Meiotic Chromosome Behavior and Capsule Setting in *Doritaenopsis* Hybrids

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ABSTRACT. The development of new cultivars in *Doritaenopsis* Guillaum. & Lami orchids is often hindered by factors such as low seed count in hybrids. Cytological study may offer the ability to develop new hybrids by revealing cultivars with good chromosome pairing and high pollen viability, which are somewhat difficult to obtain under current breeding programs. Cross pollination, pollen viability, and chromosomal behavior during meiosis were analyzed to reveal the relation between seed fertility and capsule set in *Doritaenopsis* hybrids. The number of mature capsules harvested and their relative seed content were used as indices of crossing availability. The results of meiosis were evaluated according to pollen viability detected by fluorescein diacetate and quantification of sporad types by acid fuchsin staining. Chromosome number and pairing at meiosis were observed in root tips or in samples of pollen mother cells. A positive relation was found among high seed set, high frequency of viable tetrads, high degree of chromosome pairing, and low frequency of chromosomal aberrations such as inversions and translocations. On the basis of these factors, three types of hybrids could be distinguished. In type one hybrids, chromosomes paired as bivalents, pollen mother cells divided into tetrads, and capsule setting occurred after pollination of pollen acceptors. In type two hybrids, chromosomes remained mainly as univalents that developed into micromeiocytes, pollen mother cell division was disrupted, and seed recovery was low after pollination. Type three hybrids showed chromosomes paired mostly as multivalents, chromosome bridges, pollen mother cell division with massive failure, and little fertility. In *Doritaenopsis* orchids, high pollen viability and high fertility depends on a high frequency of normal tetrads, and low seed set in cross-pollination is predicted with micronuclei in the end products of meiosis. The occurrence of chromosomal aberrations may suggest a process of genome differentiation that could compromise breeding efforts if not taken into consideration.

In general, relatively little is known about the genetics of orchids because of the high cost of growing large populations of the plant, the relatively long growing cycles (≈ 6 years) to obtain two generations, and polyploidy in advanced-generation hybrids (Kamemoto et al., 1999). Differences in ploidy are a challenge to the development of new hybrids, and differences in chromosome number may prevent zygote development after pollination (Singh and Hymowitz, 1987). Chromosome doubling is often applied to produce new cultivars of orchids (Lin et al., 2001), as has been the case with colchicine-doubled amphidiploid cultivars in *Dendrobium* Swartz (Kamemoto et al., 1999).

In *Phalaenopsis* Blume, interspecific and intergeneric hybridization has long been used for breeding superior hybrids, but as for other orchid genera, very little is known about the genetics of the species (Kao et al., 2001). The information

currently available is chromosome number ($2n = 2x = 38$), the karyotype of some representative species, the meiotic chromosome associations in several interspecific hybrids, and the genomic DNA content of several *Phalaenopsis* and *Doritis* Lindl. species (Arends, 1970; Lin et al., 2001).

Phalaenopsis species may have the same chromosome number, but genome sizes vary considerably (Kao et al., 2001). For instance, Shindo and Kamemoto (1963) compared the mean chromosome length of eight *Phalaenopsis* species and the closely related species *Doritis pulcherrima* Lindl. and they found a 2.8-fold difference in chromosome length between species with the largest (*P. violacea* Witte) and the smallest chromosomes (*P. amabilis* Blume). Arends (1970) reported two groups in *Phalaenopsis* with high genome homology distinguished on the basis of chromosomal length at somatic metaphase, as well as chromosomal configurations in metaphase I of hybrids. The first group includes species with small chromosomes, such as *P. amabilis*, *P. aphrodite* Rchb. f., *P. equestris* (Schauer) Rchb. f., *P. sanderiana* Rchb. f., and *P. stuartiana* Rchb. f. The second group contains species with large chromosomes, such as *P. amboinensis* J. J. Sm. and *P. mannii* Rchb. f. Lin et al. (2001), using flow cytometry and southern hybridization techniques, concluded that chromosome size is

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Table 1. Genome constitution and cytological characteristics of nine *Doritaenopsis* hybrids.

Hybrid ^z	Parents (pollen receptor × pollen donor) ^x	Length of buds at metaphase I (mm)	Length of buds at pachytene (mm)
<i>Doritaenopsis</i> Bin You Danseuse 'F574' ^y	<i>Doritaenopsis</i> Coral Gleam × <i>Phalaenopsis</i> Bright Danseuse	14	12
<i>Doritaenopsis</i> Chian-Huey Red Rose 'F904'	<i>Doritaenopsis</i> Taisuco Firebird × <i>Phalaenopsis</i> New Cinderella	9	7
<i>Doritaenopsis</i> Fuchsia Princess 'KHM648'	<i>Doritaenopsis</i> Coral Gleam × <i>Doritis pulcherrima</i>	8	7
<i>Doritaenopsis</i> I-Hsin Purple Jewel	<i>Doritaenopsis</i> Purple Gem × <i>Doritaenopsis</i> Taisuco Jewel	6	4
<i>Doritaenopsis</i> Jiuhbao Red Rose	<i>Doritaenopsis</i> Taisuco Firebird × <i>Doritaenopsis</i> King Shiang's Rose	8	7
<i>Doritaenopsis</i> Purple Gem	<i>Doritis pulcherrima</i> × <i>Phalaenopsis equestris</i>	7	3.5
<i>Doritaenopsis</i> Sogo Gem 'F752'	<i>Doritaenopsis</i> Purple Gem × <i>Doritaenopsis</i> Minho Diamond	16	11
<i>Doritaenopsis</i> Sogo Gem 'F859'	<i>Doritaenopsis</i> Purple Gem × <i>Doritaenopsis</i> Minho Diamond	9	ND ^w
<i>Doritaenopsis</i> Sogo Gem 'F1082'	<i>Doritaenopsis</i> Purple Gem × <i>Doritaenopsis</i> Minho Diamond	14	ND

^zHybrids are shown in alphabetical order.^yBracketed characters after grex indicate clonal name.^xRoyal Horticultural Society, 2007.^wNot determined.Table 2. Seed set in several cross pollinations in *Doritaenopsis* and *Phalaenopsis*.

Pollen receptor ^z	Pollen donor	Pollinations made (no.)	Capsules harvested (no.)	Seed set	Amount of seed (– to +++ scale) ^x
<i>Doritaenopsis</i> Chian-Huey Red Rose 'F904' (4×) ^y	<i>Doritaenopsis</i> Jiuhbao Red Rose (4×)	8	4	Viable seeds	+++ , ++
<i>Doritaenopsis</i> Chian-Huey Red Rose 'F904' (4×)	<i>Phalaenopsis equestris</i> (2×)	4	4	Aborted	–
<i>Doritaenopsis</i> I-Hsin Purple Jewel (3×)	<i>P. equestris</i> (2×)	4	4	Aborted	–
<i>Doritaenopsis</i> I-Hsin Purple Jewel (3×)	<i>Doritaenopsis</i> Jiuhbao Red Rose (4×)	4	2	Aborted	
<i>Doritaenopsis</i> Jiuhbao Red Rose (4×)	<i>Doritaenopsis</i> Sogo Gem 'F859' (2×)	5	5	Aborted	–
<i>Doritaenopsis</i> Purple Gem (2×)	<i>Doritaenopsis</i> Jiuhbao Red Rose (4×)	6	3	Aborted	
<i>Doritaenopsis</i> Sogo Gem 'F859' (2×)	<i>Doritaenopsis</i> Jiuhbao Red Rose (4×)	11	11	Aborted	–
<i>Doritaenopsis</i> Sogo Gem 'F859' (2×)	<i>Doritaenopsis</i> Chian-Huey Red Rose 'F904' (4×)	14	14	Aborted	–
<i>P. equestris</i> (2×)	<i>Doritaenopsis</i> Jiuhbao Red Rose (4×)	12	6	Viable seeds	++

^zPollen receptors are shown in alphabetical order.^yPloidy level, 2× = diploid; 3× = triploid; 4× = tetraploid.^xSeed content per capsule: lacking (–), low (+), abundant (++), very abundant (+++).Table 3. Pollen viability as detected by fluorescein diacetate in species and hybrids of *Doritaenopsis* and *Phalaenopsis*.

Hybrid/species ^z	Viable pollen cells [mean ± SD (%)] ^x
<i>Phalaenopsis equestris</i> ^y	70.0 ± 5.3
<i>Doritaenopsis</i> Chian-Huey Red Rose 'F904'	45.6 ± 11.2
<i>Doritaenopsis</i> Purple Gem	41.4 ± 8.3
<i>Doritaenopsis</i> Sogo Gem 'F859'	37.1 ± 6.3
<i>Doritaenopsis</i> Fuchsia Princess 'KHM648'	36.7 ± 3.1
<i>Doritaenopsis</i> Bin You Danseuse 'F574'	35.3 ± 2.1
<i>Doritaenopsis</i> Sogo Gem 'F1082'	33.3 ± 1.2
<i>Doritaenopsis</i> Jiuhbao Red Rose	33.0 ± 12.6
<i>Doritaenopsis</i> I-Hsin Purple Jewel	31.3 ± 12.2
<i>Doritaenopsis</i> Sogo Gem 'F752'	20.7 ± 4.0

^zHybrids are shown in descending order according to the frequency of viable cells observed.^y*Phalaenopsis equestris* was used as a standard for comparison of pollen division patterns.^xThe total scoring number per individual sample is 1000 pollen mother cells; n = 3.

indeed related to repetitive genomic DNA content. *Phalaenopsis* species such as *P. aphrodite*, *P. sanderiana*, *P. stuartiana*, and *P. lueddemaniana* Rchb. f. with low DNA values have small-sized chromosomes; those with intermediate DNA values have middle-sized chromosomes, and those with high DNA values, such as *P. amboinensis* and *D. pulcherrima*, have large-sized chromosomes.

The segregation of homologous pairs of chromosomes at meiotic anaphase I depends on prior pairing, synapsis, and recombination at earlier stages (Armstrong and Jones, 2003; Armstrong et al., 2001). Nevertheless, chromosome recognition and initial pairing in eukaryotes is not achieved by DNA–DNA interactions alone (Hiraoka, 1998; Schwarzacher,

Table 4. Distribution of sporad types for several *Doritaenopsis* and *Phalaenopsis* orchids as seen by staining of pollen mother cells with lactophenol-acid fuchsin solution.

Hybrid/species ^z	Pollen mother cells (no.) ^y						
	Monad	Dyad	Triad	Tetrad	Tetrad + 1mc ^x	Tetrad + 2mc	Tetrad +3 or more mc
<i>Phalaenopsis equestris</i>	3	0	9	928	47	6	7
<i>Doritaenopsis</i> Chian-Huey Red Rose ‘F904’	12	7	18	835	108	17	3
<i>Doritaenopsis</i> Juihbao Red Rose	19	27	50	607	226	43	28
<i>Doritaenopsis</i> Purple Gem	34	5	6	568	306	52	29
<i>Doritaenopsis</i> Fuchsia Princess ‘KHM648’	5	63	140	516	201	33	42
<i>Doritaenopsis</i> Sogo Gem ‘F1082’	115	304	76	244	23	7	221
<i>Doritaenopsis</i> I-Hsin Purple Jewel	12	56	99	225	200	129	279
<i>Doritaenopsis</i> Sogo Gem ‘F752’	127	241	219	182	53	18	56
<i>Doritaenopsis</i> Bin You Danseuse ‘F574’	241	63	56	56	18	17	590
<i>Doritaenopsis</i> Sogo Gem ‘F859’	15	16	12	15	5	30	907

^zHybrids and species are shown in descending order according to the frequency of tetrads observed.

^yThe total scoring number per individual sample is 1000 pollen mother cells.

^xmc = micronucleus.

2003), and factors such as chromosome morphology, specific sequence distribution, proteins bound to DNA, and chromatin condensation patterns may also be involved (Schwarzacher, 2003). Structurally tight heterochromatin might actually produce a self-complementary landscape that ensures partner recognition and alignment by “best-fit” mechanisms (Schwarzacher, 2003).

Heterochromatin is distinguished by its dense pattern of staining throughout the cell cycle and generally includes telomeric and pericentric regions. Heterochromatin is thought to be enriched for transposable elements and repetitive DNA; moreover, its condensed structure is repressive to transcription and recombination in this region or in nearby euchromatic, transcriptionally active areas (Huisinga et al., 2006). Heterochromatin is generally considered to replicate late in the S-phase, with two major subtypes known as facultative and constitutive heterochromatin. The term facultative refers to a reversible, epigenetically controlled state of chromatin. In contrast, in constitutive heterochromatin, the heterochromatic state is a heritable chromosomal trait (Pedrosa et al., 2001).

Phalaenopsis and *Doritis* species differ in the accumulation and distribution of heterochromatin on chromosomes, presumably as a result of the amplification of repetitive sequences during evolution (Kao et al., 2001), a process most likely driven by unequal translocation, pericentric inversion, and centric fusion and fission (Stebbins, 1971). Such differences in heterochromatin can be visualized on staining with the fluorochromes chromomycin A3 (CMA3) and 4,6'-diamino-2-phenylindole (DAPI; Pedrosa et al., 2001), because, as in most plant species, CMA3 binds to paracentromeric, early-replicating GC-rich

Fig. 1. Lactophenol-acid fuchsin staining of microspores in *Doritaenopsis* orchids. (A) *Doritaenopsis* Sogo Gem ‘F752’ monad; (B) *Doritaenopsis* Chian-Huey Red Rose ‘F904’ dyad; (C) *Doritaenopsis* Juihbao Red Rose triad; (D–F) *Doritaenopsis* Fuchsia Princess ‘KHM648’ tetrad, tetrad plus micronuclei, and tetrad plus three micronuclei. Arrowheads point to micronuclei (scale bar = 10 μ m).

Table 5. Chromosome number and meiotic chromosome configuration in *Doritaenopsis* hybrids.

Hybrid ^z	Chromosome configurations [mean $\pm$ SD (no./cell)] ^x					Chromosomes in chain [mean $\pm$ SD (no./cell)] ^w	2n chromosomes	Ploidy
	Univalents	Bivalents	Trivalents	Tetravalents	Pentavalents			
<i>Doritaenopsis</i> Bin You Danseuse 'F574'	28 $\pm$ 1	29 $\pm$ 2	3.5 $\pm$ 0.5	5 $\pm$ 1	3.5 $\pm$ 0.5	18 $\pm$ 2	76	4 $\times$
<i>Doritaenopsis</i> Chian-Huey Red Rose 'F904'		76					76	4 $\times$
<i>Doritaenopsis</i> Fuchsia Princess 'KHM648' ^y	10.5 $\pm$ 2.5	22.5 $\pm$ 2.5	3.5 $\pm$ 0.5	2.5 $\pm$ 1.5			38	2 $\times$
<i>Doritaenopsis</i> I-Hsin Purple Jewel	17.7 $\pm$ 4	30.3 $\pm$ 8.1	3.3 $\pm$ 2.3	3 $\pm$ 1		13.7 $\pm$ 6.8	57	3 $\times$
<i>Doritaenopsis</i> Juihbao Red Rose		76					76	4 $\times$
<i>Doritaenopsis</i> Purple Gem	7.2 $\pm$ 3	34.4 $\pm$ 1					38	2 $\times$
<i>Doritaenopsis</i> Sogo Gem 'F752' ^y	16 $\pm$ 2	33 $\pm$ 1	2.5 $\pm$ 0.5	4 $\pm$ 1		8.5 $\pm$ 2.5	57	3 $\times$
<i>Doritaenopsis</i> Sogo Gem 'F859'	10 $\pm$ 2	33 $\pm$ 1					38	2 $\times$
<i>Dtps</i> Sogo Gem 'F1082'	26 $\pm$ 2	36.5 $\pm$ 0.5	3 $\pm$ 1	1.5 $\pm$ 0.5			57	3 $\times$

^zHybrids are shown in alphabetical order.^yNumber of meiotic cells analyzed: n = 3; all others: n = 5.^xThe frequencies of different chromosomal configurations per cell observed at diakinesis or metaphase. A high frequency of bivalents may indicate high homology between parental genomes and low occurrence of chromosomal rearrangements. A high frequency of univalents and multivalents may indicate poor genome homology and occurrence of chromosomal rearrangements, respectively. The standard deviation indicates the range in which the configurations appeared.^wChromosomes per cell that appeared as linked in chains.

DNA, which gives an even staining through most of the euchromatic region, whereas DAPI binds to AT-rich, dense heterochromatic areas that replicate late in the S-phase (Belonogova et al., 2006; Sumner, 1991; Weiss-Schneeweiss et al., 2003). In this work, we used CMA3 and DAPI to visualize differences in chromosome structure within hybrids, as was previously reported in *Phalaenopsis* and *Doritis* species (Kao et al., 2001).

We aimed to investigate cytogenetic features, including chromosome number, chromosome pairing behavior, frequency of normal tetrads, and pollen viability, of several *Doritaenopsis* and *Phalaenopsis* hybrids and to relate that information to capsule set and overall crossing ability. Such information should assist future efforts to evaluate specific hybrids for use in breeding of seed-propagated cultivars or for line selection.

Materials and Methods

PLANT MATERIAL. Commercial hybrids of the artificial genus *Doritaenopsis* (crosses between *Phalaenopsis* and *Doritis*) were purchased from commercial nurseries and were cultivated in a greenhouse. Several hybrids were selected for being directly or indirectly derived from *D. pulcherrima*, with large chromosomes (1–3 μ m) and *P. equestris*, with minute chromosomes (less than 1 μ m). Two *Doritaenopsis* hybrids (*Doritaenopsis* Chian-Huey Red Rose 'F904' and *Doritaenopsis* Juihbao Red Rose) were included for their ease of intercrossing (Table 1). *P. equestris* was used as a standard for comparison of pollen division patterns.

POLLEN STAINING WITH FLUORESCEIN DIACETATE (FDA). Pollen viability analyzed by staining with FDA reveals cell membrane functionality (Pritchard, 1985). FDA (Fluka, Buchs, Switzerland) was dissolved at 0.5% (w/v) in absolute acetone, 1:49 diluted in 10% sucrose solution. Fresh pollinia were collected from unopened full-size flower buds and were squashed onto clean slides with FDA. Slides containing the mixture of pollens and FDA were covered for at least 15 min to allow color to develop (Pritchard, 1985). The results were observed by use of a fluorescence microscope (Labophot-2; Nikon Corp., Tokyo) with a blue light filter. Cells exhibiting a bright yellow fluorescence were considered positively stained. At least three samples from different plants of the same clone were used. Still photographs were taken by use of a digital camera (E4500; Nikon).

SPORAD QUANTIFICATION IN POLLEN MOTHER CELLS (PMCs). To quantify the frequency of different types of sporads in hybrids, PMCs were stained with lactophenol-acid fuchsin solution (Lim et al., 2001). Pollinia from full-sized flower buds were fixed with 95% ethanol-glacial acetic acid (3:1 by vol) solution for 24 h, hydrolyzed in 1 N HCl for 1 h at 60 °C, rinsed three times with distilled water, and stained with lactophenol-acid fuchsin solution containing 50 mg of acid fuchsin, 5 g of phenol, 5 mL of lactic acid, 25 mL of glycerol, 10 mL of 95% ethanol, and 60 mL of distilled water. Material for observation was examined under an inverted phase-light microscope (Diaphot; Nikon). One thousand PMCs were categorized according to the number of sporads, especially tetrads, at the end of the division II stage, and at least three different plants were used for examination. Still pictures were taken by use of a digital camera (E4500; Nikon).

CHROMOSOME SAMPLE PREPARATION AND ACETO-CARMINE STAINING. To prepare samples of somatic and meiotic

chromosomes, growing root tips were excised and anthers were taken from tightly closed flower buds at three-quarters of their final bud size (pachytene/metaphase-I stages; Table 1), as suggested by Dyer (1979). Root tips were pretreated with 2 mM 8-hydroxyquinoline (Sigma-Aldrich, St. Louis), 0.1% colchicine (Sigma-Aldrich), or paradichlorobenzene-saturated (Sigma-Aldrich) solution on a rotary shaker (100 rpm) for 5 h at 15 to 18 °C, and were then fixed in 95% ethanol-propionic acid (3:1 by vol) for 48 h at 4 °C. Samples were digested as suggested by Pedrosa et al. (2001) with 1% (w/v) cellulase (Sigma-Aldrich) and 0.5% (w/v) pectinase (Fluka) in 10 mM citrate buffer. Digested samples were squashed in a droplet of 1% aceto-carmin on a microscope slide, covered with a coverslip, and analyzed under a light microscope (BX50; Olympus Corp., Tokyo) with a CCD camera (Pro150ES; Pixera Corp., Los Gatos, CA). Permanent slides were prepared by freezing with liquid nitrogen, air-dried, and then mounted in Entellan® (Merck KGaA, Darmstadt, Germany).

ESTIMATION OF CHROMOSOME NUMBER AND SIZE. Chromosome number counting and length measurement from digital images involved use of ImageJ software, a public domain image analysis application (U.S. National Institutes of Health, 2007). Final image adjustment involved use of Adobe Photoshop CS2 (Adobe Systems, San Jose, CA).

FLUORESCENT STAINING. To observe fluorescent banding patterns in somatic chromosomes, chromosomal preparations were squashed and stained with 1% aceto-carmin on new, clean microscope slides. After the coverslips were removed with use of liquid nitrogen, the slides were air-dried and then flooded with 20 µL of a solution containing CMA3 (Sigma-Aldrich). The CMA3 solution was prepared by dissolving 0.5 mg of CMA3 per mL of 0.5× McIlvaine's buffer, consisting of 18 mL of 0.1 M citric acid plus 82 mL of 0.2 M Na₂HPO₄, pH 7, as described Friebe et al. (1996). To stain the samples with DAPI (Sigma-Aldrich), the coverslips were removed and the slides were immersed in distilled water to remove excess CMA3, and were then allowed to air dry. Once dry, the slides were flooded in DAPI-containing solution, covered, and left in the dark at room temperature for 20 min. A working solution of DAPI was prepared by dissolving a stock solution of DAPI (0.2 mg·mL⁻¹) in McIlvaine's buffer (containing 5 mM MgCl₂), at 1 µg of DAPI solution per 999 µL of buffer. Slides were observed within 24 h in the dark under a fluorescence microscope (Labophot-2; Nikon).

Results

SEED SET AND HYBRID HOMOLGY. Crosses between *Doritaenopsis* hybrids consistently showed capsule setting and production of viable seeds largely between hybrids with similar chromosome number and size that involved little or no inversions and translocations, especially for crosses of *Doritaenopsis* Chian-Huey Red Rose 'F904' and *Doritaenopsis* Jiuhbao Red Rose (Table 2). Both hybrids contained small chromosomes. Crosses of *Doritaenopsis* Sogo Gem 'F859' (as pollen recipient), and *Doritaenopsis* Jiuhbao Red Rose and *Doritaenopsis* Chian-Huey Red Rose 'F904' (as pollen donors) succeeded in the first stages of fertilization (including development of pollen tubes; data not shown), but the resulting capsules aborted 80 d after pollination.

PHYSIOLOGICAL VIABILITY OF POLLEN CELLS. FDA staining of fully developed, fresh pollen masses from hybrids and species of *Doritaenopsis* and *Phalaenopsis* revealed that a high proportion of pollen cells of *P. equestris* (70%) was stained, whereas most hybrid values ranged from 45% to 30%, as in *Doritaenopsis* Chian-Huey 'F904' (45.6%). *Doritaenopsis* Sogo Gem 'F752' showed the lowest value (20.7%; Table 3).

Fig. 2. Chromosome complement at somatic metaphase of *Doritaenopsis* orchids. (A) *Doritaenopsis* Sogo Gem 'F859'; (B) *Doritaenopsis* Sogo Gem '1082'; (C) *Doritaenopsis* Purple Gem; (D) *Doritaenopsis* Fuchsia Princess 'KHM648'; (E) *Doritaenopsis* I-Hsin Purple Jewel; (F) *Doritaenopsis* Sogo Gem 'F752'; (G) *Doritaenopsis* Bin You Danseuse 'F574'; (H) *Doritaenopsis* Chian-Huey Red Rose 'F904'; and (I) *Doritaenopsis* Jiuhbao Red Rose. Each picture displays the number of chromosomes. Arrowheads point to (L) large and (S) small chromosomes, except in (H) and (I) where all chromosomes are small (scale bar = 10 µm).

FREQUENCY OF TETRADS IN MATURE POLLINIA. Samples of mature pollinia were stained with lactophenol-acid fuchsin to quantify the frequency of tetrads per thousand pollen cells, taking tetrad formation as an indicator of even assortment of chromosomes. In *P. equestris*, *Doritaenopsis* Chian-Huey Red Rose 'F904', and *Doritaenopsis* Jiuhbao Red Rose, most PMCs divided into tetrads (928, 835, and 607 cells of 1000, respectively). The opposite occurred in hybrids *Doritaenopsis* Bin You Danseuse 'F574' and *Doritaenopsis* Sogo Gem 'F859', where less than 100 of PMCs divided into tetrads. In both hybrids, most of the other PMCs divided irregularly into sporads with a varying number of micronuclei (Table 4 and Fig. 1).

CHROMOSOME NUMBER AND PAIRING BEHAVIOR DURING MEIOSIS. Results from chromosome counts in the somatic tissue of *Doritaenopsis* and *Phalaenopsis* showed sharp differences in ploidy (Table 5 and Fig. 2). Chromosome constitutions ranged from diploidy (2 $\times$) for *Doritaenopsis* Sogo Gem 'F859', *Doritaenopsis* Purple Gem, and *Doritaenopsis* Fuchsia Princess 'KHM648'; triploidy (3 $\times$) for *Doritaenopsis* Sogo Gem 'F1082', *Doritaenopsis* Sogo Gem 'F752', and *Doritaenopsis* I-Hsin Purple Jewel; and tetraploidy (4 $\times$) for hybrids *Doritaenopsis* Jiuhbao Red Rose, *Doritaenopsis* Chian-Huey Red Rose 'F904', and *Doritaenopsis* Bin You Danseuse 'F574'.

Many hybrids showed chromosome configurations such as trivalents, tetravalents, and multivalents at metaphase I (Table 5 and Fig. 3), as well as uneven division of PMCs (Fig. 4) and chromosomal bridges (Fig. 5). Three types of hybrids based on observed chromosome configurations at metaphase I and on the meiotic disorders mentioned above were distinguished. In type one, chromosomes were completely or almost completely homologous, as evidenced by most chromosomes paired as bivalents. Type one hybrids include *Doritaenopsis* Jiuhbao Red Rose (4 $\times$), *Doritaenopsis* Chian-Huey Red Rose 'F904' (4 $\times$), and, to some extent, *Doritaenopsis* Purple Gem (2 $\times$) (Table 4). In type two hybrids, chromosomes had partial or low homology, with most chromosomes configured as univalents and, in some cases, tetravalents (Fig. 4); lagging chromosomes were common at anaphase I and the prevalence of

Fig. 3. Meiotic chromosome configurations in several *Doritaenopsis* hybrids by aceto-carmin staining. (A) *Doritaenopsis* Sogo Gem 'F859' at diakinesis; (B) *Doritaenopsis* Bin You Danseuse 'F574' at diakinesis; (C) *Doritaenopsis* I-Hsin Purple Jewel at metaphase; (D) *Doritaenopsis* Sogo Gem 'F1082' at metaphase; (E) *Doritaenopsis* Purple Gem at metaphase; and (F) *Doritaenopsis* Jiuhbao Red Rose at anaphase (BV = bivalent; M = multivalent; T = trivalent; UV = univalent; scale bar = 10 μ m).

micronucleated meiocytes was high (up to 10 in a cell; data not shown). Type two hybrids include *Doritaenopsis* Sogo Gem 'F859' (2 $\times$) and *Doritaenopsis* Bin You Danseuse 'F574' (4 $\times$). In type three hybrids, chromosomes showed aberrant configurations such as tetravalents and multivalents (chains) during meiosis, inversion loops, and translocation junctions at pachytene (Fig. 6), as well as chromosomal bridges with fragments

Fig. 4. Division patterns of pollen mother cells (PMCs) in *Doritaenopsis* hybrids by aceto-carmin staining. (A) *Doritaenopsis* Sogo Gem 'F859' at late anaphase II; (B) *Doritaenopsis* Sogo Gem 'F752' at late anaphase II; (C) *Doritaenopsis* Fuchsia Princess 'KHM 648' at early anaphase II; and (D) *Doritaenopsis* Chian-Huey Red Rose 'F904' PMCs at metaphase (scale bar = 10 μ m).

Fig. 5. Meiotic chromosome bridges in *Doritaenopsis* orchids. (A) *Doritaenopsis* Fuchsia Princess 'KHM648'; (B) *Doritaenopsis* Sogo Gem 'F859'; and (C) *Doritaenopsis* Sogo Gem 'F752.' (A) stained by 4,6'-diamino-2-phenylindole and (B and C) stained by aceto-carmin (scale bar = 10 μ m).

at anaphase (Fig. 7). Type three hybrids showed more or less serious overall micronucleation. Type three hybrids include *Doritaenopsis* Sogo Gem 'F1082' (3 $\times$), *Doritaenopsis* Sogo Gem 'F752' (3 $\times$), *Doritaenopsis* Fuchsia Princess 'KHM648' (2 $\times$), and *Doritaenopsis* I-Hsin Purple Jewel (3 $\times$).

CHROMOSOME SIZE. Because of the small size of the chromosomes (1–3 μ m), identifying individual chromosomes

at mitotic metaphase by centromere position or secondary constrictions was difficult. We classified the karyotype of each hybrid into a unimodal or bimodal arrangement on the basis of the relative size of chromosomes observed at somatic metaphase, with most hybrids having bimodal karyotypes, except for *Doritaenopsis* Chian-Huey Red Rose 'F904' and *Doritaenopsis* Jihubao Red Rose (Table 6). Additionally, we rated the formation of dicentric bridges at anaphase and the formation of micronuclei at metaphase on a scale ranging from absent (–) to very frequent (+++), with both phenomena overlapping in *Doritaenopsis* Sogo Gem 'F752', *Doritaenopsis* Sogo Gem 'F859', *Doritaenopsis* Sogo Gem 'F1082', and *Doritaenopsis* Fuchsia Princess 'KHM 648' (Table 6).

VISUALIZATION OF HETEROCHROMATIN DISTRIBUTION. Staining with fluorochromes CMA3 and DAPI was used to visualize heterochromatin distribution on chromosomes at somatic prometaphase of *Doritaenopsis* Purple Gem (Fig. 8). Cells from *Doritaenopsis* Purple Gem were amenable to fluorescent banding because of a seemingly high mitotic index (a character specific to this hybrid) and relatively large chromosomes ($\approx$ 3 μ m). DAPI staining showed strong blue fluorescence at chromosomal ends and in small intercalary areas of larger chromosomes. However, no such fluorescence was detected around centromeres. Smaller chromosomes displayed weak fluorescence by DAPI staining but remained stainable by CMA3. In comparison with aceto-carmin staining, DAPI staining was useful in visualizing abnormal phenomena such as bridges, fragments, and lagging chromosomes in meiotic samples of *Doritaenopsis* Fuchsia Princess 'KHM648' (Fig. 7).

Discussion

The chromosomal configurations observed at meiotic metaphase in orchid hybrids could reflect incompatibility caused by genomic divergence between their progenitors (Arends, 1970). Incompatible chromosomes fail to synapse at pachytene; consequently, no chiasmata are formed between them, which results in univalents observed at metaphase. Univalents may not align properly to the metaphase plate and thus segregate incorrectly at anaphase I (Ma, 2006). As a

Fig. 6. Inverted and translocated pachytene chromosomes in *Doritaenopsis* Fuchsia Princess 'KHM648' [I = inversion loop; T = translocation junction (see arrowheads), scale bar = 10 μ m].

Fig. 7. Meiotic chromosome disorders in cells of *Doritaenopsis* Fuchsia Princess 'KHM648' stained with 4,'6'-diamino-2-phenylindole. Note: (F) a chromosomal fragment, (B) a chromosome bridge, (L) and lagging chromosomes, possibly forming a micronucleus (scale bar = 10 μ m).

result, meiotic products with unbalanced chromosome constitutions may affect the functions and development of gametes (Singh, 2003). Additionally, partial homology between parent genomes in diploid hybrids and aneuploids may cause chromosomes to pair as trivalents (Swanson et al., 1981). In *Arabidopsis thaliana* (L.) Heynh., mutants of *Zip4/Sp022* genes showed unpaired univalent chromosomes and subsequent poor seed set after fertilization (Chelysheva et al., 2007). We observed a similar association of univalents and other non-bivalents with low or no fertility in *Doritaenopsis* hybrids.

The bridges and fragments we observed in meiotic anaphase I in *Doritaenopsis* Sogo Gem 'F1089', *Doritaenopsis* Bin You Danseuse 'F574', *Doritaenopsis* Sogo Gem 'F752' (3 $\times$), *Doritaenopsis* Fuchsia Princess 'KHM648', and *Doritaenopsis* I-Hsin Purple Jewel indicate that a paracentric inversion contributes to the differentiation of these hybrid genomes. Reciprocal translocations could also be frequent in *Doritaenopsis* Bin You Danseuse 'F574' and *Doritaenopsis* I-Hsin Purple Jewel and might explain the high frequency of multivalents. Interestingly, all hybrids with inversions or translocations have *D. pulcherrima* as a first- or second-generation female parent, whereas *Doritaenopsis* Chian-Huey Red Rose 'F904' and *Doritaenopsis* Jiuhbao Red Rose do not, nor do they possess any *D. pulcherrima*-like chromosomes.

In general, meiotic aberrations such as bridges/fragments, asymmetric segregation, heteromorphic bivalents, and karyotype asymmetry might result from chromosome structure mutations (Wang and Zhang, 2007). According to Levin (2002), each novel translocation and inversion initially is rare and occurs in a heterozygous condition, which is a liability because heterozygous plants typically have 50% fertility. Translocations are reportedly more common than inversions with large number of chromosomes and, in addition to altering end arrangements, may lead to loss of chromosomes (Levin, 2002).

A previous study of the distribution of heterochromatin in *Phalaenopsis* and *Doritis* (Kao et al., 2001) showed large chromosomes with a prevalence of large terminal heterochromatic blocks (common in *D. pulcherrima*), whereas small chromosomes (common in genus *Phalaenopsis* section *Phalaenopsis*) seemed to contain heterochromatin beyond the resolution capacity of cytological detection. DAPI and CMA3 staining of somatic prometaphase chromosomes of *Doritaenopsis* Purple Gem allowed for visualization of clusters of terminal, AT-rich heterochromatin in all large chromosomes, whereas small chromosomes were stained in their entirety by CMA3 (e.g., euchromatic) (Fig. 8). This finding may suggest the existence of two mechanisms influencing chromosome pairing: 1) evolutionary differences in heterochromatin distribution and content may create incompatible "best-fit" chromosomal landscapes that make pairing difficult, and 2) large chromosomes with high heterochromatin content seem more prone to breakage events (Swanson et al., 1981). Thus, hybrids with large chromosomes might undergo high rates of rearrangements that decrease homology (Levin, 2002; Swanson et al., 1981), a fitting scenario for *Doritaenopsis* Sogo Gem 'F752', 'F859', 'F1082', *Doritaenopsis* Fuchsia Princess 'KHM 648', and *Doritaenopsis* I-Hsin Purple Jewel.

Hybrids with chromosomal aberrations or micronucleation, such as in *Doritaenopsis* Sogo Gem 'F859' and *Doritaenopsis* Sogo Gem 'F1082', tended to show higher intensity of fluorescence (on FDA staining) than hybrids with otherwise fit pollen cells, such as *Doritaenopsis* Jiuhbao Red Rose. This

Table 6. Length variation and meiotic aberrations in metaphase chromosomes of several *Doritaenopsis* hybrids.^z

Hybrid ^z	Mode of length variation	Chromosome size (L or S) ^y	Dicentric bridges (– to +++ scale) ^x	Formation of micronuclei (– to +++ scale) ^w
<i>Doritaenopsis</i> Bin You Danseuse ‘F574’	Bimodal	24L + 52 S	+	++
<i>Doritaenopsis</i> Chian-Huey Red Rose ‘F904’	Unimodal	all short	–	–
<i>Doritaenopsis</i> Fuchsia Princess ‘KHM648’	Bimodal	24 L + 14 S	+++	++
<i>Doritaenopsis</i> I-Hsin Purple Jewel	Bimodal	29 L + 28 S	+	+++
<i>Doritaenopsis</i> Jiuhbao Red Rose	Unimodal	all short	–	–
<i>Doritaenopsis</i> Purple Gem	Bimodal	22 L + 16 S	–	+
<i>Doritaenopsis</i> Sogo Gem ‘F752’	Bimodal	35 L + 22 S	+++	+
<i>Doritaenopsis</i> Sogo Gem ‘F859’	Bimodal	18 L + 20 S	++	+++
<i>Doritaenopsis</i> Sogo Gem ‘F1082’	Bimodal	22 L + 35 S	+++	++

^zHybrids are shown in alphabetical order.^yL = long chromosomes (1–3 µm); S = short chromosome (less than 1 µm).^xObserved frequency of dicentric bridges at anaphase per cell: absent (–), occasional (+), frequent (++), very frequent (+++).^wObserved frequency of formation of micronuclei per cell: absent (–), occasional (+), frequent (++), very frequent (+++).

pattern is apparently at odds with the idea that chromosomal heterozygotes have reduced fertility. In cells with an intact plasma membrane, FDA can freely enter living cells and is hydrolyzed by esterase enzymes to give fluorescein; cells accumulate fluorescein and become brightly fluorescent (Pritchard, 1985). A high percentage of cells from hybrids with serious chromosomal deficiencies fluoresced may not be a contradictory result because abnormal plant meiocytes (e.g., microspores) do not undergo programmed cell death as do abnormal animal cells (Ma, 2006). Perhaps a better indication of pollen fitness is the dominant division pattern of PMCs during division II, as seen by staining with lactophenol-acid fuchsin (Table 3). In this case, hybrids with a low frequency of tetrads also tended to show asymmetric segregation, chromosomal aberrations, and poor pollen viability, and hybrids with high frequency of tetrads show the reverse.

Fig. 8. 4,'6'-Diamino-2-phenylindole/chromomycin A3-stained somatic metaphase chromosomes of *Doritaenopsis* Purple Gem. Note the prevalence of terminal blocks of heterochromatin (blue arrows) in large chromosomes and the lack of heterochromatin in smaller chromosomes (white arrows). Scale bar = 10 µm.

Our pollination results (Table 2) seem to indicate successful seed set in reciprocal crosses of hybrids with normal cell division patterns, whereas crosses involving aberrant pollen cells led to low seed yield, capsule abortion, or no production of capsules. Formation of irregular sporads had been previously reported in interspecific hybrids of *Phalaenopsis* (Arends, 1970; Shindo and Kamemoto, 1963), but inversions or translocations were not observed and pollen viability was not investigated. In our study, *Doritaenopsis* hybrids with poor chromosome pairing commonly showed failure of seed setting after cross pollination, a result similar to that reported by Arends (1970). However, the finding of inversions and translocations implies the existence of additional hybridization barriers in *Doritaenopsis* not before considered. Abnormal cytological behavior of the PMCs of an autotetraploid *Vaccinium elliotii* Chapm. also resulted with reduced pollen viability (Dweikat and Lyrene, 1991; Vorsa and Ballington, 1991). This suggests the widespread occurrence of abnormal meiotic behavior and pollen viability among plants.

For breeders, the existence of chromosomal rearrangements such as inversions and translocations in parental genomes implies that chromosome doubling in hybrids may not restore fertility (Kamstra et al., 1999); thus, careful screening of cultivars before breeding is necessary. Alternatively, the search for unreduced gametes and subsequent embryo rescue may offer the opportunity of partial fertility in cross-hybridization of *Doritaenopsis*, as implicated in other crops (Lyrene, 1991; Sugiura et al., 2000; Widrechner et al., 1982).

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