nificant *Citrus* pollinating agent (Frost and Soost, 1968). Mature fruit were harvested, and seeds were extracted and germinated in a greenhouse.

Isozyme analysis was used to confirm hybridity of the seedlings produced. Leaves were collected from 4-month-old greenhouse-grown seedlings, frozen in liquid nitrogen, and powdered with a mortar and pestle. Cold (4C) extraction buffer (50 mM Tris·HCl pH 8.4, 150 mM NaCl, 1 mM $CaCl_2$, 1% insoluble polyvinylpolypyrrolidone) was mixed with the leaf powder (0.5 ml buffer per 0.1 g leaf tissue) and stirred for 15 min. This mixture was filtered through cheesecloth, and the filtrate was centrifuged for 20 min at 20,000 x *g*. The supernatant was collected, and the proteins were precipitated in 50% v/v acetone/dH_2O (assuming additive volumes) at -20C for 1 h. Precipitated proteins were freeze-dried and stored at -20C. Proteins were resuspended in gel buffer (50 $\mu g.\mu l^{-1}$) with 10% v/v glycerol and 0.0002% w/v bromophenol blue as the tracking dye, and loaded onto the gel (150 kg/lane). Electrophoresis was performed in a Mini-Protean II 7 cm x 8 cm x 0.75 mm slab cell (Bio-Rad, Richmond, Calif.) using a 0.125 M Tris pH 8.8 polyacrylamide separation gel (8% T, 2.67% C), and cold (4C) Tris·glycine (0.025 M Tris, 0.096 M glycine, pH 8.5) electrode buffer. The proteins were separated by a MacroDrive 5 automatic crossover power supply (Pharmacia LKB Biotechnology, Piscataway, N.J.) preset to 500 V, 15 mA, and 10 W before the run. Electrophoresis was stopped when the dye front reached the end of the gel or after 40 min. Gels were stained for GOT (Torres et al., 1978) and photographed.

Anthesis and pollen dehiscence occurred 24 to 48 h after flower collection. Pollen freeze-dried for 2, 4, or 8 h failed to germinate 24 h after drying, but sporadic germination (<1%) was observed in pollen freeze-dried for 1 h. Pollen stored in anhydrous acetone for 24 h at - 20C also failed to germinate 24 h after treatment. Germination of oven-dried tachibana orange pollen was similar to that of fresh pollen, with ≈70% germination for each of the four drying times tested. Alexander's stain color contrasted well between germinated and nongerminated oven-dried pollen. Pollen tubes stained red and empty pollen grains green. From these results, tachibana orange pollen was collected as described, oven-dried for 12 h (chosen from the four drying times for convenience), stored at -20C over silica gel, and used to pollinate a hybrid of 'Temple' x 'Orlando' the following year. Frozen fresh pollen failed to germinate after 1 year and was not used.

Fifty flowers were pollinated in Spring 1989, and 36 fruit with an average of 11 seed per fruit were collected in Nov. 1989, Fifty seeds were germinated in the greenhouse, and seedlings were analyzed for GOT. Acceptable resolution was obtained by acetone precipitating the water-soluble proteins, freeze-drying, and running in PAGE with a low ionic strength compared with the Laemmli (1970) stacking gel buffer, and a high field strength of 9 V/cm^2. Resolution was decreased when the total protein concentration exceeded 3 µg/lane.

Both parents produced a unique single band; the band from tachibana orange migrated faster than the 'Temple' x 'Orlando' band (Fig. 1, lane 1). Hybrids of these clones should produce a three-band pattern, because GOT is a dimer capable of heterodimerization. All 50 seedlings analyzed had a three-band GOT profile, confirming their hybridity (Fig. 1, lanes 2-4). Confirmation of hybridity based on morphology was difficult because these two parents are quite similar, with no readily recognizable morphological markers to distinguish hybrid seedlings.

This work demonstrated that tachibana orange pollen can be oven-dried, stored at -20C, and used the following season in controlled hybridizations. *C. tachibana* exhibits significant cold hardiness for a *Citrus* sp. (Swingle and Reece, 1967) and may be useful in developing cold hardy citrus types. This technique accesses previously unavailable but potentially valuable germplasm, and may provide the means to overcome the problem of asynchronous flowering with other genetically valuable *Citrus* selections.

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DNA Restriction Fragment Length Variability in the Genomes of Highbush Blueberry

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Additional index words. RFLPs, *Vaccinium darrowi, V. ashei, V. corymbosum, V. angustifolium*

Abstract. **Restriction fragment analyses of chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) were carried out on the principal cytoplasms of northern highbush cultivars and one representative of *Vaccinium ashei* Reade. Twenty-three restriction enzymes were used to identify variation and clarify mode of organelle inheritance. All species and genotypes displayed identical cpDNA fragment patterns, but high degrees of polymorphism were observed in the mitochondrial genomes. 'Bluecrop' and 'Jersey' did not appear to have 'Rubel' cytoplasm as was previously believed. All hybrids contained maternal-type mtDNA.**

Until recently, the germplasm base of the cultivated blueberry *Vaccinium corymbosum* L. was restricted and nuclear genes could be traced primarily to three wild selections (Hancock and Siefker, 1986). This situation has improved dramatically in the last few years as breeders have released several cultivars composed of complex species backgrounds (Ballington, 1990; Lyrene, 1990). However, the cytoplasmic background of the leading cultivars grown in the northern production regions is still limited and pedigree records indicate that only four cytoplasms are represented in this material: 1) 'Brooks' and 'Rubel' of *V. corymbosum, 2)* 'North Sedgwick' lowbush of *V. angustifolium* Ait., and 3) 'Florida 4B' of *V. darrowi* Camp. (Hancock and Krebs, 1986). These selections represent a broad geographical and species range, but no attempts have been made to measure their degree of divergence. In this study, we used restriction fragment length polymorphisms (RFLPs) of mitochondrial and

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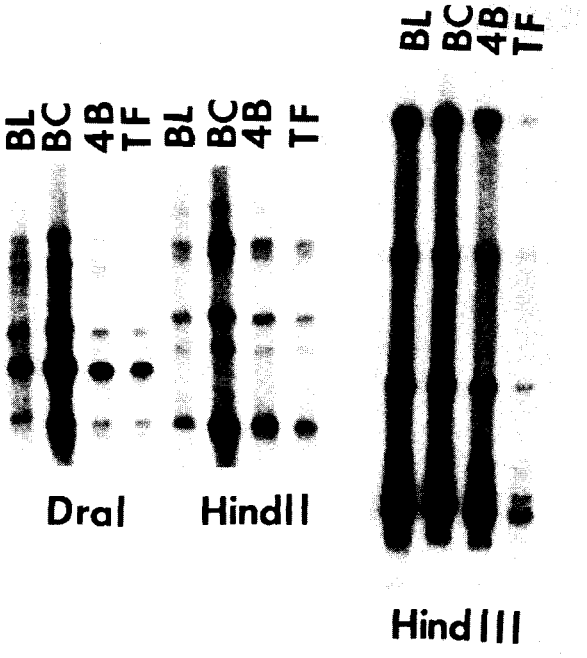

Fig. 1. Hybridization of chloroplast-specific probe of tomato (P-4) to total DNA digested with DraI, HindII, and HindIII for 'Bluetta' (BL), 'Bluecrop' (BC), 'Florida 4B' (4B), and 'Tifblue' (TF). The extra DraI band in 'Bluetta' was not present in longer digests and is assumed to be the result of incomplete digestion.

Table 1. Restriction fragment lengths of blueberry mitochondrial DNA (in kilobases) using various enzymes.

	Probe: pZmEI				
Plants	DraI	HindIII	AvaI	MspI	RsaI
Vaccinium corymbosum (4x)					
Rubel	3.6, 4.4	2.7	1.3, 1.85, 9.0	1.3, 1.4	1.4, 1.6
Bluecrop	2.7, 4.8	3.3	0.8, 2.8	1.0, 1.3, 1.6, 3.2, 4.2, 6.4	1.5, 1.9, 6.7
Bluejay	5.3	2.8	0.8, 2.8, 6.8	1.0, 1.6, 3.0, 4.2, 8.7	1.4, 1.6, 3.0
Jersey	2.7, 4.8	3.3	0.8, 2.8	1.3, 1.4, 7.3	1.5, 1.9, 2.9, 6.4, 7.8
Spartan	5.3	2.8	1.65, 2.0, 6.0	1.3, 1.4	1.4, 1.5, 2.9, 4.9
V. angustifolium x *V. corymbosum*					
Bluetta (4x)	5.1	3.8	1.5, 2.2	3.4, 4.6	1.5, 2.7
V. darrowi (2x)					
Florida 4B	3.65, 4.2	2.3, 4.9	1.85, 2.2	0.95, 1.6, 9.3	1.5, 1.9, 2.4, 7.5, 9.5
V. ashei (6x)					
Tifblue	1.8, 5.1	3.8	0.8, 1.3, 1.65, 2.0, 4.6, 6.2, 6.9, 8.1, 8.7	0.95, 1.3, 7.7, 9.2	1.6, 1.9, 2.4, 5.4, 10.5

plastid DNA to assess that variability.

RFLPs have been used as genetic markers to identify species, evaluate genetic diversity, and map genes of interest in numerous crops (Helentjaris et al., 1985, 1986; Landry et al., 1987; Osborn et al., 1987). They present heritable changes in the length of the fragments of genomic DNA arising by digestion with specific restriction enzymes. Chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) are generally inherited maternally in angiosperms (Levings and Pring, 1976; Palmer, 1985), although there are a few reports of paternal plastid inheritance (Schumann and Hancock, 1989; Wagner et al., 1987). The gene arrangement and sequence of cpDNA is typically highly conserved, while mtDNA can be variable depending on species (Ichikawa et al., 1989; Palmer, 1985; Timothy et al., 1979; Weissinger et al., 1983). Thus, analysis of cpDNA and mtDNA provides useful information for breeding programs and the study of phylogenetic and evolutionary relationships.

Five tetraploid highbush cultivars were evaluated: 'Bluetta', 'Bluecrop', 'Bluejay', 'Jersey', and 'Spartan'. Pedigree records indicate that 'Bluejay' and 'Spartan' carry the cytoplasm of the wild *V. corymbosum* selection 'Brooks', while 'Bluecrop' and 'Jersey' carry 'Rubel' cytoplasm (Hancock and Krebs, 1986). 'Bluetta' has the cytoplasm of the wild *V. angustifolium* selection 'North Sedgwick lowbush. In addition, genotypes of diploid *V. darrowi* and hexaploid *V. ashei* were examined. *Vaccinium darrowi* was represented by the wild selection 'Florida 4B' and *V. ashei* by 'Tifblue'. Several hybrids were evaluated, including US75, an interspecific hybrid between 'Florida 4B' and 'Bluecrop' that has been extensively used in the development of highbush cultivars (Draper et al., 1982), and 15 individuals from reciprocal crosses of 'Spartan' x 'Rubel' and 'Spartan' x 'Jersey'.

Total cell DNA was isolated from fully expanded leaves of greenhouse-grown plants by a modification of the CTAB procedure (Rogers and Bendich, 1985). Leaves were frozen in liquid nitrogen and ground to powder. The powder was thawed to room temperature and extracted in buffer (1:5 w/v) containing 100 mM Tris·base (pH 8.0), 1.4 M NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2% (w/v) hexadecyltrimethylammonium bromide (CTAB), 1% (w/v) polyvinylpyrrolidone (PVP), 10% (w/v) polyethylene glycol (PEG), 2% (w/v) Na sarcosine, and 0.8% (v/v) mercaptoethanol. The samples were incubated in a water bath at 65C for 1 to 2 h. The mixture was then extracted with 24 chloroform : 1 isoamyl alcohol : 1 phenol (by volume) to remove protein and pigments before phase separation by centrifugation (2600 x *g* for 10 min). The upper phase, containing the DNA, was pipetted off between extractions. DNA was precipitated with two volumes of 100% ethanol (EtOH) at -20C, rinsed with 75% EtOH to remove salt, and then 100% EtOH to speed drying. The DNA pellet was dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and further purified on cesium chloride/ethidium bromide gradients (Maniatis et al., 1982).

DNA (4 µg) from each plant was digested with the following 23 enzymes: AvaI, BamHI, BclI, BglI, BglII, CfoI, ClaI, DdeI, DraI, EcoRI, EcoRV, HindII, HindIII, KnpI, PstI, PvuI, RsaI, SalI, ScaI, SmaI, SspI, SstI, and XhoI as recommended by the supplier (Boehringer Mannheim Biochemicals, Indianapolis). Restriction digests were fractionated by electrophoresis through 0.8% or 1% (w/v) agarose horizontal slab gels in TBE buffer (0.089 M Tris·boric acid and 0.002 M EDTA, pH 8.0) at constant 2 $V \cdot cm^{-1}$ for 18 to 20 h. The gel was stained with 0.5 $\mu g \cdot ml^{-1}$ ethidium bromide (10 $mg \cdot ml^{-1}$ stock solution), illuminated with an ultraviolet light box and photographed with a Polaroid camera. The DNA was then transferred to nitrocellulose by the procedure of Southern (1975). After a minimum of 24 h, the filter was baked at 80C for 2 h in a vacuum oven.

Hybridization conditions were according to Thomashow et al. (1981). The probes were labeled with ^{32}P-dCTP using random oligonucleotides as primers according to Boehringer Mannheim recommendations. A 1 kilobase (kb) pea rbcL clone (gift from J. Palmer), and 21.8 kb (P-2), 19.4 kb (P-4), and 18.5 kb (P-5) tomato chloroplast PstI clones (Phillips, 1985) were used for the

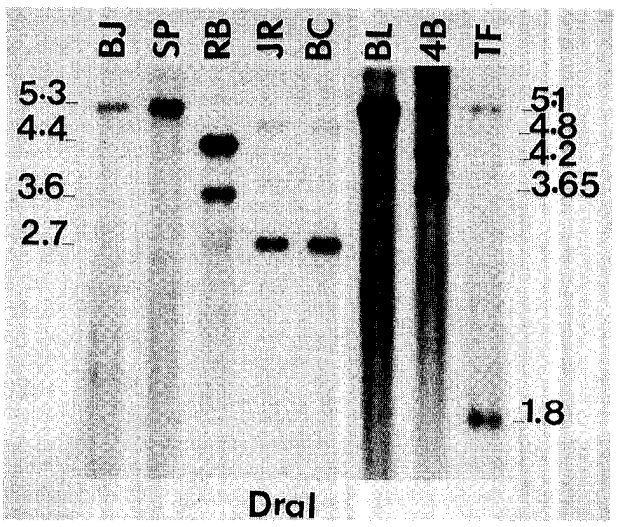

Fig. 2. Hybridization of mitochondrial probe pZmEI to DraI digests of 'Bluejay' (BJ), 'Spartan' (SP), 'Rubel' (RB), 'Jersey' (JR), 'Bluecrop' (BC), 'Bluetta' (BL), 'Florida 4B' (4B), and 'Tifblue' (TF). Fragment sizes are given in kilobases.

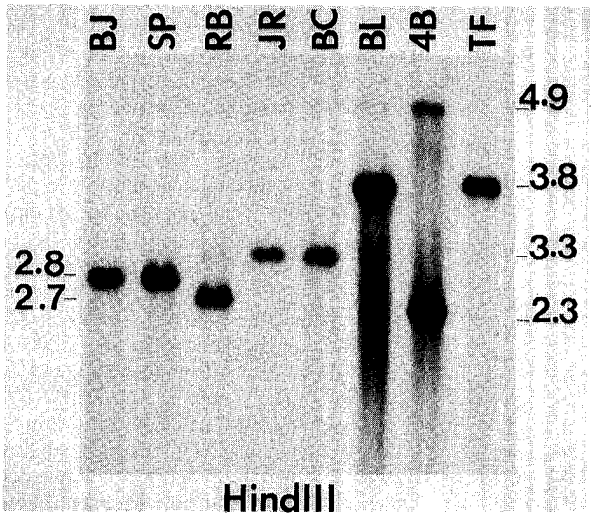

Fig. 3. Hybridization of mitochondrial probe pZmEI to HindIII digests of 'Bluejay' (BJ), 'Spartan' (SP), 'Rubel' (RB), 'Jersey' (JR), 'Bluecrop' (BC), 'Bluetta' (BL), 'Florida 4B' (4B), and 'Tifblue' (TF). Fragment sizes are given in kilobases.

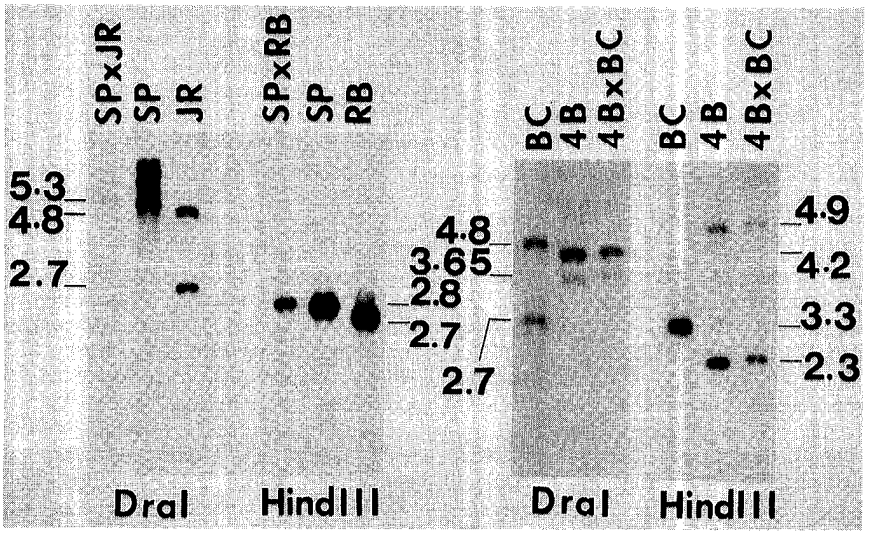

Fig. 4. Hybridization of mitochondrial probe pZmEI to total DNA digested with DraI and HindIII for inter- and intraspecific hybrids and parents. 'Spartan' x 'Jersey' (SP x JR), 'Spartan' x 'Rubel' (SP x RB), and 'Florida 4B' x 'Bluecrop' (4B x BC). Maternal parent is listed first in crosses. Fragment sizes are given in kilobases.

analysis of chloroplast DNA. The mtDNA was probed with pZmEI, a 2.4-kb EcoRI fragment encoding maize mitochondrial cytochrome c oxidase subunit II (Fox and Leaver, 1981; provided by B.B. Sears).

The same nitrocellulose filters were hybridized with cpDNA and mtDNA probes after removing the previous probe from the filter (Wight et al., 1986). Sizes of DNA fragments were estimated by comparison to Hind111 digests of lambda DNA.

Identical cpDNA restriction patterns were observed among the different cytoplasms, regardless of the restriction enzymes used. Figure 1 shows the uniform RFLP profiles of plants when the 19.4-kb chloroplast-specific probe of tomato (P-4) was hybridized to total DNA. Hybridization with the other three cpDNA probes also failed to uncover any polymorphisms (data not shown). Similarly, no variation in cpDNA was observed in any of the interspecific and intraspecific crosses.

A large degree of restriction fragment length polymorphism was observed across the mtDNAs of *Vaccinium* when digested with DraI, HindIII, AvaI, MspI, and RsaI, and probed with pZmEI (Table 1, Figs. 2 and 3). Analysis of tetraploid cultivars using the pZmEI probe uncovered several mtDNA types with DraI and HindIII restriction enzymes. Interestingly, 'Rubel' contained different fragment lengths than 'Bluecrop and 'Jersey', indicating that 'Bluecrop' and 'Jersey' do not carry 'Rubel' cytoplasm as their pedigrees imply (Hancock and Krebs, 1986). Our data are insufficient to determine the true maternal parent of these two cultivars.

When DNAs of interspecific hybrids and intraspecific hybrids were digested with DraI and HindIII and analyzed using pZmEI, they all displayed phenotypes identical to the female parent, indicating maternal inheritance of mtDNA (Fig. 4).

Our analysis of cpDNA of selected *Vaccinium* taxa revealed that the chloroplast genome has undergone little nucleotide change in the portion of the genome examined. This mirrors numerous other studies in plants (Palmer, 1985). It is possible that the chloroplast genome in *Vaccinium* first differentiated at the diploid level and has not changed after polyploidization and speciation. A similar situation was postulated in rice *(Oryza sativa* L.) (Ogihara and Tsunewaki, 1988). Blueberry species that we did not evaluate may exhibit variability in their cpDNA. Hosaka and Hanneman (1988) found uniform cpDNA types in both the common and Chilean potatoes *(Solanum tuberosum* ssp. *tuberosum)* even though the proposed progenitor, the Andean potatoes *(Solanum tuberosum* ssp. *andigena),* the South American tuber-bearing species, contained diverse patterns of cpDNA variation.

In contrast to cpDNA, the mitochondrial genome appears to have undergone substantial change. Similar patterns have been observed in many other angiosperms. Lonsdale (1984) suggested that the mtDNA of plants is larger and less stable than cpDNA because the more repetitive mtDNA may promote re-

combination and the alteration of restriction patterns. It has also been suggested that the inverted repeat found in most plastomes may suppress recombinations or that plastome mutations may be corrected by gene conversions (for review see Palmer, 1985).

The large amounts of variation observed in the mtDNA of *Vaccinium* indicate that breeders fortuitously selected variable cytoplasms in creating highbush cultivars. This is not surprising, since three divergent species at two ploidy levels are represented. It is not known if these DNA variations translate into functional differences, but the high degree of mtDNA variability among the different cytoplasmic sources suggest that RFLPs should provide useful information on the evolution and taxonomy of the group. Nuclear RFLPs should also aid in locating economically important traits through the development of linkage maps.

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Spontaneous Tetraploid Melons

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Additional index words. muskmelon, cantaloupe, *Cucumis melo,* polyploidy, virescent marker, diploid, triploid, seedless melons

Abstract. **Since 1968, three spontaneous 4x melons *(Cucumis melo* L.) plants were discovered in our field or greenhouse plantings. Two were found in the cultivar Planters Jumbo and one in the virescent marker C879-52. Each of these 4x plants had rounded cotyledons, shorter internodes, thicker stems and leaves, more hairs, and smaller fruits, with larger stem and blossom scars, than their 2x counterparts. Also, their flowers, pollen grains, stomates, and seeds were larger. The discovery of a 4x virescent plant in 1987 allows easier germplasm transfer between ploidy levels. Morphological characteristics of 2x and 4x melons will allow identification without need for chromosome counts.**

Interest in tetraploid (4x = 48 chromosomes) melons began in the 1930s with the discovery that colchicine produced polyploid plants. Shifriss (1942) began developing tetraploids of *Cucumis sativus* L. with colchicine in 1939 and then produced tetraploids of *C. melo* L. Batra (1952) developed six 4x melon cultivars with colchicine. Fruit quality in these tetraploids was superior to diploids (2x = 24 chromosomes); however, yields were lower in five of the six 4x lines. The 4x plants had larger flowers, pollen grains, and stomates in addition to thicker stems and lower fertility than the 2x plants. Tetraploid fruit were smaller and rounder than diploid fruit. Kubicki (1962) developed 4x cucumbers and melons and stated that spontaneous 4x types were lacking in the genus *Cucumis.* Dumas de Vaulx (1974) induced 4x with colchicine, studied fertility, and confirmed the morphological characteristics described by previous workers. He found that pollen tube growth was normal and did not cause poor seed set in self-pollinated 4x or crosses between 2x and 4x plants. A few viable seeds were obtained when the 4x parent was female and none when it was male. Ervin (1941) studied polysomaty in *C. melo.* He found that 4x cells occur regularly in root and stem tips of most plants. This finding suggested that polysomaty may also occur in gametes and that spontaneous 4x plants might be

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