Estimating Nuclear DNA Content in Peach and Related Diploid Species Using Laser Flow Cytometry and DNA Hybridization

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Abstract. Using laser flow cytometry, nuclear DNA amounts were estimated for 12 *Prunus* species, representing three subgenera [Prunophora (Prunus), Amygdalus, and Cerasus (Lithocerasus)], two interspecific hybrids, four cultivars, and a synthetic polyploid series of peach consisting of haploids, diploids, triploids, and tetraploids (periclinal cytochimeras). Peach nuclear DNA content ranged from 0.30 pg for the haploid nuclei to 1.23 pg for the tetraploid nuclei. The diploid genome of peach is relatively small and was estimated to be 0.60 ± 0.03 pg (or 5.8×10^8 nucleotide base pairs). The polyploid series represented the expected arithmetic progression, as genome size positively correlated with ploidy level (i.e., DNA content was proportional to chromosome number). The DNA content for the 12 diploid species and two interspecific diploid hybrids ranged from 0.57 to 0.79 pg. Genome size estimates were verified independently by Southern blot analysis, using restriction fragment length polymorphism clones as gene-copy equivalents. Thus, a relatively small and stable nuclear genome typifies the *Prunus* species investigated, consistent with their low, basic chromosome number (x = 8).

Prunus (Rosaceae), a genus of deciduous or evergreen trees or shrubs, contains >400 species adapted primarily to the temperate regions of the northern hemisphere (Krussmann, 1986). The genus, and the family as a whole, are of considerable economic importance for fruit, seed, and ornamental production (Rehder, 1940). The peach [*Prunus persica* (L.) Batsch] is a diploid species and a member of the subfamily Prunoideae (Amygdaloideae), in which the chromosome base number is x = 8 (Hesse, 1975; Sax, 1932). For commercial peach varieties, 2n = 16, although a small polyploid series exists.

Like many other tree fruit crops, peach production relies on the genetic makeup and interplay of two distinct genotypes—the scion and the rootstock. Research efforts strive to improve this germplasm and expand the adaptability of peach to new production areas and to withstand biotic and abiotic stresses in traditional production areas (Childers and Sherman, 1988). However, because of the narrow germplasm base for peach, success has been limited (Scorza et al., 1985). As a result, many breeders have turned to wild relatives and exotic germplasm in search of useful traits for developing improved cultivars (Reighard et al., 1989a, 1989b; Westcott et al., 1994).

Peach is not as well defined genetically as are many herbaceous species (e.g. corn, tomato, or *Arabidopsis*), and comparatively little is known about its cytology (Jelenkovic and Harrington, 1972). However, peach is considered to be the best-characterized tree fruit species (Mowrey et al., 1990). There are ≈40 Mendelian traits, phenotypic (Hansche, 1988; Monet, 1989; Ramming, 1991; S.A. Mehlenbacher, personal communication) and biochemical (Arulsekar et al., 1986), that have been described to date. In addition, heritability has been estimated for 20 other traits (Monet, 1989). A molecular genetic linkage map, using intraspecific F_2 populations, is being developed as a permanent resource for geneticists and breeders (Abbott et al., 1992; Chapparro et al., 1992; Eldredge et al., 1992).

Genome size is a fundamental parameter in many genetic and molecular biological studies. Knowledge of the haploid nuclear DNA content (C value) is important for basic and applied studies involving genome organization, species relationships, gene expression analysis, and germplasm improvement (Bennett, 1984). For example, genome size estimates are important when constructing and screening genomic or cDNA libraries (Clarke and Carbon, 1976; Frischauf, 1987). It is also necessary for developing linkage maps for genetic analysis and breeding purposes, and in efforts to estimate the recombinational length of nuclear genomes and correlate this genetic distance with physical distance (Meagher et al., 1988). Finally, this information can be useful in evaluating reproductive and somatic compatibility, an important parameter in scion breeding and rootstock selection programs, especially for those using interspecific crosses.

Genome size has been estimated using Feulgen scanning microspectrophotometry (microdensitometry), chemical extraction, nuclear volume ratios, reassociation kinetics, or laser flow cytometry. Although each method has its appropriate application, as dictated by inherent limitations and deficiencies (e.g., expense, time, sample size), flow cytometry is becoming the method of choice (Galbraith, 1989; Michaelson et al., 1991a). However, in many instances it is best to use more than one analytical approach to make the most reliable estimation and to validate the most convenient method (Berlyn and Miksche, 1976; Michaelson et al., 1991a).

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Recently, flow cytometric analysis has been applied to members of the Rosaceae in a survey of the four subfamilies, which included six *Prunus* species and two peach cultivars, by Dickson et al. (1992). They pointed out possible discrepancies in previously published work, again where only a single method had been used (microdensitometry). Our paper reports results using flow cytometry and differential DNA hybridization to analyze genome size for peach (four cultivars) and 11 other diploid *Prunus* species representing three subgenera [Prunophora (Prunus), Amygdalus, and Cerasus (Lithocerasus)].

Materials and Methods

The principal source of plant material was newly expanded mature leaves collected early in the summer from 2-year-old seedlings grown in greenhouses on the Clemson Univ. campus or from mature orchard trees. The orchard trees were maintained at Clemson Univ.'s Musser Fruit Tree Research Farm, Seneca, S.C., and at North Carolina State Univ.'s Sandhill Research Station, Norman, N.C. Older, late summer leaves gave large amounts of debris, gums, and very poor nuclei yields.

Suspensions of intact nuclei were prepared by slicing 50 to 150 mg of leaf material in 1.5 ml of either a Mg-HEPES buffer with dithiothreitol (Arumuganathan and Earle, 1991a) or a Mg-MOPS buffer (Michaelson et al., 1991a) containing Triton X-100 (0.2%) and a saturating amount of propidium iodide (PI) (Sigma Chemical Co., St. Louis) (Michaelson et al., 1991a; Taylor and Milthorpe, 1980). The leaf material was rinsed thoroughly with distilled water before slicing. A fresh scalpel blade was used for each preparation. When plant nuclei were to be used as reference standards (see below), their leaves were processed with the experimental material.

Nuclei suspensions were filtered (to remove tissue debris and whole cells) through two layers of Miracloth (CalBiochem, LaJolla, Calif.) or 20-μm nylon mesh held in a syringe filter unit. The nuclei

were concentrated for washing by a low-speed centrifugation at $200\times g$ (model 59A; Fisher Scientific, Norcross, Ga.) for 3 min to pellet and remove any remaining debris, followed by an additional low-speed centrifugation of the supernatant at $200\times g$ for 10 min. Finally, the nuclei were resuspended in 300 μl of the original buffer, to which ribonuclease A had been added (40 μg·ml^{-1} final concentration, DNase free), and incubated for 20 min at 37C. The final filtered and stained suspensions had a concentration of $\geq 2 \times 10^6$ nuclei/ml. All manipulations were carried out on ice or at 5C, except the RNA digestion. Each step was monitored visually using a microscope (OPTIPHOT; Nikon, Melville, N.Y.) set-up for phase-contrast or epifluorescence illumination.

Initial DNA content was estimated using four different sources of nuclei as internal calibration standards (Michaelson et al., 1991a; Tiersch et al., 1989). The first three were from plants: barley (*Hordeum vulgare* 'Sultan') with a 2C DNA content = 11.12 pg; corn (*Zea mays* ssp. mays, Va 35) with a 2C DNA content = 5.37 pg (both provided by J.D. Smith and J. Price, Texas A & M Univ); and rice (*Oryza sativa* 'Indica', IR 36) with a 2C DNA content of ≈ 1.00 pg (Arumuganathan and Earle, 1991b; Bennett and Smith, 1976) (provided by G. Kochert, Univ. of Georgia). All plants were grown in the greenhouse from seed in sandy medium, and the leaves were harvested from 3- to 4-week-old plants. The fourth standard was chicken (*Gallus domesticus*) red blood cells (CRBCs) (erythrocytes) with a reported 2C DNA content of 2.33 pg (Galbraith et al., 1983; Vindelov et al., 1983) (provided by Clemson Univ.) (Kendall et al., 1992). We settled on CRBCs as the standard for subsequent analyses of all *Prunus* species because essentially identical estimates were obtained regardless of the standard used (although the barley genome was too large to analyze our *Prunus* spp. reliably) and because a large number of high-quality CRBC nuclei could be obtained easily. Using the nucleic acid-intercalating fluorochrome PI, which unlike Hoechst, mithramycin, and DAPI shows little base-composition specificity, facilitated direct comparison between each specimen–standard
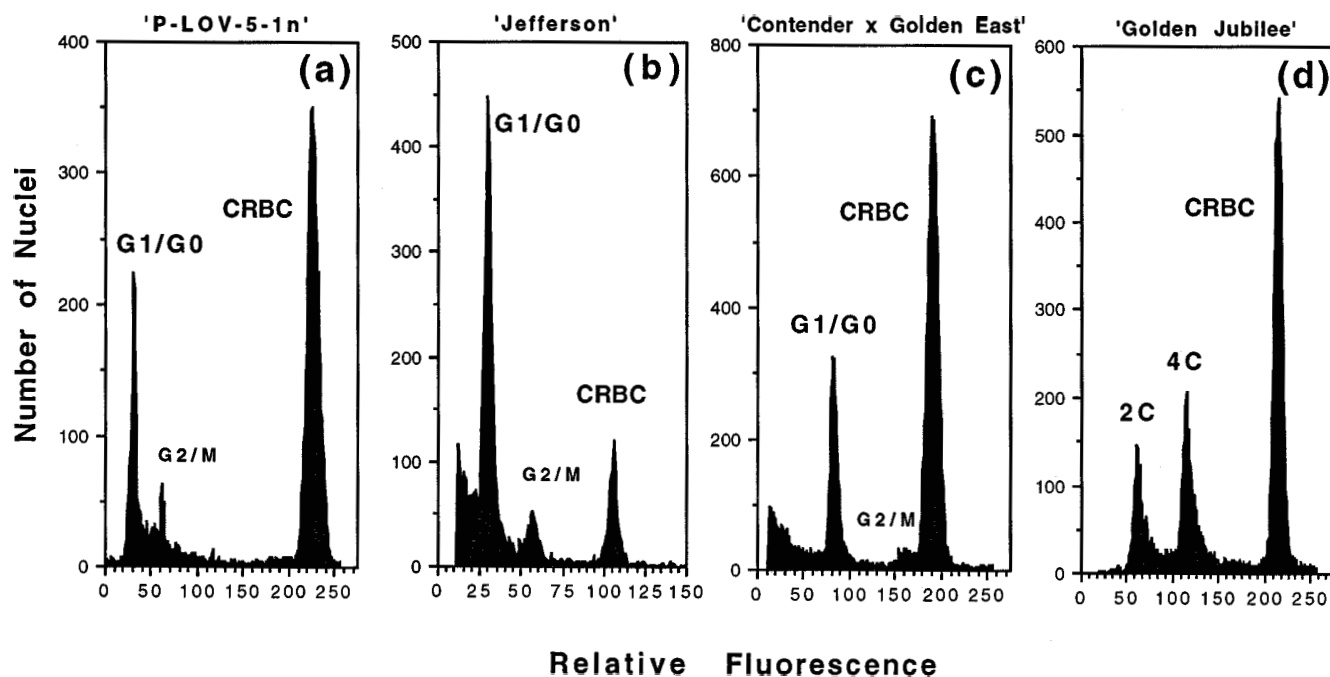
Fig. 1. Frequency distribution histograms plotting the number of nuclei as a function of their relative linear fluorescence. CRBC is the internal calibration standard. Specimen: *Prunus persica* (**a**) haploid, 'P-LOV-5-1n', mode of G1/G0 peak = 30, G2/M peak = 61, and CRBC = 225; (**b**) diploid, 'Jefferson', mode of G1/G0 peak = 30, G2/M peak = 57, and CRBC = 107; (**c**) triploid, 'Contender' x 'Golden East', mode of G1/G0 peak = 81, G2/M peak = 159, and CRBC = 191; (**d**) tetraploid cytochimera, 'Golden Jubilee', mode of 2C G1/G0 peak = 60, 4C G1/G0 peak (including some diploid nuclei in G2/M) = 114, and CRBC = 215.

combination for determining absolute DNA amounts (Gray and Langlois, 1986; Michaelson et al., 1991a; Otto, 1990).

Specimens were analyzed on a flow cytometer (EPICS 751; Coulter Electronics Corp., Hialeah, Fla.) equipped with a multiparameter data acquisition and display system and a 400-mW argon laser (model I-90; Coherent, Palo Alto, Calif.) lasing at 488 or 514 nm. Digital information from the experimental material was accumulated on >5000 fluorescent events (e.g., nuclei) per sample and stored as an accumulation of frequency distributions as single-parameter histograms. These data were displayed, in real time, as histograms of the number of nuclei (or frequency) along the y-axis vs. the relative fluorescence intensity (or channel number) on the x-axis. The procedure of Cameron (1990) was used to transfer data files to a Macintosh computer for graphical display and analysis. The translated data files were imported individually as text files into Cricket Graph (version 1.3.1; Cricket Software, Malvern, Pa.) and again displayed as histograms. Measurements of each specimen, with two replicates per sample, were repeated at least three times. Only data collected from samples with G1/G0 peaks with coefficient of variations (cvs) ≤5% were used in C-value estimates.

Genomic Southern blots were prepared by digesting purified nuclear DNA with a restriction endonuclease according to the supplier's suggestions. DNA was isolated by standard salt extraction methods (Sambrook et al., 1989). The yield, purity, and quality were evaluated by spectrophotometry, fluorometry, and gel electrophoresis. Plasmid clones (pB2D4, pB4A6, pB4A9, and pB6H11) containing fragments of peach nuclear DNA, representing single-copy restriction fragment length polymorphism (RFLP) probes previously characterized in our molecular linkage mapping project (Eldredge et al., 1992), were similarly digested, and the inserts were purified and quantified to yield solutions of known concentrations. DNA was quantified by fluorometry using a minifluorometer (model TKO 100; Hoefer Scientific Instruments, San Francisco), Hoescht dye 33258, and calf thymus DNA as a standard. In this way, and using the ratio between estimated genome size and RFLP fragment size in proportion to the amount of genomic DNA digested, a dilution series was established that represented 0.1, 0.5, 1, 5, and 10 gene-copy equivalents. The digested and quantified DNA samples were then size-fractionated in 0.8% agarose gels, transferred, and covalently bound to nylon membrane by capillary blotting using standard techniques (Sambrook et al., 1989). The membranes were differentially hybridized with each radioactively labeled (Feinberg and Vogelstein, 1984) RFLP probe. After washing the membranes at high stringency (68C; 75 mM NaCl, 7.5 mM Na citrate, and 0.2% SDS) to remove unbound probe, they were exposed to x-ray film at –70C for 1 to 3 days. The resulting autoradiograms were scanned using an enhanced laser densitometer (UltroScan XL; LKB, Piscataway, NJ) connected to an IBM 386SX computer running GelScan XL software (LKB). Quantitative evaluation, with a resolution of 50 μm and a linear range of 0 to 4 absorbance units at full scale, was accomplished by single-dimensional scanning. Results are reported as relative volume (RV = absorbency units × area).

Results and Discussion

The yield, integrity, and size of nuclei isolated using the methods of Michaelson et al. (1991a) and Arumuganathan and Earle (1991a) were determined. There was no significant difference between the two procedures when the above parameters were evaluated, and both methods provided reliable and reproducible results for the *Prunus* genotypes studied.

Figure 1 shows typical results obtained by flow cytometric analysis of isolated peach nuclei processed simultaneously with CRBC as the internal standard. Figure 1b, *Prunus persica* 'Jefferson', displays the relative linear distribution of fluorescence (channel number, on the x-axis) vs. the number of fluorescent events (nuclei counted, on the y-axis). This histogram shows a prominent peak of nuclei in the G1 (and G0) stage of interphase during the cell cycle (i.e., the chromosome cycle and nuclear division followed by cell division). This peak of fluorescent emission shows a mode, identified by distance from the origin, at channel number 30. The cv is 5.0%. These interphase nuclei, in a diploid species such as peach, are at the 2C level of DNA content.

Also in Fig. 1b, a minor peak at about twice the fluorescent value of the first can be distinguished (modal channel number 57). This later peak is interpreted as being composed of nuclei primarily in the G2 stage of interphase and in the early and mid stages of mitosis during the cell cycle. Nuclei in this small peak have a relative average DNA content of 4C. Extensive fluorescent emissions at higher channels, indicative of populations of nuclei at increased ploidy levels (e.g., 8n, 16n) or nuclear adhesion (e.g., artifactual aggregation of individual nuclei to form triplets, quadruplets), were not observed.

By comparing the level of fluorescence of the peach nuclei peak to that of the internal standard (CRBC, 2C DNA content = 2.33 pg; modal fluorescence at channel 107, cv = 4.1%), the DNA content of the peach nuclei can be determined using a simple comparative ratio. In this example, the 2C value of peach nuclei is calculated to be 0.65 pg (2.33 × 30/107).

Figure 1 also displays results from the flow cytometric analysis of three peach varieties with exceptional ploidy levels. Haploid peach trees arise spontaneously at about one in every 1000 seedlings (Hesse, 1971; Toyama, 1974). The DNA content of nuclei from a haploid peach tree was estimated to be 0.31 pg (Fig. 1a). Figure 1c represents the flow cytometric analysis of a triploid peach cultivar (e.g., Contender x Goldeneast), and shows a mean nuclear DNA content equivalent to 0.99 pg.

Certain tetraploid peach cultivars ('Golden Jubilee', 'Goldeneast') are actually periclinal cytochimeras in which the mature vegetative tissues are organized mixtures of diploid and tetraploid cells (Dermen and Stewart, 1973). Our analysis confirms the presence of two distinct populations of nuclei, one with a DNA content near the previously estimated 2C amount (e.g., 0.64 pg, cv = 4.8) and the second at about twice this amount (e.g., 4C = 1.24 pg, cv = 3.7) (Fig. 1d). The second peak is in the position predicted for 2C nuclei in G2/M, but its amplitude is distinctly larger than expected. This is because the 2C G2/M nuclei are contributing only a small portion to this peak, while most of the fluorescence is due to tetraploid nuclei in G0/G1, with a 4C DNA content. Similarly, a fluorescence peak, representing 4C nuclei in the G2/M condition, is masked by the large CRBC nuclei peak. This peak, but no others in downstream positions indicative of increased ploidy levels, is seen when nuclei isolated from the cytochimeras are analyzed without CRBCs included as an internal standard or with other nuclei standards (e.g., rice or corn; data not shown). The presence of a third peak (8C) also argues against the second peak representing a large number of 2C nuclei blocked in G2. Tables 1 and 2 summarize the estimated mean nuclear DNA content obtained for independent analyses of different peach cultivars and *Prunus* species, respectively.

Intraspecific variation in DNA content of >20% has been reported in some other diploid species (Bennett, 1987; Laurie and Bennett, 1985; Michaelson et al., 1991b; Price et al., 1980). In contrast, the range of mean values of the four peach cultivars

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Table 1. Mean nuclear DNA content of peach (*Prunus persica*) cultivars.

Peach cultivar (cytotype)	Estimated DNA content (pg)	
	Mean[z]	SD
Jefferson	0.62 a	0.05
Ferganensis	0.64 a	0.07
Lovell	0.58 a	0.03
Nemaguard	0.57 a	0.04
Haploid	0.30 b	0.02
Triploid	0.98 c	0.05
Tetraploid[y]	0.63 a	0.04
	1.23 d	0.07

[z]Means followed by the same letter are not significant at $P \leq 0.01$. Periclinal cytochimera.

Table 2. Mean nuclear DNA content of various *Prunus* species.

Species	Common name (type)	Estimated DNA content (pg)	
		Mean[z]	SD
P. angustifolia	Chickasaw plum	0.61 a	0.04
P. besseyi	Western sand cherry	0.67 ab	0.05
P. cistena	Purple-leaf plum	0.64 a	0.04
P. davidiana	King David's peach	0.62 a	0.02
P. dulcis[y]	Almond	0.66 ab	0.06
P. davidiana x *P. dulcis*	Interspecific hybrid	0.74 b	0.05
P. persica x *P. dulcis*	Interspecific hybrid	0.79 b	0.07
P. fenzliana	Almond-type	0.75 b	0.06
P. hortulana	Hortulan plum	0.60 a	0.03
P. kansuensis	Peach-type	0.60 a	0.04
P. mira	Wild Chinese peach	0.66 ab	0.07
P. tomentosa	Nanking cherry	0.57 a	0.05
P. webbii	Almond-type	0.77 b	0.08

[z]Means followed by the same letter are not significant at $P \leq 0.01$.
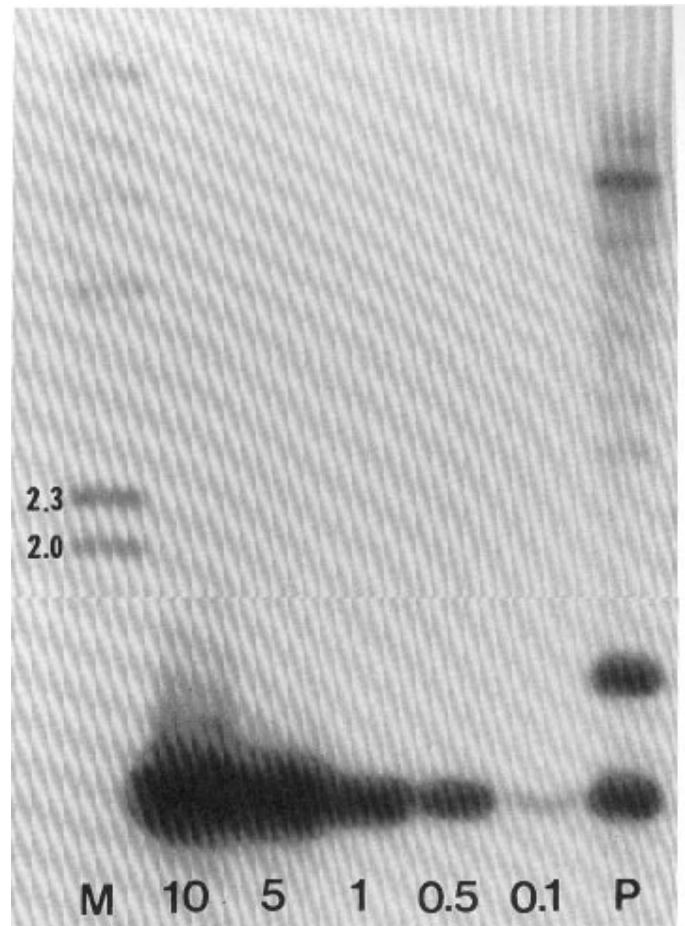[y]*Prunus dulcis* (Mill.) D.A. Webb (*P. amygdalus* Batsch); mission almond (Texas prolific).

Fig. 2. Ten micrograms of peach genomic DNA and varying amounts of plasmid pB2D4 were digested with *Eco* RI, size fractionated in 0.8% agarose and transferred to nylon membrane. This membrane was then hybridized with a solution containing a radioactively labeled RFLP clone, B2D4 (800 base pairs, bp). After hybridization the blot was washed in 0.5x SSC at 68C for 1 h and exposed to x-ray film. P = peach genomic DNA; 10, 5, 1, 0.5, and 0.1 gene-copy equivalents of B2D4; M = molecular size standards are 2.3 and 2.0 kbp (from *Hind*III digest of bacteriophage lambda DNA).

reported in Table 1 are within a single SD of one another, and the variation is not statistically significant ($P < 0.01$). The observed variation cannot be a function of the internal reference standard used, since no differences were observed regardless of source. Therefore, this small variation is likely attributable to experimental error.

Large interspecific (intrageneric) variations in nuclear DNA content, such as the 2- to 10-fold differences reported for species of *Zea, Microseris, Helianthus,* and *Vicia* (Price et al., 1983; Rees, 1984; Sims and Price, 1985), were not observed among *Prunus* species investigated here (Table 2) or in previous studies (Arumuganathan and Earle, 1991b; Dickson et al., 1992). This is not unexpected, since these species are all diploids with identical chromosome numbers (2n = 16, x = 8), some of which have been crossed to produce hybrids. Arumuganathan and co-workers (Arumuganathan and Earle, 1991b; Dickson et al., 1992) similarly found large variation only in the polyploid *Prunus* taxa they analyzed. We found larger genome sizes typically in the amygdaloid species, (e.g., with the almonds and their hybrids). Our data, taken together with that of Arumuganathan and Earle (1991b) and Dickson et al., (1992), investigating 20 members of the genus *Prunus*, documents that the small nuclear genome of *Prunus persica* and many of its diploid relatives is relatively stable with comparatively little variation in DNA content. This is consistent with the constant basic chromosome number in this genus (x = 8) and is likely related to the ability of several members to form (fertile) interspecies hybrids or serve as compatible graft partners.

Our estimates of diploid peach nuclear DNA content are in line with those estimated for the polyploid series (Table 1). These 2C values are consistent with a recent report that examined two other peach cultivars (Dickson et al., 1992). Our average estimate of 0.60 ± 0.03 pg for peach is slightly higher than, but agrees favorably with, that reported by these authors (e.g., 0.55 ± 0.06 pg for 'Red Haven'; and 0.54 ± 0.05 for 'Madison').

To test the validity of our genome size determinations, a second method was used to estimate nuclear DNA content independently. Genomic Southern blots, containing gene copy reconstructions of 0.1, 0.5, 1.0, 5.0, and 10.0 gene equivalents (assuming an average 2C value for peach), were hybridized with one of four single-copy RFLP probes. Figure 2 illustrates results from one such analysis using 10 μg of peach DNA ('Jefferson') and 0.60 pg as the average 2C value. Visual inspection of the autoradiograms indicated that the single-copy reconstructions were most similar in intensity to the homologous sequences detected in the genomic DNA lane. Densitometric analysis of these hybridization signals showed that the copy reconstructions were comparatively linear and propor-

tional as expected from the dilution series (e.g., $0.1x = 1.5\,RV$, $0.5x = 10.2\,RV$, $1.0x = 14.7\,RV$, $5.0x = 73.0\,RV$, and $10.0x = 141.2\,RV$) and that the relative volume of signal in the single-copy sequence lanes was most similar to the homologous sequences detected in the genomic DNA lanes (e.g., 800 bp sequence detected in the P lane = 15.3 RV). This close similarity in hybridization signal density supports our estimate of the 2C nuclear DNA content, as determined by flow cytometry. Nearly identical results were obtained using three other single-copy RFLP probes on two other diploid peach cultivars (i.e., 'Lovell', 'Nemaguard'), as well as using these probes on haploid and triploid samples (data not shown). For reasons of variation in DNA yield per cell, this analysis can not be meaningfully applied to the tetraploid cytochimeras. Furthermore, our estimate for peach was corroborated by microdensitometric analysis of leaf nuclei of 'Jefferson' ($2C = 0.66 \pm 0.04$ pg, cv < 6.0%; H.J. Price, personal communication). A recent investigation in our laboratory of an enigmatic tetraploid species of *Eleusine* (unpublished data), revealed major discrepancies in published nuclear DNA content measurements of all but one species in this genus (Hiremath and Salimath, 1991) and points to the need for a second, independent method to evaluate such data. Differential DNA blot hybridization, which obviates the need of specialized microspectrophotometric equipment, is a useful alternative method.

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