



Differential Accumulation of Heterochromatin as a Cause for Karyotype Variation in *Phalaenopsis* Orchids

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Karyotypes of nine *Phalaenopsis* species and the closely related *Doritis pulcherrima* were compared based on Feulgen- and DAPI-stained somatic metaphase chromosomes prepared from root tips. All species had the same chromosome number ($2n = 2x = 38$), but their karyotypes differed markedly in absolute chromosome size, relative chromosome size, and the position of the centromere. Both genome size and the amount of constitutive heterochromatin (CH) varied widely among the species studied, and there was a positive correlation between these two parameters. The distribution of CH in the genomes was non-random: one or both arms of long chromosomes usually possessed large blocks of CH, while the small chromosomes contained little or no CH. DAPI-staining revealed that most CH regions are rich in AT base pairs. We suggest that differential accumulation of CH is a major cause for karyotype variation in *Phalaenopsis* orchids. © 2001 Annals of Botany Company

Key words: *Phalaenopsis*, *Doritis pulcherrima*, karyotype differentiation, constitutive heterochromatin, total chromosome volume, nuclear DNA content.

INTRODUCTION

The genus *Phalaenopsis* (Orchidaceae) comprises approx. 45 species that are grouped into nine sections based on morphological characteristics (Sweet, 1980). The genus is of considerable interest due to its broad geographical distribution (ranging from the Himalayas, through Southeast Asia, to northern Australia) and high value as a floricultural commodity. Although interspecific and intergeneric hybridization has long been used for breeding superior cultivars, very little is known about the cytogenetics of *Phalaenopsis*. The only information currently available is the chromosome number (Sagawa and Shoji, 1968) and karyotype (Shindo and Kamemoto, 1963) of some representative species and meiotic chromosome associations in several interspecific hybrids (Arends, 1970), investigated by traditional aceto-carmine and orcein squash techniques.

An interesting result of previous studies was that although all *Phalaenopsis* species had the same chromosome number ($2n = 2x = 38$), the size of their genomes varied considerably. Shindo and Kamemoto (1963) compared the mean chromosome length of eight *Phalaenopsis* species and the closely related species *Doritis pulcherrima* and found a 2.8-fold difference between species with the largest (*P. violacea*) and smallest (*P. amabilis*) chromosomes. Lin *et al.* (2001) estimated the nuclear DNA content

of 18 *Phalaenopsis* species and *D. pulcherrima* by flow cytometry and found that 2C values ranged from 2.74 pg in *P. sanderiana* to 16.61 pg in *P. parishii*. Shindo and Kamemoto (1963) pointed out that only species with large chromosomes showed chromocentres at interphase, indicating a causal relationship between heterochromatin content and genome size.

Since the publication of the classical work of Shindo and Kamemoto (1963), Sagawa and Shoji (1968) and Arends (1970), many staining techniques have been developed that allow a finer analysis of chromosome morphology (Sumner, 1990). Using some of these techniques, we analysed karyotypes of nine representative *Phalaenopsis* species and *D. pulcherrima*, paying special attention to the amount and distribution of constitutive heterochromatin (CH). Based on these results, we propose a hypothesis for chromosome differentiation in *Phalaenopsis*.

MATERIALS AND METHODS

Plant material

Nine *Phalaenopsis* species and *Doritis pulcherrima*, representing species with large, medium and small chromosomes, were used in this study (Table 1). All plants were cultivated in a greenhouse at the Taiwan Sugar Research Institute (TSRI). Plants were either collected from their natural habitat, kindly provided by Royal Botanic Gardens, Kew, UK, or obtained from commercial breeders.

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TABLE 1. The somatic metaphase chromosomes of Phalaenopsis species and Doritis pulcherrima

Taxon*	TSRI† Accession number	2n	Chromosome size (µm)			TCL (µm)	TCV (µm ³)	Nuclear DNA content‡ (pg 2C ⁻¹)	CH (% nuclear area)
			<2.0	2.0-2.5	>2.5				
<i>Phalaenopsis</i>									
Sect. <i>Phalaenopsis</i>									
<i>P. aphrodite</i> Rehb.f.	W1-38	38	38	0	0	—	—	2.80 ± 0.06	1.87 ± 0.24
<i>P. stuartiana</i> Rehb.f.	W40-4	38	38	0	0	50.18 ± 8.61	23.70 ± 4.51	3.14 ± 0.07	1.77 ± 0.42
Sect. <i>Stauroglottis</i>									
<i>P. equestris</i> (schauer) Rehb.f.	W9-51	38	38	0	0	53.60 ± 1.22	22.29 ± 4.04	3.37 ± 0.05	2.74 ± 0.33
Sect. <i>Polychilos</i>									
<i>P. cornu-cervi</i> Rehb.f.	W8-1	38	34	4	0	55.52 ± 1.84	35.91 ± 5.75	6.44 ± 0.16	2.44 ± 0.47
<i>P. mamii</i> Rehb.f.	W25-6	38	0	6	32	106.02 ± 6.67	86.06 ± 19.06	13.50 ± 0.12	11.32 ± 1.08
Sect. <i>Amboinensis</i>									
<i>P. amboinensis</i> J. J. Smith	W2-7	38	8	4	26	—	—	14.36 ± 0.19	14.17 ± 1.08
<i>P. venosa</i> Shim & Fowl	W42-1	38	12	4	22	94.29 ± 12.75	79.76 ± 24.98	9.52 ± 0.27	13.25 ± 0.88
Sect. <i>Zebrinae</i>									
<i>P. lueddemanniana</i> Rehb.f.	W23-2	38	28	10	0	63.08 ± 2.92	44.58 ± 3.48	6.49 ± 0.22	5.51 ± 0.46
<i>P. violacea</i> Witte (Bornean form)	W43-26	38	6	6	26	112.01 ± 3.61	87.84 ± 8.32	15.03 ± 0.21	27.17 ± 2.03
<i>Doritis</i>									
<i>D. pulcherrima</i> Lindley	W46-21	38	0	6	32	122.35 ± 10.84	81.13 ± 25.80	13.49 ± 0.21	23.82 ± 2.34

* Classification of Sweet (1980)

† Taiwan Sugar Research Institute

‡ Lin et al. (2001)

TCL, Total complement length; TCV, total chromosome volume.

CH, Constitutive heterochromatin.

Feulgen staining

Somatic chromosomes were prepared from actively growing root tips. Excised root tips were split in half longitudinally and root caps and velamens (layers of dead cells with thick walls) were removed with a sharp scalpel. Meristematic tissues were pretreated with 2 mM 8-hydroxyquinoline on a rotary shaker (100 cycles min⁻¹) for 4 h at 20 °C, fixed in ethanol-glacial acetic acid (3:1) overnight, hydrolysed in 1 N HCl for 7 min at 60 °C, stained in basic fuchsin for 1 h, and treated with 1% pectinase for 1 h. The meristematic tissues were squashed in 45% acetic acid or 1% aceto-orcein.

Fluorescent staining

The meristematic tissues of root tips were collected, pretreated and fixed as described for Feulgen staining. They were then digested with 2% (w/v) cellulase Onozuka R10 (Yakult Honsha, Tokyo, Japan) and 1% (w/v) macerozyme Onozuka R10 (Yakult Honsha, Japan) in citrate buffer (4 mM citric acid and 6 mM sodium citrate, pH 4.8) for approx. 2 h at 25 °C, squashed in 45% acetic acid and air-dried. Chromosome preparations were first stained with 0.5 mg ml⁻¹ chromomycin A₃ (CMA) in half-strength McIlvaine's buffer (0.2 M Na₂HPO₄ adjusted with 0.1 M citric acid to pH 7) with 5 mM MgCl₂ and then with 0.2 µg ml⁻¹ 4'-6-diamidino-2-phenyl-indole (DAPI) in McIlvaine's buffer at room temperature for 8–10 min (Doudrick *et al.*, 1995). Chromosome preparations were mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, UK) and visualized under an Olympus AX70 fluorescence microscope with appropriate filters. The images were photographed on Fujicolor Supera 200 ASA print film.

Estimation of total chromosome volume

The length and width of Feulgen-stained metaphase chromosomes and chromatids were measured at a magnification of 5000× using a vernier caliper. The total chromosome volume (TCV) was estimated using the formula of Naranjo *et al.* (1998):

$$\text{TCV} = 2(\pi \times r^2 \times \text{TCL})$$

where r is the average radius of the chromatid and TCL the total complement length. *P. aphrodite* and *P. amboinensis* were not included in the measurement because cells with well-condensed metaphase chromosomes were difficult to obtain from these species. For the other species, four to seven metaphase plates were measured.

Determination of the proportion of CH in interphase nuclei

The amount of heterochromatin, expressed as the proportion of the total area of the interphase nucleus, was estimated as follows. Photographs of Feulgen stained interphase nuclei were scanned. The distribution of areas scanned at each optical density was plotted by computer as

a histogram of the cumulative graph using the Imaris software (Bitplane) on the SGI Octane machine (Silicon Graphics). Optical density representing the area composed of heterochromatin in each interphase nucleus was measured five times. The area was then converted to a percentage of the total nuclear area. Each mean was calculated from more than nine samples.

RESULTS

Karyotypes

Karyotypes of nine *Phalaenopsis* species and *D. pulcherrima* were compared based on Feulgen-stained somatic metaphase chromosomes prepared from root tips. All species were diploids with the same number of chromosomes ($2n = 38$), but the size and centromere position of chromosomes varied considerably. Five species, *P. aphrodite*, *P. stuartiana*, *P. equestris*, *P. cornu-cervi* and *P. lueddemanniana*, had similar and most symmetrical karyotypes. In general, their chromosomes were small and uniform in size (1–2.5 µm) and were all metacentric or submetacentric (Fig. 1A–C; Table 1). TCL ranged from 50.2 µm in *P. stuartiana* to 63.1 µm in *P. lueddemanniana*, and TCV varied from 22.3 µm³ in *P. equestris* to 44.6 µm³ in *P. lueddemanniana* (Table 1).

The two species in section *Amboinensis*, *P. venosa* and *P. amboinensis*, had strongly asymmetrical and bimodal karyotypes. Their chromosomes could be grouped into two distinct size classes, large and small, and most were subtelocentric or acrocentric (Fig. 1D). These two species differed in the number of chromosomes in each size class: *P. venosa* had 12 small chromosomes whereas *P. amboinensis* had eight. In both species, the two homologues of one small pair were often heteromorphic in size (Fig. 1D).

The karyotype of *P. violacea* was also bimodal, with six chromosomes being markedly smaller than the others (Fig. 1E). The 16 largest chromosomes were metacentric or submetacentric, while most of the remaining chromosomes were subtelocentric or acrocentric. The largest pair of chromosomes was approx. five-times longer than the smallest pair. *P. violacea* had the largest TCV (87.8 µm³) of the species studied (Table 1).

Among the species with large chromosomes, *P. mannii* and *D. pulcherrima* had comparatively more symmetrical karyotypes. Most of their chromosomes were metacentric or submetacentric, and the ratio of the longest to the shortest pair of chromosomes was less than two (Fig. 1F, G). The chromosomes of *D. pulcherrima* were more slender compared with the large chromosomes of other species (cf. Fig. 1D–G).

The amount and distribution of CH

CH and euchromatin could be distinguished in the cell cycle from interphase (Fig. 2) to early metaphase by the intensity of staining as a result of differential rates of contraction. This offers an opportunity to test whether CH may have a role in karyotype variation in *Phalaenopsis* orchids.

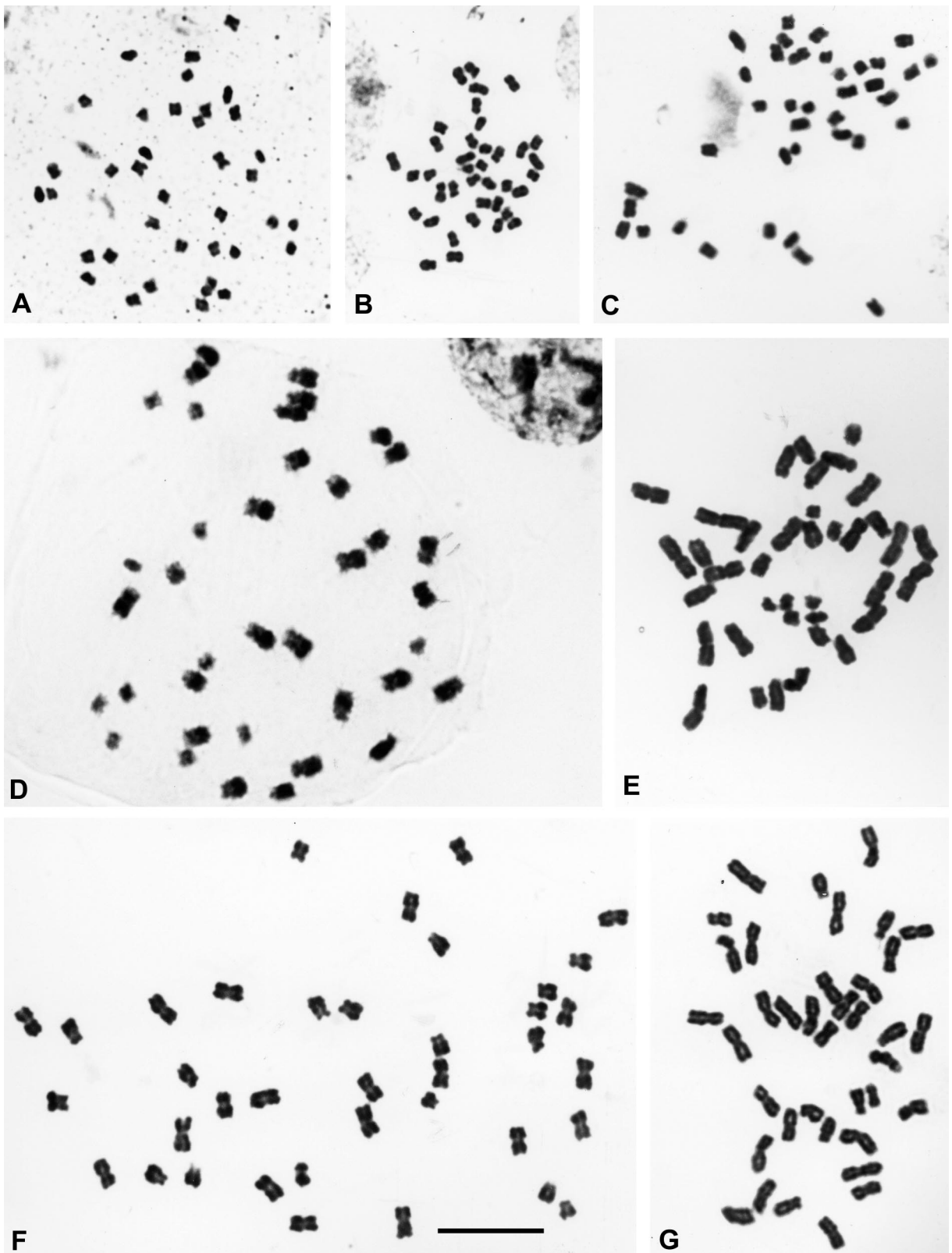


FIG. 1. Feulgen-stained somatic metaphase chromosomes. A, *Phalaenopsis stuartiana*; B, *P. equestris*; C, *P. cornu-cervi*; D, *P. venosa*; E, *P. violacea*; F, *P. mannii*; G, *Doritis pulcherrima*. Bar = 10 μ m.

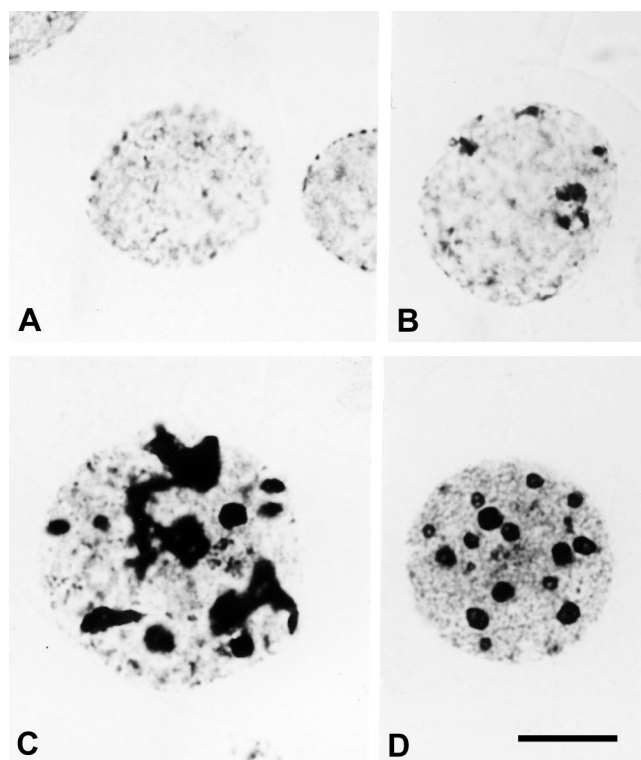


FIG. 2. Feulgen-stained interphase nuclei. A, *Phalaenopsis aphrodite*; B, *P. lueddemanniana*; C, *P. violacea*; D, *Doritis pulcherrima*. Bar = 10 μm .

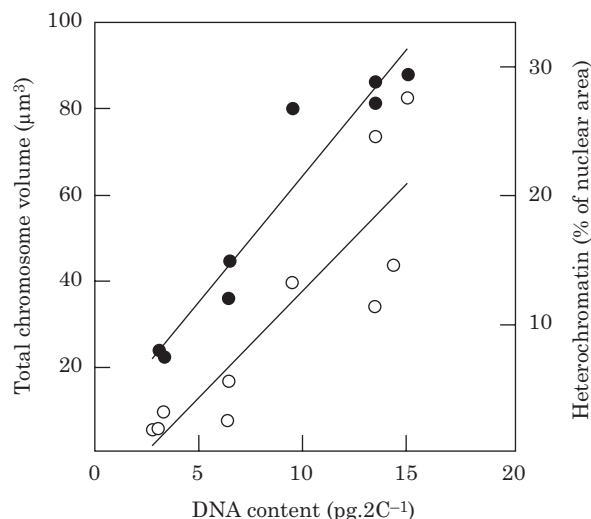


FIG. 3. Total chromosome volume (●) and the proportion of constitutive heterochromatin (○) at interphase plotted against nuclear DNA content.

The amount of CH, expressed as a percentage of the total area of the interphase nucleus, showed a range of about 15-fold from 1.77% in *P. stuartiana* to 27.17% in *P. violacea* (Table 1). The relationships between nuclear DNA content (Lin et al., 2001) and TCV, and between nuclear DNA content and the amount of CH are shown in Fig. 3. There is a high correlation between nuclear DNA content and TCV

($r = 0.96$, $P < 0.001$) and a slightly lower but significant correlation between nuclear DNA content and the amount of CH in interphase nuclei ($r = 0.88$, $P < 0.001$).

Examination of the distribution of CH on late prophase and early metaphase chromosomes (Fig. 4) indicates that large chromosomes generally contained more CH than small ones. This is true for chromosomes both within and between species. In *P. aphrodite*, *P. stuartiana*, *P. equestris*, *P. cornu-cervi* and *P. lueddemanniana*—species with relatively small chromosomes, CH mainly clusters around centromeres (Fig. 4A, B). In species with bimodal karyotypes, such as *P. venosa*, *P. amboinensis* and *P. violacea*, both ends of large metacentric/submetacentric chromosomes and the ends of the long arms of subtelocentric/acrocentric chromosomes possess large blocks of CH, whereas small chromosomes have little or no CH (Fig. 4C, D). The heterochromatin blocks in *P. mannii* and *D. pulcherrima* are smaller in size and more abundant than those in species with bimodal karyotypes. In addition, distribution of CH in these two species is not restricted to chromosome ends; the chromosome complement of *D. pulcherrima* is particularly rich in intercalary CH bands (Fig. 4E, F). Figure 5 summarizes five representative patterns of the distribution of CH.

To understand the base composition of CH in *Phalaenopsis* orchids, we stained root-tip cells with the fluorochromes CMA and DAPI and observed banding patterns on prometaphase and metaphase chromosomes. Our results show that DAPI-positive bands (Fig. 6) coincide with CH regions revealed by Feulgen staining (Fig. 4), whereas CMA-positive bands correspond to the putative nucleolus organizing regions (data not shown). Since DAPI binds preferentially to AT-rich and CMA to GC-rich regions (Sumner, 1990), these results suggest that the DNA of CH in *Phalaenopsis* orchids is rich in AT base pairs.

DISCUSSION

An interesting feature of karyotype variation is the wide difference in absolute chromosome size which, when expressed as TCV, varies approx. four-fold among the species studied (Table 1). Variation in genome size between related species has been attributed to differences in the amount of repetitive DNA sequences in their genomes (Flavell, 1982; Kubis et al., 1998). Tandemly repetitive sequences such as satellite DNAs are usually clustered in certain chromosome regions and can be detected cytologically as CH (Yunis and Yasmin, 1997) by C-banding and other techniques (Sumner, 1990). The fact that there is a very high correlation between nuclear DNA content and TCV ($r = 0.96$, $P < 0.001$) and a slightly lower, but significant, correlation between nuclear DNA content and CH ($r = 0.88$, $P < 0.001$) (Fig. 3) suggests that the amount of CH may be a major, but not the only, cause of DNA content variation among the species studied. Many plant species contain large amounts of retroelements, which are interspersed with other sequences, repetitive and non-repetitive, in the genome (Bennetzen, 1996), and are difficult to demonstrate by traditional cytological techniques.

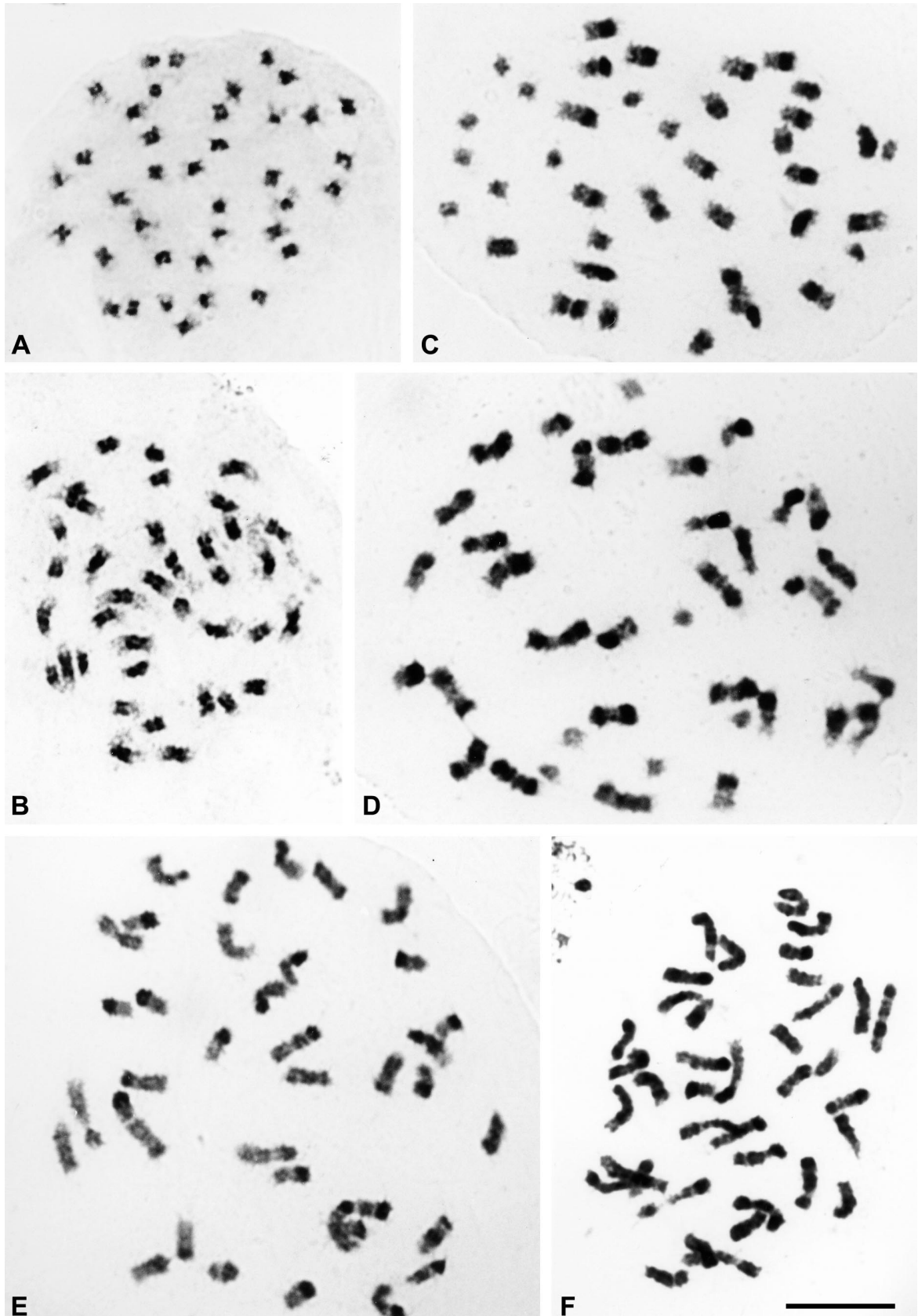


FIG. 4. Feulgen-stained late prophase/early metaphase chromosomes. A, *Phalaenopsis aphrodite*; B, *P. cornu-cervi*; C, *P. venosa*; D, *P. violacea*; E, *P. mannii*; F, *Doritis pulcherrima*. Bar = 10 μ m.

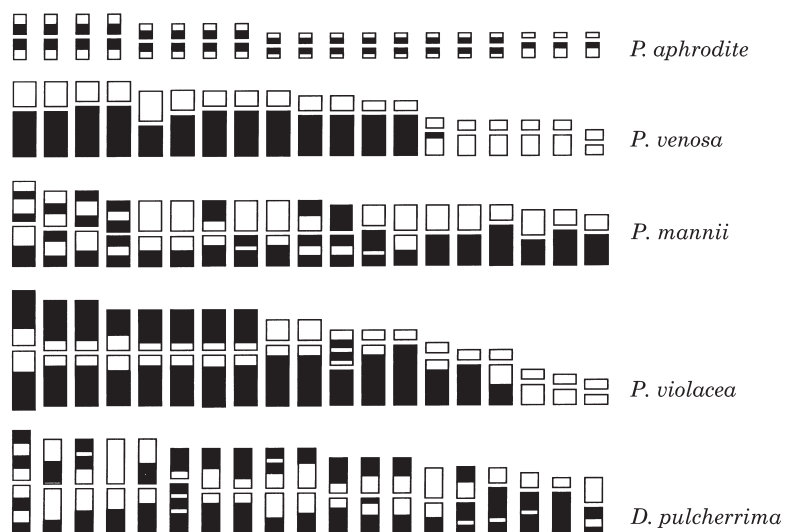


FIG. 5. Idiograms of somatic metaphase chromosomes of four *Phalaenopsis* species and *Doritis pulcherrima* showing the distribution of constitutive heterochromatin. ■, Heterochromatin; □, euchromatin.

In addition to absolute chromosome size, some species also differ in relative chromosome size distribution in the karyotype and the position of the centromere. These differences are usually brought about by unequal translocation, pericentric inversion, and centric fusion and fission (Stebbins, 1971); however, such events have not been observed in *Phalaenopsis* orchids. In the present study, analysis of the distribution of CH on late prophase/early metaphase chromosomes reveals that the lengths of chromosomes are directly proportional to the amount of CH they contain (Figs 4 and 5). For example, in species with uniformly small metacentric chromosomes, such as *P. aphrodite*, *P. stuartiana* and *P. equestris*, only small amounts of CH concentrate around centromeres. In species with bimodal karyotypes, both arms of large metacentric chromosomes and the long arms of large subtelocentric chromosomes possess large terminal CH blocks, whereas chromosomes classified as small contain little or no CH. These results support the view that CH is an additive component in genome evolution (Greilhuber, 1979; Poggio and Hunziker, 1986; D'Emérico *et al.*, 1996). Introduction and amplification of tandem repeats may be chromosome and chromosome-region specific, resulting in uneven distributions of CH in the genome and causing changes in relative chromosome size and the position of the centromere.

It is well established that repetitive DNA can have various effects on cellular and organismic characters such as nuclear volume (Baetcke *et al.*, 1967), cell size (Price *et al.*, 1973), length of mitotic cycle (Van't Hof and Sparrow, 1963), generation time (Bennett, 1972), and rate of development (Bennett, 1977). In most cases studied, variation in DNA content within and between species has been shown to be correlated with environmental factors such as temperature, light, water and soils, etc. (Furuta and Nishikawa, 1991). It appears that repetitive DNA may have an adaptive value and play a functional role in karyotype evolution. However, due to the wide geographical distribution of

Phalaenopsis orchids and the complex patterns of CH in their genomes, it would be difficult to know at this stage of investigation what factors might be responsible for the differential accumulation of CH and might therefore contribute to karyotype evolution.

Meiotic chromosome pairing and chiasma formation in interspecific hybrids are determined by the degree of chromosome homology of parental species. However, it has been shown that the presence of extra DNA in the chromosomes of one species may affect structural homology, reduce their ability to pair and recombine with the smaller homoeologous chromosomes of another species. For example, the cultivated cotton, *Gossypium hirsutum* ($2n = 4x = 52$, genome constitution AADD), is a natural amphidiploid derived from the cross between two diploid species, *G. arboreum* (AA) and *G. raimondii* (DD) (Beasley, 1942); the former has approximately twice as much DNA as the latter (Edwards *et al.*, 1974). In the F_1 hybrid, pairing between chromosomes of the A and D genomes appeared normal at pachytene. However, as meiosis proceeded, the large chromosomes of the A genome (with more heterochromatin) contracted more rapidly and, as a result, the frequency of chiasma reduced, leaving only an average of 5.9 bivalents per pollen mother cell (PMC) at metaphase I (Brown, 1954; Endrizzi and Phillips, 1960).

Another example is provided by the genus *Fescula*, in which *F. drymeja* ($2n = 2x = 14$) has approx. 50% more DNA than *F. scariosa* (also $2n = 2x = 14$) (Rees *et al.*, 1982). In their F_1 hybrid, the chiasma frequency at metaphase I was low, about 3.3 per PMC. But unlike the situation in *Gossypium*, irregularities in chromosome pairing were detected by both light and electron microscopy at pachytene. From comparison of the chromatin volume in regions of bivalents with and without synaptonemal complexes in a sectioned nucleus, the authors concluded that a massive disparity in length and amount of DNA between homoeologous chromosomes does prevent effective pachytene pairing.

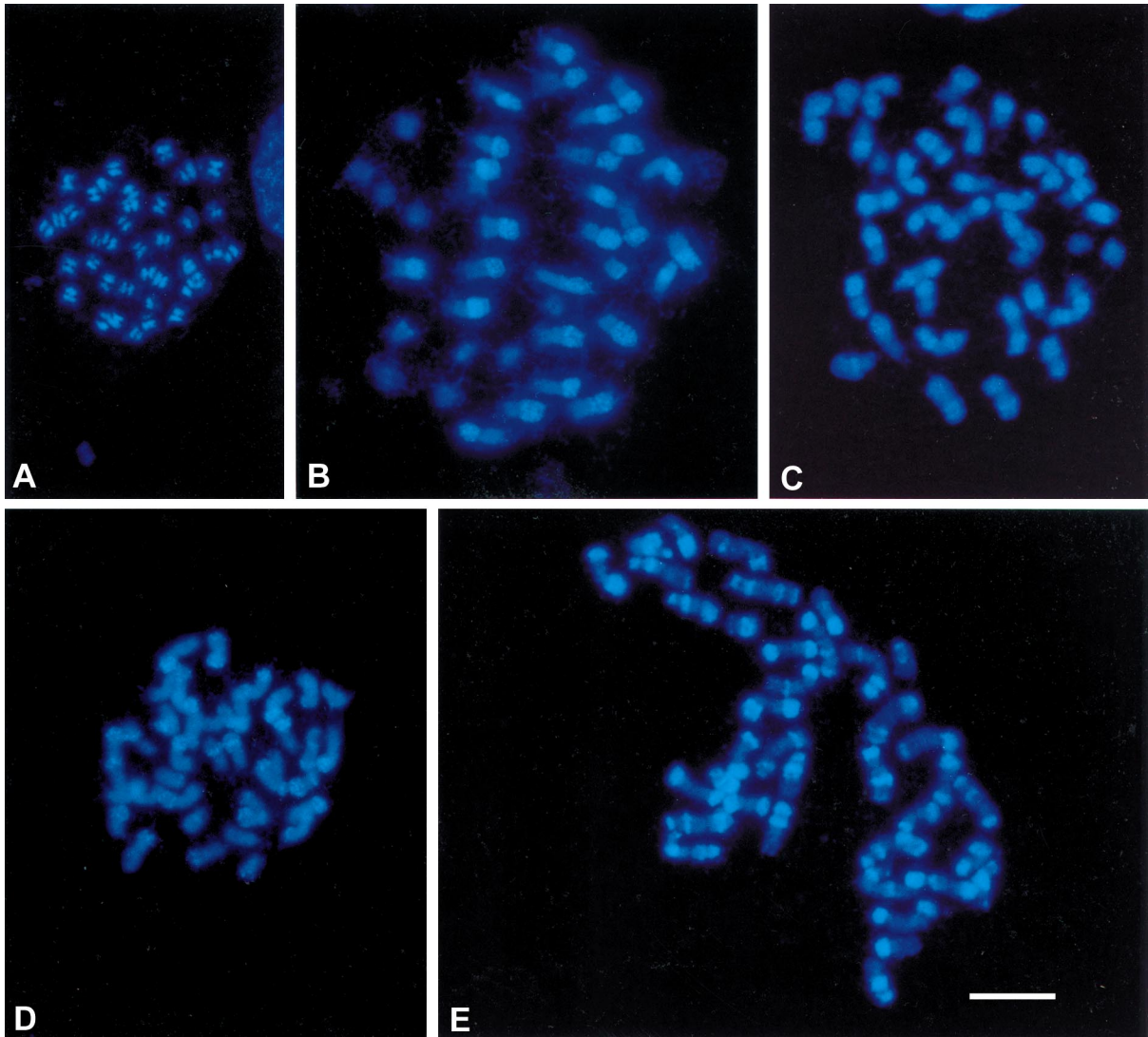


FIG. 6. DAPI-stained somatic metaphase chromosomes. A, *Phalaenopsis aphrodite*; B, *P. venosa*; C, *P. violacea*; D, *P. mannii*; E, *Doritis pulcherrima*. Bar = 10 μ m.

In this study, we have shown that genome size and the amount and distribution of CH varies widely among *Phalaenopsis* orchids. Arends (1970) studied chromosome associations at metaphase I of eight interspecific hybrids of *Phalaenopsis* and observed high frequencies of bivalents in hybrids between species both with large chromosomes (e.g. *P. amboinensis* x *P. mannii*) and between species both with small chromosomes (e.g. *P. amabilis* x *P. stuartiana*), but observed low frequencies of bivalents in hybrids between one species with large and one with small chromosomes (e.g. *P. mannii* x *P. equestris*). These observations generally agree with results obtained in *Gossypium*, *Fescula* and other species, namely that repetitive DNA may affect chromosome pairing and chiasma formation, and supports the contention of Flavell (1982) that variation in genome size caused by sequence amplification is a major cause of species divergence.

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