This is to certify that the

dissertation entitled Genomic Differentiation an Evolution in Blueberry: An Examination of an Interspecific Hybrid of Diploid Vaccinium darrowi and Tetraploid V. corymbosum.

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D degree in Plant Breeding and Genetics

Major professor

Date 12/2/1996

GENOMIC DIFFERENTIATION AND EVOLUTION IN BLUEBERRY: AN EXAMINATION OF AN INTERSPECIFIC HYBRID OF DIPLOID *VACCINIUM DARROWI* AND TETRAPLOID *V. CORYMBOSUM*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture
Plant Breeding and Genetics Program

1997

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ABSTRACT

GENOMIC DIFFERENTIATION AND EVOLUTION IN BLUEBERRY: AN EXAMINATION OF AN INTERSPECIFIC HYBRID OF DIPLOID *VACCINIUM DARROWI* AND TETRAPLOID *V. CORYMBOSUM*

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A series of experiments were conducted to study the genomic differentiation and evolution of blueberry using an interspecific hybrid, US 75, derived from Fla 4B [a wild selection of *Vaccinium darrowi* (2x)] x 'Bluecrop' [(a cultivar of *V. corymbosum* (4x)].

First, the inheritance pattern of US 75 and the mode of Fla 4B's 2n gamete production were determined using RAPD markers. US 75 was found to contain about 70% of Fla 4B's heterozygosity, a value attributed to a first division restitution (FDR) mode of 2n gamete production. Crossovers during 2n gamete formation were evidenced by the absence of 33 dominant alleles of Fla 4B in US 75, and direct tests of segregation in a diploid involving Fla 4B. RAPD markers that were present in both Fla 4B and US 75 were used to determine the mode of inheritance in a tetraploid segregating population of US 75 x *V. corymbosum* cv. 'Bluetta'. Sixty-five duplex loci were identified which segregated in a 5:1 ratio, indicating US 75 undergoes tetrasomic inheritance.

Second, a genetic linkage map of US 75 was generated. One hundred and forty markers unique for Fla 4B that segregated 1:1 in the tetraploid population were mapped into 29 linkage groups that cover a total genetic distance of 1288.2 cM, with a range 1.6 - 33.9 cM between adjacent markers. The map is essentially of *V. darrowi* because US 75 was produced via a 2n gamete from Fla 4B and only unique markers for Fla 4B were utilized.

Third, the meiotic pairing configurations and genomic similarity of US 75 and its

parents were studied using cytogenetic observations and genomic *in situ* hybridization (GISH). US 75 was found to have a lower than expected number of multivalents for an autopolyploid, but it had a significantly higher number of quadrivalents than *V. corymbosum*, and one PMC (pollen mother cell) was observed with 11 of all 12 homologous groups being paired in quadrivalents. Normal distributions of chromosomes were observed at anaphase I and II by both cytological observation and pollen viability. GISH revealed that both parents labeled all the chromosomes in the hybrid. These findings suggest that little genomic divergence has developed between the *Vaccinium* species, and that genes may be freely transferred from diploid to tetraploid species via unreduced gametes.

Finally, four new mechanisms of 2n pollen formation were found in blueberry by examining the cytogenetics of PMCs and sporad configurations of selfed progenies of US 75. These were: 1) Premeiotic doubling (PD), where chromosome doubling in a PMC during mitosis resulted in the formation of four 2n gametes; 2) Tripolar spindle (TPS), where the spindles at anaphase II were fused at one pole resulted in one 2n and two n gametes; 3) Incomplete spindle at anaphase I (IS1), where the spindle functioned briefly at anaphase I and then failed producing two 2n gametes; and 4) Incomplete spindle at anaphase II in one daughter nuclei (IS2-1), where the spindle at anaphase II in one daughter nuclei functioned briefly and then failed resulting in one 2n and two n gametes. The TPS and ISI produced FDR 2n pollen. The allelic constitution of gametes formed by PD is similar to FDR produced gametes, although the cytological process is different. The IS2-1 mechanism resulted in SDR (second division restitution)2n pollen. Surveys of mature pollen from the selfed progeny, its parent and a tetraploid grandparent revealed sporad associations consistent with all these mechanisms.

ACKNOWLEDGMENTS

I thank my committee members: Drs. James F. Hancock, Amy F. Iezzoni, James D. Kelly and David Douches for the thoughtful reviews of my manuscripts and dissertation and helpful advice during the course of my study here at Michigan State University. I thank Dr. Joanne Whallon, Peter W. Callow, Dechun Wang and Roger May for their support and valuable discussion.

I must especially express my gratitude to my major professor Dr. Hancock for his great support in every possible way over the years. From helping improvement of my English to advising research his input has been in every step of my progress, and some great help I am unable to express in words. Thanks Jim!

Last I thank my wife Jinghua and daughter Jing for their love, understanding and support throughout the course of this study.

Guidance committee:

The thesis is divided into four chapters. Chapter 1 has published in Theoretical and Applied Genetics. Chapter 2 is in press in the Journal of American Society for Horticultural Science. Chapter 3 has been submitted to American Journal of Botany. Chapter 4 has been submitted to Theoretical and Applied Genetics.

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Autopolyploids

Polyploid classification, origin, genetic consequences and evolution have long been an important focus of studies in plant science. The fact that polyploidy has played an important role in both plant evolution and breeding has been recognized for at least 50 years (Stebbins, 1950). It is estimated that 30-50% of all angiosperms and more than 70% of the crop species are polyploid (Grant, 1971; Hancock, 1992), and new polyploids are being continuously created by nature and human beings.

There are three major types of polyploids: 1) allopolyploids or amphipolyploids, 2) autopolyploids, and 3) segmental allopolyploids (Stebbins, 1947). It is generally considered that allopolyploids are of interspecific origin and can be recognized by their having a combination of characters of the parental species, meiotic pairing restricted to exactly two homologous chromosomes, and disomic inheritance. Autopolyploids are generated from an individual plant or different plants within the same species and are characterized by their having a phenotype similar to their parental species, random pairing among all the homologous chromosomes, and polysomic inheritance. Segmental allopolyploids are formed between partially divergent progenitors, display irregular pairing behavior between auto- and allopolyploids and mixed inheritance.

Conclusions and inferences of early studies (before 1980) on polyploids were mainly based on morphological, cytological and ecological observations. Much important basic information was generated in these studies on origins (interspecific for allopolyploids,

intraspecific for autopolyploids), meiotic chromosome behaviors (regular bivalents for allopolyploids, variable multivalents for autopolyploids) and segregation ratios (disomic for allopolyploids, polysomic for autopolyploids); however, considerable debate was also stimulated, especially regarding the frequency and adaptation of autopolyploids (Stebbins, 1971; Levin, 1983; Soltis and Rieseberg, 1986; Soltis and Soltis, 1993 and 1995). Autopolyploidy has been considered by a few to be a creative source of phenotypic variation in plant evolution (Levin, 1983), but most investigators have considered it to be a conservative force and a hindrance to long-term evolutionary success (Stebbins, 1950 and 1971; Briggs and Walters, 1984; Soltis and Riesberg, 1986). After reviewing the available research results from biochemical, physiological , developmental and genetical sources, Levin (1983) pointed out that autopolyploids could occupy habitats beyond the limits of its diploid progenitor. However, many investigators were convinced that autopolyploidy was maladaptive because of inherent low fertility, direct competition with progenitors, and the poor vigor of artificially produced polyploids by colchicine treatment (Sakai, 1956; Dewey, 1980; Hancock, 1996). Induced autopolyploids were usually characterized by slower development and reduced fertility (Stebbins, 1947), and autopolyploid breeding was a general failure (reviewed by Dewey, 1980; Hancock, 1996).

In recent years, the application of molecular techniques has revealed a number of more positive aspects of autopolyploidy, including: 1) their having a higher level of allelic diversity than their diploid progenitors (Soltis and Rieseberg, 1986; Krebs and Hancock, 1989; Soltis and Soltis, 1993), 2) their being repeatedly formed (Soltis and Soltis, 1993), and 3) their having high fertility through the evolution of bivalent pairing at meiosis (Crawford

and Smith, 1984; Soltis and Rieseberg, 1986; Samuel et al., 1990; Cockerham and Galletta, 1976; Krebs and Hancock, 1989). Most researchers now believe that the origin of polyploids is generally via 2n gametes (Harlan and deWet, 1975; deWet, 1980; Bretagnolle and Thompson, 1995), and as a result, raw autopolyploids can have more heterozygosity than their diploid progenitors. In some instances, natural polyploids have even been shown to outcompete their progenitors under the same conditions (Maceira et al., 1993). It is clear that many more positive features of autopolyploidy await to be discovered.

The use of molecular techniques in polyploid evolution studies began in the 1980's. Crawford and Smith (1984) surveyed 3 diploid and one hexaploid variety of *Coreopsis grandiflora* for 14 isozymes and found that all alleles detected in the hexaploid were also found in the diploids, and no fixed heterozygosity was found at any gene in any population of the hexaploid. They concluded that the hexaploid was an autoploid. Soltis and Rieseberg (1986) also used isozyme markers to document that *Tolmiea menziesii* was an autopolyploid. They found there were an average of three or four alleles at each single locus and, as a result, heterozygosity was substantially higher in the tetraploid than the diploid cytotype. These genetic consequences had been predicted for autopolyploidy, but until this work, it had not been directly demonstrated (Soltis and Rieseberg, 1986). Since these studies, many more autopolyploids have been examined by molecular markers and in all cases, high levels of heterozygosity have been found (see review of Soltis and Sotis, 1993; Gutierrez et al., 1994).

By determining if a polyploid has fixed heterozygosity or polysomic segregation patterns, molecular markers have revealed another important evolutionary aspect of

polyploidy - that predominant or even complete bivalent pairing at meiosis is not restricted to allopolyploids. Numerous studies have now shown polysomic inheritance in polyploid species that have predominant and even complete bivalent pairing (Soltis and Rieseberg, 1986; Crawford and Smith, 1984; Krebs and Hancock, 1989; Samuel et al., 1994). These findings not only challenge the traditional criteria that regular bivalent pairing signals only allopolyploidy, but also suggests that autopolyploids can achieve high levels of fertility through bivalent pairing. Indeed, autotetraploids of domesticated *Vaccinium* have higher pollen viability than related diploid species (Cockerham and Galletta, 1976; Krebs and Hancock, 1989), approximating 100% (Stushnoff and Hough,1968).

Winge's hypothesis (Winge, 1917) that zygotic and somatic chromosome doubling were the main causes of polyploidy was prevalent until the 1970s (Bretagnolle and Thompson, 1995). However, numerous species have now been found to produce 2n gametes, and it is currently believed that the driving force behind the origin and evolution of polyploids is sexual polyploidization as a result of 2n gamete formation (Harlan and De Wet, 1975). Autopolyploids produced via 2n gametes have two obvious advantages over those produced via somatic doubling. First, they have increased heterozygosity. No matter what mechanism generates the 2n gametes (see review of Bretagnolle and Thompson, 1995), the heterozygosity incorporated into the polyploids can not be lower through 2n gametes than somatic doubling and, in fact, FDR (first division restitution) and similar mechanisms can transfer as much as 80 to 100% of the parental heterozygosity to the autopolyploid (Peloquin, 1982; Hermsen, 1984; Vorsa and Ortiz, 1992; Bretagnolle and Thompson, 1995). Such high heterozygosity is often associated with higher plant vigor in both diploid and polyploid

species (Tomekpe and Lumaret, 1991; Werner and Peloquin, 1991). Secondly, the unification of 2n gametes minimizes levels of inbreeding depression in the raw autopolyploid. Since somatic doubling is the most extreme case of inbreeding, it is not surprising that autopolyploids generated in this way have displayed markedly lower fertility and vigor (Hague and Jones, 1987; Dewey, 1980; Hancock, 1996). Sexual polyploidization via 2n gametes produces autopolyploids with more genetic diversity than their progenitors which can be of direct adaptive benefit, and with polysomic inheritance this higher allelic diversity is available for future differentiation.

Probably the most important stumbling block in the establishment of a raw polyploid concerns competition with its already successful diploid progenitors. Several ways around this problem have been demonstrated including: 1) Partial separation (geographically or physiologically) between the new autopolyploids and their closely related diploid progenitors, minimizing the reductions in fertility caused by intercrossing between them (a reduction in the triploid block) (Borrill and Linder, 1971; Lumaret, 1985; Lumaret et al., 1987; Lumaret, 1988; Lumaret and Barrientos, 1990); 2) Chromosome doubling accompanied incidentally with biochemical, physiological, and developmental changes that immediately adapted the new polyploids to greater stress environments (reviewed by Levin, 1983); and 3) Heterosis resulting in the autopolyploid being competitively superior (Felber, 1991). Maceira et al. (1993) recently demonstrated using morphologically indistinguishable, sympatric diploid and tetraploid *Dactylis glomerata* that after two years of growth together, the tetraploid showed greater competitive ability than their diploid relatives. The autopolyploid substitution rate (4x : 2x) increased from 1.8 in the first year to 3.9 in the

second year. The tetraploids also had heavier seeds and faster leaf production in early spring than the diploids, and they flowered earlier. This experiment was particularly important, as the plant materials were from natural populations, rather than between colchicine-induced autopolyploids and their diploid progenitors (Sakai and Suzuki, 1955; Sakai, 1956; Hagberg and Ellerstrom, 1959). As previously mentioned, such artificially formed autopolyploids have been shown repeatedly to have reduced vigor and fertility presumably due to inbreeding depression (McCollum, 1958; Borrill, 1978;).

Polyploidy in Blueberry

Blueberry domestication began at the beginning of this century in the eastern US (Galletta, 1975; Galletta and Ballington, 1995). Through the efforts of breeders, dramatic improvements in terms of yield, berry quality, berry size, ripening season and geographical adaptation (Galletta, 1975; Galletta and Ballington, 1995) have been achieved. Interest in expanding blueberry production is now a worldwide goal (Hanson and Hancock, 1990; Galletta and Ballington, 1995).

Blueberries are in genus *Vaccinium* of the *Ericaceae*.They are all in the section *Cyanococcus* which comprises diploid, tetraploid and hexaploid species (Camp, 1945; Vander Kloet, 1983, and 1988). Three polyploids species: tetraploid lowbush, *V. anguistifolium* Ait., highbush, *V. corymbosum* L., and hexaploid rabbiteye, *V. ashei,* are the foundations of the commercial blueberry industry.

Early breeding efforts involved the selection of elite wild individuals and then intercrossing them for further selection. Concentrating on only a few elite wild selections led

initially to the development of a very narrow germplasm base. The nuclear genome of the most widely cultivated highbush blueberries were derived primarily from only 3 native selections, 'Brooks', 'Sooy' and 'Rubel' (Hancock and Siefker, 1982), and their cytoplasms came from only 4 sources (Hancock and Krebs, 1986). The predominant rabbiteye cultivars are derived from only 4 native selections, 'Myers', 'Black Giant', 'Ethel' and 'Clara' (Lyrene, 1988).

One of the negative consequences of such a narrow germplasm base is inbreeding depression (Hellman and Moore, 1983; Lyrene, 1983; Krebs and Hancock, 1988, Luby et al., 1991; Krebs and Hancock, 1990). Also, numerous traits have been noted in wild diploid species that have not been exploited in improving the current cultivars (Draper et al., 1982; Ballington, 1990; Hancock et al., 1995). The success of future breeding efforts are considered to be dependent on increasing the germplasm base to broaden climatic and soil adaptation, increase yield and disease resistance, and improve fruit quality (Galletta and Ballington, 1995). There have been several recent examples where new blueberry cultivars were developed through interspecific and interploidy crosses (Lyrene, 1990; Ballington, 1990; Hancock et al., 1995).

An important step in further promoting gene flow between the different ploidies will be a better understanding of the polyploid nature of blueberry (auto vs. allo) including: 1) the degree of genomic diversity within and between species, ploidies and newly derived interspecific polyploid hybrids, 2) modes of gene exchange between the different ploidies, and 3) elucidating the role of all the aforementioned processes on the evolution of blueberry.

Much progress has been made in studying the evolution of blueberry polyploids.

Originally, most of the tetraploid blueberries were considered to be allopolyploids (Camp, 1945; Eck, 1966; Vander Kloet, 1988) based primarily on the morphological traits. Cockerham and Galletta (1976) also suggested that *V. corymbosum* and four related tetraploid taxa were allopolyploids because the tetraploids as a group exhibited higher pollen stainability than a group of seven diploid species. Predominant bivalent associations at diakinesis and metaphase I were observed in all the tetraploids examined (Jelenkovic and Hough, 1970; Jelenkovic and Harrington, 1971; Vorsa, 1987 and 1995) except an artificially doubled genotype of *V. elloittii* (Dweikat and Lyrene, 1991). The non-randomness of chromosome association of highbush blueberry were interpreted as the result of obligatory pairing and localized distal chiasma (Jelenkovic and Hough, 1970). However, tetrasomic inheritance ratios have been demonstrated in highbush *V. corymbosum* (Draper and Scott, 1971; Krebs and Hancock, 1989) and lowbush *V. angustifolium* (Hokanson and Hancock, 1993), suggesting they are functionally autopolyploids. Tetraploid *V. angustifolium* and *V. corymbosum* are also completely interfertile, indicating the group as a whole may be autopolyploid (Krebs and Hancock, 1989).

Sexual hybridization via 2n gametes has been proposed as the origin of polyploid blueberries (Ortiz et al., 1992a) based on the findings that 2n gamete production is prevalent in blueberries. Unreduced pollen has been found in 2x (Ortiz et al., 1992b; Megalos and Ballington, 1988), 3x (Vorsa and Ballington, 1991), 4x (Ortiz et al., 1992a), 5x (Vorsa and Ortiz, 1992), 6x (Ortiz et al., 1992a) species and interspecific hybrids. Unreduced female gametes are also present in 2x species, evidenced by the production of tetraploid hybrids from 2x x 4x crosses (Megalos and Ballington, 1988; Draper et al., 1982).

While 2n gamete production in blueberry is quite common and has been successfully employed in breeding (Draper et al. 1982), research on the mechanism(s) responsible for their formation are only beginning to emerge. First division restitution with no cross over (FDR-NCO) has been proposed as the main cause of 2n pollen production in blueberries (Vorsa and Ortiz, 1992; Ortiz et al., 1992b). Study on 2n pollen formation in an aneuploid hybrid [7232-1 (2n = 4x + 9 = 57)] from [('Tifblue' x 'Darrow') x 'Rancocas'] revealed that the 2n gamete production involved three consecutive events: 1) desynapsis of paired chromosomes prior to metaphase I; 2) sister centromere disjunction in univalents at anaphase I; and 3) cytokinesis after telophase I leading to dyad formation (Vorsa and Ortiz, 1992). Similar synaptic disorders have also been reported in 2x and 4x blueberries (personal communication of Vorsa and Ortiz in Galletta and Ballington, 1995). Filler and Vorsa (personal communication in Galletta and Ballington, 1995) also found parallel and tripolar spindles, as well as synaptic irregularities, in the diploid *V. darrowi* and *V. elliottii*, and tetraploid biotypes of *V. pallidum*. Premature cytokinesis that led to the formation of 2n pollen in the 4x highbush cultivar 'Coville' was reported by Stushnoff and Hough (1968). However, numerous other types 2n gamete formation have been described in other species (Bretagnolle and Thompson, 1995) that have not been investigated in blueberry.

Information on genomic differentiation in blueberry is also limited, although a lack of reproductive barriers between similar ploidies has long been recognized (Camp, 1942), suggesting limited divergence. Fertile hybrids between the various ploidies of *V. corymbosum* are relatively easy to produce utilizing unreduced gametes (Luby et al., 1991; Ortiz et al, 1992a; Hancock et al., 1995). Cytological studies have found little chromosome

size difference among nine diploid species in *Vaccinium*, and one karyotype can be used to represent all the diploids (Hall and Galletta, 1971). Interspecific backcross hybrids of tetraploid x hexaploid crosses suggested that a minimum of two-thirds of the hexaploid chromosome complement of *V. ashei* could pair and recombine with that of the tetraploid *V. corymbosum* (Vorsa, 1987). Cytological analysis of 6 interspecific triploids derived from tetraploid x diploid *V. corymbosum* crosses revealed a range of chromosomal pairing relationship from autoploid to preferential pairing, depending on the diploid parents (Vorsa, 1989). However, because the chromosomes of blueberry are very small (1.5-2.5 μm in length) and largely indistinguishable (Hall and Galletta, 1971; Vorsa, 1989), exact pairing relationships have not been resolved.

Information on genomic differentiation and the nature of 2n gamete production would be particularly useful in utilizing the tetraploid hybrid, US 75, derived from a 2n gamete of Fla 4B (a selection of *V. darrowi* (2n = 2x = 24) and 'Bluecrop' [*V. corymbosum* (2n = 4x = 48). This particular hybrid is completely interfertile with highbush types (Draper et al., 1982) and has played an important role in the development of tetraploid highbush blueberries with a low chilling requirement (Ballington, 1990). It also has a number of additional elite traits that have the potential to further improve the northern tetraploid highbush blueberry (Erb et al., 1990 and 1993; Chandler et al., 1985; Hancock et al., 1992). However, little is known about the mode of 2n gamete formation in its diploid parent *V. darrowi* or the inheritance pattern of US 75. This information would be very useful in designing efficient breeding strategies. The type of gamete formation will determine the levels of heterozygosity transmitted to US 75, and the mode of inheritance will determine how readily that

heterozygosity will segregate. Furthermore, the information will be very useful in evaluating the phylogeny between the different ploidies.

Two features in the background of US 75 might lead to the prediction that it would act cytogenetically as an allopolyploid with disomic inheritance rather than an autopolyploid with tetrasomic inheritance. First, the two species are quite divergent, both morphologically and geographically. *V. darrowi* is an evergreen, lowbush type found in the southeastern US, while tetraploid *V. corymbosum* is a deciduous, highbush type found in the northern US. Secondly, Fla 4B's 2n genome was incorporated into US 75 via an unreduced gamete, so both sets of homologous chromosomes are available for pairing. However, the complete interfertility of US 75 with other tetraploid highbush types suggests that *V. darrowi* is little diverged from that of *V. corymbosum* and their hybrids may be autopolyploids.

The experiments in this dissertation focused on studying the genomic differentiation and evolution of blueberry using the interspecific hybrid US 75, its parents and related progeny populations. We not only determined the autopolyploid nature of US 75 employing molecular and cytogenetic methods, but also generated a genetic linkage map of US 75 and described several new mechanisms responsible for 2n gamete production in blueberry. Chapter 1 describes the inheritance patterns of RAPD markers in US 75 and the type of 2n gamete that probably produced it, by screening a population of US 75 x 'Bluetta' (a tetraploid cultivar). Chapter 2 presents a genetic linkage map of US 75 using the RAPD markers of chapter 1. Chapter 3 describes the meiotic pairing configurations and genomic similarity of US 75 and its parents using cytologenetic observations and genomic in situ hybridization (GISH). Chapter 4 presents four mechanisms of 2n gamete formation in selfed

progenies of US 75, by examining the cytogenetics of PMCs (pollen mother cells) and sporad configutations.

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CHAPTER 1

NATURE OF 2N GAMETE FORMATION AND MODE OF INHERITANCE IN INTERSPECIFIC HYBRIDS OF DIPLOID *VACCINIUM DARROWI* AND TETRAPLOID *V. CORYMBOSUM*

Abstract RAPD markers were used to determine the level of heterozygosity transmitted via 2n gametes from *V. darrowi* selection Florida 4B (Fla 4B) to inter-specific hybrids with tetraploid *V. corymbosum* cv. 'Bluecrop'. The tetraploid hybrid US 75 was found to contain about 70 % of Fla 4B's heterozygosity, a value attributed to a first division restitution (FDR) mode of 2n gamete production. Crossovers during 2n gamete formation were evidenced by the absence of 33 dominant alleles of Fla 4B in US 75, and direct tests of segregation in a diploid population involving Fla 4B. RAPD markers that were present in both Fla 4B and US 75 were used to determine the mode of inheritance in a segregating population of US 75 x *V. corymbosum* cv. 'Bluetta'. Sixty-five duplex loci were identified which segregated in a 5:1 ratio, indicating US 75 undergoes tetrasomic inheritance.

INTRODUCTION

Polyploidy has played an important role in both plant evolution and crop breeding. It is estimated that 30-50% of all angiosperms and more than 70% of the crop species are polyploid (Grant, 1971; Hancock, 1992). Breeding via 2n gametes has become an important aspect of many plant improvement programs, presumably due to the maintenance and transfer of higher levels of heterozygosity (Ballington, 1990; McCoy, 1982; Parrott et al., 1986; Ortiz and Peloquin, 1991). Most polyploids originated from sexual reproduction involving unreduced gametes (Harlan and De Wet, 1975). Unreduced gametes are formed in two primary ways: 1) an incomplete first meiotic division (first division restitution; FDR), and 2) an incomplete second meiotic division (second division restitution; SDR) (Mendiburu, 1971; Mok and Peloquin, 1975; McCoy, 1982; Hermsen, 1984). Other methods of 2n gamete production include premeiotic chromosome doubling, chromosome replication during meiotic interphase, postmeiotic chromosome doubling and apospory (Vorsa and Ortiz, 1992). Unreduced gametes via FDR are comprised mainly of the non-sister chromatids of each homologous pair of chromosomes, whereas in SDR, the sister chromatids are included in the same gametes. As a result, 2n gametes formed by FDR transmit more of the parental heterozygosity into F_1 progenies than SDR.

While few direct measurements have been made on levels of heterozygosity transmitted by SDR and FDR, theoretical calculations have been carried out. If it is assumed that there is a regular distribution of the parental heterozygous loci along chromosomes and a single crossover per pair of homologous chromosomes, then in the potato FDR has been estimated to transmit approximately 80% of the parental heterozygosity to progeny, while

SDR passes on about 40% (Hermsen, 1984). The rate at which transmitted heterozygosity ultimately assorts in polyploids is dependent on the mode of inheritance (Hancock, 1992). Segregation can be quite limited in allopolyploids with disomic inheritance, due to the maintenance of "fixed heterozygosities" on non-pairing homologous chromosomes. In autopolyploids, tetrasomic inheritance allows the variability contained in the original progenitors to segregate, but at a much slower rate than in diploids.

More and more recent attention has been paid to introducing traits from diploid to polyploid blueberry cultivars via unreduced gametes (Draper, 1977; Draper et al., 1982; Ballington, 1990; Ortiz et al., 1992). Several cultivars have been released with the genes of multiple species in their background (Ballington, 1990, Hancock et al., 1995). Particular emphasis has been placed on introducing some of the elite traits of diploid *V. darrowi* (high fruit quality, low chilling requirement, heat tolerance, high photosynthesis rate, drought resistance) into cultivars of tetraploid highbush blueberry, *V. corymbosum*. Completely interfertile F_1s are relatively easy to produce between these species because some *V. darrowi* plants produce a high number of unreduced gametes. Ortiz et al. (1992) demonstrated that about 83% of the various diploid populations of blueberries contain unreduced pollen producers and the highest producer was a genotype of *V. darrowi* with a rate of almost 20%.

Most of our research has focused upon US 75, a tetraploid hybrid (from Arlen Draper) generated by crossing a selection of *V. darrowi*, Florida 4B (Fla 4B), with a highbush cultivar, 'Bluecrop' (Draper, 1977). This particular hybrid is completely interfertile with highbush types (Draper et al., 1982) and has been used in crosses because of its high photosynthetic rate under hot and dry conditions (Hancock et al., 1992). However,

little is known about the mode of 2n gamete formation in *V. darrowi* or the inheritance patterns of US 75. This information would be very useful in designing efficient breeding strategies. The type of gamete formation will determine the levels of heterozygosity transmitted to US 75, and the mode of inheritance will determine how readily that heterozygosity will segregate.

The cytology of 2n pollen formation has not been reported for *V. darrowi* but, in inter-specific aneuploids (2n = 4x + 9 = 57) of *V. ashei* x *V. corymbosum*, Vorsa and Ortiz (1992) found the mode of 2n pollen formation to involve three steps: desynapsis, disjunction of sister chromatids, and cytokinesis. This mechanism is genetically equivalent to FDR, and therefore should result in the transmission of most of the parental heterozygosity (Vorsa and Ortiz, 1992). In the selfed progeny of US 75, few meiotic irregularities were found to lead to the production of 2n gametes including 1) premeiotic doubling, 2) tripolar spindle, 3) incomplete spindle at anaphase I, and 4) incomplete spindle at anaphase II (Qu and Hancock, in prep.). *V. corymbosum* has been shown to have tetrasomic inheritance at four enzyme loci (Krebs and Hancock, 1990), but segregation patterns have not been examined in any of the interspecific hybrids.

To determine the level of heterozygosity transmitted by 2n gametes of Fla 4B and the mode of inheritance in US 75, we selected genotype-specific RAPD markers and followed their inheritance from Fla 4B to US 75 and their segregation patterns in a US 75 x highbush cv. 'Bluetta' backcross population. The RAPD analysis allowed us to find and utilize a high number of markers in a short time span (Williams et al., 1990; Welsh and McClelland, 1990). Very few morphological (Draper and Scott, 1971), isozyme (Breuderle et al., 1991;

Van Heemstra et al., 1991; Bruederle and Vorsa, 1994), or RFLP markers (Haghighi and Hancock, 1992) have been described in blueberry and non-homologous chromosomes are not easy to distinguish (Hall and Galletta, 1971). RAPD markers have previously been utilized in blueberries to distinguish cultivars (Aruna et al., 1993) and develop a diploid linkage map (Rowland and Levi, 1994).

MATERIALS AND METHODS

Plant material

US 75, `Bluecrop', `Bluetta', Fla 4B, NC84 6-5 and two segregating populations were evaluated for their RAPD markers: 1) A tetraploid population of 61 individuals of US 75 X `Bluetta', and 2) 15 diploid individuals of Fla 4B x NC84 6-5. 'Bluetta' is a highbush cultivar and based on its pedigree is composed of 75 % *V. corymbosum* and 25% *V. angustifolium* (Draper et al., 1969). NC84 6-5 is a wild selection of *V. darrowi* kindly provided by J. Ballington.

DNA extractions and amplification conditions

Total cell DNA was isolated from young leaves using a modification of the CTAB procedure (Doyle and Doyle, 1987 as modified by Rowland and Nguyen, 1993). DNA was amplified in 12.5 µl volumes using 10 base primers (Operon Technologies Inc., Alameda, CA, and Biotechnology Laboratory, University of British Columbia). Primers were named by the initials of their source (OP and BC) and the company's lot number. Reaction conditions were: 1 ng/µl template DNA, buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.01% gelatin), 1.6 mM $MgCl_2$, 200 µM dATP, dCTP, dGTP, dTTP (Boehringer

Mannheim), 0.2 μM primer, and 0.06 units/ul Taq DNA polymerase (Gibco). DNA was amplified for 50 cycles in a Perkin Elmer thermal cycler programmed for 30 s denaturation at 94 ^{0}C, 70 s annealing at 48 ^{0}C and 120 s extension at 72 ^{0}C. The PCR products were separated through 1.2% agarose gels and visualized by ethidium bromide staining. Only reproducible fragments with strong bands were scored in our comparisons. All genotypes were subject to PCR at least twice.

Determining mode of inheritance

To determine the mode of inheritance in US 75, we first located RAPD markers that were present in both Fla 4B and US 75, but absent in `Bluecrop' and `Bluetta'. This would mean that the genotype of Fla 4B was either AA or Aa, `Bluecrop' and `Bluetta' were aaaa (nulliplex), and US 75 was either AAaa (duplex) or Aaaa (simplex). We then examined the progeny ratios in the testcross population of US 75 x `Bluetta'. Heterozygous pairs of alleles (Aa) transferred from Fla 4B to US 75 should segregate at a ratio of 1:1 (Aaaa:aaaa) for both disomic and tetrasomic inheritance, while homozygous pairs of alleles (AA) should segregate at a 5:1 ratio (Aaaa & AAaa:aaaa) for tetrasomic inheritance and a 1:0 ratio (all Aaaa) for disomic inheritance (Krebs and Hancock, 1989). For each segregating marker, a chi-square test of fit of progeny ratios was performed. We did not need to test for a 3:1 ratio as in Krebs and Hancock (1989), because we knew both dominant alleles in US 75 were contributed by Fla 4B, and as a result could not have been segregating as independent disomic loci.

Estimating heterozygosity and mode of 2n gamete production

To estimate the levels of heterozygosity maintained by 2n gamete formation in Fla 4B, we determined both the number of heterozygous allelic pairs (Aa) transferred from Fla 4B to US 75 and the number that were lost. As previously stated, FDR passes on much more heterozygosity than SDR, because FDR gametes contain the non-sister chromatids of each homologous pair of chromosomes, while SDR gametes contain only sister chromatids. The markers segregating (1:1) in the US 75 x `Bluetta' population represented the heterozygous allelic pairs that were transferred from Fla 4B to US 75.

To measure the level of heterozygosity lost through crossing over during 2n gamete production, we counted the number of unique dominant markers present in Fla 4B that were absent in US 75. The most likely way that dominant alleles can be present in Fla 4B but absent in US 75 is if a heterozygous locus was lost due to crossing over during 2n gamete production. This number was then doubled to include recessive alleles in the estimate of lost heterozygous loci. It was assumed that equal numbers of dominant and recessive alleles would be lost via crossing over during 2n gamete production.

In a few instances, we were able to directly document the loss of heterozygous loci from Fla 4B. We screened the diploid population of Fla 4B x NC84 6-5 with the primers producing markers that were present in Fla 4B and identified as duplex (AAaa) in US 75. We looked for markers which segregated 1:1 in the progeny when the marker was absent in NC84 6-5, and those which segregated 3:1 when the marker was present in NC84 6-5. These segregation ratios would only be possible if Fla 4B was heterozygous for that marker. Unfortunately, this approach was limited because most of the markers in Fla 4B and NC84

6-5 were shared.

The % heterozygosity transferred from Fla 4B to US 75 was ultimately calculated as the number of Fla 4B's heterozygous allelic pairs transferred to US 75 divided by the total number of heterozygous loci detected in Fla 4B. The total number of heterozygous loci in Fla 4B was calculated as the number of heterozygous allelic pairs transferred to US 75 and the number lost.

RESULTS AND DISCUSSION

Mode of inheritance

One hundred and forty-three of 512 primers produced a total of 267 polymorphic fragments that were present in Fla 4B and absent in 'Bluetta' and 'Bluecrop'. Of these, 234 (88%) were present in US 75, and 33 (12.4%) were absent. Of the markers found in US 75, 154 best fit a 1:1 ratio in the US 75 x 'Bluetta' population (Figure 1, Table 1), while 65 markers best fit a 5:1 ratio (Figure 2, Table 2). For all these loci, the alternate hypothesis of 1:1 or 5:1 segregation was statistically rejected ($P < 0.001$).

While the 1:1 ratios can not be used to distinguish between disomic and tetrasomic inheritance, the 5:1 segregation ratios suggest that the mode of inheritance in US 75 is tetrasomic. This is not surprising, as artificially produced interspecific hybrids between a wide range of *Vaccinium* species are highly fertile, indicating there is little genomic divergence within the genus (Hancock et al., 1995; Draper, 1977). Likewise, many different types of hybrids have been observed in nature (Vander Kloet, 1988; Breuderle and Vorsa,

Table 1. RAPD markers that fit a 1:1 (present : absent) segregation ratios in a progeny population of US 75 x 'Bluetta'

Primer	Primer Sequence	Fragment size (bases)	Observed ratio	X^2	P[a]
BC101	GCGGCTGGAG	600	28 : 33	0.41	0.55
		1340	34 : 27	0.80	0.42
BC105	CTCGGGTGGG	1840	24 : 37	2.77	0.09
BC125	GCGGTTGAGG	380	35 : 26	1.33	0.26
BC149	AGCAGCGTGG	2230	29 : 31	0.07	0.78
BC181	ATGACGACGG	2280	37 : 23	3.27	0.07
BC184	CAAACGGCAC	1390	26 : 34	1.07	0.30
BC189	TGCTAGCCTC	970	27 : 33	0.60	0.47
		1180	29 : 31	0.07	0.47
BC244	CAGCCAACCG	550	36 : 24	2.40	0.15
BC516	AGCGCCGACG	1050	28 : 33	0.41	0.55
BC523	ACAGGCAGAC	730	27 : 33	0.60	0.47
		1720	35 : 25	1.67	0.18
BC536	GCCCCTCGTC	1590	31 : 27	0.28	0.65
BC540	CGGACCGCGT	1230	34 : 26	1.07	0.30
BC546	CCCGCAGAGT	1580	36 : 24	2.40	0.15
OPA16	AGCCAGCGAA	1590	28 : 33	0.41	0.55
OPA19	CAAACGTCGG	990	28 : 33	0.41	0.55
OPC02	GTGAGGCGTC	390	30 : 29	0.02	0.85
OPC06	GAACGGACTC	290	32 : 28	0.27	0.65
		350	29 : 31	0.07	0.78
		1360	34 : 26	1.07	0.30
OPC15	GACGGATCAG	1960	29 : 30	0.02	0.85
OPC16	CACACTCCAG	1230	35 : 26	1.33	0.26
		1350	35 : 26	1.33	0.26
OPF01	ACGGATCCTG	800	34 : 26	1.07	0.30
		1300	28 : 32	0.27	0.65
		2760	27 : 33	0.60	0.47
OPF04	GGTGATCAGG	850	27 : 34	0.80	0.42
OPF05	CCGAATTCCC	1410	28 : 32	0.27	0.65
OPF05	CCGAATTCCC	2000	28 : 33	0.41	0.55
OPF08	GGGATATCGG	1410	30 : 31	0.02	0.85
OPF12	ACGGTACCAG	1230	32 : 28	0.27	0.65
OPG08	TCACGTCCAC	1520	30 : 31	0.02	0.85
OPH02	TCGGACGTGA	990	26 : 34	1.07	0.30

Table 1 (cont'd)

		2250	30 : 30	0.00	1.00
OPH03	AGACGTCCAC	2210	30 :31	0.02	0.85
OPH05	AGTCGTCCCC	1100	34 : 27	0.80	0.42
OPH07	CTGCATCGTG	2210	27 : 28	0.02	0.85
OPH12	ACGCGCATGT	180	36 : 25	1.98	0.17
OPH13	GACGCCACAC	1110	27 : 32	0.42	0.54
OPI09	TGGAGAGCAG	590	24 : 36	2.40	0.15
OPI20	AAAGTGCGGG	1590	36 : 25	1.98	0.17
OPJ04	CCGAACACGG	2330	33 : 26	0.83	0.40
		2700	33 : 26	0.83	0.40
OPJ09	TGAGCCTCAC	730	31 : 30	0.02	0.85
OPJ14	CACCCGGATG	370	26 : 35	1.33	0.26
		1020	29 : 32	0.15	0.70
OPJ17	ACGCCAGTTC	1600	28 : 31	0.15	0.70
		3690	27 : 32	0.42	0.54
OPK04	CCGCCCAAAC	700	29 : 32	0.15	0.70
OPK14	CCCGCTACAC	300	29 : 29	0.00	1.00
		680	31 : 27	0.28	0.65
OPK17	CCCAGCTGTG	680	26 : 33	0.83	0.40
OPK19	CACAGGCGGA	860	27 : 33	0.60	0.47
OPK20	GTGTCGCGAG	960	28 : 32	0.27	0.65
OPL02	TGGGCGTCAA	2090	33 : 26	0.83	0.40
		2460	27 : 32	0.42	0.54
OPL10	TGGGAGATGG	1270	25 : 32	0.86	0.39
OPL11	ACGATGAGCC	1470	24 : 36	2.40	0.15
OPL13	ACCGCCTGCT	860	34 : 26	1.07	0.30
		2150	26 : 33	0.83	0.40
OPL14	GTGACAGGCT	610	23 : 37	3.27	0.07
OPL15	AAGAGAGGGG	420	32 : 29	0.15	0.70
		550	36 : 25	1.98	0.15
		730	29 : 32	0.15	0.70
		1470	28 : 33	0.41	0.55
OPM04	GGCGGTTGTC	980	35 : 26	1.33	0.26
OPM09	GTCTTGCGGA	1420	33 : 28	0.41	0.55

Table 1 (cont'd)

OPM13	GGTGGTCAAG	3560	36 : 25	1.98	0.17
OPM16	GTAACCAGCC	650	29 : 32	0.15	0.70
OPM17	TCAGTCCGGG	1960	23 : 38	3.69	0.07
OPM19	CCTTCAGGCA	530	34 : 27	0.80	0.42
OPM20	AGGTCTTGGG	830	33 : 28	0.41	0.55
OPN11	TCGCCGCAAA	680	30 : 30	0.00	1.00
OPN16	AAGCGACCTG	570	34 : 27	0.80	0.42
	AAGCGACCTG	1110	29 : 32	0.15	0.70
OPO02	ACGTAGCGTC	1230	27 : 34	0.80	0.42
OPO04	AAGTCCGCTC	1580	33 : 28	0.41	0.55
OPO06	CCACGGGAAG	1530	34 : 27	0.80	0.42
OPO07	CAGCACTGAC	1830	29 : 32	0.15	0.70
OPO12	CAGTGCTGTG	1200	33 : 28	0.41	0.55
OPO13	GTCAGAGTCC	680	30 : 31	0.02	0.85
OPO14	AGCATGGCTC	610	37 : 24	2.77	0.09
OPO16	TCGGCGGTTC	890	26 : 35	1.33	0.26
OPP01	GTAGCACTCC	700	33 : 28	0.41	0.55
		2090	33 : 28	0.41	0.55
		2390	30 : 31	0.02	0.85
OPP04	GTGTCTCAGG	700	24 : 37	2.77	0.09
		850	30 : 31	0.02	0.85
		1230	32 : 29	0.15	0.70
OPP07	GTCCATGCCA	1350	31 : 30	0.02	0.85
OPP16	CCAAGCTGCC	620	32 : 29	0.15	0.70
OPQ03	GGTCACCTCA	1000	33 : 28	0.41	0.55
		1350	33 : 28	0.41	0.55
OPQ05	CCGCGTCTTG	860	32 : 29	0.15	0.70
OPQ09	GGCTAACCGA	2050	29 : 32	0.15	0.70
OPQ13	GGAGTGGACA	1470	27 : 34	0.80	0.42
OPR01	TGCGGGTCCT	1250	27 : 34	0.80	0.42
OPR09	TGAGCACGAG	1490	30 ; 31	0.02	0.85
		2000	29 : 32	0.15	0.70
OPR11	GTAGCCGTCT	850	27 : 34	0.80	0.42
OPR13	GGACGACAAG	480	29 : 32	0.15	0.70
OPR16	CTCTGCGCGT	240	34 : 27	0.80	0.42
OPS13	GTCGTTCCTG	810	32 : 28	0.27	0.65
OPS15	CAGTTCACGG	780	34 : 26	1.06	0.32
OPT06	CAAGGGCAGA	680	28 : 33	0.41	0.60

Table 1. (Cont'd)

OPT07	GGCAGGCTGT	730	32 : 39	0.15	0.70
OPT12	GGGTGTGTAC	360	29 : 32	0.15	0.70
		1650	27 : 34	0.80	0.42
OPT14	AATGCCGCAG	900	22 : 37	3.81	0.06
		1590	27 : 32	0.42	0.54
OPU03	CTATGCCGAC	750	27 : 34	0.80	0.42
		1750	24 : 37	2.77	0.09
OPU07	CCTGCTCATC	1840	29 : 32	0.15	0.75
OPU16	CTGCGCTGGA	1350	28 : 33	0.41	0.55
OPV04	CCCCTCACGA	2090	29 : 32	0.15	0.70
		2210	29 : 32	0.15	0.70
OPV08	GGACGGCGTT	600	35 : 26	1.33	0.26
		1370	25 : 36	1.98	0.17
OPV14	AGATCCCGCC	470	36 : 25	1.98	0.17
		1520	27 : 34	0.80	0.42
OPW03	GTCCGGAGTG	310	33 : 28	0.41	0.55
		560	28 : 33	0.41	0.55
OPW06	AGGCCCGATG	570	31 : 30	0.02	0.85
		860	31 : 30	0.02	0.85
OPW14	CTGCTGAGCA	520	26 : 35	1.33	0.26
OPX06	ACGCCAGAGG	1220	30 : 31	0.02	0.85
OPX07	GAGCGAGGCT	1830	30 : 31	0.02	0.85
OPX19	TGGCAAGGCA	580	33 : 28	0.41	0.55
		1210	27 : 34	0.80	0.42
OPZ01	TCTGTGCCAC	280	24 : 37	2.77	0.09
		2210	23 : 38	3.69	0.07
OPZ03	CAGCACCGCA	580	29 : 32	0.15	0.70
OPZ04	AGGCTGTGCT	610	33 : 28	0.41	0.55
		980	29 : 32	0.15	0.70
OPZ06	GTGCCGTTCA	610	31 : 30	0.02	0.85
OPZ07	CCAGGAGGAC	3100	31 : 29	0.07	0.78
OPZ11	CTCAGTCGCA	430	37 : 24	2.77	0.09
OPZ15	CAGGGCTTTC	2090	30 : 31	0.02	0.85
OPZ16	TCCCCATCAC	890	31 : 30	0.02	0.85
OPZ16	TCCCCATCAC	1790	32 : 29	0.15	0.70
OPAG01	CTACGGCTTC	230	31 : 30	0.02	0.85
OPAG07	CACAGACCTG	860	31 : 30	0.02	0.85
OPAG16	CCTGCGACAG	910	24 : 37	2.77	0.09

Table 1. (Cont'd)

OPAJ04	GAATGCGACC	1590	29 : 32	0.15	0.70
		2200	31 : 30	0.02	0.85
OPAJ14	ACCGATGCTG	1720	30 : 31	0.02	0.85
OPAK05	GATGGCAGTC	1590	23 : 38	3.69	0.07
OPAK15	ACCTGCCGTT	1790	33 : 28	0.41	0.55
OPAK16	CTGCGTGCTC	380	27 : 34	0.80	0.42
		610	30 : 31	0.02	0.85
OPAM01	TCACGTACGG	1590	31 : 30	0.02	0.85
		1840	33 : 28	0.41	0.55

[a] the alternate hypothesis of 5:1 was rejected at $P<0.001$ in all cases.

Figure 1. DNA amplification profiles in the tetraploid progeny population US 75 x 'Bluetta' using primer OPS13. The arrow shows a RAPD marker segregating in a 1:1 ratio (presence:absence). From right to left, first lane is a 123 bp DNA ladder, lanes 2-4 are Fla 4B, 'Bluecrop', US 75 and 'Bluetta', respectively. The remaining lanes are from 23 progeny.

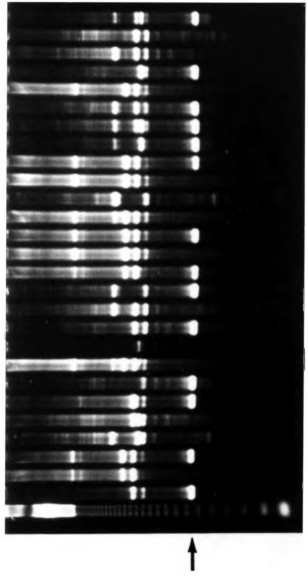

Figure 1

Table 2. RAPD Markers that fit a 5:1 (present : absent) segregation ratio in the progeny population of US 75 x 'Bluetta'

Primer	Primer sequence	Fragment size (bases)	Observation ratio	X^2	P[a]
BC189	TGCTAGCCTC	615	53 : 7	1.08	0.30
BC292	AAACAGCCCG	980	45 : 13	1.37	0.25
		1850	49 : 9	0.05	0.78
BC504	ACCGTGCGTC	1520	53 : 7	1.08	0.30
BC516	AGCGCCGACG	1960	51 : 10	0.003	0.95
BC521	CCGCCCCACT	960	51 : 10	0.003	0.95
BC540	CGGACCGCGT	350	50 : 10	0.00	1.00
OPB18	GTCCACACGG	660	54 : 7	1.18	0.29
OPC02	GTGAGGCGTC	820	46 : 13	1.22	0.28
OPC06	GAACGGACTC	620	46 : 14	1.92	0.18
OPE03	CCAGATGCAC	460	53 : 7	1.08	0.30
OPF01	ACGGATCCTG	630	49 : 11	0.12	0.75
OPFO5	CCGAATTCCC	1410	46 : 14	1.92	0.18
OPG08	TCACGTCCAC	410	52 : 9	0.09	0.78
OPH12	ACGCGCATGT	560	51 : 10	0.003	0.95
		2350	50 : 11	0.08	0.79
OPI20	AAAGTGCGGG	3180	50 : 9	0.08	0.79
OPJ01	CCCGGCATAA	730	46 : 9	0.004	0.95
OPJ09	TGAGCCTCAC	1280	50 : 11	0.08	0.79
OPJ19	GGACACCACT	490	48 : 12	0.48	0.50
OPK04	CCGCCCAAAC	610	48 : 13	0.57	0.49
OPK19	CACAGGCGGA	700	47 : 13	1.08	0.30
		2580	48 : 12	0.48	0.46
OPL07	AGGCGGGAAC	1590	50 : 9	0.08	0.79
		1840	49 : 10	0.003	0.95

Table 2. (cont,d)

OPL10	GTGACAGGCT	1580	47 : 7	0.58	0.49
OPL14	GTGACAGGCT	1150	46 : 14	1.92	0.18
OPL15	AAGAGAGGGG	700	56 : 5	3.15	0.08
		2700	48 : 13	0.57	0.49
OPL19	GAGTGGTGAC	1560	52 : 9	0.16	0.69
OPM10	TCTGGCGCAC	580	46 : 15	2.49	0.12
OPN16	AAGCGACCTG	430	50 : 11	0.08	0.79
OPO04	AAGTCCGCTC	960	48 : 13	0.57	0.49
OPO13	GTCAGAGTCC	820	47 : 14	1.86	0.18
OPO09	TCCCACGCAA	720	53 : 7	1.08	0.30
		1480	51 : 9	0.13	0.72
OPO19	CAGTTCACGG	1150	49 : 12	0.48	0.46
OPP12	AAGGGCGAGT	900	48 : 10	0.02	0.85
OPP19	GGGAAGGACA	2700	50 : 10	0.00	1.00
OPQ14	GGACGCTTCA	1650	55 : 5	3.00	0.08
OPR01	TGCGGGTCCT	950	50 : 11	0.08	0.79
OPR13	GGACGACAAG	270	51 : 10	0.003	0.95
OPS15	CAGTTCACGG	730	48 : 12	0.48	0.50
OPS19	GAGTCAGCAG	490	53 : 7	1.08	0.30
		730	51 : 9	0.04	0.80
OPT06	CAAGGGCAGA	490	53 : 8	0.55	0.51
OPT14	AATGCCGCAG	2580	50 : 9	0.08	0.79
		3440	47 : 12	0.57	0.49
OPU03	CTATGCCGAC	540	55 : 6	2.05	0.17
OPU08	GGCGAAGGTT	1420	51 : 10	0.003	0.95
OPU11	AGACCCAGAG	960	52 : 8	0.48	0.50
OPU14	TGGGTCCCTC	1890	53 : 8	0.55	0.45
OPV01	TGACGCATGG	1100	50 : 11	0.08	0.79

Table 2. (cont,d)

OPV08	GGACGGCGTT	860	52 : 9	0.16	0.69
OPV10	GGACCTGCTG	490	47 : 14	1.73	0.20
OPW03	GTCCGGAGTG	1410	51 : 10	0.003	0.95
OPX07	GAGCGAGGCT	1050	51 : 10	0.003	0.95
OPX15	CAGACAAGCC	840	52 : 9	0.08	0.79
		1610	54 : 7	0.33	0.61
OPZ12	TCAACGGGAC	980	53 : 6	1.79	0.21
OPAG15	CCCACACGCA	520	52 : 9	0.08	0.79
		1380	54 : 7	0.33	0.61
OPAJ04	GAATGCGACC	860	54 : 7	0.33	0.61
OPAK05	GATGGCAGTC	950	51 : 10	0.003	0.95
OPAL18	GGAGTGGACT	1050	52 : 9	0.16	0.69

[a] The alternate hypothesis of 1:1 was rejected at $P<0.001$ in all cases.

Figure 2. DNA amplification profiles in the tetraploid progeny population US 75 x 'Bluetta' using primer OPV06. The arrow shows a RAPD marker segregating in a 5:1 ratio (presence:absence). From right to left, first lane is a 123 bp DNA ladder, lanes 2-4 are Fla 4B, 'Bluecrop', US 75 and 'Bluetta', respectively. The remaining lanes are from 23 progeny.

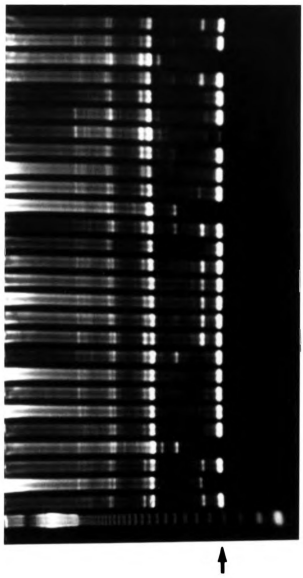

Figure 2

1994), and several studies have supported tetrasomic inheritance in both *V. corymbosum* (Draper and Scott, 1971; Krebs and Hancock, 1989) and *V. angustifolium* (Hokanson and Hancock, 1993).

Table 3. RAPD markers which did not statistically fit a 5:1 or 1:1 segregation ratio.

Primer	Primer sequence	Fragment size (bases)	observed ratio (present : absent)
BC181	ATGACGACGG	1470	40 : 21
BC239	CTGAAGCGGA	1540	42 : 18
OPH03	AGACGTCCAC	2090	43 : 18
OPJ14	CACCCGGATG	3200	43 : 18
OPR13	GGACGACAAG	290	5 : 56
OPT16	GGTGAACGCT	750	45 : 16

Fifteen markers displayed distorted segregation ratios in the tetraploid progeny population US 75 x 'Bluetta'. Six of these segregated but did not fit either a 1:1 or 5:1 ratio (Table 3). Five of the six markers (BC181, OPH03, BC239, OPL14 and OPT16) may represent independent heterozygous loci in Fla 4B that were subject to segregation distortions and may signal incomplete homology between some of the chromosomes of *V. darrowi* and *V. corymbosum*. The remaining class of the markers were found in US 75, but were absent in the progeny population (Table 4, Figure 3). The loss of these markers may have been due to mutations in heterozygous rather than homologous loci , as only single allelic alterations would be necessary to eliminate markers. It is unclear why so many mutations were observed, unless a portion of a chromosome was lost.

Figure 3. DNA amplification profiles in the tetraploid progeny population US 75 x 'Bluetta' using primer OPI09. The arrow shows a RAPD marker that was present in Fla 4B and US 75, but absent in the population. From right to left, first lane is a 123-bp DNA ladder, lanes 2-4 are Fla 4B, 'Bluecrop', US 75 and 'Bluetta', respectively. The remaining lanes are from 25 progeny.

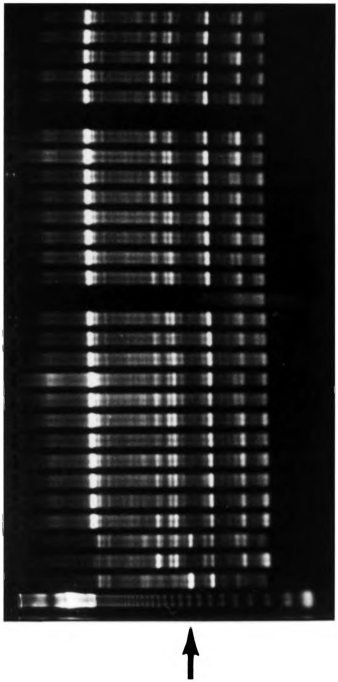

Figure 3

Table 4. RAPD markers which were present in Fla 4B and US 75, but absent in the progeny of US 75 x 'Bluetta'.

Primer	Primer sequence	Fragment size (bases)
BC101	GCGGCTGCAG	1720
OPI09	TGGAGAGCAG	1050
OPK20	GTGTCGCGAG	2260
OPM17	TCAGTCCGGG	1520
OPN14	TCGTGCGGGT	4100
OPO09	TCCCACGCAA	3300
OPS17	TGGGGACCAC	2160
OPT11	TTCCCCGCGA	1280
OPV10	GGACCTGCTG	4250

Levels of heterozygosity and mode of 2n gamete production

To calculate the level of heterozygosity transferred from Fla 4B to US 75 via unreduced gamete production, we determined the number of heterozygous loci that were transferred to US 75, plus the number that were lost via 2n gamete production. The number of Fla 4B markers segregating as simplex loci in the tetraploid population was 154, i.e. those markers that segregated in a 1:1 ratio. The number of unique markers in Fla 4B that were not present in US 75 was 33 (Table 5, Figure 5). Therefore, 66 heterozygous allelic pairs were lost during 2n gamete production in Fla 4B, if we assume that both dominant and recessive alleles were lost at the same rate. This means that 69.5 % of Fla 4B's heterozygosity was transferred to US 75 via 2n gamete production[(154 / (66 + 154)]. If we also include the markers which gave unusual segregation ratios, the level of heterozygosity transmitted was 71.9% [(169 / (66 + 169)]. Since these values are closer to the theoretical rate of heterozygosity transfer in FDR (80%) than SDR (40%), it is likely that unreduced gametes are being formed in Fla 4B via FDR or a similar mechanism.

It is not known why our levels of transferred heterozygosity were somewhat lower than the predicted level of 80% ; however, more than one crossover per arm would reduce predicted levels of transmitted heterozygosity. Also, recent studies have shown that a high proportion of the polymorphic RFLP markers commonly utilized in genetic mapping studies are found on the ends of chromosomes (Gill and Gill, 1994). This would negatively bias estimates of transferred heterozygosity via FDR 2n gamete production, since a higher proportion of heterozygosity would be lost due to crossing over than would be predicted from a random distribution of genes along a chromosome. Several other abnormal meiotic

Table 5. Primers which produced amplification products in Fla 4B that were absent in US 75.

Primer	Primer sequence	Fragment size (bases)	Number of markers
BC127	ATCTGGCAGC	730	1
BC222	AAGCCTCCCC	420	1
BC149	AGCAGCGTGG	4100	1
OPJ01	CCCGGCATAA	3200	1
OPK04	CCGCCCAAAC	2100	1
OPK11	AATGCCCCAG	2460, 2700	2
OPK14	CCCGCTACAC	1470, 1590	2
OPK20	GTGTCGCGAG	1860	1
OPM16	GTAACCAGCC	760	1
OPN01	CTCAGGTTGG	1100	1
OPN02	ACCAGGGGCA	1000	1
OPN07	CAGCCCAGAG	610	1
OPO05	CCCAGTCACT	1680, 2210	2
OPO09	TCCCACGCAA	330	1
OPO18	CTCGCTATCC	860	1
OPP05	CCCCGGTAAC	1180	1
OPP13	GGAGTGCCTC	1290, 1520	2
OPP17	GTCCATGCCA	1720	1
OPP18	GGCTTGGCCT	860	1
OPQ04	AGTGCGCTGA	3000	1
OPQ07	CCCCGATGGT	910	1
OPQ13	GGAGTGGACA	690	1
OPS18	CTGGCGAACT	450, 4200	2
OPT07	GGCAGGCTGT	3350	1
OPT12	GGGTGTGTAG	2210	1
OPT14	AATGCCGCAG	2220	1
OPZ03	CAGCACCGCA	1840	1
OPZ16	TCCCCATCAC	1060	1

Figure 4. DNA amplification reactions in the tetraploid progeny population US 75 x 'Blueetta' using primer OPP17. The arrow shows a RAPD marker that was present in Fla 4B, but absent in US 75. From right to left, first lane is a 123 bp DNA ladder, lanes 2-4 are Fla 4B, 'Bluecrop', US 75 and 'Bluetta', respectively. The remaining lanes are from 25 progeny.

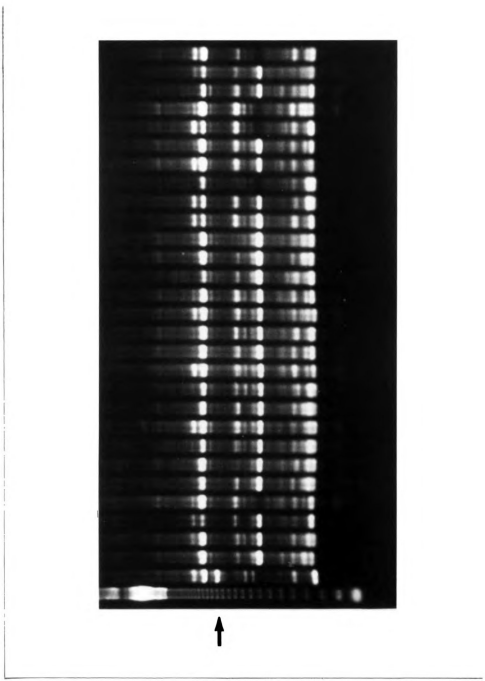

Figure 4

behaviors can produce levels of heterozygosity that mimic FDR, such as synaptic mutants, lack of homology, unbalanced ploidy or any other cause that hampers homologous pairing; but in all these instances, only 2n gametes are functional (Iwanaga, 1984; Hermsen, 1984). Vorsa et al. (1992) observed a desynaptic mechanism in an interspecific blueberry aneuploid (2n=4x+9= 57) that produced almost solely 2n pollen. This is not the case in Fla 4B, however, as the majority of its gametes are reduced and viable (Ortiz et al., 1992).

Our data also indicate that numerous crossovers have occurred during 2n gamete production in Fla 4B, which is not consistent with mechanisms associated with synaptic mutations or limited homology. We identified 33 dominant markers in Fla 4B that were absent in US 75 (Table 5, Figure 5); this most likely occurred due to crossovers between the centromere and heterozygous loci. Likewise, several recessive markers were not transferred from heterozygous loci in Fla 4B to US 75. These were discovered when the diploid population of Fla 4B x NC84 6 - 5 was screened for 31 markers that segregated 5:1 (duplex loci-AAaa) in the tetraploid population and 8 markers gave skewed segregation ratios. Of the 39 markers that fit this category, six of those that segregated 5:1 and one with a skewed tetraploid ratio were found to segregate in the diploid population (Figure 6, Table 6), signaling the loss of the recessive allele during 2n gamete production via crossing over. Four of these were present in Fla 4B and absent in NC84 6-5 and segregated 1:1 in the progeny. Three were present in both parents and segregated 3:1. One of these (OPH03) had produced an unexpected ratio in the tetraploid population. Several other heterozygous loci in Fla 4B probably remained undetected, because NC84 6-5 was homozygous for the same dominant marker as Fla 4B and could not be subjected to a segregation analysis. In total,

Table 6. Segregation ratios in the diploid population Fla b x NC84 6-5 for the RAPD markers which segregated 5:1 or had skewed segregation ratios in the tetraploid population US 75 x 'Bluetta'.

Primer	Fragment size (bases)	Observed ratio	expected ratio	X^{2a}	P
BC504	1520	6 : 9	1 : 1[b]	0.27	0.65
OPH12	2350	6 : 9	1 : 1[b]	0.27	0.65
OPC06	620	9 : 6	1 : 1[b]	0.27	0.65
OPF01	630	6 : 9	1 : 1[b]	0.27	0.65
OPH03	2090	12 : 2	3 : 1[c]	0.38	0.52
BC516	1960	8 : 6	3 : 1[c]	1.52	0.21
OPL15	2700	10 : 5	3 : 1[c]	0.20	0.68

a The chi-square tests were done using the Yates correction for small population size (Strickberger, 1985.
b Marker present in Fla4B but absent in NC84 6-5.
c Marker present in both Fla 4B and NC84 6-5.

these data provide clear evidence for the formation of multiple chiasmata during 2n gamete production.

The data presented herein can also be used to estimate where the average position of crossing over occurs on chromosomes. Since 50% of the heterozygous loci involved in crossing over can become duplex loci via FDR, the total number of heterozygous loci that were involved in cross overs in Fla 4B was 132 (2 x 66). Therefore, the percentage of the crossing-over involving heterozygous loci was 60% (132/220). If it is assumed that the heterozygous loci were evenly distributed along the chromosome and that there are two cross overs per bivalent, one on each paired arm [chromosomes in blueberries have median or submedian centromere (Hall and Galletta, 1971)], the average cross over point should be located about 2/5 of the arm length from the centromere. However, the average cross over position may actually be closer to the centromere, as more than one cross over per paired

Figure 5 DNA amplification reactions in the diploid progeny population Fla 4B x NC84 6-5 using primer OPC06. The arrow shows a RAPD marker segregating in a 1:1 ratio (presence:absence). This same marker segregated 5:1 in the tetraploid population US 75 x 'Bluetta' (Figure 6). From right to left, first lane is a 123 bp DNA ladder, lanes 2 and 3 are Fla 4B and NC84 6-5, respectively. The remaining lanes are from 15 progeny.

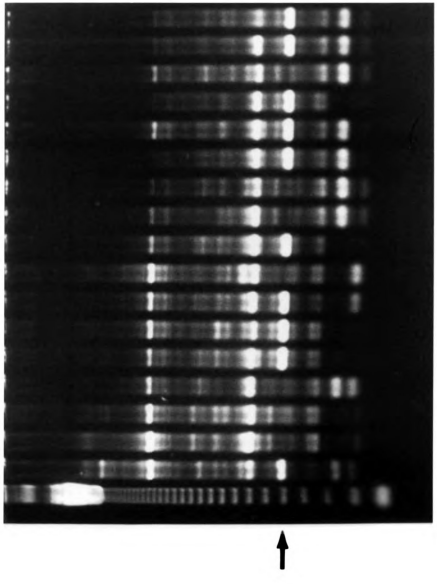

Figure 5

Figure 6. DNA amplification reactions in the tetraploid progeny population US 75 x 'Bluetta' using primer OPC06. The arrow shows a RAPD marker segregating in a 5:1 ratio (present : absent). This same marker segregated in a 1 :1 ratio (present : absent) in the diploid progeny population Fla 4B x NC84 6-5 (Figure 5). From right to left, first lane is a 123 bp DNA ladder, lanes 2-4 are Fla 4B, 'Bluecrop' US 75 and 'Bluetta', respectively. The remaining lanes are from 39 progeny.

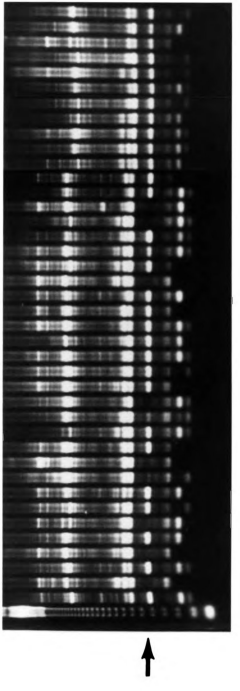

Figure 6

arm can decrease the observed value of cross over and we have found evidence that there are more than one cross over occurs per paired arm in blueberry (Chapter 3 of this dissertation).

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CONCLUSIONS

With these results, it is not surprising that Fla 4B has been such a useful parent in breeding highbush blueberries. Fla 4B is highly heterozygous and this heterozygosity was readily transmitted to its tetraploid progeny US 75. We only examined one product of an unreduced gamete, but this hybrid appeared to carry about 70% of Fla 4B's heterozygosity. This heterozygosity was shown to readily segregate in the tetraploid background through tetrasomic rather than disomic inheritance. This makes a high proportion of the genome of Fla 4B available for tetraploid highbush breeding.

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CHAPTER 2

RAPD-BASED GENETIC LINKAGE MAP OF BLUEBERRY DERIVED FROM AN INTERSPECIFIC CROSS BETWEEN DIPLOID *VACCINIUM DARROWI* AND TETRAPLOID *V. CORYMBOSUM*

Abstract. A tetraploid blueberry population resulting from a cross of US 75 {a tetraploid hybrid of Fla 4B [a selection of *Vaccinium darrowi* (2n = 2x = 24) X 'Bluecrop' [(*V. corymbosum* (2n = 4x =48)]} x 'Bluetta' (4x) was evaluated by RAPD (random amplified polymorphic DNA) analysis to generate a genetic linkage map of US 75. One hundred and forty markers unique for Fla 4B that segregated 1:1 in the population were mapped into 29 linkage groups that cover a total genetic distance of 1288.2 cM, with a range of 0.0 - 33.9 cM between adjacent markers. The map is essentially of *V. darrowi* because US 75 was produced via a 2n gamete from Fla 4B and only unique markers for Fla 4B were utilized.

INTRODUCTION

Molecular markers are being widely employed by plant breeders to assist in selection and location of genes (Gregory et al., 1993; Paran and Michelmore, 1993; Lehner et al., 1995). Isozymes and restriction fragment length polymorphisms (RFLPs) were the dominant molecular markers before 1990's. In recent years, PCR (polymerase chain reaction) based DNA marker, RAPDs (randomly amplified polymorphic DNA sequences) have been increasingly employed in mapping. RFLPs and RAPDs are popular because they generate a larger number of markers than isozymes. Although RFLPs' codominant nature makes them more attractive than RAPDs for genetic analysis, their production also requires larger quantities of template DNA and more complicated operational procedures, including the generation of genomic or cDNA probes, digesting template DNA with restriction enzymes, and southern blotting with radioactive probes. For this reason RAPDs have become popular.

To date, most molecular maps have been generated on herbaceous crops. Linkage maps have been produced for alfalfa (Echt et al., 1993), asparagus (Lewis and Sink, 1996), barley (Heun et al., 1991), carrot (Schulz et al., 1994), cole crops (Landry et al., 1991, Slocum et al., 1990; Song et al., 1991), common bean (Vallejos et al., 1992), cucumber (Kennard et al., 1994), lettuce (Landry et al., 1987), maize (Helenjaris et al., 1987), pepper (Lefebvre et al., 1995), potato (Gebhardt et al., 1989; Bonierbale et al., 1988), rice (McCouch et al., 1988), sorghum (Pereira et al., 1994), soybean (Keim el al., 1990), sugar cane (Da Silva et al., 1993; D'Hont et al., 1993), sunflower (Gentzbittel et al., 1995), tomato (Bernatsky and Tanksley, 1986; Helentjaris et al., 1986) and wheat (Gill et al., 1991; Lagudah et al., 1991). Among the woody fruit crops only apple (Hemmat et al., 1994),

blueberry (Rowland and Levi, 1994), cherry (Stockinger et al., 1996), citrus (Durham et al., 1992; Jarrel et al., 1992), grape (Lodhi et al., 1995) and peach (Chaparro et al., 1994; Rajapake et al., 1995) have been mapped using molecular markers.

The reason why woody perennial maps have lagged behind herbaceous annuals has generally been attributed to their long periods of juvenility and the inbreeding depression that often occurs in narrow crosses of outcrossing species (Rowland and Levi, 1994; Jarrel et al., 1992). However, marker-assisted selection should prove very beneficial in saving land resources and labor in woody perennials, if potentially low value genotypes can be eliminated in the seedling stage before field planting. Linked molecular markers should prove particularly useful in transferring traits from wild to cultivated species, where early generations of crosses are horticulturally poor except for the genes of interest.

In the last two decades, more and more attention has been paid to introducing traits from wild diploid blueberries into the polyploid, *V. corymbosum* (highbush) and *V. ashei* (rabbiteye) species. Several cultivars have now been released with the genes of more than one species in their background (Ballington, 1990; Hancock et al., 1995; Draper, 1977; Draper et. al., 1982). One of the most widely used breeding parents has been Fla 4B (*V. darrowi*), a diploid species native to the southeastern part of the United States. Completely fertile F_1 progeny are relatively easy to produce between *V. darrowi* and *V. corymbosum* as *V. darrowi* produces a large number of unreduced gametes (Draper et al., 1982; Ortiz et al., 1992).

The most useful inter-specific hybrid parent of *V. darrowi* x *V. corymbosum* has been US 75, a tetraploid generated by Dr. Arlen Draper at the USDA Fruit Laboratory, Beltsville,

Maryland, by crossing Fla 4B with the highbush cultivar `Bluecrop' (Draper et al., 1982). US 75 has been used primarily to reduce the chilling requirement of highbush types, although it also transmits high fruit quality, tolerance to mineral soils (Erb et al., 1990; and 1993; Chandler et al., 1985) and a high photosynthetic rate under both hot and dry conditions (Hancock et al., 1992; Moon et al., 1987).

Our current research has focused on incorporating the fruit quality and heat tolerance of Fla 4B into highbush blueberry cultivars. A recent study by Qu and Hancock (1995) found that US 75 contains about 70% of Fla 4B's heterozygous loci and its inheritance pattern is primarily tetrasomic. This would indicate that most of Fla 4B's genetic information is contained in US 75 and should readily segregate in further intercross breeding. Our previous research has shown that a small percentage of backcross progeny have improved heat tolerance (Hancock et al., 1992), but many of them are poorly adapted to cold temperatures (Hancock et al., 1995). To facilitate the identification of recombinant progeny with high fruit quality, heat tolerance and cold tolerance, a genetic linkage map of Fla 4B in the genetic background of US 75 would be very helpful. Herein, we report on a RAPD-based map generated at the tetraploid level from a cross of US 75 with another commercially important highbush cultivar, `Bluetta'.

MATERIALS AND METHODS

Plant material. US 75, `Bluecrop', `Bluetta', Fla 4B, and a tetraploid segregating population from US 75 x 'Bluetta' were screened for the presence of polymorphic RAPD markers. The crosses were made in a greenhouse in 1988 and transplanted into the field at

the Southwestern Michigan Research Experimental Center in Benton Harbor in 1990, where they were maintained under standard cultural conditions (Hanson and Hancock, 1987). 'Bluetta' is considered a highbush cultivar, but is composed of 75% *V. corymbosum* and 25% *V. angustifolium* (Draper, 1977).

DNA Extraction and Amplification Conditions. Total cell DNA was isolated from young leaves using a modification of the CTAB procedure (Doyle and Doyle, 1987; as modified by Rowland and Nguyen, 1993). DNA was amplified in 12.5-µl volumes using ten-base primers (Operon Technologies Inc., Alameda, Calif. and Biotechnology Laboratory, University of British Columbia). Primers were identified by the initials of their sources (OP and BC) and the company's lot number. Reaction conditions were: 1 ng/µl template DNA, buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.01% gelatin), 1.6 mM $MgCl_2$, 200 µM dATP, dCTP, dGTP, dTTP (Boehringer Mannheim), 0.2 µM primer, and 0.06 units/µl Taq DNA polymerase (Gibco). DNA was amplified for 50 cycles in a Perkin Elmer thermal cycler programmed for a 30 s denaturation at 94 ^{0}C, 70 s annealing at 48 ^{0}C and 120 s extension at 72 ^{0}C. The PCR products were separated through 1.2% agarose gels and visualized by ethidium-bromide staining. Only reproducible fragments with strong bands were scored in our analysis. All genotypes were evaluated using PCR at least twice.

RAPD marker selection. Only the markers unique to Fla 4B genotype were used in this study. Interestingly, very few markers were detected that distinguished 'Bluecrop' and 'Bluetta'. Since US 75 was previously determined to have tetrasomic inheritance (Qu and Hancock, 1995), there were two segregation ratios expected in the tetraploid progeny, one for duplex loci [5:1(present:absent)], and one for simplex loci (1:1). No 3:1 ratios were

observed in our progeny, since US 75 is an autopolyploid rather than an allopolyploid.

RAPD markers which segregated 1:1 ($P<0.05$) were used to create the map. We will hereafter refer to these markers as single-dose amplified fragments (SDAF), since they are equivalent to the single-dose restriction fragment (SDRF) named by Wu et al. (1992) for RFLP markers employed in mapping polyploids. The SDAF were distinguished from the double-dose (duplex loci) amplified fragments according to their different segregation ratios. A total of 61 progeny were evaluated for their RAPD patterns in the US 75 x 'Bluetta' population. According to Mather (1951), population sizes of at least 38 are necessary [$(1-\alpha_1-\alpha_2)100\%$] to distinguish between 1:1 and 5:1 ratios.

Linkage Analysis. Multimarker linkage analysis was performed using the computer program MAPMAKER V.3.0 (Lander et al., 1987), evaluating the data type as an F_2 backcross population. As previously mentioned, US 75 was assumed to be an autotetraploid with random pairing among homologous chromosomes (Qu and Hancock, 1995). The linkage groups were established with a minimum LOD of 3.0 and $\Theta = 0.30$ (Kosambi function).

RESULTS AND DISCUSSION

Marker selection The comprehensive marker segregation data have been given and discussed in chapter 1. The 154 markers that were SDAFs and best fit a 1:1 ratio in the US 75 x 'Bluetta' population (Table 1 in Chapter 1) were used to generate the map.

Linkage analysis. The MAPMAKER program assigned 140 of the 154 markers to

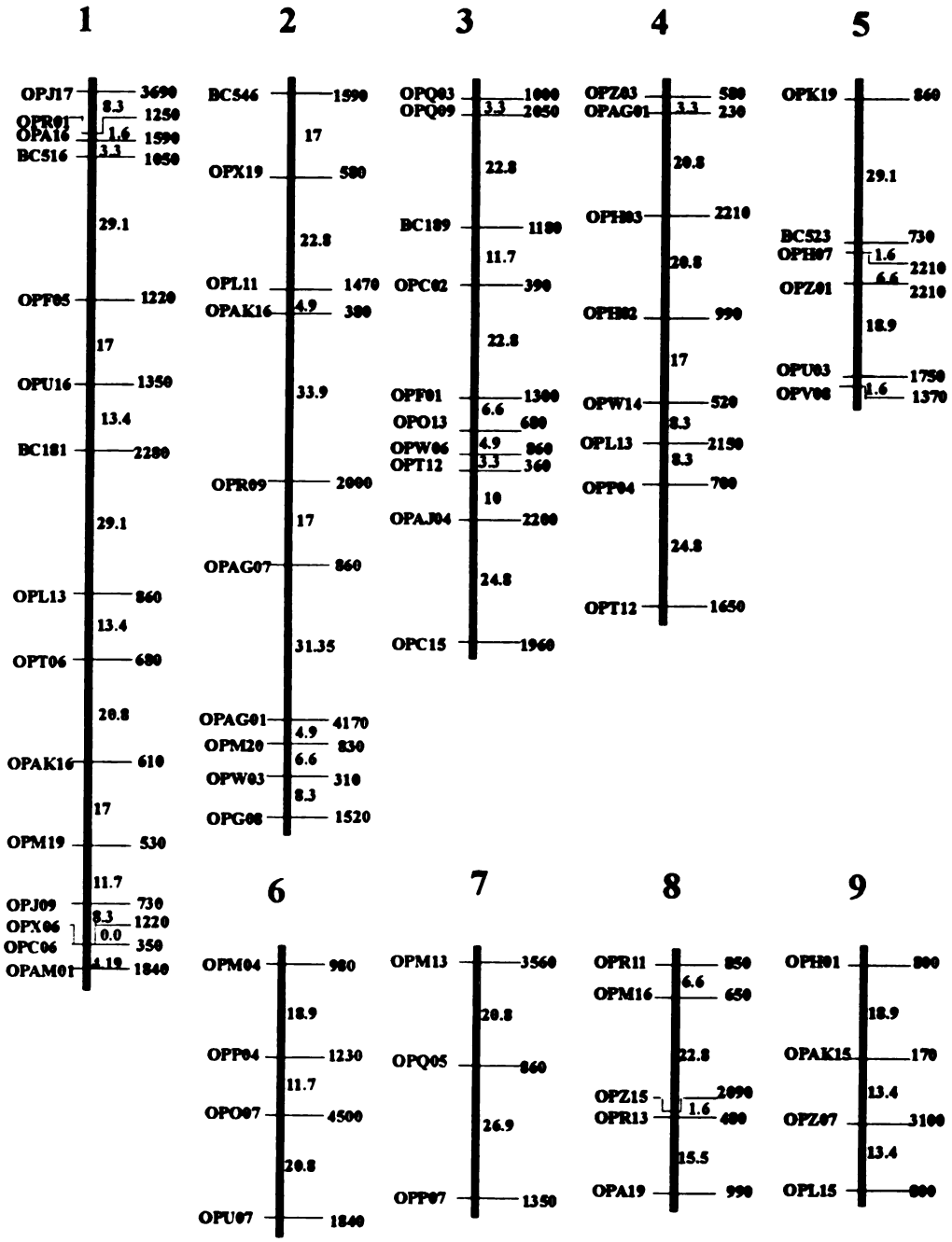

Figure 1. RAPD-based genetic linkage map of blueberry derived from a cross of US 75 (*V. darrowi* x *V. corymbosum*) x 'Bluetta' (*V. corymbosum* x *V. angustifolium*). Linkage groups are numbered from longest to shortest. Marker names are shown to the left of each linkage group and the base number of the analyzed fragments is shown on the right. Distances between adjacent markers (in cM) are indicated between the brackets. The unlinked markers and sizes were: BC244-550, BC523-1720, OPC06-290, OPF04-850, OPF12-1230, OPJ04-2700, OPJ14-1020, OPK19-640, OPM17-1960, OPQ04-5000, OPS13-860, OPX07-1830, OPZ04-610 and OPZ06-610.

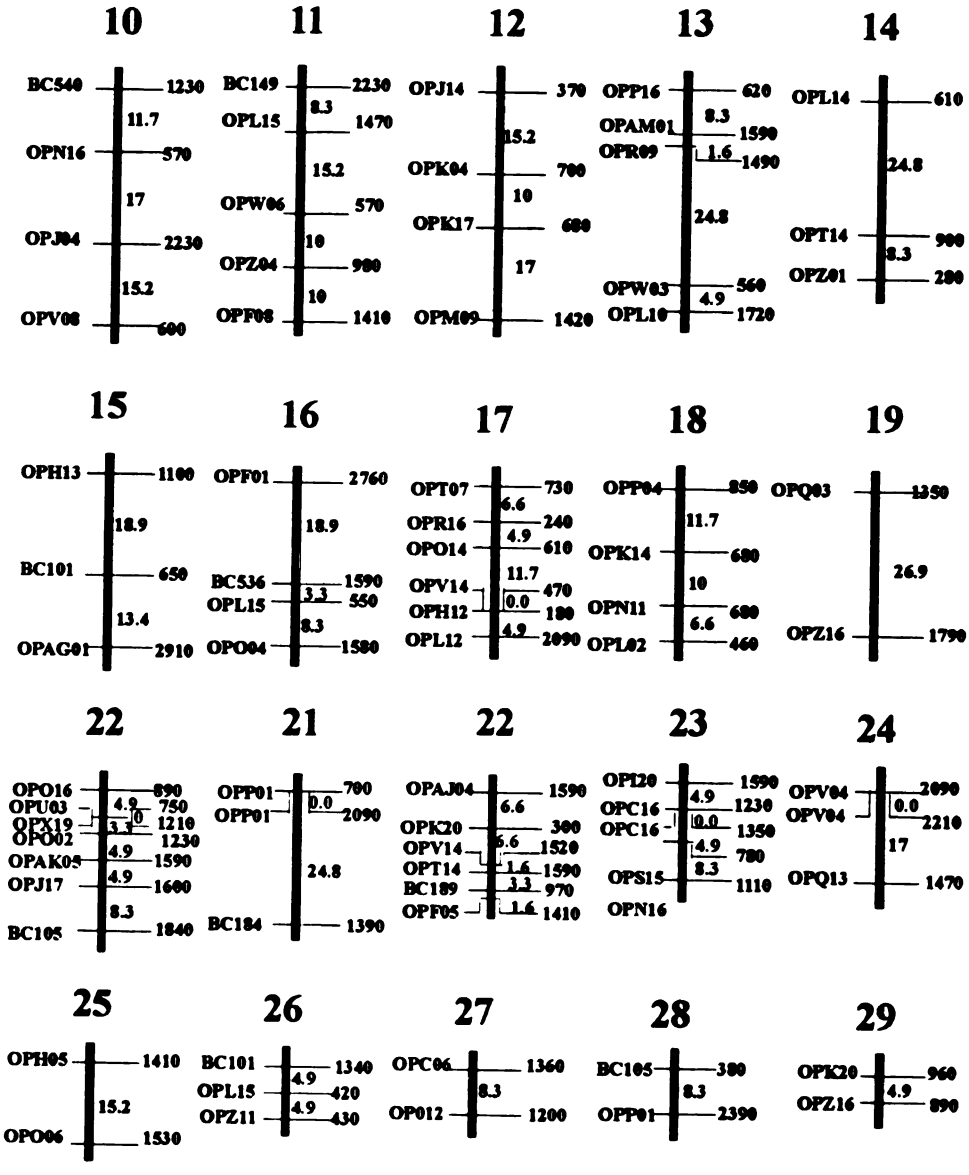

Figure 1 (continued)

29 linkage groups, leaving 14 markers unlinked (Figure 1). The number of markers assigned to individual groups ranged from 2 to 15. The total length for the map is 1288.2 cM with the linkage groups ranging in length from 4.9 to 178.1 cM. The distance between adjacent markers ranged from 1.6 to 33.9 cM, with an average distance of 9.2 cM. In six cases, two markers were mapped to the same position. Of these, three primers (OPC16, OPP01 and OPV04) amplified both markers together, while the other three pairs were amplified by two different primers (OPH12 and OPV14, OPU03 and OPX19, OPC06 and OPX06).

While this map was generated using US 75, it is essentially a linkage map of *V. darrowi,* because US 75 was produced via a 2n gamete from Fla 4B, and only unique markers for Fla 4B were utilized in this study. We detected 5 more linkage groups than the diploid number of *V. darrowi* (24). Since eleven small linkage groups were identified with only 2 or 3 markers, the evaluation of more markers may reduce the total number of linkage groups observed. It is also possible that some of the extra linkage groups represent the chromosomal pairing abnormalities suggested by Vorsa and Novy (1995).

The previous RAPD based map generated for Fla 4B was performed at the diploid level (Rowland and Levi, 1994), and as a result, can not be used to directly identify linkages with tetraploid traits. However, these associations can be located using our map of US 75 since this interspecific hybrid undergoes tetrasomic inheritance. This may make it possible to use marker-assisted selection to identify blueberry segregants with high heat tolerance and high fruit quality that also have sufficient cold tolerance and a long enough chilling requirement for northern climates. Since US 75 carries most of Fla 4B's alleles, it should prove to be a very useful parent in transferring traits from Fla 4B into the highbush

background. A similar approach to tagging agronomically important traits can probably be used in polyploid crop breeding programs utilizing unreduced gametes.

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CHAPTER 3

EVOLUTION IN AN AUTOPOLYPLOID GROUP DISPLAYING PREDOMINANTLY BIVALENT PAIRING AT MEIOSIS: GENOMIC SIMILARITY OF DIPLOID *VACCINIUM DARROWI* AND AUTOTETRAPLOID *V. CORYMBOSUM*

Abstract. The genomic relationships of diploid *V. darrowi* (2n = 2x = 24) and autotetraploid *V. corymbosum* (2n = 4x = 48) were examined utilizing an interspecific tetraploid hybrid, US 75 and representatives of the parental species. Two features in the background of US 75 led to the prediction that it would act cytogenetically as an allopolyploid: 1) the two species are quite distinct morphologically and geographically, and 2) the diploid genome was incorporated into US 75 via an unreduced gamete. However, the hybrid had been previously determined to display tetrasomic inheritance using molecular markers. In this study, US 75 was found to have a lower than expected number of multivalents for an autopolyploid, but it had a significantly higher number of quadrivalents than *V. corymbosum*, and one PMC (pollen mother cell) was observed with 11 of the 12 homologous groups being paired in quadrivalents. In spite of these high numbers of multivalents, normal distributions of chromosomes were observed at anaphase I and II by both cytological observation and pollen viability. Genomic *in situ* hybridization (GISH) revealed that both parents genomic DNA labeled all the chromosomes in the hybrid. These findings suggest that little genomic divergence has developed between the *Vaccinium* species, and that genes may be freely transferred from diploid to tetraploid species via unreduced gametes.

INTRODUCTION

The importance of autopolyploidy in plant evolution has received renewed attention in recent years (Soltis and Rieseberg, 1986; Krebs and Hancock, 1989; Soltis and Soltis, 1993 and 1995). Evidence is accumulating that autopolyploids are much more prevalent than previously thought and are considerably more dynamic (Hancock, 1992; Soltis and Soltis, 1993 and 1995; Maceira et al., 1993). Much information has been gained on polyploid genetics and evolution by producing artificial hybrids of putative diploid progenitor species. Studies on synthetic polyploids have generated knowledge on possible progenitor relationships (Song et al., 1993; Hauber, 1986; Van Dijk and Van Delden, 1990) and the nature of polyploidization via unreduced gametes or zygote doubling (Clausen and Goodspeed, 1925; Draper et al., 1982; Werner and Peloquin, 1991). Valuable information has also been gained on levels of genomic divergence through inheritance studies of such hybrids, by comparing tetrasomic to disomic ratios (Qu and Hancock, 1995). Several species have been identified that appear to be allopolyploids because they have bivalent pairing at meiosis, but are in fact autopolyploids with tetrasomic inheritance (Soltis and Rieseberg, 1986; Crawford and Smith, 1984; Krebs and Hancock, 1989; Samuel et al., 1990).

The blueberries in *Vaccinium* section *Cyanococcus* are a polyploid complex consisting of diploid (2n = 2x = 24), tetraploid (2n = 4x = 48) and hexaploid (2n = 6x = 72) species. The widest ranging species, *V. corymbosum* L., extends from southern Ontario, Quebec, and Nova Scotia across to Michigan and southward into eastern Texas and Florida. It is thought to have diploid, tetraploid and hexaploid members (Vander Kloet, 1988), although this taxonomy remains unsettled. One of the diploid forms originally recognized

by Camp (1945), *V. elliottii* Chapman, is easily recognized in the field, and the cultivated hexaploid is widely known as *V. ashei* Reade by plant breeders and horticulturalists (Luby et al., 1991).

Fertile hybrids between the various ploidies of *V. corymbosum* are relatively easy to produce by utilizing unreduced gametes (Luby et al., 1991; Ortiz et al., 1992; Hancock et al., 1995), and considerable information has been generated on the chromosomal behavior of inter-ploid hybrids. Cytological analysis of 6 interspecific triploids derived from tetraploid x diploid *V. corymbosum* crosses revealed a range of chromosomal pairing relationships from autoploid to preferential pairing, depending on the diploid parent (Vorsa, 1989). Interspecific backcross hybrids of tetraploid x hexaploid crosses suggested that a minimum of two-thirds of the hexaploid chromosome complement of *V. ashei* could pair and recombine with that of the tetraploids (Vorsa, 1987). However, because the chromosomes of blueberry are very small (1.5 - 2.5 μm in length) and largely indistinguishable (Hall and Galletta, 1971; Vorsa, 1989), exact pairing relationships have not been resolved.

Tetraploid *V. corymbosum* have been shown to have tetrasomic inheritance (Krebs and Hancock, 1989), even though bivalent pairing dominates during meiosis (Jelenkovic and Hough, 1970; Jelenkovic and Harrington, 1971; Vorsa, 1987). The meiotic pairing configurations were shown to deviate significantly from the random association patterns expected when there are four homologous chromosomes in a cell (Durant, 1960; Jackson and Casey, 1980 and 1982). Predominantly bivalent associations at diakinesis and metaphase I have been observed in all the tetraploid materials examined except an artificially doubled genotype of *V. elliottii* (Dweikat and Lyrene, 1991). The non-randomness of highbush

chromosome associations were interpreted to be the result of obligatory pairing and localized distal chiasma (Jelenkovic and Hough, 1970). A few meiotic abnormalities such as secondary pairing and lagging of chromosomes during anaphase in *V. corymbosum* were observed in other studies, but the production of viable pollen was not severely impaired (Newcomer, 1941; Rousi, 1967). It was proposed that either the parental tetraploid species (*V. corymbosum*) had been almost completely diploidized cytogenetically, or unconscious selection against quadrivalent pairing had occurred during breeding for the most fruitful plants (Jelenkovic and Hough, 1970).

Synthetic hybrids of blueberries have been utilized by plant breeders to transfer elite traits from lower to higher ploidy levels (Draper et al., 1982; Lyrene and Sherman, 1983; Laverty and Vorsa, 1991). One of the most successful breeding parents has been the tetraploid hybrid, US 75, derived from a 2n gamete of Fla 4B [a selection of *Vaccinium darrowi* (2n = 2x = 24)] and 'Bluecrop' [*V. corymbosum* (2n = 4x = 48)] (Draper et al., 1982). It has played an important role in the development of tetraploid highbush blueberries with a low chilling requirement (Ballington, 1990) and several of its elite traits have the potential to further improve the northern tetraploid highbush blueberry (Erb et al., 1990 and 1993; Chandler et al., 1985; Hancock et al., 1992).

Two features in the background of US 75 might lead to the prediction that it would act cytogenetically as an allopolyploid with disomic inheritance rather than an autopolyploid with tetrasomic inheritance. First, the two species are quite divergent, both morphologically and geographically. *V. darrowi* is an evergreen, lowbush type found in the southeastern US, while tetraploid *V.corymbosum* is a deciduous, highbush type found in the northern US.

Secondly, Fla 4B's 2n genome was incorporated into US 75 via an unreduced gamete, so both sets of homologous chromosomes are available for pairing. However, a recent study by Qu and Hancock (1995) showed that the inheritance pattern of RAPD markers in US 75 is indeed tetrasomic, indicating that it is acting as an autopolyploid. This suggests that very little genomic divergence has occurred in the subgenus *Cyanococcus* as a whole.

With the RAPD inheritance information available and knowing the parental background and history of the interspecific hybrid US 75, we felt that a study of its meiotic behavior would provide more insight into the nature of polyploidy in *Vaccinium*. Herein, we describe the meiotic configurations of US 75, two of its selfed progeny, its male parent 'Bluecrop' and a wild plant of *V. corymbosum*. We also used GISH (genomic *in situ* hybridization) (Bailey et al., 1993; De Jong et al., 1995) to estimate the level of genomic homology between *V. darrowi* and *V. corymbosum*. Our findings provide further support that little genomic divergence has occurred between *V. corymbosum* and *V. darrowi*, and suggest that genes can be readily transferred from diploid to tetraploid species via unreduced gametes.

MATERIALS AND METHODS

Cytogenetics. Five to seven year old plants of 'Bluecrop' (*V. corymbosum*), Fla 4B (*V. darrowi*), US 75, two selfed progeny of US 75 (US 75s), and a wild tetraploid *V. corymbosum* collected at Otis Lake (CEL) were grown together for a year in an unheated greenhouse at Michigan State University. Flower buds undergoing meiosis were collected and fixed in methanol : acetic acid (3:1) for at least 48 hours in a refrigerator. For long-term

storage, the fixed buds were transferred to 70% ethanol and returned to the refrigerator. Anthers were separated from the rest of the flower buds, and pollen mother cells (PMC) were squeezed out and squashed in either a drop of acetocarmine : 45% acetic acid (1:5) or 45% acetic acid alone. The somatic chromosome numbers of the tetraploid materials were also determined at the mitotic metaphase of the PMCs. Fla 4B's somatic chromosome number was determined using young pistils which were treated in 1 N HCL for 4 min at 60 ^{0}C, and then washed and squashed in carbol fuchsin. Observations and photomicrographs were made with phase contrast or laser confocal scanning microscopes.

Pollen viability. US 75 pollen viability was tested by both vital staining and germination. For staining, fresh pollen were placed in acetocarmine for 45 min. and counts of viable and non-viable pollen were made in randomly selected fields under the microscope. To measure pollen germination, fresh pollen was dispersed onto a thin layer of medium (15% sugar, 1% agar and 100 ppm boric acid) spread over the bottom of a petri dish that was sealed with parafilm and kept at room temperature. Pollen in blueberry is shed in tetrads representing all four meiotic products, and a tetrad was considered viable if any pollen tube emerged from it and grew at least ten times longer than its diameter. Counts of viable tetrads and photographs were made in random fields under the microscope.

***In situ* hybridization**: Mitotic chromosomes of US 75 and 'Bluecrop' were prepared as described above in 45% acetic acid on grease free slides. The slides were then either quickly frozen on a slab of dry ice for 5 min before the coverslips were removed with a razor blade, or stored at -70 ^{0}C and the coverslips were removed 4-7 hr before hybridization. The chromosomes on the slides were dehydrated in a series of ethanol concentrations (70, 95 and

100%) for 15 min each immediately after removing the coverslips, air dried for 3-6 hr, and either used immediately for hybridization or stored at -70 ^{0}C.

For probe preparation, DNA was isolated according to Qu and Hancock (1995). The genomic DNA of `Bluecrop' and Fla 4B was labeled with biotin-14-dATP using the BioNick Labeling System (GibcoBRL, Gaithersburg, MD) as described by the supplier. A Sephadex G-25 column (Pharmacia, Piscataway, NJ) was used to separate the unincorporated nucleotides from the labeled DNA. The labeled DNA probes were adjusted to a concentration of 30 ng/µl with distilled water and stored at -20 ^{0}C.

In situ hybridization was performed using the protocol of Vector Labs (Burlingame, CA). Mitotic chromosomes of US 75 were hybridized directly with the labeled probe of `Bluecrop', and Fla 4B individually, or were blocked with one of the parent genomic DNA and then hybridized with the labeled probe of the other. As a control, the `Bluecrop' and Fla 4B mitotic chromosomes were also hybridized with their own DNA probe.

For *in situ* hybridization, the slides were incubated in 70% formamide, 2x SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) for 2 min at 70 ^{0}C for denaturation, and dehydrated in an ethanol series (70, 95 and 100%) for 5 min each at -20 ^{0}C. The hybridization mixture (20 µl/slide) consisted of 50% formamide, 2x SSC, 10% dextran sulphate, 2 µg sonicated herring sperm DNA, and either 3000 ng of sheared (autoclaved for 4 min) unlabeled DNA of one parent and 30 ng labeled DNA probe of the other parent (for blocking and hybridization), or 180 ng labeled DNA probe of one parent (for control). The preparations were denatured for 5 min at 75 ^{0}C and quickly cooled on ice for 5 min. After applying the hybridization mixture to the slides, they were covered with coverslips, sealed

with rubber cement, and kept in a humid chamber at 37 ^{0}C overnight.

After hybridization, the coverslips were carefully removed and the slides were washed for 5 min. in three changes of 50% formamide, 2x SSC and 2 changes of PN buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1% nonidel P-40, pH 8.0) at 45 ^{0}C. The slides were then incubated for 20 min at room temperature in 5 μg/ml Fluorescein Avidin DCS (Vector Labs) in PNM buffer (5% non-fat dried milk, 0.1% sodium azide in PN buffer, centrifuged to remove solids). After two 3 min washes in PN buffer at room temperature, the slides were incubated for 20 min at room temperature in 5 μg/ml Biotinylated Anti-Avidin D (Vector Labs) in PNM buffer and washed twice in PN buffer. The slides were then incubated in 5 ng/ml Fluorescein Avidin DCS for 20 min at room temperature following a brief wash in PN buffer. Finally, the slides were mounted in VECTASHIFI D™ Mounting Medium (Vector Labs) containing 0.2-0.5 μg/ml propidium iodide for viewing.

Microscopy. Preparations were visualized on a laser confocal microscope (Carl Zeiss, Inc., Thornwood, NY) with oil immersion objectives. For transmitted images the 488 (blue) or the 633 (red) laser lines were employed. For detection of FITC, the 488 (blue) line was used in conjunction with an LP 520 or a BP 520-560 barrier filter, depending on the sample. Similarly, for samples stained with propidium iodide the 633 laser line was used with LP 520 or BP 590-620 barrier filters.

RESULTS

Chromosome configurations. Somatic chromosome numbers (2n = 4x = 48) of the tetraploid materials are shown in Figure 1. The diploid chromosome number (2n = 2x = 24) of Fla 4B is displayed in Figure 2.A. Fla 4B commonly showed twelve ring bivalents at metaphase I (Figure 2.B).

Typical chromosome configurations from pachytene through anaphase in the tetraploids are shown in Figures 3. A-K. The general chromosomal configurations observed for US 75 (Figure 3. A-F), 'Bluecrop' (Figure 3. G and H), US 75s (Figure 3. I and J) and CEL (Figure 3. K) were very similar to those previously reported in blueberry (Jelenkovic and Hough, 1970; Jelenkovic and Harrington, 1971 and Vorsa, 1987; Vorsa and Novy, 1995). From diakinesis to metaphase I predominantly bivalent pairing was observed, with very few univalents and trivalents being present. Quadrivalents were almost always the only forms of multivalents found.

Ring, chain, bar and x configured bivalents were observed in the tetraploid material (Figure 3. J). The frequency of ring and chain bivalents was very similar in cells undergoing diakinesis, each accounting for about 25% of the total bivalents. The remaining 50% bivalents, bar and x, were sometimes difficult to distinguish. In a few cases 24 bar bivalents were observed at diplotene (Figure 3.B). When assorted by size (Figure 4), bar bivalents of both large and small chromosomes appeared to have more than 3 or 4 pairing chiasmata. While pseudo-multivalent associations between bivalents was not as obvious in this study as in others (Jevenkovic and Hough, 1970), bivalent numbers did appear to increase as meiosis proceeded.

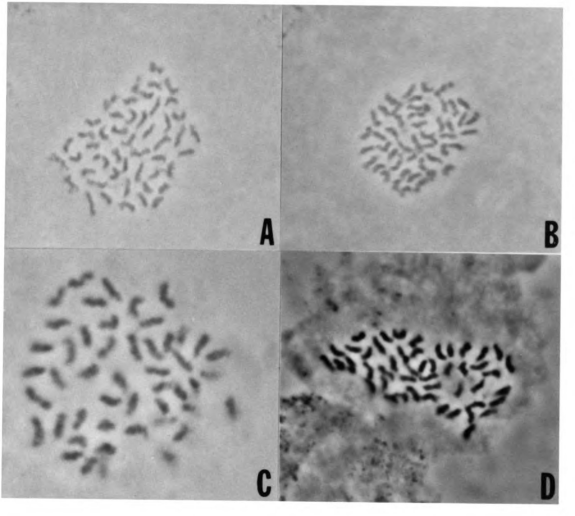

Figure 1. Somatic chromosome numbers of the tetraploids (2n = 4x = 48). A) US 75, B) 'Bluecrop', C) US 75s, and D) CEL.

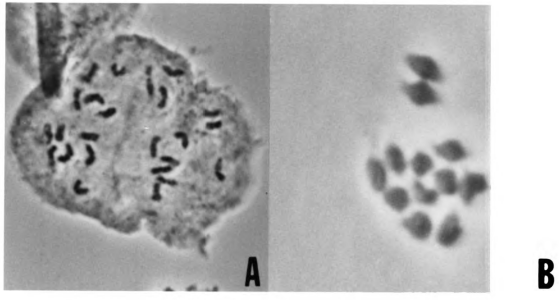

Figure 2. A) Somatic chromosomes of Fla 4B (2n=2x=24), and B) A metaphase I configuration with 12 ring II.

Figure 3. Representatives of the meiotic chromosome configurations in the tetraploids. US 75 (A - F): A) Diplotene, exhibiting 11 quadrivalents (IV) and 2 bivalents (II, arrow), B) Late diplotene with 24 II, C) Diakinesis with 6 ring IV, 1 chain IV(large arrow), 1 III (small arrow), 7 II and 3 I (arrow head), D) Diakinesis with 5 ring IV and 14 II, E) Late anaphase I, showing lagging chromosomes (arrow), F) Early telophase I with 24 chromosomes/pole; 'Bluecrop' (G and H): G) Diakinesis with 2 IV [one possible broken ring IV (arrow)] and 20 II, H) Early anaphase I with 24 II; US 75s (I and J): I) Diakinesis with 3 ring IV and 18 II, J) Metaphase I with 24 II with ring (large arrow), chain (small arrow), bar (large arrow head) and x (small arrow head) configurations; and CEL: K) Metaphase I with 1 IV and 22 II.

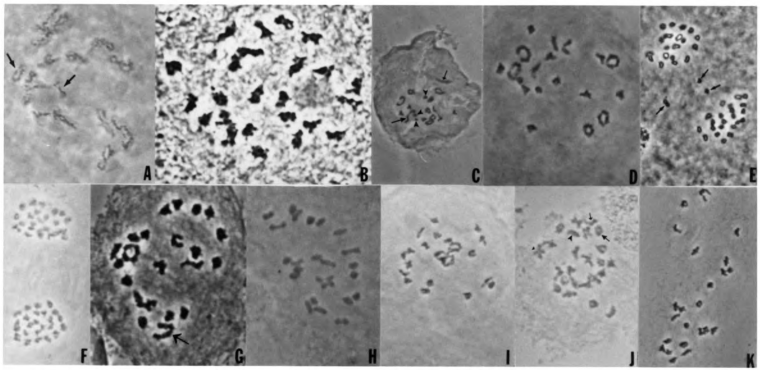

Figure 3

Figure 4. Rearrangement of Figure 2-C by chromosome size. Note high numbers of attachment sites in both large and small chromosomes.

While percentages of quadrivalents were low in all the tetraploid materials examined, US 75 did have significantly higher percentages of quadrivalents than `Bluecrop' or CEL (Table 1). In one case, eleven quadrivalents was observed in a diplotene PMC of US 75 (Figure 3. A). Seven quadrivalents (six ring and one chain) were found in one PMC of US 75 in diakinesis (Figure 3. C).

Ring quadrivalents were the most common form of multivalents observed consisting of about 80% of the total. The remaining 20% of the quadrivalents were chain style, although many of them appeared to be broken ring quadrivalents (Figure 3. G). Clear III + I and I + I (Figure 3. C) configurations were observed in one diakinesis PMC of US 75. No multivalents higher than four were observed.

Although exact counts of chromosome numbers at anaphase I and II were often not possible, the relative distribution of chromosomes to the poles appeared to be equal at both anaphase I (Figure 3. F) and II. In only a few cases were lagging chromosomes observed (Figure 3. E).

Pollen viability. The staining test indicated that 98% of the US 75 pollen was viable (Figure 5. A), and 94% (1785/1892) of the tetrads produced at least one pollen tube (Figure 5.C). About 10% of the sporads were shaped as dyads (Figure 5. B), suggesting they might be unreduced gametes (Ortiz et al., 1992)

***In situ* hybridization**. When the `Bluecrop' probe was applied to the chromosomes of US 75 without blocking, all the chromosomes were completely labeled (Figure 6. A). The signal appeared comparable to 'Bluecrop' probed with its own DNA (Figure 6. B). Probe

Table 1. Chromosome pairing configurations at diakinesis and metaphase I.

		Pairing Configurations								
Genotype	PMC	24II	22II+1IV	20II+2IV	18II+3IV	16II+4IV	14II+5IV	12II+6IV	10II+7IV	Average IV/PMC
US 75	72	8	11	13	16	16	5	2	1	2.6111a**
US 75s	42	7	7	10	9	7	2			2.1904a**
'Bluecrop'	33	15	11	4	2	1				0.8788b
CEL	7	3	2	2	1	1				1.0000b

** Means with different letters are significantly different at the P<0.001 level using the Duncan's multiple range test.

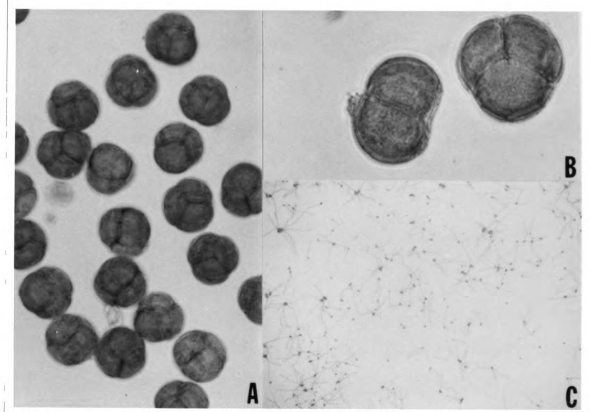

Figure 5. Pollen viability of US 75: A) Typical microscope field of pollen stained with acetocarmine, B) A dyad pollen sporad (left) which may represent 2n gametes, and C) Typical microscope field of germinated pollen.

from Fla 4B also labeled all US 75 chromosomes evenly when not blocked, although the signal was not as strong as with probe of 'Bluecrop' (data not shown). When the chromosomes of US 75 were blocked with either 'Bluecrop' or Fla 4B DNA and hybridized with the probe of other genome, none of the chromosomes were labeled (Figure 6.C).

DISCUSSION

The origins of blueberry polyploids and whether they are autopolyploid or allopolyploids has long been of interest to both blueberry taxonomists and breeders (Camp, 1942, Vander Kloet, 1983; Krebs and Hancock, 1989; Qu and Hancock, 1995). Originally, most of the tetraploid blueberries were considered to be allopolyploids (Camp, 1945; Eck, 1966; Vander Kloet, 1988) based primarily on morphological traits. Cockerham and Galletta (1976) also suggested that *V. corymbosum* and four related tetraploid taxa were allotetraploids because the tetraploids as a group exhibited higher pollen stainability than a group of seven diploid species. However, tetrasomic inheritance ratios have now been demonstrated in highbush *V. corymbosum* (Draper and Scott, 1971; Krebs and Hancock, 1989), lowbush *V. angustifolium* (Hokanson and Hancock, 1993) and the interspecific hybrid US 75 (Qu and Hancock, 1995), suggesting that they are autopolyploids. *V. angustifolium* and tetraploid *V. corymbosum* are completely interfertile, and fertile hybrids have been generated from most diploid x polyploid combinations (Hancock et al., 1995). The work described here and previous studies have found only limited chromosome size and genome content differentiation in blueberries (Hall and Galletta, 1971; Costich et al., 1993). Considering that *V. darrowi* is thought by many to be an ancient diploid blueberry species

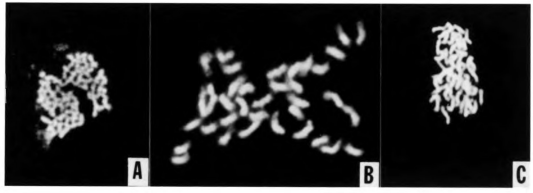

Figure 6. Genomic *in situ* hybridization. A) US 75 chromosomes hybridized by the 'Bluecrop' labeled genomic probe. Before it was transmitted, the color is green yellow; B) The 'Bluecrop' labeled genomic probe hybridized on its own chromosomes (some chromosomes were lost during the slide treatment). Before it was transmitted, the color is yellow green; and C) US 75 chromosomes blocked with 'Bluecrop' genomic DNA and hybridized with labeled Fla 4B probe. Before it was transmitted, the color is brown red.

(Vander Kloet, 1983; Vorsa et al., 1988; Bruederle and Vorsa, 1994), it appears that the subgenus *Cyanococcus* of *Vaccinium* can be thought of a compilospecies.

The prevalence of bivalent configurations observed in this study is in agreement with the earlier studies of meiosis in *V. corymbosum* (Jelenkovic and Hough, 1970; Jelenkovic and Harrington, 1971; Vorsa et al., 1987). Over 90% of the chromosomes in 'Bluecrop', CEL and over 70% in US 75 were in bivalent associations (Table 1). Since these tetraploid blueberries display tetrasomic inheritance, it is suggested that their bivalents are being formed from random selections of the 4 homologous chromosomes. This is further evidenced by the fact that no 1:0 (present : absent) segregation ratios were found in the 65 homologous RAPD markers unique to Fla 4B (Qu and Hancock, 1995 and 1996).

From this work, it should be clear that chromosomal configurations (bivalent vs. multivalent) can not be used as a reliable indicator of whether a species is genetically an autoployploid with tetrasomic inheritance or an allopolyploid with disomic segregation. Blueberry is apparently not unusual in this regard as predominantly bivalent pairing at diakinesis and metaphase has been observed in a number of artificial and naturally occurring autotetraploid species (Jones, 1961; Soltis and Rieseberg, 1986; Crawford and Smith, 1984; Samuel et al., 1990).

Several lines of evidence indicate that while striking morphological, geographical and ploidy differences exist between *V. darrowi* and *V. corymbosum*, little divergence has developed between their genomes, or at least between the APS (autonomous paring sites) which are involved in initiating homologous chromosome pairing (Callow and Gladwell, 1984; Jones, 1994). These include: 1) tetrasomic inheritance in US 75, 2) the significantly

higher number of quadrivalents observed in US 75 than in 'Bluecrop' and CEL, and 3) the observation that 11 quadrivalents formed from the 12 homologous groups in at least one PMC in US 75 (Figure 3. A). The genomic similarity of diploid *V. darrowi*, Fla 4B, and tetraploid *V. corymbosum*, 'Bluecrop' is further evidenced by the finding that genomic DNA from both 'Bluecrop' and Fla 4B hybridized with all of the chromosomes of US 75.

Some chromosomal divergence must exist in the group as RAPD analysis revealed unique fragments for both Fla 4B and 'Bluecrop'(Qu and Hancock, 1995 and 1996), although the fragments were not divergent enough to prevent hybridization with the other species' genomic probe. Vorsa et al. (1995) also found a number of RAPD markers in another cross of *V. darrowi* x tetraploid *V. corymbosum* that deviated significantly from tetrasomic ratios, and in other work found that 2 out of 6 artificially produced triploids (4x x 2x *V. corymbosum*) displayed preferential pairing (Vorsa, 1989). However, the genomic diversity in *Vaccinium* section *Cyanococcus* is surprisingly little considering the morphological differentiation that has occurred between the species. Other members of the *Ericaceae* also display limited genomic divergence, as Krebs (1996) found normal segregation patterns in a broad array of interspecific *Rhododendron* crosses.

While the number of chiasmata per bivalent is often assumed to be a maximum of 2 (Jackson and Casey 1980, 1982; Jackson and Jackson, 1996), many bivalent configurations we observed in blueberry were bars with 3 or 4 attachment sites (Figure 3. B). Both large and small chromosomes appeared to have multiple chiasmata (Figure 4). Vorsa (1990) also found evidence for more than 2 chiasmata per bivalent in inter-specific triploid blueberries. We did observe numerous rings with only two terminal attachments at diakinesis, but these could

have been the end result of the terminalization of multiple chiasmata scattered along the chromosomes. This is supported by the observation of Qu and Hancock (1995) that 32 of the 102 heterozygous loci detected in Fla 4B were duplex in US 75 and therefore had been subjected to crossing over. Since only 50% of the recombination products generated during FDR are detected in a single progeny (Hermsen, 1984; Hancock, 1992), this means that 64 loci were actually affected by crossing-over in Fla 4B, which gives a total recombination frequency of 62.7% (64/102). It is unlikely that such a high recombination frequency could have occurred if chiasmata were located primarily at the ends of chromosomes. The x configuration of bivalents also suggest that there were chiasmata close to centromere.

Genomic diploidization is thought by many to be an important step in the adaptation of autopolyploids (DeWet, 1980; Watanabe, 1983; Jackson and Jackson, 1996), but there have been no cytogenetic or inheritance studies that directly document this process, except for a controlled experiment where a statistically significant reduction in quadrivalent frequency was observed in artificially produced tetraploid maize after ten generations of sexual reproduction (Lewis, 1980). Jones (1961) found a stable level of quadrivalents in both natural and artificially produced autotetraploids of *Dactylis*, leading him to remark that "it would be wise to talk of the stabilization of meiosis rather than of its diploidization". There is circumstantial evidence in blueberry that chromosomal diploidization may have occurred, as US 75 had significantly more multivalents than native *V. corymbosum*, and a colchicine induced tetraploid of *V. elliottii* was previously shown to have significantly more multivalents than tetraploid *V. corymbosum* (Dweikat and Lyrene, 1991). However, the role of these two species in the evolution of polyploid *Vaccinium* is unresolved.

It may be that chromosome pairing relationships in polyploids are diploidized in two ways. In the one classically described, bivalent pairing becomes restricted to a specific set of two homologous chromosomes, while in the other, bivalents are formed by random pairing of all homologues. *V. corymbosum* fits the second class best, as it is mostly bivalent pairing but displays tetrasomic inheritance. Over time, sufficient chromosomal divergence and/or changes in pairing control (PC) (Jackson and Hauber, 1994) alleles might occur to change a species from random to preferential pairing, however, selection pressure for preferential pairing would be minimal, once complete fertility was achieved through the evolution of complete random bivalent formation. The specific ph-like gene which prevents the pairing between homoeologous chromosomes in wheat has been well studied (Riley and Chapman, 1958; Waines, 1976; Feldmen, 1993; Gill et al., 1993), but related PC genes regulating bivalent formation of homologous chromosomes in autopolyploids have not been identified. There is evidence that they exist in natural populations, however, as Jackson and Hauber (1994) found PC mutations that led to both auto- and alloploid pairing behavior in natural populations of *Helianthus ciliaris*.

Stebbins (1947) suggested that raw autopolyploids would have reduced fertility due to: (1) irregular distribution of chromosomes caused by unequal separation of multivalents; (2) irregular distribution caused by meiotic abnormalities of a physiological nature, presumably controlled genetically; and (3) genetic-physiological sterility of an unexplained nature, but not associated with meiotic irregularity." These factors appear to be of limited importance in US 75, as it has very high pollen stainability (98%) and germination rate (94%), and is readily crossed with itself and other tetraploids (Ballington, 1990; Hancock et

al., 1995). This suggests that in some instances autopolyploids can be sufficiently fertile to be successful immediately after they are generated. Most artificially produced autopolyploids are maladapted (DeWet, 1980; Hancock, 1996), but it is not surprising that numerous autopolyploids have become established, if one considers how many times polyploids have been formed in nature with a variety of parents (Soltis and Soltis, 1993 and 1995).

The present study of chromosomal associations and genomic divergence in *V. corymbosum* adds to the growing body of knowledge that suggests evolution in autopolyploids is much more dynamic than was previously thought (Soltis and Soltis, 1993; Soltis and Soltis, 1995), and that many species may be incorrectly assumed to be allopolyploids with disomic inheritance based on bivalent pairing. Rather than being an "evolutionary dead-end", autoployploidy may actually allow for enhanced divergence, if diploids can freely transfer new genes into related autoployploids via unreduced gametes. In North America, a series of diploid *Vaccinium* species are found along the eastern seaboard from Florida to Maine. The autotetraploid *V. corymbosum* overlaps this entire range. One wonders if the wide adaptive range of *V. corymbosum* may be due to the assimilation of diploid genes as it moved north from its presumed southeastern origin (Vander Kloet, 1988). This would have been facilitated by its autopolyploidy, particularly if raw hybrids had high fertility due to regular chromosomal pairing. Such interactions may be an important component of evolution in all autopolyploids.

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CHAPTER 4

FOUR MECHANISMS OF 2N POLLEN FORMATION IN INTERSPECIFIC BLUEBERRY (*VACCINIUM*) HYBRIDS

Abstract. Mechanisms responsible for 2n pollen formation in blueberry were studied cytogenetically using selfed progeny of a tetraploid interspecific hybrid, *Vaccinium darrowi* (2x) x *V. corymbosum* (4x). Four meiotic irregularities were found to lead to the production of 2n gametes: (1) Premeiotic doubling (PD), where chromosome doubling in a pollen mother cell (PMC) during mitosis resulted in the formation of four 2n gametes; (2) Tripolar spindle (TPS), where the spindles at anaphase II were fused at one pole resulting in one 2n and two n gametes; (3) Incomplete spindle at anaphase I (IS1), where the spindle functioned briefly at anaphase I and then failed producing two 2n gametes; and (4) Incomplete spindle at anaphase II in one daughter nuclei (IS2-1), where the spindle at anaphase II in one daughter nuclei functioned briefly and then failed resulting in one 2n and two n gametes. The TPS and IS1 produced FDR (first division restitution) 2n pollen. The allelic constitution of gametes formed by PD is similar to FDR produced gametes, although the cytological process is different. The IS2-1 mechanism resulted in SDR (second division restitution) 2n pollen. Surveys of mature pollen from the selfed progeny, its parent and a tetraploid grandparent revealed sporad associations consistent with all 4 mechanisms of 2n gamete formation.

INTRODUCTION

Unreduced gametes have played an important role in natural speciation through the sexual production of polyploids (Harlan and deWet, 1972; deWet, 1980; Bretagnolle and Thompson, 1995), and in crop improvement as a vehicle to unilaterally transfer traits from 2x to 4x species. The mechanisms associated with 2n gamete production have been actively researched in many species, since the relative frequency of 2n gamete production determines how efficiently 2n gametes can be employed in breeding and the way 2n gametes are formed determines how much heterozygosity is transferred to progeny. There are two major modes of 2n gamete formation: FDR (first division restitution) and SDR (second division restitution) (Peloquin, 1982; Hermsen, 1984). On the average, FDR is thought to transmit 80% of a species heterozygosity, while SDR passes on only about a 40% (Peloquin, 1982; Hermsen, 1984).

The blueberries in section *Cyanococcus* of the genus *Vaccinium* are a polyploid complex consisting of diploid (2n = 2x = 24), tetraploid (2n = 4x = 48) and hexaploid (2n = 6x = 72) species. Cultivated blueberries were derived from three polyploid taxa: tetraploid *V. corymbosum* (highbush blueberry), hexaploid *V. corymbosum* (syn. *V. ashei*, rabbiteye blueberry), and tetraploid *V. angustifolium* (lowbush blueberry). Polyploid speciation in blueberry probably occurred via unreduced gametes, as a high percentage of 2n pollen has been found in species and interspecific hybrids at different ploidy levels including 2x (Ortiz et al., 1992a; Megalos and Ballington, 1988), 3x (Vorsa, 1991), 4x (Ortiz et al., 1992b), 5x (Vorsa and Ortiz, 1992) and 6x (Ortiz et al., 1992b). Unreduced female gametes are also produced by 2x species, as evidenced by tetraploid hybrids being formed from 2x x 4x

crosses (Megalos and Ballington, 1988; Draper et al., 1982) and DNA marker segregation (Qu and Hancock, 1995).

An important aspect in blueberry breeding has been to combine genes from different ploidy levels, since numerous horticulturally important traits have been described in both wild diploid species and the higher ploidies (Hancock et al., 1995; Galletta and Ballington, 1995). The most useful interspecific hybrid parent has been US 75, a tetraploid of Fla 4B (a wild selection of diploid *V. darrowi*) x 'Bluecrop' (a cultivar of tetraploid *V. corymbosum*) presumably produced when a pollen grain of "Bluecrop' fertilized a 2n egg of Fla 4B (Draper et al., 1982). It has played an important role in the development of tetraploid highbush blueberries with a low chilling requirement (Ballington, 1990) and several of its other elite traits such as high fruit quality, mineral soil tolerance and photosynthetic heat tolerance, have the potential to further improve the northern tetraploid highbush blueberry (Erb et al., 1990 and 1993; Chandler et al., 1985; Hancock et al., 1992).

While 2n gametes are quite common in blueberry and have already been successfully employed in breeding, research on the mechanism(s) responsible for their formation are only beginning to emerge. First division restitution with no crossing over (FDR-NCO) has been proposed as the main cause of 2n pollen production in blueberries (Vorsa and Ortiz, 1992; Ortiz et al., 1992a). Studies of 2n pollen formation in diploid, tetraploid and aneuploid hybrids ($2n = 4x + 9 = 57$) revealed that 2n gamete production commonly involved three consecutive events: (1) desynapsis of paired chromosomes prior to metaphase I; (2) sister centromere disjunction in univalents at anaphase I; and (3) cytokinesis after telophase I leading to dyad formation (Vorsa and Ortiz, 1992; Vorsa and Ortiz, personal communication

in Galletta and Ballington, 1995). Filler and Vorsa (personal communication in Galletta and Ballington, 1995) have also found parallel spindles and tripolar spindles in the diploid *V. darrowi* and *V. elliottii*, and tetraploid biotypes of *V. pallidum*. Stushnoff and Hough (1968) observed premature cytokinesis leading to the formation of 2n pollen in the 4x highbush cultivar 'Coville'. Qu and Hancock (1995) demonstrated that a 2n female gamete of Fla 4B was probably produced via the mechanism of FDR using RAPD markers. In this report, we discuss the mechanisms responsible for the 2n pollen production in selfed progenies of US 75 (US 75s) and give frequencies of 2n pollen production in 'Bluecrop' , US 75 and US 75s.

MATERIALS AND METHODS

Cytogenetics

Two 7-year old plants of US 75s were transplanted from the field in 1995 and grown in an unheated greenhouse at Michigan State University for a year. Flower buds undergoing meiosis were collected in the spring of 1996 and fixed in methanol : acetic acid (3:1) for at least 48 hr in a refrigerator (Qu and Hancock, 1996). For long term storage, the fixed buds were transferred to 70% ethanol and held in a refrigerator. Anthers were separated from the rest of the flower buds and squashed in 10% acetocarmine. Observations and photomicrographs were made with a phase contrast microscope.

Pollen survey

Pollen from the two US 75s plants were collected from fixed buds. Pollen of US 75 and 'Bluecrop' was collected fresh in 1993 from greenhouse grown plants and stored for 3 years at 0 ^{0}C for later analysis. The pollen were stained with acetocarmine (Qu and Hancock,

1996) and counts of unreduced pollen gains were made in a series of random fields under the phase contrast microscope.

RESULTS

Representative stages of normal meiosis in US 75s are shown in Figure. 1. Four abnormal meiotic process were observed that led to the production of unreduced gametes: (1) Premeiotic doubling (PD): Twice the tetraploid number of chromosomes were observed in PMCs which would have resulted in four 2n gametes following normal meiosis [Figure 2.A, B (large arrow)]; (2) Tripolar spindles (TPS): Spindles were fused at one pole in anaphase I, which would have produced one 2n and two n gametes [Figure 2. C, D]; (3) Incomplete spindle at anaphase I (IS1): the spindle at anaphase I failed and left 48 univalents forming one pole. This would have produced two 2n gametes (Figure 2. E, F); and (4) Incomplete spindle at anaphase II (IS2): Spindles failed in one of the daughter nuclei in anaphase II (IS2-1), which would have resulted in one 2n and two n gametes (Figure 2. G, H). Incomplete spindle at anaphase II could also have occurred in both daughter nuclei (IS2-2), but its frequency would have been extremely low and we failed to observe it. Since only 2% of IS2-1 was observed, the frequency of IS2-2 would have been 0.0004% (0.02 x 0.02).

The overall frequency of 2n gametes observed cytogenetically was 12% (39/318), PD(1.2%), TS(4%), IS1(5%) and IS2-1(2%), with both US 75s plants having about the same percentage. The products (pollen grains) of a PMC remain attached in blueberry and in normal meiosis form a tetrad (Stushnoff and Hough 1968; Stushnoff and Palse 1969). The PD mechanism should produce a tetrad of large 2n gametes, tripolar spindles and IS2-1

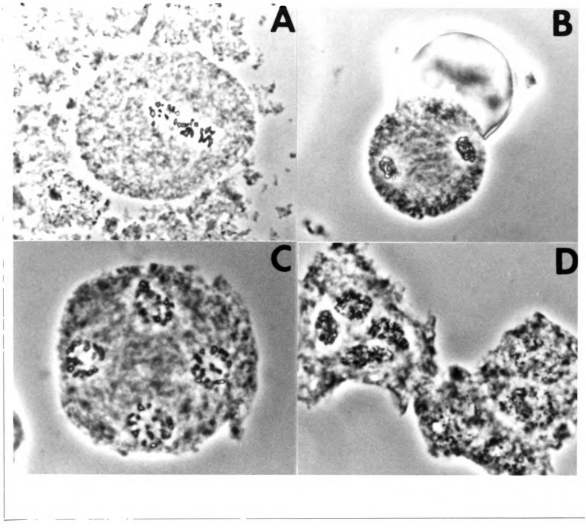

Figure 1. Representatives of normal meiotic stages of US 75s. A) Metaphase I, B) Telophase I, C)Telophase II, and D) Immature tetrads.

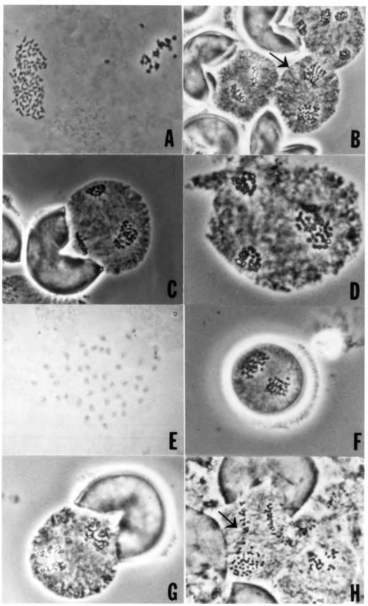

Figure 2. Representatives of irregular meiotic stages of US 75s which resulted in 2n pollen formation. 1) Premeiotic doubling (PD): A) Chromosome doubling before meiosis, showing 96 chromosomes, B) A possible PD PMC at early anaphase I (arrow); 2) Tripolar spindle (TPS): C) Late anaphase II of a TPS PMC, D) Telophase II of a TPS PMC; 3) Incomplete spindle at anaphase I (IS1): E) Chromosomes at anaphase I which were well separated by IS, but remain in one pole (two chromosomes were lost during slide preparation), F) A possible IS1 PMC at telophase II; and 4) Incomplete spindle in one daughter nuclei (IS2-1): G) Early anaphase II, chromosomes at one pole undergoing normal disjunction, but the spindle at the other pole has failed, H) Another example of spindle failure in one pole during anaphase II.

should produce triad pollen with one large 2n and two small n gametes, IS1 and IS2-2 (if it exists) would result in dyad composed of large 2n gametes. The pollen surveys revealed all three types of sporads in US 75s, 'Bluecrop' and US 75 (Figure 3). In Figure 3, dyad and giant tetrad pollens are shown which probably represent two 2n and four 2n gametes, respectively. Normal tetrad and triad pollens are difficult to distinguish depending on their orientation, but likely triads are also shown in Figure. 3.C. A few monads were also found (Figure 3.D).

The pollen survey of US 75s revealed about 10% (236/2358) putative 2n gametes : 1.4% were giant tetrads which probably represented PD and 8.6% were dyads which probably represented IS1. Few unambiguously triad 2n pollen associations were observed, but based on the cytogenetic data, about 6% of them [2% (IS2-1) + 4% (TPS)] would have been present in US 75s. Therefore, the percentage of 2n sporads was probably about 16% (1.4% PD + 8.6% IS1 + 6% TPS and IS2-1), which is slightly higher than that by cytological observation (12%). About 1% monad were also observed.

The frequency of 2n sporads in US 75 was about 14% [giant tetrads (1.3%) + dyads (11.3%) + monads (1.2%)] and in 'Bluecrop' was about 7% [giant tetrads (1.1%) + dyads (5.1%) + monads (0.8%)].

DISCUSSION

At least thirteen different types of cytological abnormalities have been described that lead to 2n gamete formation (Bretagnolle and Thompson, 1995). Two or more anomalies have been found to occur simultaneously in both a single species (Lelley et al., 1987;

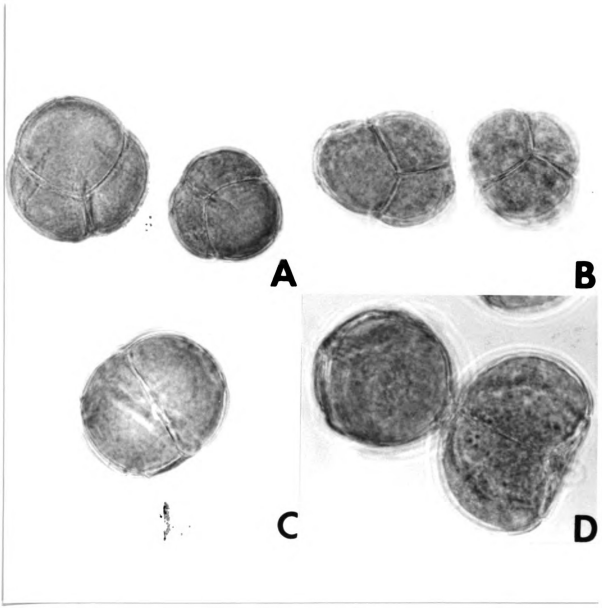

Figure 3. Representatives of unreduced sporads. A) Giant tetrad (left), which probably represented PD product; B) Triad with one large pollen grain (left), which probably represented TPS product; C) Dyad, which probably represented IS1 product, and D) A monad (left).

Werner and Peloquin 1987 and 1991; Tavoletti et al., 1991) and a single plant (Parrot and Smith, 1984; Tavoletti et al., 1991; Werner and Peloquin, 1991).

In this study we observed at least 4 mechanisms of 2n gamete formation in single individuals of blueberry - PD, TPS, IS1 and IS2-1. The IS mechanism found in this study is cytologically similar to the previously described synaptic mutants (Riley and Law, 1965; Iwanaga, 1983;) where the chromosome pairing irregularities result in asynapsis or desynapsis during meiosis I. However, three lines of evidence support a unique mechanism in our material: 1) In anaphase II, two separate planes of division were observed in the PMCs [Figure 2. G, H], rather than the triangular arrangement expected in TPS (Figure 2. C, D). The PMCs with IS had at one pole a single large set of well-separated chromosomes that appeared to be in the final stages of separation before the spindle failed, and at the other pole two small sets of chromosomes were observed parallel to each other and apparently being pulled apart by a fully functional spindle. 2)The germination rate of US 75 pollen was 94% and no dyads were unstained (Qu and Hancock, 1996), while the desynaptic aneuploid blueberry studied by Vorsa and Ortiz (1992) produced only 50% viable 2n pollen; and 3) The parental species of US 75 have high genomic similarity making synaptic irregularity unlikely (Qu and Hancock, 1996).

The lower frequency of IS1 observed cytologically (5%) than that based on the pollen survey 8.6% in US 75s was probably due to it being more difficult to distinguish all the meiotic irregularities cytologically than to determine it morphologically by pollen shape and size. How monad sporad formed remains unsolved.

While tripolar spindles were observed in this study, parallel and fused spindles were

not unambiguously observed, even though they are considered to be different phenotypic expressions of the same gene (Mok and Peloquin, 1975; Veilleux and Lauer 1981; Myers et al., 1984). This is apparently not the case in blueberry, or we missed their rare occurrence.

Premeiotic doubling similar to what we observed has been described in other species at a very low frequency. In potato (Lam, 1974) and *Secale cereale* (Lelley et al., 1987), approximately 0.2% and 1% of this abnormality have been observed. The formation of 4n PMC in *Secale cereale* was thought to be due to the failure of spindle formation during the last premeiotic mitosis (Lelley et al., 1987). In our material, IS could also be a possible mechanism, if the error causing IS happens in both meiosis and mitosis.

After examining levels of transmitted heterozygosity via a 2n gamete of Fla 4B we previously suggested that mechanisms genetically equivalent to FDR was much more prevalent than SDR in blueberry (Qu and Hancock, 1995). In the present study, IS1 and TPS are forms of FDR and the allelic constitution of PD gametes would be similar to that produced by FDR, while only IS2-1 would have resulted in SDR gametes. The frequency of PD, TPS and IS1 gametes in US 75s was 10.2%, while only 2% of IS2-1 were observed. This confirms that mechanisms genetically equivalent to FDR are the primary mode of 2n gamete formation in blueberry.

Based on their study of an aneuploid 2n pollen producer, Vorsa and Ortiz (1992) suggested that at least two loci were involved in the genetic control of 2n pollen production in the section *Cyanococcus*. Our study indicates that there may be at least two more loci, one for IS and one for TPS. Many of the genes responsible for the 2n gamete production in US 75 and US 75s were likely transmitted from Fla 4B since (1) the frequency of 2n gamete

production in Fla 4B is 18% (Ortiz et al., 1992a), which is much closer to that of US 75 (15%) than 'Bluecrop' (7%), and (2) the whole 2n genome of Fla 4B was incorporated into US 75 via a 2n gamete.

The concept that sexual polyploidization via 2n gametes is the main driving force for polyploid formation and evolution has gained widespread acceptance (Harlan and DeWet, 1975; deWet, 1980; Bringhurst, 1990; Rade and Haufler, 1992; Bretagnolle and Thompson 1995). From the work described herein, it is clear that numerous mechanisms of 2n gamete production exist in blueberry and it is indeed likely that the 4x and 6x blueberry species resulted from sexual polyploidization (Ortiz et al., 1992). It has been suggested that FDR x SDR and FDR x FDR are the most favorable combinations of 2n gametes due to the high levels of heterozygosity transferred (Watanabe et al., 1991).

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