DETERMINATION OF NUCLEAR DNA CONTENT IN ORCHIDS BY FLOW CYTOMETRY

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Abstract

Flow cytometric analysis was found useful for determining ploidy and DNA content values for orchid species and hybrids. Immature leaf tissue yielded the most reliable results for all orchids sampled. Leaf endopolyploidy was common, with levels as high as 16N found in mature *D. gouldii* leaves. DNA content values for 37 *Dendrobium* species, 11 *Dendrobium* hybrids, 33 additional orchid species and 8 hybrids were determined. Values for *Dendrobium* species ranged from 1.53 pg to 4.23 pg/2C nuclei. C-values for the remaining orchid species ranged from 1.91 pg to 15.19 pg/2C nuclei, with those of *Cattleya* alone ranging from 3.29 pg to 9.29 pg/2C nuclei. The highest variation within *Cattleya* corresponded to different specimens of the same species, suggesting polyploidy. An analysis of *Dendrobium* hybrids and parent species showed that DNA content values could be reliably predicted in cases where parentage was indefinite.

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List of Abbreviations

Orchid Authorities

Ames, Oakes ..... Ames
Banks, Joseph ..... Banks
Bailey, Fredrick Manson ..... Bail.
Bateman, James ..... Batem.
Blume, Carl Ludwig von ..... Bl.
Brown, Robert ..... R.Br.
Buchanan-Hamilton, ... Buch.-Ham.
Cribb, Phillip ..... Cribb
Deane, Henry ..... Deane
Dodson, Calaway H. ..... Dodson
Dressler, Robert L. ..... Dressler
Fischer, Friedrich Ernst Ludwig ..... Fisch.
Fitzgerald, Robert David ..... Fitzg.
Fritz, G. J. ..... Fritz
Gardner, Charles Austin ..... Gardn.
Griffith, William ..... Griff.
Henfrey, Arthur ..... Henfr.
Holttum, Richard Eric ..... Holt.
Hooker filius, Joseph Dalton ..... Hook.f.
Hortorum ("of the gardens") ..... Hort.
Hu ..... Hu
Jackson, George ..... Jacks.
Knoweles, George B. ..... Knowles
Kranzlin, Fritz Wilhelm Ludwig ..... Kranzl.
Kuntze, Carl Enrst Otto ..... Kuntze
(Linnaeus) Linne, Carl von ..... L.
Lindley, John ..... Lindl.
Lourero, Joao de ..... Lour.
Miquel, Friedrick Anton Wilhelm ..... Miq.
Moir, W.W. Goodale ..... Moir
Moore, Fredrick William ..... Moore
Mueller, Ferdinand Jakob Heinrich ..... F. Muell.
O'Brien, James ..... O'brien
Pfitzer, Ernst Hugo Heinrich ..... Pfitz.
Reichenbach filius, Heinrich Gustav ..... Rchb.f.
Reinwardt, Casper George Carl ..... Reinw.
Richard, Achille ..... A.Rich.
Ridley, Henry Nicholas ..... Ridl.
Rolfe, Robert Allen ..... Rolfe
Roxburgh, William ..... Roxb.
Rupp, Herman Montaque Rukker ..... Rupp
Schiede, ... Scheide
Schlechter, Friedrich Richard Rudolf ..... Schlr.
Seidenfaden, Gunnar ..... Seidenf.
Senghas, K. ..... Sengh.
Shauer, Johann Conrad ..... Shauer
Sims, John ..... Sims
Smith, Johann Jacobus ..... J.J.Sm.
Stein, B ..... Stein
Steudel, Ernst Gottieb von ..... Steud.
Swartz, Olaf Peter ..... Sw.
Teijsmann, Johannes Elias ..... Teijsm.
Thomson, Thomas ..... Thomson
Thunberg, Carl Peter ..... Thunb.
Wallich, Nathaniel ..... Wall.
Westcott, Fredric ..... Westc.
Willdenow, Karl Ludwig von ..... Willd.

List of Abbreviations (Continued)

Chemicals

Acridine orange ..... AO
Chicken erythrocyte nuclei ..... CEN
Chromomycin A3 ..... A3
4, 6-diamidino-2-phenylindole ..... DAPI
4, 6-(diimidizolin-2-yl)-2-phenylindole ..... DIPI
Dithiothreitol ..... DTT
Ethidium bromide ..... EB
Sodiun ethylene-diamine tetraacetic acid .... EDTA
Hoechst ..... HO
Hydroxyethylpiperazine
ethane sulfonic acid ..... HEPES
Mithramycin ..... MI
Morpholino propanesulfonic acid ..... MOPS
Propidium iodide ..... PI
Tris hydroxymethyl aminomethane ..... TRIS
Triton X-100 ..... TrX 100

Terminology

Coefficient of Variation CV
Constant (haploid genome compliment) C
Diploid 2N
Flow cytometry ..... FCM
Genome designations for *Dendrobium*:
Callista ..... Ca
Dendrobium (Eugenanthe) ..... Eu
Eleutheroglossum ..... El
Formosae (Nigrohirsutae) ..... N
Latorea L
Pedilonum ..... Ped
Phalaenanthe ..... P
Rhopholanthe ..... R
Spatulata (Ceratobium) C
Haploid genome set 1n
Pollen mother cell ..... PMC
Protocorm-like body ..... PLB
Somatic genome set 2n
Tetraploid 4N
Triploid 3N

Chapter 1: Literature Review

I. Introduction

The family Orchidaceae consists of over 25,000 known species in over 800 genera (Dressler 1993). Species classification is complicated by too diverse floral and vegetative characteristics used by taxonomists to distinguish species. Plant breeders may disregard taxonomic classifications when they are contrary to their own classifications based on breeding characteristics and chromosome numbers. However, in the genus *Dendrobium* with over 1000 known species, chromosome number is of little use as a diagnostic feature because the majority of the species have 2n = 2N = 38 chromosomes. Cytologists have found significant differences among the appearances of individual chromosome sets, referred to as karyotypes. However, no correlation has been found between karyotype and species relationship in *Dendrobium* (Kamemoto 1987). A different approach is required to assist with species classification. Quantitative DNA content measurements are reported to improve karyotype character evaluation and aid in studies on speciation (Greilhuber and Ehrendorfer 1988). Many methods have been used to quantify genomic DNA content, but the most recent and most accurate is flow cytometry (FCM) (reviewed by Bennett et al. 1982).

A complication to cytology and DNA content analysis is the existence of tissues with varying numbers of chromosome sets depending on developmental stage. This phenomenon, referred to as endopolyploidy, has been reported in orchids (see sections III and VI of this review). Analysis of DNA content using endopolyploid tissues would yield elevated values for DNA. This makes it necessary to survey a variety of tissues within a plant to determine the baseline DNA content level.

The following literature review provides the necessary background information for understanding FCM and how it can be applied to orchid cytology and genetic research.

This review begins with orchid breeding, with special emphasis on the genus *Dendrobium*. The next section describes FCM, including the relevant stain technologies, data acquisition and analyses. This is followed by a discussion of past technologies for DNA content and ploidy determination. Also, a listing of references that specifically pertain to FCM analysis of DNA content in orchids and other horticulturally important plants is provided.

II. Orchid Breeding

A. History

Orchid hybridization began during the mid 1800s with the first successful orchid hybrid credited to J. Dominy (Lenz and Wimber 1959). Currently, most of the commercial orchids used for cut sprays and potted plants are hybrids. Generally, an orchid hybrid is a plant derived from breeding two different species. Early hybridization was performed by orchid hobbyists whose main goals were to see if interspecific and intergeneric crosses could be achieved and to create new and interesting orchids. However, there are several disadvantages with random selection of parents. Interspecific breeding often results in no, or low, seed set. It can often also produce reduced fertility in the resulting offspring, preventing subsequent hybridizations. Both are a result of incompatibility due to chromosome pairing difficulty during meiosis. Low seed set is due to the low percentage of successful pairing. A further problem with breeding species of unknown compatibility is that assessment of fertility for the resulting offspring is very time consuming since time to first flowering may take several years.

It was not until cytology was applied to orchids that orchidologists began to understand what determines success in hybridization. Beginning in the late 1940s, extensive cytological studies were undertaken on orchids of the Cattleya alliance (Kamemoto and Randolf 1949). Two significant findings were made. First, within the Cattleya alliance comprised of genera such as *Cattleya*, *Epidendrum* and *Laelia*, most species were found to

have a diploid chromosome number of 2n = 40 with a few aneuploids at 2n = 41 or 42. Second, outstanding orchid hybrids were found to be polyploid (Kamemoto 1950). Further studies revealed that the majority of award winning *Cattleya* hybrids were either triploids or tetraploids (Kamemoto 1952).

B. Breeding Compatibility

Breeding compatibility is measured by the percentage of fertile offspring resulting from a cross between two different species. The chromosome compatibility is determined from the meiotic behavior of the chromosomes in the resulting offspring. Several intensive breeding and cytological studies were performed on species hybrids within the Cattleya, Vanda and Dendrobium alliances (reviewed by Kamemoto 1987). Hybrids between more distantly related species produced fewer fertile offspring than those between more closely related species.

Cytological observations showed that the degree of species relatedness could be determined by the meiotic behavior of chromosomes in the pollen mother cells (PMCs) of the hybrids. A normal diploid PMC contains two sets of chromosomes derived from each of the parents. A diploid hybrid resulting from two closely related species will show normal meiotic chromosome behavior. This means that the two sets of chromosomes will pair correctly, each chromosome with its appropriate homologue, then separate at anaphase to become two equal daughter cells. The daughter cells then undergo reduction division resulting in four haploid pollen cells.

If the hybrid is a result of more distantly related species, meiotic behavior may be abnormal due to dissimilar chromosome sets. Some or all chromosomes within a set may be unable to find a homologous match in the other set. When this occurs, a chromosome may either remain unpaired or may pair up with one or more other chromosomes that share similar regions. A PMC from such a hybrid would be quite distinctive from a normal PMC. Instead of the normal side by side pairing of chromosomes, the cell may have single

chromosomes, a ring of two or more chromosomes joined at the ends, or even chains of varying numbers of chromosomes. When this abnormal pairing occurs, the reduction division phase is aberrant. Abnormal pairs are not subject to the same divisional forces and do not migrate as readily to the poles in early anaphase. As a result, the daughter cells may have unequal numbers of chromosomes and be nonequivalent in terms of the genetic material. This type of meiotic behavior results in aneuploid and polyploid pollen which may not be viable.

C. Breeding Objectives

Early orchid breeding was performed without the knowledge of orchid genetics and with unpredictable results. However, occasionally breeding would result in offspring with highly desirable characteristics earning awards at orchid shows. Current breeding strategies used by commercial orchidists for the production of cut flowers and flowering plants are more methodical and utilize the information gained from cytological research. For example, less effort is applied towards attempts to hybridize species which are found to be distantly related by cytogenetic studies.

The objectives of most commercial orchid breeding programs is to produce improved flower appearance including increased floriferousness, and more compact plant structure. This often involves the use of polyploids (reviewed by Kamemoto 1987). Polyploid orchid plants are known to have thicker tissues, larger flowers and stronger compact stems. Many of the award winning orchids were discovered to be polyploids. Although polyploid plants may have desirable characteristics, there are also some disadvantages. Polyploid plants can have tissues that are too thick, making them heavy. They can also have slow growth habits and poor fertility if ploidy is very large, or of an odd level. However, polyploidization can be used to restore fertility in some hybrids. Primary hybrids with low or no fertility can be subjected to polyploidization treatments to increase fertility.

D. Production of Polyploids

Although orchid species are predominantly diploid, polyploids can result naturally from the union of a normal gamete with an unreduced gamete. For example, a normal monoploid ovule (1N) can be fertilized by an unreduced diploid gamete (2N) resulting in a triploid (3N) offspring. Generally, triploids have low fertility because of the unequal chromosome sets. Gametes produced by triploids are either 2N, 3N, or mixed aneuploid. The latter type, often the most abundant, usually cannot survive. A cross involving a triploid tends to be more successful when it is used as the seed parent (Kamemoto 1972). A triploid crossed with a diploid would result in a mix of tetraploid (4N) and aneuploid (2N+) offspring. The resulting ploidy levels of offspring from crosses between parents of different ploidy levels can be seen in Table 1.1. Commercially, triploids are primarily produced for superior potted plants and lei flowers (Kamemoto 1987). Outstanding plants can be vegetatively propagated by shoot tip, bud, or meristem culture to maintain characteristics. However, most triploid University of Hawaii cultivars are seed propagated and are listed in Table 1.2 along with their commercial use.

Tetraploids result from a triploid by diploid cross, or from the union of two unreduced diploid gametes. Tetraploidy can be perpetuated by crossing two tetraploid parents. Tetraploids (and high ploidy level) can also be induced artificially through colchicine treatments. Colchicine arrests chromosome division during mitosis. This is a strategy used to regain fertility in diploid hybrids resulting from the union of two distantly related parents (Sanguthai et al. 1973). As mentioned in the previous section, non-homologous chromosome sets undergo abnormal meiosis. However, if a plant has undergone colchicine treatment, the somatic chromosome set then has two copies of each genome type. Therefore, gametes produced by such a plant will contain one copy of each genome set derived by normal division.

The *Dendrobium* breeding program at the University of Hawaii involves extensive use of polyploids for the production of commercial quality cut flower and potted plants. Induction of amphidiploids is achieved by treating protocorm-like bodies (PLBs) of diploid species hybrids in liquid tissue culture medium with 0.1% (w/v) colchicine for 5 to 10 days (Sanguthai et al. 1973). Following treatment, PLBs are cultured on solid tissue culture medium until plantlets are large enough for greenhouse planting. Treatment yields a mixture of ploidy levels, including aneuploids. Amphidiploid plantlets are identified and selected based on growth characteristics and general appearance. The largest plantlets are generally found to be diploids, while stunted or slow growing plantlets tend to have ploidy levels higher than 4N. The tetraploid plantlets are generally slower growing than diploids and more robust, but otherwise of normal appearence. Aneuploids often appear distorted or abnormal. Up to now the only way to confirm ploidy levels is to count mitotic chromosomes in root tips.

E. Dendrobium Distribution and Taxonomy

The genus *Dendrobium* is one of the largest in the Orchidaceae comprising approximately 1000 identified species (Dressler 1993). Members are predominantly epiphytic and have a broad geographic distribution over several continents and latitudes (Schelpe and Stewart 1990). *Dendrobium* species have adapted to distinct habitats, ranging from subalpine grasslands to tropic sea level, and show a tremendous diversity in growth habit and form. Size and shape of pseudobulbs, leaf forms, and flowering characteristics are extremely variable and have been reviewed (Cribb 1983, 1986; (Upton 1989)Upton 1989; Schelpe and Stewart 1990; Baker and Baker 1995).

In an attempt to bring order to this large genus, Schlechter (1914) created four subgenera comprised of 41 sections based on vegetative characteristics. Cribb (1983, 1986) further subdivided many sections into groups based on floral characteristics. However, classification of species with this morphology-based system is frequently

dynamic. Some assignments are questioned by breeders based on their observations of chromosome pairing behavior in hybrids (Wilfret et al. 1979). Additional information about genome size may aid taxonomists and systematists.

F. Genome Breeding Strategies for Dendrobium

The genus *Dendrobium* contains approximately 1000 species subdivided into numerous sections. The horticulturally important sections are Phalaenanthe, Spatulata (formerly Ceratobium), Eleutheroglossum, Latourea, Eugenanthe, Nigrohirsutae, and Callista. The genome designations of these sections are P, C, E, L, Eu, N, and Ca, respectively (Kamemoto and Wilfret 1971). Extensive research on breeding compatibility and meiotic chromosome behavior of intersectional hybrids has helped to explain species relationships within this genus (Kamemoto and Wilfret 1971; Wilfret et al. 1979). Sections which can be intercrossed with a high seed viability or seed germination are considered to be more closely related. Low seed viability, i.e., low crossability, results from mismatched or non-pairing (non-homologous) chromosome sets.

Studies have shown that hybrids with only moderate crossability can recover fertility through colchicine induction of amphidiploidy (polyploidization) (Sanguthai et al. 1973). Amphidiploidy is desirable for two reasons. First, polyploid characteristics of resulting offspring are highly prized, as mentioned previously. Second, successful chromosome pairing during gamete formation allows for production of fairly uniform seed populations, with one or both parents being amphidiploids.

Useful amphidiploid parents and commercial cultivars have been produced from the intersectional hybrids involving Phalaenanthe x Ceratobium (PPCC), Ceratobium x Eleutheroglossum (CCEE), and Phalaenanthe x Eleutheroglossum (PPEE). However, in the case of the intersectional hybrid between *D. phalaenopsis* (PP) x *D. discolor* (CC), which occurs naturally as the diploid hybrid *D. superbiens* (PC), genome doubling (PPCC) can occur on its own without treatment (Kamemoto 1951). Fertile amphidiploids are used

as cultivars or for further hybridization and seed production. One of the earliest amphidiploid cultivars was *D.* Jaquelyn Thomas O580, which later served as a stud plant for University of Hawaii seed propagated cultivars. The successful cut flower cultivars UH44, UH232, UH306, UH503, UH507, and UH800 arise from both male and female amphidiploid parents.

III. DNA Content and Ploidy Analysis: General Methods

Early methods for quantifying nuclear DNA involved chemical properties of nucleic acids extracted from a large quantity of cells. These included chemical extraction, reassociation kinetics and microdensitometry (Bennett and Smith 1976) . In the chemical extraction method total DNA is extracted by dissolving in a known volume of solvent. The concentration of DNA was measured colorimetrically. Reassociation kinetics made use of DNA extracted from a tissue sample of known mass and subjected to denaturation to separate the strands. The single strands were then hybridized to a specific amount of labeled DNA. The DNA content of the unknown sample was determined by measuring the amount of bound labeled material and estimating the per cell content from the weight of the starting tissue (Rothsfeld et al. 1966). However, the use of both chemical extraction and reassociation kinetics for DNA content analysis gave way to microdensitometry after the 1970s (Bennett and Smith 1991).

Microdensitometry involves an in situ chemical reaction of reagents with chromatin that can be visualized microscopically. This method was made possible through a combination of the Feulgen DNA stain reaction (Feulgen and Rossenbeck 1924) and quantitative microspectrophotometry (Ris and Mirsky 1949). This method was later improved as quantitative cytofluorimetry by using DNA specific fluorochromes (Ruch 1966). These methods were in contrast to reassociation kinetics because they made it possible to estimate variation in DNA content at the level of individual cells. This also made it possible to

recognize different populations of cells in the same tissue. However, these microscopic methods are slow, requiring scanning of individual cells on a slide, and thus hinders analysis of large numbers of cells.

Another problem with microscopic techniques is that they require cells with condensed chromatin for best visualization. However, highly condensed chromatin does not bind as readily to certain stains. This makes comparison of results obtained from studies using different stains unreliable. Variation in DNA content values may also result from cells involved in differentiation with sections of the genome undergoing DNA amplification (Nagl 1972; Nagl et al. 1972; Nagl and Rücker 1974; Nagl 1976; Nagl and Capesius 1977). Compared to microdensitometry and cytofluorimetry, flow cytometry is much more convenient, rapid and, more importantly, more precise (Dolezel 1991). Using the aforementioned techniques, nuclear DNA content has been determined for 1% of all angiosperms, and less than 2% of that includes members of the Orchidaceae (Bennett and Leitch 1995).

IV. Introduction to Flow Cytometry

A. General Principles

Cytometry refers to the measurement of physical and chemical characteristics of cells or cellular components (Melamed et al. 1979). Flow cytometry is a specific procedure for making such measurements while the cells pass in single file through the measuring apparatus in a fluid stream (Shapiro 1988). Flow cytometry was first reported by Louis Kamentsky, at the New York Academy of Science, for cervical cell classification by cell size and nucleic acid content (Kamentsky et al. 1967). However, FCM was not applied to plants until the early 1980s. A comprehensive review on the use of FCM in plants was written by Dolezel (1991).

The basic principle of FCM is relatively straightforward. A liquid suspension of single cells or specific subcellular components are drawn up into an alignment stream and passed through a sizing orifice to flow past a light beam and a reflective light receptor. As the light reflects off the cell, it is collected by a photoreceptor and passed through a series of optical filters. Specific wavelengths of light are transmitted to a photomultiplier for translation of the light signal into an electrical impulse. The impulse is converted into numerical data that are displayed as a scatter plot on a CRT screen and stored by computer for future retrieval. Many instruments have the added capability of sorting and collecting cells after characterization.

The different physical and chemical characteristics measured by FCM are referred to as parameters. Table 1.3 shows a list of measurable parameters categorized as either extrinsic or intrinsic. Extrinsic parameters require reagents or stains for visualization; intrinsic parameters involve autofluorescence or characteristics which involve reflected light instead of emitted light (Shapiro 1988). Both types of parameters have been further separated as being structural, or functional in nature. Specific parameters are discussed later in this review (see FCM Methodologies Section V). In addition to flow cytometry, microspectrophotometry and image analysis can be employed to measure these parameters. However, the time expended and the degree of accuracy may be quite different among methods (Bennett et al. 1982).

Flow cytometric analysis is dependent upon the measurement of light scattering off and emitted by the sample as it passes through a laser beam. This leads to the true uniqueness of the flow cytometer as a tool which can be seen in some of its other applications, such as flow sorting and multiparameter analysis (Shapiro 1988). In flow sorting, cells or cellular components are selected for collection based according to their size and fluorescence and diverted from the main flow stream, by either electrical or mechanical means, into a separate receptacle. This procedure can be used to collect viable cells, separate different cell

types (e.g. erythrocytes from lymphocytes), select cells with a specific DNA content, or even to sort chromosomes. Depending on the nature of the reagents used for staining and isolation, cells obtained in this way can be re-cultured, or re-analyzed.

Multiparametric analysis is a technique in which a subpopulation of cells defined by a specific characteristic can be analyzed for one or more parameters. An example of this type of analysis would be to determine the DNA content as well as the percent base pair composition though use of two discriminating fluorochromes emitting at discrete wavelengths.

B. History

The modern flow cytometer is a product of several decades of research looking for a reliable automated method of identifying and separating different cell populations. W. H. Coulter is credited for the first such apparatus, which was designed to distinguish erythrocytes from lymphocytes in a diluted saline solution (Coulter 1956). The two cell types were identified by their volumes generating differential electrical impulses when they passed through an orifice. This method required no staining and was purely mechanical in nature. However, many researchers were interested in developing an instrument to characterize the physiochemical nature of cells.

In 1950, T. Caspersson described the use of microspectrophotometric measurement of the absorption of unstained cells in ultraviolet and visible spectra to study nucleic acid and protein metabolism during normal and abnormal cell growth (Caspersson 1950). A major limitation at this time was that RNA and DNA could not be distinguished within an intact cell. This technique also had restricted range of application due to the nature of absorption measures. The photodetector in a microspectrophotometer measured light transmitted through the specimen. This method had problems discriminating between the light lost due to absorption and light lost by scattering. T. Caspersson showed that precise measurements required optics with a high numerical aperture in order to collect as much of the scattered

light as possible. An additional drawback was that the refractive index of the sample needed to match that of the mounting medium and the immersion fluid to minimize scattering at the interfaces. There was also a problem with samples containing refractive granules that could not be analyzed with any accuracy (Shapiro 1988).

A series of discoveries were brought together during the 1960s which made microspectrophotometry more useful. The two most significant were the finding of a correlation between doubling of nucleic acid content and mitotic activity (Caspersson and Schultz 1938), and use of acridine orange (AO) for Feulgen staining to identify and quantify DNA and RNA content in tissue (von Bertalanffy and Bickis 1956). Acridine orange binds to both DNA and RNA; due to differences in the resulting structural configurations, each emits light at different wavelengths. Prior to the use of AO, the Feulgen staining method was not considered to be reliable or quantitative (Shapiro 1988). These two discoveries lead to the ability to discriminate normal from malignant cells (von Bertalanffy et al. 1956). This was because malignant cells fluoresced more intensely, indicating higher nucleic acid content than the normal cells.

Additional discoveries by L. A. Kamentsky were incorporated into the first flow cytometer (Kamentsky et al. 1967). Although fairly primitive compared to today's models, Kamentsky's flow sorter employed all of the principles currently used. Kamentsky built a flow cytometer which was capable of using nucleic acid content and cell size to characterize cervical cells. This early instrument measured light absorption at 260 nm followed by light scattering at 410 nm. The main limitation of this early unit was the level of electronic technology. Computer components were large and expensive and limited in data handling capabilities compared to computers available now. H. M. Shapiro discusses this point in history with reference to the primitive FCM computer devices built from the cathode ray tubes scavenged from broken arcade games and pieced together with scraps of wire (Shapiro 1988).

V. Modern Flow Cytometry

A. Instrumentation

There are two major textbooks on flow cytometry instrumentation that include full explanation of the components and the differences between the equipment currently available, Flow cytometry and cell sorting by Melamed et al. (1979) and Practical flow cytometry by Shapiro (1988). Therefore, this review will only list the major components of the flow cytometer and briefly discuss their functions .

A flow cytometer has one or two laser light sources (commonly argon ion gas) to provide the light intensity and wavelength necessary for fluorochrome excitation. The wavelength of the excitation light is controlled by regulating the power input and using a set of coated mirrors which can be adjusted to allow for a precise monochromatic light beam. Argon ion gas is a popular choice among laboratories because it can be excited to produce lasers of variable wavelengths. Although argon is usually run at 488 nm (blue-green) or 515 nm (green) emission, it can also emit light at 457 nm (violet-blue), 465 nm (blue), 472 and 476 nm (blue-green), 496 and 501 nm (green). With the use of additional mirror sets emission can also be produced at 528 nm (green) and in the ultraviolet at 351 and 363 nm. Ultraviolet light emissions require a much higher power input to acheive that level of ionic excitation (Shapiro 1988).

Once the monochromatic light contacts the sample, two events occur. First, light is scattered from the object in a manner specific to the object's size and shape. This phenomenon is referred to as forward angle light scatter. This is an important feature because it can be used to sort out (gate) objects of the wrong size. The second event is that particles capable of fluorescent excitation will emit a different light energy. The emitted light is directed to another set of filters by a mirror. The function of this filter set is to block out background light and pass only a specified range of wavelengths.

The desired wavelength is passed through one filter (short band pass) and directed to a photomultiplier. The photomultiplier amplifies the light signal and translates it into an electrical impulse that later becomes a data point. For dual wavelength applications involving two different fluorescent emissions, such as simultaneous detection of DNA RNA, a second filter set (long band pass) is in place to collect the second wavelength of interest.

The other feature of a flow cytometer is the sample delivery system. The sample is drawn up into a fluid stream (sheath fluid) and directed into the center of the flow (hydrodynamic focusing) where the sample particles move in single file past the excitation light opening. The rate of the flow can be adjusted. However, there are trade-offs between high flow rates and precision of sample image to take into consideration. Samples flowing too quickly will warp. That is, the faster they flow, the more elongate they become. Elongating distorts the forward light scatter readings and may interfere with light emission. This results in higher coefficients of variation and thus decreases accuracy of the data. However, some samples may be particularly sensitive to changes in osmolarity, pH, or temperature of the sheath fluid. In this case samples should be run as quickly as possible. For this reason it is also important to use a sheath fluid which closely duplicates the sample's native environment.

B. Methodologies

As mentioned previously, characteristics measured by FCM are categorized as intrinsic or extrinsic (Table 1.3). This study is concerned with extrinsic features. All extrinsic parameters require staining for analysis to proceed. Several fluorescent dyes have been discovered over the years which lend themselves to use in flow cytometry. An extensive list of fluorochromes is included in Table 1.4. Each of the fluorochromes differs in terms of how they bind to nucleic acids and in their excitation and emission spectra.

Flow cytometric analysis for ploidy is closely linked to cell cycle analysis. The phases of the cell cycle appear as peaks on a histogram. Cells at the static G0/G1 phase of the cell cycle are represented by the first peak. Cells undergoing replication, in the G2 phase, are represented by the second peak. Cells synthesizing DNA are located between the two peaks. The size and shape of the peaks are determined by the proportion of the cell population actively dividing. Cells extracted from young developing tissues will have a large G2 peak. Cells taken from fully differentiated mature tissue will have a large G0/G1 peak.

Another way of looking at the peaks is in terms of genome copy number. The first peak represents a diploid state, better referred to as having two copies of the haploid genome. This is an important distinction when referring to polyploid material. Therefore, rather than calling the first peak the diploid or 2N cell population, the term constant was coined. Constant, shortened to C (= 1N), refers to the single copy genome condition, independent of ploidy. The G0/G1 peak for somatic nuclei is the 2C peak and the G2 peak is equal to 4C peak. For example, in diploid somatic cells the nuclei at the G0/G1 phase are 2N = 2C, but tetraploid somatic nuclei at the same phase are 4N = 2C.

Ploidy is determined using relative values or using absolute DNA values. The ploidy of a sample can be determined by comparing the relative position of the fluorescent peak, expressed in arbitrary units (AU) of fluorescent intensity on a 256 unit (or channel) scale, to an equivalent sample of known ploidy. The ratio of the AU values reflects the difference in ploidy. The absolute value for DNA content is determined in a similar manner except the 2C peak value, expressed in AU, is compared to a known DNA sample (standard). To calculate the DNA content, the 2C sample value is divided by the standard value then multiplied by the known absolute DNA content of the standard. Ploidy can also be determined using the ratio of the DNA content values instead of AU. The reliability of the measurement is determined by the width of the peak which represents the coefficient of

variation (CV). A lower CV value indicates a high level of reliability in the calculated DNA content value. A clear and concise discussion of ploidy determination and DNA content analysis can be found in the works by Melamed et al. (1975), Shapiro (1988), Dolezel (1991) and Givan (1992).

C. Plant Nuclei Extraction Procedures

In general, procedures for isolation of nuclei involve either hypotonic lysis using a detergent and various osmolarities of salts, or enzymatic degradation, or a combination of the two. The components of a nuclear extraction buffer are determined by the requirement for preserving the integrity of the nuclei and inhibiting nuclease activity (Dolezel 1991). Several extraction protocols have been developed in the last decade. Each method varies by the type of salt buffer used, concentration of magnesium ions, and type and concentration of nuclear stabilizing agents. Most of the extraction methods were derived from protocols for extracting nuclear DNA and were not designed to keep the nuclei intact (Dolezel 1991). However, with modifications to the orignal buffer components nuclear integrity was maintained. The components of a few of the popular extraction buffers can be seen in Table 1.5.

There are three major classes of extraction buffers in use for plant material. The first type is a polyamine buffer containing a metal chelator, such as sodium ethylene-diamine tetraacetic acid (EDTA), in combination with a reducing agent and polyamines. The function of the EDTA is to bind divalent cations that serve as nuclease cofactors. Reducing agents, such as dithiothreitol (DTT) and mercaptoethanol, along with polyamines, such as spermine, act to stabilize chromatin. The second class of buffers contain hexylene glycol with divalent cations, usually Mg^{+2} or Ca^{+2}, to stabilize chromatin. The third type is a $MgCl_2$-based buffer often containing sodium citrate, a mild chelator, for added chromatin stability. Buffers containing divalent cations must be used at ice cold temperature to prevent destabilization of the nuclei, especially when used in the absence of a metal chelator.

However, it is generally recommended that all buffers be ice cold to decrease nuclease activity. Several other lysis buffers used are made principally of a protoplast culture medium with a non-ionic detergent, such as Triton X-100 (TrX 100) or Tween 20 in concentrations ranging from 0.1% to 2%. An additional component used in buffers is DNase-free RNase. This is used to remove RNA from samples stained with either of the intercalating dyes propidium iodide (PI) and ethidium bromide (EB) stains, which bind to both RNA and DNA. Extraction protocols utilizing other types of fluorochromes do not have this requirement.

An examination of the buffers seen in Table 1.5 shows that each differs slightly. The Galbraith buffer contains an extremely high concentration of Mg^{+2} ions (Galbraith et al. 1983). This is because the dye mithramycin (MI), one of the DNA binding fluorescent antibodies, requires Mg^{+2} ions to form complexes with DNA. Because of the high divalent cation concentration, the entire extraction procedure must be performed in a cold room with ice cold buffer and equipment. Michaelson et al. (1991) circumvented this problem by halving the $MgCl_2$ concentration and using PI to stain nuclei in the presence of RNase. One other noticeable difference in the buffers listed is the concentration of salts. It is cautioned that either too high or too low salt concentrations may interfere with dye binding, resulting in poor resolution of nuclei as indicated by a high CV (Dolezel 1991). Therefore, whenever switching dyes between protocols, it is important to watch for an otherwise inexplicable increase in CVs. The final difference in buffers is the actual buffer base used. Some protocols call for tris hydroxymethyl aminomethane (TRIS), another for morpholino propanesulfonic acid (MOPS), and still another for hydroxyethylpiperazine ethane sulfonic acid (HEPES). The difference is due to the pH requirement of the particular plant material for which the author designed the protocol.

VI. Application of FCM to Orchids and Other Horticultural Crops

Since the introduction of FCM in the early 1970s, 1851 flowering plant species have been analyzed for DNA content. This resulted in a number of published plant C-value lists (Bennett and Smith 1976; Bennett et al. 1982; Galbraith et al. 1983; Ulrich et al. 1988; Brown and Bergounioux 1989; Hammatt et al. 1990; Lucretti et al. 1990; Arumuganathan and Earle 1991; Bennett and Smith 1991; Michaelson et al. 1991; Dickson et al. 1992; Dolezel et al. 1992; Nath et al. 1992; Brandham and West 1993; Marie and Brown 1993; Bennett and Leitch 1995). The species contained in these lists are predominantly ornamentals, cereals and other crop plants. Many of these references include studies which trace the ancestral origin of certain crop plants (Rayburn et al. 1989; McMurphy and Rayburn 1991; Nath et al. 1992; Bashir et al. 1993; Biradar and Rayburn 1993; Ceccarelli et al. 1993; Costich et al. 1993; Rayburn et al. 1993; Ohri et al. 1994). Other studies have involved the relationship between genome size and maturity group of crops selected for cold tolerance and short season adaptation (Rayburn et al. 1989; McMurphy and Rayburn 1991; Biradar and Rayburn 1993; Biradar et al. 1994; Graham et al. 1994).

Several early studies on endopolyploidy and quantification of DNA content (relative and absolute values) in orchid species were made using reassociation kinetics, Feulgen microdensitometry, or microfluorimetry (reviewed by Kuehnle 1996). Each of these studies involved measuring DNA from in vitro cells under artificial hormonal influence. Only one study reported absolute DNA values for orchid species using *Allium cepa* as an external DNA standard (Capesius et al. 1975). Cell ploidy in protocorms was determined by measuring DNA content arbitrary units and comparing the values measured at different stages in development. It was found that as cells undergo differentiation the DNA content increases both by endoreplication of the entire genome, and by amplification of repetitive DNA sequences (Alvarez 1968; Alvarez 1969; Nagl 1972; Nagl et al. 1972; Nagl and

Rücker 1972, 1974; Nagl 1976). This would mean that the amount of DNA measured is dependent on the stage of development. However, it is possible that the values estimated using the early methods are not entirely accuraate. Bennett and Smith (1976) list several problems and sources of error asssociated with microdensitometry including misuse of the method, inconsistent staining, variability in DNA density, optical errors and variability among tissues.

DNA content estimations and method used for 41 previously analyzed orchid species, including five *Dendrobium* species, are listed in Table 1.6. The values ranged from 5.1 to 11.7 pg DNA / 2C nuclei. Not included are values for two hybrids, *Cymbidium ceres* and *Cattleya schrombocattleya* (both Epidendroideae) misnamed in Capesius and Nagl (1978) as reviewed by Kuehnle (1996). Notably absent are any species of *Cattleya* or *Vanda,* two genera of high commercial interest. Other major genera with few or no representative species analyzed are *Phalaenopsis, Epidendrum, Bulbopyhllum,* and *Cypripedium.* Only one study to date involves flow cytometric analysis of DNA content of an orchid species, *Vanilla planifolia* (Arumuganathan and Earle 1991). This study was part of a survey of DNA content of different plants. The orchid nuclei samples were taken from mature leaves and yielded a C value of 15.90 pg DNA.

VII. Specific Research Goals

Orchids, highly valued by commercial flower growers and plant enthusiasts alike, comprise one of the world's largest and most evolutionarily advanced botanical families. Orchid sales in Hawaii were recorded at an estimated $11.2 million in 1994 with about $7.7 million from *Dendrobium* alone (HASS 1995). Despite the importance of orchids, very little is understood about their genetics. Genetic information is essential to address controversies in orchid taxonomy and evolution, and to aid applied research in breeding, including genetic engineering. Recent advances in technology now offer the opportunity to

take an innovative approach to orchid genetics. Questions that could only be pursued with difficulty, if at all, can now be addressed.

The goal of my masters project is to use flow cytometric DNA analysis to obtain information on orchid genetics with special emphasis on *Dendrobium* species and hybrids. DNA C-values are known for approximately 1100 higher plants, for which FCM has been applied to 10.7% including only one species of orchid (Bennett and Leitch 1995). By performing nuclear DNA content analysis, a number of questions can be answered. For example, are there any quantitative differences in amount of DNA found among different *Dendrobium* species, independent of differences in chromosome number? If quantitative differences exist, can these be detected in hybrid offspring to trace parental contribution?

Additionally, I propose to use FCM to identify ploidy levels in individuals which have undergone polyploidization by natural or artificial means. I would like to assess the practicality of using this method to screen in vitro material which has undergone colchicine treatment. Seedling offspring from amphidiploid breeding that might be diploid could also be screened in the same manner. Identification of polyploid nuclei will be useful for studying endopolyploidy in different tissues at the plant level. Such a study is necessary to insure that ploidy differences found between individuals are not due to tissue sampling discrepancies.

Specifically, there are four objectives of my masters project. The first objective is to determine if orchid plants exhibit endopolyploidy in different tissues. Early studies on DNA content in orchids resulted in evidence that a change of DNA content occurs during cellular differentiation (Nagl 1972; Nagl et al. 1972; Nagl and Rücker 1974). The methodology used in those early studies may not have been accurrate, as indicated in section VI, and requires further study. Determining the presence of endopolyploidy in orchid tissues is important to verify the 2C peak values from the histograms used to

calculate DNA content. If tissues exhibit different ploidy levels, sampling consistency can be crucial for accurate results.

In conjunction with this first objective, an attempt will be made to identify ploidy levels of in vitro material. This objective is important for several reasons. To begin with, in vitro material undergoes rapid multiplication and is subject to the control of growth regulators which may lead to partial (aneuploidy) or complete multiplication (polyploidy) of the chromosome compliment. Closely related to this is the fact that induction of amphidiploids by colchicine results in a population of mixed ploidy plants. A quick and precise method for ploidy determination would assist in screening plant samples. Individuals with increased ploidy level could be distinguished from a larger population of plants. It is hoped that both ploidy level and aneuploidy can be determined in this material. In addition to in vitro material, seedlings resulting from polyploid parents can also be screened for ploidy level. FCM screening of polyploid material would be an improved method for identification that would save time and labor compared to current methods.

The second objective is to determine DNA content for each of the major *Dendrobium* species representing the major taxonomic sections used in breeding programs. Previous studies involving *Dendrobium* species have shown that karyotypes are variable and not correlated with sectional designations (Wilfret and Kamemoto 1969). DNA content will also be measured for species within other closely related genera. Data on DNA content could be used to study phylogeny of this group of orchids and would contribute to the overall knowledge of orchid genetics.

The third objective involves using DNA content values of parent species to establish the lineage of hybrid plants. A similar study was conducted on *Brassica* in which the parent species of natural hybrids in the wild were identified (Sabharwal and Dolezel 1993). This method may also be applicable to cases involving polyploid breeding with parents of odd ploidy levels (3N, 5N) resulting in an offspring population with different genome

combinations. The current method to determine genome make up is strictly qualitative and involves matching certain key floral (mostly) characteristics with a known genome type. For example, hybrids containing the Phalaenanthe (P) genome should have rounded sepals often twice as wide as the petals. However, from this method the number of copies of the P genome can only be assumed by the degree in which the floral characteristic is expressed along with knowledge about the parents. Flow cytometry may be able to give a more precise representation of the genome profile for a hybrid with mixed genome parentage.

VIII. Tables

Table 1.1. Number of offspring of differing ploidy levels resulting from crosses involving diploid, triploid, and tetraploid *Dendrobium* parents (adapted from Kamemoto et al. 1972).

Cross	No. offspring examined	No. offspring at each ploidy level						
		2N	2-1/2N	3N	3-1/2N	4N	4-1/2N	5N
2N X 2N	45	43 (96%)[a]		2 (4%)				
4N X 4N	25					25 (100%)		
2N X 4N	37			37 (100%)				
4N X 2N	99			99 (100%)				
3N selfed	16			1 (6%)	2 (13%)		13 (81%)	
2N X 3N	40		2 (5%)			38 (95%)		
3N X 2N	96					96 (100%)		
4N X 3N	13				9 (69%)			4 (31%)
3N X 4N	127				111 (87%)			16 (13%)

[a] Percentage of total number observed

Table 1.2. Seed propagated triploid *Dendrobium* cultivars used commercially for potted plants and lei flower production.

Cultivar ID Number	Cultivar Name	Commercial Use
UH613	*D*. Lynne Horiuchi	potted plant
UH1041	*D*. Nanae 'Uniwai Beauty'	potted plant
UH1101	*D*. Sylvia Yuen	potted plant
UH1182	*D*. Pua'ala	potted plant, lei flower
UH1208	*D*. Betty Nakada	potted plant
UH1221	*D*. Cathy Beck	potted plant
UH1307	*D*. Remy Hartmann	potted plant
UH1382	*D*. Lim Chong Min	potted plant
UH1392	*D*. Louis Bleriot	lei flower

Table 1.3. Cellular parameters measurable by flow cytometry (adapted from Shapiro 1988).

Structural	Category [a]	Functional	Category [a]
Cell size	Intrinsic	Redox	Intrinsic
Cell shape	Intrinsic	Membrane integrity	Extrinsic
Cytoplasmic granularity	Intrinsic	Membrane permeability	Extrinsic
Pigment content	Intrinsic	Enzyme activity	Extrinsic
Protein fluorescence	Intrinsic	Endocytosis	Extrinsic
DNA content	Extrinsic	Surface charge	Extrinsic
DNA base ratio	Extrinsic	Intracellular receptors	Extrinsic
Chromatin structure	Extrinsic	DNA synthesis	Extrinsic
RNA content	Extrinsic	Membrane fluidity / microviscosity	Extrinsic
Total protein	Extrinsic	Cytoplasmic matrix structure	Extrinsic
Basic protein	Extrinsic	Surface receptors	Extrinsic
Sulfhydryl groups	Extrinsic	Cytoplasmic / mitochondrial membrane potential	Extrinsic
Antigens	Extrinsic	Membrane bound Ca^{+2}	Extrinsic
Surface sugars	Extrinsic	Cytoplasmic Ca^{+2}	Extrinsic
Cytoskeletal organization	Extrinsic	Intracellular pH	Extrinsic

[a] Intrinsic characteristics are those which require no stains or reagents for visualization.

Table 1.4. Fluorochromes used in flow cytometry for nucleic acid staining with the operational spectra (adapted from Shapiro 1988 and Givan 1992).

Fluorochrome	Nucleic Acid	Binding Affinity	Excitation Spectra λ_{abs} (nm)	Emission Spectra λ_{em} (nm)
Ethidium Bromide (EB)	RNA & DNA	Intercalator	526 blue-green	604 red
Propidium Iodide (PI)	RNA & DNA	Intercalator	536 blue-green	620 red
Acridine Orange (AO)	RNA & DNA	Intercalator	440 - 470/502 [a] blue	650/526 [a] red / green
Hoechst 33258 (HO258)	DNA (externally)	A-T binding (G-C, nonspecifically)	346 UV	460 blue
Hoechst 33342 (HO342)	DNA (externally)	A-T binding (G-C, nonspecifically)	343 UV	482 blue
DAPI [b]	DNA	A-T binding	359 UV	461 blue
DIPI [c]	DNA	A-T binding	345 UV	455 blue
Mithramycin (MI)	DNA	G-C binding	390 - 460 violet-blue	495 - 625 green
Chromomycin A3	DNA	G-C binding	390 - 460 violet-blue	495 - 625 green
Olivomycin	DNA	G-C binding	390 - 460 violet-blue	495 - 625 green

[a] Multiple wavelengths indicate RNA / DNA spectra.

[b] 4, 6-diamidino-2-phenylindole, dihydrochloride.

[c] 4, 6-(diimidazolin-2-yl)-2-phenylindole, dihydrochloride.

Table 1.5. Components of different buffers used to extract plants nuclei.

Arumuganathum and Earle (1991)	Dolezel et al. (1989)	Galbraith et al. (1983)	Michaelson et al. (1991)	Rayburn et al. (1989)	Ulrich et al. (1988)
1.5 mM $MgSO_4$	15 mM TRIS	45 mM $MgCl_2$	21 mM $MgCl_2$	10 mM $MgCl_2$	0.1 M Citric acid
7.5 mM KCl	2 mM Na_2EDTA	30 mM Na citrate	30 mM Na citrate	10 mM TRIS	
0.75 mM HEPES	80 mM KCl	20 mM MOPS	20 mM MOPS	1 M Hexylene glycol	
6.48 mM DTT	20 mM NaCl				
	0.5 mM Spermine				
	15 mM Mercaptoethanol				
0.2% TrX 100	0.1% TrX100	1.0% TrX 100	1.0% TrX 100	0.5% TrX 100	0.5 % Tween 20
100 ug / ml PI	50 ug / ml PI	100 ug / ml MI	50 ug / ml PI	20 ug / ml DAPI	5 ug/ml DAPI
2.5 ul / ml RNase	50 ug / ml RNase		20 ug / ml RNase		
pH 8.0	pH 7.5	pH 7.0	pH 7.0	pH 8.0	pH 7.0

Table 1.6. DNA content of Orchid species with method of C-value determination.

Subfamily	Tribe	Subtribe	Species	2n [a]	Ploidy	pg DNA /2C	Method [b]	Reference
Cypripedioideae	-	-	*Paphiopedilum insigne* (Wall. ex Lindl.) Pfitz.	26+ 2B	2N	41.2	Fe	Cox et al. 1993
Cypripedioideae	-	-	*Paphiopedilum villosum* (Lindl.) Stein	26	2N	45.0	Fe	Narayan et al. 1989
Epidendroideae	Arethuseae	Bletiinae	*Acanthephippium sylhetense* Lindl.	40	unk	15.0	Fe	Narayan et al. 1989
Epidendroideae	Arethuseae	Bletiinae	*Calanthe tricarinata* Lindl.	40	unk	26.5	Fe	Narayan et al. 1989
Epidendroideae	Arethuseae	Bletiinae	*Phaius tankervillieae* (Banks) Bl.	42	unk	17.0	Fe	Narayan et al. 1989
Epidendroideae	Coelogyneae	Coelogyninae	*Coelogyne barbata* Griff.	40	2N	13.2	Fe	Narayan et al. 1989
Epidendroideae	Coelogyneae	Coelogyninae	*Coelogyne flaccida* Lindl.	40	2N	8.9	Fe	Narayan et al. 1989
Epidendroideae	Coelogyneae	Coelogyninae	*Otochilus lancilabius* Seidenf.	unk	unk	9.7	Fe	Narayan et al. 1989
Epidendroideae	Coelogyneae	Coelogyninae	*Pholidota imbricata* (Roxb.) Lindl.	40	unk	6.2	Fe	Narayan et al. 1989

Table 1.6. (continued) DNA content of Orchid species with method of C-value determination.

Subfamily	Tribe	Subtribe	Species	2n [a]	Ploidy	pg DNA /2C	Method [b]	Reference
Epidendroideae	Epidendreae	Bulbophyllineae	*Bulbophyllum reptans* Lindl.	38	unk	8.3	Fe	Narayan et al. 1989
Epidendroideae	Dendrobieae	Dendrobiinae	*Dendrobium aphyllum* (Roxb.) Fisch.	38	2N	6.7	Fe	Narayan et al. 1989
Epidendroideae	Dendrobieae	Dendrobiinae	*Dendrobium densiflorum* Wall. ex Lindl.	40	2N	5.1	Fe	Narayan et al. 1989
Epidendroideae	Dendrobieae	Dendrobiinae	*Dendrobium fimbriatum* Hook.f.	38, 40	2N	6.3	Fe	Narayan et al. 1989
Epidendroideae	Dendrobieae	Dendrobiinae	*Dendrobium hookeriana* Lindl.	40	2N	11.7	Fe	Narayan et al. 1989
Epidendroideae	Dendrobieae	Dendrobiinae	*Dendrobium moschatum* (Buch.-Ham.) Sw.	38	2N	9.3	Fe	Narayan et al. 1989
Epidendroideae	Malaxideae	-	*Liparis rostrata* Rchb.f.	28, 30	unk	19.4	Fe	Narayan et al. 1989
Epidendroideae	Malaxideae	-	*Microstylis wallichii* Lindl.	36	unk	5.0	Fe	Narayan et al. 1989
Vandoidea	Cymbideae	Cyrtopodiinae	*Cymbidium floribundum* Lindl. (as: *C. pumilum* cv. Gareth Latangor)	40	unk	8.7	RK	Nagl and Capesius 1977

Table 1.6. (continued) DNA content of Orchid species with method of C-value determination.

Subfamily	Tribe	Subtribe	Species	2n [a]	Ploidy	pg DNA /2C	Method [b]	Reference
Vandoidea	Cymbidieae	Cyrtopodiinae	*Cympidium pendulum* Sw.	40	2N	9.3	Fe	Narayan et al. 1989
Vandoideae	Maxillarieae	Oncidiinae	*Brassia maculata* R.Br.	24	2N	7.1; 7.4	RK; M:DAPI	Nagl and Capesius 1977; Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Leochilus oncidioides* Knowles & Westc.	42	2N	2.4	Fe	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Notylia barkeri* Lindl.	42	2N	2.2	M:DAPI	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Oncidium ampliatum* Lindl.	44	2N	5.0	M:DAPI	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Oncidium ascendens* Lindl.	unk	unk	3.4	M:DAPI	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Oncidium flexuosum* Sims	56	2N	2.2	M:DAPI	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Oncidium leucochilum* Batem. ex Lindl.	56	2N	1.2	M:DAPI	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Oncidium serratum* Lindl.	52	2N	5.4	M:DAPI	Bennett & Leitch 1995 [c]

Table 1.6. (continued) DNA content of Orchid species with method of C-value determination.

Subfamily	Tribe	Subtribe	Species	2n [a]	Ploidy	pg DNA /2C	Method [b]	Reference
Vandoideae	Maxillarieae	Oncidiinae	*Psygmorchis pusilla* (L.) Dodson & Dressler	10	2N	3.0	M:DAPI	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Trichocentrum capistratum* Rchb.f.	28	2N	13.8	M:DAPI	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Trichocentrum capistratum* Rchb.f. (as: *T. panamense* Rolfe)	28	2N	8.6	M:DAPI	Bennett & Leitch 1995 [c]
Vandoideae	Maxillarieae	Oncidiinae	*Trichopilia marginata* Henfr. ex Moore	56	2N	5.6	M:DAPI	Bennett & Leitch 1995
Vandoideae	Vandeae	Sarcanthinae	*Aerides odorata* Lour.	38, 40	2N	7.6	Fe	Bennett & Leitch 1995 [c]
Vandoideae	Vandeae	Sarcanthinae	*Gastrochilis dasypogon* (J.J.Sm.) Kuntze	38	unk	11.2	Fe	Narayan et al. 1989
Vandoideae	Vandeae	Sarcanthinae	*Phalaenopsis amabilis* (L.) Bl.	38	2N	2.4	RK	Capesius and Nagl 1977
Vandoideae	Vandeae	Sarcanthinae	*Rhynchostylis retusa* (L.) Bl.	38	unk	5.2	Fe	Narayan et al. 1989
Vandoideae	Vandeae	Sarcanthinae	*Schoenorchis gemmata* (Lindl.) J.J.Sm.	40	unk	6.4	Fe	Narayan et al. 1989

Table 1.6. (continued) DNA content of Orchid species with method of C-value determination.

Subfamily	Tribe	Subtribe	Species	2n [a]	Ploidy	pg DNA /2C	Method [b]	Reference
Vandoideae	Vandeae	Sarcanthinae	*Trudelia cristata* (Lindl.) Sengh. (as: *Vanda cristata* Lindl.)	38	2N	8.8	Fe	Narayan et al. 1989
Epidendroideae	Vanilleae	Vanillinae	*Vanilla planifolia* Jacks.	32	2N	15.9	FCM:PI	Arumugananthum & Earle 1991
Orchidoideae	Orchideae	Habenariinae	*Habenaria edgeworthii* Hook.f.	42	2N	15.4	Fe	Narayan et al. 1989
Orchidoideae	Orchideae	Habenariinae	*Habenaria pectinata* Hook.f.	42	2N	31.0	Fe	Narayan et al. 1989
Orchidoideae	Orchideae	Habenariinae	*Herminium gramineum* Lindl.	40	2N	14.8	Fe	Narayan et al. 1989
Spiranthoideae	Erythrodeae	Goodyerinae	*Goodyera repens* (L.) R.Br.	30, 32	2N	9.7	Fe	Narayan et al. 1989

[a] Chromosome counts were not necessarily determined with samples used for DNA content analysis.

[b] Methods of DNA content determination : Fe = Feulgen microdensitometry; M = fluorescent microdensitometry (DAPI used); FCM = flow cytometry (PI used); RK = reassociation kinetics. DNA standards were *Allium cepa* cv. Ailsa Craig (pg DNA/2C = 67) for Fe; *Pisum sativum* cv. Mineria Maple (pg DNA/2C = 19.5) for M; chicken red blood cells (pg DNA/2C = 2.33) for FCM. No standard was identified for RK samples.

[c] Personsal comminication to Bennett and Leitch (1995) from Nagl and Capesius.

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Chapter 2: Characterization of Dendrobium Tissues and Identification of Ploidy Levels Using Flow Cytometry

I. Introduction

Flow cytometry (FCM) has been used by researchers to determine ploidy level (Brandham and West 1993; Grace et al. 1993), calculate DNA content (reviewed by Bennett and Leitch 1995) and study developmental endopolyploidy (DeRocher et al. 1990; Galbraith et al. 1991; Gilissen et al. 1993; Smulders et al. 1994) in a variety of plants. However, the orchid genus *Dendrobium* has never been studied using FCM. Over the years, the need has arisen for development of a method to easily determine the ploidy level and DNA content of *Dendrobium* species and cultivars. This is because polyploid *Dendrobium* cultivars of high commercial value are prone to mutations during micropropagation that may lead to changes in ploidy.

The current method to determine ploidy involves direct microscopic examination of mitotic chromosomes in actively growing root tip cells (Kosaki 1958). This method is tedious and time-consuming. Moreover, it is limited in the amount of information provided because it only reflects the condition of root cells, not of all somatic cells. Galbraith et al. (1991) reported that *Arabidopsis thaliana* somatic tissue can have cells with a range of ploidy levels depending on the type of tissue and developmental stage. Previous studies on orchids using Feulgen microdensitometry (Nagl 1972; Nagl et al. 1972; Nagl and Rücker 1974) have shown that endopolyploidy may exist for in vitro material and that it is under developmental control. However, the results from those studies may be considered inconclusive primarily due to the limitations of the technique used (limitations reviewed by Bennett and Smith 1976). Flow cytometry has proven to be a more reliable method than microdensitometry for research on developmental endopolyploidy because of fewer chances for error (Galbraith et al. 1983).

When using FCM for ploidy determination or DNA content analysis, it is important to know if endopolyploidy is present in the sample tissues. If cells with elevated ploidy levels are present the baseline ploidy level may be misidentified, and DNA content values could be overestimated. Therefore, it is important to characterize different tissues prior to making statements regarding ploidy or DNA content of a plant.

Of value to breeders and propagators is a rapid and accurate assessment of changes in ploidy in material maintained in vitro for the purpose of increasing propagation stock and production of polyploids. The commonly used method of assessing ploidy in tissue cultures is based on visual identification of associated morphological and growth characteristics of plantlets (Nakasone and Kamemoto 1961). The ploidy level of select individuals is verified by chromosome counts. This method is not practical for large scale screening required by production facilities. FCM can be used in the place of chromosome counting to detect changes in ploidy level due to the effects of long-term in vitro culturing (Moyne et al. 1993) or intentional chromosome doubling by colchicine treatment (Sanguthai et al. 1973).

In the present study, a variety of *Dendrobium* tissues were sampled and characterized in terms of ease of extraction of nuclei. Presence of endopolyploidy was determined and ability to identify the baseline ploidy level assessed. Tissue found to have a high yield of identifiable baseline ploidy nuclei was used to test the reliability of FCM analysis for determination of ploidy level. This was performed by comparing the histograms for clonal plants known to be either diploid or tetraploid. The same method was used to determine if changes in ploidy level had occurred in plantlets kept in long term in vitro culture as compared to greenhouse-grown clones of known ploidy. Results of this study indicate that FCM is a reliable method for screening in vitro plantlets and greenhouse breeding material.

II. Materials and Methods

A. Plant Material

Four *Dendrobium* species, each from a different taxonomic section with different vegetative and floral characteristics, were selected based on abundance of different tissue types. Species included *D. phalaenopsis* var. 'compactum' from the section Phalaenanthe, *D. gouldii* from the section Spatulata, *D. moschatum* from the section Dendrobium and *D. smillieae* from the section Pedilonum. The four tissues sampled from each greenhouse-grown species included pollinia from six newly opened flowers, root tips from 10 -12 actively growing roots, and mature and immature leaves (100 mg each). An immature leaf was defined as being newly developed and fully expanded, but still soft in texture. Conversely, a mature leaf was defined as a healthy, well-hardened leaf on either the growing stem or a flowering stem. In addition to the material listed above, *D. moschatum* was found to have a juvenile growth form. The morphology of the juvenile form pseudobulb differed from other pseudobulbs of the same plant in texture, size and shape of the leaves. An immature leaf from a juvenile stem was included for comparison.

The accuracy of FCM ploidy level determination was tested by comparing immature leaf material from both diploid (2n = 2N = 38) and tetraploid (2n = 4N = 76) plants of the hybrid *Dendrobium* Neo-Hawaii. Ploidy level was previously determined by direct microscopic examination of chromosomes from mitotic root tip cells (Kosaki 1958). The ploidy level was assessed by comparing the ratio of the relative linear red fluorescence value for each of the 2C peaks on the resulting flow cytometric histograms .

Immature leaves from the hybrid *Dendrobium superbiens* 'D184' were used to test the effect of long term in vitro culture on ploidy level. Long term in vitro propagation was achieved by initially culturing diploid protocorm-like bodies (PLBs) in liquid modified Vacin and Went (VW^+) medium supplemented with 15% coconut water (Sagawa 1991) for

2 years. Cultures were transferred to VW^+ supplemented with 7.5% green banana and solidified with 0.75% agar for an additional 2 years. Two months prior to analysis, a subset of plantlets were removed from in vitro media and potted up communally (compot) in tree fern medium. Both in vitro and compot plantlets (three each) were analyzed by flow cytometry. Ploidy level was determined by comparison to 2N and 4N greenhouse plants derived from the same starter PLBs previously propagated on the same solid medium for only 4 - 6 months. The 4N greenhouse plants were dervived by colchicine treatment of a subset of the original PLBs prior to solid media culturing (Sanguthai et al. 1973). Ploidy level was previously determined for the 4-year-old greenhouse plants by direct microscopic examination of chromosomes from mitotic root tip cells, where diploid and tetraploid plants had 38 and 76 chromosomes, respectively (Koskai 1958).

B. Nuclei Extraction Methods

Extraction methods were taken from Arumuganathan and Earle (1991) and are detailed in Chapter 3 and in the appendix. Procedural differences occurred with extraction from pollinia and root tips. Root tip nuclei were extracted by using the same extraction buffer. However, instead of chopping, root cuticles were removed by peeling and macerating the remaining cap cells in buffer. Because of the relatively small number of cells, a minimum of 10 to 12 tips was required for analysis. Pollen nuclei were released from pollinia in a similar manner. Twelve pollinia were placed in buffer and sliced in half, followed by gentle maceration. All tissues used were collected in the afternoon of the day before use, except for pollinia. Pollinia were collected as they became available and were stored at 4 C. All samples were analyzed three times to insure accurracy.

C. FCM and Data Analysis

Nuclei were run on a Coulter EPICS 753 flow cytometer equipped with a 488 nm argon gas laser. Results were collected and analyzed by computer using CYCLOPS

software (Cytomation, Inc., Fort Collins, CO). DNA content was calculated by comparison to chicken erythrocyte nuclei (CEN; BioSure, Inc.) which was added to select samples as a known standard. The DNA content value used for CEN was 2.33 pg / 2C nuclei (Galbraith et al. 1983). Some samples had 2C peaks too close to the CEN peak for clear discrimination. In those cases, CEN was run as an external standard run preceding and following the plant sample. DNA content and all coefficients of variation (CV) reported for mean peak values were calculated from linear scale data. Histogram data were saved in text format and imported into Cricket Graph III software (Computer Assoc. Inc., Islandia, NY) on a Macintosh computer. Histograms presented for display of ploidy levels present in different tissues are from log transformed data produced by CYCLOPS. By using transformed data, higher ploidy levels could easily be seen rather than being eliminated from the linear scale. The remaining histograms are reported using a linear scale.

D. Interpretation of Histograms

When comparing diploid and tetraploid samples, the meaning of the 2C and 4C peaks is important. The 'C' stands for constant and refers to the number of parental genome sets present (Swift 1950). Therefore, the peak for pollen is designated as 1C because only one set of parental chromosomes are present. The 2C value is twice the 1C value. If the 1C nuclei are from diploid plants then the 2C nuclei are 2N. However, if the 1C nuclei are from tetraploid plants then the 2C nuclei are 4N. It is important not to confuse C value with the ploidy designation 'N'. This concept was used to interpret the results for *D.* Neo-Hawaii and *D. superbiens*.

III. Results

Nuclei were successfully obtained from immature and mature leaf samples, root tips and pollinia. A preliminary trial of newly developing leaf tissue was unsuccessful. A thick exudate was produced during cutting and interfered with collection of nuclei. There was no

difference in the number of extracted nuclei from in vitro or greenhouse-grown leaves. The recovery of nuclei was approximately 1 x 10^6 nuclei per 100 mg fresh tissue, with the size of the diploid nuclei measuring about 6 μm across. The number of nuclei collected from root tips was relatively low, but yield appeared to be relative to the low number of cells present. Since the number of actively growing root tips are limited on a plant at any given time, overall collection of nuclei could not be improved. Collection of pollen nuclei was more successful, except in the case of *D. smillieae*. The pollinia produced by this species were soft and sticky and prevented extracted nuclei from floating freely. The result was a suspension of nuclear aggregates that could not be separated, even by filtration. When the suspension was run through the flow cytometer in three separate attempts, no discernible peaks could be identified. *D. smillieae* pollen nuclei were subsequently eliminated from the study.

Three distinct peaks (2C, 4C and 8C) were present on the histograms for the somatic tissues of most species sampled. The exceptions were *D. moschatum* leaf samples (Figure 2.1) and *D. smillieae* root tips (Figure 2.2), which only showed two peaks, and *D. gouldii* mature leaf tissue (Figure 2.3) which had an additional fourth peak. However, the 8C peak for *D. smillieae* immature leaf nuclei was extremely small, representing only 1.7% of the total nuclei present (Table 2.1). All three peasks were present on the somatic tissue histograms for *D. phalaenopsis* var. 'compactum' (fiure 2.4). Although the 2C peak for the mature leaf tissue was less distinct. All three pollen samples yielded a single peak on the histogram designated as 1C. Table 2.1 shows the distribution of nuclei that exist at each peak for each of the tissues sampled. Although three peaks were present for most somatic samples, the distribution of nuclei for the peaks was variable. The distribution of root tip nuclei at the 2C peak ranged from 10% for *D. moschatum* to 60% for *D. smillieae*. The 2C peak for *D. moschatum* were barely discernible from background signal produced by

debris, plastids and broken nuclei. These nuclei were discriminated by the use of side scatter gating of smaller sized particles during flow cytometric analysis.

The resulting distributions of nuclei in leaf material were also variable. The percentage of nuclei at the 2C peak in immature leaves ranged from 37.8% for *D. phalaenopsis* var. 'compactum' to 73.0% for both *D. moschatum* and *D. smillieae*. The percentage of 2C nuclei in mature leaves ranged from 19.6% for *D. gouldii* to 40.5% for *D. moschatum*. The percentage of 2C nuclei were consistently higher in immature leaves compared to mature leaves, regardless of species.

DNA content values were calculated for *D. phalaenopsis* var. 'compactum' var. 'compactum', *D. gouldii*, *D. moschatum* and *D. smillieae* were 2.35 ± 0.05 pg, 2.09 ± 0.06 pg, 3.48 ± 0.14 pg and 3.15 ± 0.13 pg, respectively. Since the 2C peaks for *D. phalaenopsis* var. 'compactum' (Figure 2.4) and *D. gouldii* (Figure 2.3) were so close to the CEN peak, it was necessary to run CEN with pollen nuclei to insure resolution of both peaks. CEN were also run as an external standard just before and after the sample and the resulting peak value compared to the 2C value from the immature leaf nuclei. The resulting DNA content values were comparable. This indicates that, in cases where the 2C sample peak corresponds closely with the CEN peak, external comparison can be used when pollen is not available or cannot be extracted.

Ploidy level of *D.* Neo-Hawaii was interpreted from the histograms shown in Figure 2.5 using the 2C peak from the diploid plants as a baseline. The 2C peak corresponds to the G1/G0 phase of the normal cell cycle. For diploid cells, nuclei in this phase are diploid. Nuclei present in the 4C and 8C peaks on the histograms are two and four times the size as the 2C nuclei, respectively. Nuclei present in the 4C peak are a mixture of G2 nuclei in the process of mitotic division and G1/G0 nuclei with double the chromosome complement. The G2 phase nuclei corresponding to the G1 nuclei at 4C are contained in the 8C peak. Also contained within the 8C peak are doubled 4C nuclei which were arrested at division.

Both the diploid and tetraploid *D*. Neo-Hawaii samples had three peaks present corresponding to 2C, 4C and 8C nuclei. However, each peak on the tetraploid histogram was twice the value of the corresponding peak on the diploid histogram. The 8C peak of the tetraploid histogram was off the linear scale, but could be seen at the upper end of the log scale histogram (results not shown).

Ploidy level of leaves sampled from *D. superbiens* was interpreted by using the 2C peak for the diploid greenhouse histogram (Figure 2.6). DNA content values for nuclei were calculated using CEN as a standard. The 2C peak for the tetraploid greenhouse histogram was found to be twice the value of the diploid 2C peak, at 3.88 ± 0.04 pg and 1.91 ± 0.04 pg, respectively. Both of the 2C values for the in vitro (3.17 ± 0.05 pg) and compot (3.57 ± 0.03 pg) samples were found to be closer to the tetraploid 2C peak value than to the diploid 2C peak value. Both samples were thought to be diploid because the original PLBs were from diploid plants. The same PLBs later produced the diploid greenhouse-grown plants in this study. Unlike the PLBs used t o create the tetraploid greenhouse-grown plants, the PLBs producing these in vitro and compot plantlets were never subjected to colchicine treatment. This means that spontaneaous chromosome doubling must have ocurred during long term culturing.

IV. Discussion

The somatic tissues of four *Dendrobium* species tested, exhibited multiple ploidy levels, referred to as endopolyploidy. This phenomenon has been identified in other plant species such as *Arabidopsis thaliana* (Galbraith et al. 1991) and also in germinating orchid seeds (Nagl et al. 1972). This is the first time that mature plant tissues were examined cytogenetically in orchids. In order to determine DNA content values for *Dendrobium* species, it was necessary to discover a reliable source of 2C nuclei. If a peak corresponding to higher ploidy levels were misidentified as a 2C peak, the resulting DNA content

estimates would be elevated. In a survey of different tissues, immature leaf material was found to be the most abundant in 2C nuclei. Other tissues had a large proportion of nuclei at higher ploidy levels. Although root tips and mature leaf tissue from some species also had 2C nuclei, they were not reliable sources for all species. Pollen also proved to be a poor universal source for nuclei because of extraction problems as seen for *D. smillieae*.

However, some difficulties apply to immature leaf material. If tissue was too young, a mucous polysaccharide gum interfered with nuclei extraction. The species which exhibited the worst problem was *D. phalaenopsis* var. 'compactum'. Immature *D. phalaenopsis* var. 'compactum' leaves produced exudate all the way up to the point in development where the tissues began to thicken and harden. At that point, there was a shift in the distribution of nuclei at the different ploidy levels. Upon maturation of the leaf, the 2C peak became nearly undifferentiatable from background. Even though the total number of extractable nuclei was reduced in the immature *D. phalaenopsis* var. 'compactum' leaf tissue, the 2C peak could still be identified.

There are a number of possible solutions to the problem of interfering exudate during extraction. Arumuganathum and Earle suggested using protoplasts rather than fresh tissue (1991). Other authors have suggested altering the concentrations of the existing buffer components to alleviate the problem (Galbraith 1990; Cheung et al. 1993; Dolezel et al. 1994; Poulin et al. 1994). Several extraction buffers have been successfully used on other plant species (Coleman and Goff 1985; Ulrich et al. 1988; Brown and Bergounioux 1989; Dolezel 1991; Dolezel et al. 1992; Marie and Brown 1993). However, the problem with altering the existing buffer, or switching to another, is that any change in the components can interfere with the dye binding efficiency. Many of the other researchers use dyes such as mithramycin and acridine orange which perform differently from PI (reviewed by Dolezel 1991). At this time, it is more feasible to try different tissues rather than changing

the constituents of the extraction buffer. However, in the case of a species where no tissue yields reliable results, the only recourse would be to experiment with the buffer.

A comparison of the different species showed that each had a different DNA content. As mentioned above, two species had 2C peaks in close proximity to the CEN standard. It was found in this study that CEN could be used as an external standard, thereby avoiding the problem of overlapping peaks on the histograms. The use of external standards is generally not as precise as an internal standard, but is used by several researchers (reviewed by Bennett and Leitch 1995). The alternative is to use a different standard with a different peak value. This becomes problematical when dealing with endopolyploid samples. Use of a standard with a larger value would only shift the standard peak to the 4C or 8C peak position of the sample. A smaller standard would be difficult to resolve because of the small sized debris produced by extraction from *Dendrobium* leaves.

The most important finding of this study was that ploidy level of a plant sample could be determined by comparison to similar material of known ploidy. However, the limitations of this procedure must be explained. As determined by this study, *Dendrobium* species and hybrids can have different DNA content values. This means that ploidy determinations must be performed using a genetically similar control sample. In other words, *D. superbiens* could not be used as a control for comparison to *D.* Neo-Hawaii because they differ in 2N DNA content. However, if the correct comparisons are made, this method is reliable.

Modifications of this study's ploidy determination method are described in the literature (DeLaat et al. 1987; Dolezel 1991; Galbraith et al. 1991; Moyne et al. 1993; Smulders et al. 1994). For example, instead of calculating DNA content and comparing the resulting values, both samples are mixed together and analyzed simultaneously. If the ploidy levels differ, there will be a separate peak produced by each sample. This technique gives a relative comparison of ploidy levels of each sample. Ploidy is determined by taking the

ratio of each peak position. This method could not be used for *Dendrobium* somatic samples because of the presence of endopolyploidy. Mixing diploid and tetraploid samples would result in a histogram with overlapping peaks. The 4C would probably be oversized and the 2C peak corresponding to the baseline diploid nuclei may be proportionally too small to be recognizable. Under these conditions, accurate interpretation would be impossible.

Instead of mixing samples, it is also possible to analyze both separately and to take the ratio of the relative peak positions measured in arbitrary units (AU). The problem with this method is that with a separate analysis the researcher must take steps to insure that 'drift' is accounted for. Drift refers to shifting of the position of the peaks due to minor fluctuations in laser alignment, power fluctuations, or quenching of sample fluorescence with time. The best way to control for this problem is to include an internal standard with both samples. In the present study the inclusion of CEN served this purpose. CEN were mixed with the 4x samples, as well as being analyzed separately throughout the FCM session. By monitoring drift in this fashion all samples were insured to be directly comparable.

This study tested the usefulness of FCM ploidy determination. Samples with known ploidy were compared to in vitro plantlets of unknown ploidy. It was discovered that long term in vitro culturing resulted in specimens with DNA content values intermediate to the diploid and tetraploid controls. A possible explanation for this phenomenon is that the in vitro samples are aneuploids, approaching tetraploidy. However, to verify aneuploidy it would be necessary to count chromosomes. In vitro plantlets posses only a few small roots making chromosome counts difficult, due to lack of material. In a plant propagation setting, screening individual in vitro plants for desired ploidy level would not require an absolute knowledge of chromosome number to decide if a plant should be discarded. The decision could be based on the degree of variance from a known DNA content value equivalent to

the desired ploidy level for that particular material. Time and expense could be saved by eliminating undesirable plants early.

This study shows that ploidy level and DNA content can be accurately assessed using FCM. This method has been used to study the regeneration capacity of long term *Rosa hybrida* cultures by Moyne et al. (1993). Similar studies could be performed on *Dendrobium* hybrids, as well as other commercially propagated orchids. With this method, extensive studies on developmental endopolyploidy in orchids can now be carried out . And finally, with the increase of plant molecular studies, information regarding the DNA content measurement of genome size has become valuable to researchers. For instance, by knowing genome size, the number of clones required for genetic mapping can be determined (Brown and Bergounioux 1989). This information will also be of interest to systematists studying genomic variability. The future of this technique lies with demand for the type of information generated by FCM.

V. Summary

Nuclei were successfully extracted from orchid leaves, roots and, to a lessor extent, pollinia. FCM analysis showed the presence of endopolyploidy with ploidy levels up to 16C in somatic tissues. Immature leaf tissues resulted in the best overall yield of 2C nuclei. In vitro leaf material gave similar results to greenhouse grown material. Pollinia and roots gave poor results based on low yield or indistinct peaks measured by high CV values. Because of the decline in 2C nuclei present in mature leaf tissue, immature leaves were preferred for DNA content and ploidy analysis to avoid overestimation errors. FCM analysis of immature leaf nuclei resulted in accurate detection of ploidy level in the hybrid *D.* Neo-Hawaii.

VI. Tables

Table 2.1. Distribution of nuclei at different ploidy levels found in *Dendrobium* tissues.

Species	Tissue	% Nuclei at each histogram peak (CV)[b]				
(section)	type[a]	1C	2C	4C	8C	16C
D. phalaenopsis Fitzg.	p	100 (5.0)	-	-	-	-
(Phalaenanthe)	r	-	27.1 (4.3)	42.4 (3.3)	30.5 (2.8)	-
	il	-	37.8 (3.2)	43.1 (4.1)	19.2 (3.4)	-
	ml	-	21.9 (5.3)	43.3 (2.7)	34.8 (3.0)	-
D. gouldii Rchb. f.	p	100 (3.5)	-	-	-	-
(Spatulata)	r	-	22.5 (4.4)	41.8 (4.1)	35.7 (3.2)	-
	il	-	48.8 (4.7)	42.7 (4.2)	8.5 (4.7)	-
	ml	-	19.6 (5.3)	26.1 (4.0)	42.5 (3.3)	11.8 (2.3)
D. moschatum Sw.	p	100 (3.6)	-	-	-	-
(Dendrobium)	r	-	10.5 (3.6)	47.8 (4.4)	41.7 (4.7)	-
	jl	-	66.4 (3.6)	27.3 (2.9)	6.3 (2.3)	-
	il	-	73.0 (4.5)	27.0 (5.3)	-	-
	ml	-	26.9 (3.2)	46.4 (3.2)	26.7 (2.6)	-
D. smillieae F. Muell.	r	-	60.6 (4.5)	39.4 (3.2)	-	-
(Pedilonum)	il	-	73.0 (5.0)	25.3 (4.5)	1.7 (3.7)	-
	ml	-	40.5 (5.0)	49.2 (4.7)	10.3 (4.5)	-

[a] abbreviations for tissue types sampled: p = pollen; r = root tips; il = immature leaf; ml = mature leaf; jl = juvenile leaf.

[b] Coefficient of variation (CV) for each histogram peak shown after percent values are derived from linear red fluorescence data analysis.

VII. Figures

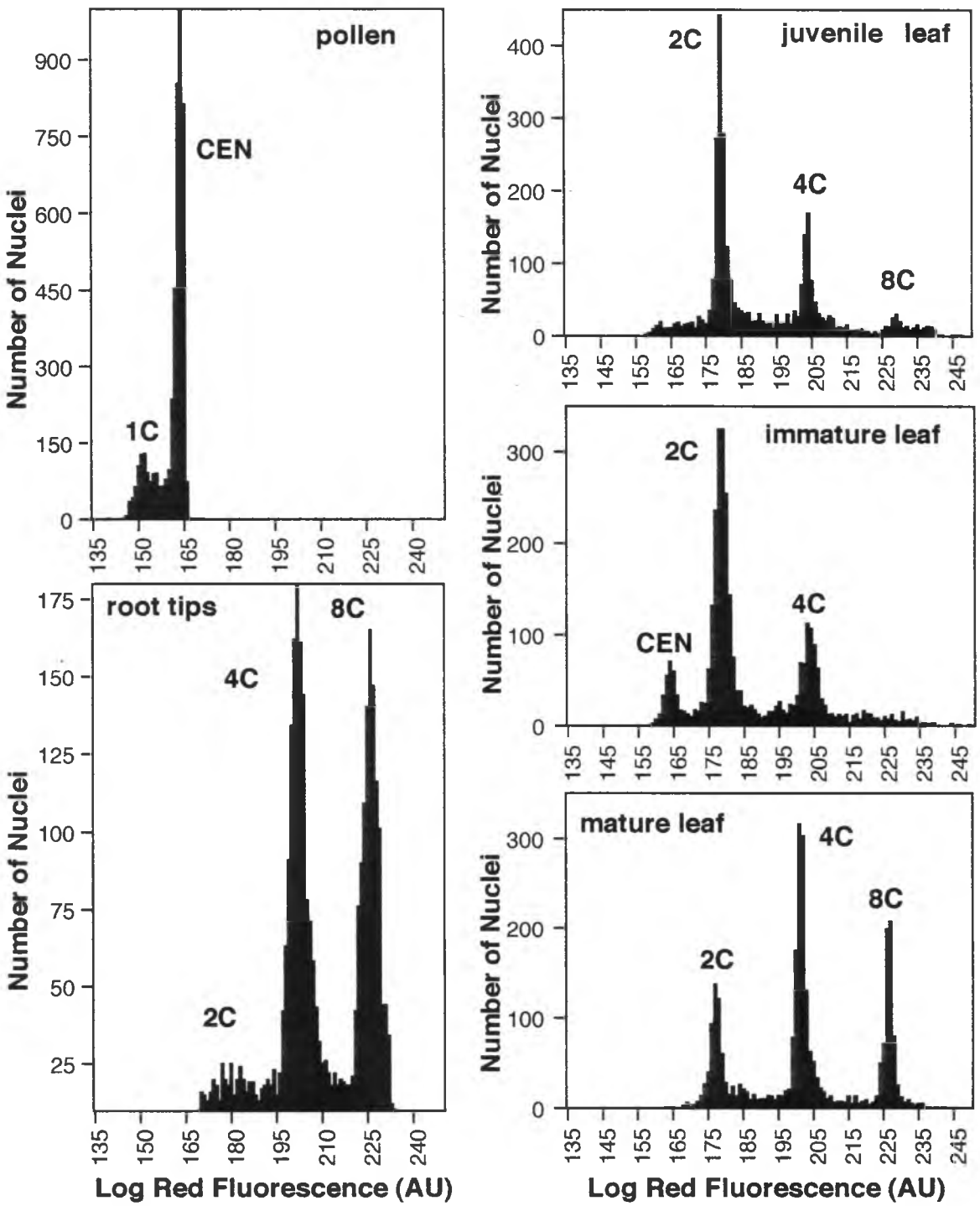
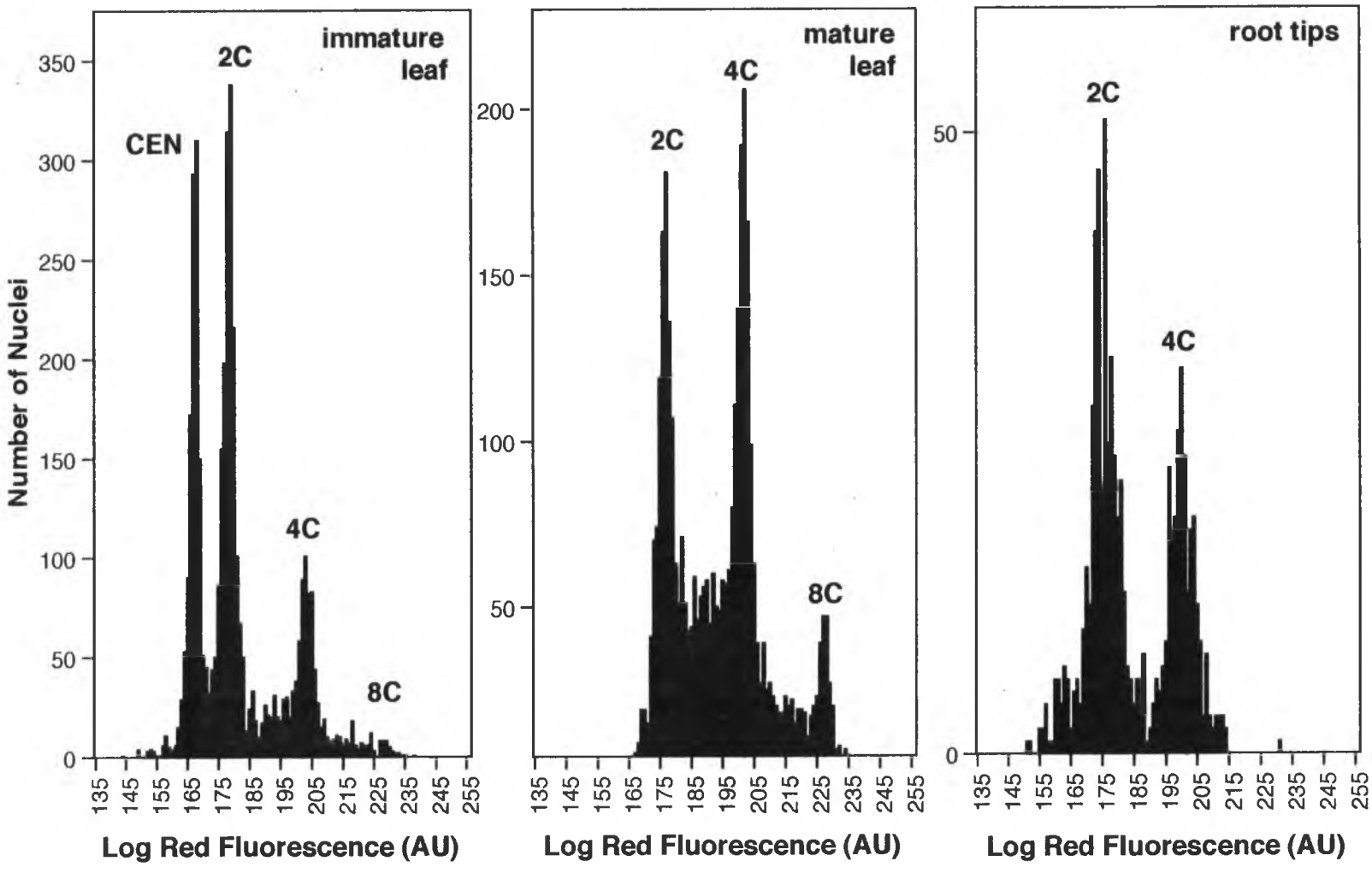


Figure 2.1. Frequency histograms of extracted nuclei from *D. moschatum* tissues showing endopolyploidy. CEN was included as an internal DNA reference standard with pollen and immature leaf samples.


immature leaf
CEN
2C
4C
8C
Number of Nuclei
Log Red Fluorescence (AU)
mature leaf
2C
4C
8C
Log Red Fluorescence (AU)
root tips
2C
4C
Log Red Fluorescence (AU)

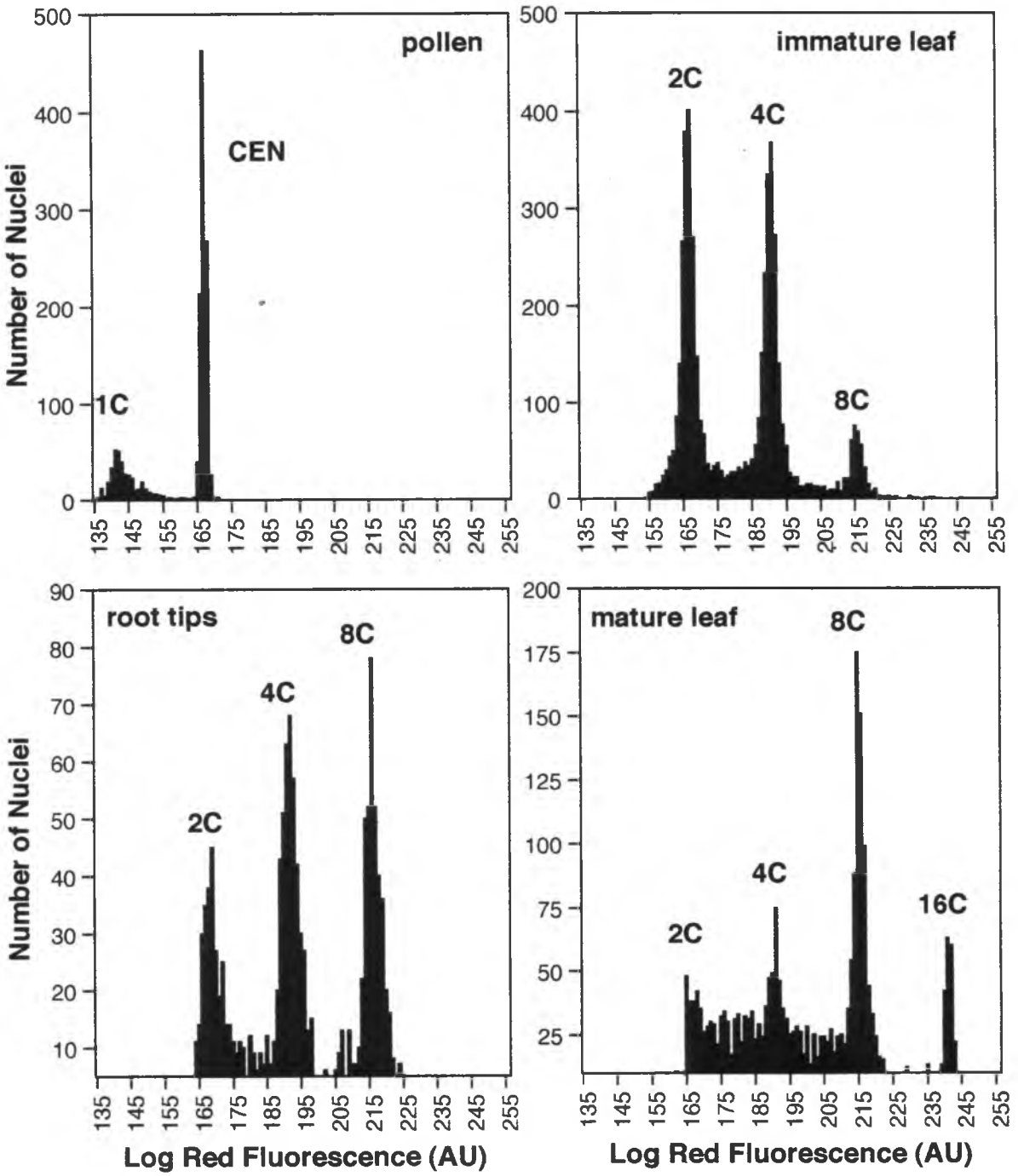


Figure 2.3. Frequency histograms of extracted nuclei from different *D. gouldii* tissues showing endopolyploidy. CEN was included as an internal DNA reference standard with pollen and immature leaf samples.

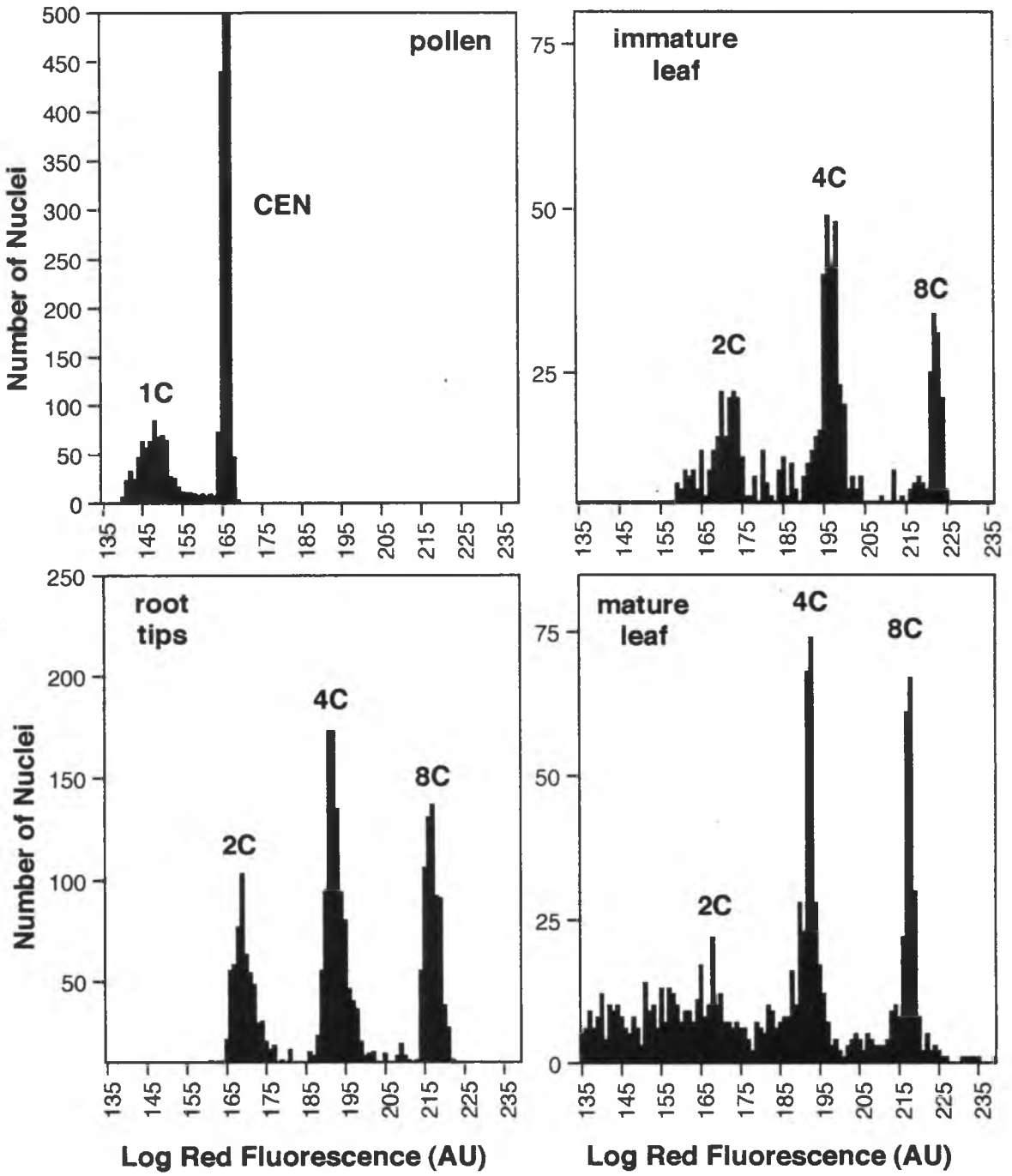


Figure 2.4. Frequency histograms of extracted nuclei from different *D.phalaenopsis* var. 'compactum' tissues showing endopolyploidy. CEN was included as an internal DNA reference standard with pollen.

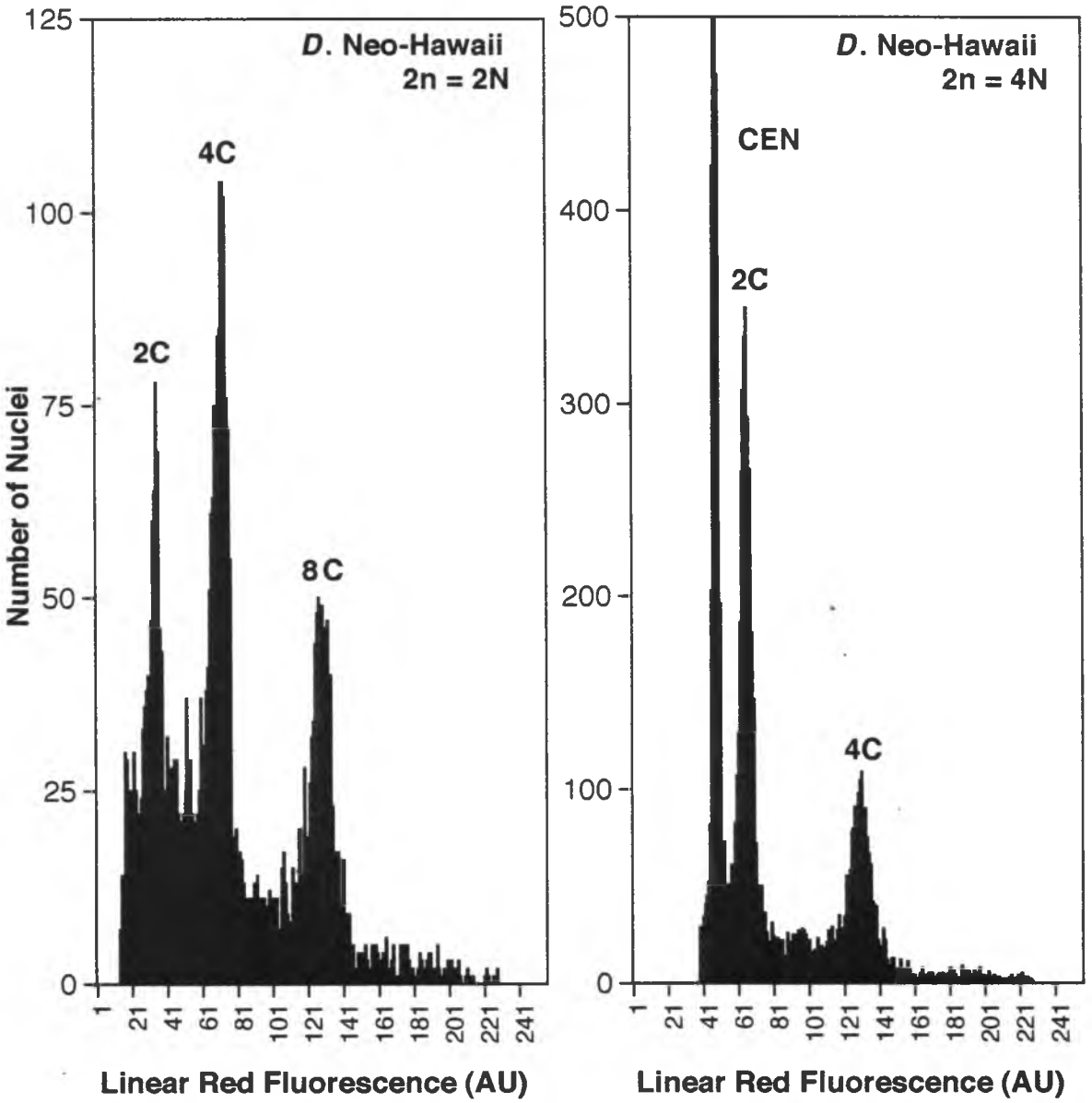


Figure 2.5. Frequency histograms of diploid (2N) and tetraploid (4N) *D.* Neo-Hawaii extracted nuclei from immature leaf samples. CEN was included with 4N samples as an internal DNA reference standard. The 8C peak is off-scale for the 4N histogram.

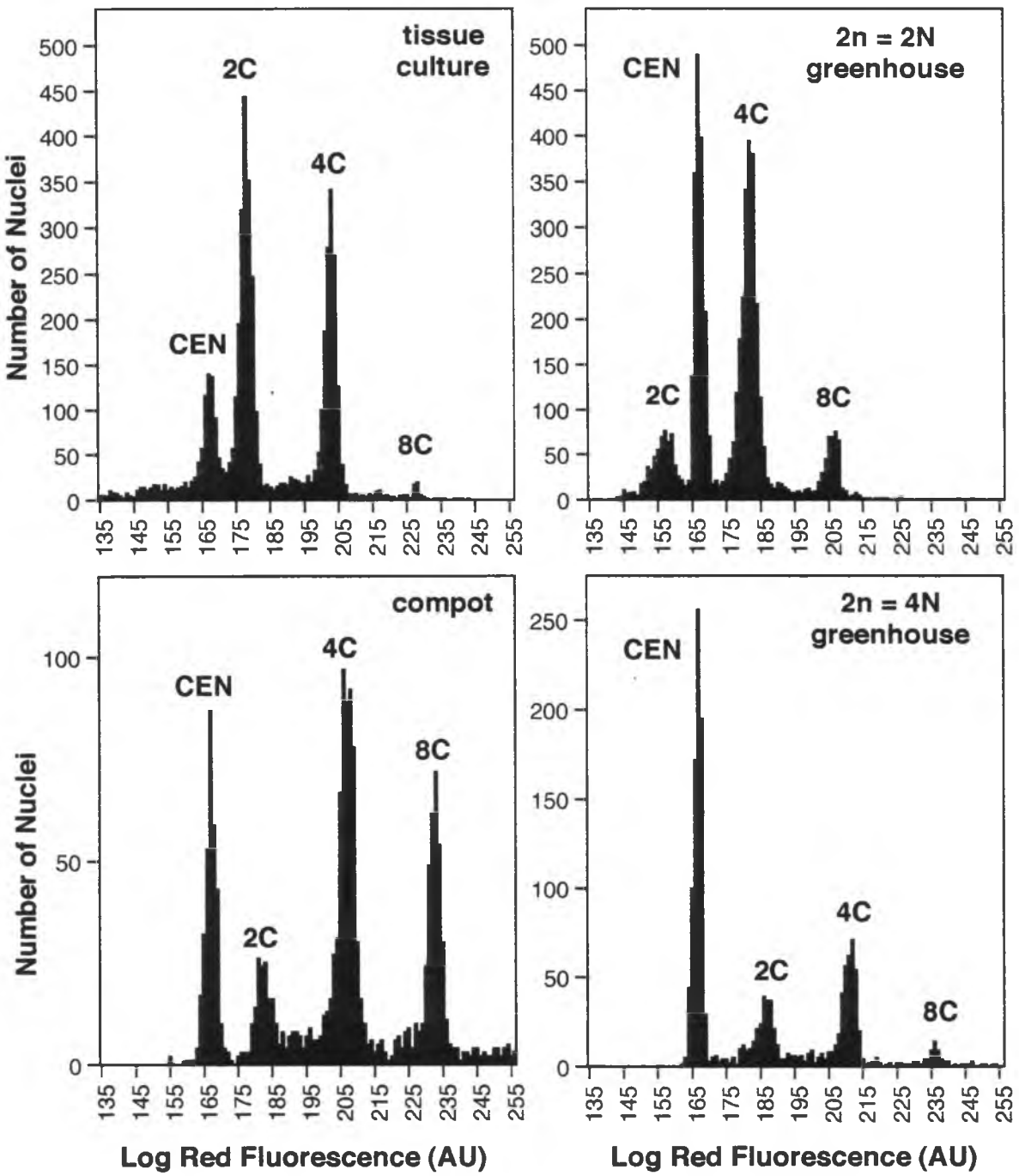


Figure 2.6. Frequency histograms of extracted nuclei from *D. superbiens* 'D184' in vitro, compot and diploid (2N) and tetraploid (4N) greenhouse-grown clonal samples showing peaks relative to CEN, an internal DNA reference standard.

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Chapter 3: Nuclear DNA Content of 26 Orchid (Orchidaceae) Genera with Emphasis on Dendrobium

I. Introduction

Demand for comparative studies of angiosperm genome size has increased steadily since the discovery of methods to measure DNA content during the 1950s (Bennett and Smith 1976, 1991; Bennett and Leitch 1995). Knowledge of genome size has application in fields of diverse interest such as cell and molecular biology, ecology, phytogeography, and systematics. For example, genome size has been used to study the effect of gain (De Azkue and Martinez 1988) or loss (Srivastava and Lavania 1991) of DNA on speciation within a genus. DNA C-values have been used to confirm taxonomic schemes, as for species within the genus *Vicia* (Maxted et al. 1991), or to separate taxa into different species as in the case of *Tephrosia* taxa (Raina et al. 1986). In molecular studies, knowing the genome size of a test organism is useful for estimating the number of clones required to create a genomic library containing the genes of interest. To date there are only about 1200 species for which C-values have been published (Bennett and Leitch 1995). For the Orchidaceae, considered the largest plant family with estimates ranging from 17,000 to 35,000 species, DNA content is reported for only 41 species (Nagl and Capesius 1977; Narayan et al. 1989; Arumuganathan and Earle 1991; Cox et al. 1993; Bennett and Leitch 1995). C-values are notably absent or lacking for several of the commercially valuable genera including *Cattleya, Cypripedium, Encyclia, Epidendrum Vanda* and *Dendrobium*. The latter is the largest orchid genus, comprising approximately 1000 identified species (Dressler 1993), and hybrids are heavily traded as tropical cut flowers (Laws 1995).

Dendrobium is of considerable interest due to broad geographic distribution and tremendous diversity in growth habits and form (Schlechter 1914; Cribb 1983, 1986; Upton 1989; Schelpe and Stewart 1990; Baker and Baker 1995). However, classification

of species with this morphology-based system is frequently dynamic. Some assignments are questioned by breeders based on their observations of chromosome pairing behavior in hybrids (Wilfret et al. 1979; Kamemoto 1987). Currently, the DNA content is known for only five *Dendrobium* species, with value ranging from 5.1 to 11.7 pg DNA/2C nuclei (Narayan et al. 1989). Determination of genome sizes of additional species may aid taxonomists and systematists.

The most precise method for determining genome size is flow cytometry (FCM) (Dolezel 1991). In this study, nuclear DNA contents of 33 representative species from 25 orchid genera were evaluated, of which three species had been previously analyzed using other methods. Additionally, 37 species of *Dendrobium* from eight taxonomic sections were selected and their DNA content compared. Only one of the five previously analyzed *Dendrobium* species was included. Variation in genome size for orchid species was assessed. Differences in the amount of DNA between each *Dendrobium* species, as well as among species within each section, were evaluated.

II. Materials and Methods

A. Plant Material

Leaf samples of 37 *Dendrobium* species and 33 species from 25 other genera and were obtained from the University of Hawaii at Manoa orchid collection, or were donated by R. Tokunaga (H & R Nurseries, Inc. Waimanalo, Hawaii). The source of each sample is indicated in Tables 3.1 and 3.2. The available 2n chromosome counts for the non-*Dendrobium* species are also shown in Table 3.1. All *Dendrobium* species analyzed were previously determined to have 2n = 2N = 38 chromosomes, except for *D. formosum* with 2n = 2N = 40 chromosomes (Tanaka and Kamemoto 1984). Table 3.2 lists the examined *Dendrobium* species and their sectional designations according to Schlechter (1914; English translation by Blaxell et al. 1982).

B. Nuclei Extraction Methods

Nuclei for DNA content analysis were extracted from fully expanded young leaves or from healthy mature leaves on new season pseudobulbs. Extraction of nuclei and DNA staining were performed according to Arumuganathan et al. (1991). Approximately 100 mg of tissue, excluding midrib, were sliced into 0.5 mm or less strips in 1 ml $MgSO_4$ extraction buffer at 4 C. The extraction buffer contains 1 mg/ml dithiothreitol (DTT), 100 µg/ml propidium iodide (PI) and 2.5 µg/ml Triton X-100. After filtration through a 33 µm nylon mesh (Fisher Scientific) and precipitation at 15,000 rpm, nuclei pellets were resuspended in 400 µl of extraction buffer plus 2.5 µl/ml DNase-free RNase and incubated at 37 C for 15 min. Samples were placed on ice following incubation until FCM analysis.

A minimum of 10,000 nuclei per sample were analyzed on a Coulter EPICS 753 argon laser flow cytometer (Marine Sciences Department, University of Hawaii at Manoa) exciting at 488 nm and recording 510 nm emissions. Preliminary samples were run at the University of Nebraska, Biotechnology Laboratory with the assistance of K. Arumuganathan using an identical EPICS 753 flow cytometer. Data collected by FCM were analyzed simultaneously using CYCLOPS software (Cytomation, Inc., Fort Collins, CO). The 2C population of nuclei on resulting histograms contained at least 500 nuclei with CV values $\leq 5\%$. A minimum of two preparations for each species were run separately to confirm results. Chicken erythrocyte nuclei (CEN; BioSure, Inc., San Jose, CA) with a predetermined DNA content value of 2.33 pg per 2C nuclei were used as an internal DNA content standard (Arumuganathan et al. 1991). C-values were calculated using the mean 2C fluorescent value in arbitrary units (AU) for the sample divided by that of the CEN multiplied by 2.33 pg. A more detailed nuclei extraction protocol can be found in the appendix.

III. Results

The mean DNA content per 2C nuclei measured for 33 orchid species from genera other than *Dendrobium* ranged from 2.12 pg for *Broughtonia sanguinea* to 18.59 pg for *Doritis pulcherrima* (Table 3.1). Two varieties of *Cattleya bowringiana* were analyzed and gave nearly identical results. Four varieties of *C. walkeriana* were analyzed and resulted in a DNA content range of 5.31 pg to 9.29 pg.

The mean DNA content per 2C nuclei measured for 37 *Dendrobium* species ranged from 1.53 pg for *D. cruentum* to 4.23 pg for *D. spectabile* (Table 3.2). Values for DNA content were also variable for species within each taxonomic section. DNA content values for the four species within the section Dendrobium ranged from 2.41 pg for *D. parishii* to 3.48 pg for *D. moschatum*. Three species within the section Formosae ranged from 1.53 pg for *D. cruentum* to 2.67 pg for *D. bellatulum*. Within the section Latouria, values for six species ranged from 1.88 pg for *D. macrophyllum* to 4.23 pg for *D. spectabile*. Three Pedilonum species ranged from 3.15 pg for *D. smillieae* to 3.60 pg for *D. bullenianum*. Three species within Phalaenanthe ranged from 1.79 pg for *D. bigibbum* to 1.98 pg for *D. phalaenopsis*. Sixteen species within Spatulata ranged from 1.69 pg for *D. discolor* to 4.05 pg for *D. samoense*. Only a single species was analyzed for each of the sections Callista (*D. lindleyi*) and Rhopalanthe (*D. crumenatum*), yielding values of 2.40 pg and 2.61 pg, respectively.

IV. Discussion

This study presents 2C values for 33 species outside the genus *Dendrobium* as determined by flow cytometry. Of these species, DNA contents of four were previously analyzed using other methods. 2C values were determined for *Brassia maculata* (2C = 7.1 pg) using reassociation kinetics (Capesius and Nagl 1978), *Phaius tankervilleae* (2C = 17.0

pg) and *Rhynchostylis retusa* (2C = 5.2 pg) using Feulgen microdensitometry (Narayan et al. 1989), and *Oncidium ampliatum* (2C = 5.0 pg) using fluorescent microdensitometry (Bennett and Leitch 1995). Only one of these species, *O. ampliatum*, resulted in a value comparable to that obtained in the present FCM study. This is likely due to similarity of the two analysis methods. Both techniques involve quantitative fluorescent DNA staining. Provided that researchers using fluorescent microdensitometry work within the limitations of the technique (reviewed by Bennett and Smith 1976), the end results should be comparable to those produced by FCM. Previous authors have found FCM and fluorescent microdensitometry to yield similar results and have used this fact to justify the use of the easier method of FCM (Arumuganathan et al. 1991; Galbraith 1990). The only factor preventing FCM from replacing fluorescent microdensitometry is the initial cost of the instrumentation.

The method least similar to FCM is reassociation kinetics. The previously reported value for *Brassia maculata* using reassociation kinetics is twice that found in this study by FCM. The former method was designed to assess the base pair ratio of a genome; its accuracy in estimating the total amount of DNA per nucleus is limited. This is due to the lack of precision for determining the number of nuclei from which the DNA was extracted. The advantage of FCM is that the calculations for DNA content are independent of the actual number of nuclei.

The two species previously analyzed by fluorescent microdensitometry were determined to have DNA content values different from the values found by FCM in this study. The two techniques produced *R. retusa* and *P. tankervilleae* 2C values that differed by 4.4 pg and 6 pg, respectively. It is possible that the different values are a reflection of varietal differences. Similar differences in the amount of DNA have been reported in maize varieties selected for cold tolerance, or early flowering (Rayburn et al. 1993, 1994). However, the differences could be due to the techniques used. A lower value from

fluorescent microdensitometry may be due to understaining, poor orientation of the chromosomes in relation to the detector, to the amount of chromosome condensation, or to differences in cell type or developmental stage. Any of these error factors alone, or in combination, could account for the discrepancy in values (Bennett and Smith 1976). None of these factors are important in FCM. The difference in values could also be due to the choice of DNA standard used in that study. *Allium cepa*, with an estimated DNA content of 67.0 pg, was used as an external standard. The vast difference between the sample and standard genome sizes can lead to inaccurate estimations (Galbraith 1990). Ideally, the standard and sample should be as close to each other as resolution of the method allows, while retaining discrete peaks without overlap. Using an external standard also contributes to inaccurate estimates because instrument fluctuations between sample and standard runs may alter the readings.

Chromosome counts previously recorded for *Cattleya bowringiana* and *C. walkeriana* were for diploid specimens. The actual counts for the samples in this study are unknown, but were thought to be diploid. However, values for nuclear DNA content for the four *C. walkeriana* cultivars ranging from 7.30 pg to 9.29 pg, are larger than the measured value of 5.97 pg for the wild collected *C. walkeriana* 'Coerulea' specimen. Although none of the values for selected varieties are precise multiples of the wild species, they may be near triploids. Differences could also be due to varietal variation as reported in maize (Rayburn et al. 1994). The two *C. bowringiana* varieties have comparable genome sizes, but the value alone was insufficient to determine ploidy.

DNA contents for nine Cymbidiodeae species sampled ranged from 3.44 pg to 9.34 pg. The largest species was *Peristera elata*, in the tribe Maxillarieae, with 9.34 pg of DNA. The next closest in size was *Cymbidium sinense* with 6.31 pg DNA. The remaining species analyzed from this tribe were found to have half the DNA compared to *P. elata*. It is possible that the specimen of *P. elata* analyzed was polyploid. However, large tribal

genome size variation has been reported for other families (reviewed by Bennett and Leitch 1995).

The genus *Dendrobium* is in the tribe Dendrobineae within the subfamily Dendrobioideae (Dressler 1981). The closest related species analyzed in this study outside the genus was *Bulbophyllum cocoinum* . This species is in the same tribe, but a different subtribe. *B. cocoinum* has over twice the amount of nuclear DNA than the average amount for *Dendrobium* species. This may lend support to the proposal by Yukawa et al. (1993) that *Bulbophyllum* be removed from Dendrobineae.

Five species of *Dendrobium* were previously analyzed using microdensitometry, *D. aphyllum* (6.7 pg), *D. densiflorum* (5.1 pg), *D. fimbriatum* (6.3 pg), *D. hookeriana* (11.7 pg), and *D. moschatum* (9.3 pg) (Narayan et al. 1989). Of these, *D. moschatum* is the only one also reanalyzed in the present study. The DNA content value for this species , derived by Feulgen microdensitometry, is more than twice that found using FCM, presumably due to use of an inappropriate external standard (*Allium cepa* 2C = 67 pg).

Results in Table 3.2 show a variety of differences and similarities among *Dendrobium* species, despite uniform chromosome number of 2n = 2N = 38. Previous work by Wilfret and Kamemoto (1971) showed that karyotype could be used to differentiate species that were morphologically very similar. Their study also found that *D. bigibbum* was distinct from *D. phalaenopsis*, with the latter having three more pairs of large subterminal chromosomes and three less pairs of small median chromosomes than the former. The larger mean chromosome size of *D. phalaenopsis* is supported by FCM data showing *D. phalaenopsis* to have a larger genome size than *D. bigibbum* . This is strong evidence that flow cytometry can be used in some cases to distinguish species nearly identical in morphological appearance.

Another example of using FCM to distinguish species can be seen in the case of the specimen of *D. streblocerus*, which is thought to be misidentified *D. tangerinum* (H.

Kamemoto, Univ. of Hawaii, personal communication). FCM analysis shows that *D. streblocerus* has a larger genome size than *D. tangerinum*. This could indicate that either the samples are correctly identified, or that the species is highly variable. This method has also shown a slight difference between the species *D. strepsiceros* and *D. stratiotes*, which are very similar morphologically, but were found to differ in DNA content by at least 0.24 pg. An additional comparison can be made between *D. stratiotes* and *D. antennatum*. The former is often referred to as a larger form of the latter (Schelpe and Stewart 1990). This holds true for genome size, with *D. stratiotes* being larger than *D. antennatum* by at least 0.43 pg.

D. canaliculatum, previously classified in the section Eleutheroglossum, groups mid-range with the other Spatulata species in genome size. Even though *D. canaliculatum* appears to fit in the new section, the range for the values within that section is large and overlaps the values for species in the other sections analyzed. The results of this study show that nuclear DNA content cannot be used to determine the appropriate section for a particular species. This parallels the observation by Wilfret and Kamemoto (1971) that karyotypes could not be used to assign species to sections.

Morphological similarities used to assign species to sections are not necessarily an indication of similarity in nuclei DNA content. Often species expected to be similar turned out to be different, as was seen for *D. tangerinum* and *D. strepsiceros*. It is possible that a more extensive survey of more representatives per species, including a geographical sampling, would reveal some insight into the true variation in genome size for this genus.

Further analysis of species from genera related to *Dendrobium*, such as *Epidendrum*, would also be useful. However, a few problems need to be overcome first. The main problem involves extraction of nuclei from some genera conspicuously missing from this study. Four separate attempts at isolating and analyzing nuclei from *Aerides*, *Epidendrum* and several *Encyclia* failed. Removal by Sephadex filtration of the viscous polysaccharide,

produced by chopping leaf tissue, may improve recovery of isolated nuclei. Another option is to extract nuclei from other tissues, such as pollen or root tips, or from protoplasts.

V. Summary

DNA content values for 33 representative orchid species from 25 genera and 37 *Dendrobium* species from eight taxonomic sections, were analyzed using FCM. The resulting C-values for non-*Dendrobium* species ranged from 2.12 to 18.56 pg/2C nuclei for *Broughtonia sanguinea* and *Doritus pulcherrima*, respectively. The *Dendrobium* C-values ranged from 1.53 to 4.23 pg/2C nuclei for *D. cruentum* and *D. spectabile*, respectively. Within the genus *Dendrobium,* the greatest variance of C-values was found for the sections Latouria and Spatulata. The range of C-values for the six species analyzed within Latouria was 1.88 pg/2C nuclei for *D. macrophyllum* to 4.23 pg/2C nuclei for *D. spectabile*. The C-values for the 16 species within Spatulata ranged from 1.69 pg/2C nuclei for *D. discolor* to 4.05 pg/2C nuclei for *D. samoense*. The least variation in DNA content was found within the section Phalaenanthe, with C-values ranging from 1.79 to 1.98 pg/2C for *D. bigibbum* and *D. phalaenopsis*, respectively.

VI. Tables

Table 3.1. Mean DNA content for species of 25 orchid genera, excluding *Dendrobium* .

Species [Previous synonym]	Subfamily / tribe[a]	Chrm. # per 2n nuclei[b]	Mean DNA content ± SE (pg)	Mbp/1C nuclei [c]
Anselia africana Lindl. [d]	Vandoideae / Cymbidieae	42	3.70 ± 0.16	1785
Barkeria lindleyana Batem. ex Lindl. [d]	Epidendroideae / Epidendreae	unk	3.29 ± 0.22	1587
Brassia maculata R.Br. [d]	Vandoideae / Maxillarieae	60	3.74 ± 0.31	1804
Broughtonia sanguinea (Sw.) R.Br. [d]	Epidendroideae / Epidendreae	40	2.12 ± 0.09	1023
Bulbophyllum cocoinum Batem. ex Lindl.[d]	Epidendroideae / Epidendreae	38	5.35 ± 0.28	2581
Cadetia taylori (F.Muell.) Schltr. [d]	Epidendroideae / Dendrobieae	unk	1.91 ± 0.08	922
Cattleya bowringiana O'brien var. 'alba' [d]	Epidendroideae / Epidendreae	40, 42, 60	4.98 ± 0.23	2403
Cattleya bowringiana O'brien var. 'Coerulea' [d]	Epidendroideae / Epidendreae	unk	4.99 ± 0.26	2408
Cattleya forbesii Lindl. [d]	Epidendroideae / Epidendreae	54 - 60	3.29 ± 0.16	1587
Cattleya walkeriana Gardn. [d]	Epidendroideae / Epidendreae	40	5.97 ± 0.24	2880
Cattleya walkeriana var. alba 'Pendentive' Holt. [e]	Epidendroideae / Epidendreae	unk	8.55 ± 0.20	4005
Cattleya walkeriana var. alba 'Limerick' Holt. [e]	Epidendroideae / Epidendreae	unk	8.13 ± 0.22	3923
Cattleya walkeriana var. 'Coerulea' Holt. [e]	Epidendroideae / Epidendreae	unk	5.31 ± 0.26	2562
Cattleya walkeriana var. 'Coerulea Chouju' Holt. [e]	Epidendroideae / Epidendreae	unk	9.29 ± 0.60	4482

Table 3.1 (continued). Mean DNA content for species of 25 orchid genera, excluding *Dendrobium*

Species [Previous synonym]	Subfamily / tribe[a]	Chrm. # per 2n nuclei[b]	Mean DNA content ± SE (pg)	Mbp/1C nuclei [c]
Cattleya walkeriana var. semi-alba 'Puanani' Holt.[e]	Epidendroideae / Epidendreae	unk	7.30 ± 0.15	3522
Cleisostoma subulatum Bl. [d] [*Sarcanthus subulata* Lindl.]	Vandoideae / Vandeae	38	6.40 ± 0.27	3088
Coelogyne pastulata Pfitz. [d]	Epidendroideae / Coelogyneae	unk	5.48 ± 0.22	2644
Cymbidium sinense (Jacks.) Willd. [e]	Vandoideae / Cymbidieae	unk	6.31 ± 0.21	3045
Doritis pulcherrima Lindl. [d]	Vandoideae / Vandeae	38	9.25 ± 0.28	4463
Epidendrum steinbachii Ames [d]	Epidendroideae / Epidendreae	unk	2.87 ± 0.11	1385
Grammatophyllum scriptum (L.) Bl. [d]	Vandoideae / Cymbidieae	38,40	3.44 ± 0.11	1660
Laelia rubescens Rolfe [d]	Epidendroideae / Epidendreae	40	2.45 ± 0.07	1182
Laelia tenebrosa Rolfe [d]	Epidendroideae / Epidendreae	unk	3.51 ± 0.20	1694
Neofinetia falcata (Thunb.) Hu [d]	Vandoideae / Vandeae	38	4.73 ± 0.35	2282
Oncidium ampliatum Lindl. [d]	Vandoideae / Maxillarieae	44	4.78 ± 0.20	2306
Oncidium sphacelatum Lindl. [d]	Vandoideae / Maxillarieae	56	4.74 ± 0.20	2287
Oncidium varuelum Moir [d]	Vandoideae / Maxillarieae	63	3.85 ± 0.19	1858
Peristera elata Hook.f. [d]	Vandoideae / Maxillarieae	40	9.34 ± 0.16	4507

Table 3.1 (continued). Mean DNA content for species of 25 orchid genera, excluding *Dendrobium*

Species [Previous synonym]	Subfamily / tribe[a]	Chrm. # per 2n nuclei[b]	Mean DNA content ± SE (pg)	Mbp/1C nuclei [c]
Phalaenopsis equestris (Shauer) Rchb.f. [d]	Vandoideae / Vandeae	38	5.53 ± 0.28	2668
Phalaenopsis luedemanniana Rchb.f.[d]	Vandoideae / Vandeae	38	8.65 ± 0.41	4174
Phaius tankervillae (Banks) Bl. [d]	Epidendroideae / Arethuseae	46	11.38 ± 0.49	5491
Rhynchostylis gigantia (Lindl.) Ridl.[d]	Vandoideae / Vandeae	38	6.02 ± 0.26	2905
Rhynchostylis retusa (L.) Bl. [d]	Vandoideae / Vandeae	38	9.65 ± 0.48	4656
Schomburgkia lyonsii Lindl. [d]	Epidendroideae / Epidendreae	unk	3.89 ± 0.14	1877
Smitinandia micrantha (Lindl.) Holt. [d] [*Ascocentrum micranthum* Lindl.]	Vandoideae / Vandeae	38	4.19 ± 0.32	2022
Trichopilia maculata Rchb.f. [d]	Vandoideae / Maxillarieae	unk	4.67 ± 0.09	2253
Vanilla phaeantha Rchb.f. [d]	Epidendroideae / Vanilleae	32	15.19 ± 0.96	7329
Vanilla pompona Schiede [d]	Epidendroideae / Vanilleae	32	14.45 ± 0.71	6972
Vanda lamellata Lindl. [e]	Vandoideae / Vandeae	38	4.10 ± 0.22	1978

[a] Classification according to Dressler (1981).

[b] Chromosome numbers are taken from Tanaka and Kamemoto (1984) and came from different specimen plants than those analyzed in this study; unk = unknown count.

[c] Megabase pairs per haploid genome (Mbp / 1C nuclei) calculated based on the equivalent of 1 pg DNA = 965 Mbp (Strauss 1971).

[d] Source of plant material: University of Hawaii, Honolulu, HI.

[e] Source of plant material: R. Tokunaga, H & R Nurseries, Waimanalo, HI.

Table 3.2. Mean nuclear DNA content for *Dendrobium* species from eight taxonomic sections.

Species [Previous synonym]	Subgenus[a]	Section [Previous synonym or classification]	Mean DNA content ± SE (pg)	Mbp/1C nuclei [b]
D. affine (Deane) Steud. [c] [*D. dicuphum* F.Muell.]	Dendrobium	Phalaenanthe	1.86 ± 0.10	897
D. antennatum Lindl. [c] [*D. d'albertsii* Rchb.f.]	Dendrobium	Spatulata [Ceratobium]	2.77 ± 0.10	1336
D. atroviolaceum Rolfe[d]	Athecebium	Latouria	2.57 ± 0.07	1240
D. bellatulum Rolfe [d]	Dendrobium	Formosae [Nigrohirsutae]	2.67 ± 0.09	1288
D. bicaudatum Reinw. [c] [*D. rumphianum* Teijsm.]	Dendrobium	Spatulata [Ceratobium]	2.41 ± 0.03	1163
D. bigibbum Lindl.[c]	Dendrobium	Phalaenanthe	1.79 ± 0.05	864
D. bracteosum Rchb.f. [d]	Dendrobium	Pedilonum	3.53 ± 0.09	1703
D. bullenianum Rchb.f. [c] [*D. topaziacum* Ames]	Dendrobium	Pedilonum	3.60 ± 0.16	1737
D. canaliculatum R.Br. [c]	Dendrobium	Spatulata [Eleutheroglossum]	2.71 ± 0.00	1308
D. conanthum Schltr. [c]	Dendrobium	Spatulata [Ceratobium]	2.25 ± 0.11	1086
D. cruentum Rchb.f. [d]	Dendrobium	Formosae [Nigrohirsutae]	1.53 ± 0.09	738
D. crumenatum Sw. [c]	Rhopalobium	Rhopalanthe	2.61 ± 0.17	1259
D. discolor Lindl. [c] [*D. undulatum* R.Br.]	Dendrobium	Spatulata [Ceratobium]	1.69 ± 0.13	815
D. forbesii Ridl. [d]	Athecebium	Latouria	1.91 ± 0.11	922
D. formosum Roxb. ex Lindl. [d] [*D. infundibulum* Rchb.f.]	Dendrobium	Formosae [Nigrohirsutae]	1.73 ± 0.11	835
D. gouldii Rchb.f.[c]	Dendrobium	Spatulata [Ceratobium]	2.09 ± 0.06	1008

Table 3.2 (continued). Mean nuclear DNA content for *Dendrobium* species from eight taxonomic sections

Species [Previous synonym]	Subgenus[a]	Section (Previous synonym or classification)	Mean DNA content ± SE (pg)	Mbp/1C nuclei [b]
D. helix Cribb[c]	Dendrobium	Spatulata [Ceratobium]	2.32 ± 0.08	1119
D. lasianthera J.J.Sm. [c] [*D. ostrinoglossum* Rupp]	Dendrobium	Spatulata [Ceratobium]	2.12 ± 0.14	1023
D. lindleyi Steud. [c] [*D. aggregatum* Roxb.]	Athecebium	Callista	2.40 ± 0.06	1158
D. macrophyllum A.Rich. [c]	Athecebium	Latouria	1.88 ± 0.06	907
D. moschatum (Buch.-Ham.) Sw.[c]	Dendrobium	Dendrobium [Eugenanthe]	3.48 ± 0.14	1679
D. parishii Rchb.f. [c]	Dendrobium	Dendrobium [Eugenanthe]	2.41 ± 0.13	1163
D. phalaenopsis Fitzg.[c] [*D. bigibbum* var. *superbum* Hort. ex Rchb.f.] [*D. bigibbum* var. *phalaenopsis* (Fritz.) Bail.]	Dendrobium	Phalaenanthe	1.98 ± 0.05	955
D. polysema Schltr. [d]	Athecebium	Latouria	3.03 ± 0.06	1462
D. pulchellum Roxb. ex Lindl. [c]	Dendrobium	Dendrobium [Eugenanthe]	3.18 ± 0.01	1534
D. rhodostictum F.Muell. & Kranzl. [d]	Athecebium	Latouria	2.94 ± 0.10	1418
D. samoense Cribb [c]	Dendrobium	Spatulata [Ceratobium]	4.05 ± 0.15	1954
D. schulleri J.J.Sm. [c]	Dendrobium	Spatulata [Ceratobium]	1.96 ± 0.09	946
D. signatum Rchb.f. [c] [*D. hildebrandii* Rolfe]	Dendrobium	Dendrobium [Eugenanthe]	2.92 ± 0.10	1408
D. smillieae F.Muell. [c]	Dendrobium	Pedilonum	3.15 ± 0.13	1520

Table 3.2 (continued). Mean nuclear DNA content for *Dendrobium* species from eight taxonomic sections

Species [Previous Synonym]	Subgenus[a]	Section [Previous synonym or classification]	Mean DNA content ± SE (pg)	Mbp/1C nuclei [b]
D. spectabile (Bl.) Miq. [c]	Athecebium	Latouria	4.23 ± 0.23	2041
D. stratiotes Rchb.f. [c]	Dendrobium	Spatulata [Ceratobium]	3.35 ± 0.05	1616
D. streblocerus Rchb.f. [c]	Dendrobium	Spatulata [Ceratobium]	3.73 ± 0.36	1800
D. strepsiceros J.J.Sm. [c]	Dendrobium	Spatulata [Ceratobium]	2.92 ± 0.14	1409
D. tangerinum Cribb [c]	Dendrobium	Spatulata [Ceratobium]	2.19 ± 0.17	1057
D. taurinum J.J.Sm. [c]	Dendrobium	Spatulata [Ceratobium]	1.92 ± 0.06	926
D. violaceoflavens Thomson [c]	Dendrobium	Spatulata [Ceratobium]	2.69 ± 0.24	1298

[a] Classification according to Baker and Baker (1995).

[b] Megabase pairs per haploid genome (Mbp / 1C nuclei) calculated based on the equivalent of 1 pg DNA = 965 Mbp (Strauss 1971).

[c] Source of plant material: University of Hawaii, Honolulu, HI.

[d] Source of plant material: R. Tokunaga, H & R Nurseries, Waimanalo, HI.

Table 3.3. Range of DNA content values found within each *Dendrobium* section.

Section [Previous synonym] [a]	# Species [b]	DNA content range
Callista	1	2.40 pg
Dendrobium [Eugenanthe]	4	2.41 pg - 3.48 pg
Formosae [Nigrohirsutae]	3	1.53 pg - 2.67 pg
Latouria	6	1.88 pg - 4.23 pg
Pedilonum	3	3.15 pg - 3.60 pg
Phalaenanthe	3	1.79 pg - 1.98 pg
Spatulata [Ceratobium]	16	1.69 pg - 4.05 pg
Rhopalanthe	1	2.61 pg

[a] Classification according to Baker and Baker (1995).

[b] Number of *Dendrobium* species sampled for each taxonomic section.

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Chapter 4: Evaluation of Dendrobium Species and Hybrids Using Flow Cytometry

I. Introduction

Although the genus *Dendrobium* is one of the largest in the Orchidaceae, comprising approximately 1000 identified species (Dressler 1993), it is the interspecific hybrids that are of commercial importance. The sale of *Dendrobium* hybrids in Hawaii were recorded at an estimated $7.7 million out of a total of $11.2 million for all orchid sales in 1994 (HASS 1995). Because of the value of *Dendrobium* hybrids, many studies concerning the cytogenetics and crossability of *Dendrobium* species, and the fertility of hybrids, were performed over the past four decades (Kamemoto 1987). The advent of flow cytometric methods allows for a different approach to studying hybrids.

Flow cytometry was previously used to study interspecific hybrids within the Brassicaceae (Fahleson et al. 1988; Sabharwal and Dolezel 1993) and F1 hybrids of maize (Rayburn et al. 1993). In each of those studies, the contribution of parental genome to the hybrid was determined. In the present study, *Dendrobium* species and their resulting interspecific hybrids were analyzed by FCM to determine if hybrid genome size could be predicted from parental DNA content. DNA content values for six *Dendrobium* species and three corresponding interspecific hybrids were determined by FCM analysis. Parental C-values species were used to calculate the expected hybrid DNA content values. The accuracy of hybrid genome size predictions are discussed with explanations for any deviations from the expected values.

II. Materials and Methods

A. Plant Material

Leaf samples of five *Dendrobium* species and four interspecific hybrids were obtained from the University of Hawaii at Manoa orchid collection. A leaf from a sixth species, *D. canaliculatum,* was donated by R. Tokunaga (H & R Nurseries, Inc. Waimanalo, Hawaii). All samples are listed in Table 4.1. The hybrid *D.* Macrobig was derived by crossing *D. macrophyllum* by *D. bigibbum.* The tetraploid form was used as a parent along with *D. spectabile* to make the triploid hybrid *D.* Pua'ala. Diploid and tetraploid *D.* Autumn Lace resulted from crossing *D. canaliculatum* with *D. streblocerus.* Diploid and tetraploid *D.* Mini Pearl resulted from crossing *D. canaliculatum* with *D. bigibbum* var. 'compactum'. However, the *D. canaliculatum* and *D. spectabile* samples analyzed by FCM were not the same specimens used as parents. This was due to the fact that the original specimens died. The *D. canaliculatum* specimen used was selected based on the similarity in floral features to the actual parent. The *D. spectabile* specimen was selected based on availability. The available chromosome counts for the *Dendrobium* species and hybrids were previously determined and are also shown in Table 4.1 (Amore and Kamemoto 1993; Kamemoto 1985). The exceptions are *D. canaliculatum* and *D. spectabile* where counts were performed on the original specimens, but not on the FCM samples due to lack of available root tips.

B. Nuclei Extraction Methods

The extraction methods were taken from (Arumuganathan and Earle 1991) and is detailed in the appendix. Immature leaves was collected for each sample the afternoon prior to use and stored at 4 C. Approximately 100 mg of tissue, excluding midrib, were sliced into 0.5 mm, or less strips, in 1 ml $MgSO_4$ extraction buffer (solution A) at 4 C. Solution A contains 1 mg/ml dithiothreitol (DTT), 100 µg/ml propidium iodide (PI) and 2.5 µg/ml

triton X-100. After filtration through a 33 µm nylon mesh (Spectrum, Houston, TX) and precipitation at 15,000 rpm, nuclei pellets were resuspended in 400 µl of extraction buffer plus 2.5 µl/ml DNase-free RNase (solution B) and incubated at 37 C for 15 min. Samples were placed on ice following incubation until FCM analysis.

C. FCM and Data Analysis

Nuclei were run on a Coulter EPICS 753 flow cytometer equipped with a 488 nm argon gas laser located at the Marine Sciences Department at the University of Hawaii, Manoa campus. Results were collected and analyzed by computer using CYCLOPS software (Cytomation, Inc., Fort Collins, CO). DNA content was calculated by comparison to chicken erythrocyte nuclei (CEN; BioSure, Inc.), which was added to select samples as a known standard. The DNA content value used for CEN was 2.33 pg / 2C nuclei (Galbraith et al. 1983). Some samples had 2C peaks too close to the CEN peak for clear discrimination. In those cases, CEN was run as an external standard run preceding and following the plant sample. DNA content and all coefficients of variation (CV) reported for mean peak values were calculated from linear scale data. Histogram data were saved in text format and imported into Cricket Graph III software (Computer Assoc. Inc., Islandia, NY) on a Macintosh computer.

III. Results

DNA content values and ploidy levels for the *Dendrobium* species and hybrids are listed in Table 4.1. Expected DNA content values for each hybrid was estimated as the square root of the product of the two parental genome sizes. The results were multiplied by two for tetraploid samples. Frequency histograms of relative DNA content measured in arbitrary units (AU) of linear red fluorescence for parental species and the resulting hybrids are shown in Figures 4.1 to 4.4. The DNA content value for the hybrid *D.* Macrobig had a variance between the actual and expected values of less than 1%. The results for *D.* Pua'ala

varied from the expected value by 25%. However, if the *D. spectabile* specimen was considered to be a tetraploid, then the variance from the expected value became less than 1%. The diploid and tetraploid *D.* Autumn Lace samples varied from the expected values by 36% and 28%, respectively. However. if the *D. canaliculatum* sample was actually tetraploid, then the variance for the diploid and tetraploid hybrids became 9% and 2%, respectively. The variance from the expected values for the diploid and tetraploid samples of hybrid *D.* Mini Pearl were 27% and 31%, respectively. However, if *D. canaliculatum* was again considered to be tetraploid, the variance from the expected values became 2% for both diploid and tetraploid samples.

IV. Discussion

Previous studies involving interspecific hybrids have shown that flow cytometric analysis is useful for determining parentage and ploidy of questionable specimens (Sabharwal and Dolezel 1993). In the present study, the DNA content values for interspecific hybrids of *Dendrobium* species were compared to the expected values estimated from the values for the parental species. The first comparison was between the tetraploid *D.* Macrobig and the parents *D. bigibbum* and *D. macrophyllum*. Since the chromosome numbers for all three samples were known in advance of FCM, there was no question regarding ploidy. The DNA content for *D.* Macrobig was found to vary from expected by less than 1%. This means that the accuracy for this technique was high and could be used to distinguish parentage in *Dendrobium*.

The next comparison was between the triploid *D.* Pua'ala and the tetraploid parent *D.* Macrobig and diploid specimen *D. spectabile*. This comparison was less clear because the *D. spectabile* specimen analyzed was not the actual parent plant, but a representative specimen with an unknown chromosome number. An examination of the expected DNA value showed that the value for *D. spectabile* was too high by two times, indicating that it

was really a tetraploid. Assuming this to be the case, a different expected value of 3.95 pg was calculated and compared to the value for *D*. Pua'ala resulting in a variance of less than 1%.

The diploid and tetraploid hybrids *D*. Autumn Lace and *D*. Mini Pearl contain the species *D. canaliculatum*. However, the specimen used in this study was not the actual parent and the chromosome count was unknown. Fortunately, the other parent species were available and were used in the comparison to determine the ploidy of the *D*. canaliculatum specimen and assess the accuracy of this specimen being similar to the real parent. When *D. canaliculatum* was treated as a diploid, the comparison to the expected values for both hybrids resulted in variances ranging from 27% to 36%. However, if it was assumed to be tetraploid, the variances are reduced to 2% for, except for the diploid *D*. Autumn Lace with a variance of approximately 9%. This indicates that the *D. canaliculatum* specimen was probably tetraploid and predicts reliable diploid DNA content value for the missing parent.

Although the results show strong evidence that both specimens of *D. spectabile* and *D. canaliculatum* are tetraploids, the only ways to prove the ploidy is either to count chromosomes or analyzed by FCM in a side by side comparison with another specimen of each species with known ploidy. This method was used successfully in chapter 2 to identify intraplant ploidy differences.

V. Summary

The DNA content for six *Dendrobium* species representative of the parents of four hybrids was determined by FCM. Predicted C-values for the hybrids were then calculated based on the values for those species. Actual C-values were then determined by FCM and compared to the predicted values. The actual value for *D*. Macrobig was virtually equal to the predicted value. However, the values predicted for the remaining hybrids initially were

different from the actual values. The reason for the discrepancy was likely due to one of the representative parental species being tetraploid rather than diploid. When the DNA content for the suspect parent was halved and predicted hybrid values recalculated, the actual hybrid values were closer in comparison. The remaining difference in value could be accounted for by intraspecific DNA content variation. The results of this study indicate that FCM analysis can be used to verify or identify parentage of *Dendrobium* hybrids. FCM was also found to be reliable for identification of ploidy level without requiring chromosome counts. This is the first time flow cytometric analysis has been successfully employed on *Dendrobium* species and hybrids for this purpose.

VI. Tables

Table 4.1. Listing of *Dendrobium* hybrids and the parental species showing actual and expected DNA content values

Parental species	Resulting hybrid	Ploidy level	Actual DNA content (adjusted)[a]	Expected DNA content (adjusted)[b]
D. bigibbum		2N = 38	1.72 pg ± 0.08	
D. macrophyllum		2N = 38	3.95 pg ± 0.10	
	D. Macrobig	4N = 76	5.24 pg ± 0.26	5.21 pg
D. spectabile		2N = ?	5.32 pg ± 0.23 (2.66 pg)	
D. Macrobig		4N = 76	5.24 pg ± 0.26	
	D. Pua'ala	3N = 57	3.94 pg ± 0.14	5.28 pg (3.95 pg)
D. streblocerus		2N = 38	2.05 pg ± 0.10	
D. canaliculatum		2N = ?	4.94 pg ± 0.21 (2.47 pg)	
	D. Autumn Lace	2N = 38	2.05 pg ± 0.06	3.18 pg (2.25 pg)
	D. Autumn Lace	4N = 76	4.61 pg ± 0.14	6.36 pg (4.50 pg)
D. bigibbum var. 'compactum'		2N = 38	1.64 pg ± 0.06	
D. canaliculatum		2N = ?	4.94 pg ± 0.21 (2.47 pg)	
	D. Mini Pearl	2N = 38	2.06 pg ± 0.08	2.84 pg (2.01 pg)
	D. Mini Pearl	4N = 76	3.92 pg ± 0.15	5.69 pg (4.02 pg)

[a] Actual DNA content values in pg DNA / 2C nuclei with adjusted parenthetical value estimates based on parental species being a tetraploid sample.

[b] Expected values are calculated from the equation: expected = SQRT (species A value * species B value). In the case of 4N hybrids, the result is multiplied by 2. Parenthetical values are adjusted estimates based on parental species being a tetraploid sample.

VII. Figures

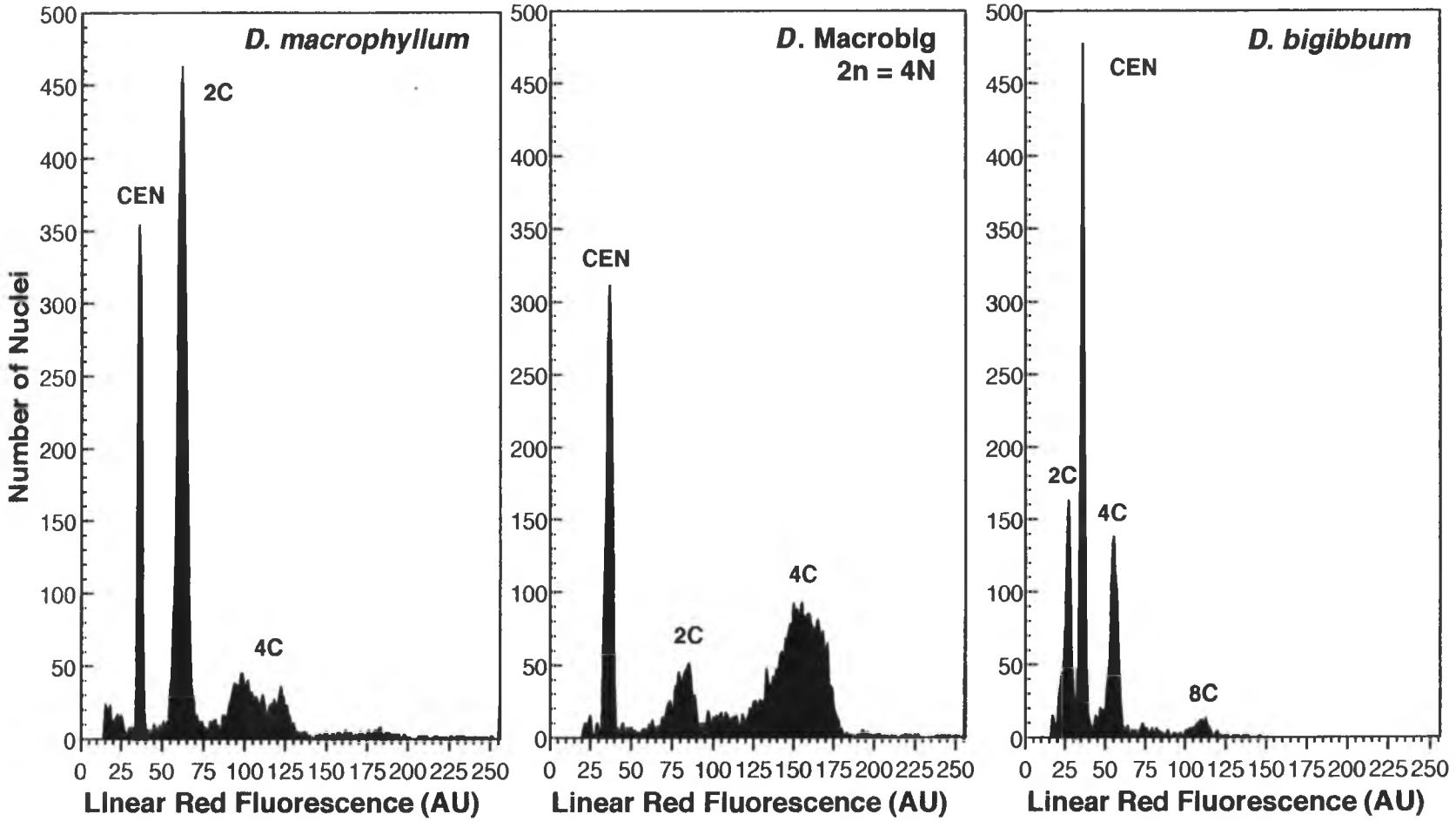

Figure 4.1. Frequency histograms showing extracted nuclei from immature leaf tissue for the diploid species *D. macrophyllum* and *D. bigibbum* and their tetraploid hybrid offspring *D.* Macrobig. CEN was included as an internal DNA reference standard.

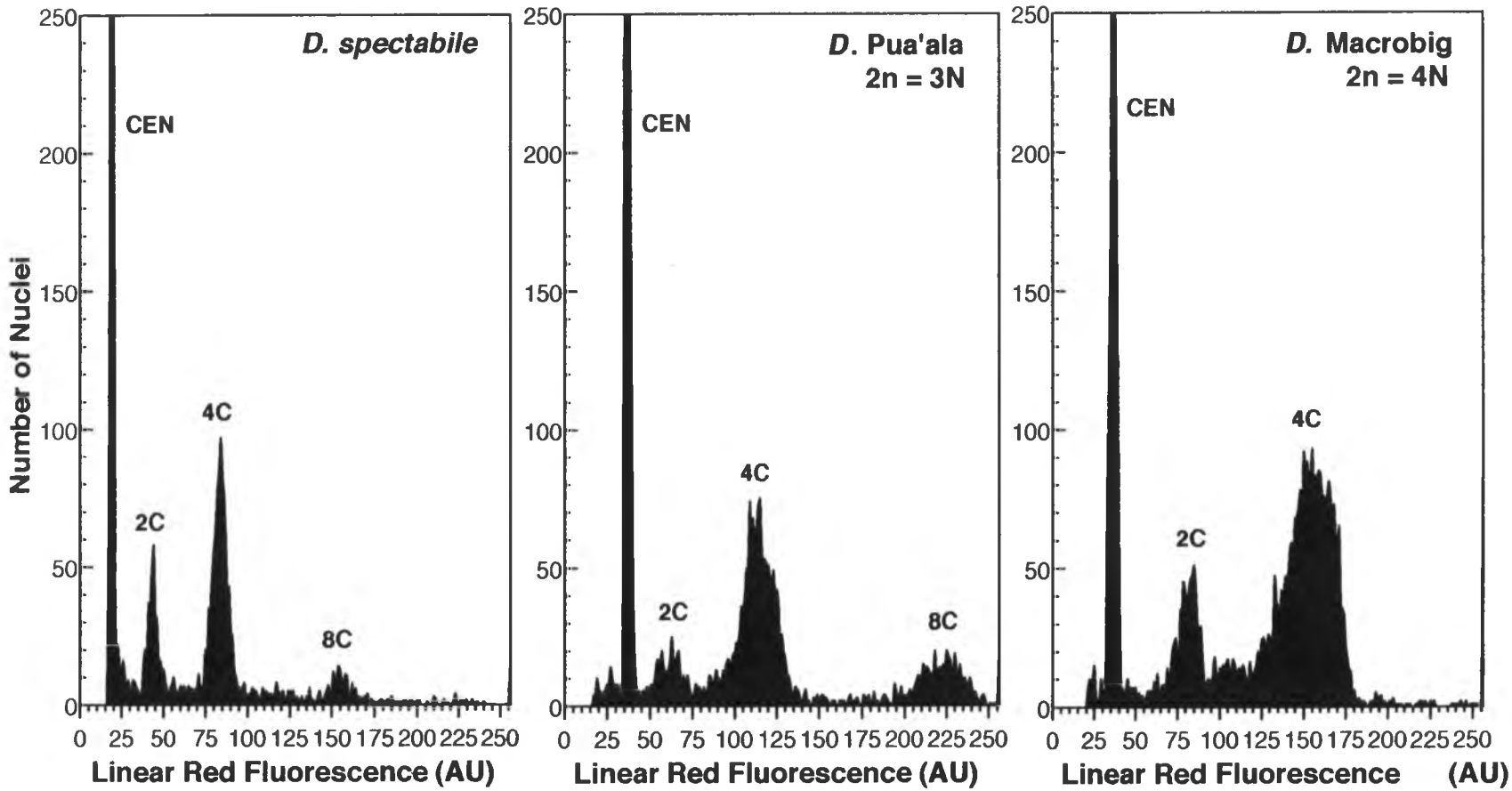

Figure 4.2. Frequency histograms showing extracted nuclei from immature leaf tissue from the species *D. spectabile* (of unknown ploidy) and tetraploid hybrid *D.* Macrobig and their triploid offspring *D.* Pua'ala. CEN was included as an internal DNA reference standard.

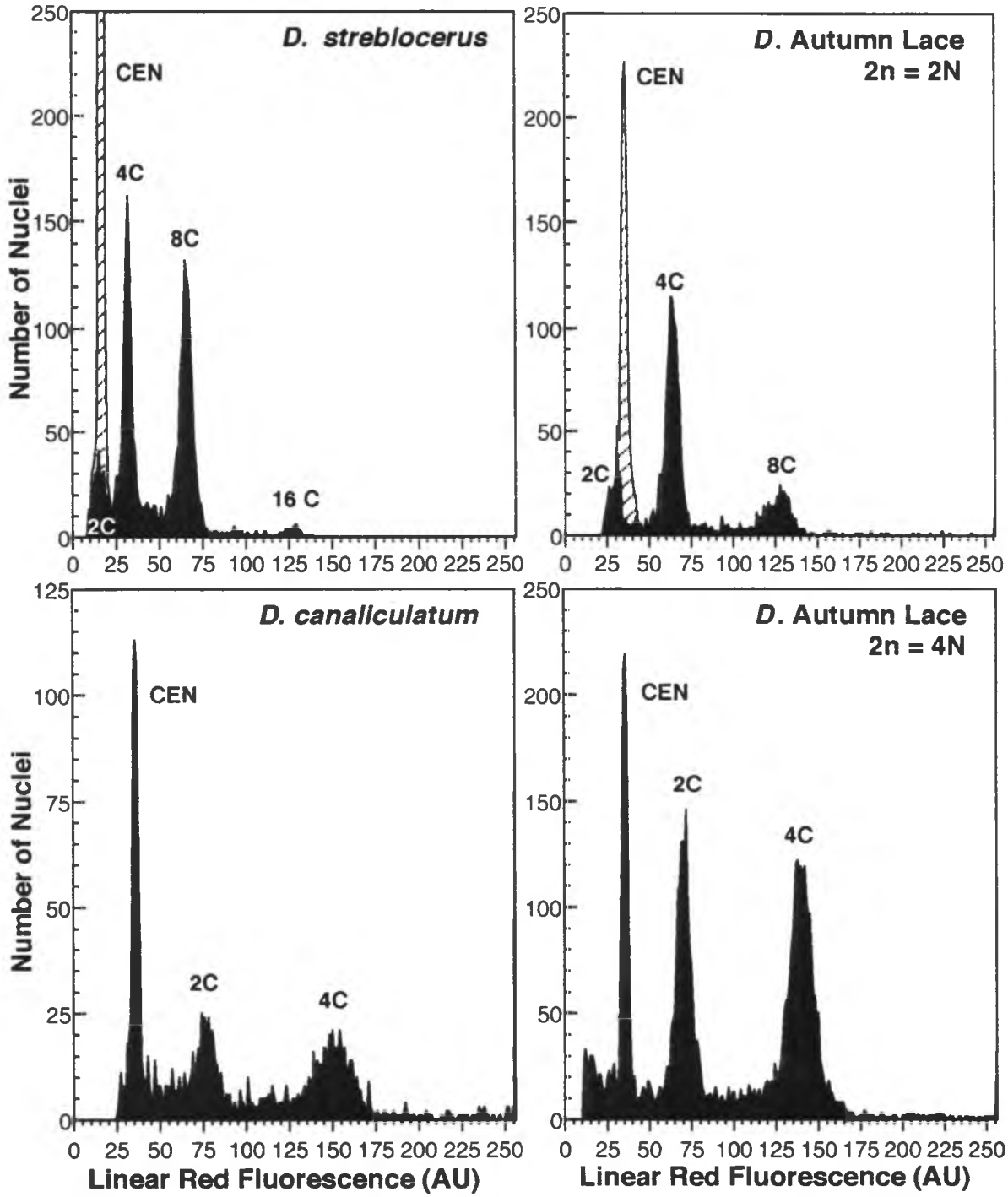

Figure 4.3. Frequency histograms of extracted nuclei from immature leaf tissues for the species *D. streblocerus* and *D. canaliculatum* and the their hybrid offspring *D.* Autumn Lace in both 2N and 4N forms. CEN was included as a DNA reference standard. Red stippled peaks indicate that CEN was run externally.

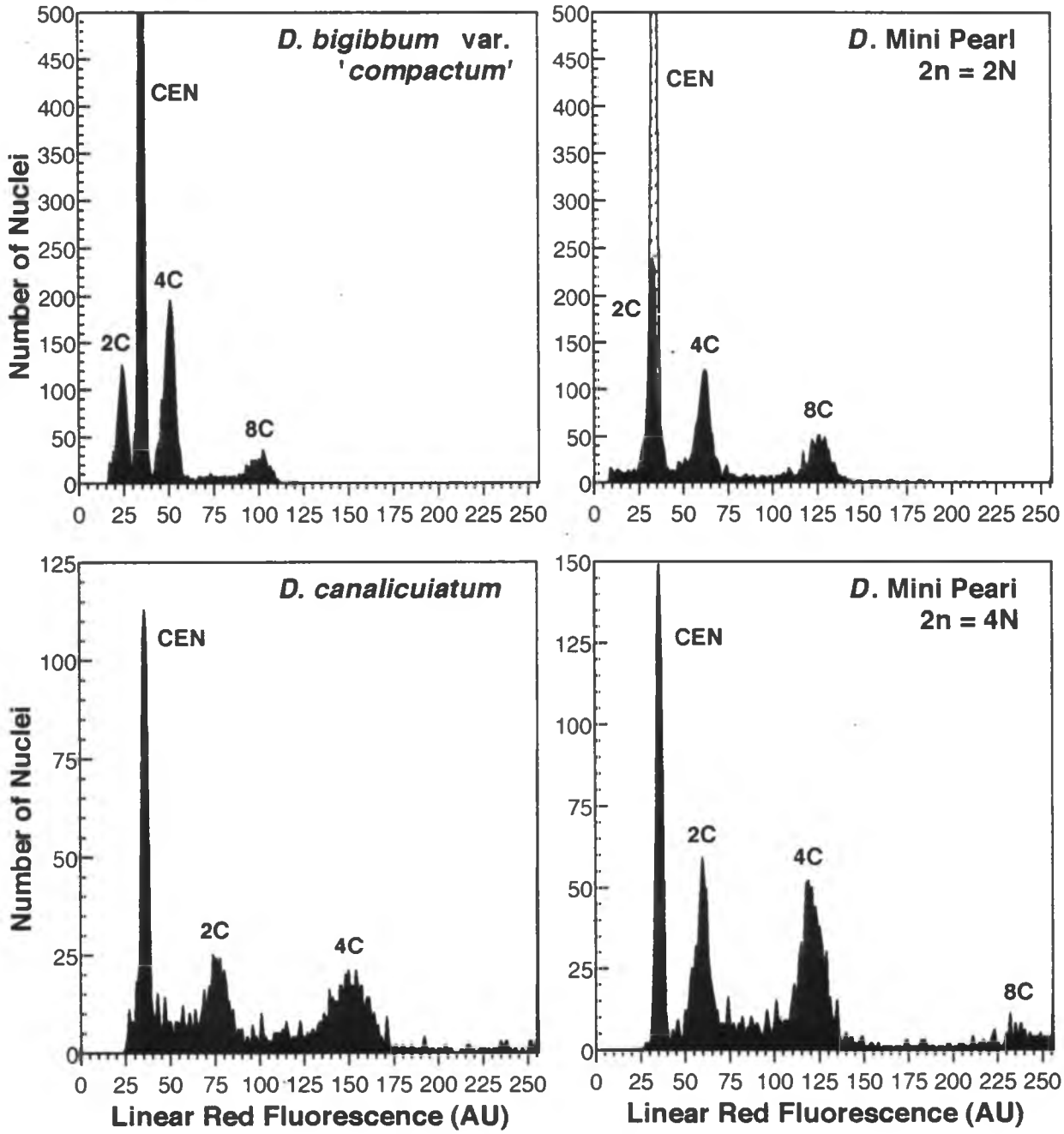

Figure 4.4. Frequency histograms of extracted nuclei from immature leaf tissues for the species *D. bigibbum* var. *compactum* and *D. canaliculatum* and the their hybrid offspring *D.* Mini Pearl in both 2N and 4N forms. CEN was included as a DNA reference standard . The red stippled peak indicates that CEN was run externally.

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Chapter 5: Perspectives on Orchid Flow Cytometric Analysis: Is it of value to taxonomists, breeders and propagators?

I. Introduction

The results of the experiments in the present thesis showed that laser flow cytometry (FCM) analysis can be successfully performed on many species of *Dendrobium* and other genera. This study has been valuable for providing important baseline data on genome size for 70 orchid species, for which sizes for only three had been previously reported. It was also shown that, in addition to DNA content, ploidy could be identified by this technique. The ability to determine ploidy quickly by FCM, without resorting to counting chromosomes, may be of considerable interest to orchidologists. Knowledge of ploidy is of value in predicting breeding success, and to propagators concerned with quality of mericlones. Unfortunately, the industry lacks the ability to make this determination with certainty, as few growers have the means or training to count chromosomes. Thus commercial feasibility of using FCM analysis to solve problems for the orchid industry requires assessment.

This chapter addresses the question of whether or not FCM is a feasible replacement of chromosome counting for ploidy determination. A discussion of additional potential applications of FCM to orchidology and genetic improvement is presented.

II. Materials and Methods

Sixteen hybrid orchid samples (Tables 5.1) from the University of Hawaii at Manoa collection and from R. Tokunaga (H & R Nurseries, Waimanalo, HI) were analyzed for DNA content and ploidy using the methods summarized in Chapter 3. Most samples submitted by R. Tokunaga were of an unknown ploidy level. These specimens were analyzed because they were candidates for a breeding program due to their desirable floral

characteristics and because clones of these specimens were being sold as a particular ploidy.

III. Results and Discussion

The FCM results for eight interspecific hybrids *Dendrobium*, one interspecific hybrid of *Angraecum,* and seven intergeneric hybrids involving genera of the Cattleya alliance are listed in Table 5.1. The DNA content values for all hybrids examined ranged from 2.86 pg to 16.57 pg, which is similar to the range of values found in Chapter 3. Two values are listed for *Dendrobium* Caesar. The first value is approximately half that of the second value, and belongs to a sample known to be diploid. The second *D*. Caesar sample was thought to be tetraploid, but may have been mislabeled. Because of the doubled DNA content value, the ploidy of the second sample is confirmed to be tetraploid. This validates the use of FCM to determine ploidy.

The values for the remaining *Dendrobium* hybrids in this study ranged from 2.86 pg to 4.25 pg. These values fall within the range of values found for *Dendrobium* species. All *Dendrobium* hybrids included in this study, with the exception of *D*. Caesar, were derived from three or more species. This makes predictions of the DNA content values more difficult. Chapter 4 showed that the value for a hybrid, or a corresponding parent, could be predicted mathematically. However, to predict the value for a polyploid complex hybrid, the percent of parental species genome contribution would need to be factored into the calculations. Additional studies are required to determine if this can be done accurately.

Unfortunately, chromosome counts were lacking for all samples acquired from R. Tokunaga. No correlation could be made between DNA content and ploidy for those samples. However, future comparisons can be made between the values listed in Table 5.1 and new samples of the same hybrids, provided that this study is followed up with chromosome counts. For example, if another specimen of *Brassiolaelia* Richard Mueller is

analyzed, the resulting DNA content can be compared to the value for the sample used in this study to determine ploidy.

Values for the non-*Dendrobium* hybrids listed in Table 5.1 can be compared indirectly to values found in Chapter 3. The value for *Brassolaelia* Richard Mueller is three times higher than the value for a species of *Brassia* (3.74 pg) and *Laelia* (2.45, 3.51 pg). The values for the *Laelia* hybrids were also three times higher than the *Laelia* species analyzed in Chapter 3. The values for *Cattleya* species previously analyzed, ranged from 3.29 to 9.26 pg. The values for the *Cattleya* hybrids in this study ranged from 6.99 to 12.56 pg. It is interesting that the values are multiples, indicating the possible presence of ploidy ranging from diploid to tetraploid. However, the only way to verify this would be to perform chromosome counts on the hybrids or the parental species involved.

This study serves to illustrate the limitations of DNA content analysis for commercial use. The samples submitted by R. Tokunaga were for ploidy analysis. However, the only information gained was genome size for each sample. In order to determine ploidy, a reference sample is required for comparison, as was the case with *D*. Caesar. Before screening commercial samples can be performed, a data base should be established for comparison purposes. An additional limitation of FCM is the lack of ability to detect aneuploids using current methods.

As long as the limitations are understood, FCM is a feasible replacement for chromosome counting. Chromosome counting is tedious, often requiring several hours to complete a single sample. On the other hand, 20 - 30 samples can be analyzed in a single day by FCM. Samples submitted for FCM analysis must be fresh and clean. Samples must then be stored dry in the refrigerator to inhibit degradation and prevent bacterial growth. This will help to insure quality results. At the present time, the only thing preventing commercial use of FCM is the lack of a service center for processing samples. There is strong need by the orchid industry in Hawaii, as well as for mainland states, to establish a

facility to perform ploidy analysis. The major drawback of this is the initial cost of the instrumentation. The equipment, software and technical support can cost tens of thousands of dollars. However, once the equipment is acquired, a technician can be trained in a relatively short period of time. Similar FCM diagnostic facilities have been in use for the medical profession since the 1970s.

IV. Future Applications of FCM to Orchidology

The experiments presented in this thesis utilized 37 *Dendrobium* species from eight taxonomic sections. This is only a small fraction of the 1000 known species for this genus. The information gained from the DNA content analysis is valuable because it reflects yet another variable characteristic of the genus. It was determined in Chapter 3 that there was considerable difference among species within the same section, and that some species could be differentiated by the amount of nuclear DNA. However, there is still much information to be gained by further FCM studies.

A more extensive sampling of the genus *Dendrobium* is required. More species representing all the sections need to be analyzed to determine the amount of variation within the taxonomic groupings. A geographic survey of species should also be performed to determine if intraspecific variation exists due to altitude or latitude differences as have been discovered in *Pinus* (Wakamiya et al. 1993) and certain crop plants such as maize (Biradar et al. 1994, McMurphy and Rayburn 1991, Rayburn et al. 1994). Along this same line of experimentation, a survey of varieties and larger populations should be performed to determine the extent of variation within a population.

Another study that would have important implications for breeders and systematists involves correlating DNA content among species of the different *Dendrobium* sections to existing data on breeding compatibility and relationships (reviewed by Kamemoto 1987). Previous breeding compatibility studies looked at the cross compatibility and meiotic

chromosome pairing behavior in intersectional hybrids. This produced information on the percentage of bivalent chromosome pair formation, tetrad production and seed viability among hybrids. This information has increased the knowledge about the degree of relationship among species. DNA content may help to explain the karyotype variation seen for these species as it has for maize (Rayburn et al. 1985). This could help to explain breeding compatibility problems that exist between species of some sections. Differences in DNA content may be correlated with sectional designation, or may help to determine the appropriate sectional assignment for species with ambiguous taxonomic characteristics.

Flow cytometry is not limited to ploidy and DNA content determination. Another application is flow karyotyping. Flow karyotyping is a method in which either individual chromosomes, or sets of chromosomes, are differentially stained for base pair content and amount of heterochromatin. In this method, a profile for a specific genome can be defined for the A-T or G-C% out of the total DNA. And by measuring heterochromatin, the amount of repetitive sequence DNA can be added to the profile. This method has been used in maize, *Vicia* and *Crepsis* (Rayburn et al. 1985, 1992; Hammatt et al. 1990; Biradar and Rayburn 1993; Godelle et al. 1993; Lucretti et al. 1993).A study involving flow karyotyping might shed some light on why there is so much variation within *Dendrobium*, A species flow karyotype could also be used as a taxonomic characteristic.

A recent method developed utilizing FCM is chromosome isolation and sorting. This method involves identification and separation of specific chromosomes based on size and staining characteristics. Currently this method is used in tomato, maize, wheat, *Brassica*, *Vicia* and *Arabidopsis thaliana* (Arumuganathan et al. 1991; Bashir et al. 1993; Dolezel et al. 1994; Fahleson et al. 1988; Lucretti et al. 1993; Van Devanter et al. 1994). However, this method may not be feasible yet for *Dendrobium* species because the chromosomes are extremely small, and currently a protocol for single cell cultures is lacking. The latter is

necessary for production of synchronous cultures of metaphase chromosomes (Arumuganathan et al. 1991).

Methods are being developed for protoplast cultures of *Dendrobium* and other orchid genera (Kuehnle and Nan 1990; Oshiro and Steinhart 1991; Steinhart and Renvyle 1993) which would make production of metaphase chromosomes possible. *Cattleya, Phalaenopsis* and *Vanda* would be good candidates for chromosome isolation studies because of the relatively large chromosomes found in many species in those genera (Kamemoto and Randolf 1949; Storey 1952; Tanaka and Kamemoto 1960; D'Emerico et al. 1993). Cell suspension cultures of *Phalaenopsis* are available and may be suitable for chromosome analysis. Once isolated, specific chromosomes can be used in molecular sytematics and bioengineering studies. For example, individual chromosomes can be cloned and sequenced (Arumuganathan et al. 1994), or inserted whole into other cells (Bashir et al. 1993).

The future use of flow cytometry in orchidology will involve additional ploidy and DNA content determination. However, the usage should not be limited to that application. The ease of use and versatility FCM will help to overcome the limitations, discussed above, making FCM an ideal tool for the study of orchids.

V. Tables

Table 5.1 DNA content values for orchid hybrids.

Orchid hybrid	DNA content pg/2C ± SE	Mbp/1C nuclei [a]
Angraecum Longiscott [b]	16.57 ± 0.68 pg	7995
Brassolaelia Richard Mueller [b]	11.61 ± 0.63 pg	5602
Dendrobium Jaquelyn Thomas 'Uniwai Blush' 4N [c]	3.69 ± 0.17 pg	1911
Dendrobium Pompadour 4N [c]	4.25 ± 0.20 pg	2051
Dendrobium Caesar 2N [c]	4.08 ± 0.17 pg	1969
Dendrobium Caesar 4N [c]	8.42 ± 0.39 pg	4063
Dendrobium Theodore Takaguchi 2N [c]	4.05 ± 0.19 pg	1954
Dendrobium Mae Teramoto 4N [c]	3.81 ± 0.18 pg	1835
Dendrobium D' Bush Pansy 2N [c]	3.33 ± 0.18 pg	1607
Dendrobium Dawn Maree [b]	2.86 ± 0.13 pg	1380
Laeliocattleya Jungle Elf [b]	9.98 ± 0.62 pg	4815
Laeliocattleya Love Knot [b]	12.56 ± 0.90 pg	6060
Miltonidium Pupukea Sunset [b]	7.63 ± 0.30 pg	3681
Sophrocattleya Beaufort 'Luna Rousse' [b]	6.99 ± 0.24 pg	3373
Sophrocattleya Beaufort 'Elizabeth' [b]	7.03 ± 0.36 pg	3392
Sophrolaelia Orpetii 'East Wind' [b]	10.86 ± 0.52 pg	5240

[a] Megabase pairs per haploid genome (Mbp/1C nuclei) calculated base on the equivalent of 1 pg DNA = 965 Mbp (Strauss 1971).

[b] Source of plant material: R. Tokunaga, H & R Nurseries, Waimanalo, HI.

[c] Source of plant material: University of Hawaii, Honolulu, HI.

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Appendix: Protocol for Isolation of Plant Nuclei

Adapted from: **Arumuganathan, K. and E. D. Earle.** 1991. Estimation of nuclear DNA of plants by flow cytometry. Plt Mol Rep 9:229-233.

Stock Solutions:

$MgSO_4$ buffer: Dissolve in distilled deionized water (ddH_2O):

0.246 g 10 mM $MgSO_4 \cdot 7\ H_2O$

0.370 g 50 mM KCl

0.120 g 5 mM N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid (HEPES; #BP310-100, Fisher Scientific Co., 2170 Martin Ave., Santa Clara, CA 95050) **(CAUTION: irritant)**

Adjust volume to 100 ml with ddH_2O

Adjust final pH to 8.0 store at 4°C

Triton X-100 stock (10% w/v):

1 g Triton X-100 in 10 ml ddH_2O

store at 4°C

Propidium iodide (PI) stock (5 mg/ml): (see note on special handling)

5.0 mg PI in 1 ml ddH_2O (# 537059, Calbiochem Corp., P.O.Box 12087, La Jolla, CA 92039-2087)

Cover vial with aluminum foil or use amber glass and protect from light

Store at 4°C **Hazardous material: wear gloves**

RNase (DNase-free):

Ready to use from Boehringer Mannheim Corp. (#1119-915; 9115 Hague Rd., P.O. Box 50414, Indianapolis, IN 46250-0414)

Store at -20°C (non-frost-free freezer)

Chicken Erythrocyte Nuclei (CEN):

BioSure® CEN Singlets from Riese Enterprises (# 1013; P.O. Box 9523, San Jose, CA 95157)

Working Solutions:

The following solutions should be prepared fresh from the stock solutions, protected from light with aluminum foil and kept on ice. **Wear gloves.**

Extraction Solution A: (30 ml should be enough for up to 12 samples)

14.3 ml $MgSO_4$ buffer (ice cold)

30 mg dithiothreitol (DTT; # V315A, Promega Corp., Madison, WI 53711-5399)

(CAUTION: irritant)

600 ul PI stock (final conc. 0.1 mg/ml) (see notes on special handling)

750 ul Triton X-100 stock

RNase Solution B: (8 ml should be enough for up to 12 samples)

8 ml Solution A

20 ul RNase (DNAse-free)

DNA controls: dilute 1 drop of BioSure CENs in 1 ml of Solution B.

Use 10-25 ul diluted CENs per plant sample depending on final volume and concentration of resuspended plant nuclei (10^5 - 10^6).

Preparation of Nuclei Suspensions by Chopping of Plant Tissues:

- Excise tissues from healthy plant
- Place tissues (100 mg) in plastic petri dishes (35 mm x 10 mm) on ice
- Add 1 ml Solution A and slice tissue (< 0.5 mm) with sharp scalpel
- Filter the homogenate through 33 um nylon mesh into microcentrifuge tube (Spectra/Mesh #146506; Spectrum, 1100 Rankin Rd. Houston, TX 77073-4716)
- Centrifuge at high speed (15,000 rpm) for 15-20 seconds
- Discard supernatant into PI waste vessel
- Resuspend pellet in 400 ul Solution B
- Incubate for 15 min at 37°C then return to ice
- Run the sample on the flow cytometer adding CENs as needed

Notes on Special Handling and Helpful Hints:

- PI is a dye which intercalates with DNA and RNA. It is classified as a possible carcinogen. Therefore, careful handling is required. Wear gloves when handling dye, solutions with dye, and samples stained with dye. All dye contaminated materials should be treated as hazardous waste and disposed of according to local regulations. PI is light sensitive so must be protected from direct light while in both concentrated and dilute forms.
- Filters can be constructed by carefully heat-welding nylon mesh onto the cut end of a 1 cc syringe. Avoid using plunger to force liquid through mesh. Excess friction or agitation may cause nuclei to aggregate.
- Be careful when pipetting nuclei out of the petri dish. Avoid drawing up air bubbles with the sample. This can cause rupturing of nuclei or make them aggregate. It is also advisable to cut the end of the pipette tip to widen the orifice. Repeated pipetting can also lead to rupturing or aggregation. Widening the tip will reduce this hazard. Aggregation of the nuclei is mostly due to static electrical attraction.