2 (see Mowrey et al., 1990, for origin of peach plant introductions). A similar trend was found for the Mdh 1-3 and Mdh 1-2 alleles in peach (Mowrey et al., 1990).

This study identified a new genetic marker in peach and further substantiates that the North American germplasm base of peach is very narrow. Further, allozyme variation revealed in this and a previous study (Mowrey et al., 1990) of the PI collection suggests that this collection should be examined for other valuable qualitative and quantitative traits.

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Use of Stored Pollen to Hybridize a Mandarin Hybrid and *Citrus tachibana*

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Additional index words. fruit breeding, isozymes, tachibana orange, glutamate-oxaloacetate transaminase

Abstract. **Fresh pollen from *Citrus tachibana* Macf. was oven-dried (37C), freeze-dried, or placed into anhydrous acetone, and stored at -20C over silica gel. Pollen freeze-dried or stored in anhydrous acetone did not germinate 24 hours after treatment; oven-dried pollen germinated in 1 hour and was comparable to fresh pollen. Pollen that was oven-dried for 12 hours and stored for 1 year was used to pollinate a monoembryonic hybrid of 'Temple' (origin unknown) x 'Orlando' *(C. paradisi* Macf. 'Duncan'xC. *reticulata* Blanco 'Dancy'). Glutamate-oxaloacetate transaminase (GOT) isozyme profiles verified progeny hybridity.**

Citrus species are all sexually compatible, although some inter- or intraspecific hybrids are difficult to create because of varying degrees of facultative apomixis (Barrett and Rhodes, 1976). Use of C. *tachibana* (tachibana orange) germplasm in the U.S. Dept. of Agriculture-Agricultural Research Service (USDA-ARS) breeding program has not been possible for two reasons. First, C. *tachibana* is highly apomictic (C.J.H. and D.J. Hutchison, unpublished); most seedlings are nucellar in origin, thus precluding its use as a seed parent. Second, it flowers in the Orlando area 4 to 6 weeks later than most other *Citrus* spp. and hybrids, thus preventing its use as a pollen parent. The purpose of this work was to determine if tachibana orange pollen could be stored for 1 year with sufficient viability to be used in controlled hybridizations.

Unopened flowers approaching anthesis were collected in Apr. 1988 from a mature *C. tachibana* tree growing in the germplasm collection at the USDA-ARS, A.H. Whitmore Research Farm near Leesburg, Fla. Flowers were stored in covered polystyrene dishes at 25C until anthesis and pollen dehiscence, and anthers were cut from the filaments with a scalpel. Fresh anthers were oven-dried at 37C for 3, 6, 12, or 24 h; or freeze-dried (-40C, 10 millitorr) for 1, 2, 4, or 8 h. Dried anthers were stored in 1.5 ml microcentrifuge tubes over silica gel at -20C. Pollen and anthers were also stored at -20C in anhydrous acetone prepared by mixing S-mesh Drierite (W.A. Hammond Drierite, Xenia, Ohio) with acetone for 1 h, and then filtering through Whatman no. 1 filter paper (Thomas Scientific, Swedesboro, N.J.) to remove the hydrate. The entire process was repeated two more times. This pollen was also removed 24 h later, washed three times in distilled water (dH_2O), rehydrated, germinated, and stained.

Anthers were removed from cold storage and tested for pollen viability 24 h later. They were poured onto a glass slide and placed into a polystyrene dish over wet filter paper for 1 h to rehydrate the pollen. Pollen was germinated in Brewbaker's medium (Sahar and Spiegel-Roy, 1980) for 4 h at 27C, stained with Alexander's stain (Alexander, 1980), and counted to determine viability. At least 1000 pollen grains were counted per treatment. A pollen grain was considered viable if tube length exceeded grain diameter. Stored pollen was used the next season to pollinate emasculated flowers of a seedy, monoembryonic 'Temple' x 'Orlando' hybrid. Flowers were bagged after pollination, although this is generally unnecessary in *Citrus* breeding as honeybees, the primary *Citrus* pollen vectors, do not visit flowers after the petals have been removed (C.J.H. and D.J. Hutchison, unpublished), and wind is not a sig-

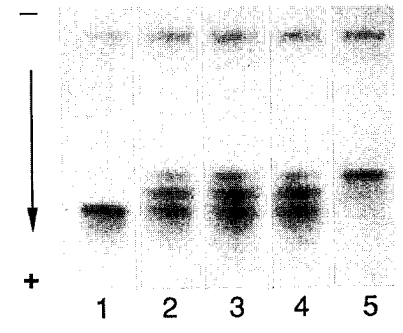

Fig. 1. GOT isozyme profiles of *C. tachibana,* 'Temple' x 'Orlando' hybrids, and three hybrid seedlings. Lane 1, *C. tachibana;* lanes 2–4 hybrids of ('Temple' x 'Orlando') x *C. tachibana;* lane 5, 'Temple' x 'Orlando' seed parent.

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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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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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