within squash flesh as it relates to color might expedite selection for color and nutrient content in squash. Additional knowledge of gene number and inheritance of flesh color would enable plant breeders to more effectively design populations to manipulate the desired trait. Finally, availability of markers associated with flesh color and a genetic linkage map identifying regions of the genome linked with flesh color would help breeders who wish to increase flesh color and study its relation to other phenotypic traits. The objectives of this research project were to:

1. Attempt to correlate flesh color in *Cucurbita* with carotenoid content as measured by HPLC,
2. Measure flesh color segregation in multiple *Cucurbita* populations using L*a*b* color space values and determine the heritability of these components of color, and
3. Develop a linkage map for *C. moschata* and perform QTL analyses for L*a*b* color space values in the population.

CHAPTER 2
CORRELATION BETWEEN L* A* B* COLOR SPACE VALUES AND CAROTENOID CONTENT IN PUMPKINS AND SQUASH (*Cucurbita* spp.)

Introduction

Carotenoids are the principle pigments responsible for the many colors of leaves, fruits, and flowers in plants[*] (Gross, 1991). They act as photoprotective agents and accessory light-harvesting complexes. Carotenoids also play an important role in human health by acting as sources of pro-vitamin A or by acting as protective antioxidants required for proper reproduction, growth and development, a normal functioning ocular system, epithelial cell integrity, and immune system functionality (FAO/WHO, 2002; Murkovic et al., 2002). In vegetables, common pro-vitamin A carotenoids include β-carotene, α-carotene, and β-cryptoxanthin (ODS/NIH, 2006). Other common carotenoids such as lycopene, lutein and zeaxanthin do not have vitamin A activity but serve as antioxidants.

Pumpkins and squash (*Cucurbita* spp.) are excellent dietary sources of carotenoids (Gross, 1991) and, in 2007, ranked 11th in amount produced among other vegetables produced around the world (FAOSTAT, 2008). The predominant carotenoids found in pumpkins and squash include lutein, α-carotene, and β-carotene (Gross, 1991). Based on reports by Holden et al. (1999) and Murkovic et al. (2002), the concentrations of lutein, α-carotene, and β-carotene found within *Curcurbita* species and their various fruit-types can vary dramatically. In their studies, the fresh weight (FW) range of lutein, α-carotene, and β-carotene in summer-type squash (*C. pepo*) were 0.0

[*] Article reproduced with permission from: Itle, R.A. and E.A. Kabelka. 2009. Correlation between L* a* b* color space values and carotenoid content in pumpkins and squash (*Cucurbita* spp.). HortSci., 44(3):633 – 637.

to 21.3 µg/g, 0.3 to 1.7 µg/g, and 0.6 to 23.0 µg/g, respectively. Within winter-type squash (*C. moschata* and *C. maxima*), lutein ranged from 0.8 to 170.0 µg/g FW, α-carotene from 0.0 to 75.0 µg/g FW, and β-carotene from 7.1 to 74.0 µg/g FW.

The mesocarp (flesh) colors of pumpkins and squash generally include a wide range of whites, yellows, and oranges (Gross, 1991). This color is based on the particular carotenoid types and concentrations which are influenced by genetic and environmental factors. Over a dozen genes that affect the rind and flesh color of squash have been described (Paris and Brown, 2005) and include *D* (*Dark*), *l*-1 (*light coloration*-1), *l*-2 (*light coloration*-2) and *B* (*Bicolor*). Tadmor et al. (2005) studied the effects of these genes in different combinations within near-isogenic lines (NILs) of *C. pepo*. In genetic backgrounds that lacked either the dominant *D* or dominant *L*-2 alleles, a yellow flesh color developed. In genetic backgrounds with either dominant *D* or *L*-2, a yellow-orange flesh color developed, and when the dominant allele of *B* interacts with the dominant allele of *L*-2, an intense orange flesh color will occur. One additional gene that affects squash flesh color is the dominant *Wf* (*White flesh*) which confers a white flesh color by preventing yellow pigment accumulation (Paris and Brown, 2005).

The broad range in carotenoid types and concentrations among and within *Cucurbita* species indicates the potential for genetic improvement of these compounds through plant breeding. Accuracy in breeding will require estimates of carotenoid types and their concentrations that are precise enough to distinguish genotypic differences among breeding material. One obstacle is that the extraction and analysis of carotenoid content is time consuming and expensive. In practical breeding programs, it is not realistic to analyze the carotenoid content of even a small segregating population for

selection of genotypes with high levels of carotenoids. An alternative reliable method to estimate carotenoid content and concentration would be beneficial.

High performance liquid chromatography (HPLC) is used to chemically analyze tissues for carotenoid types and concentrations (Gross, 1991). It is labor-intensive and expensive but is a reproducible and highly sensitive process that can separate, identify, purify, and quantify carotenoid levels. In contrast, colorimeters, which objectively measure and describe visible color, are relatively inexpensive and easy to use. The preferred methods to objectively measure color are the tristimulus Hunter and the CIE L*a*b* systems (Seroczynska et al., 2006). Tristimulus color measurement systems have three color sensors with spectral sensitivity curves similar to that of the human eye and are referred to as color-matching functions (Konica Minolta Photo Imaging, U.S.A., Inc., Mahwah, NJ). These three functions are detected as x, y, and z coordinates which can be converted into the desired color measurement value system.

Previous studies have correlated color measurement systems with carotenoid content in vegetable crops such as tomato (Arias et al., 2000; D'Souza et al., 1992), sweet potato (Ameny and Wilson, 1997; Simonne et al., 1993), pepper (Reeves, 1987), and winter-type squash (Francis, 1962; Seroczynska et al., 2006). Interestingly, the authors of these studies differ in their opinions as to how reliable colorimetric analysis are at estimating carotenoid content and concentrations. If a fair estimate of carotenoid content and concentration could be obtained, this rapid and inexpensive method could be very useful in breeding pumpkins and squash for enhanced carotenoid levels. The objective of this research was to determine if the carotenoid content and concentration

of pumpkin and squash (*C. moschata* and *C. pepo*) can be correlated with colorimetric analysis using the CIE L*a*b* color value system.

Materials and Methods

Plant Material

An initial selection of 15 *C. moschata* (Figure 2-1) and 15 *C. pepo* (Figure 2-2) cultigens [cultivars, heirlooms, and plant introductions (PI)] was made based on subjective descriptions of flesh color with samples representing a range of whites, yellows, and oranges. Seeds of cultivars and heirlooms were purchased from commercial seed producers while PIs were obtained from the USDA-ARS North Central (Ames, IA) and Southern (Athens, GA) Regional Plant Introduction Stations.

Field Trials

A preliminary field study was conducted 6 April - 15 June 2007 at the Plant Science Research and Education Center (PSREC) located in Citra, FL to evaluate and select from the 15 *C. moschata* and 15 *C. pepo* cultigens those that would provide a range of white, yellow, and orange flesh color based on colorimetric analysis using the method described below. A randomized complete block design with two replications per cultigen, eight plants per plot, was used. Temperatures recorded by the Florida Automated Weather Network (FAWN) recorded 22°C avg., 38°C max., and 5°C min. over the course of the growing season.

From the preliminary study, six *C. moschata* (Figure 2-3) and five *C. pepo* (Figure 2-4) cultigens were selected for both colorimetric and HPLC analysis following plantings at two locations, PSREC and the Gulf Coast Research and Education Center (GCREC), Wimauma, FL, during 11 March - 27 June 2008. Average shelter air temperatures at 60cm recorded by FAWN were 23°C avg., 39°C max., and 2°C min. and 23°C avg.,

36°C max., and 4°C min. for PSREC and GCREC, respectively. A randomized complete block design with two replications per cultigen, eight plants per plot, was used at each location. Recommended conventional cultural practices and fertility rates for Florida squash were followed for both 2007 and 2008 (Olson and Simonne, 2007).

Colorimetric and HPLC analysis

A total of 220 mature fruit (11 cultigens x 5 fruit x 2 replications per location x 2 locations) were harvested for colorimetric and HPLC analysis. Fruit were considered mature at the time they were ready for harvest based on common commercial practices for each squash type. Color was recorded using a Minolta CR-400 Colorimeter (Minolta Camera Co., Ltd., Ramsey, NJ) tristimulus color analyzer, equipped with an 8 mm diameter measuring area and diffuse illumination of a 2° Standard Observer. The L* coordinate indicates darkness or lightness of color and ranges from black (0) to white (100). Coordinates, a* and b*, indicate color directions: +a* is the red direction, -a* is the green direction, +b* is the yellow direction and –b* is the blue direction. Chroma is the saturation or vividness of color. As chromaticity increases, a color becomes more intense, as it decreases a color becomes more dull. Hue angle is the basic unit of color and can be interpreted, for example, as 0° = red and 90° = yellow. Both chroma and hue are derived from a* and b* using the following equations: Metric chroma: $C= \sqrt{[(a^*)^2 + (b^*)^2]}$ and metric hue angle: $h = \tan^{-1} (b^*/a^*)$ [degrees].

Each fruit was sliced transversely, and L*a*b* color space measurements from the edible flesh (mesocarp) of each fruit were recorded within five minutes to avoid discoloration. Our preliminary study revealed replicate measurements of the mesocarp of each fruit per cultigen to be not significantly ($P \geq 0.05$) different from one another. Therefore, avoiding the seed cavity and surrounding tissue (approx. 10 mm), three

random measurements per fruit were recorded. A total of 660 (11 cultigens x 5 fruit x 3 colorimeter measurements per fruit x 2 replications per location x 2 locations) colorimetric values were recorded.

The separation and quantification of carotenoids were accomplished by HPLC. A total of 22 samples (11 cultigens per location) were prepared for HPLC analysis, directly following colorimetric measurements. From each of five fruit harvested per replication, a 10.0 +/- 0.1 g FW cubed flesh sample was cut and combined to make a 100.0 g +/- 0.5 g sample per cultigen per location. Samples were vacuum sealed in plastic storage bags within 50 minutes of initial fruit slicing, wrapped with aluminum foil, labeled and held at -20°C. Samples were then sent frozen to Craft Technologies, Inc. (Wilson, NC) for saponification and HPLC processing. Carotenoids measured included lutein, zeaxanthin, cis-lutein-zeaxanthin, α-carotene, β-carotene, cis-β-carotene, and total carotenoids. All fruit was processed with the colorimeter and prepared for HPLC within three days of field harvest and underwent HPLC analysis within four weeks of harvest.

Data analysis

Color space values and carotenoid content of the *Cucurbita* cultigens, across locations, were subjected to analysis of variance by the GLM procedure of SAS (Statistical Analysis System version 9.2, SAS Institute, Cary, NC). Cultigens, locations, replications, fruit, and replicate measurements within fruit were considered as random effects. Least significant differences (LSD) among cultigens were determined at a 5% significance level. Cultigen means across locations were calculated for each trait. Components of variance were estimated by using the VARCOMP procedure of SAS. Broad-sense heritabilities, based on cultigen means, were calculated for each trait according to Fehr (1987). Spearman's coefficient (r_s) of rank correlations (Steel, Torrie

and Dickey, 1997) were calculated to test differences in rank order among the cultigens between the two locations. Pearson correlation coefficients (r) and linear regression (R^2) between color space values and carotenoid content were calculated from the means of the cultigens across locations using the CORR and REG procedure of SAS. Scatter plots of the data indicated that all relationships between color space values and carotenoid content were linear.

Results and Discussion

Colorimetric Evaluation

Significant ($P \leq 0.0001$) differences were observed among cultigens for flesh color represented by the five color space values L*, a*, b*, chroma, and hue. This demonstrates that genetic variation for flesh color is present among the cultigens tested. Locations were not significantly ($P \geq 0.60$) different for any of the color space values, but there was a significant ($P < 0.0001$) cultigen by location interaction. Based on Spearman's rank correlation tests, no significant ($r_s \geq 0.89$) change in rank order of the cultigens between locations was noted. This indicates that the flesh color of each cultigen was consistent despite different environments. Replicate measurements within fruit of each cultigen were not significantly ($P \geq 0.18$) different but significant ($P \leq 0.02$) differences were observed for the color space values among fruit within cultigens. Variance component analysis, however, revealed that estimates of variance among fruit within cultigens was less than 1.5% of the phenotypic variance for each color space value. With such a small effect, little would be gained by evaluating more fruit per cultigen. Broad-sense heritability (H) estimates of the five color space values (Table 2-1) ranged from 0.81 to 0.93 indicating that genetic improvement and effectiveness of selection for color would be moderate to high.

Subjective observations of flesh color were consistent with the color space value hue in that lower hue angles corresponded to more orange-red flesh and higher hue angles corresponded to more yellow flesh (Table 2-1). ‘Butterbush’ (*C. moschata*), with a mean hue angle of 77.5°, represented orange-red flesh color in this study and was significantly ($P \leq 0.05$) different from all other cultigens evaluated. ‘PI 314806’ (*C. pepo*) reflected a whitish-yellow flesh color with a mean hue angle of 102.9°. Differences in hue of the cultigens tested may be due to the various types and ratios of pigments present.

Mean color space values a* (+a* red direction; -a* green direction) ranged from -4.9 for ‘Tennessee Sweet Potato’ (*C. moschata*) and ‘Fordhook Acorn’ (*C. pepo*) to 14.8 for ‘Butterbush’ (*C. moschata*) (Table 2-1). The lowest mean color space value b* (+b* yellow direction; -b* blue direction) was 9.8 found within PI 314806 (*C. pepo*) and the highest, 71.4, found within ‘Waltham Butternut’ (*C. moschata*). Cultigens with hue angles ≥ 90° had yellow flesh, mean a* values ranging from 0.2 to -4.9, and mean b* values ranging from 9.8 to 47.8. Cultigens with hue angles ≤ 90° had yellow-orange flesh, mean a* values ranging from 6.6 to 14.8, and mean b* values ranging from 47.3 to 71.4.

A range of L*, lightness (83.8) to darkness (70.5) of color, and chroma, dullness (10.0) to vividness (72.2) of color was present within the cultigens evaluated (Table 2-1) and may reflect different concentrations of pigments. Three yellow-orange cultigens with hue values not significantly ($P \geq 0.05$) different from each other included ‘Waltham Butternut’ (*C. moschata*), ‘Sucrine DuBerry’ (*C. moschata*), and ‘Table Gold Acorn’ (*C. pepo*). The flesh color of ‘Waltham Butternut’ is lighter (mean L* = 73.4) and more vivid

(mean chroma = 72.2) compared to ‘Sucrine DuBerry’s flesh color which is darker (mean L* = 70.5) and duller (mean chroma = 47.8). The yellow-orange flesh and rind color of ‘Table Gold Acorn’ is due to the introgression of the *B* (*Bicolor*) gene. Typically, acorn-type squash have light-yellow flesh color at maturity as reflected by ‘Table King Bush’ (mean L* = 83.8; mean hue angle = 93.3), ‘Thelma Sander’s Sweet Potato’ (mean L* = 81.6; mean hue angle = 93.8) and ‘Fordhook Acorn’ (mean L* = 83.4; mean hue angle = 97.7). Several genes that affect rind as well as the flesh color of squash have been described (Paris and Brown, 2005; Tadmor et al., 2005) including *D* (*Dark*), *l*-1 (*light coloration*-1), *l*-2 (*light coloration*-2) and *B* (*Bicolor*). Tadmor et al. (2005) studied the effects of these particular genes in different combinations within near-isogenic lines (NILs) of *C. pepo* and reported that when the dominant allele of *B* interacts with the dominant allele of *L*-2 an intense orange rind and flesh color will develop. The flesh color of ‘Table Gold Acorn’ is lighter (mean L* = 76.5) than both ‘Waltham Butternut’ and ‘Sucrine DuBerry’ and falls between these two cultigens for color vividness (mean chroma = 65.3).

Carotenoid Content

Among cultigens tested, significant ($P \leq 0.03$) differences were observed for α-carotene, β-carotene, cis-β-carotene, and total carotenoids suggesting genetic variation in carotenoid accumulation. No significant differences were observed for lutein ($P = 0.08$), zeaxanthin ($P = 0.07$), or cis-lutein-zeaxanthin ($P = 0.06$) but this may be a reflection of restricted sampling for analysis. In this study, locations were not significantly ($P > 0.05$) different for any carotenoid measured. Broad-sense heritability (*H*) estimates of the carotenoids measured ranged from 0.37 to 0.85 (Table 2-2),

implying that genetic improvement and effectiveness of selection would be most effective for α-carotene (H^2 = 0.85) and β-carotene (H^2 = 0.74).

On average, orange-red and yellow-orange flesh colored cultigens contained 21.5 µg/g FW total carotenoids while yellow flesh colored cultigens contained 2.4 µg/g FW total carotenoids (Table 2-2). This represented a 9-fold increase in total carotenoids in orange-red and yellow-orange flesh colored cultigens versus yellow flesh colored cultigens. The most abundant carotenoids within the orange-red and yellow-orange flesh colored cultigens were lutein (average = 9.8 µg/g FW), α-carotene (average = 4.5 µg/g FW), and β-carotene (average = 5.4 µg/g FW). The most abundant carotenoid within the yellow flesh colored cultigens was lutein (average = 1.7 µg/g FW). Zeaxanthin (range = 0.1 - 0.2 µg/g FW), cis-lutein-zeaxanthin (range = 0.1 - 1.2 µg/g FW), and cis-β-carotene (range = 0.1 - 1.7 µg/g FW), were measured but not found in all cultigens evaluated.

Tadmor et al. (2005) evaluated the carotenoid content of NILs of *C. pepo* differing in fruit pigmentation loci *B* (*Bicolor*), *D* (*Dark*), *l*-1 (*light coloration*-1), and *l*-2 (*light coloration*-2). In their study, genetic backgrounds that lacked either the dominant *D* or dominant *L*-2 alleles had yellow flesh color. On average, these NILs contained 1.2 µg/g FW lutein, 0.6 µg/g FW α-carotene and β-carotene, and 1.8 µg/g FW total carotenoids. Within our yellow flesh colored *C. pepo* cultigens, similar carotenoid concentrations were obtained with an average lutein concentration of 1.8 µg/g FW, an average α-carotene and β-carotene concentration of 0.6 µg/g FW, and an average total carotenoid concentration of 2.7 µg/g FW (Table 2-2). In our study, no significant ($P \geq$

0.05) differences among the yellow flesh colored *C. pepo* cultigens for any of the carotenoids were identified.

In this study we evaluated ‘Table Gold Acorn’, a yellow-orange flesh colored *C. pepo* cultigen. The yellow-orange flesh color of this cultigen is due to the presence of the *B* pigmentation allele. In our study, this cultigen had at least a 3-fold increase in total carotenoid content compared to other *C. pepo* cultigens (Table 2-2). In the Tadmor et al. (2005) study, the effect of *B* on carotenoid content within their *C. pepo* NILs was dependent on the genetic combinations of pigmentation loci *D*, *I*-1 and *I*-2. In genetic backgrounds with either dominant *D* or dominant *L*-1, the *B*/*B* genotype had an approximate 2-fold increase in total carotenoid content. In NILs with *B*/*B* and *L*-2/*L*-2 genotypes total carotenoid content increased 10-fold. The genetic makeup of ‘Table Gold Acorn’ will need to be further investigated to determine its allelic state at the pigmentation loci *D*, *I*-1 and *I*-2 and the cause of its increased carotenoid content.

The *C. moschata* cultigen ‘Butterbush’ contained the highest total carotenoid content (42.3 µg/g FW) and significantly ($P \leq 0.05$) more α-carotene (14.9 µg/g FW) and β-carotene (15.3 µg/g FW) than to other *C. moschata* cultigens evaluated in this study (Table 2-2). This suggests genetic variability is present within *C. moschata* that leads to elevated carotenoid content and concentrations. Additional evidence for variability in carotenoid content and concentration within *C. moschata* is provided by Murkovic et al. (2002). The *C. moschata* cultigens in their study averaged 86.7 µg/g FW total carotenoids (range = 41.6 to 143.0 µg/g FW), 28.2 µg/g FW α-carotene (range = 9.8 to 59.0 µg/g FW), and 51.0 µg/g FW β-carotene (range = 31.0 to 70.0 µg/g FW). The carotenoid averages and their ranges were higher than those found in our study. It

would be interesting to investigate the genetic basis that leads to elevated carotenoids within *C. moschata* and to compare this with the *C. pepo* findings of Tadmor et al. (2005).

Correlations between Color Space Values and Carotenoids

Pearson correlations coefficients (r) between color space values, L*, a*, b*, chroma, and hue, with lutein, α-carotene, β-carotene and total carotenoids, the four most prominent carotenoids measured in this study, were calculated (Table 2-3). The color value L* (lightness or darkness) correlated ($P < 0.03$) negatively with lutein (r = -0.68) and total carotenoids (r = -0.66). A negative correlation between L* and certain carotenoids would be expected since any increase in pigment would increase the darkness and thereby decrease L*. The strength of our two linear relationships, however, would be considered weak (r ≤ -0.70). The color value a* (color direction in red or green) was strongly (r ≥ 0.85) correlated with total carotenoids (r = 0.91) followed by moderate (0.75 ≤ r ≤ 0.84) to weak correlations with lutein (r = 0.84), β-carotene (r = 0.77), and α-carotene (r = 0.70).

The color value b* (color direction in yellow or blue) and chroma (saturation or vividness of color) correlated strongly with lutein (r = 0.87 for both) and moderately with total carotenoids (r = 0.75 and r = 0.76). Hue (tint of color; an angular measure) was weakly correlated with α-carotene (r = -0.62) and β-carotene (r = -0.69), but moderately correlated with lutein (r = -0.80) and total carotenoids (r = -0.83). The negative correlation observed between hue and the carotenoids measured in this study suggests that as hue angles decrease, carotenoid concentrations increase.

Studies relating colorimetric values with total carotenoids and/or β-carotene in winter-type squash have previously been reported. Francis (1962) evaluated cultigens of

C. maxima and *C. moschata* and identified moderate to strong correlations between L* (r = -0.78), a* (r = 0.83), b* (r = 0.79), chroma (r = 0.93), and hue (r = -0.96) with total carotenoids. In a study evaluating *C. maxima* germplasm, Seroczynska et al. (2006) reported poor to fair correlations between L* (r = -0.53), a* (r = 0.77), b* (r = 0.76), and chroma (r = 0.77) with total carotenoids and poor to fair correlations between L* (r = -0.54), a* (r = 0.74), b* (r = 0.66), and chroma (r = 0.67) with β-carotene. Comparing our findings with those of Francis (1962) and Seroczynska et al. (2006) revealed similar correlations and strengths except our correlation between a* (r = 0.91) and total carotenoids was stronger.

In other vegetable crops, studies have reported correlations between colorimetric values and carotenoids. For example, in tomato, both D'Souza et al. (1992) and Arias et al. (2000) found L* (r = -0.91 and r = -0.92) and a* (r = 0.87 for both) to be strongly correlated with lycopene content. In sweet potato, Simonne et al. (1993) reported a strong positive correlation between hue and β-carotene (r = 0.99) whereas Ameny and Wilson (1997) reported a moderate correlation between b* and β-carotene (r = 0.74). All four reports viewed colorimetric analysis as an appropriate estimator of carotenoid concentrations. A report by Reeves (1987), however, questioned the wisdom of assessing carotenoid concentrations by tristimulus colorimetry. He stated that while correlations may show statistical significance, the variation explained by the correlation may be too low to be of practical use. In his evaluation of pepper color and carotenoid data, the best parameter to predict total carotenoid content was a negative correlation (r = -0.713) with L* from pureed peppers. In his view, a mathematical equation based on

this correlation would, at best, explain only 51% (R^2) of the observed variation between L* and total pigment content.

The strongest linear relationship, in our study, was found between a* and total carotenoids (r = 0.91; R^2 = 0.83). The R^2 value indicates that 83% of the variation in total carotenoids can be accounted for by the change in a*. The color values b* and chroma were also strongly correlated with lutein (r = 0.87; R^2 = 0.76 for both). The R^2 values indicate that 76% of the variation in lutein can be accounted for by the change in b* or chroma. While regression equations based on these correlations may account for only 76-83% of the variation for lutein and total carotenoids, respectively, they may still be useful for estimating these concentrations. Regression equations for the prediction of lutein and total carotenoids based on a*, b* and chroma, based on our findings, are provided in Table 2-4.

Conclusion

In this study, we found a range of color and carotenoid types and concentrations within pumpkins and squash. This genetic variation should make it possible to increase the nutritional value through crossing and selection from within and among the different types with high levels of carotenoids. Based on this study, strong correlations between colorimetric values and carotenoid content were identified. These close associations will assure that indirect selection for high carotenoid content within pumpkin and squash breeding material will be successful, easy to implement, and inexpensive.

Table 2-1. Means and broad-sense heritability (*H*) estimates of color space values, L*, a*, b*, chroma and hue[a], measured in fruit flesh of 11 *Cucurbita* cultigens.

Cultigen[b]	Species	L*[c]	a*	b*	Chroma	Hue	Color (subjective)
Butterbush	*C. moschata*	73.7	14.8	65.9	67.7	77.5°	orange-red
Waltham Butternut	*C. moschata*	73.4	10.4	71.4	72.2	81.8°	vivid yellow-orange
Sucrine DuBerry	*C. moschata*	70.5	6.6	47.3	47.8	82.2°	dull yellow-orange
Ponca Butternut	*C. moschata*	74.8	7.3	68.1	68.5	83.9°	yellow-orange
PI 458728	*C. moschata*	75.4	0.2	47.8	47.8	90.0°	dull yellow
Tennessee Sweet Potato	*C. moschata*	83.2	-4.9	31.5	31.9	99.0°	light yellow
Table Gold Acorn	*C. pepo*	76.5	9.4	64.5	65.3	81.9°	yellow-orange
Table King Bush	*C. pepo*	83.8	-2.4	43.4	43.5	93.3°	light yellow
Thelma Sander's Sweet Potato	*C. pepo*	81.6	-2.5	40.8	40.9	93.8°	light yellow
Fordhook Acorn	*C. pepo*	83.4	-4.9	36.3	36.6	97.7°	light-yellow
PI 314806	*C. pepo*	82.2	-2.2	9.8	10.0	102.9°	light whitish-yellow
$LSD_{0.05}$		0.8	0.8	1.5	1.6	0.8°	
H		0.81	0.87	0.93	0.93	0.91°	

[a]Numeric description of color using L*a*b* CIELAB color space. L* (lightness or darkness) - ranges from black (0) to white (100); a* color direction in red (a* > 0) or green (a* < 0); b* color direction in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more vivid; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red and 90° = yellow.

[b]Cultigens include cultivars, heirlooms, and plant introductions (PI) previously selected to represent a range of color from white to orange based on L*a*b* color spaces values.

[c]Values shown are based on 5 fruit x 3 colorimetric measurements per fruit x 2 replications per location x 2 locations per cultigen.

Table 2-2. Means and broad-sense heritability (*H*) estimates of carotenoids (µg/g FW) measured in 11 *Cucurbita* cultigens.

Cultigen[a]	Lutein[b]	Zeaxanthin	cis-Lutein/ Zeaxanthin	α-Carotene	β-Carotene	Cis β-Carotene	Total Carotenoids	Color (subjective)
Butterbush	9.2	0.2	0.9	14.9	15.3	1.7	42.3	orange-red
Ponca Butternut	8.7	0.1	0.6	1.8	2.1	0.3	13.9	vivid yellow-orange
Waltham Butternut	17.3	0.1	1.2	3.0	3.8	0.9	26.8	dull yellow-orange
Sucrine DuBerry	4.3	0.0	0.3	2.6	2.1	0.4	9.7	yellow-orange
PI 458728	3.9	0.0	0.3	0.4	0.6	0.1	5.3	dull yellow
Tennessee Sweet Potato	0.6	0.0	0.3	0.0	0.2	0.0	1.1	light yellow
Table Gold Acorn	9.3	0.2	0.9	0.0	3.6	0.6	14.7	yellow-orange
Table King Bush	2.6	0.0	0.2	0.0	0.9	0.1	3.9	light yellow
Thelma Sander's Sweet Potato	1.4	0.0	0.1	0.0	0.6	0.1	2.2	light yellow
Fordhook Acorn	1.4	0.0	0.1	0.0	0.4	0.0	1.9	light-yellow
PI 314806	0.1	0.0	0.0	0.0	0.0	0.0	0.1	light whitish-yellow
$LSD_{0.05}$	10.4	0.2	0.8	4.1	5.6	0.9	20.4	
H	0.37	0.49	0.42	0.85	0.74	0.59	0.59	

[a]Cultigens include cultivars, heirlooms, and plant introductions (PI) previously selected to represent a range of color from white to orange based on L*a*b* color spaces values. Cultigens 'Butterbush' through 'Tennessee Sweet Potato' are *C. moschata.* Cultigens 'Table Gold Acorn' through 'PI 314806' are *C. pepo*.

[b]Separation and quantification of carotenoids were accomplished by HPLC. Values shown based on 100 gram fresh weight samples per cultigen per location.

Table 2-3. Pearson correlation coefficients (r) between color space values (L*, a*, b*, chroma, and hue) and carotenoids calculated from the means of 11 *Cucurbita* cultigens.

Traits[a]	L*		a*		b*		Chroma		Hue	
	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value	r	*P*-value
Lutein	-0.68	0.0209	0.84	0.0013	0.87	0.0006	0.87	0.0006	-0.80	0.0033
α-Carotene	-0.49	0.1246	0.70	0.0155	0.45	0.1609	0.47	0.1438	-0.62	0.0433
β-Carotene	-0.49	0.1303	0.77	0.0052	0.55	0.0809	0.56	0.0703	-0.69	0.0198
Total Carotenoids	-0.66	0.0285	0.91	0.0001	0.75	0.0076	0.76	0.0062	-0.83	0.0017

[a]Numeric description of color using L*a*b* CIELAB color space. L*, lightness or darkness; a* color direction in red or green; b* color direction in yellow or blue; chroma, saturation or vividness; hue, tint of color.

Table 2-4. Best fit (r ≥ 0.85) regression equations for pairs of related carotenoids with color space values.

Pairs of relationships[a]	Regression equations	r	R^2
Total Carotenoids and a*	Total Carotenoids = 6.1226 + 1.7106*a*	0.91	0.83
Lutein and b*	Lutein = -6.3881 + 0.2446*b*	0.87	0.76
Lutein and Chroma	Lutein = -6.3743 + 0.2418*Chroma	0.87	0.76

[a]Lutein and total carotenoids in µg/g FW.

Figure 2-1. A sampling of the range of fruit shapes and rind colors present in *C. moschata* fruit from the initial screen in 2007.

Figure 2-2. A sampling of the range of fruit shapes and rind colors present in *C. pepo* fruit from the initial screen in 2007.

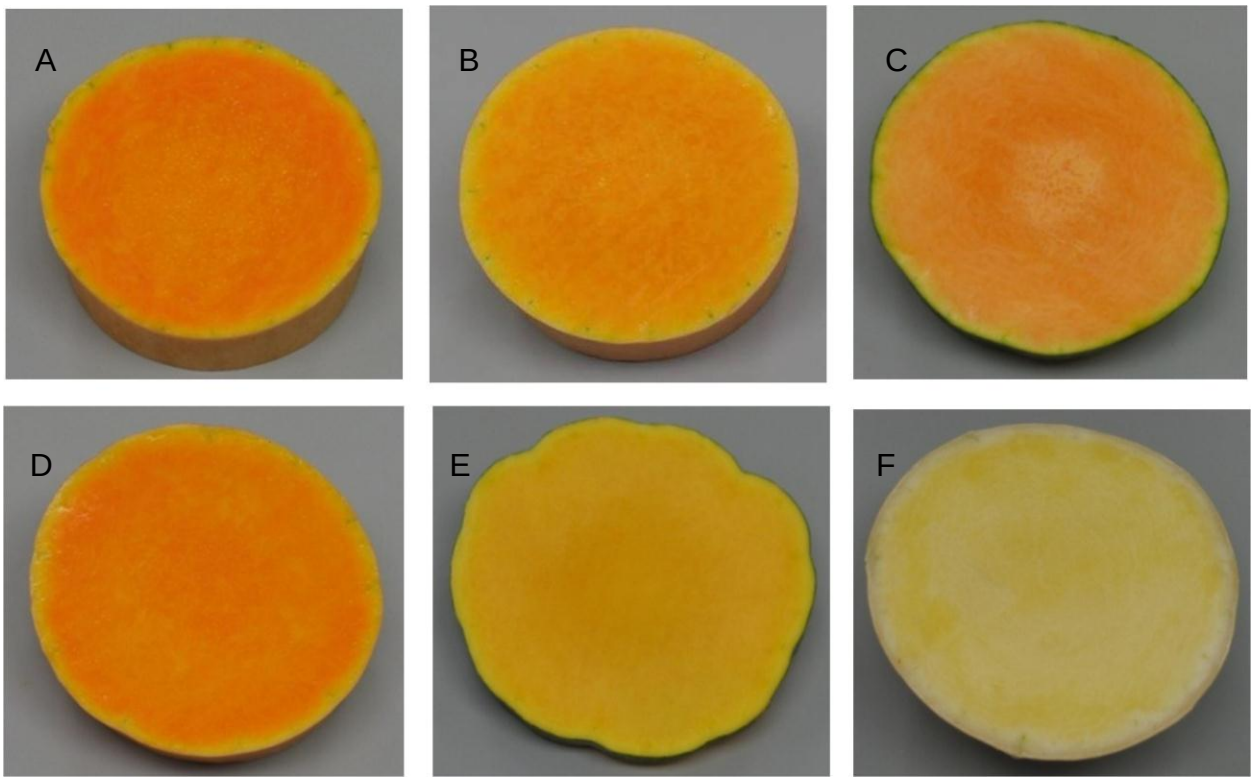

Figure 2-3. Cross sections of the mesocarp of six *C. moschata* cultigens selected from the pre-screen in 2007 for the 2008 correlation study. A) 'Butterbush' B) 'Waltham Butternut' C) 'Sucrine DuBerry' D) 'Ponca Butternut' E) PI 458728 and F) 'Tennessee Sweet Potato.'

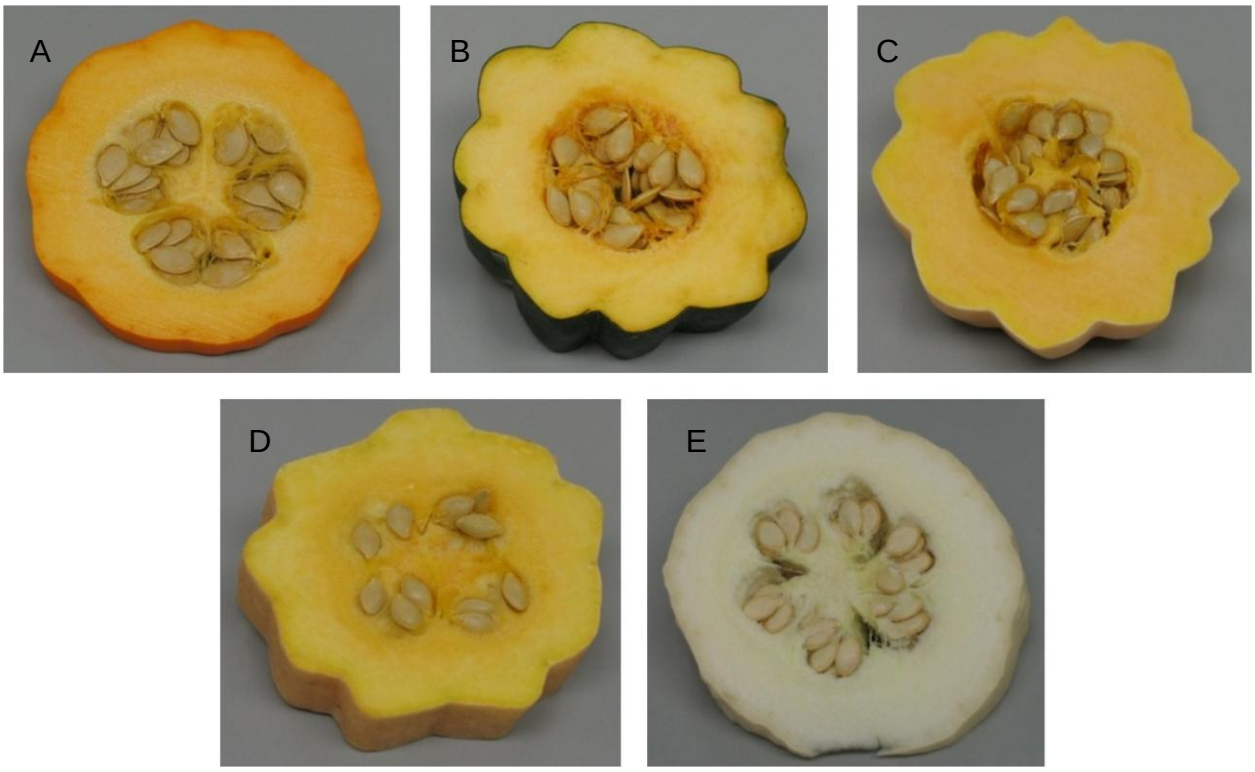

Figure 2-4. Cross sections of the mesocarp of five *C. pepo* cultigens selected from the pre-screen in 2007 for the 2008 correlation study. A) 'Table Gold Acorn' B) 'Table King Bush' C) 'Thelma Sander's Sweet Potato' D) 'Fordhook Acorn' and E) PI 314806

CHAPTER 3
HERITABILITY ESTIMATES OF L* A* B* COLOR SPACE VALUES IN WINTER SQUASH (*Cucurbita* spp.)

Introduction

The mesocarp tissue of squash (*Cucurbita* spp.) has a wide range of color, from white to orange, which is conditioned by different carotenoid types and concentrations. In addition, carotenoids contribute to the nutritional value of squash (Gross, 1991), as an antioxidant source that protects the body against cancer and degenerative diseases (Murkovic et al., 2002). Certain carotenoids within squash can also serve as a source of pro-vitamin A, a precursor of vitamin A available in plant tissues, which functions to promote normal vision, bone growth, cell division and differentiation, and reproduction (ODS/NIH, 2006).

In chapter 2, correlations between L* a* b* color space values and carotenoid content within flesh color in squash (*Cucurbita* spp.) were identified (Table 2-3). Increase in color space values a* (+a* red direction; -a* green direction), b* (+b* yellow direction; -b* blue direction), and chroma (vividness or saturation of color) were correlated with an increase in total carotenoid content and lutein, respectively. Collectively, these results indicate that L* a* b* color space values can be used as indirect estimates of carotenoid content. These relationships are beneficial due to the reduced time and cost associated with characterization of nutrient content by color analysis as compared with chemical analysis. Correlation results from this study indicate that squash with dark (lower L*) and more vivid (higher chroma) flesh color has more nutritional value than lighter colored varieties.

Previous studies have determined the genetic control of rind and flesh color in members of the Cucurbitaceae. In *C. pepo* (Paris, 2000) and *C. maxima* (Lopez-Anido

et al., 2003) thirteen and three loci determining light and dark green rind color were identified, respectively. In other cucurbit crops, only flesh color has been examined. In watermelon, *Citrullus lanatus*, both canary yellow and scarlet red flesh color were found to be controlled by a single, dominant gene, dominant to white flesh and coral red, respectively. However, populations segregating for salmon yellow and red flesh lacked progeny with clear parental phenotypes and had a range of intermediate colors. This deviation from a one gene model was suggestive of quantitative traits controlling flesh color (Gusmini and Wehner, 2006). Flesh color in melon (*Cucumis melo*) is controlled by both major and minor genes (Cuevas et al., 2010). In melon the flesh color in both F_2 and BC_1 populations fit an epistatic two gene model. Narrow-sense heritabilities of flesh color, a reflection of β-carotene content in these populations were moderate; 0.55 in the F_1, F_2, and BC populations and 0.68 in the F_3 populations.

Previous observations suggest inheritance of flesh color of squash is quantitative, influenced by genetic and environmental factors. Thus, knowledge of the heritability and gene action of flesh color genes would give the plant breeder an idea of the population size and time frame needed to manipulate flesh color and thus, nutritional content in squash. To date, there are no available population studies examining the gene number or the inheritance of genes controlling flesh color in squash (*C. moschata* and *C. pepo*). The objective of this study was to determine heritability and gene action of flesh color measured by L*a*b* color space values in one *C. moschata* and two *C. pepo* populations.

Materials and Methods

Plant Material

Results from an initial screening of *C. moschata* and *C. pepo* cultigens (Chapter 2) were used to identify parents to cross for population development. Within each species in the preliminary screen, cultigens were selected based on flesh color extremes and similar fruit shape for crossing. One cross was made per plant. Unsuccessful crosses were removed before new crosses were attempted. Fruit was harvested 45 days after successful cross completion, and seed was extracted two weeks after the fruit was removed from the plant. Reciprocal crosses were also attempted for all cross combinations. From successful crosses, two *C. pepo*, ['Table Gold Acorn,' TGA, (yellow-orange) x PI 314806 (light whitish-yellow)] and ['Table King Bush,' TKB, (light yellow) x PI 314806], and one *C. moschata*, ['Butterbush,' BB, (orange-red) x 'Sucrine DuBerry,' SDub, (dull yellow-orange)] F_1 individuals were randomly selected and selfed to create three F_2 populations. Additionally, F_1 individuals were reciprocally backcrossed to each parent. All crosses were made in a greenhouse.

Field Trials

The field trial used for selecting parents for crossing was described in Chapter 2. F_2 and backcross (BC) screens were conducted 9 April – 3 July 2008 and 6 April – 4 July 2009, respectively. One hundred seeds (genotypes) for each F_2 and BC population described previously were planted in a completely random design at PSREC. Eight seeds and six each of parent 1, parent 2 and the F_1 were also included within each F_2 and BC population, respectively. A maximum of three mature fruit were harvested from each plant for colorimetric readings. Fruit of the F_2 and related BC populations were harvested along the same timeline in 2008 and 2009 (Table 3-1). During the 2008 field

trials, temperatures recorded by the Florida Automated Weather Network (FAWN) were 24°C avg., 39°C max., and 2°C min. In 2009, temperatures recorded by FAWN were 24°C avg., 38°C max., and 1°C min. over the course of the growing season. Recommended conventional cultural practices and fertility rates for Florida squash were followed for all field trials for both 2008 and 2009 (Olson and Simonne, 2007).

Colorimetric Analysis

Color was recorded using a Minolta CR-400 Colorimeter (Minolta Camera Co., Ltd., Ramsey, NJ) tristimulus color analyzer, equipped with an 8 mm diameter measuring area and diffuse illumination of a 2° Standard Observer. Color measurements include L* a* b*, chroma, and hue. The L* coordinate indicates darkness or lightness of color and ranges from black (0) to white (100). Coordinates, a* and b*, indicate color directions: +a* is the red direction, -a* is the green direction, +b* is the yellow direction and –b* is the blue direction. Chroma is the saturation or vividness of color. As chromaticity increases, a color becomes more intense, and as it decreases a color becomes duller. Hue angle is the basic unit of color and can be interpreted, for example, as 0° = red and 90° = yellow. Both chroma and hue are derived from a* and b* using the following equations: Metric chroma: $C = \sqrt{[(a^*)^2 + (b^*)^2]}$ and metric hue angle: $h = \tan^{-1}(b^*/a^*)$ [degrees].

Using fruit that had been sliced transversely, L*a*b* color space measurements from the edible flesh (mesocarp) of each fruit were recorded in succession to avoid discoloration. The preliminary study revealed replicate measurements of the mesocarp of each fruit per cultigen were not significantly ($P \geq 0.05$) different. Therefore, avoiding the seed cavity and surrounding tissue (approx. 10 mm), three random measurements per fruit were recorded.

Variance, Heritability and Standard Error Estimations by Generation Means Analysis

Variances were calculated for each color space value within a generation for each of the nine populations. Additive variances were calculated as (Warner 1952; Wright 1968):

$$\sigma^2_A = 2\sigma^2_{F2} - (\sigma^2_{BC1P1} + \sigma^2_{BC1P2}) \quad (3\text{-}1)$$

where σ^2_{F2} equals the total variance present in the F_2 population, and σ^2_{BC1P1} and σ^2_{BC1P2} are the variances in the backcross populations, with the F_1 crossed back to parent 1 and parent 2 of the original population cross, respectively.

Environmental variances were calculated as (Warner 1952; Wright 1968):

$$\sigma^2_E = (1/2)\sigma^2_{F1} + (1/4)\sigma^2_{P1} + (1/4)\sigma^2_{P2} \quad (3\text{-}2)$$

Phenotypic variances equal the variance among plants of each F_2 population, for each trait (Mahmud and Kramer, 1951):

$$\sigma^2_P = \sigma^2_{F2} \quad (3\text{-}3)$$

Genetic variances were calculated as (Wright 1968):

$$\sigma^2_G = \sigma^2_P - \sigma^2_{E'} \quad (3\text{-}4)$$

where σ^2_P equals phenotypic variance among the F_2 plants. Broad-sense heritabilities were calculated using genetic variance and phenotypic variance:

$$H = \sigma^2_G / \sigma^2_P \quad (3\text{-}5)$$

Narrow-sense heritabilities were calculated as (Sleper and Poehlman 2006):

$$h^2 = \sigma^2_A / \sigma^2_P \quad (3\text{-}6)$$

Standard errors were calculated as (Lynch and Walsh, 1998):

$$SE = \mathrm{Sqrt}[\mathrm{Var}(V_x)], \text{ with } \mathrm{Var}(V_x) \quad (3\text{-}7)$$

where Var(Vx) equals the variance of the desired parameter. Estimates of Var(V_x) were calculated as:

$$\Sigma\ [2[\mathrm{Var}(k)^2] / (n+2)] \quad (3\text{-}8)$$

where Var (k) is each of the variance components used to calculate V_x. Standard error calculations for broad-sense heritability weres calculated as (Hallauer and Miranda, 1988):

$$SE(H) = [SE(\sigma^2_G)] / \sigma^2_P \quad (3\text{-}9)$$

Estimates of standard error for narrow-sense heritability estimates were calculated as (Hallauer and Miranda, 1988):

$$SE(h^2) = [SE(\sigma^2_A)] / \sigma^2_P \quad (3\text{-}10)$$

Epistatic Interaction Estimates

Epistatic interactions were calculated for each color space within a generation for each of the three crosses and were estimated based on a six parameter non-weighted model as described by Hayman (1958) and Gamble (1962). Means (*m*) and additive (*a*), dominance (*d*), additive x additive (*aa*), additive x dominance (*ad*) and dominance x dominance (*dd*) effects were calculated as:

$$m = \mu_{F2} \quad (3\text{-}11)$$
$$a = \mu_{BC1P1} - \mu_{BC1P2} \quad (3\text{-}12)$$
$$d = -(1/2)\mu_{P1} - (1/2)\mu_{P2} + \mu_{F1} - 4\mu_{F2} + 2(\mu_{BC1P1} + \mu_{BC1P2}) \quad (3\text{-}13)$$
$$aa = -4\mu_{F2} + 2(\mu_{BC1P1} + \mu_{BC1P2}) \quad (3\text{-}14)$$
$$ad = -(1/2)\mu_{P1} + (1/2)\mu_{P2} + \mu_{BC1P1} - \mu_{BC1P2} \quad (3\text{-}15)$$
$$dd = \mu_{P1} + \mu_{P2} + 2\mu_{F1} + 4\mu_{F2} - 4(\mu_{BC1P1} + \mu_{BC1P2}) \quad (3\text{-}16)$$

All calculations including estimates derived from parent 1, parent 2, and F_1 were calculated using 2008 data after ANOVA revealed significant genotype by year interactions present for nearly all color space values all three populations. Variances were calculated as (Gamble, 1962):

$$m = \sigma^2_{F2} \quad (3\text{-}17)$$
$$a = \sigma^2_{BC1P1} - \sigma^2_{BC1P2} \quad (3\text{-}18)$$
$$d = -(1/4)\,\sigma^2_{P1} - (1/4)\,\sigma^2_{P2} + \sigma^2_{F1} - 16\,\sigma^2_{F2} + 4(\sigma^2_{BC1P1} + \sigma^2_{BC1P2}) \quad (3\text{-}19)$$
$$aa = -16\,\sigma^2_{F2} + 4(\sigma^2_{BC1P1} + \sigma^2_{BC1P2}) \quad (3\text{-}20)$$
$$ad = -(1/4)\,\sigma^2_{P1} + (1/4)\,\sigma^2_{P2} + \sigma^2_{BC1P1} - \sigma^2_{BC1P2} \quad (3\text{-}21)$$
$$dd = \sigma^2_{P1} + \sigma^2_{P2} + 4\,\sigma^2_{F1} + 16\,\sigma^2_{F2} - 16(\sigma^2_{BC1P1} + \sigma^2_{BC1P2}) \quad (3\text{-}22)$$

Standard errors were calculated for each of the six parameters by the sum of the square root of the variances for each factor within the equation (Gusmini et al., 2007).

Data Analysis

Color space values for all F_2 and BC populations were subjected to analysis of variance by the GLM procedure of SAS (Statistical Analysis System version 9.2, SAS Institute, Cary, NC). Fruit and replicate measurements within a fruit were considered as random effects. Least significant differences (LSD) among genotypes were determined at a 5% significance level. Additionally, color space values for parent 1, parent 2, and F_1 used to create each population were analyzed by SAS GLM to assess differences between years. The RANK and Spearman's CORR procedures in SAS were used to examine all parent 1, parent 2 and F_1 individuals from all three crosses over two years. Generation means, standard deviations, and ranges were calculated for each trait using the MEANS procedure of SAS. Least significant differences among generations within each year were determined at a 5% significance level.

All heritability estimates were initially calculated based on a maximum of three fruit per genotype with three color measurements per fruit within the F_2, BC_{1P1} and BC_{1P2} generations. However, ANOVA indicated highly significant differences between fruit within a genotype for nearly all color space values for all populations. Therefore, estimates were also calculated using one fruit per genotype due to variable fruit maturity within a genotype. Fruit within a genotype were selected based upon highest (a*, b*,

and chroma) or lowest (L* and hue) average replicate measurements within a fruit. Selection of fruit with highest or lowest average fruit replicate measurements for each color space value was based upon the direction of each respective color space value correlated with carotenoid levels (Table 2-3) and represent the genetic potential of the genotype examined. Selected single fruit measures of heritability are thus reported in this chapter.

Epistatic interactions were also initially calculated using both three and one fruit, with one fruit selection estimates reported here. Estimates were then tested to determine if values obtained were significantly different from zero using Fisher's t-test. Color space values with the highest correlations to carotenoid levels, a*, b* and Chroma, were also examined for the presence of transgressive segregants using LSD at a 5% significance level. Additionally, all hue angle values were converted from degrees to radians for mean and variance calculation used for heritability and epistatic interaction estimates.

Results and Discussion

Analysis of variance

('Table Gold Acorn' x PI 314806) (*C. pepo*) populations

For the parents and F_1, significant interactions existed between year and genotype for all color space values. Genotypes were significantly different from one another for all color space values, with the exception of L* ($P = 0.0523$). Fruit within a genotype were significantly different from one another for all color space values. Replications within a fruit for all color space values were not significantly different from one another, with *P*-values ranging from 0.9208 (L*) to 0.9990 (a*). Analyses of variance for the F_2 and the BC_1 populations developed from this cross are summarized in table 3-2.

('Table King Bush' x PI 314806) (*C. pepo*) populations

For the parents and F_1, significant interactions existed between year and genotype for L*, a*, and b* (*P* = 0.0031, *P* = 0.0039, and *P* = 0.0489, respectively), while color space values chroma and hue showed no interaction between year and genotype. Genotypes were significantly different for all color space values except L* (*P* = 0.1338). Fruit within a genotype was significantly different for all color space values. Replications within a fruit were not found to be significantly different from one another for all color space values. Analyses of variance for the F_2 and the BC_1 populations developed from this cross are summarized in table 3-3.

('Butterbush' x 'Sucrine DuBerry') (*C. moschata*) populations

For the parents and F_1, significant interactions existed between year and genotype for all color space values except for L* (P = 0.2214). Genotypes were significantly different from one another for color space values L*, b* and chroma, and showed no significant differences for a* (*P* = 0.1947) and hue (*P* = 0.4544). Fruit within a genotype were significantly different for all color space values (*P* < 0.0001), however, replications within a fruit were not significantly different for all color space values. Analyses of variance for the F_2 and the BC_1 populations developed from this cross are summarized in table 3-4.

L* a* b* Color Space Means and Ranges

('Table Gold Acorn' x PI 314806) (*C. pepo*) populations

For color space value L*, PI 314806 had the lightest flesh in both 2008 and 2009, while TGA had the darkest flesh in both years; the F_1 L* values fell in between both parents (Table 3-5). Means of L* for each backcross were between the F_1 and the respective backcrossed parent measured in the same year (Table 3-6). Means of color

space value a* were highest in TGA in both 2008 and 2009, and were higher by over 10 units for all a* mean values. In 2008, mean a* values of the F_1 and F_2 population were lower than PI 314806 by 0.8 units, and in 2009 the mean of the F_1 was lower than PI 314806 by 1.0 unit. This indicates that TGA had more red color than did any subsequent generation or population developed using TGA. Similar to L* mean values, both BC_{1P1} and BC_{1P2} a* mean values were between their respective parents.

For color space value b*, TGA had the highest values in 2008 and 2009, indicating greater yellow coloration (Table 3-5). TGA was notably higher in chroma than all others, indicating a more saturated and vivid color. Mean color space values for chroma and b* were nearly identical for all generations, with the largest difference being 0.9 units between the mean of b* for TGA in 2009 and the mean of chroma for TGA in 2009. All generation mean hue angles, with the exception of TGA values in both years, were over 90°. Hue angle means were highest for F_1 plants in 2008 and 2009, indicating a greener hue. Lowest hue angle means were recorded for TGA plants in both years, indicating a yellow-orange hue.

('Table King Bush' x PI 314806) (*C. pepo*) populations

Mean L* values of F_1 and F_2 generations from crosses with TKB were very similar to crosses with TGA, with the exception of backcross populations (Tables 3-5, 3-7). Both backcross means fell outside of the means of their respective parents. Mean a* color space values of F_1 and F_2 were lower than PI 314806 in 2008, and the F_1 generation mean was lower than PI 314806 in 2009. Similar to the F_1 means, the BC_{1P2} mean a* value was lower than PI 314806 a* mean. This indicates that both the F_1 and the BC_{1P2} population had the greenest color.

TKB in both 2008 and 2009 had the highest mean values of b*, indicating the greatest yellow color, while PI 314806 P_2 had the lowest b* mean values in both years. The highest chroma value was in TKB, while PI 314806 had the lowest value.

('Butterbush' x 'Sucrine DuBerry') (*C. moschata*) populations

L* mean color space values for all *C. moschata* populations were lower than all generation means for both the TGA and TKB populations, indicating the BB x SDub populations had the darkest flesh (Tables 3-5, 3-6, 3-7, and 3-8). In 2008, mean L* values of the F_2 and the F_1 generation were higher than BB. In 2009, mean L* values of the F_1 and the BC_1 generations were also higher than mean values of BB. Mean color space a* values were highest in BB in both years, indicating it had the greatest red color. Means of color space value b* were highest for BB and lowest for SDub in both years, indicating that BB had yellower flesh than SDub. For chroma, BB had the highest value, while SDub had the lowest value. Results from b* and chroma mean values indicate that BB was more yellow and more vivid in color than SDub for both years.

For both color space values b* and chroma, both TGA and TKB were the most yellow and most vivid of the generations in their respective populations. This could be due to the genetic composition of each genotype. Over a dozen genes that affect the rind and flesh color of squash have been described (Paris and Brown, 2005), including *D* (*Dark*), *l*-1 (*light coloration*-1), *l*-2 (*light coloration*-2) and *B* (*Bicolor*). The effects of these genes were studied within near-isogenic lines (NILs) in *C. pepo* by Tadmor et al. (2005). In genetic backgrounds that lacked either the dominant *D* or dominant *L*-2 alleles, a yellow flesh color developed. In genetic backgrounds with either dominant *D* or *L*-2, a yellow-orange flesh color developed and when the dominant allele of *B*

interacts with the dominant allele of *L*-2, an intense orange flesh color will occur. The interaction of these genes may be responsible for the increased yellow-orange and yellow pigmentation, respectively, of both TGA and TKB as they relate to the other generations within their populations.

In both years, TGA had a lower hue angle indicating a more orange flesh color, and TKB had a higher hue angle, indicating a more yellow flesh color. White flesh, (*Wf*) in *C. pepo* is documented as dominant to colored flesh and prevents accumulation of yellow in mesocarp tissues (Paris and Brown, 2005). This is evident in the distribution of mean hue angles for F_1 and BC_{1P2} generations in both the TGA and TKB populations. For the BB populations, mean hue angle values were highest in the F_2 and F_1, generations in 2008, and BC_{1P1} had the highest hue angle in 2009, indicating that these generations had the most yellow-orange hue within the population. Additionally, these three generation means had higher hue angle values than did BB for each respective year. All generation mean color space a*, b*, and hue angles were also lower than both the TGA and TKB populations. This indicates that the *C. moschata* populations had the most orange flesh.

Rationale for Selecting Single Fruit Color Space Value Measurements

Variation for fruit within a genotype is problematic for obtaining accurate heritability and epistatic interaction estimates. This is due to the error variance being confounded within the genotypic variance estimate, and will result in inaccurate estimates of heritability and epistatic interactions. Nearly all color space values within each population of each cross were found to be significantly different for fruit within a genotype. This is likely due to the difficulty in obtaining three fruit from an individual plant that were at a similar maturity, as squash has indeterminate flowers that can vary

by several days in maturity along the vine. As flesh color development continues until maturity, this non-genetic variation is problematic for our analyses. Due to this variation, single fruit selections for each color space value within a genotype were made and used for heritability and epistatic interaction analysis.

In addition, ANOVA analyses revealed significant genotype × year interactions for nearly all color space values for the respective parent 1, parent 2, and F_1 in all crosses when examined separately. Exceptions were chroma (*P* = 0.0534) and hue (*P* = 0.1910) in the TKB x PI 314806 cross and L* (*P* = 0.2214) in the BB x SDub cross. Parent 1, parent 2 and F_1 data for each cross were therefore not able to be combined for 2008 and 2009 and only data from 2008 for P_1, P_2 and F_1 for each cross were used for heritability calculations. When these crosses were examined collectively, all color space values had a highly significant genotype × year interaction (Table 3-9). To further examine the genotype × year interactions, the year variation was tested by using Spearman's rank correlation for all of the same genotypes that were grown in both 2008 and 2009 (TGA, TKB, PI 314806, BB, SDub, and the F_1 created from each cross of the former). Spearman's rank correlation revealed no significant change in rank of genotypes for L* (r = 0.97, *P* = <0.0001), a* (r = 0.97, *P* = <0.0001), b* (r = 0.98, *P* = <0.0001), chroma (r = 0.97, *P* = <0.0001), and hue (r = 0.93, *P* = 0.0002) between years, These results indicate that although the variation in fruit measurements within a genotype were significant, the relative differences between genotypes were not altered when examined in multiple growing seasons.

Broad and Narrow-Sense Heritability Estimates

Estimates of negative broad and narrow sense heritabilities were obtained for some populations. Negative estimates of narrow-sense heritability arose from negative

additive variance estimates, and are to be regarded as zero (Robinson et al. 1955) and all negative estimates are reported as such herein. Obtaining negative variance components for heritability calculations may occur due to failure to meet assumptions of the model, the presence of additional data correlations present, or due to sampling error (Thompson and Moore, 1963). In addition, negative heritability estimates can occur if true heritability is low to moderate (0.10 – 0.25) and the number of observations is not large enough (Gill and Jensen, 1968). All variance estimates used to calculate broad and narrow-sense heritability are reported to aid explaining the source(s) of variation causing the negative estimates and prevent reporting bias.

('Table Gold Acorn' x PI 314806) (*C. pepo*) populations

Broad-sense heritability for color space value L* was good (0.82) and similar to the broad-sense heritability estimate obtained for hue angle (0.86) (Table 3-10). Narrow-sense heritability estimates were negative for L* and all other color space values in the population, with the exception of hue, due to large variation in backcross populations to parent 1 for all color space values (Table 3-11). Broad-sense heritability was low for color space value a*(0.32) and was the lowest for the TGA populations. Color space values b* and chroma had similar broad-sense heritability estimates (0.93 and 0.92, respectively) and were the highest broad-sense heritability estimates for the TGA populations. Additionally, color space value b* had the highest broad-sense heritability for all populations from all three crosses (Tables 3-10, 3-12, and 3-14).

('Table King Bush' x PI 314806) (*C. pepo*) populations

Color space value L* had a broad-sense heritability estimate of 0.59 and a narrow sense heritability estimate of 1.00 (Table 3-12). This was the highest narrow-sense heritability estimate for all color space values for all three crosses (Tables 3-10, 3-12,

and 3-14). This indicates that in the TKB cross, control of color space value L* has little environmental control.

Color space value a* had the lowest broad-sense heritability estimate for the TKB populations and is the lowest reported broad-sense heritability estimate for all populations derived from TGA, TKB, and BB crosses (Tables 3-10, 3-12, and 3-14). Color space values b* and chroma had the same broad-sense heritability estimate (0.88) and were the highest broad-sense heritabilities obtained for the TKB populations. Hue angle had a moderate broad-sense heritability (0.67) and a high narrow-sense heritability (0.92), which was the highest estimate for the TKB. All other narrow-sense heritability estimates for the TKB populations were considered zero due to negative variance estimates (Table 3-13).

('Butterbush' x 'Sucrine DuBerry') (*C. moschata*) populations

Color space value L* had a broad-sense heritability estimate of 0.19 and was the lowest positive broad-sense estimate for the BB populations (Table 3-14). Additionally, color space value L* had the highest narrow-sense heritability estimate for the BB populations. Color space values a* and hue were the only color space value to have both negative broad and narrow-sense heritability estimate for the BB populations due to negative variance additive and genetic variance estimates calculated from large variance estimates (Table 3-15) . Color space values b* and chroma had similar broad-sense heritabilities (0.40 and 0.36, respectively) and moderate narrow-sense heritabilities (0.76 and 0.60 respectively). Color space value chroma also had the lowest positive narrow-sense heritability for all three crosses (Tables 3-10, 3-12, and 3-14). Additionally, narrow-sense heritability estimates obtained for b* and chroma were the most similar from all crosses to narrow-sense estimates obtained for beta-carotene

associated fruit color in melon (*Cucumis melo*) with F_2 and BC_1 generations, 0.55, and F_3 families, 0.68 (Cuevas et al. 2010).

Flesh color heritability estimates obtained in other cucurbit crops, namely cucumber (*Cucumis sativus)* (Cuevas et al. 2009), and watermelon (*Citullus lanatus*) (Bang et al. 2010) were able to be evaluated using chi-square goodness of fit test for gene segregation ratios. Flesh color in cucumber associated with beta-carotene indicated a two recessive gene model, and flesh color in watermelon also indicated control by a two gene model. Data obtained in this study were not able to be easily classified into scorable groups and this calculation method was therefore not an option.

Epistatic Gene Effect Estimates

('Table Gold Acorn' x PI 314806) (*C. pepo*) populations

All color space value means (m) were found to be significantly different than zero for all epistatic gene effects ($P \leq 0.04$) (Table 3-16) with the exception of hue angle. For hue, this may be due in part to values being on a smaller scale due to conversion to radians. No significant gene effects were identified for color space values L* or a*.

For color space value b*, the additive x additive interaction was significant at a 10% level ($P = 0.09$). The gene effect was in the direction of TGA, the more yellow parent. This epistatic effect was observed as a larger difference from the F_1 to BC_{1P1} means (21.8 units) as compared to 0.8 units between the F_1 and the BC_{1P2} means (Table 3-6). In addition, LSD analysis revealed the F_1 mean was significantly different from the BC_{1P1} mean, and was not different from the BC_{1P2} mean.

Color space value chroma had the same significant gene effect, at the same significance level as did color space value b*. The effect was also noted in the direction of TGA, the more vivid parent (Tables 3-16). There was also a larger difference present

between the F_1 mean and the BC_{1P1} mean (21.8 and 21.7 units for b* and chroma, respectively) than the F_1 mean and the BC_{1P2} mean (0.8 and 0.9 unitis for b* and chroma, respectively). Significant differences were also found between F_1 means and BC_{1P1} means (Table 3-6).

Hue angle had significant gene effects for additive (*P* = 0.00), additive x additive (*P* = 0.00), and additive x dominance (*P* = 0.00) interactions. All gene effects were toward the parent with the lower hue angle, TGA. Differences were smaller between F_1 and BC_{1P2} (1.5 units) than F_1 and BC_{1P1} (7.4 units) (Table 3-6). Additionally, the mean of PI 314806 was not significantly different than F_1 or BC_{1P2} when examined by LSD analysis. Among these genotypes, additive effects were involved in control of hue angle color space values (Tables 3-16).

('Table King Bush' x PI 314806) (*C. pepo*) populations

All color space value means (m) were found to be significantly different than zero, for all epistatic gene effect estimates (*P* ≤0.02) (Table 3-17), with the exception of hue angle. No significant gene effects were noted for color space values L* and a*.

Additive and additive x additive gene effects were also significant for color space values b* and chroma, at the 10% significance level (*P* = 0.09 and 0.07 for each effect, respectively, for both b* and chroma), both in the direction of TKB, the more yellow and vivid parent (Table 3-17). Differences between F_1 and BC generations were similar for both b* and chroma, with differences of 1.7 and 1.8, respectively, between F_1 and BC_{1P2} and 17.8 and 17.9, respectively, between F_1 and BC_1 (Table 3-7). In addition, both color space values showed significant differences between the F_1 and the BC_{1P1} means and not between the F_1 and BC_{1P2} means, displaying the effect of additive gene effects for both b* and chroma color space values.

Significant dominance gene effects (P = 0.00) in the direction of the parent with the higher hue angle, PI 314806, and additive x dominance (P = 0.00), and dominance x dominance (P = 0.00) gene effects in the direction of parent with the lower hue angle, TKB, were identified in hue angle (Table 3-17). Similar to the distribution of generation means for F_1, BC_{1P1}, and BC_{1P2} populations in the TGA x PI 314806 cross, the TKB populations also had a smaller difference between the F_1 and the BC_{1P2} population than between the F_1 and the BC_{1P1} population. However, unlike the TGA cross, the mean of F_1 was significantly different than the mean of P_2, but not different than BC_{1P2} in LSD analysis. Therefore dominance effects in the direction of PI 314806 are not observed with the present data. However, the F_1 is significantly different than BC_{1P1}, suggesting the presence of both additive x dominance and dominance x dominance effects. Additionally, the only gene effects found to be significant at the 5% significance level within this population were for hue angle.

('Butterbush' x 'Sucrine DuBerry') (*C. moschata*) populations

All color space value means (m) were found to be significantly different than zero, for all epistatic gene effect estimate calculations (P = 0.00) (Table 3-15), with the exception of hue angle. Dominance x dominance interaction was found to be significant (P = 0.00) for color space value L* in the direction of the darker parent, SDub. Differences from F_1 to BC_{1P2} and from F_1 to BC_{1P1} means were almost equal (1.1 and 1.0 units, respectively) (Table 3-8), and the F_1 mean was significantly different than both BC population means. However, the mean of the F_1, is higher than BB in both 2008 and 2009, and the means of the F_2 and BC_{1P1} generations, are higher than BB in each respective year. Therefore the present data do not provide evidence of a dominance effect in the direction of SDub.

Color space value b* had significant dominance x dominance gene effects at the 10% significance level (*P* = 0.08) (Table 3-15). The gene effect in b* was in the direction of the less yellow and less vivid parent, SDub. Distance from the F_1 to BC_{1P1} mean in b* was 2.1 units, and distance from the F_1 to BC_{1P2} mean was 4.3 units (Table 3-8). However, all genotypic means were significantly different from one another in LSD analysis within the backcross populations, and BC1P1 was closer to BB (4.5 units) than BC1P2 was to SDub (5.9 units). The data does not support dominance x dominance interaction.

No significant gene interactions were noted for color space value chroma. Hue angle had significant dominance x dominance gene effects (*P* = 0.00) in the direction of the parent with the lower hue angle, SDub (Table 3-18). A greater difference was observed between means of the F_1 and BC_{1P1} population (4.0 units) than between the F_1 and BC_{1P2} population (0.3 units). Additionally, the mean of the F_1 was significantly different from BC_{1P1}, but not different from BC_{1P2} or parent 2, SDub, as determined by LSD analysis. These results support a dominance gene effect in the direction of SDub.

Overall, only dominance x dominace gene effects were found to be significant within this population. Additionally, the type of epistatic interaction could not be determined for the BB cross, but could be determined for Hue angle in both the TGA and TKB crosses. Interaction type is determined by comparing signs of dominance (d) and dominance x dominance (dd) interactions; like signs indicate complementary epistasis while unlike signs indicate duplicate epistasis (Kearsey and Pooni, 1996). It is also a requirement that both dominance (d) and dominance x dominance (dd) interactions must be significant to determine epistasis type, which was only met in the

TGA and TKB crosses. For both crosses, duplicate epistasis was observed for hue angle, at the 10% significance level in the TGA cross and at the 5% significance level in the TKB cross (Tables 3-14 and 3-16).

Breeding for Increased Flesh Color in Winter Squash

Transgressive segregation was examined using LSD analysis for fruit(s) with the highest average replicate measurements for a*, b*, and chroma color space values for each plant of F_2 and BC_1 generations of all three populations. Transgressive segregation was examined only for these color space values due to the strength of correlations observed between these color space values and carotenoid content (Chapter 2, Table 2-3). No transgressive segregation was observed for color space values a*, b*, and chroma for the TGA populations examined. TGA had the highest of the aforementioned color space values among both F_2 and BC_1 populations derived from the genotype. This lack of transgressive segregation could be a reflection of limited homogenous data collected for each genotype, the necessity for a larger number of individuals within the population, or the relatedness of the parents. These data suggest that individuals within these populations cannot be used to exceed the values of their respective parental generations, in either color or carotenoid content. However, reported correlations between L* a* b* color space values and carotenoid levels could be strengthened by keeping replications per location separate and examining more samples with HPLC analysis. Correlation between color and carotenoid content could then be re-examined. Strengthening correlations may provide additional color space values to examine for transgressive segregants as they relate to carotenoid content.

Transgressive segregation was observed for color space values a* in the BC_{1P1} population of the TKB cross. Additionally, transgressive segregation was observed in

the BB cross for color space values a* and b* in the F_2 population, and for color space values a* and b* in the BC_{1P1} population. By selecting these individuals that are significantly higher than the parent 1 means (TKB and BB, respectively), it is possible to rapidly improve color and thereby carotenoid content in squash through plant breeding.

Although there was an absence of transgressive genotypes in the TGA and TKB populations for color space values b* and chroma, there is the potential to shift population means in the direction of the highest value parent (TGA and TKB, respectively) in all populations for these color space values. This is illustrated by the additive gene effects of these traits and distribution of generation means. In addition, high broad-sense heritabilities for these color space values for both populations suggest breeding for color as it relates to nutritional content can be done successfully.

Transgressive segregation was observed for color space values b* and chroma for the F_2 population and a and b* for the BC_{1P1} population of the BB cross. Data suggest that populations within this cross have the highest potential for genetic improvement for these three color space values. However, of the three crosses, the BB cross has the lowest heritabilities for these three color space values. This indicates that while the potential for improvement is there, it would be the most difficult of the three crosses to breed.

To obtain more accurate heritability estimates, more measurements per genotype could be used if fruit maturity was controlled. Additionally, beta-carotene levels in some muskmelon cultivars have been shown to be influenced by soil type in different locations (Lester and Eischen, 1995). This suggests that other carotenoids controlling color in cucurbit crops may also be affected by different environments. Planting F_2 and BC

generations in either multiple years and/or multiple locations may enable partitioning out the environmental variation and aid in providing better estimates of heritability and epistatic gene effects.

Table 3-1. Harvest outline for all populations in two *Cucurbita pepo* and one *Cucurbita moschata* families in 2008 and 2009.

Genotype	Plant Number[a]	Fruit Number[b]	Harvest after Planting (weeks)
Table Gold Acorn (TGA)	8(9)	24(27)	10
PI 314806	5(7)	15(19)	10
F_1 (TGA x PI 314806)	8(10)	24(30)	10
F_2 (TGA x PI 314806)	96	276	10
BC_{1P1} (TGA x F_1)	74	210	10
BC_{1P2} (F_1 x PI 314806)	91	260	10
Table King Bush (TKB)	8(10)	22(26)	11
PI 314806	6(7)	16(18)	11
F_1 (TKB x PI 314806)	8(11)	22(32)	11
F_2 (TKB x PI 314806)	91	260	11
BC_{1P1} (F_1 x TKB)	75	219	11
BC_{1P2} (F_1 x PI 314806)	84	246	11
Butterbush (BB)	8(12)	21(36)	12
Sucrine DuBerry (SDub)	8(11)	23(29)	12
F_1 (BB x SDub)	8(10)	23(30)	12
F_2 (BB x SDub)	86	249	12
BC_{1P1} (F_1 x BB)	67	201	12
BC_{1P2} (F_1 x SDub)	88	232	12

[a] Number proceeding parentheses indicates P_1, P_2, and F_1 plants from which fruit were harvested in the 2008 season. Number in parentheses indicates P_1, P_2, and F_1 plants from which fruit were harvested in the 2009 season; this number is a combination of plants within both BC_{1P1} and BC_{1P2} populations.

[b] Number proceeding parentheses indicates P_1, P_2, and F_1 fruit harvested from respective plants in the 2008 season. Number in parentheses indicates P_1, P_2, and F_1 fruit harvested from respective plants in the 2009 season; this number is from the combination of plants within both BC_{1P1} and BC_{1P2} populations.

Table 3-2. Examination of fixed effects due to genotypes and expected mean squares due to random effects in three populations of *Cucurbita pepo* derived from 'Table Gold Acorn' (TGA) x PI 314806.

Sources	Df	L*	a*	b*	Chroma	Hue
F_2(TGA x PI 314806)						
		Test of fixed effects (F values)				
Genotype [a]	95	3.08***	14.26***	49.76***	50.35***	12.46***
		Expected mean squares				
Fruit(Genotype)[b]	178	15.51***	0.63***	11.63***	11.50***	6.27***
Rep(Fruit)[b]	6	3.87	0.45	2.86	3.12	2.89*
Residual	546	2.55	0.31	2.53	2.65	1.20
BC_{1P1} (TGA x F_1)						
		Test of fixed effects (F values)				
Genotype [a]	70	6.34***	24.31***	42.57***	42.50***	33.25***
		Expected mean squares				
Fruit(Genotype)[b]	137	17.13***	6.49***	52.13***	52.17***	11.15***
Rep(Fruit)[b]	6	2.34*	3.72	70.53*	69.19*	9.58
Residual	414	3.91	3.20	25.21	25.18	4.86
BC_{1P2} (F_1 x PI 314806)						
		Test of fixed effects (F values)				
Genotype [a]	90	1.84***	5.15***	4.56***	4.57***	4.93***
		Expected mean squares				
Fruit(Genotype)[b]	167	11.44	0.24***	5.39***	5.37***	3.95***
Rep(Fruit)[b]	6	21.82	0.2183*	3.88**	3.98**	1.23*
Residual	514	3.44	43.96	1.28	1.32	0.62

[a]Residual fixed effects: MS(Fruit(Genotype)).
[b]Residual: MS(Residual).
*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the ≤0.001 probability level.

Table 3-3. Examination of fixed effects due to genotypes and expected mean squares due to random effects in three populations of Cucurbita pepo derived from 'Table King Bush' (TKB) x PI 314806.

Sources	Df	L*	a*	b*	Chroma	Hue
F_2(TKB x PI 314806)						
		Test of fixed effects (F values)				
Genotype [a]	90	7.47***	11.38***	39.77***	39.68***	18.50***
		Expected mean squares				
Fruit(Genotype)[b]	167	10.40***	0.66	11.36***	11.26***	5.15***
Rep(Fruit)[b]	6	2.52	0.20	2.19	2.29	0.86
Residual	514	3.44	0.13	2.60	2.63	0.88
BC_{1P1} (F_1 x TKB)						
		Test of fixed effects (F values)				
Genotype [a]	74	4.47***	6.17***	63.31***	63.27***	16.40***
		Expected mean squares				
Fruit(Genotype)[b]	142	10.50***	1.98***	19.03***	18.79***	5.60***
Rep(Fruit)[b]	6	3.54	0.17	3.48	3.52	0.39
Residual	432	4.14	0.39	5.95	5.78	1.01
BC_{1P2} (F_1 x PI 314806)						
		Test of fixed effects (F values)				
Genotype [a]	83	21.33***	4.09***	3.98***	3.96***	4.48***
		Expected mean squares				
Fruit(Genotype)[b]	160	10.75***	0.28***	3.25***	3.31***	2.81***
Rep(Fruit)[b]	6	9.89*	0.54***	4.10*	3.93*	12.55***
Residual	486	3.64	0.11	1.80	1.81	1.87

[a]Residual fixed effects: MS(Fruit(Genotype)).
[b]Residual: MS(Residual).
*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the ≤0.001 probability level.

Table 3-4. Examination of fixed effects due to genotypes and expected mean squares due to random effects in three populations of Cucurbita pepo derived from 'Butterbush' (BB) x 'Sucrine DuBerry' (SDub).

Sources	Df	L*	a*	b*	Chroma	Hue
F_2(BB x SDub)						
		Test of fixed effects (F values)				
Genotype [a]	85	10.43***	11.48***	9.07***	9.07***	11.10***
		Expected mean squares				
Fruit(Genotype)[b]	161	4.42***	9.39***	16.78***	17.23***	7.58***
Rep(Fruit)[b]	6	1.94	5.88***	5.77	3.48	5.90***
Residual	492	1.63	1.31	3.00	2.75	1.17
BC_{1P1} (F_1 x BB)						
		Test of fixed effects (F values)				
Genotype [a]	67	4.56***	9.68***	10.65***	11.50***	9.41***
		*Expected mean squares15.19***				
Fruit(Genotype)[b]	134	6.22***	14.84***	15.05***	15.19***	11.52***
Rep(Fruit)[b]	28	1.57*	2.61**	2.94	3.40	1.99*
Residual	402	1.02	1.42	2.56	2.52	1.13
BC_{1P2} (F_1 x SDub)						
		Test of fixed effects (F values)				
Genotype [a]		4.91***	6.79***	10.12***	11.40***	5.46***
		Expected mean squares				
Fruit(Genotype)[b]		5.99***	18.42***	18.27***	6.27***	15.44
Rep(Fruit)[b]		1.59	3.16***	4.34	1.28	2.90**
Residual		1.52	1.57	3.31	3.04	1.58

[a]Residual fixed effects: MS(Fruit(Genotype)).
[b]Residual: MS(Residual).
*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the ≤0.001 probability level.

Table 3-5. Means, standard deviations (SD), and ranges of colorimetric values (L*, a*, b*, chroma and hue)[a] of individuals from measured in two *Cucurbita pepo* and one *Cucurbita moschata* families.

		L*		a*		b*	
Genotype[b]	n	Mean[c] (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Table Gold Acorn (TGA)	8(24)[d]	76.7 (2.7)	66.0 – 82.0	8.3 (2.7)	0.9 – 15.7	61.8 (7.3)	44.4 – 74.6
PI 314806	5(15)	82.7 (2.3)	77.8 – 87.7	-2.0 (0.3)	-2.4 – -1.4	8.9 (1.3)	6.7 – 13.2
F_1 (TGA x PI 314806)	8(24)	80.5 (1.8)	76.9 – 84.5	-2.8 (0.5)	-4.3 – -1.6	12.3 (2.1)	9.3 – 17.0
F_2 (TGA x PI 314806)	96(276)	81.3 (3.3)	69.4 – 88.8	-2.8 (1.2)	-8.7 – -0.4	17.1 (8.4)	5.1 – 44.7
Table King Bush (TKB)	8(22)	80.4 (2.3)	75.8 – 85.1	-0.6 (1.7)	-3.0 – 3.6	49.4 (5.1)	40.3 – 60.1
PI 314806	6(16)	83.7 (2.0)	78.1 – 87.4	-2.1 (0.3)	-2.8 – -1.5	9.1 (1.7)	6.8 – 13.9
F_1 (TKB x PI 314806)	8(22)	81.3 (3.0)	73.9 – 87.2	-2.4 (0.4)	-3.4 – -1.7	12.1 (2.1)	7.4 – 18.0
F_2 (TKB x PI 314806)	91(260)	81.4 (3.7)	68.6 – 90.6	-2.7 (1.1)	-7.1 – 0.9	18.9 (7.5)	7.1 – 43.6
Butterbush (BB)	8(21)	67.4(3.3)	59.2 – 73.2	22.4(3.6)	15.0 – 30.9	66.9(3.4)	58.4 – 75.2
Sucrine DuBerry (SDub)	8(23)	65.1(2.4)	61.3 – 72.3	18.6(4.3)	6.5 – 24.2	54.2(3.4)	47.1 – 64.5
F_1 (BB x SDub)	8(23)	68.9(2.1)	65.4 – 75.0	18.4(4.3)	2.7 – 22.3	61.5(3.8)	50.5 – 72.3
F_2 (BB x SDub)	86(249)	68.3(2.7)	60.3 – 78.2	18.1(3.9)	-1.2 – 28.4	62.1(4.8)	49.7 – 77.8

Table 3-5. Continued

		Chroma		Hue	
Genotype[b]	n	Mean (SD)	Range	Mean (SD)	Range
Table Gold Acorn (TGA)	8(24)[d]	62.4 (7.5)	44.4 – 75.6	82.6 (2.4)	76.5 – 88.8
PI 314806	5(15)	9.1 (1.3)	6.9 – 13.4	102.7 (1.5)	99.9 – 105.1
F_1 (TGA x PI 314806)	8(24)	12.7 (2.1)	9.5 – 17.5	103.1 (1.7)	96.7 – 106.7
F_2 (TGA x PI 314806)	96(276)	17.4 (8.4)	5.2 – 44.7	100.2 (3.3)	90.7 – 110.7
Table King Bush (TKB)	8(22)	49.4 (5.1)	40.3 – 60.2	90.9 (1.9)	86.5 – 93.9
PI 314806	6(16)	9.3 (1.7)	7.0 – 14.1	103.4 (1.8)	99.6 – 106.6
F_1 (TKB x PI 314806)	8(22)	12.4 (2.0)	7.6 – 18.1	101.6 (2.3)	95.4 – 104.9
F_2 (TKB x PI 314806)	91(260)	19.0 (7.5)	7.3 – 43.6	99.1 (3.6)	88.7 – 108.5
Butterbush (BB)	8(21)	70.1(2.6)	63.2 – 76.8	71.5(3.4)	64.2- 78.7
Sucrine DuBerry (SDub)	8(23)	57.5(3.6)	50.5 – 67.2	71.1(4.2)	66.7 – 83.1
F_1 (BB x SDub)	8(23)	64.3(4.3)	50.7 – 75.1	73.5(3.6)	70.5 – 86.8
F_2 (BB x SDub)	86(249)	64.8(4.8)	50.2 – 79.8	73.8(3.5)	66.3 – 91.3

[a]Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.
[b]*C. pepo* families include TGA x PI 314806, and TKB x PI 314806. BB x SDub is the *C. moschata* family.
[c]Values shown are based on 3 colorimetric measurements per fruit.
[d]Sample value ‘n’ preceding parentheses is plant number. Value within parentheses is fruit number collected from ‘n’ plants.

Table 3-6. Means, standard deviations (SD), and ranges of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Table Gold Acorn' (TGA) x PI 314806 (*Cucurbita pepo*) BC populations.

		L*		a*		b*	
Genotype	n	Mean[b] (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Table Gold Acorn (TGA)	9(27)[c]	72.9(3.6)	61.4 – 80.5	11.1(4.0)	-0.2 – 19.5	68.5(5.4)	54.7 – 79.1
PI 314806	7(19)	81.9(2.8)	74.0 – 87.2	-2.5(0.5)	-4.2 – -1.9	11.3(1.9)	7.8 – 16.7
F_1 (TGA x PI 314806)	10(30)	80.3(2.4)	73.2 – 86.9	-3.5(0.6)	-5.8 – -2.2	15.4(2.0)	11.6 – 21.0
BC_{1P1} (TGA x F_1)	74(210)	79.4(4.3)	66.2 – 87.7	-1.9(4.6)	-11.7 – 15.2	37.2(16.6)	9.8 – 77.0
BC_{1P2} (F_1 x PI 314806)	91(260)	80.9(2.7)	7.7 – 87.2	-2.9(0.5)	-5.0 – -1.5	14.6(2.2)	8.2 – 21.3

Table 3-6. Continued

		Chroma		Hue	
Genotype	n	Mean[b] (SD)	Range	Mean (SD)	Range
Table Gold Acorn (TGA)	9(27)[c]	69.4(5.6)	53.7 – 80.8	80.9(3.1)	74.5 – 90.2
PI 314806	7(19)	11.6(1.9)	8.1 – 17.2	102.5(1.4)	99.2 – 105.1
F_1 (TGA x PI 314806)	10(30)	15.8(2.0)	11.9 – 21.8	102.8(2.1)	98.0 – 107.1
BC_{1P1} (TGA x F_1)	74(210)	37.5(16.6)	10.1 – 78.4	95.4(6.9)	77.9 – 109.5
BC_{1P2} (F_1 x PI 314806)	91(260)	14.9(2.2)	8.5 – 21.5	101.3(1.9)	96.0 – 108.0

[a]Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.
[b]Values shown are based on 3 colorimetric measurements per fruit.
[c]Sample value ‘n’ preceding parentheses is plant number. Value within parentheses is fruit number collected from ‘n’ plants.

Table 3-7. Means, standard deviations (SD), and ranges of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Table King Bush' (TKB) x PI 314806 (*Cucurbita pepo*) BC populations.

		L*		a*		b*	
Genotype	n	Mean[b] (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Table King Bush (TKB)	10(26)	80.5(2.3)	72.9 – 83.8	-0.4(1.6)	-3.5 – 6.3	53.1(3.6)	44.8 – 61.9
PI 314806	7(18)	81.5(2.6)	-3.7 – -1.6	-2.4(0.5)	-3.8 – -1.6	11.2(1.4)	8.6 – 14.7
F_1 (TKB x PI 314806)	11(32)	80.7(2.8)	73.3 – 86.9	-2.9(0.5)	-5.2 – -1.7	14.9(1.6)	12.0 – 18.8
BC_{1P1} (F_1 x TKB)	75(219)	81.2(3.2)	68.5 – 89.5	-3.2(1.4)	-7.6 – 3.8	32.7(12)	11.1 – 59.7
BC_{1P2} (F_1 x PI 314806)	84(246)	80.3(2.7)	70.6 – 88.2	-2.5(0.5)	-5.9 – -1.0	13.2(1.8)	8.8 – 20.5

Table 3-7. Continued

Genotype	n	Chroma		Hue	
		Mean[b] (SD)	Range	Mean (SD)	Range
Table King Bush (TKB)	10(26)	53.1(3.6)	44.9 – 61.7	90.5(1.7)	84.1 – 94.0
PI 314806	7(18)	11.4(1.5)	8.8 – 15.0	102.3(1.3)	99.4 – 105.2
F_1 (TKB x PI 314806)	11(32)	15.2(1.6)	12.2 – 19.5	100.9(1.6)	95.8 – 105.3
BC_{1P1} (F_1 x TKB)	75(219)	33.1(11.9)	11.3 – 59.7	96.6(3.5)	85.5 – 105.4
BC_{1P2} (F_1 x PI 314806)	84(246)	13.4(1.9)	9.1 – 21.3	100.8(1.8)	93.7 – 106.0

[a]Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.
[b]Values shown are based on 3 colorimetric measurements per fruit.
[c]Sample value ‘n’ preceding parentheses is plant number. Value within parentheses is fruit number collected from ‘n’ plants.

Table 3-8. Means, standard deviations (SD), and ranges of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Butterbush' (BB) x 'Sucrine DuBerry' (SDub) (*Cucurbita moschata*) BC populations.

		L*		a*		b*	
Genotype	n	Mean[b] (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Butterbush (BB)	12(36)	69.6(2.2)	64.9 – 76.0	16.8(4.5)	2.8 – 26.4	69.0(3.4)	59.1 – 77.7
Sucrine DuBerry (SDub)	11(29)	66.9(1.9)	63.6 – 72.4	14.5(4.5)	5.0 – 21.8	53.2(4.1)	38.7 – 60.4
F_1 (BB x SDub)	10(30)	69.8(1.9)	65.4 – 74.0	16.6(3.2)	9.3 – 25.2	63.4(3.1)	56.7 – 69.8
BC_{1P1} (F_1 x BB)	67(201)	70.8(2.3)	64.6 – 80.0	12.5(4.5)	-5.5 – 22.9	65.5(4.4)	43.3 – 76.1
BC_{1P2} (F_1 x SDub)	88(232)	68.7(2.4)	62.0 – 77.1	15.2(4.3)	1.2 – 24.1	59.1(4.6)	42.8 – 71.0

Table 3-8. Continued

		Chroma		Hue	
Genotype	n	Mean[b] (SD)	Range	Mean (SD)	Range
Butterbush (BB)	12(36)	71.2(3.4)	59.2 – 79.8	76.3(3.7)	68.4 – 87.3
Sucrine DuBerry (SDub)	11(29)	55.3(4.6)	41.6 – 63.4	74.9(4.2)	68.4 – 84.1
F_1 (BB x SDub)	10(30)	65.6(3.3)	58.1 – 72.5	75.4(2.5)	69.0 – 81.5
BC_{1P1} (F_1 x BB)	67(201)	66.8(4.7)	43.4 – 77.0	79.4(3.9)	70.3 – 96.7
BC_{1P2} (F_1 x SDub)	88(232)	61.2(5.0)	43.4 – 72.4	75.7(3.7)	68.0 – 88.6

[a]Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.
[b]Values shown are based on 3 colorimetric measurements per fruit.
[c]Sample value ‘n’ preceding parentheses is plant number. Value within parentheses is fruit number collected from ‘n’ plants.

Table 3-9. Expected mean squares due to random effects in respective parent 1, parent 2, and F1 of three Cucurbita populations resulting from C. pepo 'Table Gold Acorn' (TGA) x PI 314806, C. pepo 'Table King Bush' (TKB) x PI 314806, and C. moschata 'Butterbush' (BB) x 'Sucrine DuBerry' (SDub) over 2008 and 2009.

Sources	Df	L*	a*	b*	Chroma	Hue
		Expected mean squares				
Year [a]	1	34.57	420.79	1733.45**	1320.57*	172.73
Genotype [b]	8	5501.41***	12274***	81560***	86997***	22719***
Genotype x Year[c]	8	116.77***	189.77***	132.44***	215.85***	164.88***
Fruit(Genotype)[c]	203	13.52***	22.04***	24.62***	26.61***	21.33***
Rep(Fruit)[c]	72	2.40	2.00	5.50	5.73	1.62
Residual	983	4.89	5.04	9.98	10.51	4.51

[a]Residual(Year): 0.9761*MS(Year*Genotype)+0.0239*MS(Residual).
[b]Residual(Genotype): 0.9964*MS(Year*Genotype)+0.9012*MS(Fruit(Genotype))-0.8976*MS(Residual).
[c]Residual: MS(Residual).
*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.
***Significant at the ≤0.001 probability level.

Table 3-10. Genetic means estimates for heritability of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Table Gold Acorn' (TGA) x PI 314806 (*Cucurbita pepo*) populations with one fruit per genotype[b].

Genetic parameter[c]	L*	±SE	a*	±SE	b*	±SE	Chroma	±SE	Hue[d]	±SE
σ^2_A	0.00	---	0.00	---	0.00	---	0.00	---	0.0000	---
σ^2_P	7.93	2.82	0.98	0.99	77.29	8.79	77.11	8.78	0.0036	0.0596
σ^2_E	1.45	0.53	0.67	0.29	5.69	1.82	5.79	1.86	0.0005	0.0001
σ^2_G	6.48	1.25	0.32	0.32	71.60	11.19	71.32	11.17	0.0031	0.0005
H	0.82	0.16	0.32	0.32	0.93	0.14	0.92	0.14	0.86	0.15
h^2	0.00	---	0.00	---	0.00	---	0.00	---	0.00	---

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.

[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.

[c] σ^2_A, σ^2_P, σ^2_E, σ^2_G, H, h^2 are additive variance = $2\sigma^2_{F2} - (\sigma^2_{BC1P1} + \sigma^2_{BC1P2})$, phenotypic variance = σ^2_{F2}, environmental variance = $(1/2)\sigma^2_{F1} + (1/4)\sigma^2_{P1} + (1/4)\sigma^2_{P2}$, genotypic variance, broad-sense heritability, narrow-sense heritability estimate = σ^2_A / σ^2_P, respectively.

[d] Values converted from degrees to radians for calculation.

Table 3-11. Variance estimates for calculating heritability of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Table Gold Acorn' (TGA) x PI 314806 (*Cucurbita pepo*) populations with one fruit per genotype[b].

Variance estimate	L*	a*	b*	Chroma	Hue[c]
σ^2_{P1}	4.56	2.55	14.51	14.74	0.0005
σ^2_{P2}	0.13	0.01	0.90	0.84	0.0003
σ^2_{F1}	0.56	0.05	3.68	3.79	0.0006
σ^2_{F2}	7.93	0.98	77.29	77.11	0.0036
σ^2_{BC1P1}	13.55	22.88	285.79	285.62	0.0869
σ^2_{BC1P2}	3.55	0.17	3.46	3.42	0.0009

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.

[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.

[c] Values converted from degrees to radians for calculation.

Table 3-12. Genetic means estimates for heritability of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Table King Bush' (TKB) x PI 314806 (*Cucurbita pepo*) populations with one fruit per genotype[b].

Genetic parameter[c]	L*	±SE	a*	±SE	b*	±SE	Chroma	±SE	Hue[d]	±SE
σ^2_A	13.21	3.50	0.00	---	0.00	---	0.00	---	0.0036	0.0013
σ^2_P	11.33	3.37	1.04	1.02	60.97	7.81	60.14	7.75	0.0039	0.0628
σ^2_E	4.59	1.44	0.92	0.39	7.15	2.56	7.11	2.57	0.0014	0.0004
σ^2_G	6.74	2.20	0.12	0.42	53.82	9.30	53.03	9.19	0.0026	0.0007
H	0.59	0.19	0.12	0.40	0.88	0.15	0.88	0.15	0.66	0.18
h^2	1.00	---	0.00	---	0.00	---	0.00	---	0.92	0.00

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.

[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.

[c] σ^2_A, σ^2_P, σ^2_E, σ^2_G, H, h^2 are additive variance = $2\sigma^2_{F2} - (\sigma^2_{BC1P1} + \sigma^2_{BC1P2})$, phenotypic variance = σ^2_{F2}, environmental variance = $(1/2)\sigma^2_{F1} + (1/4)\sigma^2_{P1} + (1/4)\sigma^2_{P2}$, genotypic variance, broad-sense heritability, narrow-sense heritability estimate = σ^2_A / σ^2_P, respectively.

[d] Values converted from degrees to radians for calculation.

Table 3-13. Variance estimates for calculating heritability of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Table King Bush' (TKB) x PI 314806 (*Cucurbita pepo*) populations with one fruit per genotype[b].

Variance estimate	L*	a*	b*	Chroma	Hue[c]
σ^2_{P1}	3.83	3.49	22.34	22.50	0.0012
σ^2_{P2}	2.57	0.03	2.01	2.01	0.0006
σ^2_{F1}	5.98	0.08	2.13	1.96	0.0018
σ^2_{F2}	11.33	1.04	60.97	60.14	0.0039
σ^2_{BC1P1}	6.00	1.99	148.60	146.15	0.0036
σ^2_{BC1P2}	3.45	0.11	2.00	2.05	0.0006

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.

[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.

[c] Values converted from degrees to radians for calculation.

Table 3-14. Genetic means estimates for heritability of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Butterbush' (BB) x 'Sucrine DuBerry' (SDub) (*Cucurbita moschata*) populations with one fruit per genotype[b]

Genetic parameter[c]	L*	±SE	a*	±SE	b*	±SE	Chroma	±SE	Hue	±SE
σ^2_A	4.83	1.92	0.00	---	17.09	7.47	13.46	7.64	0.0000	---
σ^2_P	5.84	2.42	13.06	3.61	22.48	4.74	22.48	4.74	0.0029	0.0540
σ^2_E	4.76	1.39	20.40	6.15	13.44	3.80	14.37	4.75	0.0048	0.0014
σ^2_G	1.08	1.64	0.00	---	9.05	5.09	8.12	5.84	0.0000	---
H	0.19	0.28	0.00	---	0.40	0.23	0.36	0.26	0.00	---
h^2	0.83	0.33	0.00	---	0.76	0.33	0.60	0.34	0.00	---

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.

[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.

[c] σ^2_A, σ^2_P, σ^2_E, σ^2_G, H, h^2 are additive variance = $2\sigma^2_{F2} - (\sigma^2_{BC1P1} + \sigma^2_{BC1P2})$, phenotypic variance = σ^2_{F2}, environmental variance = $(1/2)\sigma^2_{F1} + (1/4)\sigma^2_{P1} + (1/4)\sigma^2_{P2}$, genotypic variance, broad-sense heritability, narrow-sense heritability estimate = σ^2_A / σ^2_P, respectively.

Table 3-15. Variance estimates for calculating heritability of colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Butterbush' (BB) x 'Sucrine DuBerry' (SDub) (*Cucurbita moschata*) populations with one fruit per genotype[b].

Variance estimate	L*	a*	b*	Chroma	Hue[c]
σ^2_{P1}	11.04	12.13	12.94	4.93	0.0027
σ^2_{P2}	3.99	19.53	11.57	12.08	0.0060
σ^2_{F1}	2.00	24.97	14.62	20.23	0.0053
σ^2_{F2}	5.84	13.06	22.48	22.48	0.0029
σ^2_{BC1P1}	2.96	16.29	12.48	13.34	0.0035
σ^2_{BC1P2}	3.89	13.82	15.40	18.16	0.0029

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.

[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.

[c] Values converted from degrees to radians for calculation.

Table 3-16. Epistatic gene effect estimates for colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Table Gold Acorn' (TGA) x PI 314806 (*Cucurbita pepo*) populations with one fruit per genotype[b].

Gene effect[c]	L*	±SE	*P*-value	a*	±SE	*P*-value	b*	±SE	*P*-value
m	79.54	2.82	0.00	-2.48	0.99	0.01	18.47	8.79	0.04
a	-1.88	4.14	0.65	1.91	4.80	0.69	24.78	17.01	0.15
d	-3.85	14.04	0.78	-3.36	10.42	0.75	13.73	49.00	0.78
aa	-3.06	8.80	0.73	3.51	9.64	0.72	59.77	34.87	0.09
ad	0.79	4.27	0.85	-3.91	4.87	0.42	-2.68	17.12	0.88
dd	5.96	20.19	0.77	6.44	19.68	0.74	-49.17	76.78	0.52

Table 3-16. Continued

Gene effect[c]	Chroma	±SE	*P*-value	Hue	±SE	*P*-value
m	18.74	8.78	0.04	1.74	3.42	0.61
a	24.81	17.00	0.15	-1.04	0.30	0.00
d	13.77	48.97	0.78	-1.05	0.64	0.10
aa	59.58	34.88	0.09	-2.21	0.60	0.00
ad	-2.87	17.12	0.87	-1.66	0.30	0.00
dd	-48.90	76.74	0.53	2.27	1.21	0.06

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.
[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.
[c] Gene effects were calculated using calculations described by Hayman (1958) and Gamble (1962).

Table 3-17. Epistatic gene effect estimates for colorimetric values (L*, a*, b*, chroma and hue)[a] measured in 'Table King Bush' (TKB) x PI 314806 (*Cucurbita pepo*) populations with one fruit per genotype[b].

Gene effect[c]	L*	±SE	*P*-value	a*	±SE	*P*-value	b*	±SE	*P*-value
m	79.87	3.37	0.00	-2.39	1.02	0.02	20.08	7.81	0.01
a	0.84	3.07	0.79	-0.39	1.45	0.79	21.13	12.27	0.09
d	-4.00	15.06	0.79	-1.55	5.10	0.76	0.67	39.83	0.99
aa	0.49	11.55	0.97	-0.95	3.11	0.76	45.90	25.23	0.07
ad	2.39	3.32	0.47	-1.39	1.73	0.43	0.40	12.52	0.97
dd	5.04	19.05	0.79	3.74	7.36	0.61	-29.35	58.46	0.62

Table 3-17. Continued

Gene effect[c]	Chroma	±SE	*P*-value	Hue	±SE	*P*-value
m	20.32	7.75	0.01	1.71	3.60	0.63
a	21.09	12.17	0.09	-0.08	0.07	0.24
d	0.70	39.54	0.99	0.86	0.29	0.00
aa	45.75	24.98	0.07	-0.24	0.21	0.26
ad	0.47	12.42	0.97	-0.78	0.07	0.00
dd	-29.35	58.02	0.61	-1.52	0.37	0.00

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.
[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.
[c] Gene effects were calculated using calculations described by Hayman (1958) and Gamble (1962).

Table 3-18. Epistatic gene effect estimates for colorimetric values (L*, a*, b*, chroma and hue)[a] measured in measured in 'Butterbush' (BB) x 'Sucrine DuBerry' (*Cucurbita moschata*) populations with one fruit per genotype[b].

Gene effect[c]	L*	±SE	*P*-value	a*	±SE	*P*-value	b*	±SE	*P*-value
m	67.41	2.42	0.00	19.12	3.61	0.00	63.68	4.74	0.00
a	1.93	2.62	0.46	-2.40	5.49	0.66	6.11	5.28	0.25
d	7.54	11.25	0.50	-17.29	19.03	0.37	2.24	22.18	0.92
aa	3.55	7.71	0.65	-12.52	22.80	0.58	4.84	18.59	0.80
ad	1.06	3.26	0.75	-4.89	6.17	0.43	-0.10	5.83	0.99
dd	-73.99	15.04	0.00	33.18	28.67	0.25	-52.17	29.81	0.08

Table 3-18. Continued

Gene effect[c]	Chroma	±SE	*P*-value	Hue	±SE	*P*-value
m	66.37	4.74	0.00	1.2704	3.0929	0.68
a	5.32	5.61	0.35	0.0603	0.0804	0.46
d	-2.52	22.59	0.91	0.2708	0.2827	0.34
aa	0.23	21.20	0.99	0.1913	0.3328	0.57
ad	-1.14	5.98	0.85	0.0671	0.0930	0.47
dd	-44.87	31.01	0.15	-1.7409	0.4242	0.00

[a] Numeric description of color using L*a*b* CIELAB color space. L* (lightness) ranges from black (0) to white (100); a* intensity in red (a* > 0) or green (a* < 0); b* intensity in yellow (b* > 0) or blue (b* < 0). Chroma (saturation or vividness) - as chromaticity increases a color becomes more intense; as it decreases a color becomes more dull. Hue (tint of color) - an angular measurement where 0° = red, 45° = orange-red, and 90° = yellow.
[b] Fruit with overall highest a*, b* and chroma values were selected due to strong correlations with total carotenoids, lutein and lutein, respectively. Fruit with lowest L* and hue due correlation direction of all measured carotenoids.
[c] Gene effects were calculated using calculations described by Hayman (1958) and Gamble (1962).

CHAPTER 4
CONSTRUCTION OF A RAPD AND SSR-BASED GENETIC LINKAGE MAP AND QTL MAPPING OF L*A*B* COLOR SPACE VALUES IN WINTER SQUASH (*Cucurbita moschata*)

Introduction

Carotenoids in squash, such as α and β-carotene, serve as vitamin A (retinol) precursors that function in many body processes including vision, bone growth, cell division and differentiation, and reproduction (ODS/NIH, 2006). Carotenoid content in squash fruit, a main component of its nutritional value (Gross, 1991) can be indirectly increased through phenotypic selection for fruit with increased flesh pigmentation. The range of color in squash flesh is indicative of a quantitative trait, controlled by many genes, rather than a qualitative trait controlled by few genes (Chapter 3, Table 3-5). By marking the genomic regions involved in flesh color of squash fruit, plant breeders may use the information as a tool to more effectively select for an increase in color and thereby nutritional content.

Currently, linkage map development within the *Cucurbita* genus is limited; there exists only five genetic maps for the *Curcubita* genus to date. The first two maps constructed were of interspecific crosses between *C. moschata* and *C. pepo* and composed of random amplified polymorphic DNA (RAPD) markers. The first, used an F_2 population (Lee et al.,1995), while a BC_1 population was used for the second map that included morphological markers (Brown and Myers, 2002). The third map in *Cucurbita* was created in 2004 by Zraidi and Lelley in *C. pepo* using an F_2 population. It was created primarily with RAPD markers, but included simple sequence repeat (SSR) markers, morphological, and phenotypic characters. This map was expanded upon and compared to a second *C..pepo* map reported in 2007. Zraidi et al. (2007) used two F_2

populations, created from two intraspecific crosses, to create the first consensus map for *C. pepo* using RAPD, amplified fragment length polymorphism (AFLP), SSR, and sequence characterized amplified region (SCAR) markers.

More recently, due to the utility of SSR markers in mapping and diversity studies, and the limited number of SSRs available for *Cucurbita*, over 500 SSR primers polymorphic in *C. pepo*, *C. moschata*, and *C. ecuadorensis* were developed (Gong et al., 2008a). This group used these SSRs to update their first *C. pepo* map (Zraidi *et al*.,2007) and constructed a fourth published map in *Cucurbita* and the first SSR-based map in *C. pepo* in 2008. The fifth and most recent *Cucurbita* map was the first SSR-based map of *C. moschata* using an F_2 population, and examined its synteny with the most recent *C. pepo* map (Gong et al, 2008b).

The current limited availability of genetic maps within *Cucurbita* is due in large part to the limited funding available for research since crops within this genus are minor and do not rank among the most economically important vegetable crops. Currently within Cucurbitaceae, genetic mapping is most developed in cucumber (*Cucumis sativus*) and melon (*Cucumis melo*) (Paris, 2008). Of the two, *Cucumis sativus* has had more marker development including single nucleotide polymorphism (SNP) and expressed sequence tag (EST) marker systems (Staub, et al., 2008). Yet *Cucumis melo*, using SSR markers, is the most advanced genetic map and species in the Cucurbitaceae family, serving as the family's model system (Pitrat, 2008). Both *Cucumis sativus* and *Cucumis melo* have had numerous quantitative trait loci (QTLs) identified. Major areas of QTL identification in *Cucumis sativus* include fruit quality traits, disease resistance, and components of yield (Staub et al., 2008), while *Cucmis melo* QTL identification

similarly includes fruit quality and disease resistance, in addition to flower biology QTLs (Pitrat, 2008). To date, no QTLs have been identified within the *Cucurbita* genus.

The objectives of this experiment were to create a genetic linkage map in *C. moschata* that could be compared with existing *C. moschata* and *C. pepo* linkage maps (Gong et al, 2008b) and would have sufficient marker density to perform quantitative trait loci (QTL) studies to map regions of the genome associated with mesocarp coloration.

Materials and Methods

Plant Material

The U.S. cultivar 'Butterbush' (BB) and French heirloom variety 'Sucrine DuBerry' (SDub) were selected from germplasm screening for color and carotenoid correlation conducted 6 April - 15 June 2007 at the University of Florida Plant Science Research and Education Center (PSREC) in Citra, Fl. Selections were based on differences in subjective flesh color observations (see Chapter 2). BB was crossed with SDub in a greenhouse to produce F_1 seedlings, one of which was selected to self to create an F_2 population segregating for flesh color. Ninety F_2 individuals were grown in a completely randomized design with eight individuals each of BB, SDub and F_1 plants at the PSREC during 9 April – 3 July 2008. The plot was grown under fertility rates and cultural practices as outlined by Olson and Simonne (2007) for both field experiments conducted in 2007 and 2008.

DNA Extraction

Newly emerging disease and insect free true leaves from three week old plants were collected for DNA extraction from the field planting at the PSREC. Leaves were frozen and held at -80^{0}C prior to lyophilization using a Labconco freeze dryer (Kansas

City, MO) for 48 hours prior to extraction, for which a modified CTAB DNA extraction protocol was used (Kabelka and Young, 2010).

Marker Selection, Polymorphism Screening, and Genotyping

Molecular marker advancement within *C.moschata* has been limited, and randomly amplified polymorphic DNA (RAPD) markers have been the primary marker system used for detecting genetic differences. BB and SDub were screened against 1,200 RAPD primer combinations (Eurofins MWG Operon Technologies, U.S.) to identify those that exhibited polymorphism between the parents. Simple sequence repeat (SSR) markers are currently the most useful marker system available for identifying polymorphism below the species level in Cucurbitaceae (Lebeda et al., 2007). However, SSR markers identified from genomic sequences of *C. moschata,* and *C. pepo* have only recently become available (Gong et al., 2008a). As these SSR markers became available, BB and SDub were screened for polymorphism with 262 derived from *C. moschata* and 193 from *C. pepo* (455 total).

For RAPD PCR, the final reaction volume of the parental screens was 26 µl, and contained 5 µl of template DNA, 13.44 µl of DNA grade water (Fisher, Pittsburgh, PA), 2.5 µl of 10x PCR buffer, 2.0 µl of 25 mM $MgCl_2$ (Promega, Madison, WI), 2.0 µl of 2.5 µM dNTPs (from where?), 0.06 µl of *Taq* polymerase, and 1 µl 20 pm of forward and reverse primers. PCR amplification parameters used an initial denaturation step at 95°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 1 min, 43°C annealing for 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 5 min. All amplifications were run on an Eppendorf Mastercycler (Hauppauge, NY). For amplification of SSR markers, the PCR protocol for parental screens was changed to 33 cycles, and primer-specific annealing temperatures (Gong et al., 2008a) were used.

Additionally, for both the RAPD and SSR PCR protocols, the final PCR reaction volume was 25 µl for the F_2 progeny screens, with the amount of DNA grade water per reaction reduced from 13.44 to 12.44 µl.

RAPD PCR products were separated on a 1.5% agarose gel at 100 volts for 5 hours; SSR PCR products used a six percent polyacrylamide gel at 260 volts for two hours. Both gel types were run in 0.5 x (tris base, boric acid, EDTA) TBE buffer. For RAPD PCR products, 15uL of ethidum bromide (EtBr) was added to the agarose gel, while the acrylamide gels for SSR PCR products were stained with 50 µl ethidium bromide (EtBr) diluted in 200mL 0.5x TBE buffer for 25 minutes Gels of both marker-types were viewed under UV light and images of each gel were recorded in digital format.

Additionally, a subset of four SSR markers initially determined to be monomorphic by polyacrylamide gel electrophoresis, M120, M042, M009, and P098, were genotyped using high resolution melting (HRM) (see Chapter 5).

Linkage Map Construction and QTL Mapping Analyses

Polymorphic markers identified for both marker types in BB and SDub were used to genotype the entire F_2 population of 90 individuals, (Figure 4-1). All marker data were analyzed in JoinMap 3.0 (Ooijen and Voorrips, 2001) using Kosambi's mapping function with a LOD score threshold of 3.0 and REC threshold of 0.45. Skewed markers determined based on the Chi-square test in JoinMap, evaluated at a significance level of $P \leq 0.05$, were included in map calculations and are indicated on the map (Figure 4-2.)

Phenotypes for the color space values L* a* b* chroma and hue to be used for QTL mapping were obtained as outlined in Chapter 3. Due to highly significant variation

between fruit from the same genotype, the highest value measurement (genetic potential) for each of the color space values were used for QTL analysis. MapQTL 5.0 (Ooijen, 2004) was used to perform interval mapping (IM) and composite interval mapping (MQM), with a mapping step size of 1 cM, for each of the five color space values. Only MQM results are reported. Cofactors for MQM analyses were selected manually based upon both genome-wide and linkage group specific LOD score thresholds as determined by a 1,000 iteration permutation test, at $P \leq 0.05$. Cofactors were used to reduce residual variance of multiple segregating QTLs, thereby increasing the genetic effect of the associated map position and increasing the significance of the test. QTLs were determined significant when LOD values exceeded either the genome-wide or linkage group specific LOD score threshold.

Results and Discussion

Polymorphic Marker Detection

BB and SDub were screened against 1,200 RAPD primer combinations, 700 of which were found to amplify at least one polymorphic locus (Table 4-1). Both parents were subsequently re-screened for the 700 primer combinations for repeatability. Five hundred thirty-eight of the 700 primers (77%) were confirmed polymorphic and used for genotyping 90 F_2 individuals. SSR marker parental screens revealed a high level of monomorphism, with 177 identified from markers derived from *C. moschata* (68%) and 121 from markers derived from *C. pepo* (63%) being unusable in the mapping population. Of the 262 SSR markers derived from *C. moschata* genomic DNA, only 49 (19%) were polymorphic, with 36 (14%) that did not amplify, and 177 (68%) that were monomorphic. Of the 193 SSR markers derived from *C. pepo* genomic DNA, only 12 (6%) were polymorphic, with 60 (31%) that did not amplify, and 121 (63%) that were

monomorphic. In total, only 61 SSR markers had polymorphisms between the two parents and were able to be used for genotyping. The low level of polymorphism illustrates the high level of inbreeding present among *C. moschata* germplasm. Additionally, 46 of the 298 markers that were found to be monomorphic in the parents were also found on the SSR-based synteny map of *C. moschata* and *C. pepo* (Gong et al., 2008b).

Linkage Map Composition

A linkage map was constructed in *C. moschata* (2n=2x=40) from an F_2 population of 90 individuals derived from an intraspecific cross between BB and SDub (Figure 4-2). Three hundred nineteen RAPD and 39 SSR loci were used to construct the linkage map. The total SSR loci include four monomorphic *C. moschata* SSR loci that were recovered using HRM, and are included in the total number of SSR markers. Of these, 269 loci (235 RAPD and 34 SSR), were placed on the linkage map after analysis (Tables 4-1 and 4-2).

A total of 21 linkage groups were obtained (Table 4-2) with a total map length of 1086.9 cM, and averages of 51.8 cM per linkage group and 4.0 cM between markers (Table 4-3). The number of mapped linkage groups should equal the base chromosome number of 20 for *C. moschata*. It is likely that insufficient marker density in our population reduced our ability to identify the correct number of linkage groups. Only 75% of the marker loci genotyped could be placed on linkage groups (Table 4-1).

Cucurbita Map Comparisons

The first SSR-based linkage map for *C. moschata* was published by Gong et al. (2008a), and is currently the most current published map for the species. Within the same publication, Gong et al. (2008a) also examined the synteny of the SSR-based *C.*

moschata map with the most current and singular SSR-based *C. pepo* linkage map (Gong et al., 2008b). Comparing the linkage map produced in this study with both the previously reported *C. moschata* and *C. moschata* - C. *pepo* synteny map revealed both similarities and differences in linkage group homology between the three maps (Table 4-4). Linkage groups in this study that have homology with only one linkage group of the previous *C. moschata* map are LG3, LG5, LG6, LG11, LG13, LG14, and LG 17. Of these, LG 11, LG 13 and LG 14 have only one shared SSR between the two groups. Linkage groups LG3, LG5, LG6, and LG17 have two shared SSRs, which increases our confidence in the homology between the linkage groups examined.

However, when more than two SSR markers were shared between the linkage map produced in this study and the current SSR-based *C. moschata* linkage map, homology was often found to multiple linkage groups. This is displayed most notably in LG1, the largest linkage group. The same splitting of linkage group homology also occurred for shared SSRs greater than two for the *Cucurbita* synteny map in LG1. Identification of SSR loci on different linkage groups compared to previously published maps is likely due to additional SSR loci located in the *C. moschata* genome. Alternatively, chromosomal translocations may have occurred. Additional comparative mapping studies with different *C. moschata* genotypes will be necessary to elucidate these differences. Due to multiple linkage groups having more than one homologous group in the two previously reported maps, the numbering of linkage groups for the present map were arbitrarily assigned.

It was not possible to demonstrate further map linkage group homology with other published *Cucurbita* maps. Just three maps were constructed prior to the works by

Gong et al. (2008a) and these maps only contain RAPD and AFLP markers (Lee et al., 1995; Brown and Myers, 2002; and Zraidi et al., 2007). No AFLPs are on the map created in this study, and RAPDs are not suitable markers to serve as anchor points for map alignment.

QTL Analyses

QTL detection of L* a* b* chroma and hue color space values

All QTL analyses were performed using phenotypes from only 1 fruit per genotype due to highly significant amounts of variation for fruit within a genotype for all generations in all color space values ($P < 0.0001$), likely due to variable fruit maturity within a genotype. Fruit within a genotype were selected based upon highest (a*, b*, and chroma) or lowest (L* and hue) average replicate measurements within a fruit. Selection of fruit with highest or lowest average fruit replicate measurements for each color space value was based upon the direction of each respective color space value correlated with carotenoid levels (Chapter 2, Table 2-3) and represent the genetic potential of the genotype examined.

Analyses of QTLs were performed separately for each color space value. All associations reported were identified above the individual linkage group LOD threshold for each color space value, unless otherwise noted. QTLs were identified on linkage groups 7 and 18 for color space value L* (Figure 4-3, Table 4-5). QTLs associated with color space a* value were identified on linkage groups 3, 10, and 18 (Figure 4-4, Table 4-5). Linkage groups 5 and 17 had QTLs associated with both color space values b* and chroma. The LOD score distribution of markers was nearly identical when comparing b* to chroma on the same linkage group (Figures 4-5 and 4-6, Table 4-5). Color space value hue angle had the most QTL identified, on linkage groups 3, 13, 14,

15, and 19. Additionally, genome-wide LOD score significance was detected on linkage groups 3, 13, and 15 (Figure 4-7, Table 4-5).

Implications for population selection on QTL detection

Of the three populations used to evaluate heritability in chapter three, the 'Butterbush' (BB) population was selected due to the presence of transgressive F_2 segregants for color space value, hue, the overall angular measurement of color. Unfortunately, two drawbacks to using this rationale mapping and QTL population selection were subsequently identified.

First, populations with transgressive segregation were identified prior to correlation between color space values and carotenoid content. This was necessary to allow sufficient time for genotyping and linkage mapping. Therefore, it remained unknown which color space value(s) would give the best estimate of the relationship between color and carotenoid content of squash flesh. After the correlations were calculated, it was found that color space values a*, b* and chroma would have been more appropriate to examine transgressive segregation (Chapter 2, Table 2-3).

Although evidence of transgressive segregation for a quantitative trait is valuable from a breeding standpoint, the identification of genomic regions contributing to the quantitative trait is often better accomplished by selecting parents with a large amount of variation for the trait. Therefore, use of transgressive segregation as selection criteria for which population to map and perform QTL analyses is not as important as selecting parents with the largest differences for the trait in question; the wider phenotypic range for the trait of interest increases power of QTL in progeny. Unfortunately, the only intraspecific *C. moschata* cross we performed was between parents with little variation for flesh color. Based on variation for flesh color, the *C. pepo* Table Gold Acorn (TGA)

would have most likely increased our probability of QTL detection (Chapter 3, Table 3-1). However, these QTL assignments should be viewed with caution, as heritability estimates for hue angle in this population indicated very little genetic variance present for this trait (Table 3-14). Therefore, validation needs to be performed using different genetic backgrounds to confirm these QTL locations.

Additionally, nutritional carotenoid levels do not need to be increased within butternut squash (*C. moschata*), but could be increased in acorn squash (*C. pepo*). No official recommended dietary intake levels for carotenoids, other than vitamin A precursors, have been reported (Murkovic et al., 2002). Therefore, the only carotenoid within squash with a recommended dietary intake is pro-vitamin A, which is converted into retinol activity units, RAE (Trumbo et al., 2001). In 1.00 cup (205g) of baked butternut squash (*C. moschata*) served without salt, there are 1,144 RAE. For adult females, over the age of 19, this serving accounts for 163% of the minimum daily value. For adult males, this is 127%. In 1.00 cup (205g) of baked acorn squash (*C. pepo*) served without salt, there are 43 RAE. For adult females, over the age of 19, this serving accounts for 6% of the percent daily value. For adult males, this is 5% (USDA-ARS, 2010). Although butternut squash vitamin A levels do not need to be increased, this work may be used to identify genomic flesh color associations in *C. pepo*. Evidence of linkage group homology between *C. moschata* and *C. pepo* (Gong et al., 2008b) suggests that *C. moschata* QTLs may be used to identify syntenic genomic regions in *C. pepo* controlling flesh color and be used to increase its color and nutrient content through plant breeding.

Implications and Practical Applications of QTL Analysis in Breeding for Color and Carotenoid Content in *Cucurbita*

Alignment of homologous groups between the *C. moschata* map produced in this study with SSR-based maps produced in *C. moschata* and the synteny map of *C. moschata* and *C.pepo* (Table 4-4) show promise. However, due to the high level of monomorphic SSR markers identified in the *C. moschata* linkage map presented in this chapter, complete alignment other *C. moschata* and *C. pepo* populations would not be possible. With increased recovery of monomorphic SSR markers using high resolution melting (HRM) real-time PCR analysis (see Chapter 5), QTL information obtained here could be compared with other *Cucurbita* populations through map alignment. This would enable similar regions associated with color to be identified in other populations. In addition, as more QTL studies and map alignments become available within the *Cucurbita* genus, multiple traits can be used in squash breeding efforts.

Table 4-1. Loci summary in *C. moschata* linkage map.

	Marker Type	
	RAPD	SSR
Total primer combinations	1200	455
Total polymorphic primer combinations	700	61[a]
Total rescreened	538	---
Total polymorphic primer combinations in F_2	222	39
Total polymorphic loci[b] in F_2	319	39
Total loci on linkage map	235	34

[a] Count excludes polymorphic markers identified using real-time PCR high resolution melting.
[b] The number of polymorphic loci differs from the number of markers because some markers had multiple polymorphic loci on a gel.

Table 4-2. Individual linkage group summaries for marker composition, length, density of markers and average marker distance in *C. moschata*.

Marker type	LG 1	LG 2	LG 3	LG 4	LG 5	LG 6	LG 7	LG 8	LG 9	LG 10	LG 11	LG 12	LG 13
RAPD	77	35	19	14	7	9	12	11	8	6	6	7	3
SSR	12	4	2	0	2	3	1	1	1	0	2	0	2
Total	89	39	21	14	9	12	13	12	9	6	8	7	5
Map statistics													
Length (cM)	162.2	105.1	63.9	37.5	76.8	75.6	49.1	108.7	48.8	47.2	53.6	42.8	59.7
Marker density (markers/cM)	0.55	0.37	0.33	0.37	0.12	0.16	0.26	0.11	0.18	0.13	0.15	0.16	0.08
Average marker distance (cM)	1.82	2.69	3.04	2.68	8.53	6.30	3.78	9.06	5.42	7.87	6.70	6.11	11.94
Largest gap (cM)	23.0	14.6	16.7	6.1	21.6	36.3	15.1	21.8	14.2	16.1	21.3	20.3	23.6
Gaps >15cM (n)	2	0	1	0	2	1	1	3	0	1	1	1	2

Table 4-2. Continued

Marker type	LG 14	LG 15	LG 16	LG17	LG 18	LG 19	LG 20	LG 21
RAPD	4	5	3	0	3	2	2	2
SSR	1	0	1	2	0	0	0	0
Total	5	5	4	2	3	2	2	2
Map statistics								
Length (cM)	33.8	18.1	27.5	17.4	4.5	13.4	22.5	18.7
Marker density (markers/cM)	0.15	0.28	0.15	0.11	0.67	0.15	0.09	0.11
Average marker distance (cM)	6.76	3.62	6.88	8.70	1.50	6.70	11.25	9.35
Largest gap (cM)	24.2	6.0	12.5	17.4	2.5	13.4	22.5	18.7
Gaps >15cM (n)	1	0	0	1	0	0	1	1

Table 4-3. Linkage map summary in *C. moschata.*

Entire Map	
Total map length (cM)	1086.9
Total markers	269.0
Average marker density (Marker/cM)	4.0
Largest gap (cM)	36.3
Total no. linkage groups	21
Average cM/linkage group	51.8

Table 4-4. Common simple sequence repeat (SSR) markers on linkage groups between the *C. moschata* F_2 population of 'Butterbush' x 'Sucrine DuBerry', 'Waltham Butternut' (WB) x 'Nigerian Local' and ZHOU x WB integrated F_2 populations, and the *C. moschata* and *C. pepo* synteny map[a].

C. moschata 'Butterbush' population	Linkage group homology in *C. moschata*	Common SSRs	Linkage group homology in *Cucurbita* synteny map	Common SSRs
LG1	LGm7, LGm2, LGm20, LGm1b, LGm25, LGm4, LGm22, LGm1a	13	LG7, LG11, LG20	5
LG2	LGm14, LGm19	5	LG 13	2
LG3	LGm3	2	LG 16	1
LG5	LGm1b	2	LG10	1
LG6	LGm15	2	---	---
LG7	LGm10,LGm13	2	---	---
LG11	LGm8	1	---	---
LG13	LGm5b	1	---	---
LG14	LGm5	1	---	---
LG17	LGm21a	2	LG 14	1

[a] Population and map information for integrated F_2 *C. moschata* and *C. moschata* and *C. pepo* synteny maps were taken from Gong et al., 2008b.

Table 4-5. QTL detected for flesh color using L*a*b* color space values in a *Cucurbita moschata* F_2 population of 'Butterbush' x 'Sucrine DuBerry.'

	QTL[a]	Linkage group	Interval length (cM)[b]	LOD peak position (cM)	Maximum LOD	Nearest marker	R^2 (%)[c]	Genetic effect: a[cd]
L*	*lightness1*	7	1.90	29.95	2.43	OPAB03A	12.3	-2.52
	lightness2	7	3.00	36.96	2.06	OPAA09	16.9	1.03
	lightness3	18	2.00	1.00	3.38	OPL11	17.9	-1.33
a*	*red/green1*	3	2.37	55.40	3.55	OPT20	14.3	-1.80
	red/green2	10	8.10	27.12	2.50	OPN11	10.7	-1.62
	red/green3	18	4.57	3.03	2.58	OPAD10A	10.3	-2.21
b*	*yellow/blue1*	5	6.00	72.94	3.35	OPI17	28.9	3.77
	yellow/blue2	17	5.00	0.00	1.77	M235	8.2	1.73
Chroma	*colorsaturation1*	5	6.00	72.94	3.27	OPI17	27.8	3.79
	colorsaturation2	17	11.00	3.00-5.00	1.97	M235	9.8	1.94
Hue	*hueangle1*	3	3.77	63.18	5.85*	OPAF14A	28.5	1.80
	hueangle2	13	16.58	4.00	4.49*	OPY13A	15.5	-1.63
	hueangle3	14	16.00	4.00	2.43	OPAI13	21.9	-1.32
	hueangle4	15	9.66	14.55	3.44	OPM03	14.7	1.02
	hueangle5	19	3.39	13.39	1.68	OPBG18B	21.2	-2.39

[a] QTLs reported are based on LOD scores above linkage group specific LOD score threshold.
[b] Interval length based upon linkage group region with LOD scores above linkage group specific LOD score threshold.
[c] Percent explained variance and genetic effect reported at interval with the highest LOD score.
[d] Additive genetic effect.
* Denotes QTL associations with genome-wide LOD score threshold.

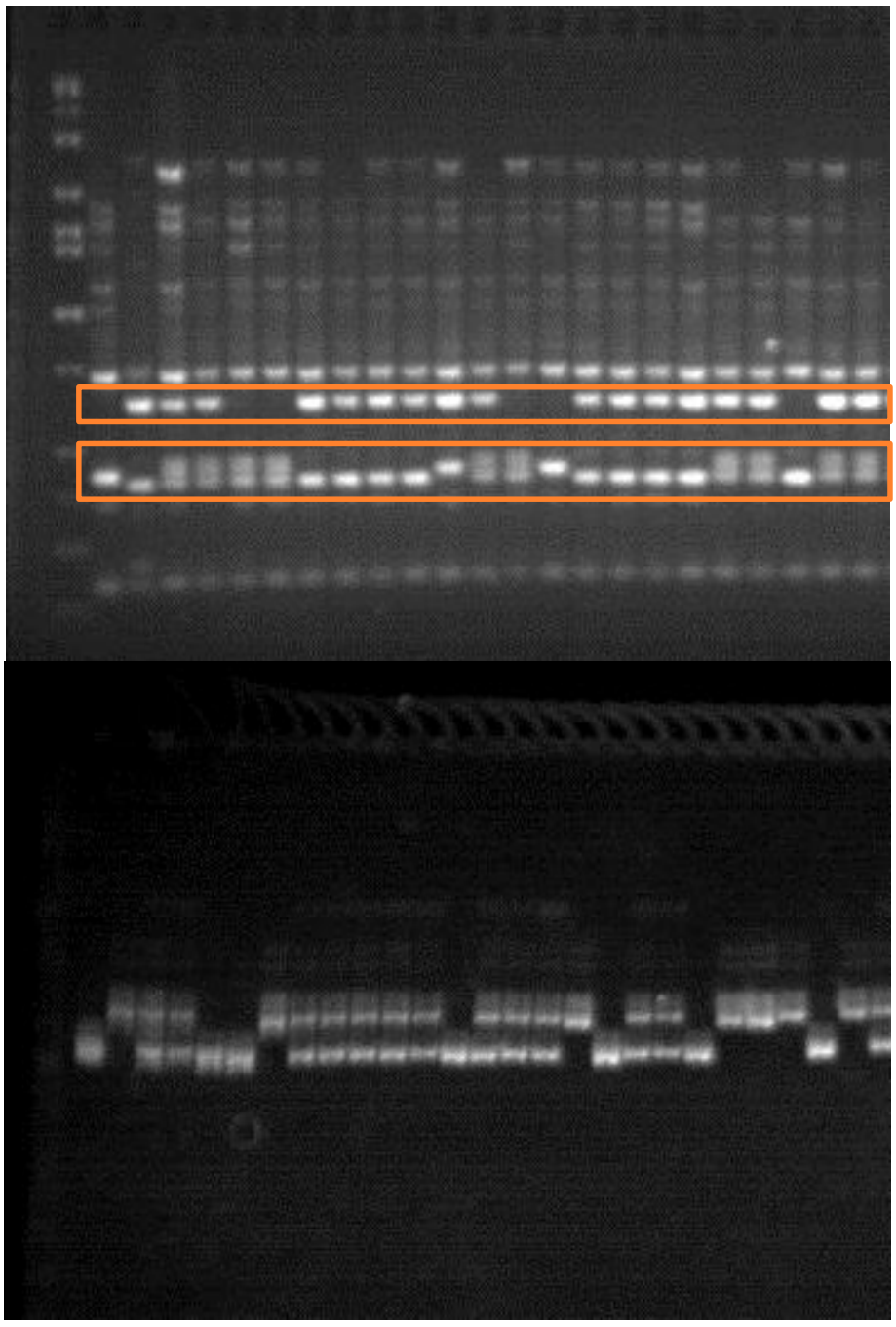

Figure 4-1. Example gel images imaged under UV light with ethidium bromide stain. A) A RAPD marker (OPQ15) with multiple polymorphic loci, both dominant and codominant, on a 1.5% agarose gel, B) A SSR marker (M005) with a codominant locus on a 6% polyacrylamide gel In both gel images, lane 1 is 'Butterbush', lane 2 is 'Sucrine DuBerry', lane 3 is the F_1, and the following lanes are the F_2 progeny.

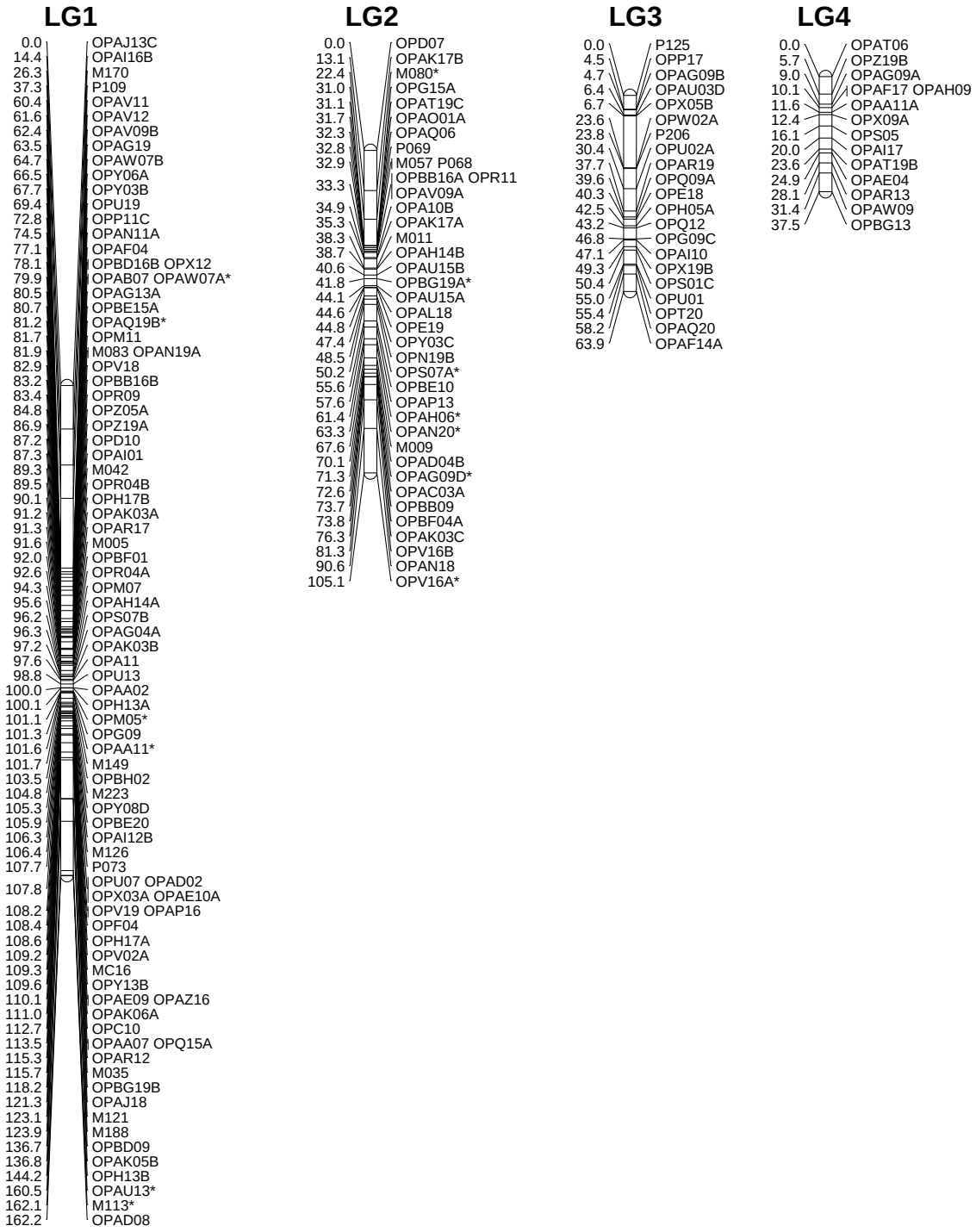

Figure 4-2. Linkage map of *Cucurbita moschata* using both random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers. Markers beginning with an 'OP' designation indicate RAPD markers. Markers ending with a letter designation such as "A,B,C, or D" indicate one of the polymorphic sites identified by an individual marker. Markers beginning with 'M' are SSR markers that were derived from *C. moschata* while those beginning with beginning with 'P' were derived from the *C. pepo* genome. Asteriks (*) indicate significantly skewed at the 0.05 probability level.

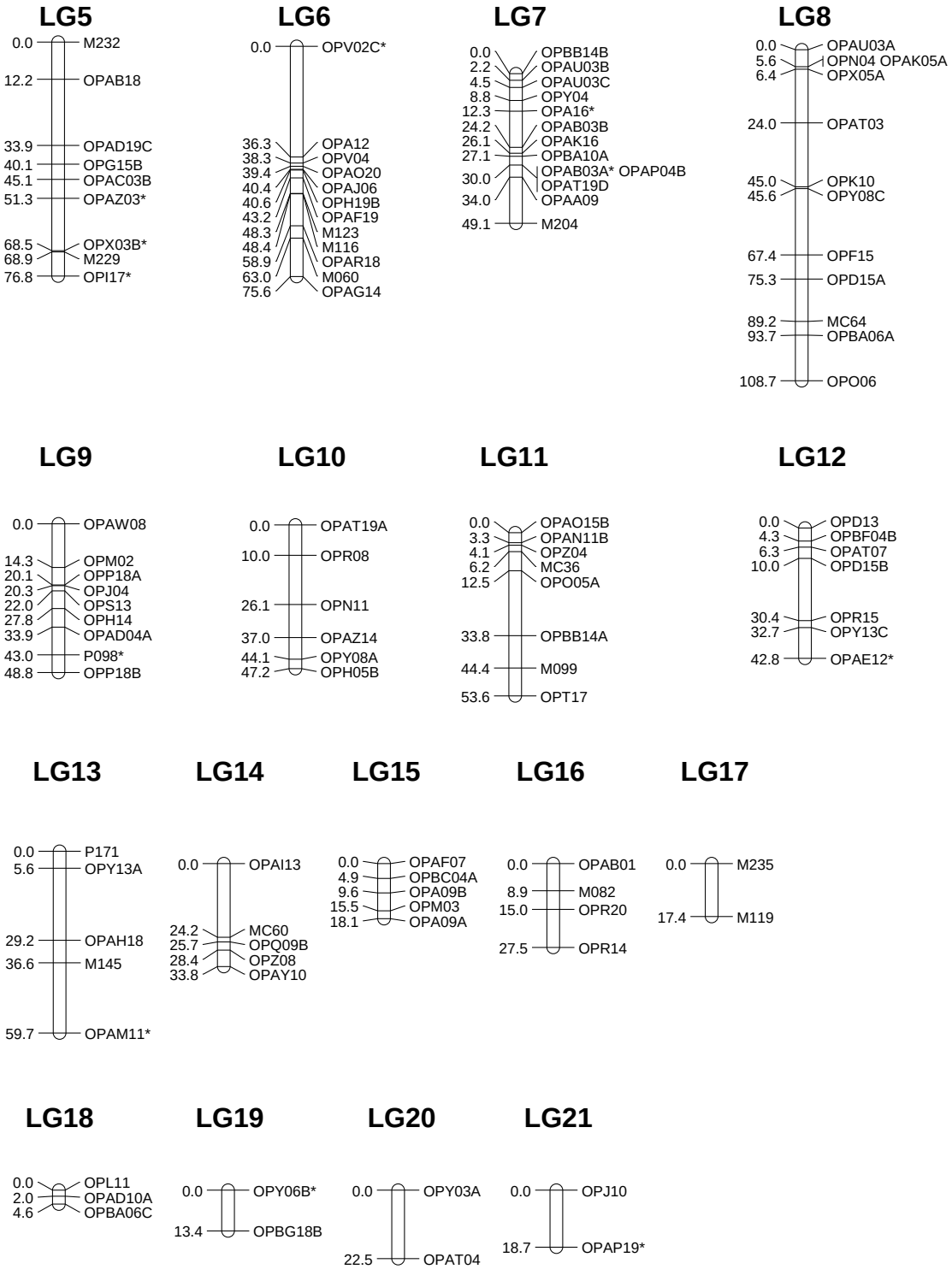

Figure 4-2. Continued

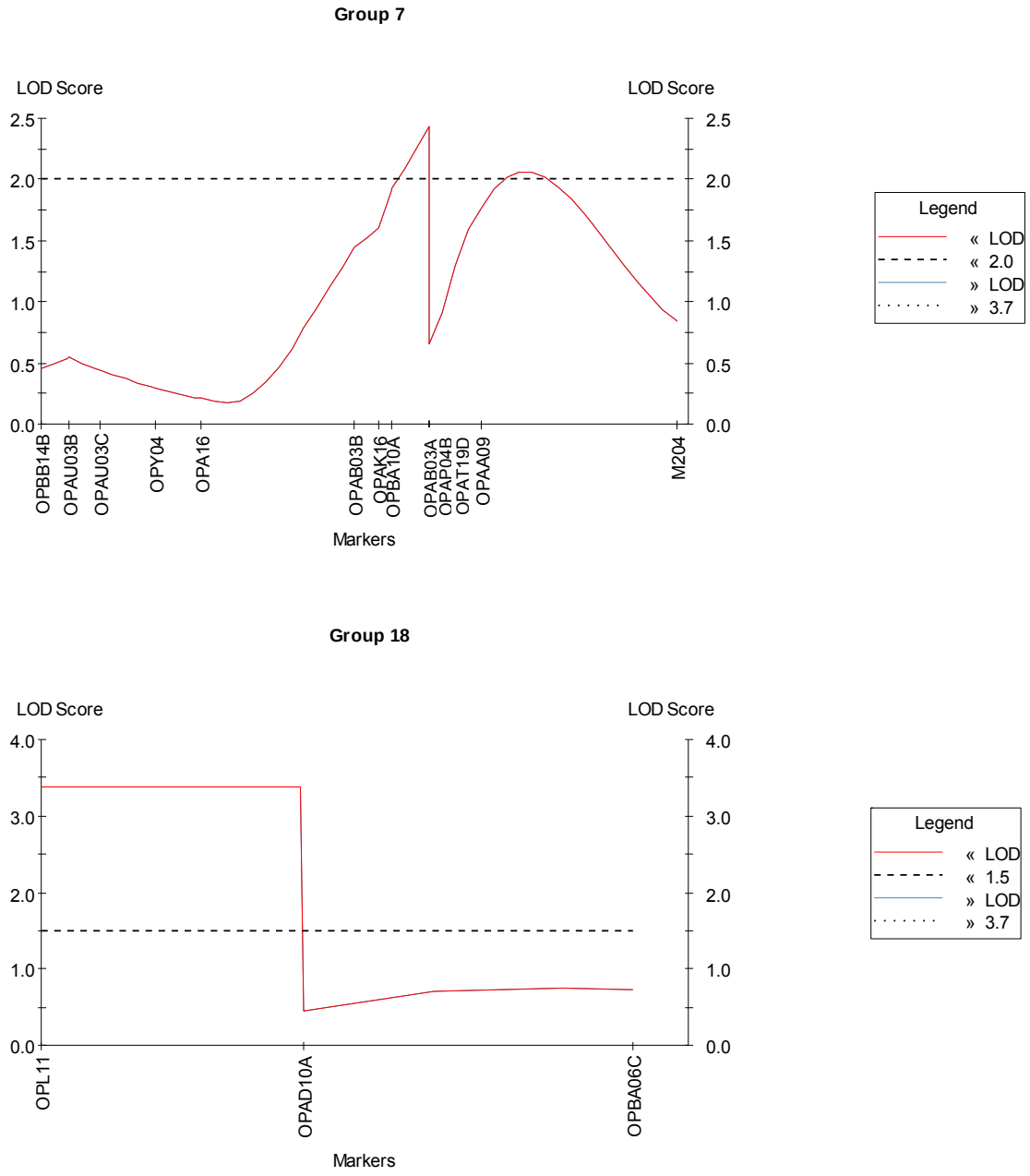

Figure 4-3. Color space value L* quantitative trait loci (QTL) linkage group associations evaluated using both genome wide and individual linkage group LOD scores ($P \leq 0.05$).

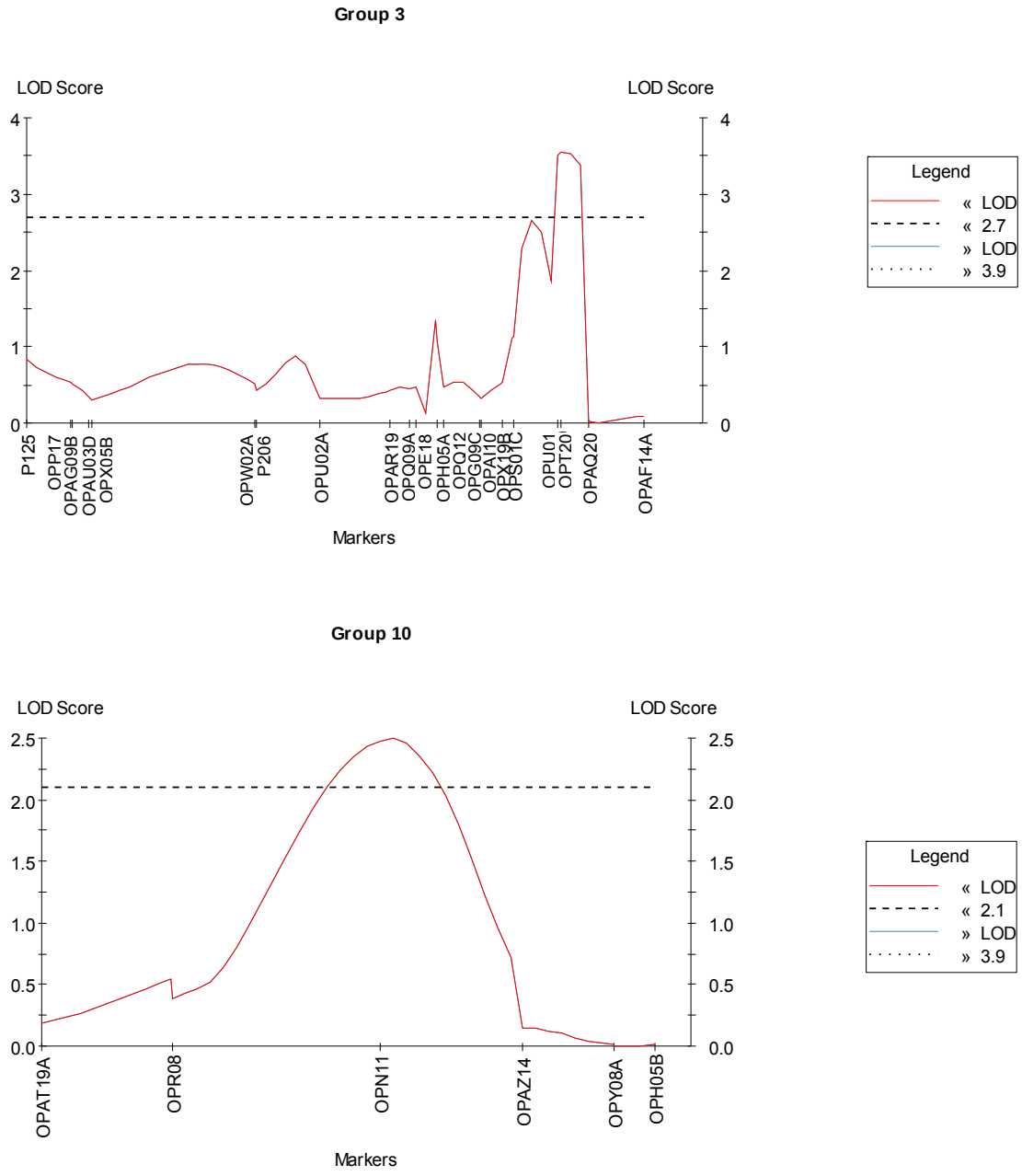

Figure 4-4. Color space value a* quantitative trait loci (QTL) linkage group associations evaluated using both genome wide and individual linkage group LOD scores ($P \leq 0.05$).

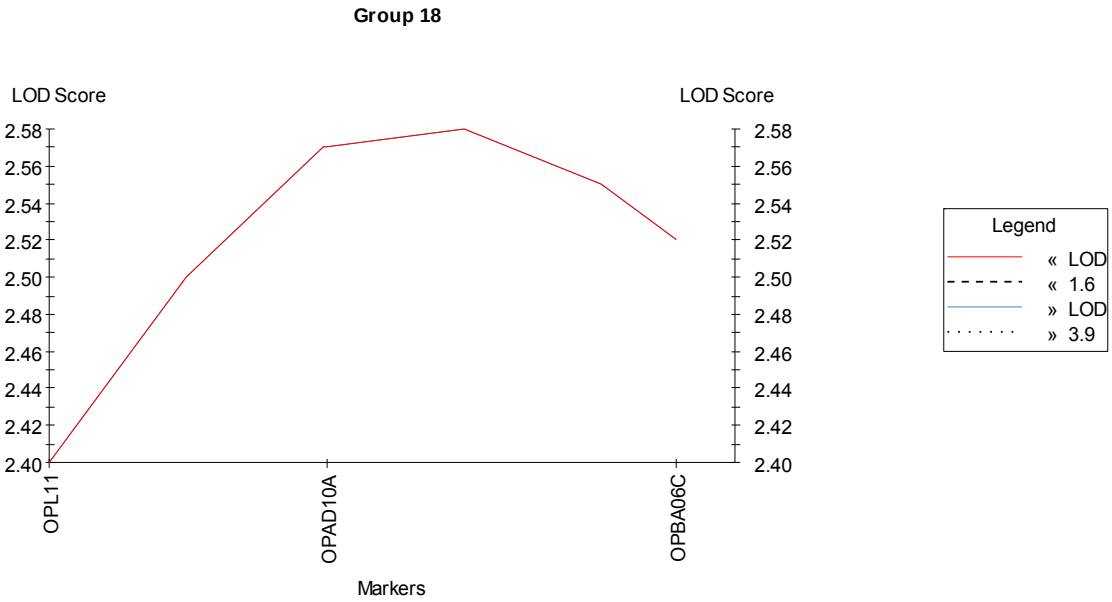

Figure 4-4. Continued

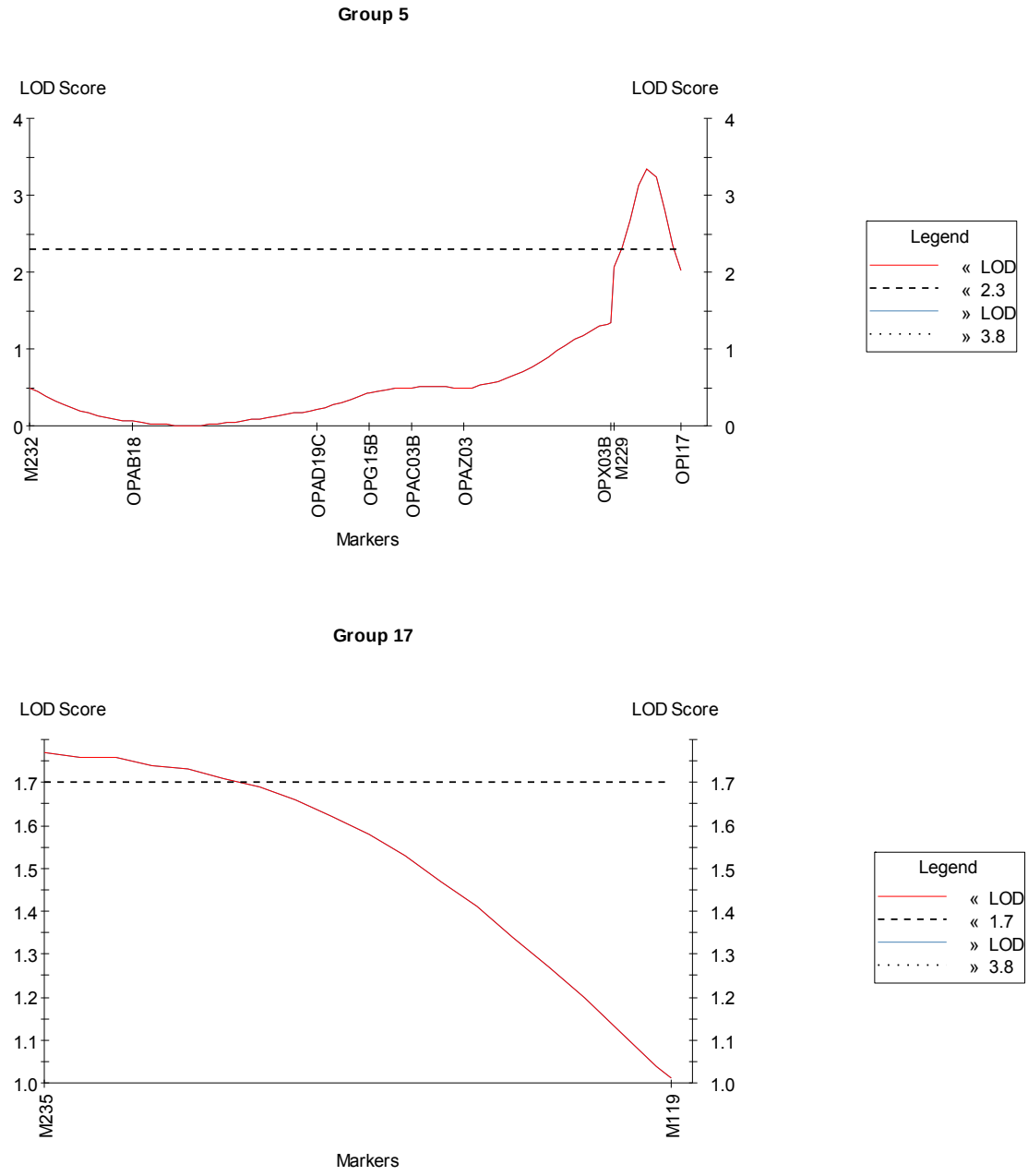

Figure 4-5. Color space value b* quantitative trait loci (QTL) linkage group associations evaluated using both genome wide and individual linkage group LOD scores ($P \leq 0.05$).

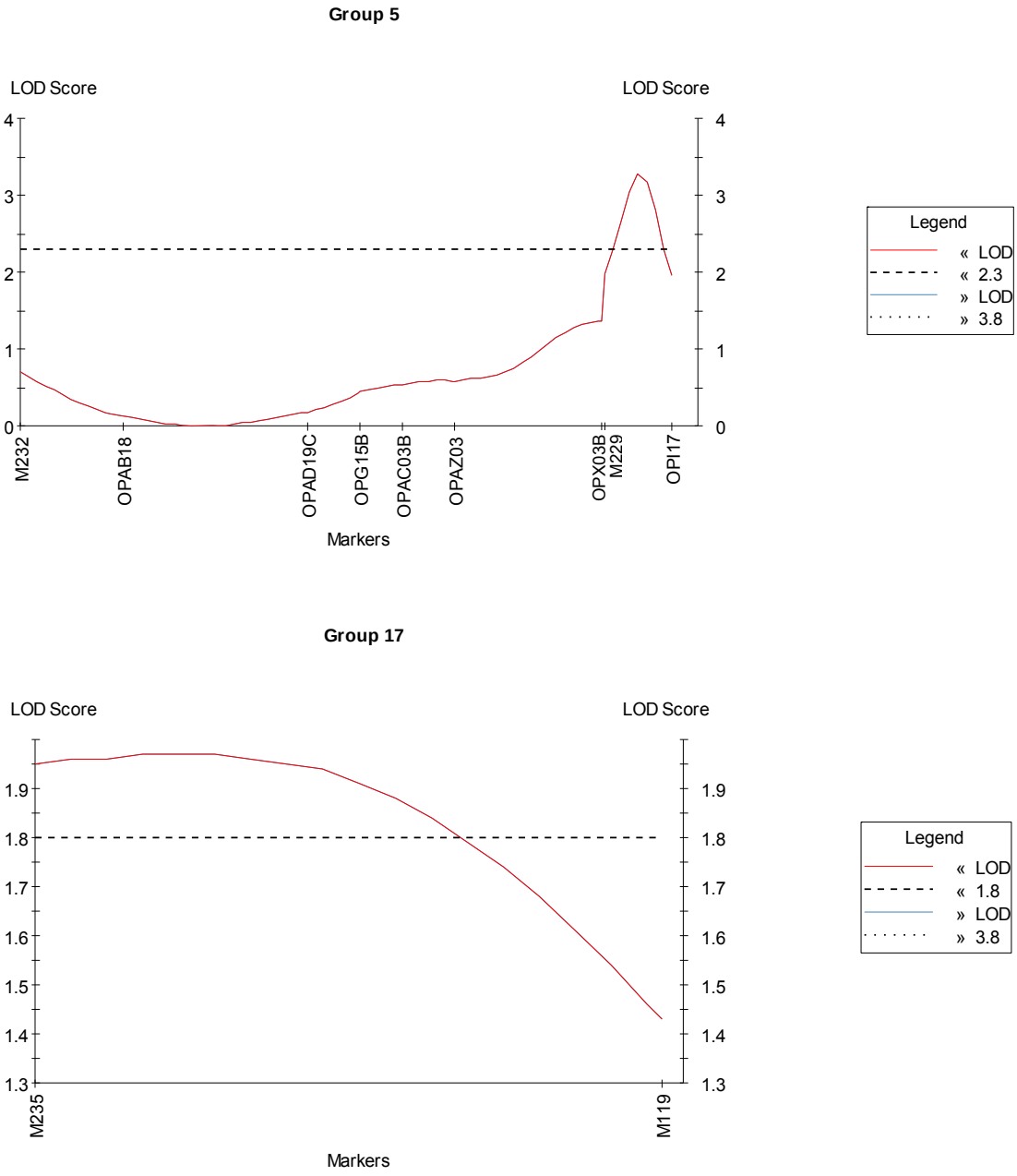

Figure 4-6. Color space value chroma quantitative trait loci (QTL) linkage group associations evaluated using both genome wide and individual linkage group LOD scores ($P \leq 0.05$).

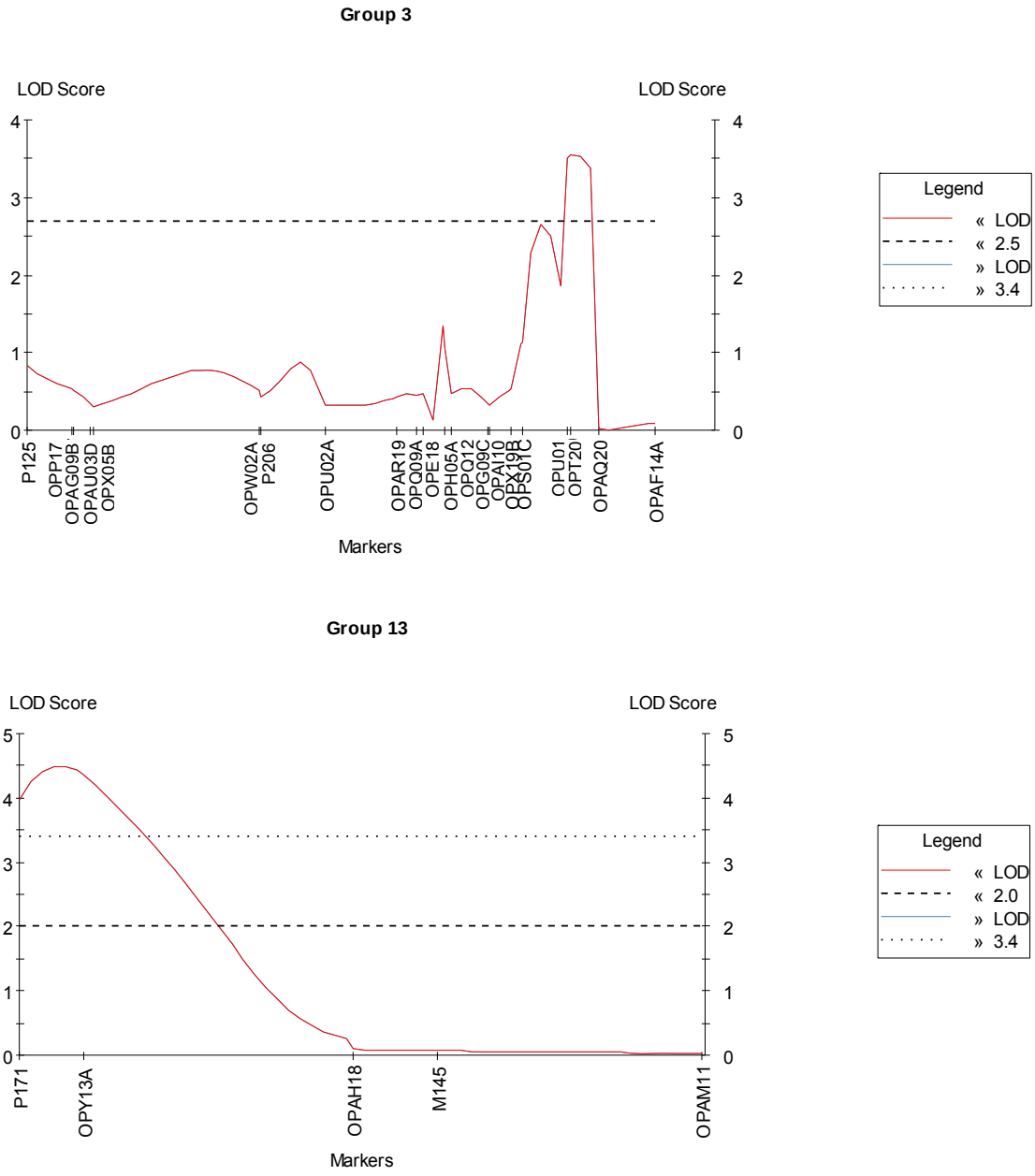

Figure 4-7. Color space value hue angle quantitative trait loci (QTL) linkage group associations evaluated using both genome wide and individual linkage group LOD scores ($P \leq 0.05$).

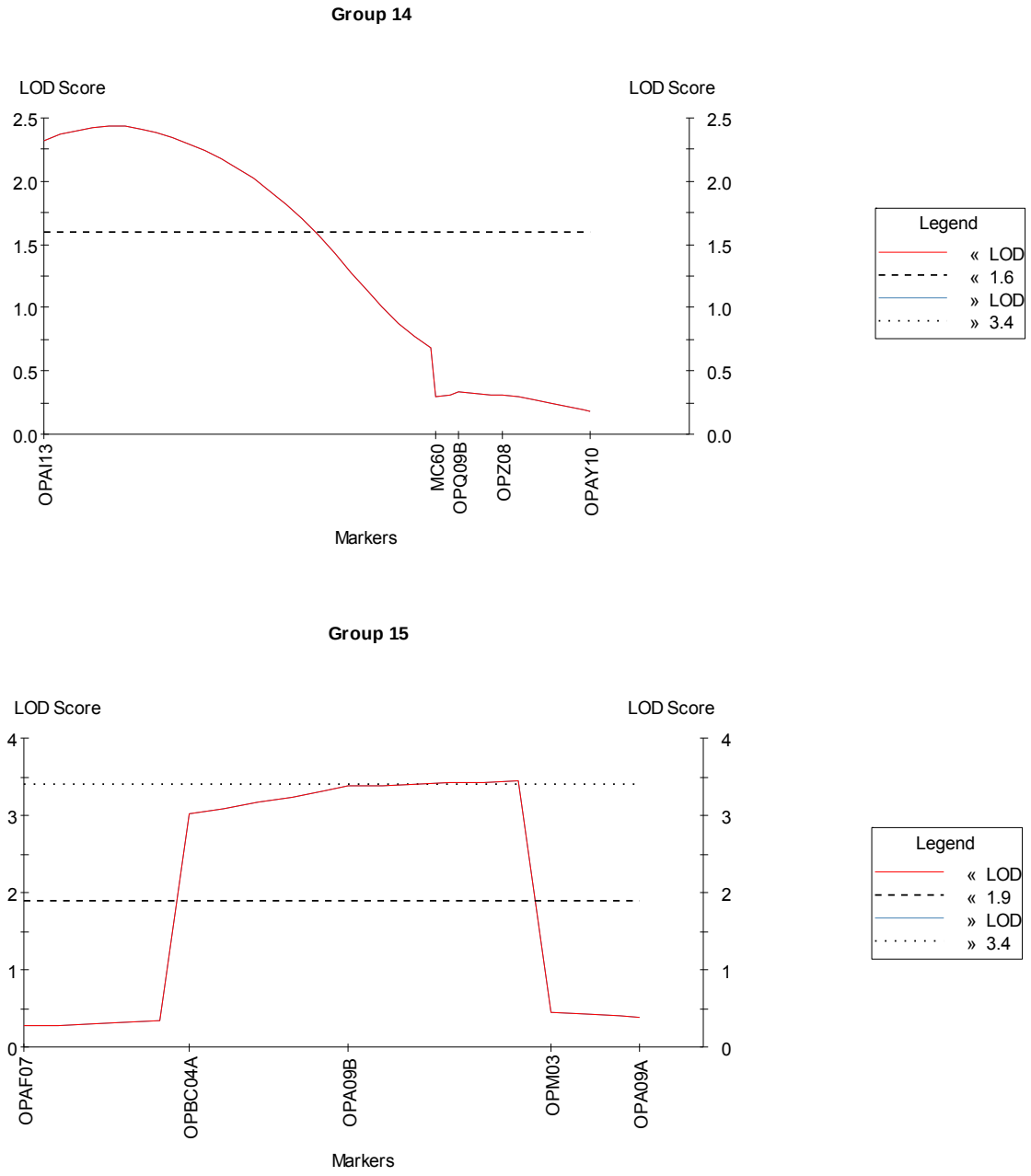

Figure 4-7. Continued

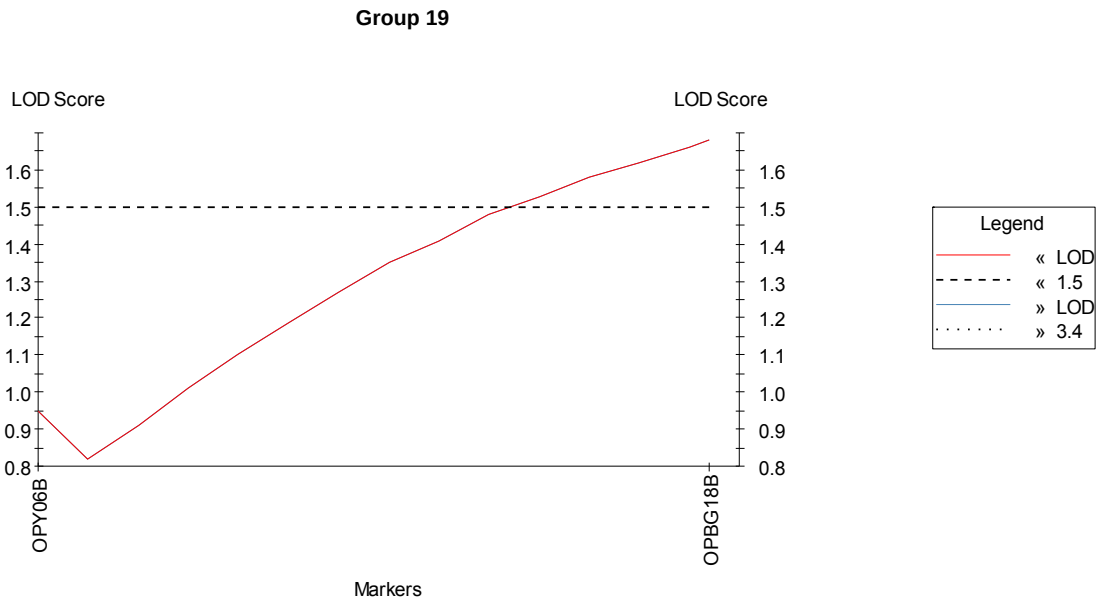

Figure 4-7. Continued

CHAPTER 5
DEVELOPMENT AND UTILIZATION OF A PROTOCOL FOR SMALL DNA SEQUENCE DIFFERENCE DETECTION IN MONOMORPHIC SIMPLE SEQUENCE REPEAT (SSR) MARKERS IN WINTER SQUASH (*Cucurbita moschata*) USING HIGH RESOLUTION MELTING CURVE ANALYSIS

Introduction

DNA markers have proven to be valuable tools in plant breeding and genetics. The utility of molecular markers depends on the presence of polymorphisms in target material, and the method of polymorphism detection. Detection methods can vary in complexity, and the nature of polymorphism under scrutiny. For example, restriction fragment length polymorphism (RFLP) markers are a hybridization-based method that requires the availability of a previously sequenced clone, and detects polymorphisms due to variable distances between adjacent restriction sites. Variable distances can be due to base substitutions in a restriction site, insertion and/or deletion of DNA sequence (indels), or other chromosomal rearrangements (Xu, 2010).

In contrast, polymerase chain reaction (PCR)-based methods are more expedient than hybridization-based methods, and do not require previously sequenced clones or large amounts of DNA. PCR-based markers utilizing random oligonucleotide primers such as amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) are often used in minor plant species with a lack of prior genome level information (Schlotterer, 2004). These markers do not require any sequence information prior to analysis, and for this reason they lack locus specificity. However, PCR-based markers derived from sequence-tagged sites have the advantage of targeting specific genome regions. Polymorphism resulting from these markers takes advantage of indels, either in the polymerase chain reaction-amplified region between two molecular marker primer binding sites, or in the primer binding sites themselves

resulting in a loss of amplification products (Xu, 2010). For example, allele variation at simple sequence repeat (SSR) loci results from variable numbers of repeated DNA units, typically in di- or tri-nucleotide motifs (Ellegren, 2004).

As the wealth of DNA sequence information for plant species continues to grow, through expressed sequence tag library development, transcriptional profiling, and whole genome sequencing, the availability of markers based on single nucleotide polymorphisms (SNP) is increasing. SNP sites are the most abundant molecular markers in plant genomes, widely and evenly distributed throughout the genome, and occurring as frequently as every 60-120 base pairs in some species (Agarwal et al., 2008). SNP alleles result from a single base pair change rather than large indels or repeat sequence variation, thus polymorphisms are not typically differentiated through traditional visualization methods such as agarose gel electrophoresis. Specialized detection methods such as DNA sequencing, primer extension, and enzymatic cleavage are often employed in high throughput assays (Chagne et al., 2007). An alternative to these approaches is the analysis of the melting behavior of double-stranded PCR-amplified DNA fragments at high temperatures, which depends very much on the sequence and the sequence composition.

The thermal stability of PCR product results from its nucleotide sequence (Montgomery et al., 2007). Small differences in sequence composition, such as SNP allele differences leading to variation in GC nucleotide content, result in differing stability of double stranded DNA (Reed et al., 2007). High resolution melting analysis utilizes dyes that fluoresce in the presence of double-stranded DNA coupled with instruments that monitor the fluorescence during heating of the PCR amplification product

(Montgomery et al., 2007). As temperature increases and melting of double-stranded DNA occurs, and fluorescence signal decreases. This leads to a characteristic melting profile that has been used to genotype diverse plant species such as grape (Mackay et al., 2008), apple (Chagne et al., 2008), barley (Lehmensiek et al., 2008), lupin (Croxford et al., 2008), almond (Wu et al., 2008), and potato (De Koeyer et al., 2010).

Breeding populations resulting from crosses between parents with a high level of inbreeding often result in a low level of allele polymorphism at a given locus. Although a marker locus may be present and amplifies during PCR, parents with the same alleles at the locus will not produce segregating progeny, resulting in monomorphic loci. The high frequency of SNP markers in plant genomes will help in situations such as this by increasing the pool of available markers. However, in minor agricultural species such as *Cucurbita moschata*, the lack of available genome sequence limits the use of SNP markers. In some cases, monomorphic marker loci can be "recovered" by the presence of a SNP in the amplicon that results in the addition or deletion of a restriction enzyme cleavage site. However, these cleaved amplified polymorphic sequence (CAPS) markers require an additional restriction digestion step prior to genotyping, and require sequencing of the original amplification product to determine the appropriate enzyme to use (Agarwal et al., 2008). Similar to CAPS markers, high resolution melting curve analysis may be used to recover monomorphic marker loci by allowing genotyping of sequence variation that does not result in the loss of primer binding sites or presence of indels large enough for traditional electrophoretic analysis The objective of these experiments was to develop a protocol to genotype the BB x SDub F_2 population for 10

SSR markers previously discarded due to lack of segregation between the parents and progeny using high resolution melting curve analysis.

Materials and Methods

Plant Material and DNA Extraction

For these experiments, an F_2 population of 90 individuals was developed from a cross between the U.S. cultivar 'Butterbush' (BB) and French heirloom variety 'Sucrine DuBerry'(SDub) as described previously (see Chapters 3 and 4). The F_2 population, and eight individuals each of BB, SDub and F_1 plants were grown during 9 April – 3 July 2008 in a completely randomized design at the University of Florida Plant Science Research and Education Center (PSREC) in Citra, Fl. Newly emerging disease and insect free true leaves from three week old plants were collected for DNA extraction using the same protocol described in Chapter 4.

Marker Selection and Polymorphism Screening

Molecular marker development within *C.moschata* has been limited, and randomly amplified polymorphic DNA (RAPD) markers have been the primary marker system used for detecting genetic differences. Simple sequence repeat (SSR) markers are currently the most widely used marker system in Cucurbitaceae, but only recently have become available for the *Cucurbita* genus in 2008 (Gong et al., 2008). Initially, the parents BB and SDub were screened for polymorphism with 455 SSR markers, 262 derived from *C. moschata* and 193 from *C. pepo* genomes, respectively. The PCR reaction total volume was 26 µl, and contained 5 µl of template DNA , 13.44 µl DNA grade water (Fisher, Pittsburgh, PA), 2.5 µl 10 x PCR buffer, 2.0 µl 25 mM $MgCl_2$ (Promega, Madison, WI), 2.0 µl of 2.5 µM dNTPs, 0.06 µl *Taq* polymerase, and 1 µl of 20 pm F/R primer. PCR amplification parameters used an initial denaturation step of

95°C for 5 min, followed by 33 cycles of denaturation at 94°C for 1 min, primer-specific annealing temperature for 1 min, and DNA extension at 72°C for 2 min, with a final extension step of 72°C for 5 min. All PCR reactions were run on an Eppendorf Mastercycler (Hauppauge, NY). PCR products were subjected to electrophoresis on a six percent polyacryalmide gel at 260 volts for two hours in 0.5 x TBE buffer and stained with 50 µl ehidium bromide diluted in 200mL 0.5x TBE buffer for 25 minutes and imaged under an ultraviolet light source.

DNA Sequencing, Alignment and Comparison

A set of ten SSR markers all determined to be monomorphic in BB and SDub by acrylamide gel electrophoresis, were selected for sequencing of the amplification products of both parents. DNA samples consisting of 45 µl of PCR amplification product were purified using the QIAquick PCR Purification Kit from Qiagen (Valencia, CA) and sent for Sanger sequencing at the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR). Sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, v. 3.1 (Perkin-Elmer/Applied Biosystems, Foster City, CA), analyzed on an ABI 3130 DNA sequencer (Applied Biosystems, Inc., Fullerton, CA), and edited with Sequencher 3.1.1. (Gene Codes,1998). Sequence reads of both parents were aligned using the complete alignment function in ClustalX (Conway Institute, Dublin, Ireland) and quality values determining the probability of the correct base call were calculated using FinchTV software (Seattle, WA).

High Resolution Melt Curve Analysis Protocol Development

All melt curve analyses were performed using a LightCycler 480 (Roche, Indianapolis, IN). The instrument utilizes real-time PCR technology to quantify

fluorescence signal changes resulting from double-stranded DNA denaturation during a post-amplification temperature gradient applied to the total PCR reaction volume. The PCR final reaction volume was 10 µl, contained 2 µl of template DNA, 1.4 µl PCR-grade water (Roche, Indianapolis, IN), 5 µl 2 x Master Mix (Roche, Indianapolis, IN), 0.8 µl 25 mM $MgCl_2$ (Roche, Indianapolis, IN), 2.0 µl 2.5 µM dNTPs, and 0.8 µl 20 pm F/R primer. All reactions were run using a modified version of the LightCycler 480's standard protocol, which was used as the starting point for determining small genotypic differences with the initial ten sequenced markers. Protocol parameters were altered to align with the current PCR protocol in squash, and included a decrease in amplification cycle number from 45 to 35, altering denaturing, annealing and extension times to 1, 1, and 2 min respectively. Further modifications to the standard protocol made as a result of experimentation, are outlined in the results and discussion section.

Markers with the largest deletions were screened first, and markers with only SNP differences screened last. In addition, as recommended by Roche, a large range of 65 - 95°C was initially used for the high resolution melting step. Markers were screened with BB, SDub, F_1, and the first 5 F_2 individuals to determine an optimized melting curve temperature (Tm) for detecting genotypic difference based on actual PCR product melting. Melting curve program temperatures were optimized for each marker with a 20°C range centered on the expected Tm value for each marker. However, plates that were prepared for troubleshooting with multiple markers maintained a large range of temperatures for melting conditions. All optimization experiments were run in an effort to design protocol parameters to detect polymrophisms between parental genotypes and the expected 1:2:1 co-dominant allele segregation ratio in the F_2 progeny. All

primers were analyzed using the gene scanning genotyping analysis option in the LightCycler 480 software, based on melting curve at a baseline sensitivity of 0.30. Higher sensitivity values were examined on an individual marker basis in an attempt to match the expected segregation ratio for an F_2 population.

Results and Discussion

Parental screens revealed a high level of monomorphic SSR markers, with 190 identified from markers derived from *C. moschata* (73 percent) and 125 from markers derived from *C. pepo* (65 percent). The degree of genetic similarity between BB and SDub for available SSR markers necessitated the use of RAPD markers to develop a linkage map suitable for quantitative trait locus (QTL) analysis. However, RAPD markers are not useful for comparing synteny between previous *C. moschata* and *C. pepo* linkage maps. In an effort to align our linkage map with previously developed maps, we identified 46 markers found to be monomorphic in BB and SDub, but present on the SSR-based synteny map of *C. moschata* and *C. pepo* (Gong et al., 2008). For these experiments, a total of 10 of these 46 markers were used to develop a protocol to "recover" monomorphic SSR markers in this population.

DNA Sequence Differences

Sequence analysis of alleles of monomorphic SSR markers revealed small sequence differences in nine of the ten initial markers sent for sequencing. Both deletions and SNPs were identified (Table 5-1). The largest sequence difference was observed in marker M042 with a six base pair deletion in BB. Markers M120, P098, and M066 also had deletions over one base pair in size with four, three, and two base pair deletions identified, respectively. Four potential SNPs were identified in maker M120, but in each case the base-calling software was unable to accurately assign a nucleotide

difference at the SNP site. However, seven out of eight quality values determining accurate readings of base calls were over the Q=20 threshold, perhaps indicating that these SNPs are wobble locations. Only one marker, M261, had no sequence differences between BB and SDub. All other markers had at least one SNP present.

Genotyping DNA Sequence Differences Using the LightCycler 480

Of the nine markers exhibiting sequence variation between BB and SDub, three (33%) were able to be genotyped utilizing high resolution melt curve analysis. Marker M042, with the largest sequence difference between the two parents (a six base pair deletion), showed three distinct melting curve groups (Figure 5-1), with genotypic classes segregating in the expected 1:2:1 ratio for a codominant marker in an F_2 population (Table 5-2). Given the relatively large deletion present in BB, this marker may have been scorable utilizing higher resolution genotyping and/or staining methods (i.e., higher percentage gels, silver staining, or capillary electrophoresis). Four distinct melting curve groupings were identified for marker M009 (Figure 5-2). Progeny scored as a fourth genotypic class were evaluated using melting curve temperature-shifted difference plot graphs (data not shown) and determined to have a similar curve to those progeny homozygous for the allele inherited from SDub (Table 5-2). Interestingly, M009 contained a single SNP between the two parents, a genotypic difference that would not be scorable using higher resolution genotyping methods due to a lack of size polymorphism.

Progeny did not segregate as expected for marker P098 (3 base pair deletion in SDub), Evaluation of P098 at 0.40 sensitivity revealed closer adherence to a 1:2:1 segregation pattern than did progeny evaluation at 0.30 (χ^2 = 16.667, 26.689, respectively) (Table 5-2). Of the 269 loci on the *C. moschata* linkage map reported in

Chapter 4, 24 (9%) showed significant deviation from the expected segregation ratio. P098 is likely an example of distorted segregation due to factors such as gametophytic selection. The six remaining markers did not display genotypic differences that were initially scorable using high resolution melting curve analysis.

Evaluating DNA Concentration Effects on Genotyping With High Resolution Melt Curve Analysis

High resolution melting curve analysis is particularly sensitive to variation in template DNA concentration (N. Bassil, personal communication). With a thermocycler capable of real-time PCR quantification, we were able to develop a method to experimentally define the working concentration of the template DNA samples using crossing point (Cp) values. The Cp value indicates the cycle during PCR where the sample fluorescence enters an exponential growth phase. Thus, difference in the Cp value of one sample compared to another can be used as an indication of the starting DNA concentration of the reaction mixture. Since the amplification product grows exponentially during PCR, relatively small differences in Cp values may indicate large differences in template DNA concentration. For example, using the SSR marker M066, the Cp values for BB and SDub varied by over four cycles (19.13 and 23.71, respectively), indicating the starting DNA concentration for BB was 16x more concentrated than SDub.

Cp values of BB, an F_1 individual, and three F_2 progeny were selected for comparison between DNA concentration as measured by absorbance at 260 nm using two instruments; an Eppendorf BioPhotometer 6131 v.35 (Hauppauge, NY) and a Genesys 10 spectrophotometer v.2 (Thermo Scientific, Waltham, MA) (Table 5-3). Using the SSR marker M066, average Cp values from replicate experiments indicated

that BB and the F_1 individual should have significantly higher DNA concentrations than the F_2 progeny. However, the relationship between Cp value and DNA concentration measured by absorbance was not linear. This may be an indication of contaminants in the DNA, such as RNA, proteins, polysaccharides, and/or polyphenols that may be inhibiting a true measure of DNA concentration by absorbance.

To further examine the effect of DNA concentration based on dilutions on relative Cp values, four markers (M120, M256, M261, and P224) were selected to create serial dilutions for concentration differences. None of these markers showed a difference between BB and SDub at sensitivity of 0.30 for melting curve analysis, although each contained a deletion and/or SNP between the two parents. Serial dilutions, ranging from 1:10 to 1:320, were prepared for each parent for each marker in two replicates. PCR amplification was allowed to continue until all samples reached saturation, as indicated by cessation of exponential growth of amplification product, For each marker, both parents showed a distinct progression from most concentrated to most dilute in terms of number of cycles required to reach Cp. There was approximately a once cycle difference to Cp in neighboring serial dilutions for each parent replicate for each marker.

Analysis of variance indicated the only significant difference was among dilutions for each parent (P <0.001). No significant differences were identified between markers (P = 0.9941), replicates within a maker (P = 0.9523), or between parents (P = 0.0767), suggesting that the only factor affecting Cp cycle number difference was dilution, representative of DNA concentration. The one cycle difference in Cp value between serial dilutions is due to the sample having half the amount of DNA as compared to its

earlier counterpart. This is the basis for using Cp value as a measurement of relative DNA concentration.

To examine the effect of DNA concentration on genotyping by high resolution melting, the SSR marker M120 was used. Serial dilutions of each parent were prepared in replicate, and amplification cycle number was selected based upon the point where all dilutions had the largest separations between one another. This cycle was expected to display the highest detection of polymorphic differences in comparison between the melt curves produced. Cycle 29 was selected for ending amplification in BB, followed with genotyping by high resolution melting. Two distinct groups were seen in replicates of BB (Figure 5-3). Dilutions of 1:10, 1:20, 1:40, and 1:80 all genotyped the same, while further dilutions were genotyped together or fell into unknown genotypic classes. A similar trend for Cp values was seen in SDub (data not shown). These data suggest a point where samples with the same genotype are genotyped differently based upon differences in DNA concentration; it appears to be a range of approximately 4 cycles of difference. This indicates that Cp values based on DNA concentration for all samples need to be relative to one another to minimize DNA concentration effect and maximize the potential to identify the correct genotypic class.

Using the SSR marker M120, which we were initially unable to genotype using melting curve analysis, we attempted to genotype the population again after adjusting the DNA concentration of each F_2 individual based on the Cp values from the initial PCR. Amplification was terminated at 29 cycles while all samples still displayed exponential growth. At a sensitivity of 0.30, differences were observed in Cp values of the samples, reflective of genotypic differences rather than concentration differences

(Figure 5-4). Although skewed for the expected segregation ratio ($\chi^2 = 28.311$), the marker still presented three distinct genotypic classes. To test for the artificial creation of genotypic classes by preventing PCR from running to saturation, marker M261 (no sequence difference) was run for the population and amplification was stopped at 30 cycles during exponential growth. At a sensitivity of 0.30, the parents and 89 of 90 F_2 progeny were placed in the same genotypic class, indicating artificial differences were not being created.

Table 5-1. DNA sequence differences detected between 'Butterbush' and 'Sucrine DuBerry' for initial ten monomorphic simple sequence repeat (SSR) markers in winter squash, *Cucurbita moschata*.

SSR primer	Polymorphism detected	Base pair difference	DNA sequence	Base pair number	Quality Value Q[a]	
					P_1	P_2
M009[b]	SNP	1	CTG CCG	24	11	57
M042	Deletion, P_1[c]	6	TCGTCG	P_2, 86 -91		
M066	Deletion, P_2[d]	2	CT	P_1, 21-22		
M120	Deletion, P_2	4	TCTC	P_1, 42-45		
	SNP	1	ANT[e] ATT	60	49	49
	SNP	1	TNT TTT	73	52	59
	SNP	1	TNT TTT	77	35	10
	SNP	1	GNG GTG	80	36	45
M256	SNP	1	TCT TGC	23	8	9
M261	none					
P039	Deletion, P_2	1	A	P_1, 4		
P098	Deletion, P_2	3	AAG	P_1, 39-41		
P224	SNP	1	CACT CCAT	14	37	18
	SNP	1	CACT CCAT	15	14	17
P235	SNP	1	TGT GTA	3	4	1
	SNP	1	TGT GTA	4	4	1
	SNP	1	TGT GTA	5	7	4

[a] Quality values exceeding the baseline value of Q=20 indicate that correct base pair identification has a probability greater than 99%.
[b] Markers beginning with 'M' are SSR markers that were derived from the *C. moschata* genome. Markers beginning with 'P' are SSR markers that were derived from the *C. pepo* genome.
[c] 'P_1' denotes parent 1 as 'Butterbush.'
[d] 'P_2' denotes parent 2 as 'Sucrine DuBerry.'
[e] N indicates base calling software could not assign a base at this location. The site is a putative SNP.

Table 5-2. Summary of melting curve genotyping of scorable simple sequence repeat (SSR) markers, prior to DNA concentration adjustments based on Cp value, in winter squash (*Cucurbita moschata*) with 90 F_2 progeny resulting from a cross between 'Butterbush' and 'Sucrine DuBerry.'

	F2 individuals										
SSR primer[a]	P1 allele[b]	P1 χ^2 value	Adjusted P1 χ^2 [c]	F1 allele	F1 χ^2 value	Adjusted F1 χ^2	P2 allele	P2 χ^2 value	Adjusted P2 χ^2	Total χ^2 value	Adjusted Total χ^2	Total F2'scored[d]
M042	25	0.278		44	0.022		21	0.100		0.400*		89
M009	16	1.878		50	0.556		4(20)[e]	15.211	0.100	17.644	2.533*	90
P098	3(1)	16.900	15.211	65(1)	8.889	9.800	18	0.900		26.689	27.600	88
	7(1)[0.4]	10.678	9.344	56(6)	2.689	6.422	17(1)	1.344	0.900	14.711	16.667	88

* No significant difference of expected segregation ratio of 1:2:1, $\chi^2 < 5.991$.

[a] All markers run under standard LightCycler 480 protocol with modifications made to align protocol to standard PCR protocol for squash, *C. moschata* and *C. pepo.*

[b] Sensitivity level of 0.30 was used to determine number of individuals for genotyping in each class, unless otherwise marked.

[c] Adjusted chi-square value with different genotypes with similar melt curve shape included.

[d] Total F_2 individuals scored differ from total of 90 when 'negative' genotypes were present.

[e] Values in parentheses indicate number of individuals genotyped as 'unknown' or in another genotypic class, yet could be genotyped into one of the three main genotypic classes using difference plot analysis.

Table 5-3. Estimates of DNA concentration in winter squash, *Cucurbita moschata* and F_2 progeny resulting from a cross between 'Butterbush' and 'Sucrine DuBerry' as measured by crossing point (Cp) value in the LightCycler 480 software using simple sequence repeat (SSR) marker M066 and compared to absorbance readings from the Eppendorf Biophotometer and the Thermo Scientifc Genesys spectrophotometer.

Sample	Average Cp Value[a]	Difference in Cp value from P_1	Concentration difference estimate based on CP value	BioPhotometer Concentration[b]	Genesys Concentration[b]
P_1	18.675	0	0	302.5	517.5
F_1	19.7	1.025	2x	523.1	625.0
F_2(95)	23.005	4.33	16x	61.8	77.5
F_2(80)	22.52	3.845	16x	213.1	652.5
F_2(11)	22.83	4.155	16x	467.8	605.0

[a] Average based on Cp values determined from M066 A and M066 B.
[b] Concentration readings (ng/µl) were determined from absorbance of 260mn.

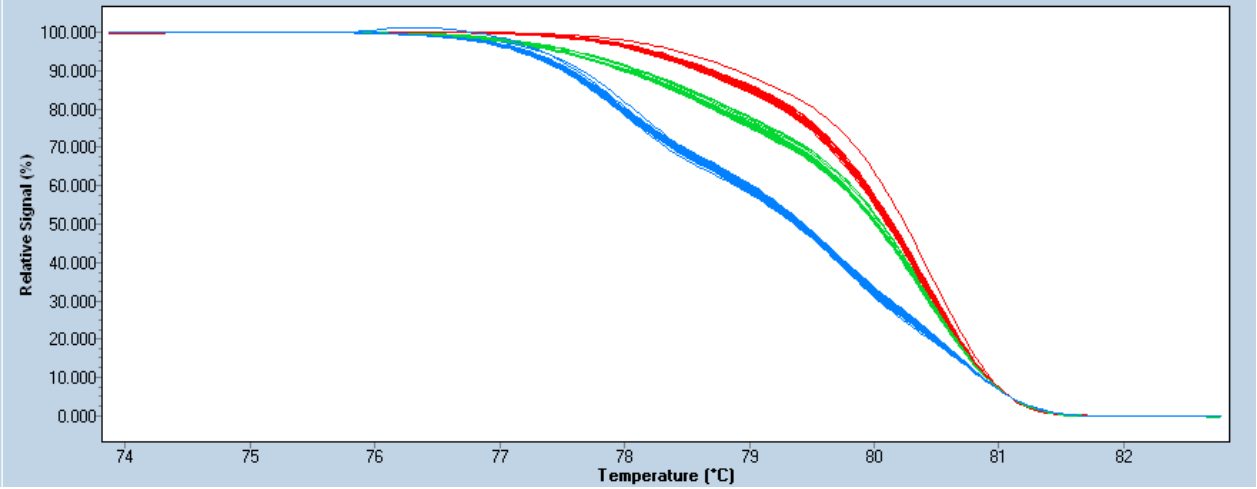

Figure 5-1. Normalized and temperature-shifted melting curves for simple sequence repeat (SSR) marker M042 showing three genotypic classes at sensitivity of 0.30 for 90 F_2 progeny resulting from a cross between 'Butterbush' (BB) and 'Sucrine DuBerry' (SDub) in *Cucurbita moschata*. F_2 individuals that inherited the BB allele are denoted by red, the heterozygotes by blue, and the SDub alleles by green.

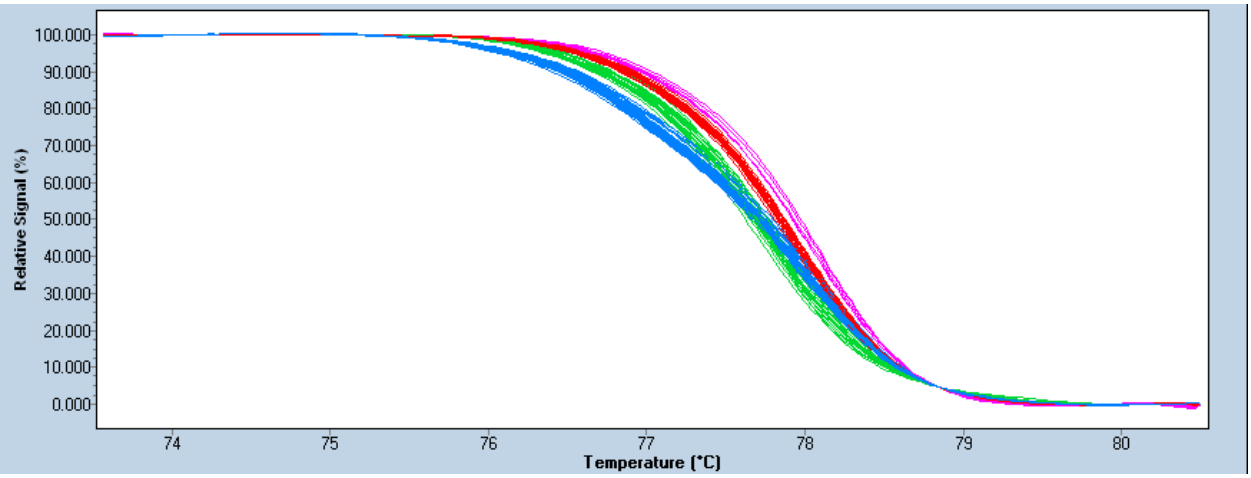

Figure 5-2. Normalized and temperature-shifted difference plot of melt curve shape showing three genotypic classes at sensitivity of 0.30 for 90 F_2 progeny resulting from a cross between 'Butterbush' (BB) and 'Sucrine DuBerry' (SDub) for simple sequence repeat (SSR) marker M009 in *Cucurbita moschata*. F_2 individuals that inherited the BB allele are denoted by green, the heterozogotes by blue, and the SDub alleles by red. Genotypes, grouped separately, indicated by the purple curves, aligned with the shape of individuals with the SDub allele and were added to that genotypic class.

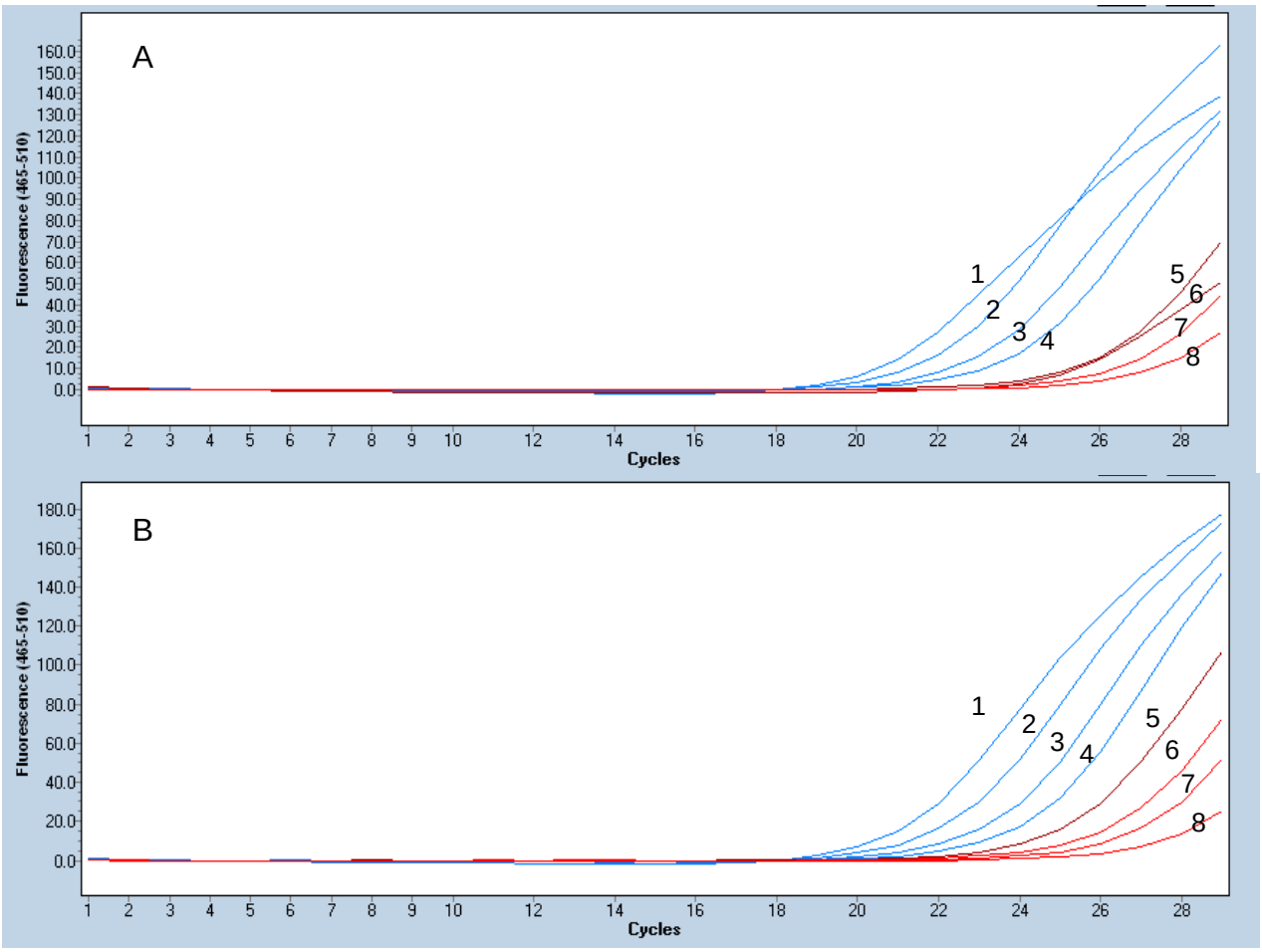

Figure 5-3. Fluorescence history of 'Butterbush' (BB) at serial dilutions of (1) 1:10, (2) 1:20, (3) 1:40, (4) 1:80, (5) 1:160, (6) 1:320, (7) 1:640, (8)1:1280 for simple sequence repeat (SSR) marker M120. Amplification was stopped during mid-exponential growth. A) BB replicate 1, B) BB replicate 2.

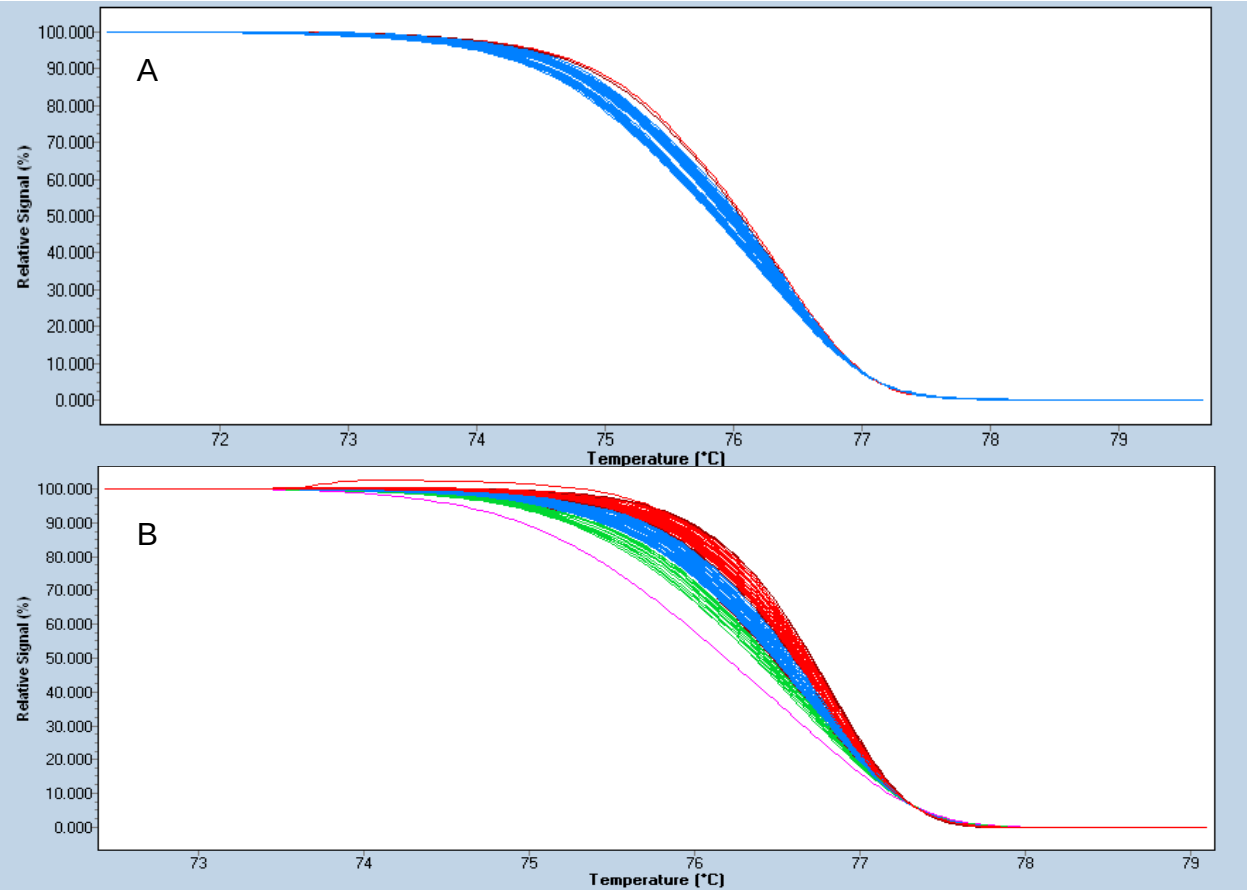

Figure 5-4. Normalized and shifted melting curves for simple sequence repeat (SSR) marker M120 at sensitivity of 0.30 for 90 F_2 progeny resulting from a cross between 'Butterbush' (BB) and 'Sucrine DuBerry' (SDub). A) Parents, F_1 and F_2 progeny prior to adjusting relative DNA concentrations based on Cp value and ending amplification during mid-exponential amplification and B) Parents, F_1 and F_2 progeny after adjusting relative DNA concentrations based on Cp value and ending amplification during mid-exponential amplification. F_2 individuals that inherited the BB allele are denoted by blue, the F_1 alleles by red, and the SDub alleles by green. Unknown individuals, indicated by maroon curves, were genotyped based on similarity of melt curve to the above known genotypes.

CHAPTER 6
CONCLUSIONS

A range of color within pumpkins and squash was identified, using L*a*b* color space values, and these color space values were correlated with differentcarotenoid types and concentrations. Strong correlations were found between color value a* and total carotenoids (r = 0.91) and color value b* and chroma with lutein (r = 0.87). Genetic variation should make it possible to increase the nutritional value through crossing and selection from within and among the different types with high levels of carotenoids. These close associations will assure that indirect selection for high carotenoid content within pumpkin and squash breeding material will be successful, easy to implement, and inexpensive.

Heritability and gene action was determined for flesh color in winter squash in both *Cucurbita moschata* and *Cucurbita pepo* F_2, BC_{1P1} and BC_{1P2} segregating populations. Broad-sense heritabilities ranged from 0.19 to 0.82 for L*, 0.12 to 0.32 for a*, 0.40 to 0.93 for b*, 0.36 to 0.92 for chroma, and 0.14 to 0.15 for hue across all three crosses. Additionally, transgressive segregation for color space values a*, b* and chroma was examined and was identified in one *C. pepo* and the *C. moschata* population. Color space values that do not have transgressive segregation in crosses suggest that the population means could be shifted and color within squash flesh can be increased over time. Color space values that do have transgressive segregation in crosses suggest that transgressive individuals can be used to increase flesh color through breeding with fewer generations. However, a breeding challenge is presented due lower broad-sense heritabilities for color space values and the presence of narrow-sense heritabilities with negative, essentially zero, values. This indicates that flesh color has a large non-

genetic variance which needs to be more adequately examined and controlled. Planting F_2 and backcross populations within the same year, in the same location, or over multiple years and locations may allow better estimates of heritability.

A linkage map of a F_2 population segregating for flesh color in *C. moschata* (2n=2x=40) was created from 235 RAPD and 42 SSR markers. A total of 21 linkage groups were obtained at a LOD score threshold of 3.0. Creation of this linkage map was then used to identify regions of the genome associated with squash flesh color. QTLs were detected on LG7 and 18 for L*, LG3, LG10, LG18 for a*, LG 5 and LG17 for b* and chroma, and LG 3, LG13, LG14, LG15, and LG19 for hue. However, given the low heritability estimates for some of the traits, these QTL assignments are tentative and await validation in different populations and environments. Additionally, one way to strengthen the linkage map and thereby strengthen the associations identified in the QTL analysis would be to use the candidate gene approach. Sequences identified in the carotenoid pathway in other plant crops and/or in related species within *Cucubitaceae*, such as *Cucumis sativus* (cucumber) or *Cucumis melo* (melon), could be used to genotype the current F_2 population.

Homology between the linkage groups obtained in this study was also examined with the most recently published SSR-based map in *Cucurbita*, which examined the synteny of the *C. moschata* with the *C. pepo* genome (Gong, et al., 2008b). Homology was identified for several linkage groups. However, due to a large number of SSR markers on the Gong et al. *C. moschata* map identified as monomorphic in the ‘Butterbush’ x ‘Sucrine DuBerry’ population, complete alignment was not possible. Recovery of these monomorphic SSR markers is needed before sufficient alignment

can occur with the published reference linkage map. With increased recovery of monomorphic SSR markers using high resolution melting (HRM) real-time PCR analysis, QTL information obtained here could be compared with other *Cucurbita* populations through map alignment. This would enable similar regions associated with color to be identified in other populations. In addition, as more QTL studies and map alignments become available within the *Cucurbita* genus, multiple traits can be used in squash breeding efforts.

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BIOGRAPHICAL SKETCH

Rachel obtained her Bachelor of Science degree in horticulture with dual minors in biology and plant pathology from The Pennsylvania State University in May 2006. While pursuing her undergraduate degree, Rachel had the opportunity to have an undergraduate research experiences and two internship positions in horticulture. She served as a summer intern at Phipps Conservatory and Botanical Gardens in Pittsburgh, PA (2004), worked as a research assistant in Dr. Majid Foolad's Tomato Breeding and Genetics Program at Penn State (2004 – 2005), and interned at Ball Helix, a genetic research division on Ball Horticultural Company in West Chicago, IL (2005). She also studied under Dr. Paul Backman in the Plant Pathology Department and Penn State where she examined the effect of biological and cultural management strategies in orchard soils with apple replant disease (2005-2006).

Rachel was awarded the Alumni Fellowship and began her Doctor of Philosophy degree in plant breeding and genetics in the Horticultural Sciences Department at the University of Florida in August 2006 in Dr. Eileen Kabelka's Cucurbit Breeding and Genetics Program. While studying for her doctorate, she had the privilege of teaching the Vegetable Gardening class for non-major undergraduate students and found a passion for teaching in addition to plant breeding. After graduation, Rachel will continue her career in plant breeding and genetics with a postdoctoral research position in the Blueberry Breeding and Genetics Program at the University of Florida under the direction of Dr. James Olmstead. Rachel's career interests lie in plant breeding and genetics.