Induction of polyploid and analysis of natural polyploidization in the Orchidaceae

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2022 年 1 月

千葉大学大学院園芸学研究科

環境園芸学専攻生物資源科学コース

近藤　悠

（千葉大学審査学位論文）

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General Introduction

Polyploids have three or more complete sets of chromosomes, and 70% of angiosperms experience polyploidization (Leitch & Bannett, 1997; Soltis & Soltis, 1999). Novelty and phenotypic diversity of polyploid may provide adaptive capacity, particularly in times of high environmental stress (Soltis *et al.,* 2015). Specifically, there is a higher frequency of polyploid plants at higher latitudes and higher altitudes, with particularly high proportions of polyploid taxa in arctic populations (Brochmann *et al* 2004). Thus, polyploidy contributes greatly to plant genome evolution via genetic variability, which may lead to selection for evolution and adaptation (Alix *et al.,* 2017). Polyploids arise in nature through somatic doubling and unreduced reproductive cells (Sattler *et al.,* 2016). Somatic doubling is associated with mitotic events such as endomitosis or endoreduplication (Sattler *et al.,* 2016). Unreduced 2*n* reproductive cells are pollen or eggs having somatic chromosome numbers rather than gametophytic ones, and they predominantly lead to polyploidy in plants (De Storme & Geelen, 2013a; Sattler *et al.,* 2016; Loginova & Silkova, 2017). Polyploids are also useful in horticulture because polyploid individuals exhibit larger organs and deeper flower color (Takamura & Miyajima, 1996; Sattler *et al.,* 2016). Artificial chromosome doubling has been usually conducted by the application of antimitotic reagent, such as colchicine, oryzalin, trifluralin and amiprophos-methyl (APM) (Dhooghe et al. 2011). Among these, colchicine has been successfully applied to seeds, seedlings, buds and meristematic regions for chromosome doubling of many plant species (Dhooghe et al. 2011). Sexual polyploidization, namely polyploidization via 2*n* gametes is an efficient way to produce polyploids

(Ramanna & Jacobsen, 2003). Breeding utilizes 2*n* gametes to produce interspecific hybrids with different genome ratios between parental species as well as intraspecific triploids with vigorous growth rates and lack of seeds (Barba-Gonzalez *et al.,* 2004; Mai *et al.,* 2019). The following pathways are used to induce triploids in breeding: i) fertilization between reduced and 2*n* gametes, ii) cross between diploid and tetraploid artificially induced by treatment with an antimitotic agent, and iii) endosperm culture (Nakano *et al.,* 2021). Triploid induction via reduced and 2*n* gametes is the most efficient method because it can be obtained via manipulation of one generation, namely hybridization, although the frequencies of 2*n* gametes are relatively low.

In ornamental plants, intra- as well as inter- specific hybridizations have commonly been conducted to obtain novel characters in flower traits such as color and size as well as other important characteristics for commercial production such as altered flowering period, environmental stress tolerance and disease resistance (Nimura et al. 2006; Vendrame et al. 2007; Nimura et al. 2008; Laskowska et al. 2015; Zhang et al. 2017). Interspecific hybrids are usually sterile partially or completely, which made further breeding programs difficult. Hence, induction of amphidiploid by artificial chromosome doubling has been applied to restore the fertility of interspecific hybrids, which enables further hybridization for their genetic improvement (Ishizaka and Uematsu 1994; Ishiki and Taura 2003; Nimura et al. 2006; Nimura et al. 2008; Laskowska et al. 2015; Zhang et al. 2017).

Orchidaceae is the largest family of flowering plants, which is divided into five subfamilies,

namely Apostasioideae, Cypripedioideae, Orchidoideae, Vanilloideae, and Epidendroideae (Chase, 2005). Orchids have a very wide variety of lifestyle, flower morphologies, polyploidy and Genome size, and have been successful colonisers of a wide variety of different habitats (Leitch *et al.,* 2009; Felix and Guerra., 2010; Givnish *et al.,* 2015). Furthermore, chromosome number varied from 2*n* = 24 in *Malaxis pubescens* to 2*n* = 240 in *Epidendrum cinnabarinum* (Felix and Guerra., 2010). In orchids, interspecific hybridization is relatively easy, and it has been extensively employed in breeding novel cultivars. Thus, polyploidy and chromosome numbers of cultivars are more complex. Previous studies indicated polyploidy in commercial cultivars e.g., *Phalaenopsis* are consisted of diploid, triploid, tetraploid and hexaploid (Lee *et al.,* 2020), *Dendrobium* are consisted of diploid, triploid and tetraploid (Sakai *et al.,* 1998). 2*n* gametes should be involved the breeding of *Phalaenopsis* cultivars of Orchidaceae (Lee *et al.,* 2020). In almost all orchids, mature sporads maintain tetrads post meiosis (Pacini & Hesse, 2002). Owing to this intrinsic trait of orchids, we could estimate the process of meiosis, that is, tetrads form due to normal meiosis and dyads form due to irregular meiosis. In present study, I focused on the polyploidy of orchid, and two pathways of polyploidization namely, artificial chromosome doubling and sexual polyploidization. Polyploidy in orchids contributes greatly to plant genome evolution via genetic variability as well as orchid breeding. Chapte1 mentioned about method of efficient chromosome doubling by the treatment of antimitotic agent *in vitro* for breeding. Chapter 2-4 mentioned natural polyploidization caused by unique characters of *Epidendrum*. Furthermore, I

mentioned the potential of using natural polyploidization for polyploid breeding methods that are more efficient than artificial chromosome doubling via antimitotic agent.

Chapter 1

Efficient artificial chromosome doubling of an interspecific hybrid *Dendrobium* Stardust 'Fire Bird' by treatment of amiprofos- methyl to PLBs

Introduction

Dendrobium is one of the largest genera in Orchidaceae and includes more than 1,200 species in the Australasian, Indo- Asian and Pacific regions (Adamus *et al.,* 2011). Some novel species or hybrids in the given genus are used as cut flowers and potted plants commercially (Kamemoto *et al.,* 1996). *D. nobile* is one of the most popular species as potted plants in this genus, with wide range of flower colors in a plant of 60-90 cm in height (Baker and Baker 1996; Kamemoto *et al.,* 1996).

Dendrobiums, interspecific hybridization is relatively easy and has extensively been employed for the breeding of novel cultivars (Kamemoto *et al.,* 1996). However, interspecific hybrids are usually sterile partially or completely, which made further breeding programs difficult. Hence, induction of amphidiploid by chromosome doubling has been applied to restore the fertility of interspecific hybrids, which enables further hybridization for their genetic improvement (Ishizaka and Uematsu 1994; Ishiki and Taura 2003; Nimura *et al.,* 2006; Nimura *et al.,* 2008; Laskowska *et al.,* 2015; Zhang *et al.,* 2017). Furthermore, chromosome doubling has been recognized to provide other advantages in some ornamental plants, e.g. increase of flower size and the number of petals in rose (Allum *et al.,* 2007), increased flower size and smaller plant foliage in *Echinacea* (Abdoli *et al.,* 2013) and increase in flower size and content of chalcone which would contributed to produce deeper flower color in *Cyclamen* (Takamura and Miyajima 1996).

Chromosome doubling has been conducted by the application of antimitotic reagent, such

as colchicine, oryzalin, trifluralin and amiprophos-methyl (APM) (Dhooghe *et al.,* 2011). Among these, colchicine has been successfully applied to seeds, seedlings, buds and meristematic regions for chromosome doubling of many plant species (Dhooghe *et al.,* 2011). However, some undesirable effect, such as inhibition of initial growth of post treatment and decrease in survival rate after treatment, have also been reported in various species such as *Dianthus* (Nimura *et al.,* 2006) and *Echinacea* (Abdoli *et al.,* 2013). In contrast, APM has been used for chromosome doubling in *Dianthus* with less damages of plants (Nimura *et al.,* 2006). Moreover, in Colchicaceae plants more than 25% of shoots obtained after application of APM at various concentrations were amphidiploid and ploidy chimera (Yamakawa *et al.,* 2015).

In the previous reports, various types of explants such as buds, calli, and shoot tips have been used for chromosome doubling (Dhooghe *et al.,* 2011). In the present study protocorm like bodies (PLBs) were used as plant materials. PLB is characterized by its embryogenic nature due to the close similarity to the protocorm which is an intrinsic structure formed soon after seed germination of Orchidaceae plants (Arditti 2008; Lee et al 2013). Because of its high proliferation rate in *in vitro* culture, it has efficiently been utilized for the micropropagation of economically important species and cultivars of orchid including *Dendrobium* (Teixeira da Silva *et al.,* 2006; Hossain *et al.,* 2013). Since a mass of PLB has many meristematic regions, it can be expected to be a useful material for the efficient induction of large number of amphidiploids in the present study.

D. Stardust ‘Fire Bird’ (Fig. 1) is an interspecific hybrid between *D. unicum* and *D.* Ukon ‘Arai’, which is originated from the crosses of four wild species i.e. *D. nobile*, *D. moniliforme*, *D. signatum* and *D.heterocarpum* (Fig. 2). This cultivar has shoots of 15-20 cm in length and vivid orange multiple flowers with strongly veined lips. These unique flower characters have been introduced from *D. unicum* used as female parent for *D*. Stardust ‘Fire Bird’. Although further improvement of horticultural traits in this cultivar has been strongly desired to meet the demands of marketability, almost all of the trials to obtain the next generation by selfing, crosses with several cultivars and related wild species have failed with fruit drop at early stages after pollination or with harvest of fruits without viable seeds in my preliminary study. Sterility have been reported to occur several factors such as imbalance of genome composition, anisoploid and incompatibility of pollen-stigma interaction (Johansen 1990; Zhao *et al.,* 2007; Pinheiro *et al.,* 2015; Sattler *et al.,* 2016). However, *D.* Ukon used as male parents of *D.* Stardust ‘Fire Bird’ is originated from the crosses of four wild species i.e. *D. nobile. D. moniliforme, D. signatum and D. heterocarpum*. However, *D. unicum* used as female parent is distant in genetic relationship from these four wild species (Takamiya *et al.,* 2014). Thus, sterility of the hybrid might be caused by the strong imbalance of the genome composition.

In the present study I have attempted to achieve chromosome doubling by the treatment of APM *in vitro*, in terms of exploration of optimal conditions of treatment such as concentrations of APM, exposure time and timing of the treatment of APM. At the same time, I have made comparison

between amphidiploid and original diploid to find morphological marker for discriminating chromosome-doubled plants at early stages after APM treatment *in vitro*. Furthermore, I made cytological observation of tetrad pollen at anthesis in *D.* Stardust 'Fire Bird' to evaluate their pollen fertility or abnormality to estimate the reason for the failure to next generation.

Materials and Methods

Plant materials

These *Dendrobium* hybrids, namely. *D*. Stardust 'Fire Bird and *D*. Ukon 'Arai' were kindly provided by Mr. Nobuyuki Asai, (Asai Daikeien Co., Ltd. Aichi, Japan) and *D*. *unicum* was purchased from nursery of Kitakaru Garden (Gunma, Japan). *D*. Fairyflake 'Carmen', which is a typical nobile-type *Dendrobium*, was purchased from Tokyo Orchid Nursery (Tokyo, Japan). These plant materials were cultivated in a greenhouse which were kept lower than 35℃ in summer and higher than 11℃ in winter. They were potted with sphagnum in good drainage and Sumitomo No.2 (Sumitomo Chemical Co., Ltd. Tokyo, Japan) as aqueous fertilizer, which was diluted to contain 0.05% nitrogen was applied regularly. Four fungicides i.e. Benlate® (Sumitomo Chemical Co., Ltd.), Daconil® (Sumitomo Chemical Co., Ltd.), Topsin M® (Nippon Soda Co., Ltd. Tokyo, Japan) and Bellkute® (Nippon Soda Co., Ltd.) and Four pesticides i.e. Agri-Mek® (Syngenta Japan Co., Ltd. Tokyo, Japan), Actara® (Syngenta Japan Co., Ltd.), Marathon® (Sumitomo chemical garden products Inc. Tokyo, Japan) and Hachi-Hachi® (Nihon Nohyaku Co., Ltd. Tokyo, Japan) and pesticide were sprayed every one month.

PLBs of *D.* Stardust 'Fire Bird' were induced from meristematic regions of shoots that were collected from *ex vitro* plants. Shoots of the plant were sterilized for 10 minutes with hypochlorite (ca. 1% available chlorine) contained 2-3 drops of Tween 20 for surfactant. After the sterilizing, shoots were rinsed three times with distilled water. Explants were excised 1.5×1.5 cm length from shoots and

transferred to ND medium (Tokuhara and Mii 1993) supplemented with 10 g L^{-1} maltose, 1 mg L^{-1} BA and 0.1 mg L^{-1} to induce PLBs. PLBs were cultured in Petri dish (9cm in diameter) containing solidified 20 mL ND medium for multiplication, supplemented with 10 g L^{-1} maltose, 1mg L^{-1} BA, 0.1 mg L^{-1} NAA, and 2 g L^{-1} Gelrite®. The pH of the medium was adjusted to 5.4 before autoclaving at 121℃ for 15 min. Sub-culture and division of PLBs were conducted every three weeks.

Growth of PLBs

To reveal the mode of growth of PLBs, fresh weight of PLBs was measured every two days on sterilized Petri dish. Four replications were made for each experimentation, which was conducted at 25 ± 2℃ under 24 h-illumination (35 μmol m^{-2} s^{-1}).

APM treatment

In this study, two series of experiment of APM (Bayer Crop Science Japan, Tokyo, Japan) treatments were conducted. In the first experiment, 500 mg PLBs were treated *in vitro* in combination of concentrations at 10, 20, 30, 40 and 50 mg L^{-1} for different durations of 3, 6, 9, 12 and 24 hours. Six months after each treatment of APM, thirty plantlets were randomly collected from each treatment with the use of random numbers and 750 plantlets in total were obtained. These plantlets were divided into two groups based on the difference morphology. One type was almost identical to the features of

the plantlets without APM treatment, whereas another type had swelled and stunted stems. Aspect ratio of the swelled stems approximately one. Thirty plantlets each that were collected randomly from each type with the use of random numbers and group without treatment in control were their ploidy levels were determined by flow cytometry (FCM).

In second experiment, PLBs were treated by APM at concentrations of 10, 20, 40, 80 and 160 mg L^{-1} in combination with four treatment durations including 6, 12, 24 and 48 hours. Division of PLBs was conducted two weeks prior to the series of experiments. Five hundred mg PLBs were divided into several masses of approximately 4-7 mm in diameter and transferred onto 20 mL ND liquid medium, supplemented with 10 g L^{-1} maltose and APM at each concentration, in 9 cm Petri dish. They were incubated on a reciprocal shaker at 120 rpm at 25℃ ± 3℃. After the treatment of APM, PLBs were rinsed three times with distilled water and transferred to 20 mL ND medium solidified with 2 g L^{-1} Gelrite® medium and sub-cultured every four weeks till 8 weeks after the treatment. PLBs without treatment of APM were transferred to ND medium and subcultured in the same manner and were used as a control. Each treatment was replicated five times.

Assessment of treatment

Eight weeks after APM treatment, PLBs were photographed from right above the culture dishes and classified into two categories by their colors, namely, green as survived and brown as dead,

respectively. Rate of green area per all PLBs area was calculated and defined as survival rate. Plantlets of 1 cm or more in height were isolated from PLBs during the period of 9 to 21 weeks after APM treatment and sub-cultured. The total number of plantlets was used as another index for damage. Twenty plantlets at maximum for each treatment were measured for the ploidy level by FCM. Then frequency of chromosome-doubled plantlets was calculated as percentage of chromosome doubled plantlets per survived plantlets. Finally, as the index to evaluate the appropriate combination of APM concentrations and exposure time, the efficiency of chromosome doubling was obtained; survival rate (%) × total number of plantlets obtained × frequency of chromosome doubled plantlets (%), in the present study

Determination of ploidy levels by flow cytometry analysis

The youngest leaves of plantlets were analyzed by FCM. Fresh leaves of approximately 2 × 2 mm in length were chopped with a razor blade for 20 seconds in Petri dish containing 200μL extraction buffer (solution A of the Quantum Stain NA UV 2 Germany) which was added with 800μL buffer for DAPI staining (solution B of the Quantum Stain NA UV2, Germany) and then passed through a nylon sieve (40 μm mesh). After two-minutes of stain by DAPI, flow cytometric analysis was conducted using CA II cytometer (Partec, Munster, Germany). For each sample, 700-1,500 nuclei were analyzed with a mean value of coefficient of variation < 4%. *Eustoma grandiflorum* which has a

2C DNA amount = 3.26 pg (Lindsay et al. 1994) was used as the internal standard throughout the analysis.

Evaluation of morphological and growth characters of plantlets of two ploidy levels

Both the plantlets of original cultivar and the ones with doubled chromosome number (referred to as amphidiploid here after) were grown on ND medium. To promote the growth of axillary buds, these plants which had six or more leaves were cut into nodal segment and laid on ND medium, supplemented with 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA. After two weeks of incubation *in vitro,* the nodal explants were transferred to ND medium without phytohormone and cultured for two weeks. After the incubation, plantlets of approximately 1 cm in length were removed from nodal section and transferred to test tube containing solidified 10 mL ND medium without phytohormone. After three months of culture, plantlets were subjected to evaluate several characters such as number of leaves and roots, length and width of leaves, width of stems and fresh weights.

Counting the chromosome number

Method of chromosome counting by Koehler *et al.,* (2008) was followed, otherwise stated. Chromosome number of *Dendrobium* hybrids and induced amphidiploid was counted using green root tips from at least three plants. Root tips of 5-8 mm long were excised at around 10:30 a.m. and

pretreated in 2 mM 8-hydroxyquinoline for 36 h at 4℃. They were then fixed with a mixture of ethanol and acetic acid in 3:1 (v/v) for 5h at room temperature. After fixation, root tips were stored in 70% ethanol at 4℃ before use. For chromosome observation, stored root tips were soaking in distilled water to remove ethanol for one hour. The root tips were cut in to less than 0.5mm on slide glass and 10 μL enzyme solution containing 2% (w/v) Celluluase Onozuka RS (Yakult Pharmaceutical Industry Co., ltd. Tokyo, Japan) and 2% (w/v) Pectolyase Y-23 (Kikkoman Corporation Chiba, Japan) was dropped on it. The root tips on slide glass were incubated for 20 minutes at 37℃ in tight box with saturated moisture provided by water contained at the bottom. Then, enzyme solution was wiped and a drop of 45% acetic acid was put on it. After 1-2 minutes, cover glass was put on the root tips and squashed with finger with accuracy. Cover glass on the slide glass was removed by razor blade one day after freezing in -80 ℃. The cells were stained with 5 μg mL^{-1} DAPI with mounting medium (VECTASHILD Burlingame, USA), observed by fluorescence microscope at x1000 (Model BX53; Olympus, Tokyo, Japan) and photographed by camera (Cool SNAP Myo, Arizona, USA). Chromosome number determined from at least 10 mitotic cells with well spread chromosomes.

Cytological observation of microspores development

Development and viability of microspores were observed by the methods of Aoyama (1994). Pollinia obtained from five flowers at anthesis of four genotypes i.e. *D*. Stardust 'Fire Bird', *D*. Ukon

'Arai', *D*. *unicum*, *D*. Fairyflake 'Carmen' were divided into pieces by the razor blade followed by the treatment with a mixture of 1 N HCl and 45% acetic acid (2:1 v/v), and stained with 2% acetic orcein. Microspores were classified into seven types i.e. monad, dyad, triad, tetrad (normal type), unequalled tetrad, with micronucleus and polynucleus according to the previous studies (Aoyama 1994; Lee et al .2011). For each sample, at least one hundred microspores were counted, and frequencies of each type were calculated.

Statistical analysis

Data were evaluated by R Version 3.4.2 statistical software (R Development Core Team, Australia, https://www.r-project.org/). Analysis of variance (ANOVA) were calculated by aov function and multiple comparison were calculated by Tukey HSD function. Differences were regarded as significant at p value < 0.05.

Results

Mode of growth of PLBs

Thirty days after culture on ND medium, fresh weight of PLBs increased approximately twice as much as that at culture initiation (Fig. 3). The weight increased in a manner of sigmoid curve consisted by slow growth of PLB at initial phase, followed by the phase of rapid growth. Three weeks after subculture of PLBs, some of them developed into shoots with small leaves. Based on these results, APM treatment was applied two weeks after the division of PLBs which was at early phase of rapid growth without development of small leaves.

Morphology of plantlets and their ploidy level

In my first experiment, two types of plantlets, which were different in morphology were recognized six months after APM treatment. One type was almost identical to the features of the plantlets without APM treatment (Fig. 4a), whereas another type had swelled and stunted stems (Fig. 4b). All the plantlets of former type and the control ones were revealed to be diploid, whereas ca. 60% of plantlets in the latter type were amphidiploid

Effect of APM treatment on the induction of chromosome doubling

All the treatment of APM did not show any necrosis of PLBs in at initial phase, i.e. four

weeks after the treatment (data not shown). Survival rates that were recorded eight weeks after treatment of APM were decreased with the increase of APM concentrations, whereas exposure period of APM gave no significant difference (Fig. 5a). In the treatment of APM with lower concentrations at 10 and 20 mg L^{-1}, survival rate was almost the same as that of the control.

The number of plantlets that were recoded 9-21 weeks after the treatment of APM. Necrosis as well as delay of growth were observed in those periods. In almost all the combinations of concentrations and exposure time except for six and twelve hours of treatment at APM of 20 mg L^{-1}, the number of plantlets obtained was less than that of control (Fig. 5b). Furthermore, treatment of APM of 40 mg L^{-1} or higher concentrations greatly decreased the number of plantlets to ca. 30% of the control, irrespective of the duration of treatment. Although a clear-cut tendency was not found in all the combinations of concentrations and exposure time of APM, the number of the plantlets obtained significantly decreased with the prolongation of exposure time in each APM concentrations compared to that of the control (0 mg L^{-1})

Higher frequencies of amphidiploid were found in longer treatment, such as 12-48 h in each concentration (Fig. 5c). The highest frequencies of amphidiploids reached to almost 80% by the treatment of APM at 160 mg L^{-1} for 12 h. Frequencies of amphidiploids evaluated 9, 13, 17 and 21 weeks after APM treatment were not significantly different (data not shown).

Efficiency of chromosome doubling calculated from the 3 parameters, i.e., survival rate,

total number of plantlet and frequency of amphidiploid as described in Materials and Methods is shown in (Fig. 5d).The results showed that low concentration treatment such as 10 and 20 mg L^{-1} was more efficient than higher concentrations to induce chromosome doubling and that the highest efficiency of ca.5 was found in treatment of APM at 10 mg L^{-1} for 12 h or 48 h. However, in most of the treatment with higher concentrations, such as 40 mg L^{-1} or more, the efficiency was less than one, due to the low survival rate as well as number of plantlets, even with higher frequencies of amphidiploid.

Morphological analysis

Amphidiploid plants had wider leaves, larger stems and heavier fresh weight than those of diploid plants (Table 1). In seven diploid plants out of eleven, formation of a new shoot from the base of the original stem was observed. On the contrary, only one out of fifteen amphidiploid plants formed a new shoot.

Chromosome number and flow-cytometric analysis

Chromosome number of *D*. Stardust 'Fire Bird' was $2n = 38$ (Fig. 6a), whereas that of amphidiploid was $2n = 76$ (Fig. 6b). Moreover, both diploid and amphidiploid had two peaks. The larger peak at smaller DNA value showed the DNA content of G1 cells and another one with twice the

DNA contents of G2 cells (Fig. 6c, d).

Morphology of microspore

The frequencies of normal tetrad pollen formation in all the 4 genotypes examined in the present study were in a range of 66.2-87.5% and *D.* Stardust 'Fire Bird' showed the lowest frequency of 66.2% followed by *D.* Ukon 'Arai' of 74.9% (Table 2; Fig. 7). These two cultivars were characterized to have more triad microspores (more than 20%) than other two genotypes.

Discussion

APM Treatment to PLBs

Chromosome doubling could have been achieved by antimitotic reagents through inhibiting cell cycle at M-phase (Dhooge *et al.,* 2011). Thus, for the efficient induction of chromosome doubling, it is essential to select the target tissue with high mitotic activity and appropriate time for the APM treatment. Actually, in the genus Rosa, selection of appropriate timing of exposure with antimitotic reagent successfully yielded tetraploid by 34.7% (Allum *et al.,* 2007). In the present study, I have succeeded to obtain amphidiploids by treating PLBs with APM at the stage between 10 to 20 days after sub-culture, which was estimated to contain actively dividing cells at high frequencies based on the growth curve of PLBs (Fig. 3). In *Phalaenopsis*, chromosome doubling often occurred during multiplication by cutting an individual PLB (Chen *et al.,* 2009). However, in the present study, no amphidiploid plant was obtained without APM treatment.

Effect of APM concentrations and time of exposure

Treatment of APM at higher concentrations eventually induced severe necrosis of PLBs and inhibited subsequent growth of plantlet in the present study. High concentrations of antimitotic reagents and prolongation of exposure time induced serious damages to explants after treatment in cyclamen, carnation and rose (Takamura and Miyajima 1996; Nimura *et al.,* 2006; Khosravi *et al.,*

2008). In the previous studies, APM treatment was not used at concentration of 80 mg L^{-1} or higher (Nimura *et al.,* 2006; Yamakawa 2015; Grosso *et al.,* 2018). But present study suggested that high APM concentration at 80 mg L^{-1} or higher caused serious damage on PLBs (Fig. 5a). Long time of exposure with APM was also revealed give serious damage as assessed by number of plantlets obtained from 500 mg PLBs (Fig. 5b). Although survival rate was recorded only once at initial phase of culture for chromosome doubling (Takamura and Miyajima 1996; Nimura *et al.,* 2006; Khosravi *et al.,* 2008), present study suggested that APM concentration and time of exposure affected that damage of PLBs at different phase of culture after the treatment, i.e. the former strongly affected at an initial phase, whereas the latter at later phase. APM concentrations and time of exposure are important two factors to affect the efficiency of tetraploid (Dhooghe *et al.,* 2011). In the present study more than 20% of the plantlets obtained from all of the APM treatments applied were found to be amphidiploid. However, no clear-cut tendency was found on the effects of concentrations of APM and time of exposure on the chromosome doubling in the present study, which was consistent with previous work using PLBs of *Cattleya* (De Mello e Silva *et al.,* 2000). On the contrary, clear correlations were reported between the frequencies of chromosome-doubled plants and concentrations of antimitotic reagents as well as exposure time by using nodal sections or apical buds as target materials (Chen *et al.,* 2007; Khosravi *et al.,* 2008). In the present study I used relatively large numbers of PLBs in a mass, in which meristematic regions might have been in different physiological conditions. At the same time, each

PLB might have different conditions in term of the contact with culture medium, which should also have affected the incorporation of APM. Thus, it is necessary to develop the method to evaluate the comprehensive effects of several important factors on chromosome doubling of physiologically non-uniform target materials such as PLBs used in the present study. For this purpose, I employed 'index the chromosome doubling efficiency' to evaluate the combine effects of 3 important data set, i.e. survive rate, total number of plantlets obtained, and frequency of chromosome doubled plantlets, as described in Materials and Methods. As the results, the index indicated that APM treatment at low concentrations, such as 10 and 20 mg L^{-1} was more efficient than that at high concentrations of 80 and 160 mg L^{-1} for chromosome doubling (Fig. 5d). Importance of APM concentrations for chromosome doubling of PLBs was in line with a previous study in which PLBs of another variety of *Dendrobium* was treated with oryzalin (Miguel *et al.,* 2011). Although, in the previous studies, survival rate and frequencies of chromosome doubling were evaluated independently (Takamura and Miyajima 1996; Nimura *et al.,* 2006; Allum *et al.,* 2007; Abdolo *et al.,* 2013; Yamakawa et al; 2015), 'index for chromosome doubling efficiency' employed in the present study will be useful for evaluating the total effect of the related factors on the chromosome doubling of various target plant materials.

Phenotypic difference between diploid and tetraploid

Putative amphidiploid plantlets at 2-3 leaves phase showed stunted growth with swelled

stems compared with diploid plantlets with normal growth (Fig.4). Mitotic chromosome doubling leads various morphological change in several organ such as leaf size, flower color, plant height and pollen grain length in many plant (Podwyszyriska et al 2018; Ghanbari et al 2019) Difference in stem morphology between diploid and amphidiploid was consistent with that previously reported in carnation (Nimura *et al.,* 2006) and echinacea (Abdoli *et al.,* 2013). Furthermore, in the present study three months after the initiation of cutting of nodal section, the emergence of new shoot and further growth of amphidiploid in vitro was delayed compared to that of diploid. This growth could be utilized as features for identifying amphidiploid.

Fertility of interspecific hybrid

Interspecific hybrids are often sterile or low in ability of seed production which is induced by failure of chromosome pairing at meiosis as shown in *Paphiopedilum* (Lee *et al.,* 2011). Abnormality of microspore of *D*. Stardust 'Fire Bird' was found to be higher than those of other cultivars and species of *Dendrobium* examined in the present study which might be one of the reasons for the failure of seed production. Although nonhybrid plants produce only 0.1-2.0% unreduced gametes, Interspecific hybrids often produce more unreduced gametes than nonhybrid plants (Ramsey 2007; Kreiner et al 2017). Some interspecific hybrids produce reduced non-viable gametes and unreduced viable gametes (Barba-Gonzalez et al 2004; Fakhri et al 2016). Microspores of dyad and

triad of *D*. Stardust ‘Fire Bird’ (Fig. 7c, d) could be unreduced gametes. Chromosome doubling often drastically increased the viability in of pollen interspecific hybrids. For example, frequency of pollen viability restored from 0 to 66.8% in eggplant (Isshiki and Taura 2003) and that of pollen germination increased from 0 to 52.6% in lily (Zhang *et al.,* 2017) in amphidiploids respectively. Next generation was also obtained by self-pollination of amphidiploid interspecific hybrid in *Cyclamen* (Ishizaki and Umetsu 1994), *Dianthus* (Nimura *et al.,* 2006; Nimura *et al.,* 2008) and *Nicotiana*. (Laskowska *et al.,* 2015). The protocol established in the present study could be also applied for the chromosome doubling of not only other *Dendrobium* genotypes but also wide range of orchid species and genera (Fig. 8). Since amphidiploids obtained in the present study were expected to be useful to obtain next generation in the breeding program in this novel group of dendrobium orchids, restoration of their pollen fertility should be confirmed as early as possible. However, the members of the genus *Dendrobium* generally have long juvenile phase of 2-3 years, which inhibits the early assessment of reproductive characters of amphidiploid plants. Recently, the juvenile phase of *Dendrobium* plant was shortened by application of cytokinins in the medium (Kim *et al.,* 2007, Wang *et al.,* 2009). I am now attempting to induce *in vitro* flowering of amphidiploid of given variety to shorten juvenile phase.

Fig. 1 *Dendrobium* Stardust ‘Fire Bird’ used as plant material. Scale bar = 5cm

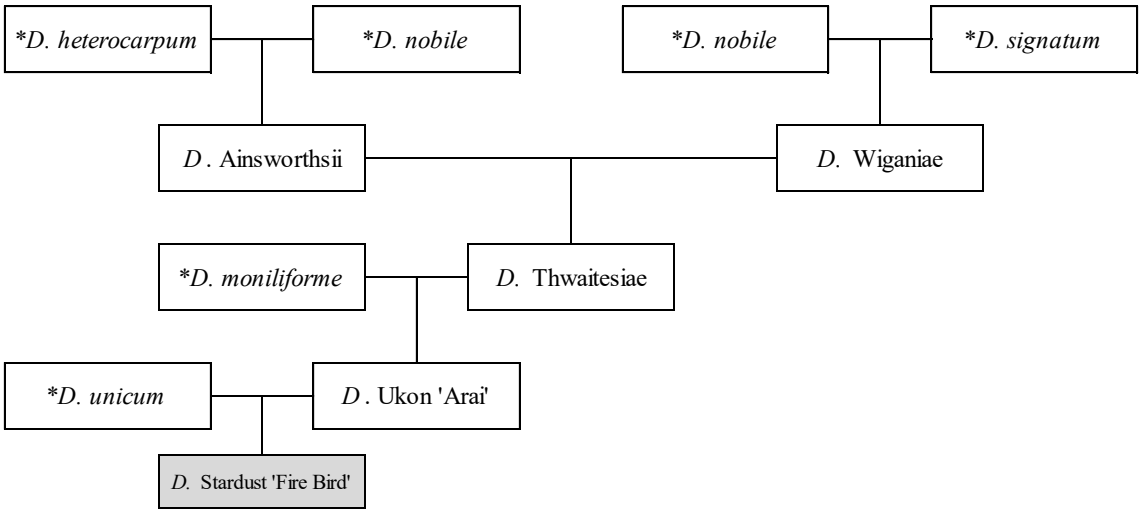


Fig. 2 Genealogy of *Dendrobium* Stardust ‘Fire Bird’. Asterisks indicate wild species.

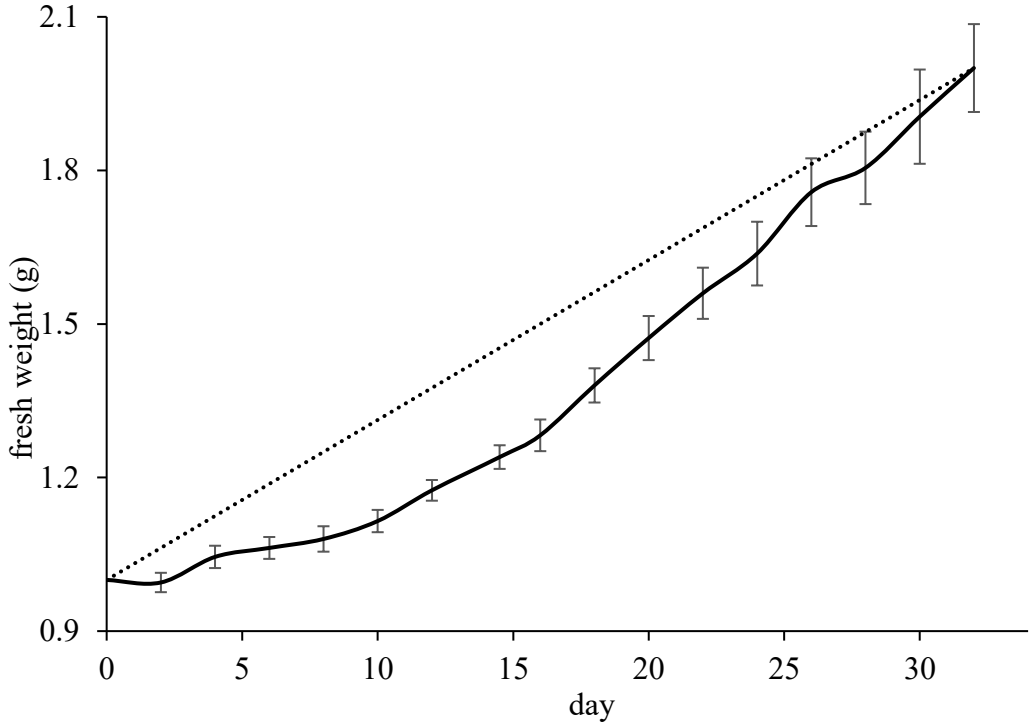


Fig. 3 Average of growth rate of protocorm like body (PLBs) of *Dendrobium* Stardust 'Fire Bird' cultured for 30 d on New Dogashima medium. One gm of PLBs were inoculated onto 20 mL medium and cultures weighed aseptically every two d. Dotted line connected weights 0 to 30 d after inoculation to indicate PLB growth rate. Four replications were made for each experimentation. Vertical bars indicated standard error.

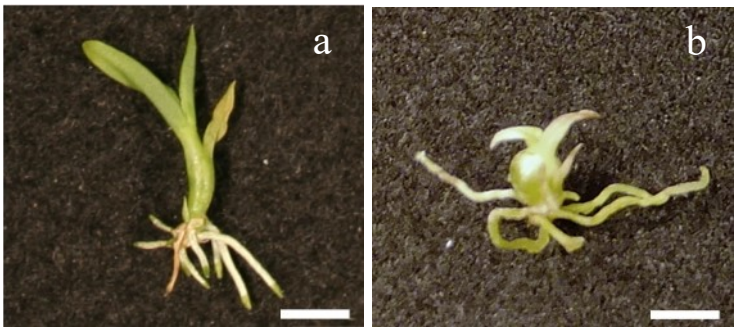
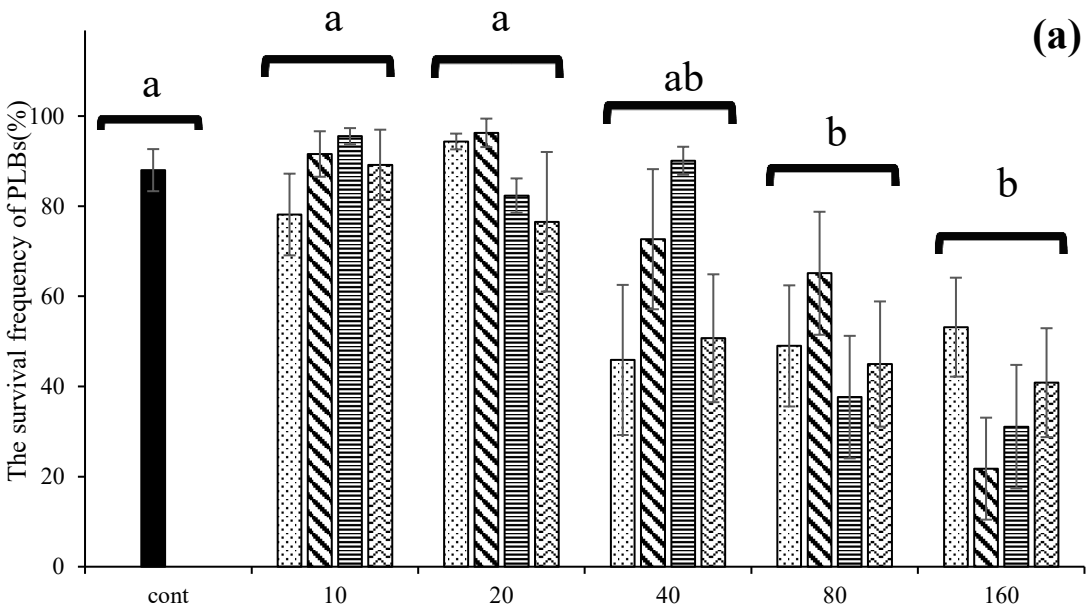
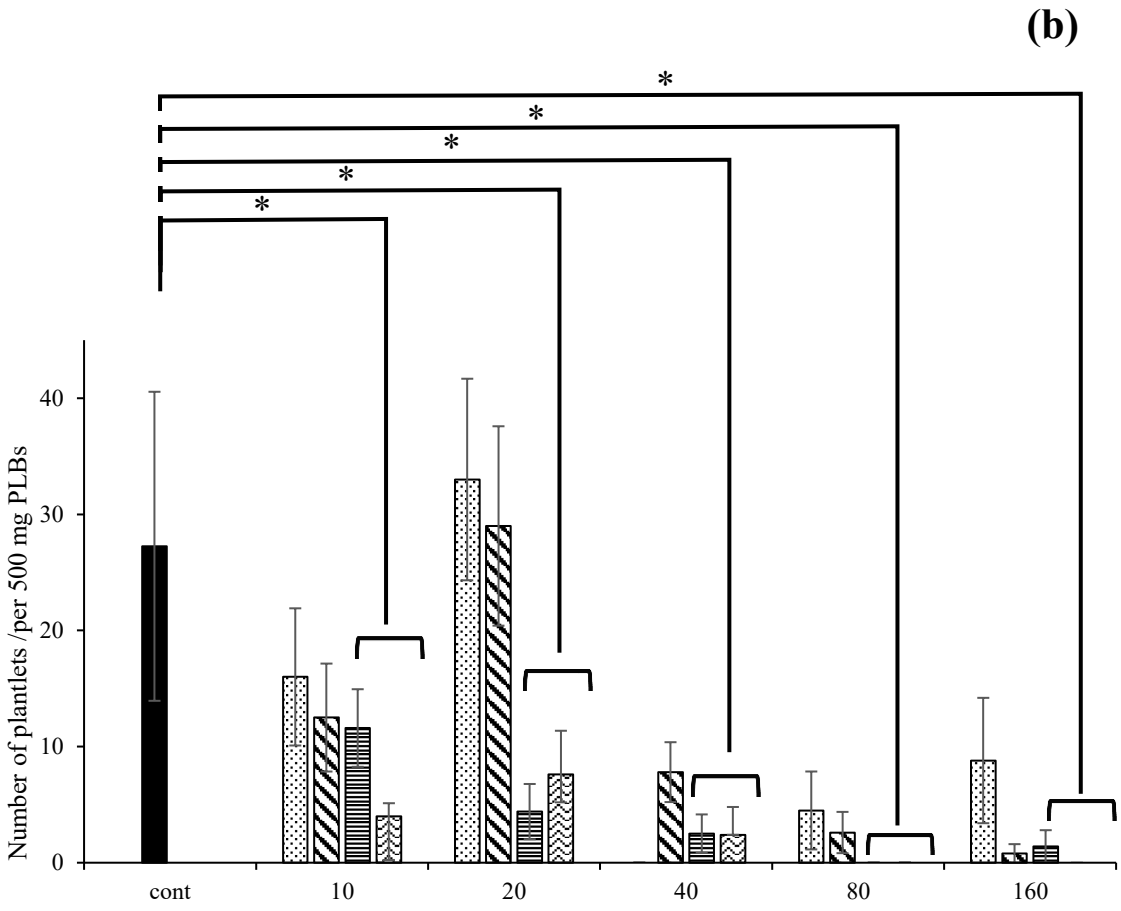


Fig. 4 Two types of *Dendrobium* Stardust 'Fire Bird' plantlets cultured for six months on New Dogashima medium after treatment with 30 mg L^{-1} amiprofos-methyl for 24 h. 'b' subsequent swollen and stunted stem of 'a'. Scale bars = 2.5 mm.


(a)
a
a
a
ab
b
b
The survival frequency of PLBs(%)
0
20
40
60
80
100
cont
10
20
40
80
160


(b)
*
*
*
*
*
Number of plantlets /per 500 mg PLBs
0
10
20
30
40
cont
10
20
40
80
160

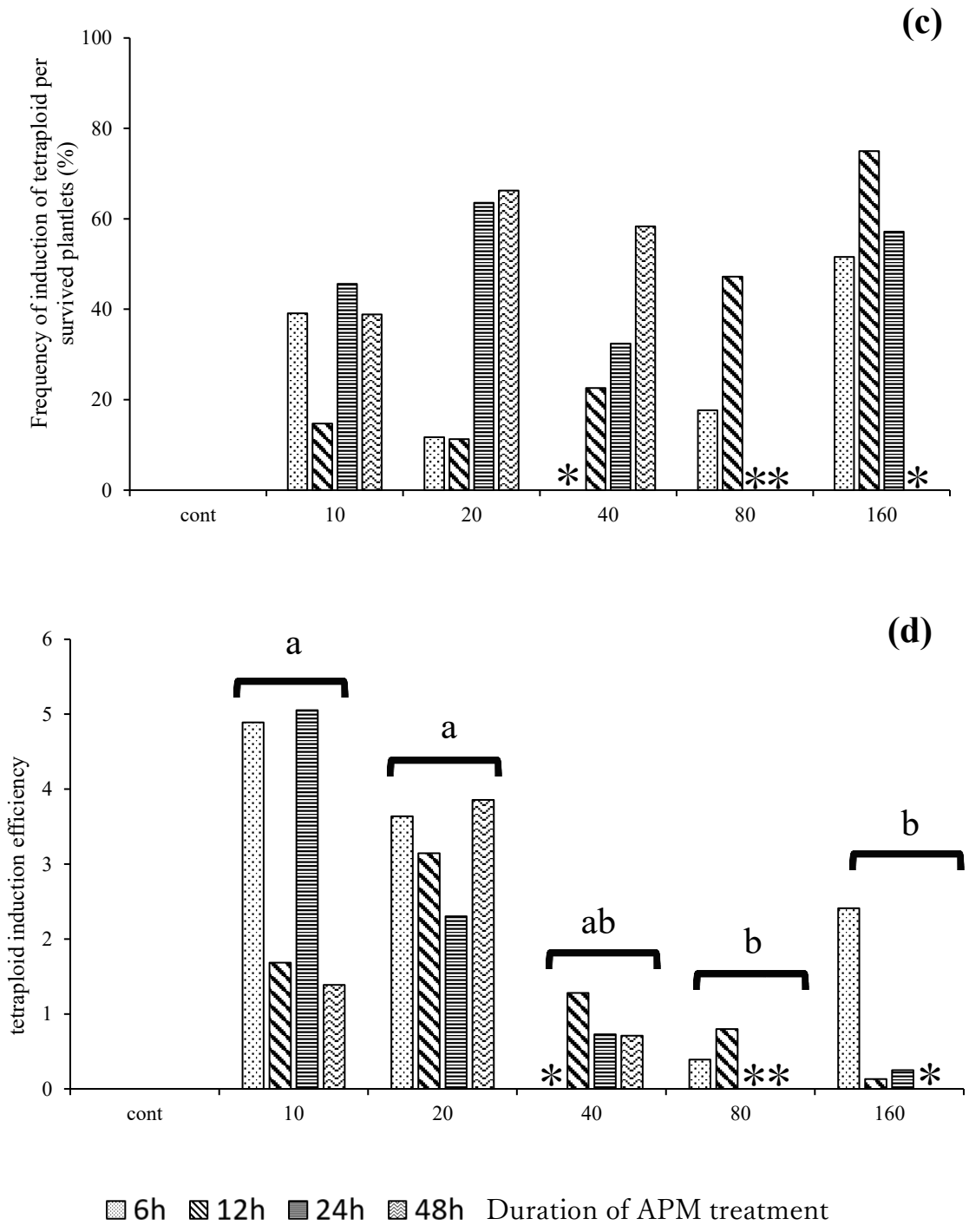

(c)
Frequency of induction of tetraploid per survived plantlets (%)
100
80
60
40
20
0
cont
10
20
40
80
160
*
**
*
(d)
tetraploid induction efficiency
6
5
4
3
2
1
0
a
a
ab
b
b
cont
10
20
40
80
160
*
**
*
6h
12h
24h
48h
Duration of APM treatment

Fig. 5 Effects of amiprofos-methyl (APM) concentration and treatment time on survival rate of protocorm like body (PLB), plantlet development and amphidiploid formation of *Dendrobium* Stardust 'Fire Bird'

(a): The survival rate of PLBs evaluated 8 weeks after APM treatment. Statistical analysis was conducted among APM concentrations. Different letters show significant differences for percentages of at the p value < 0.05. (b): Total number of plantlets obtained from 500 mg PLBs cultured 21 weeks after APM treatment. Statistical analysis was conducted between control and each duration time of APM treatment. Asterisks indicate significant difference by t test analysis * $p < 0.05$. (c): Frequencies of amphidiploid per total number of plantlets obtained. Asterisks indicate that no plantlets were obtained. (d): Efficiency of APM treatment as assessed by the chromosome doubling efficiency index; survival rate (%) × total number of plantlets obtained × frequency of chromosome doubled plantlets (%). Asterisks indicate that no plantlet were obtained. Different letters show significant differences among APM concentrations at the p value < 0.05. Vertical bars of (a) and (b) indicate standard errors.

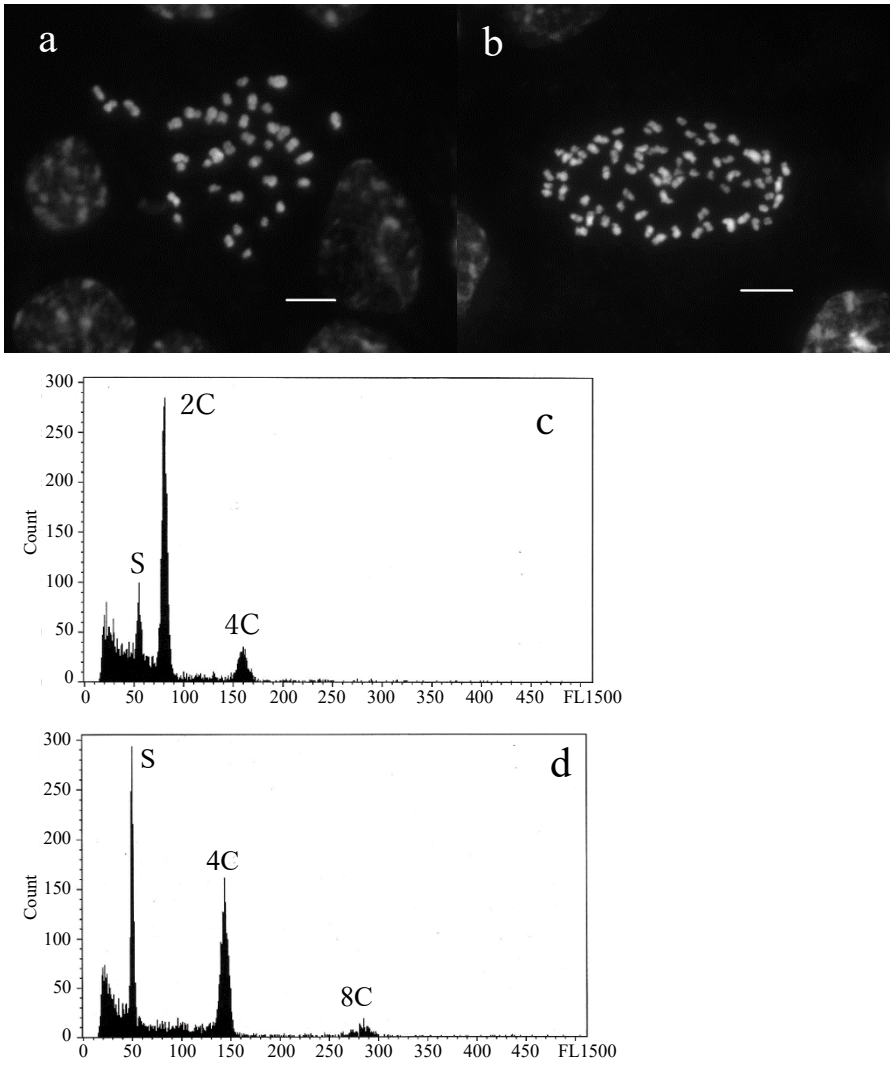


Fig. 6 Somatic chromosomes from root tip cells, a: *Dendrobium* Stardust 'Fire Bird' (2*n* = 38), b: amphidiploid (2*n* = 76). Scale bars = 5μm. Flow-cytometric profiles of DNA contents, c: 2C and 4C peaks of *D.* Stardust 'Fire Bird', d: 4C and 8C peaks of amphidiploid. 'S' indicated peaks of *Eustoma grandiflorum* of internal standard which has a 2C DNA amount = 3.26 pg.

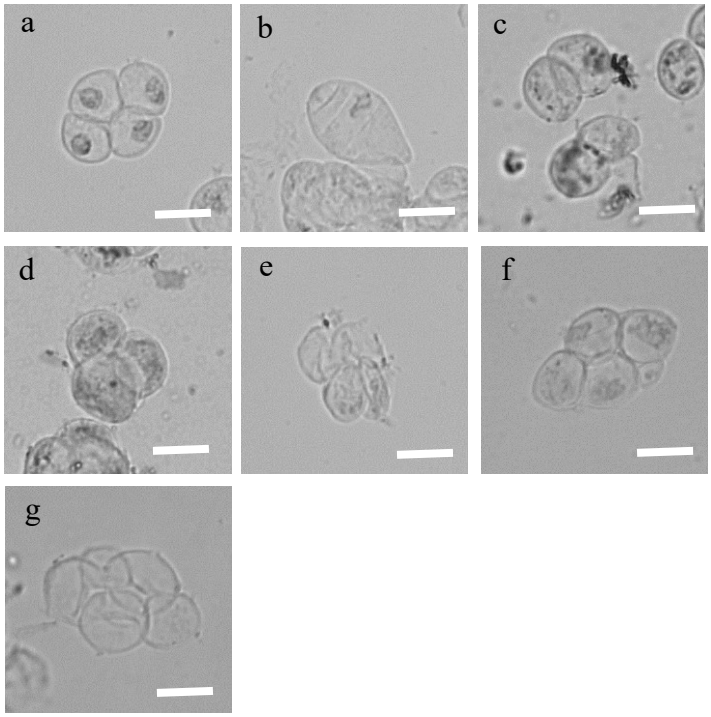


Fig. 7 Seven types of microspores of *Dendrobium* Stardust 'Fire Bird' from diploid stained with acetic orcein. a: tetrad (normal), b: monad, c: dyad, d: triad, e: unequal tetrad, f: with micronucleus and g: polynucleus. Scale bars = 5 μm.

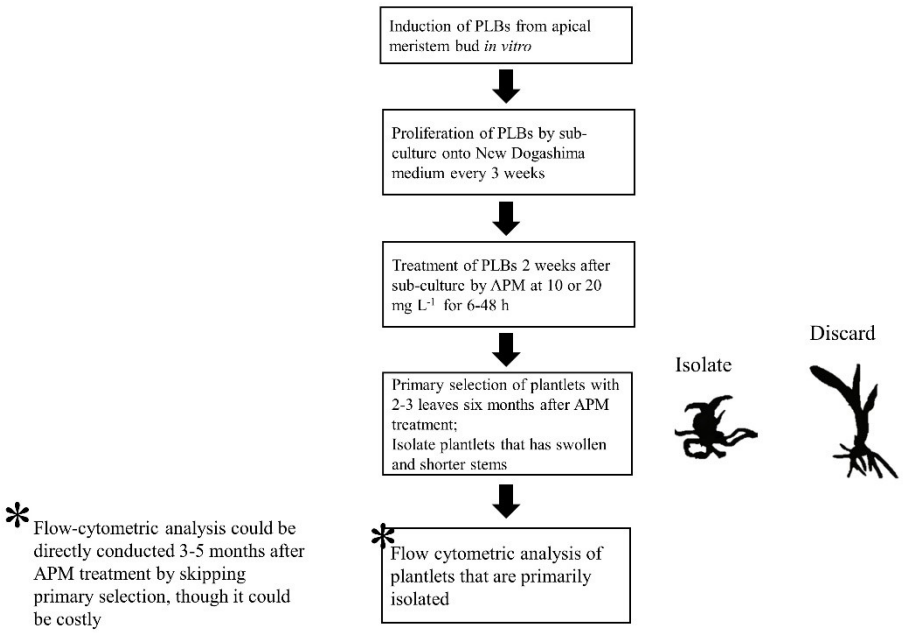


Fig. 8 The protocol of chromosome doubling of *Dendrobium* Stardust 'Fire Bird' through the treatment of protocorm like body (PLB) by amiprofos-methyl (APM) combined with primary isolation of plantlets *in vitro*.

Table1 Morphological difference between original diploid and APM-induced amphidiploid plants of *D*. Stardust 'Fire Bird' four months after propagation from nodal section

Ploidy	No. of plants	Total number of second stem plants	No. of leaves *	No. of roots	Leaf length (cm)[a]	Leaf width (cm)*[a]	Internode length (cm)	Stem width (cm)*[b]	Fresh weight (g)*
2*x*	11	7	5.0±0.3	5.8±0.4	10.81±1.02	2.48±0.30	2.86±0.31	2.27±0.31	0.19±0.02
4*x*	15	1	5.8±0.3	5.9±0.6	11.84±1.31	4.03±0.23	3.29±0.21	3.67±0.24	0.32±0.03

* Significantly different at the α=0.05 level by two-sample *t*-test

[a] Measurment secondary bottom leaf

[b] Measurment part of widest stem

Table 2 Frequencies of irregular microspores in *D*. Stardust ‘Fire Bird’ and three genotypes.

Plant materials	normal (tetrad)	Irregular					
		monad	dyad	triad	unequal tetrad	with micronucleus	polynucleus
D. Fairyflake 'Carmen'	87.5 [a]	0.2	3.3	9.5	0.0	0.0	0.0
D. unicum	83.9 [a]	0.0	4.0	14.9	0.2	0.0	0.0
D. Ukon 'Arai'	74.9 [ab]	0.6	7.0	20.7	0.4	0.0	0.9
D. Stardust 'Fire Bird'	66.2 [b]	2.2	3.7	23.7	0.7	1.1	0.2

Chapter 2

Chromosome number of *Epidendrum*

Introduction

The genus *Epidendrum*, which belongs to Epidendroideae, is a neotropical genus comprising approximately 1500 species with a distribution ranging from southeastern United States to northern Argentina (Pridgeon *et al.,* 2005). The genus shows wide morphological diversification (Pinherio *et al.,* 2009). Interspecific and interploidy hybrids have been detected between some wild species in the sympatric zone (Moraes *et al.,* 2013; Marques *et al.,* 2014). Furthermore, in orchids such as epidendrums, interspecific hybridization is relatively easy, and it has been extensively employed in breeding novel cultivars. Over 800 combinations of interspecific hybrids in the genus *Epidendrum* have been registered in Orchid Wiz version 12.3 (OrchidWiz, LLC. Louisville, Colorado, USA. The chromosome numbers of wild species in the genus *Epidendrum* range from $2n = 24$ to $2n = 240$, and some wild species have polyploids (Aoyama 1994; Pinheiro *et al.,* 2009; Assis *et al.,* 2013).

In ornamental plants, intraspecific and interspecific hybridizations have commonly been conducted to obtain novel characteristics in flowers, such as color and size, as well as other important traits for commercial production, including altered flowering periods, environmental stress tolerance, and disease resistance (Nimura *et al.*, 2006, Vendrame *et al.,* 2007, Nimura *et al.,* 2008, Laskowska *et al.,* 2015, Zhang *et al.,* 2017, Kondo *et al.,* 2020). Interspecific cultivars of *Epidendrum* mainly originated from the breeding of several wild species, *i.e.*, *E. radicans*, *E. secundum*, *E. cinnabarinum,* and *E. jamiesonis*. Those cultivars have larger flowers and thicker leaves than the wild species. The

most common consequence of polyploidy is the increase in plant organs caused by the augmented number of copied genes, referred to as the ‘‘gigas’’ effect (Sattler *et al.,* 2016, Hlaing *et al.,* 2020). I postulated that the *Epidendrum* cultivars, which have larger flowers and thicker leaves, are polyploid. However, to the best of my knowledge, there is no information on their number of chromosomes. The information regarding ploidy in *Epidendrum* is essential for future breeding programs because a reproduction barrier in interploid crosses has been reported (Behrend *et al.,* 2015).

In this study, I first confirmed the variation of chromosome numbers among interspecific cultivars. I also analyzed the nuclear DNA amount in interspecific cultivars and wild species in *Epidendrum*. I revealed a correlation between them, enabling the estimation of chromosome numbers and ploidy levels easily by measuring the amount of DNA through flow-cytometry (FCM). I postulated the ploidy levels of interspecific cultivars in *Epidendrum* and compared them with the data obtained from these two analyzes.

Materials and Methods

Plant materials

Ten *Epidendrum* hybrids, including *E*. Narrative Genji 'Fujitsubo', *E*. Venuspiars 'Akatsuki', 'Towaremon' and 'Towaorange', *E*. Sunny Girl 'Toki' and *E*. 'White 1', were gifts from Floriculture Niyodo Co., Ltd. (Kochi Pref., Japan). *E. radicans* 'Col' was collected *in situ* in Colombia, and *E. radicans* 'Lavender', *E. secundum* var. *secundum* and *E. cinnabarinum* were purchased from Matsumoto Orchid (Tokyo, Japan). *E. radicans* 'Miura' was purchased from Orchid Valley Miura (Kanagawa Pref., Japan). *E. secundum* var. *purpureum* was purchased from the Mochizuki Orchid Nursery (Ibaraki Pref., Japan). *E. radicans, E. secundum*, and *E. cinnabarinum* were used to produce hybrids used in the present study. Plant materials were cultivated in a greenhouse maintained below 38 °C in summer and higher than 11 °C in winter. They were potted with sphagnum in good drainage or bark and Sumitomo No. 2 (Sumitomo Chemical Co., Ltd. Tokyo, Japan), and an aqueous fertiliser, which was diluted to contain 0.05% nitrogen, was applied regularly. Eight fungicides, namely Benlate® (Sumitomo Chemical Co., Ltd. Tokyo, Japan), Daconil® (Sumitomo Chemical Co., Ltd.), Topsin M® (Nippon Soda Co., Ltd. Tokyo, Japan), Zampro® (BASF Japan Ltd. Tokyo, Japan), Revus® (Syngenta Japan Co., Ltd.), Kinondo- Hydration No 40® (Agro-Kanesho Co., Ltd. Tokyo, Japan), Ridomil Gold MZ® (Syngenta Japan Co., Ltd. Tokyo, Japan), and Bellkute® (Nippon Soda Co., Ltd.), and five pesticides, namely Agri-Mek® (Syngenta Japan Co., Ltd.), Actara® (Syngenta Japan Co., Ltd.),

Marathon® (Sumitomo Chemical Garden Products Inc. Tokyo, Japan), Gracia® (Nissan Chemical Co., Ltd. Tokyo, Japan), and Hachi-Hachi® (Nihon Nohyaku Co., Ltd. Tokyo, Japan), were sprayed.

Counting the chromosome number

Method of chromosome counting by Koehler *et al.,* (2008) was followed, otherwise stated. Chromosome numbers were counted using a minimum of eight yellow root tips collected from three or more vegetatively propagated plants. The root tip sampling was conducted at least one week after spraying agricultural chemicals to avoid unexpected adverse effects. Root tips of 5-8 mm long were excised at around 10:30 a.m. and pretreated in 2 mM 8-hydroxyquinoline for 48 h at 10℃. They were then fixed with a mixture of ethanol and acetic acid at a 3:1 (v/v) ratio for 3-5 h at room temperature. After fixation, the root tips were stored in 70% ethanol at 4℃. In preparation for chromosome observation, the stored root tips were soaked in distilled water for one hour to remove the ethanol. Then, the root segments were cut on a glass slide to isolate the distal 0.5 mm portion of the root tips and an addition of a 10 μL enzyme solution containing 2% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Ltd. Tokyo, Japan) and 2% (w/v) Pectolyase Y-23 (Kikkoman Corporation Chiba, Japan) was added to them on the slide. The slides were incubated for 20-30 minutes at 37℃ in a sealed container humidified with water. Then, enzyme solution was wiped and a drop of

45% acetic acid was put on it. After 1-2 minutes, cover glass was put on the root tips and squashed with finger with accuracy. Cover glass on the slide glass was removed by razor blade one day after freezing in -80℃. The cells were stained with 5 μg/mL DAPI (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) along with mounting medium (VECTASHIELD Burlingame, USA), observed using a fluorescence microscope at ×1000 magnification (Model BX53; Olympus, Tokyo, Japan), and photographed using a camera (Cool SNAP Myo, Arizona, USA). The chromosome lengths of six wild taxa were measured using Image J (https://imagej.nih.gov/ij/).

Measurement of the nuclear DNA amount

The youngest leaves of plantlets were analyzed by flow cytometry (FCM). Fresh leaves of approximately 2×2 mm in length were chopped with a razor blade for 20 seconds in Petri dish containing 200μL extraction buffer (solution A of the Quantum Stain NA UV 2 Germany) which was added with 800μL buffer for DAPI staining (solution B of the Quantum Stain NA UV2, Germany) and then passed through a nylon sieve (40 μm mesh). After two-minutes of stain by DAPI, flow cytometric analysis was conducted using CA II cytometer (Partec, Munster, Germany). For each sample, approximately 3,000 nuclei were analyzed for a mean value with a coefficient variation of < 4% (data with a coefficient variation of more than 4% was not included). *Eustoma grandiflorum*, which has a 2C DNA amount = 3.26 pg (Lindsay *et al.,* 1994), was used as the internal standard throughout the

analysis. DNA amounts were calculated by comparing *Eustoma grandiflorum* and expressed in 'units' instead of 'picograms' because the DNA amount was analyzed only by DAPI staining (Nimura *et al.,* 2008). Amount of DNA per chromosome number (DNA amount of chromosome) was calculated by dividing the nuclear DNA amount by the number of chromosomes.

Results

Chromosome numbers of the six wild species plant materials consisting of three taxa in *E. radicans*, two taxa in *E. secundum*, and a taxon of *E. cinnabarinum* were examined in this study and ranged from $2n$ = 30-80 (Fig. 1, Table 1). *E. radicans* 'Col' had $2n$ = 38, *E. radicans* 'Lavender' had $2n$ = 60, *E. radicans* 'Miura' had $2n$ = 80, *E. secundum* var. *secundum* had $2n$ = 30, *E. secundum* var. *purpureum* had $2n$ = 60, and *E. cinnabarinum* had $2n$ = 64. The chromosome number of *E. secundum* var. *purpureum* was two times that of *E. secundum* var. *secundum*. The resultant DNA amount of chromosome were consistent in *E. radicans* 'Lavender' and *E. radicans* 'Miura' as well as *E. secundum* and *E. secundum* var. *purpureum* (Table 1). However, the value was inconsistent among *E. radicans* 'Lavender', *E. radicans* 'Miura', and *E. radicans* 'Col', no significant difference in chromosome length was shown among the six wild species plant materials.

The six cultivars examined in this study showed chromosome numbers in a range of $2n$ = 84-164 (Fig. 2, Table 1). The chromosome numbers were different among plants having the same hybrid name with common parentage; *E.* Venuspiars: *E.* Venuspiars 'Akatsuki', Venuspiars 'Towalemon', and Venuspiars 'Towaorange'. The resultant DNA amount of chromosome of 6.89-8.94 (10^{-2} units) was comparatively higher than that of wild species 5.30-7.13 (10^{-2} units) (Table 1). A significant positive correlation of R^2 = 0.98 in the nuclear DNA amount and chromosome number of wild species and cultivars was observed (Fig. 3).

Discussion

The *Epidendrum* varies in chromosome number. Ranges from $2n = 24$ to $2n = 240$, and basic number of $x = 12$ and $x = 20$ were observed in most of the representative samples of the given genus (Assis *et al.,* 2013). *E. radicans* showed variation in chromosome numbers with $2n = 38$, 60, and 80. However, the DNA amount of chromosome of *E. radicans* 'Col' was 5.3; it was lower than those of *E. radicans* 'Lavender' and *E. radicans* 'Miura' of 6.55 and 6.39, respectively. In previous studies, *E. radicans* showed assorted chromosome numbers with $n = 19$ and $2n = 40$, 57, 60, 62, 64 (Mehra *et al.,* 1970, Tanaka and Kamemoto, 1984, Pinheiro *et al.,* 2009) that could be divided into three groups. The first group had a basic number of $x = 19$, and included *E. radicans* 'Col' with $2n = 38$ and $n = 19$, $2n = 57$ in the previous studies (Mehra *et al.,* 1970, Tanaka and Kamemoto, 1984). The second group had a basic number of $2n = 20$ and included *E. radicans* 'Lavender' with $2n = 60$ and *E. radicans* 'Miura' with $2n = 80$ and $2n = 40$, 60 in the previous studies (Tanaka and Kamemoto, 1984, Pinherio *et al.,* 2009). In the third group, the other chromosome numbers of $2n = 62$ and 64 were suggested to be aneuploids of the two groups above. Based on the results of chromosome number and nuclear DNA amount, *E. radicans* 'Col' in the first group, *E. radicans* 'Lavender,' and *E. radicans* 'Miura' could be cryptic species or non-pure species produced *via* introgression of any other species. *E. secundum* exhibited a wide variation in chromosome number, having $2n = 28$, 30, 40, 42, 48, 50, 52, 56, 58, 68, 80, and 84 because hybridization and introgression may have occurred among other closely related

species described in the previous studies (Pinheiro *et al.,* 2009, Assis *et al.,* 2013). In the present study, *E. secundum* showed two chromosome numbers, $2n = 30$ and $2n = 60$. Furthermore, the DNA amount of chromosome of *E. secundum* var. *secundum* and *E. secundum* var. *purpureum* were almost identical. If the basic number of *E. secundum* is $x = 15$, then *E. secundum* var. *secundum* is diploid and *E. secundum* var. *purpureum* is tetraploid when both taxa of *E. secundum* are pure species. *E. cinnabarinum* showed $2n = 64$, which is inconsistent with $2n$ = ca. 240, n = 108-124 in the previous studies (Conceição *et al.,* 2006, Felix *et al.,* 2010). I could postulate that the *E. cinnabarinum* analyzed in the present study were of a lower ploidy than those in previous reports. Another possibility could be that the plant materials used in the present study or previous are a cryptic species within *E. cinnabarinum*. Further observation is needed using additional plant materials with passport data verifying their *in situ* collections. It is also possible that the *E. cinnabarinum* analyzed in the present study is a cryptic species or has a lower ploidy than in previous reports (Conceição *et al.,* 2006, Felix *et al.,* 2010).

Cultivars examined in the present study showed more chromosomes than wild species in a range of $2n$ = 84-164, suggesting that the cultivars' polyploid levels were high. The formation of polyploids could have been achieved through two processes: polyploidization in wild species *in situ* and/or from hybridization during breeding. Unreduced reproductive cells having somatic chromosome numbers rather than gametophytic ones, are considered predominant pathways leading to

polyploidization in plants (De Storme and Geelen, 2013, Sattler *et al.,* 2016, Loginova and Silkova, 2017). Although nonhybrid plants usually produce only 0.1-2.0% of unreduced 2*n* gametes, interspecific hybrids often produce more unreduced 2*n* gametes than nonhybrids, in the frequencies of 5.1% in *Rosa*, 6.99-39.93% in *Lilium*, and 0.06-3.29% in *Brassica* (Crespel and Gudin, 2003, Barba-Gonzalez *et al.,* 2005, Ramsey, 2007, Mason *et al.,* 2011, Kreiner *et al.,* 2017). In contrast, in *Epidendrum*, wide variations of chromosome numbers in wild species were reported in previous studies (Pinheiro *et al.,* 2009, Assis *et al.,* 2013). Thus, it is plausible that unreduced 2*n* gametes play crucial roles in the formation of polyploids both in *in situ* wild species and also in the development of cultivars. The present study suggests that we could estimate chromosome number and ploidy by a measurement of nuclear DNA amount using.

I have revealed that the occurrence of polyploids in wild species and cultivars in *Epidendrum* could have been induced *via* unreduced 2*n* gametes. For further experimentation, aiming to elucidate the mechanisms for the induction of unreduced 2*n* gametes, plant materials examined in the present study would be suitable. Furthermore, the series of ploidy levels in the plants shown here could also be appropriate materials for revealing the mechanisms leading to barriers in interploidy hybridization, with an emphasis on recovery provided by unreduced 2*n* gametes.

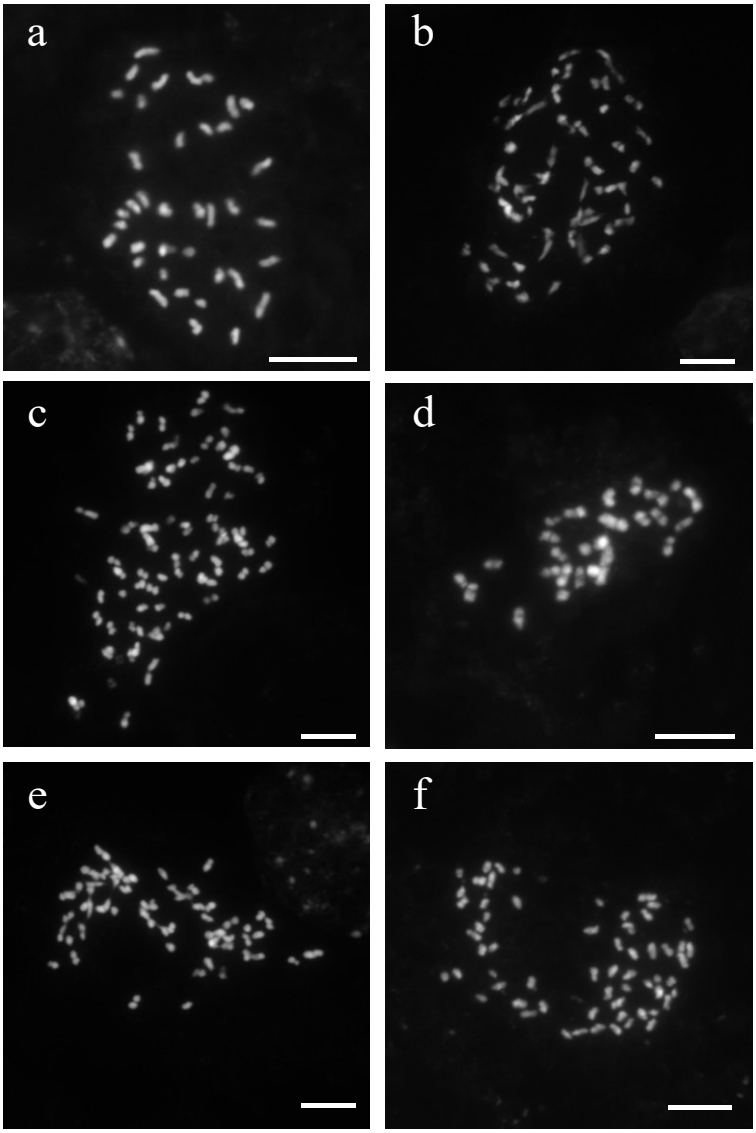


Fig. 1 Metaphase chromosomes of *Epidendrum* wild species. a. *E. radicans* 'Col' (2*n* = 38). b. *E. radicans* 'Lavender' (2*n* = 60). c. *E. radicans* 'Miura' (2*n* = 80). d. *E. secundum* (2*n* = 30). e. *E. secundum* var. *purpureum* (2*n* = 60) f. *E. cinnabarinum* (2*n* = 64). Scale bar = 10μm.

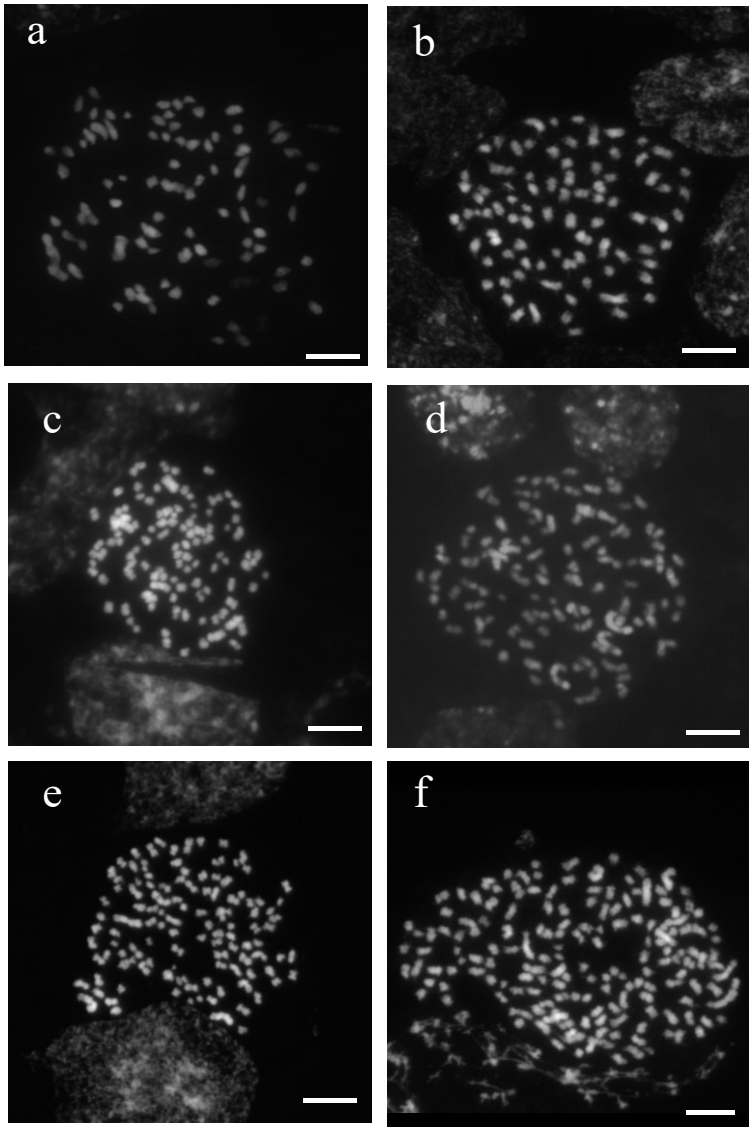


Fig. 2 Metaphase chromosomes of *Epidendrum* cultivar. a. *E.* Narrative Genji 'Fujitsubo' (2*n* = 84). b. *E.* 'White1' (2*n* = 88). c. *E.* Sunny Girl 'Toki' (2*n* = 102). d. *E.* Venuspiars 'Akatsuki' (2*n* = 106). e. *E.* Venuspiars 'Towalemon' (2*n* = 120). f. *E.* Venuspiars 'Towaorange' (2*n* = 164). Scale bar = 10μm.

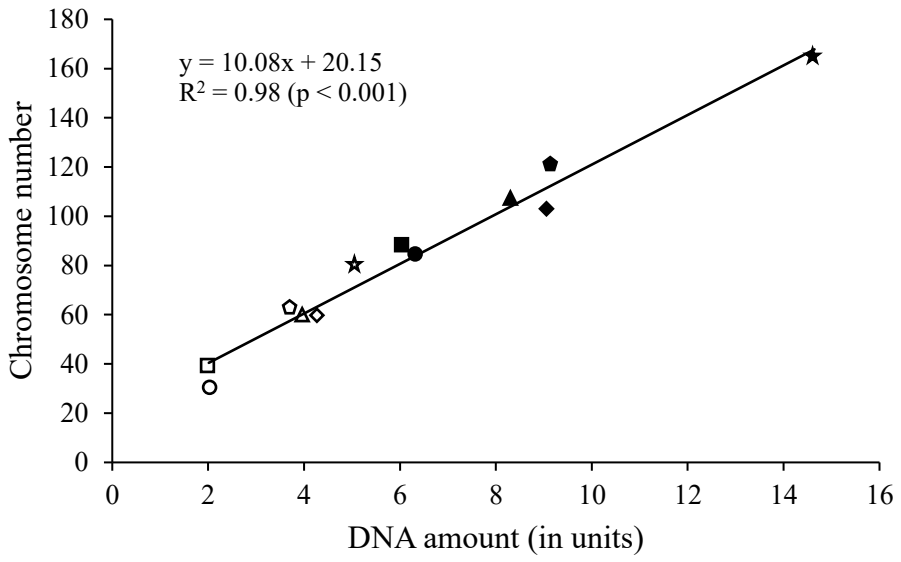


Fig. 3. Relationship between the nuclear DNA amount (axis x) and chromosome number (axis y) of *Epidendrum* wild species and cultivar. Open markers indicate wild species (Circle = *E. secundum*, Square = *E. radicans* 'Col', Diamond = *E. secundum* var. *purpureum*, Triangle = *E. radicans* 'Lavender', Pentagon = *E. cinnabarinum*, Star = *E. radicans* 'Miura'); solid shapes indicate cultivars (Circle = *E.* Narrative Genji 'Fujitsubo', Square = *E*. 'White1', Diamond = *E*. Sunny Girl 'Toki', Triangle = *E*. Venuspiars 'Akatsuki', Pentagon = *E*. Venuspiars 'Towalemon', Star = *E*. Venuspiars 'Towaorange'). The statistical analysis shows a linear correlation (R^2 = 98%) between DNA amount (in units) and chromosome number.

Table1 number of chromosome and DNA amount.

Plant materials			2*n* chromosome number	DNA amount (units)	DNA amount/chromosome number (x100)	Chromosome length (μm)	Previous reports*	
E. radicans	'Col'	wild species	38	2.01	5.30	1.99 ± 0.10		
	'Lavender'	wild species	60	3.93	6.55	2.57 ± 0.24	*n* = 19; 2*n* = 40, 57, 60, 62, 64	ME70, TK1984, PI09
	'Miura'	wild species	80	5.11	6.39	2.29 ± 0.18		
E. secundum	var. *secundum*	wild species	30	2.05	6.82	2.06 ± 0.13	2*n* = 28, 30, 40, 42, 48, 50, 52, 54, 56, 58, 68, 80, 84	PI09, FG10, AS13
	var. *purpureum*	wild species	60	4.28	7.13	2.58 ± 0.14		
E. cinnabarinum		wild species	64	3.73	5.83	2.01 ± 0.09	*n* = 108, 124; 2*n* = 240	FG10, AS13
E. Narrative Genji	'Fujitsubo'	cultivar	84	6.36	7.57	n.d.		
E.	'White1'	cultivar	88	6.06	6.89	n.d.		
E. Sunny Girl	'Toki'	cultivar	102	9.12	8.94	n.d.		
E. Venuspiars	'Akatsuki'	cultivar	106	8.34	7.87	n.d.		
	'Towalemon'	cultivar	120	9.16	7.63	n.d.		
	'Towaorange'	cultivar	164	14.64	8.93	n.d.		

*ME70 = Mehra *et al*. 1970, TK 1984 = Tanaka and Kamemoto 1984, PI09 = Pinheiro *et al.* 2009, FG10 = Felix and Guerra 2010, AS13 = Assis *et al.* 2013

Chapter 3

Two pathways of 2*n* gamete formation and differences in the frequencies of 2*n* gametes between wild species and interspecific hybrids

Introduction

Many studies have reported cytological mechanisms for the induction of 2*n* gametes. Although the most common pathways for induction of 2*n* gametes are meiotic defects, the defects vary in phase and mechanism. Brownfield & Köhler (2011) proposed: (i) defects in sister chromatid cohesion and recombination in prophase I, (ii) omission of meiosis in metaphase I or metaphase II, (iii) disruption of spindle orientation in meiosis II, and (iv) nuclear restitution due to defects in cytokinesis after nuclear division. As an alternative mechanism that generates 2*n* gametes from tetraploid pollen mother cells (PMCs) in the pre-meiotic phase, pre-meiotic chromosome doubling has only been reported for a small set of isolated species in three genera, namely, *Turnera, Avena*, and *Solanum* ssp. (Katsiotis & Forsberg, 1995; Fernandez & Neffa, 2004; De Storme & Geelen, 2013b). The genetic implication of gametes should be emphasised in association with the mechanism of 2*n* gamete formation (Brownfield & Köhler, 2011). Furthermore, 2*n* gametes that contain non-sister chromatids and provide first division restitution (FDR) during meiosis retain much of the heterozygosity. In contrast, gametes containing sister chromatids that provide second division restitution (SDR) during meiosis show less heterozygosity (Brownfield & Köhler, 2011). Moreover, the genetic implication of 2*n* gametes from tetraploid PMCs is almost identical to that of gametes from autotetraploids due to normal meiosis.

In ornamental plants, intraspecific as well as interspecific hybridiations have commonly

been conducted to obtain novel characteristics of flower traits, such as color and size, as well as other important characteristics for commercial production, such as an altered flowering period, tolerance to environmental stress, and disease resistance (Nimura *et al.,* 2006; Vendrame *et al.,* 2007; Nimura *et al.,* 2008; Laskowska *et al.,* 2015; Zhang *et al.,* 2017; Hlaing *et al.,* 2020). Plants that originate without interspecific hybridization usually produce only 0.1–2.0% of 2*n* gametes (Ramsey, 2007; Kreiner *et al.,* 2017b). In contrast, interspecific hybrid plants produce substantially more 2*n* gametes, and the frequencies of 2*n* gametes estimated by observing dyads during microsporogenesis have been reported to be 5.1% and 0.06–3.29% in *Rosa* and *Brassica*, respectively (Crespel & Gudin, 2003, Mason *et al.,* 2011). Some interspecific hybrids produce reduced non-viable gametes and 2*n* viable gametes (Barba-Gonzalez *et al.,* 2004; Fakhri *et al.,* 2016). Although many studies have been conducted on 2*n* gametes of non-hybrid wild species and some hybrids, only few studies have compared 2*n* gametes between wild species and hybrids.

Unreduced 2*n* gamete production is influenced by environmental factors, genetic factors, reproductive mode, life history, and ploidy level (Crespel *et al*. 2006; Brownfield and Köhler 2011; Kreiner *et al*. 2017a; Kreiner *et al*. 2017b). Some genes related to the production of unreduced genes have been reported in previous studies, e.g., *el1* in *Zea*, *Atps1* in *Arabidopsis*, and *ps* in *Solanum* (d'Erfurth *et al*. 2008; Loginova and Silkova 2017). High or low temperatures as environmental stress factors can stimulate the production of 2*n* gametes in various plants (Mason *et al*. 2011; Stome *et al.*

2012; Zhou *et al*. 2015; Mai *et al*. 2019).

The chromosome numbers of wild species in the genus *Epidendrum* range from 2*n* = 24 to 2*n* = 240, and some wild species have polyploids (Pinheiro *et al.,* 2009; Assis *et al.,* 2013; Kondo *et al.,* 2021). In addition to wild species, cultivars originating from interspecific hybridization have more chromosomes than the wild species, thus, indicating the high polyploid levels of cultivars. Therefore, polyploids of wild species and cultivars in *Epidendrum* could have been induced via 2*n* gametes. In almost all orchids, including those belonging to the genus *Epidendrum*, mature sporads maintain tetrads post meiosis (Pacini & Hesse, 2002). Owing to this intrinsic trait of orchids, we could estimate the process of meiosis, that is, tetrads form due to normal meiosis and dyads form due to irregular meiosis.

The frequencies of unreduced 2*n* gametes were estimated using a variety of methods, including flow cytometry, observation of meiosis, and measurement of pollen sizes (Dweikat & Lyrene, 1988; Dewitte *et al.,* 2009; Laere *et al.,* 2009). Pollen size is an indicator of unreduced 2*n* gametes (Lerne *et al.,* 2009; Nikoloudakis *et al.,* 2018; Mai *et al.,* 2019). Unreduced 2*n* pollen is larger than normal reduced pollen because unreduced 2*n* pollen contains a larger amount of DNA in cells, for example, the size ratio of diameters of normal versus 2*n* gametes has been reported to be 1:1.42 and 1:1.5 in *Achillea* and *Brassica*, respectively (Ramsay, 2007; Mason *et al.,* 2011). Although various cytological mechanisms have been reported for the induction of 2*n* gametes, one plant species

generally has one predominant pathway of 2*n* gamete formation (Fernandez & Neffa, 2004; Brownfield & Köhler, 2011; De Storme & Geelen, 2013b). In the present study, I revealed two pathways of production of 2*n* gametes and difference(s) in the frequencies of different types of sporads through the observation of several stage of meiosis and the measurement of sporads sizes, viabilities, and DNA content. The flowering periods of *Epidendrum* hybrids were very long, *e.g*., from January to November in 'Akatsuki'. Thus, I compared the frequencies of unreduced 2*n* gametes in spring, summer, and winter for the assessment of a relationship between environmental factors and irregular meiosis.

Materials and Methods

Plant materials

Ten *Epidendrum* hybrids, namely *E*. Narrative Genji 'Fujitsubo', *E*. Cosmo Dream Color 'Towaakane' and 'Kochimomo', *E*. Venuspiars 'Akatsuki', 'Towaremon' and 'Towaorange', *E*. Sunny Girl 'Toki', *E*. Yoko Yokohama, *E*. 21st century 'Red', *E*. 'White 1', and ten *Epidendrum* wild species, namely *E. radicans* 'Col', *E. radicans* 'Lavender', *E. radicans* 'Miura', *E. secundum* var. *secundum*, *E. secundum* var. *purpureum, E. cinnabarinum*, *E. xanthinum, E. ibaguense*, *E. calanthum*, and *E. calanthum* 'White', were used as plant materials. Four *Epidendrum* hybrids, including *E*. Narrative Genji 'Fujitsubo', *E*. Cosmo Dream Color 'Towaakane' and 'Kochimomo', and *E*. 21st century 'Red', were gifts from Floriculture Niyodo Co., Ltd. (Kochi Pref., Japan). *E. xanthinum* were purchased from Matsumoto Orchid (Tokyo, Japan). *E. calanthum*, and *E. calanthum* 'White', were purchased from Orchid Valley Miura (Kanagawa Pref., Japan). Source of other plant materials and cultivation conditions were the same as those mentioned in Chapter 2.

Cytological observation of sporad development

The development and viability of sporads were observed using the methods described by Aoyama (1994). Pollinia obtained from five flowers during anthesis of each plant were fixed with a mixture of ethanol and acetic acid in 3:1 (v/v) at room temperature of 20-28 ℃ overnight.

Subsequently, 45% acetic acid was dropped on pollinia on the slide to remove the fixation solution. Pollinia were divided into pieces using razor blades, and this was followed by treatment with a mixture of 1 N HCl and 45% acetic acid (2:1 v/v) and staining with 2% acetic orcein at room temperature. Furthermore, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining was performed as described by Kondo *et al*. (2021) unless stated otherwise. Cells were stained with 5 µg/mL DAPI (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) along with mounting medium (VECTASHIELD Burlingame, USA), observed using a fluorescence microscope at ×1000 magnification (Model BX53; Olympus, Tokyo, Japan), and photographed using a camera (Cool SNAP Myo, Arizona, USA). Sporads were classified into five types, namely monad, dyad, triad, tetrad (normal type), and others (e.g., micronucleus and polynucleus) according to previous studies (Aoyama, 1994; Lee *et al.,* 2011). The predicted frequencies of 2*n* gametes were calculated using the following formula by Zhou *et al*. (2015): Frequencies of 2*n* gametes (%) = (2 × dyad + triad) / (monad + 2 × dyad + 3 × triad + 4 × tetrad). For each sample, at least 100 sporads were counted, and their frequencies were calculated. Microscope 'BA210E1080' (Shimazu Rika Co., Ltd., Tokyo, Japan) and Camera 'Moticam 1080BMH' (Shimazu Rika Co., Ltd.) were used for observation and imaging. The sizes of dyads and tetrads of 10 *Epidendrum* wild species were measured using ImageJ software (https://imagej.nih.gov/ij/ National Institutes of Health, Bethesda, USA). Microgamete sizes of dyads were calculated according to the following formula: whole areas of dyad/2. Similarly, microgamete

sizes of tetrads were calculated as follows: whole areas of tetrad/4. At least 50 dyads and 100 tetrads from three flowers were measured for each replication.

Unreduced 2n gametes formation under various environmental conditions

To assess of a relationship between environmental factors and irregular meiosis, the pollinia were collected at different timings of the year 2020, namely March 5th, April 14th, July 7th, August 25th and September 8th. The temperature data between January to October were shown in Fig.2. Table 1 indicated mean value of daily maximum temperature, daily average temperature, daily minimum temperature and day length between 4 weeks and 2 weeks before flowering date, because the timing of meiosis is approximately 3 weeks before flowering in *Epidendrum* (data not shown).

To assess relationship between temperature and unreduced 2*n* pollen formation, a growth chambers were used. Five pots of 'Fujitsubo' were placed in each growth chambers. Each chamber was set to a 14-h photoperiod and one of three day/night temperature regimes: 35/25 (High temperature), 25/15 (Cool temperature), and 20/10 (Low temperature). The day/night temperature change began when the lights were turned on or off. Pollinia were collected every one weeks between 2 weeks and 4 weeks after treatment.

In vitro germination of pollinia

Pollen germination test was conducted by culturing pollinia on BK medium (Brewbaker *et al.,* 1963) supplemented with 100 g L^{-1} sucrose and 2% agar (FUJIFILM Wako Pure Chemical Corporation. Osaka, Japan). The pH of the medium was adjusted to 4.0, and the medium was warmed in a microwave until the agar melted perfectly. After 48 h of incubation at 25 °C, pollinia were fixed with a mixture of ethanol and acetic acid in 3:1 (v/v) at room temperature overnight. After fixation, pollinia were transferred to 70% ethanol for storage at 4 °C. Pollinia incubated on the medium was observed in the same manner as for sporad observation. Pollinia germination was defined when the pollen tube had developed in length over the diameter of original sporad and at least 100 sporads per pollinia were counted. The germination test of sporads was replicated five times.

Determination of ploidy levels via flow cytometry analysis

The methods were the same as those mentioned in Chapter 2 unless stated otherwise. Fresh pollinia or fresh somatic tissues of the leaf and ovary wall were analyzed via flow cytometry. To analyze the ploidy levels of uninuclear microspores, nuclei were extracted from immature pollinia isolated from inflorescence buds at the appropriate stage (Fig. 1) without mixing with other somatic tissues. Peak analysis of the PA cytometer (Partec, Munster, Germany) equipped with a filter combination of UG-1 and GG435 was used to calculate the mean value of each peak. For each sample of mature pollinia, 1,500–3,000 nuclei were analyzed. *Eustoma grandiflorum*, which has a 2C DNA

amount of 3.26 pg (Lindsay *et al.,* 1994), and *Antirrhinum majus*, which has a 2C DNA amount of 1.30 pg (Zaitlin & Pierce, 2010), were used as biological internal standards. DNA amounts were calculated by comparing *Eustoma grandiflorum* or *Antirrhinum majus*, and they were expressed in 'units' instead of 'picograms' as the DNA amount was analyzed only via DAPI staining (Nimura *et al.,* 2008).

Statistical analysis

Data were evaluated using R version 3.4.2 statistical software (R Development Core Team, Australia, https://www.r-project.org/). Analysis of variance was performed using the aov function, and multiple comparisons were calculated using the Tukey HSD function. Differences were considered significant at $p \leqq 0.05$. Normality of data of sporad sizes was calculated using the Shapiro-Wilk normality test.

Results

Morphology of sporads

Irregular sporad, namely, monads, dyads, and triads, were produced using both wild species and hybrids (Fig. 3; Fig. 4). Mature pollinia always showed a characteristic binuclear configuration with one highly condensed, generative nucleus exhibiting bright fluorescence and one vegetative nucleus exhibiting weak fluorescence in DAPI staining (Fig. 3d-f). The dyad and triad had two and one large sporads, respectively. Nuclei in such large sporads indicated a higher amount of DNA content than that in normal tetrads. The frequencies of irregular sporad formation in all 10 wild species examined in the present study were in the range of 4.9–42.8% (Fig. 4a). In contrast, the frequency of irregular sporad formation in the 10 hybrids showed a wider range of 27.8–99.1%, with that in 'Fujitsubo' being 27.8% and in 'Kochimomo' being 99.1% (Fig. 4b). Additionally, the present study showed that hybrids produced higher frequencies of irregular sporads than all 10 wild species examined except for 'Fujitsubo' and 'Toki'. The frequency of dyads was the highest among the three types of irregular sporads produced using both wild species and hybrids. The frequencies of 2*n* gametes were in the range of 1.7–21.7% and 9.1–83.9% in wild species and hybrids, respectively.

Estimation of ploidy level of pollinia via flow-cytometric analysis

Flow-cytometric analysis of somatic tissues of the ovary wall showed three peaks of 2C, 4C,

and 8C (Fig. 5a). Similar to previous studies, many orchid species, including present materials, exhibited polysomaty in aged or ageing organs (Fukai *et al.,* 2002; Kondo *et al.,* 2020). In contrast, flow-cytometric analysis of immature pollinia at the uninuclear stage showed one large 1C peak and two small peaks of 2C and 4C in *E radicans* 'Miura' (Fig. 5c). As nuclei in the uninuclear stage indicate that only 1C peak of cells of G_1 phase and 2C peak of cells of G_2 phase could be observed in the uninuclear stage of normal meiosis (Table 2), the small 4C peak in immature pollinia suggested the occurrence of 2*n* gametes. Flow-cytometric analysis of mature pollinia showed two peaks of 1C and 2C in almost all wild species and hybrids (Fig. 6a, b). Mature pollinia of orchids are binucleate pollen (Travnicek *et al.,* 2015), and two peaks of 1C and 2C indicated vegetative and generative nuclei, respectively. Unfortunately, no 4C peaks indicating 2*n* gametes in mature pollen were observed due to a difficulty in measuring flow cytometry in mature pollinia in orchids (Travnicek *et al.,* 2015). However, flow-cytometric analysis of pollinia of 'Kochimomo' that produced almost only 2*n* gametes showed only one peak, which nearly corresponded with the peak of leaves of 'Kochimomo' (Fig. 6c).

Germination frequencies of microspores

Except for *E. cinnabarinum*, the germination frequencies of sporads of wild species were higher than 50% (Fig. 7a) whereas the germination frequency of sporads of hybrids were in the range of 0–87.5% (Fig. 7b). No sporads germinated in 'Kochimomo', which produced the largest number of

irregular sporads. No clear-cut relationships were observed between the germination frequency, frequencies of irregular meiosis, and DNA value (Fig. 4–6), e.g., the frequencies of irregular sporads in ‘Towaakane’ were higher than 70% whereas the germination frequency was over 60%, and in ‘Kochimomo’, the frequency was over 90% whereas the germination frequency was nearly zero.

Sizes of microspores and pre-meiotic chromosome doubling

Within the same wild species, the average of the whole area of dyads and tetrads showed no significant difference; however, a microspore of the dyads of wild species were 1.45–1.76 times larger than that of tetrads (Fig. 8).

The DNA value of wild species showed a positive correlation with the size of tetrads of wild species (coefficient of correlation $R^2 = 0.47$) (Table 3). The size of each tetrad within the same wild species varied, and the Shapiro-Wilk normality test did not reveal a normal distribution in size distributions (Fig. 9; Table3). The asymmetric distribution of tetrad size was generally skewed right. Microspore size distribution of *E. radicans* ‘Miura’ showed a multitude of normal tetrad sizes and only few enlarged tetrads (Fig. 9; Fig. 10). Six out of ten wild species showed similar tendencies. In contrast, *E. secundum* var. *purpureum* sporads, which is $2n = 60$ (Kondo *et al*. 2021), contained relatively many enlarged tetrads (Fig. 9c). Moreover, tetraploid meiocytes and enlarged tetrads with two larger nuclei were observed in the pre-meiotic stage and mature pollinia, respectively (Fig. 10 b, d). The frequencies

of tetrads, which were 1.5-fold and 2.0-fold larger than the average size of tetrad, were in the range of 1.7–12.7% and 0-3.0%, respectively, in the 10 wild species (Table 3). Furthermore, seven out of ten *Epidendrum* hybrids, namely, *E*. Narrative Genji 'Fujitsubo', *E*. Cosmo Dream Color 'Towaakane', *E*. Venuspiars 'Akatsuki', 'Towaremon' and 'Towaorange', *E*. Sunny Girl 'Toki', *E*. Yoko Yokohama, examined also produced enlarged tetrad (data not shown).

Unreduced 2*n pollen formation under various environmental conditions*

Seven out of ten *Epidendrum* hybrids showed significant difference of frequencies of unreduced 2*n* pollen between the different seasons (Fig. 11). In general, irregular meiosis of pollinia which collected in spring (April 14th) were lowest. Additionally, the present study showed that the frequencies of unreduced 2*n* pollen formation were influenced by seasonal effect in both summer and winter, e.g. 'Akatsuki' produced three times as much unreduced 2*n* pollen in summer (August and September) as in spring, 'Towaakane' produced three times as much unreduced 2*n* pollen in winter (March 5th) as in spring, and 'Fujitsubo' produced 2-3 times as much unreduced 2*n* pollen in winter and summer as in spring.

Although the frequency of unreduced 2*n* pollen production was higher in the high temperature treatment than the cool temperature treatment, no significant difference was observed between high temperature treatment and cool temperature treatment (Fig.12 a). Furthermore,

frequency of dyad and triad increased from 7 days to 21 days after the heat temperature treatment, whereas abnormal microspores, namely polynucleus increased instead of dyad and tetrad in the 28 days after the high temperature treatment (Fig. 12b and c). Thus, the frequency of unreduced 2*n* pollen decrease in the 28 days after the high temperature treatment. No difference was observed between the cool temperature treatment and low temperature treatment (Fig. 13).

Discussion

2n gamete formation via two pathways

Many studies have reported cytological mechanisms for the induction of 2*n* gametes. In the present study, unreduced 2*n* gametes such as irregular sporads, namely, dyads and triads, via meiotic defects, were produced by both wild species and hybrids. Pollen size is an indicator of unreduced 2*n* gametes (Lerne *et al.,* 2009; Nikoloudakis *et al.,* 2018; Mai *et al.,* 2019), for example, the size ratio of diameters of normal versus 2*n* gametes has been reported to be 1:1.42 and 1:1.5 in *Achillea* and *Brassica*, respectively (Ramsay, 2007; Mason *et al.,* 2011). Here, the area ratios of tetrad (normal) versus dyad (2*n* gametes) were 1:1.45–1.76 (Fig. 8), and the ratio of diameters 1:1.20–1.33 that were calculated using area ratios in this study could support that the dyad of *Epidendrum* was an unreduced 2*n* pollen.

The formation of 2*n* gametes mostly involves a restitution of meiotic cell cycle; however, pre-meiotic chromosome duplication in PMCs has rarely been reported in few plant species (De storme & Geelen, 2013a; Nikoloudakis *et al.,* 2018). Tetraploid meiocytes of *E. secundum* var. *purpureum* that were observed in the pre-meiotic stage indicated 2*n* gamete formation via pre-meiotic chromosome doubling (Fig. 10b). Ten wild species produced 1.7–12.7% of enlarged tetrad, which were 1.5-fold larger than the normal tetrad. Based on the size differences between dyad and normal tetrad in *Epidensrum*, the enlarged tetrads indicated that the 10 wild species produced 2*n* tetrads via

pre-meiotic doubling. In contrast, in *E. secundum* var. *purpureum*, the frequency of enlarged tetrads should be estimated by considering the distribution of tetrad size because the average size of all tetrads does not represent the average size of the normal tetrad, with the occurrence of enlarged tetrads with a relatively high ratio (Fig. 9c).

Although various cytological mechanisms have been reported for the induction of 2*n* gametes, one plant species generally has one predominant pathway of 2*n* gamete formation (Fernandez & Neffa, 2004; Brownfield & Köhler, 2011; De Storme & Geelen, 2013b). However, coexistence of two meiotic restitution mechanisms, FDR and SDR, were reported in few plant species of *Populus* (Liesebach *et al.,* 2015) and *Citrus* (Rouiss *et al.,* 2017). Notably, I showed that *Epidendrum* produced 2*n* gametes via two pathways, that is, the main pathways of meiotic defects contained FDR and/or SDR and rare pathways of pre-meiotic chromosome doubling in plants. The genetic implication of gametes is associated with the mechanism of 2*n* gamete formation (Brownfield & Köhler, 2011). Specifically, FDR during meiosis retains much of the heterozygosity, and SDR during meiosis reduces heterozygosity levels (Brownfield & Köhler, 2011). Furthermore, the genetic structure of 2*n* gametes from tetraploid PMCs is similar to that of gametes from autotetraploid normal meiosis (De storme & Geelen, 2013b). Thus, especially in *Epidendrum*, polyploid progeny with various genetic backgrounds might be formed via fertiliszation of 2*n* gametes via three mechanisms, namely, meiotic defeats (FDR and/or SDR) and pre-meiotic chromosome doubling, although further studies are needed to distinguish

FDR and SDR through meiosis observation or molecular analysis of offspring by tracing molecular markers. The unique characteristic of *Epidendrum* with multiple pathways of formation of 2*n* gametes, which leads to polyploidization, can contribute to its adaptation and speciation *in situ*.

Frequencies of unreduced 2*n gametes*

The frequencies of 2*n* gametes were 1.7–21.7% and 9.1–83.9% in wild species and hybrids, respectively. In previous studies, the frequencies of unreduced 2*n* gametes have been estimated to be 0.1–2.0%, 1.21%, and 0.06–2.17% in wild species of *Achillea borealis* (Ramsey, 2007), *Avena ventricosa* (Nikoloudakis *et al.,* 2018), and *Brassica napus* (Sora *et al.,* 2016), respectively, and the frequencies of 2*n* gametes of *Epidendrum* wild species were higher than those of other non-hybrid species. The genus *Epidendrum* shows extensive variations in chromosome number within species (Pinherio *et al.,* 2009; Kondo *et al.,* 2021). According to the present study, polyploidy of wild species in the given genus arose due to 2*n* gametes. Furthermore, the frequencies of 2*n* gametes of *Epidendrum* hybrids were generally higher than those of *Epidendrum* wild species examined. According to previous studies, the percentage of unreduced 2*n* gametes in hybrids of *Rosa* (Crespel & Gudin, 2003), *Hibiscus* (Larne *et al.,* 2009), *Brassica* spp. (Mason *et al.,* 2011), and Lilium (Chung *et al.,* 2013) are 5.1%, 0.35–4.14%, 0.06–3.29%, and 44.6%. In the present study, the frequencies of unreduced 2*n* gametes in many *Epidendrum* hybrids were higher than those in hybrids of other genera. Thus, the present

study suggests that *Epidendrum* wild species have a high productive capacity for 2*n* gametes, and the capacity could be inherited and enhanced in *Epidendrum* hybrids. This suggestion is inconsistent with the results of *Phalaenopsis* orchids, which suggests that the *Phalaenopsis* cultivars of hybrids produce irregular gametes that can be estimated as unreduced 2*n* gametes, although wild species have no productive capacity for unreduced 2*n* gametes (Bolaños-Villegas *et al.,* 2008). The contrasting results indicated the production of unreduced 2*n* gametes in *Epidendrum* hybrids caused due to a combination of production capacity of *Epidendrum* wild species and effects of hybrids, for example, chromosomal behaviour during meiosis, whereas it is only caused due to the hybrid effect in Phalaenopsis.

Unreduced 2*n* gamete production is influenced by environmental factors, genetic factors, reproductive mode, life history, and ploidy level (Crespel *et al.,* 2006; Brownfield & Köhler, 2011; Kreiner *et al,* 2017a; Kreiner *et al.,* 2017b). Some genes related to the production of unreduced genes have been reported in previous studies, e.g., *el1* in *Zea*, *Atps1* in *Arabidopsis*, and *ps* in *Solanum* (d'Erfurth *et al.,* 2008; Loginova & Silkova, 2017). Some of these genes may be involved in the production of unreduced 2*n* gametes in *Epidendrum*. Interspecific hybrids often produce more unreduced 2*n* gametes than non-hybrid plants (Ramsey, 2007; Kreiner *et al.,* 2017). Hybridization has been reported among wild species of *Epidendrum* in the sympatric zone, and it is difficult to distinguish pure species based on morphological characteristics (Moraes *et al.,* 2013; Marques *et al.,* 2014). Thus, hybridization due to the possibility of introgression in wild species might have affected

the production of 2*n* gametes in *Epidendrum* wild species in the present study. Polyploidy affects meiosis, and triploids produce more triads than tetraploids in *Rosa* (Crespal *et al.,* 2006). The *Epidendrum* hybrids used as plant materials in the present study comprise a wide range and high ploidy levels (Kondo *et al.,* 2021). However, the present study did not show a relationship between ploidy levels based on DNA values and irregular meiosis. High or low temperatures as environmental stress factors can stimulate the production of 2*n* gametes in various plants (Mason *et al*. 2011; Stome *et al*. 2012; Zhou *et al*. 2015; Mai *et al*. 2019). The frequencies of unreduced 2*n* gametes were variable seasonally and between genotypes in *Epidendrum*, which suggested that the variation in unreduced 2*n* pollen production is of both environmental and genetic factors (Lim et al., 2004, Chung et al., 2013). Additionally, the present study showed that some cultivars produced higher frequency of unreduced 2*n* pollen in winter than in spring, whereas some cultivars produced higher frequency of unreduced 2*n* pollen in summer than in spring. Thus, unreduced 2*n* pollen formation in genus *Epidendrum* also influenced by high temperature and low temperature. Nevertheless, comparison of high, cool, and low temperature treatment in growth chamber showed no significant difference of production of unreduced 2*n* pollen. Thus, the present study suggested that the unreduced 2*n* pollen production was related to other environmental factors and plant conditions, namely day length, humidity, age of inflorescence and photosynthesis as well as temperature stress.

Fertilities of sporads

Genome constitution and genomic similarity are closely related to the formation of unreduced 2*n* gametes in interspecific hybrids (Zhao *et al.,* 2007; Fakhri *et al.,* 2016). For example, unreduced 2*n* gametes were more viable than normal pollen in *Brassica* spp. hybrids (Mason *et al.,* 2011). Thus, the present study suggested that the fertility of sporads was related to the genomic constitution of *Epidendrum* hybrids as well as frequencies of irregular meiosis. The germination frequencies of almost all wild species and many hybrids were very high, suggesting that unreduced 2*n* gametes had the capacity to engage in fertilization. Mature sporads of many orchids, including the genus *Epidendrum*, maintain tetrad after meiosis, and the plasmodesmata persists through pollen maturation (Yeung, 1987; Pacini & Hesse, 2002). Thus, the mechanism of pollinia formation specific to Orchidaceae may have contributed to the viability of 2*n* gametes in both wild species and hybrids.

Evaluation of unreduced 2*n gametes*

Two peaks were observed using flow cytometry in mature pollenia of orchids with binucleate pollen, suggesting that vegetative nuclei and generative nuclei indicate 1C and 2C peaks, respectively (Travnicek *et al.* 2015). Although the two peaks were observed using flow cytometry, almost 2C peaks with the same DNA contents as that of leaf tissues were wide in range, and their coefficient values were high, ranging from 2.73% to 15.65%, despite nearly 3% of the coefficient

values of *Eustoma* as the internal standard (Fig. 6). Based on these results, two factors could be associated with the incorrect coefficient values of second peaks. First, aneuploidy of microspores could have existed in the sporads of *Epidendrum* hybrids. Allotriploids produce various gametic chromosome numbers with irregular meiosis (Lim *et al*. 2003; Leflon *et al*. 2006). *Epidendrum* hybrids used as plant materials in previous studies can be polyploid or aneuploid and interspecific hybrids because of DNA values (Kondo *et al.* 2021). Thus, second peaks of flow cytometry of *Epidendrum* hybrids could indicate aneuploidal gametes with various DNA values. However, an accurate analysis of flow cytometry could be impeded by folding of impurities, debris, RNA, and microgametes. An improvement in methods is necessary for accurate analysis, for example, using RNAse and ultrasonic treatment for nuclear isolation (Dewitte *et al*. 2006; Kreiner *et al*. 2017; Nikoloudakis *et al*. 2018). Unfortunately, no 4C peaks indicating 2*n* gametes in mature pollen were observed due to a difficulty in measuring flow cytometry in mature pollinia in orchids (Travnicek *et al.* 2015). Therefore, improvement of the flow cytometry method for mature pollinia would also be a prerequisite in future experimentations.

The present results suggested that wild species in the genus *Epidendrum* and their hybrids produced 2*n* gametes which frequencies were higher in hybrids than wild species. Additionally, the formation of 2*n* gametes could have arisen from multiple pathways, namely irregular meiosis, and pre-

meiotic chromosome doubling. Further studies to compare fertilization between normal gametes and 2*n* gametes and investigate genes related to the production of 2*n* gametes are needed for the utilization of 2*n* gametes for breeding and to reveal the formation of 2*n* gametes in Orchidaceae in detail. Furthermore, *Epidendrum* produces a high frequency of 2*n* gametes via multiple pathways, and thus, they would be appropriate materials for revealing plant evolution related to polyploidization and formation of 2*n* gametes.

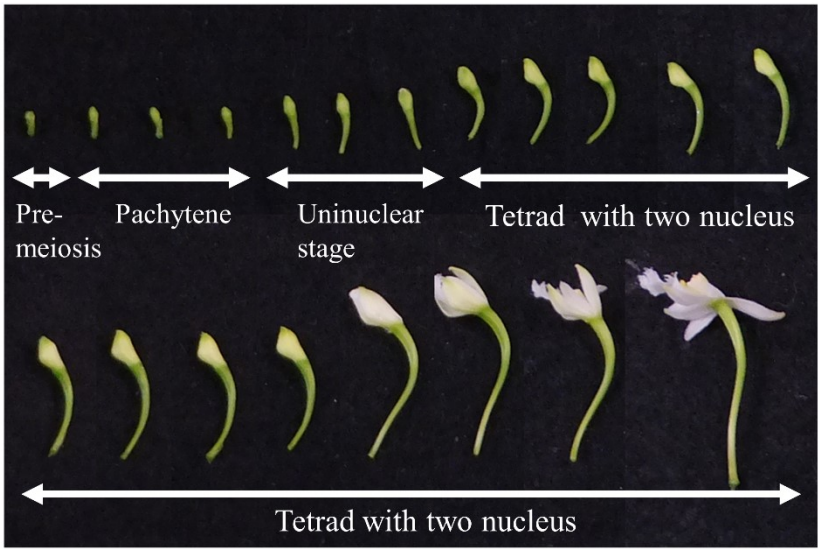


Fig. 1 Stage of inflorescence buds of *Epidendrum.*

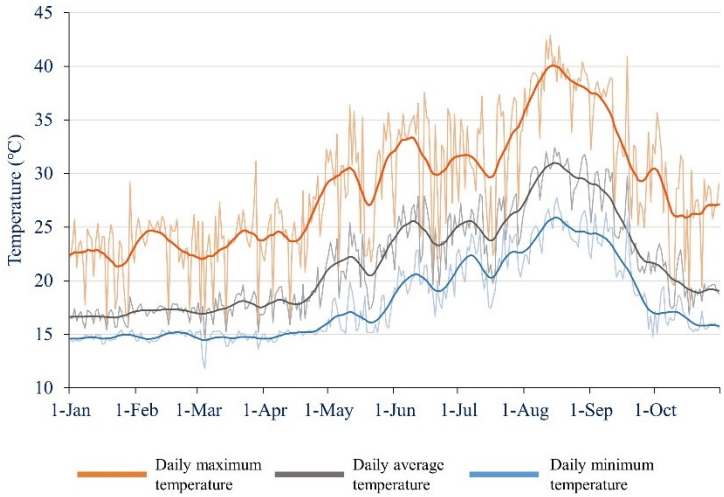


Fig. 2 Temperature from January 1st to October 31st, 2020. Dark lines indicate moving average, light lines indicate daily data.

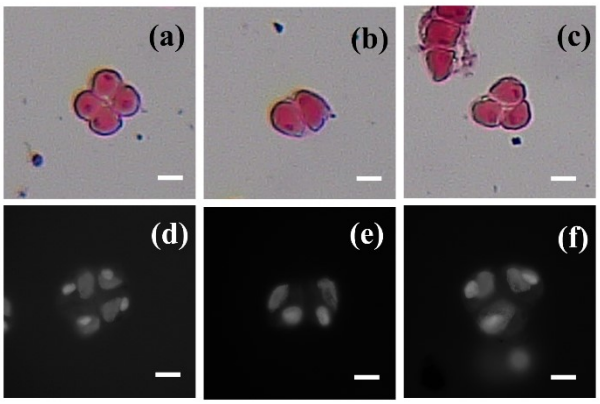


Fig. 3 Three types of microspores of *E. calanthum* 'white' stained with acetic orcein (a-c) and DAPI (d-f). (a) and (d): tetrad (normal), (b) and (e): dyad, (c) and (f): triad. Scale bars = 10 μm.

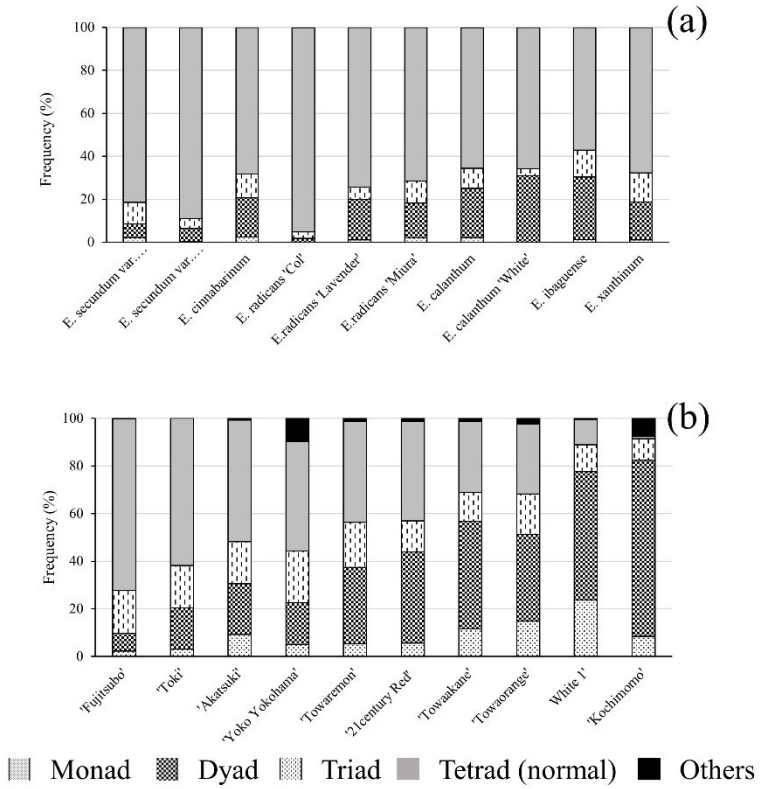


Fig. 4 Frequencies of irregular microspores in six wild species and ten hybrids. Average of pollinia from five flowers was analyzed for each genotype. (a): 10 wild species, (b): 10 hybrids.

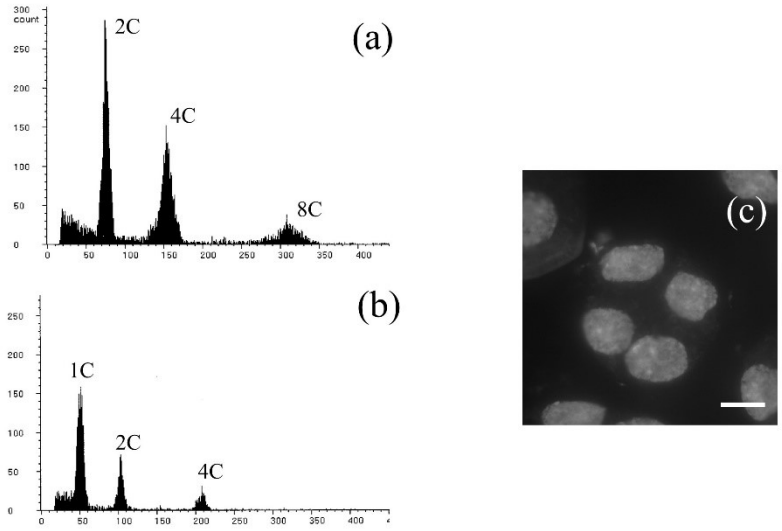


Fig. 5 Flow-cytometric analysis of immature pollinia of *E. radicans* 'Miura'. (a): Flow-cytometric profiles of DNA contents of somatic cells from ovary walls; (b): Flow-cytometric profiles of DNA contents of immature uninuclear microspores; and (c): DAPI staining of immature uninuclear microspores. Scale bars = 10 μm.

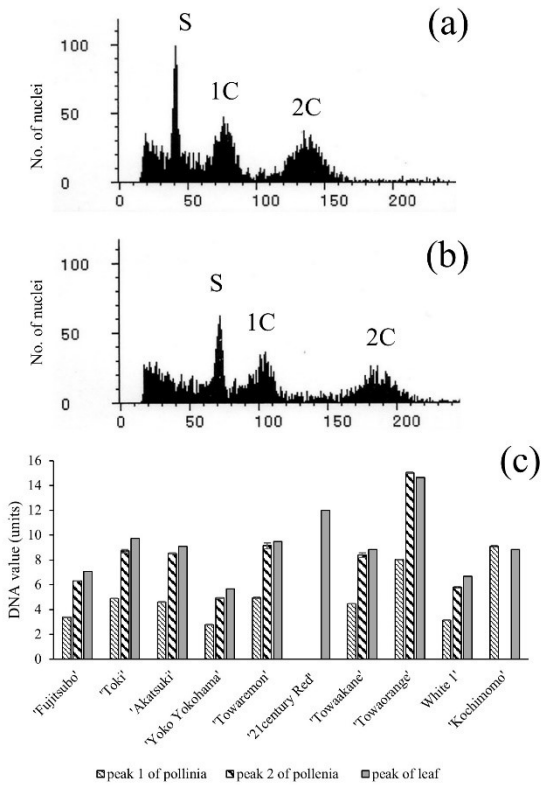


Fig. 6 Flow-cytometric analysis of pollinia and leaves of hybrids. (a): Flow-cytometric profiles of DNA contents of *E. radicans* 'Miura' using *Antirrhinum majus* as an internal standard. (b): Flow-cytometric profiles of DNA contents of 'Akatsuki' using *Eustoma grandiflorum* as an internal standard. IS indicates each internal standard. Peaks were calculated using PA cytometer. (c): DNA value of pollinia and leaf. DNA value calculated using mean value of peak analysis in PA cytometer. Four replications were made for pollinia experimentation. Vertical bars indicated standard error.

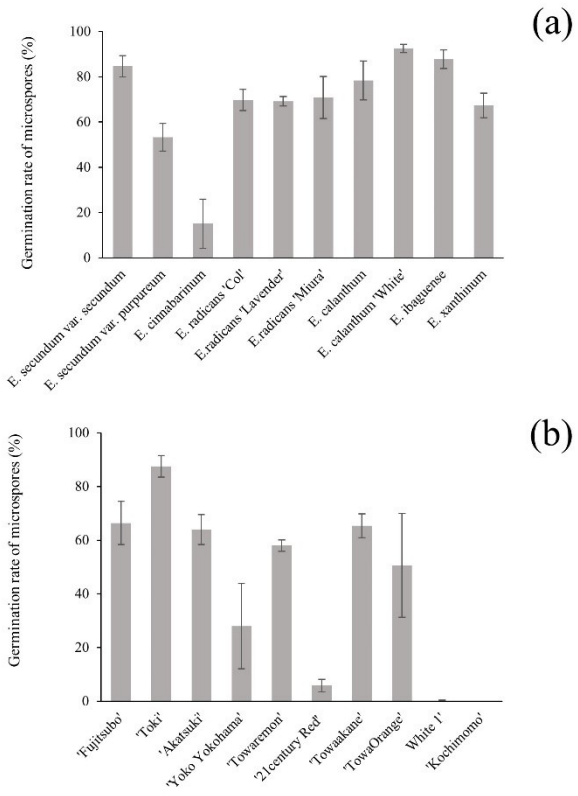


Fig. 7 Germination rate of microspores. Five replications were made for each experimentation. (a): 10 wild species, (b): 10 hybrids. Vertical bars indicated standard error.

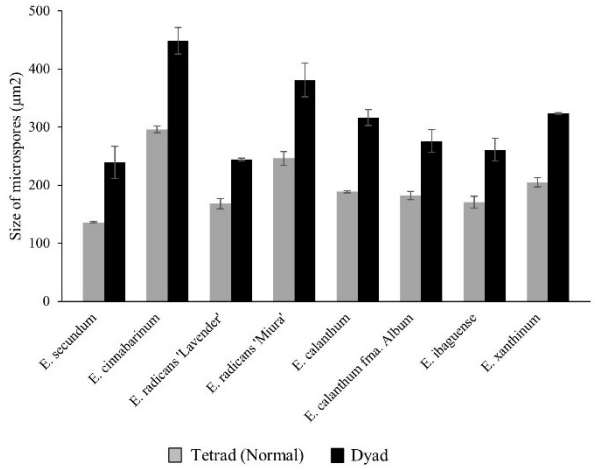


Fig. 8 Comparison size of dyad and tetrad. Microgamete sizes of dyad were calculated; whole areas of dyad / 2. Microgamete sizes of tetrad were calculated; whole areas of tetrad / 4. Three replications were made for each experimentation. At least 50 dyads and one hundred tetrads from three flowers were measured at each replication. Vertical bars indicate standard error.

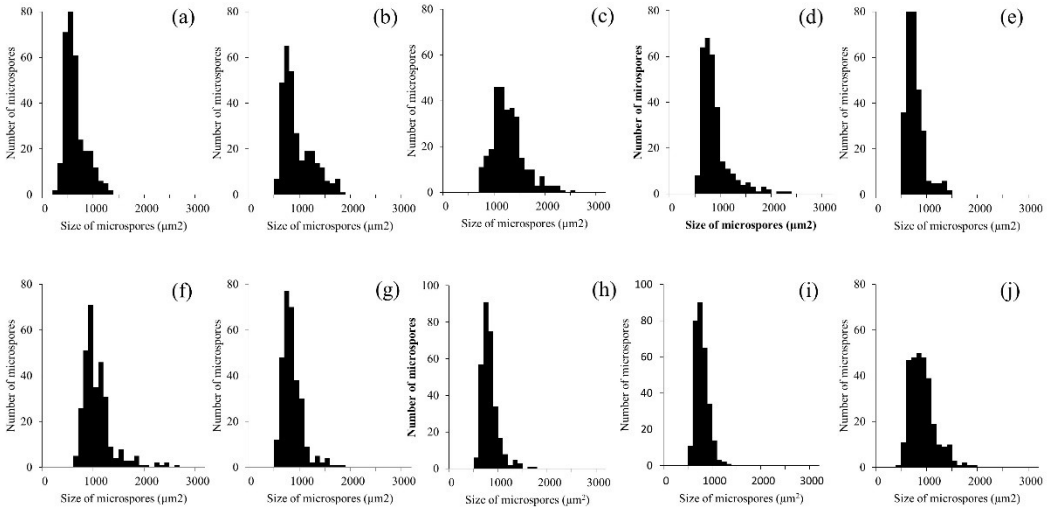


Fig. 9 Histogram of size of microspore of tetrad. Total 300 of microspores measured from three flowers, (a); *E. secundum* var. *secundum*, (b); *E. secundum* var. *purpureum,* (c); *E. cinnabarinum*, (d); *E. radicans* 'Col', (e); *E. radicans* 'Lavender', (f); *E. radicans* 'Miura', (g); *E. calanthum*, (h); *E. calanthum* 'White', (i); *E. ibaguense*, (j); *E. xanthinum,*

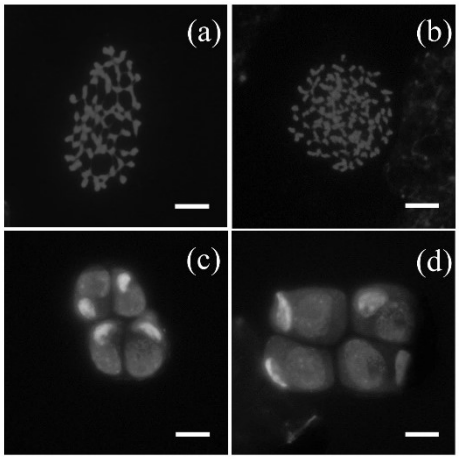


Fig. 10 Meiocyte and pollinia cytology of *E. secundum* var. *purpureum*. (a): normal meiocyte (2*n* = 2*x* = 60), (b): tetraploid meiocyte (2*n* = 4*x* = 120), (c): normal tetrad, D: enlarged tetrad. Scale bar = 10 μm.

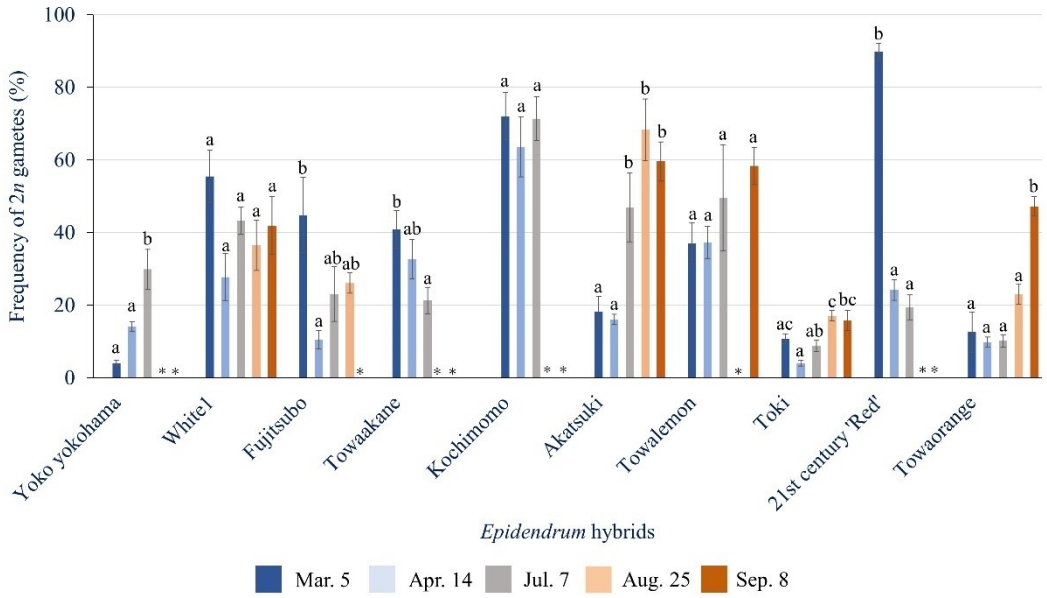


Fig. 11 Unreduced 2*n* pollen formation under different seasons. Five replications were made for each experimentation. Vertical bars indicate standard error. Asterisks indicate that no pollinia were obtained. Different letters show significant differences among the each *Epidendrum* hybrids at the *p* value < 0.05.

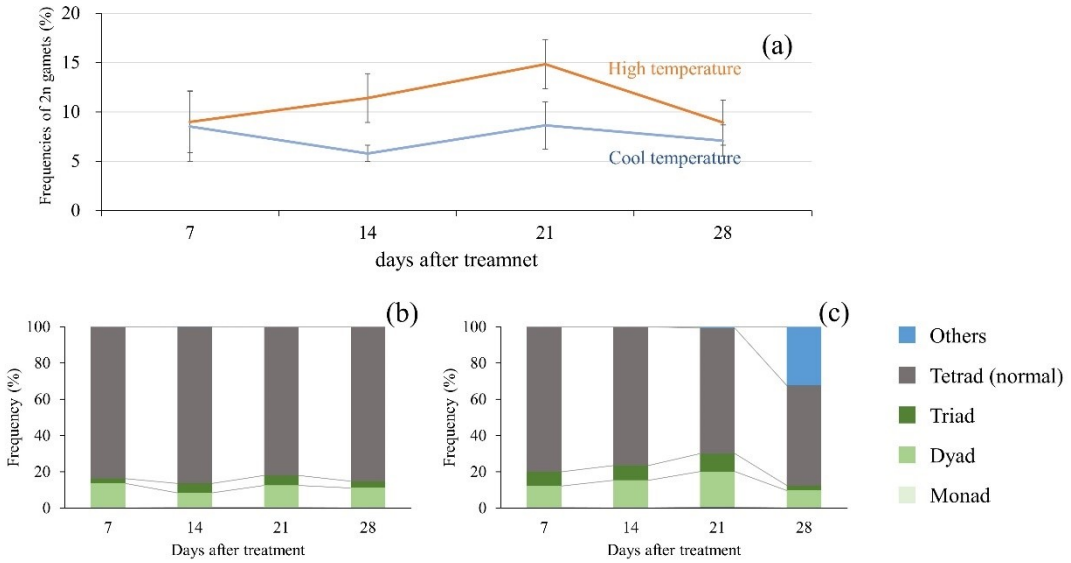


Fig. 12 Relationship between temperature and unreduced 2*n* pollen formation under growth chamber conditions. (a) frequency of 2*n* pollen in different growth chamber conditions. Vertical bars indicate standard error. (b) and (c) frequency of irregular meiosis of microspores in different growth chamber conditions. (b) Cool temperature condition (25/15℃). (c) High temperature condition (35/25℃).

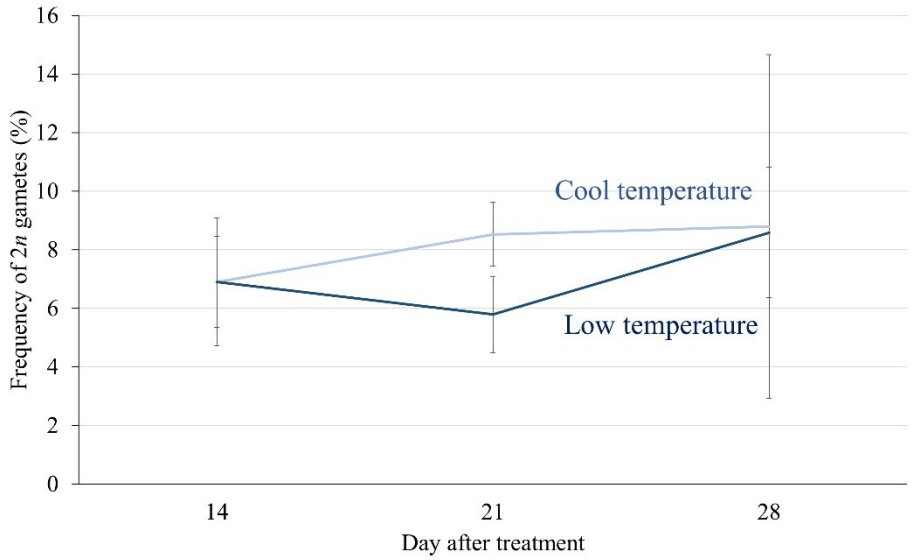


Fig. 13 Relationship between temperature and unreduced 2*n* pollen formation under growth chamber conditions. Day/night temperature regimes: 25/15 (Cool temperature), and 20/10 (Low temperature).

Table 1 mean value of daily maximum temperature, daily average temperature, daily minimum temperature and day length between 4 weeks and 2 weeks before flowering date.

Sampling day	Max. temperature(℃)	Ave. temperature(℃)	Min. temperature(℃)	Day length
Mar. 5	24.5	17.3	14.8	9h 42min
Apr. 14	24.4	17.9	14.7	11h 38min
Jul. 7	31.0	24.4	19.9	14h 10min
Aug. 25	36.8	28.3	23.4	13h 48min
Sep. 8	39.7	30.5	25.5	13h 23min

Table 2 The peaks that can be observed at each stage in flow cytometric analysis.

	nucleus types	uninuclear			binuclear			Mature
		G_1 phase	S phase	G_2 phase	G_1 phase	S phase	G_2 phase	
n gamates	microspore nucleus	1C	1C-2C	2C	-	-	-	-
	generative nucleus	-	-	-	1C	1C-2C	2C	2C
	vegetative nucleus	-	-	-	1C	1C	1C	1C
2*n* gametes	microspore nucleus	2C	2C-4C	4C	-	-	-	-
	generative nucleus	-	-	-	2C	2C-4C	4C	4C
	vegetative nucleus	-	-	-	2C	2C	2C	2C

Table 3 The size of tetrads of wild species.

Species	DNA value (units)	Average of pollen sizes[*z] (μm^2)	Median of pollen sizes (μm^2)	Skewness[*y]	Kurtosis[*y]	Normality[*x]	>1.5-fold of average (%)	>2.0-fold of average (%)
E. secundum var. *secundum*	2.05	543±5	484	1.13	3.96	F	12.7	2.8
E. secundum var. *purpureum*	4.28	852±47	752	1.03	3.21	F	12.7	0.3
E. cinnabarinum	3.73	1184±24	1146	0.98	4.30	F	6.7	0.3
E. radicans 'Col'	2.01	789±24	714	2.15	8.62	F	9.0	3.0
E. radicans 'Lavender'	3.93	673±34	642	1.52	5.85	F	5.7	0.3
E. radicans 'Miura'	5.11	985±46	896	2.05	8.68	F	6.9	2.0
E. calanthum	2.36	755±8	730	1.70	7.26	F	5.3	1.0
E. calanthum 'White'	2.38	729±28	692	1.77	7.89	F	4.3	0.7
E. ibaguense	4.04	683±40	657	0.96	6.33	F	1.7	0.0
E. xanthinum	4.32	818±31	782	1.03	4.27	F	8.7	1.0

*z In normal ditiribution, skewness is 0, kurtosis is 3.

*y Calculated from Shapiro-Wilk normality test. $p<0.001$. 'F' indicated the groups is not normal distribution.

Chapter 4

F_1 progeny via fertilization of 2*n* gametes

Introduction

In the previous studies, polyploidization has been recognized to provide advantages in some ornamental plants, e.g. increase of flower size and the number of petals in rose (Allum *et al.,* 2007), increased flower size and smaller plant foliage in *Echinacea* (Abdoli *et al.,* 2013) and increase in flower size and content of chalcone which would contributed to the production of deeper flower color in *Cyclamen* (Takamura and Miyajima, 1996). However, plants with excessive ploidy levels, namely auto-octoploid indicate lower growth than diploid and tetraploid (Tsukaya, 2008; Niu *et al.,* 2016; Mo *et al.,* 2020).

Artificial chromosome doubling has been conducted by the application of antimitotic reagent, such as colchicine, oryzalin, trifluralin and amiprophos-methyl in ornamental breeding (Dhooghe *et al.,* 2011). However, sexual polyploidization, namely polyploidization via 2*n* gametes is an efficient way to produce polyploids (Ramanna & Jacobsen, 2003). Breeding utilizes 2*n* gametes to produce intraspecific triploids with vigorous growth rates and with lack of seeds (Barba-Gonzalez *et al.*, 2004; Mai *et al.*, 2019). The following pathways are used to induce triploids in breeding: i) fertilization between reduced and 2*n* gametes, ii) cross between diploid and tetraploid artificially induced by treatment with an antimitotic agent, and iii) endosperm culture (Nakano *et al.*, 2021). Triploid induction via reduced and 2*n* gametes is the most efficient method because it can be obtained via manipulation of one generation, namely hybridization. However, few polyploid progeny is

obtained in breeding using 2*n* gametes because many plant produce low frequencies of 2*n* gametes (De storme *et al.,* 2013b; Fakhri *et al.,* 2016; Zeng *et al.,* 2020).

Cultivars of *Epidendrum* originating from interspecific hybridization have more chromosomes than the wild species, thus, indicating the high polyploid levels of cultivars (Kondo *et al.*, 2021). *Epidendrum* wild species and cultivars produces 2*n* pollen with high frequency through multiple pathways namely meiotic defeat and pre-meiotic chromosome doubling (Chapter 3). Unreduced 2*n* gametes should be involved the breeding of *Phalaenopsis* cultivars of Orchidaceae because majority of phalaenopsis cultivars consist of triploid and tetraploid (Lee *et al.,* 2020). However, in *Epidendrum*, unreduced 2*n* gametes may be involved the breeding more than once in breeding because the cultivar of *Epidendrum* consist of wider and higher polyploids than phalaenopsis.

Incompatibility of interspecific crosses and interploid crosses may be due to pre-zygotic or post-zygotic barrier (Behrend *et al.,* 2015; Sutherland and Galloway, 2017; Walter *et al.,* 2019). Pre-zygotic barrier is caused by defeat or inhibition of pollen germination and pollen tube growth, and post-zygotic barrier result in non-germination of seeds due to embryo death which caused by endosperm degeneration (Rebernig *et al.,* 2015; Walter *et al.,* 2019). 2*n* gametes also undergo each process of pre-zygotic and post-zygotic event and compete against normal 1*n* pollen to seed formation. Thus, the frequency of 2*n* gametes is inconsistent with frequency of progeny originated by fertilization of 2*n* gametes (De storme *et al.,* 2013b; Fakhri *et al.,* 2016; Zeng *et al.,* 2020). Some interspecific

hybrids produce reduced non-viable gametes and 2*n* viable gametes (Ramanna *et al.,* 2003; Barba-Gonzalez *et al.,* 2004; Fakhri *et al.,* 2016). 2*n* pollen have a competitive advantage against normal 1*n* pollen while it traversed the stigma and entered into style in *Rosa*. In previous study, the formation of polyploid seeds may be impaired by imbalances in the endosperm balance number in the fertilization between normal gametes and 2*n* gametes (De storme *et al.,* 2013a).

The objectives of this study were production of polyploid progeny via hybridization of 2*n* gametes and assessment of ornamental value through morphological evaluation.

Materials and Methods

Plant materials

Ten *Epidendrum* hybrids, namely *E*. Narrative Genji 'Fujitsubo', *E*. Cosmo Dream Color 'Towaakane' and 'Kochimomo', *E*. Venuspiars 'Akatsuki', 'Towaremon' and 'Towaorange', *E*. Sunny Girl 'Toki', *E*. Yoko Yokohama, *E*. 21st century 'Red', *E*. 'White 1', and five *Epidendrum* wild species, namely *E. ibaguense*, *E. radicans* 'Lavender', *E. radicans* 'Miura', *E. secundum* var. *secundum*, *E. secundum* var. *purpureum.* Source of plant materials and cultivation conditions were the same as those mentioned in Chapter 2 and 3.

Pollination and seed sowing

Flowers of wild species of *Epidendrum* were hand-pollinated within 4 days after anthesis. Capsules containing mature seed were collected at the initiation of dehisce. Seeds were surface sterilized in solution of NaClO containing 1% active chlorine and 0.1% polyoxyethylene sorbitan monolaurate (= Tween® 20) (w/v) (Kanto Chemical Co. Inc., Tokyo, Japan) for 30 min. After five rinses with sterilized distilled water, seeds were transferred to solidified ND medium (Tokuhara & Mii, 1993) with 20 g L^{-1} of sucrose and 2.5 g L^{-1} of Gellan gum (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The pH was adjusted to 5.4 before being autoclaved. The cultures were maintained at 25 °C for a photoperiod of 24 h at a light intensity of 30 μmol m^{-2} s^{-1} under a cool white

fluorescent light.

In vitro germination of pollinia

The methods were the same as those mentioned by Chapter 3 unless stated otherwise. After 24 h of incubation at 25 °C, pollinia were collected. Pollinia germination was defined when the pollen tube had developed in length over the diameter of original sporad and at least 50 microspores were counted. The germination test of sporads was replicated five times.

Determination of ploidy levels via flow cytometry analysis

The methods were the same as those mentioned by Chapter 1 unless stated otherwise. The youngest leaves and protocorms of plantlets were analyzed by FCM. For each sample 700–1,500 nuclei were analyzed. *Eustoma grandiflorum*, which has a 2C DNA amount of 3.26 pg (Lindsay *et al.,* 1994) were used as biological internal standards. DNA amounts were calculated by comparing *Eustoma.* Individuals with an amount of DNA which matches the estimated amount of DNA when only normal *n* gametes are fertilized were defined as Normal Ploidy (NP). In contrast, Individuals with an amount of DNA which clearly exceed estimated amount of DNA when only normal *n* gametes are fertilized were defined as High Ploidy (HP). We did not distinguish whether 2*n* egg, 2*n* pollen or both involved fertilization in individuals of HP.

Plant production

Progeny obtained from two crosses of ‘Towaakane × ‘Fujitsubo’, namely ‘Towaakane’ using as ovule parent and ‘Fujitsubo’ × ‘Towaakane’ namely ‘Fujitsubo’ using as ovule parent were used for the morphological characterization. The plantlets, cultured in vitro at a height of 5cm, were rinsed with water, and then transplanted into tray filled with medium mixture half vermiculate and half sphagnum. Plantlets were growing plastic box which covered with plastic films to maintain high humidity. After 3 weeks of acclimatization, they were transplanted into 9cm plastic bottle filled with bark. The growing condition were the same as those mentioned in plant materials.

Chromosome counting

The methods were the same as those mentioned by Kondo *et al.,* (2021) unless stated otherwise. Root tips of 5-8 mm long were excised at around 1:30 p.m. and pretreated in 2 mM 8-hydroxyquinoline for 24 h at 10℃.

Evaluation of ornamental character

The morphological characteristics of NP and HP plants except for flower character were measured ten months after acclimatization (Apr. 2018). Characteristics of flower, namely flower color,

size, and frequency of 2*n* gametes were measured when each individual had first flowering in the seasons of 2020. Characteristics of flower thickness were measured in 2021.

Color analysis

To evaluate flower color objectively, color components of the Commission Internationale de l'Eclairage (CIE) L*a*b* coordinates, namely lightness (L*), green(−)–red(+) component (a*), blue(−)–yellow(+) component (b*), and chroma [C*: calculated as C*= $\sqrt{a^{*2} + b^{*2}}$], were measured with a hand-held spectrophotometer CM-10 plus Spectro Color Meter (Konica-Minolta Co., Ltd, Tokyo, Japan). the adaxial surface of one sepal were subjected to color measurement. Scores of five sepals were averaged.

Statistical analysis

Data were evaluated by R Version 3.4.2 statistical software (R Development Core Team, Australia, https://www.r-project.org/). Analysis of variance (ANOVA) were calculated by aov function and multiple comparison were calculated by Tukey HSD function. Differences were regarded as significant at p value < 0.05.

Results

Germination rate; tetrad vs. dyad

The germination frequencies of dyad 24 h after incubation were higher than that of tetrad in all six *Epidendrum* (Fig. 1). Germination frequencies of dyad of three hybrids which produced high frequencies of 2*n* gametes, namely 'Towaakane' 'Towaremon' and 'Towaorange', were significantly higher than that of tetrad. After 48 h incubation, we could not distinguish each type of microspores (data not shown).

Estimation of Ploidy level of F_{1s} by measurement of DNA amounts

F_1 progeny plants were obtained from self-pollination of *E. secundum* var. *purpureum* and from cross pollination of *E. radicans* 'Lavender' × *E. radicans* 'Miura'. One polyploid progeny, which had two-fold DNA value than the parent, existed in F_1s from self-pollination of *E. secundum* var. *purpureum* (n = 60) (Table1). However, no high ploidy progeny was obtained in other cross combinations. The polypoid progeny could have arisen from fertilization 2*n* pollen and 2*n* egg. Furthermore, eight polyploid progeny from fertilization between *n* egg of *E. radicans* 'Lavender' and 2*n* pollen of *E. radicans* 'Miura' were observed in the cross pollination of *E. radicans* 'Lavender' × *E. radicans* 'Miura' (n = 60).

The DNA amount of F_1s examined in the present study were in the range of 5 –23 units

(Table 2). As the DNA amount of the parents ranged from 5.4 to 14.6 in the cross experiment, the range of DNA amount of F_1 progeny became two-fold. Two or more populations based on DNA amount were observed in almost all cross combinations. In general, DNA amounts in the populations of low DNA amount were lower than estimated DNA amount of progeny fertilized by a *n* egg and *n* pollen. DNA amounts in the populations of higher DNA amount almost consisted with the estimated DNA amount of progeny fertilized 2*n* pollen, 2*n* egg or both. However, individuals or populations which deviated from the estimated DNA amount were observed in some cross combinations e.g., Yoko Yokohama × 21st century 'Red', 'Towaakane' × 'Towaorange' and 'Towaorange' × 'Toki'. The frequencies of High ploidy (HP) production were in the range of 0-100% (Fig. 2). High frequencies of HP progeny production were observed when 'Towaakane' was used as pollen parent. In contrast, a few HP plants were obtained in the crosses using 'Toki' as pollen parent. In crosses with 'Towaakane' as pollen parent, HP progeny were obtained with high frequency in crosses with ovule parents of low DNA amounts. Furthermore, in the self-pollination of 'Towakane', no individuals, which were presumed to have been fertilized by 2*n* pollen and 2*n* eggs, were observed.

Morphological analysis

Two populations with different ploidy level were obtained in the crosses and reciprocal crosses using 'Towaakane' as the maternal parent and 'Fujitsubo' as the paternal parent. The DNA

amount of NP progeny and HP progeny were in the range of 7.5-8.5 and 10.5-12 in the cross of ‘Towaakane’ × ‘Fujitsubo’. Similarly, the DNA amount of NP progeny and HP progeny were in the range of 7.0-9.0 and 11.5-13 in the reciprocal cross. HP plants exhibited vigor growth, especially in terms of longer leaves and wider stems, in the cross of ‘Towaakane’ × Fujitsubo’ (Table 3). Furthermore, leaves of HP were significantly wider and thicker than those of NP progeny in the cross of ‘Fujitsubo’ × ‘Towaakane’ (Table 4). Significant difference in flower color between NP and HP was observed in the cross of ‘Towaakane’ × ‘Fujitsubo’ (Table 5). Areas of flowers of HP were larger than NP although no significant difference was observed in the cross of ‘Fujisubo’ × ‘Towaakane’ (Table 5 and 6). However, other parameters were not significantly altered in both crosses. Dispersion of the flower color of NP was spread out over a wider range than that of HP (Fig. 3 and 4). Interestingly, fourteen out of eighteen and ten out of eighteen standard deviations of each parameter of NP were higher than HP progeny in the crosses of ‘Towaakane’ × ‘Fujitsubo’ and ‘Fujitsubo’ × Towaakane’ respectively (Table 3-6).

Inheritance of production capacity of irregular microspores via meiotic defeat

The frequencies of containing irregular microspores in monad, dyad and triad in ‘Fujitsubo’ and ‘Towaakane’ were 27.8% and 70.6% respectively. The frequencies of irregular microspores productions of F1*s* were in the range of 13.0-80.6% over the parents (Fig. 5). The average of irregular

microspores production frequency of F_1 progeny were higher in NP progeny than in HP progeny in the both crosses.

Chromosome number of F_1s

'Fujitsubo' and 'Towaakane' had 2*n* = 84 and ca. 95 respectively. Chromosome numbers of six F_1s in the cross of 'Towaakane' × 'Fujitsubo' were ranged from 2*n* = 87 to 136 (Fig. 6). NP progeny defined by FCM analysis had 2*n* = 84 and 102. Chromosome number of HP progeny were 2*n* = 135, 136 and 136. The chromosome number of HP progeny almost corresponded with chromosome number, which fertilized 2*n* egg of 'Towaakane' and *n* pollen of 'Fujitsubo'.

Discussion

Ploidy level of F_1 progeny and frequencies of HP progeny via fertilization of 2n gametes

The germination frequencies of dyad 24 h after incubation were higher than that of tetrad in all six *Epidendrum* (Fig. 1). Some interspecific hybrids produce reduced non-viable gametes and 2*n* viable gametes which has full, balanced complement of chromosomes (Barba-Gonzalez *et al.,* 2004; Fakhri *et al.,* 2016). Thus, the present study suggests that chromosomes balance of dyad of *Epidendrum* hybrids were stable because the *Epidendrum* hybrids originated some wild species. Germination frequencies of dyad of hybrids, which produced high frequencies of 2*n* gametes, were significantly higher than that of tetrad. Unreduced 2*n* gamete production is influenced by environmental factors, genetic factors, reproductive mode, life history, and ploidy level (Crespel *et al.,* 2006; Brownfield & Köhler, 2011; Kreiner *et al,* 2017a; Kreiner *et al.,* 2017b). 2*n* gametes formation via deviations of meiosis can occur in plants with disturbed chromosome pairing as, for example, in distant interspecific hybrids (Ramanna and Jacobsen, 2003; Ramanna *et al.,* 2003; Barba-Gonzalez *et al.,* 2004). Furthermore, genomic constitution affects unreduced 2*n* gametes formation e.g. high homologous chromosome pairing interfered with meiotic restitution and the formation of unreduced 2*n* gametes in the allotetraploid with the genomic formula ABDD of Triticeae (Wang *et al.,* 2010). Thus, chromosome instability of *Epidendrum* hybrids which produce high frequency of dyad induces high frequency of meiotic disturbance. However, *Epidendrum* hybrids which produce few dyads may

have stabler chromosome constitution e.g. high ratio of homologous chromosomes. In this case, the *n* gametes may exhibit normal viability because chromosome constitution of *n* gametes may also be stable. Furthermore, difference in frequencies of irregular microspore production between NP and HP F_1s progeny may be explained by the amount of homologous chromosome.

The frequencies of 2*n* gametes were in the range of 1.7–21.7% and 9.1–83.9% in wild species and in hybrids, respectively (Kondo unpublished). The frequencies of HP progeny production were inconsistent with that of 2*n* gametes. The estimated frequencies of HP progeny production based on frequencies of 2*n* gametes were 39.7 % when 'Towaakane' was used as pollen parent. Nevertheless, the frequency of HP progeny production in the cross of Yoko Yokohama × 'Towaakane' was 67.5%, which is higher than the estimated frequency, whereas the frequency of HP progeny production in the cross of 'Toki' × 'Towaakane' was 7.5%, which is lower than the estimated frequency. Incompatibility of interspecific crosses and interploid crosses may be due to pre-zygotic or post-zygotic barrier (Behrend *et al.,* 2015; Sutherland and Galloway, 2017; Walter *et al.,* 2019). Pollen germination and pollen tube growth are affected by pistil/pollen interactions on the stigma surface and in transmitting tissue (Lee *et al.,* 2008; Alves *et al.,* 2019). Post-zygotic reproductive barriers between higher order polyploids may not be as strong as in diploid–tetraploid systems because parental genomic imbalance in the seeds which attributed to aberrant development of endosperm may be lower in the high polyploids crosses than diploid-tetraploid crosses (Sutherland and Galloway, 2017). Cross

direction in the interploidy crosses also affect the fitness component, namely germination was higher in interploid crosses in which the maternal ploidy was greater, whereas fruit set and seed number were greater in interploidy crosses in which the paternal ploidy was greater (Sutherland and Galloway, 2017). In the combinations where the measured frequencies of HP progeny production are lower than the estimated frequencies, various negative factors may have caused the inhabitation of HP progeny, namely low germination rate of 2*n* gametes, low pollen tube growth rate and embryo abortion or non-germinated seeds due to genome imbalance. In contrast, 2*n* gametes may be more competitive than n gametes in some factors. Larger pollen grains contain more energy stores that may facilitate pollen tube germination and growth through the stigma and style (McCallum and Chang, 2016). 2*n* gametes of dyads were 1.45–1.76 times larger than *n* gametes of tetrads (Chapter 3). Thus, the pollen tube growth rate could be higher in 2*n* gametes than in n gametes although further studies are needed to evaluate pollen tube growth rate of 2*n* gametes and n gametes by tracing pollen tube growth in the style. Furthermore, polyploidy-associated breakdown of gametophytic self-incompatibility (SI) were reported in Solanaceae and subfamily Maloideae of the Rosaceae (Tao and Iezzoni, 2010). 2*n* pollen isinvolved in hybridization and has a competitive advantage while it traverses the stigma and enteres into style in *Rosa* (Gao *et al.,* 2019). 2*n* gametes may have a promoting or inhibiting effect on SI system of *Epidendrum* although the mechanism of SI system in orchids is still unclear, with few reports (Miillner *et al.,* 2015; Niu *et al.,* 2017).

There are four methods to detect 2*n* gametes: (i) screening based on pollen grain size, (ii) screening by FCM, (iii) progeny analysis, (iv) analysis of micro- and megasporogenesis (Bretagnolle and Thompson, 1995). Many studies report 2*n* pollen formation because detection of 2*n* eggs is more difficult than that of 2*n* pollen. In the progeny analysis by FCM in the present study, the presence of 2*n* eggs in the *Epidendrum* is indicated. However, we could not determine whether the 2*n* egg or the 2*n* pollen was involved in fertilization by using FCM when the DNA amounts of parents were close to each other. However, chromosome counting indicated fertilized 2*n* egg of 'Towaakane' and n pollen of 'Fujitsubo' although DNA amounts of 'Towaakane' and ''Fujitsubo' were close to each other. Elucidation of genome constitution via genomic *in situ* hybridization (Barba-Gonzalez *et al.,* 2004) should provide stronger proof of fertilization of 2*n* egg F_1s showed wide range of DNA amount, which was divided into several populations in each cross. Triploid hybrids -derived gametes have various number of chromosome although chromosome number of progenies show a skewed distribution depending on the cross combination (Leflon *et al.,* 2006). Iindividuals or populations which deviated from the estimated DNA amount were observed among some cross combinations in *Epidendrum*. These results may depend on the viability of the different gametes and embryos according to their structure. Specifically, progeny fertilized with gametes of imbalanced genome which were inconsistent with *n* or 2*n* gametes may have been stabler than progeny fertilized with balanced genome. Polyploidization via unreduced 2*n* gametes are limited in high ploidy e.g. octoploid is maximal ploidy

in *Arabidopsis* (Wang *et al.,* 2010). In present study, ploidy level of *Epidendrum* expanded beyond parental ploidy level. That suggested that indicate unique character of *Epidendrum* although further studies are needed to elucidate the factor of sustainable polyploidization.

Morphology of NP and HP progeny

HP progeny of *Epidendrum* exhibited vigour plant growth and larger area of flower. The effect of polyploidy in plants is the increase in cell size, caused by the larger number of gene copies and referred to as the ‘‘gigas’’ effect (Sattler *et al.,* 2016). Cell-size enlargement of polyploid plays a pivotal role in increasing the plant organs when the cell number is not decreased (Zhang *et al.,* 2019). Higher ploidy enhances cell expansion but inhibits leaf cell division (Tsukaya, 2008). The positive effect of polyploidy may be larger than that of negative effect although *Epidendrum* hybrids used as parents are already polyploid. Rapidly formed auto-octoploid via artificial chromosome doubling of diploid express much lower growth than diploid (Tsukaya, 2008; Niu *et al.,* 2016; Mo *et al.,* 2020). In contrast, substantial reduction in growth is not observed in allo-octoploid via artificial chromosome doubling of allo-tetraploid e.g. *Panicum* and *Calendula*, and hexadecaploid via artificial chromosome doubling of natural octoploid which experienced speciation associated with polyploidization e.g. *Prunus* and *Pinellia* (He *et al.,* 2012; Schlze and Contreras, 2017; Yoon *et al.,* 2017; Esmaeili *et al.,* 2020). Thus, *Epidendrum* cultivars are high polyploidy; however, HP progeny via 2*n* gametes

fertilization may have exhibited more vigorous growth than NP progeny because *Epidendrum* cultivars are complex allopolyploid. Larger flowers could have novel marketability as cut flower and potted flower. Furthermore, differences in color between NP and HP progeny are useful for primary selection by ploidy analysis using FCM in the *Epidendrum* breeding.

Aneuploid may have been contained in the progeny of both NP and HP because the DNA amount of each population had wide range. Imbalance in gene dosage of aneuploid has phenotypic consequences that are specific to each karyotype (Henry *et al.,* 2005; Henry *et al.,* 2010). Thus, this experiment may not be a simple comparison between normal ploidy and high ploidy. Although abnormal morphologies e.g., waving leaves, were observed, the frequencies of individuals which exhibited abnormal morphology were low.

Interestingly, NP progeny showed wide range of morphology. Furthermore, standard deviations of each parameter of NP progeny were higher than those of HP progeny. Based on these results, two factors could be associated with the deviation. Firstly, frequency of aneuploid of NP progeny may be higher than that of HP progeny. Chromosome counting of NP and HP progeny in the cross of 'Towaakane' × 'Fujitsubo' showed wide range of chromosome number ($2n$ = 84,102) although we were able to observe only two individuals of each progeny. Secondly, there is difference in the contribution of each genome between NP progeny and HP progeny. NP progeny and HP progeny which inherited genomes from 'Towaakane' (T genome) and 'Fujitsubo' (F genome) showed TF

genome and TTF or TFF genome, respectively. In HP progeny, emergence of extreme phenotypes is low because the contribution of each genome may be reduced than NP progeny.

Many parameters of morphology were not significantly altered in both of crosses. In contrast to the small effects of genome doubling on gene regulation in autopolyploids, the combination of two divergent genomes in allopolyploid by interspecific hybridization induces genomewide nonadditive gene regulation (Wang *et al.,* 2006). Epigenetic changes via DNA methylation and histone modifications may also reprogramme gene expression and developmental patterns of newly synthesized polyploid (Anssour *et al.,* 2009; Song and Chen, 2015). Thus, the phenotypic changes were scarcely observed, because gene regulation were regulations brought by the epigenetic changes.

Inheritance of production capacity of irregular microspores via meiotic defeat

Some genes related to the production of unreduced genes have been reported in previous studies, e.g., *el1* in *Zea*, *Atps1* in *Arabidopsis*, and *ps* in *Solanum* (d'Erfurth *et al.,* 2008; Loginova & Silkova, 2017). Some of these genes may be involved in the production of unreduced 2*n* gametes in *Epidendrum*. Identification of the gene by molecular analysis in the F_1s progeny is expected in the future study.

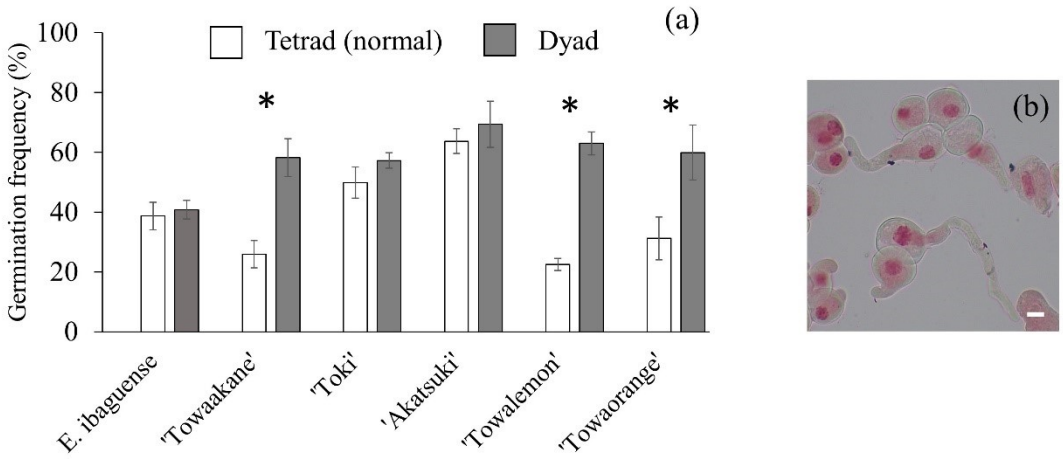


Fig. 1 Germination frequencies of dyad and tetrad of microspores 24 h after incubation. Five replications were made for experimentation. (a): One wild species and five hybrids. Asterisks indicate significant difference by t test analysis * $p < 0.05$. (b) Germination of microspores of dyad and tetrad. Scale bar indicates 10 µm. Vertical bars indicate standard error.

			Pollen donor									
	DNA amount		5.41	6.06	6.36	7.95	8.34	8.46	9.12	9.16	11.43	14.64
	DNA amount	Cultivar	Yoko Yokohama	'White 1'	'Fujitsubo'	'Towaakane'	'Akatsuki'	'Kochimomo'	'Toki'	'Towalemon'	21st century 'red'	'Towaorange'
Ovule donor	5.41	Yoko Yokohama	**6.3**		**20.0**	**67.5**	**0.0**	**87.5**	**6.0**		**92.3**	
	6.06	'White 1'			**16.3**	**46.2**	**4.4**			**0.0**		**25.6**
	6.36	'Fujitsubo'			**10.0**	**50.0**	**0.0**		**0.0**			**0.0**
	7.95	'Towaakane'			**52.5**	**67.5**	**5.0**		**2.6**	**22.5**		**15.0**
	8.34	'Akatsuki'			**2.5**	**15.0**	**7.7**		**0.0**	**15.0**		**2.5**
	8.46	'Kochimomo'		**27.5**	**27.5**	**32.5**	**7.7**	**100.0**	**0.0**	**22.5**		**12.5**
	9.12	'Toki'				**7.5**	**15.0**		**0.0**			
	9.16	'Towalemon'	**0.0**		**12.5**	**18.0**			**6.5**	**23.9**	**3.3**	
	11.43	21st century 'red'			**2.9**	**17.5**	**11.6**	**7.7**	**2.9**	**23.8**		
	14.64	'Towaorange'				**35.0**			**5.0**			

Fig. 2 Frequencies of high ploidy (HP) progeny (%) in the crosses of hybrids. Grey boxes indicate the crosses which no progeny was obtained. Individuals with an amount of DNA which matches the estimated amount of DNA obtained when only normal n gametes are fertilized were defined as Normal Ploidy (NP). In contrast, Individuals with an amount of DNA which clearly exceed estimated amount of DNA obtained when only normal n gametes are fertilized were defined as High Ploidy (HP). We did not distinguish whether 2*n* egg, 2*n* pollen or both were involved in fertilization in individuals of HP.

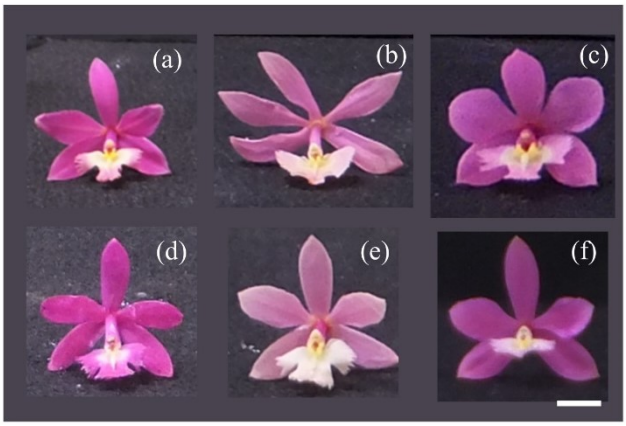


Fig. 3 Flower of F_1 progeny. (a), (b) and (c); Crosses of 'Towaakane' × 'Fujitsubo'. (d), (e) and (f); Crosses of 'Fujitsubo' × 'Towaakane'. (a), (b), (d) and (e) are normal ploidy (NP), and (c) and (f) are high ploidy (HP). (a) and (d) showed the deepest flower color in the NP progeny. (b) and (e) showed the lightest flower color in the NP progeny.

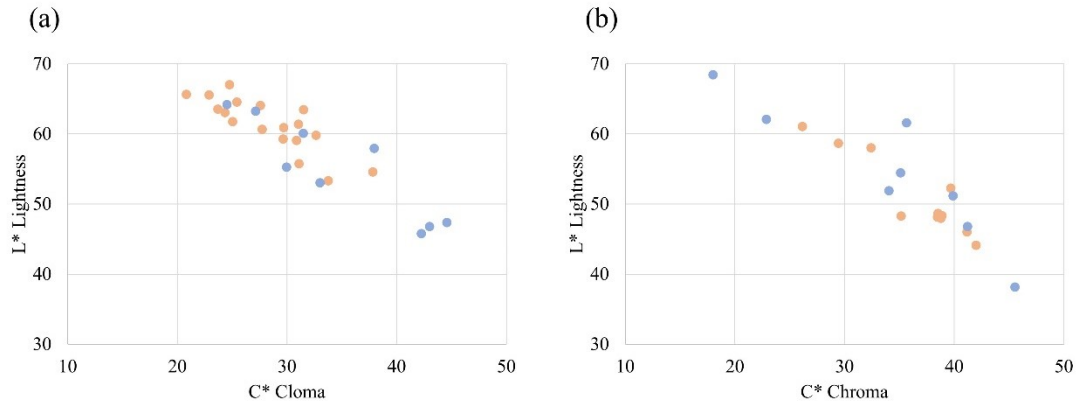


Fig. 4 Scatter plot of flower color of F_1 progeny. (a); Crosses of 'Towaakane' × 'Fujitsubo'. (b); Crosses of 'Fujitsubo' × 'Towaakane'. Blue dots and orange dots indicate normal ploidy and high ploidy respectively.

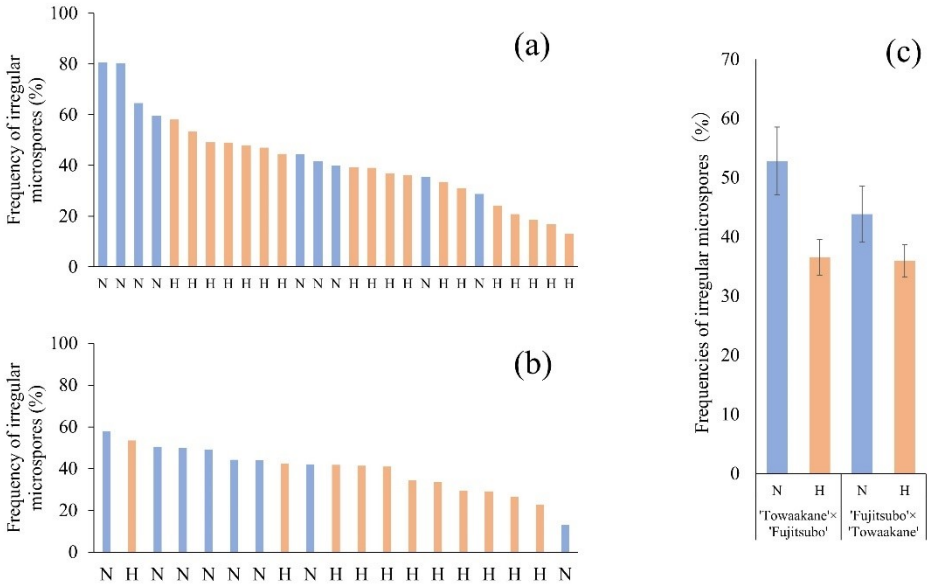


Fig.5 Frequencies of irregular microspores of F_1 progeny. (a); Crosses of 'Towaakane' × 'Fujitsubo'. (b); Crosses of 'Fujitsubo' × 'Towaakane'. (c); Average of frequencies of irregular microspores. Blue bars and orange bars indicate normal ploidy and high ploidy respectively.

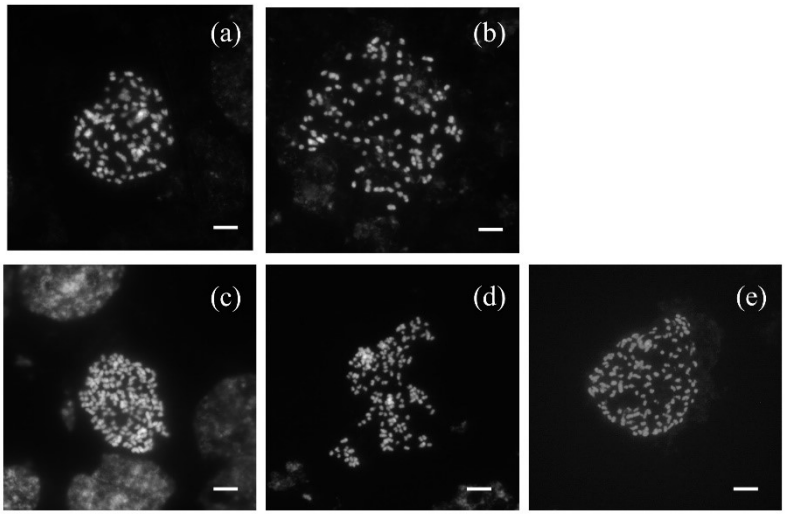


Fig. 6 Metaphase chromosomes of F_1 progeny of Crosses of 'Towaakane' × 'Fujitsubo' (a) and (b); Progeny of normal ploidy (2*n* = 84 and 102 respectively). (c), (d) and (e); Progeny of high ploidy (2*n* = 135, 135, 136 respectively). Scale bar = 10μm.

Table1 Ploidy level of F_1 progeny of wild species.

Ovule parents (chromosome number)	Pollen parent (chromosome number)	No. of analysed plantlets	No. of plantlets			
			Normal ploidy (n ovule × n pollen)	High ploidy ($2n$ ovule × n pollen)	High ploidy (n ovule × $2n$ pollen)	High ploidy ($2n$ ovule × $2n$
E. radicans 'Lavender' ($2n$ = 60)	*E. radicans* 'Col' ($2n$ = 38)	60	60	0	0	0
E. radicans 'Lavender' ($2n$ = 60)	*E. radicans* 'Miura' ($2n$ = 80)	60	52	0	8	0
E. radicans 'Miura' ($2n$ = 80)	*E. radicans* 'Lavender' ($2n$ = 60)	63	63	0	0	0
E. secundum var. *purpureum* ($2n$ = 60)	*E. secundum* var. *secundum* ($2n$ = 30)	60	60	0	0	0
E. secundum var. purpureum ($2n$ = 60)	*E. secundum* var. *purpureum* ($2n$ = 60)	60	59	0	0	1

Table2 DNA amount of F_1 progeny. Grays columns indicate the estimated DNA amount of progeny fertilized by a *n* egg and *n* pollen. Yellows columns indicate the estimated DNA amount of progeny fertilized by a 2*n* egg and *n* pollen. Blues columns indicate the estimated DNA amount of progeny fertilized by a *n* egg and 2*n* pollen. Greens columns indicate the estimated DNA amount of progeny fertilized by a 2*n* egg and 2*n* pollen.

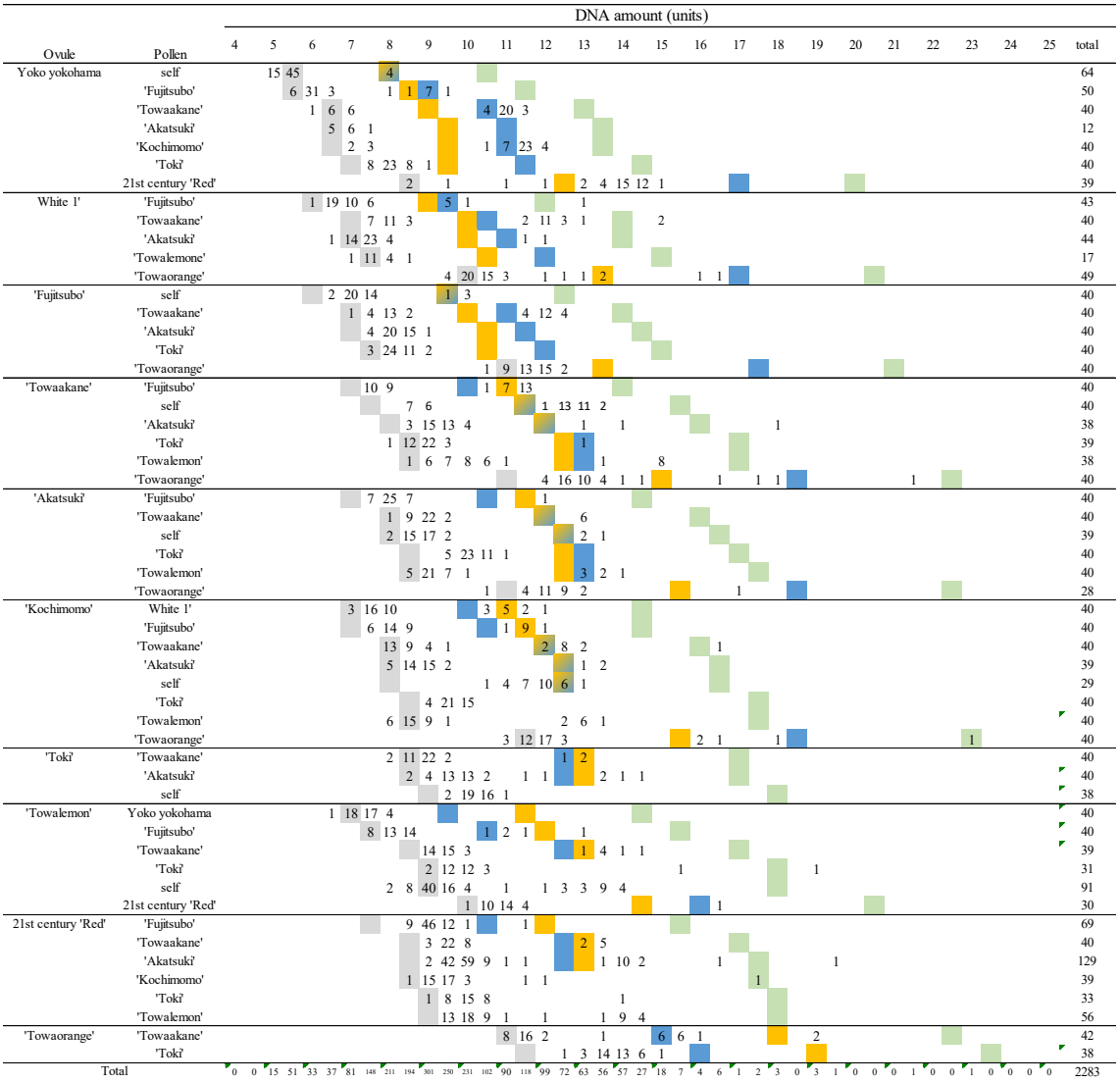


Table3 Morphological characteristics of NP and HP plants. F_1 progeny of Crosses of 'Towaakane' × 'Fujitsubo'.

	Ploidy (No. of plants)	Leaf					Node length	Average of node length	Width of bulb	Number of bulbs
		Length (mm)	Width (mm)	Thickness (mm)	Number	Aspect	(mm)	(mm)	(mm)	
Average	Normal (n = 14)	32.2 ± 3.3	17.5 ± 1.4	1.7 ± 0.1	14.3 ± 0.8	1.9 ± 0.2	64.3 ± 7.4	4.4 ± 0.4	5.8 ± 0.3	2.4 ± 0.3
	High (n = 21)	42.9 ± 2.6	19.5 ± 0.7	1.9 ± 0.1	13.7 ± 0.5	2.2 ± 0.1	70.6 ± 5.8	5.0 ± 0.3	6.6 ± 0.2	2.4 ± 0.4
	$p<0.05$	*							*	
Standard deviation	Normal	12.2	5.2	0.5	2.8	27.8	1.5	1.1	1.3	0.8
	High	11.9	3.4	0.4	2.3	26.6	1.4	1.1	1.9	0.6

± indicate standerd error

Table4 Morphological characteristics of NP and HP plants. F_1 progeny of Crosses of 'Fujitsubo' × 'Towaakane'.

	Ploidy (No. of plants)	Leaf					Node length	Average of node length	Width of bulb	Number of bulbs
		Length (mm)	Width (mm)	Thickness (mm)	Number	Aspect	(mm)	(mm)	(mm)	
Average	Normal (n = 12)	31.3 ± 4.5	14.0 ± 1.0	1.9 ± 0.1	11.9 ± 0.5	2.2 ± 0.3	53.8 ± 5.9	4.5 ± 0.4	5.7 ± 0.2	2.6 ± 0.7
	High (n = 16)	35.1 ± 2.6	19.6 ± 0.9	2.2 ± 0.1	13.2 ± 0.6	1.8 ± 0.1	65.9 ± 4.8	4.9 ± 0.2	6.5 ± 0.3	2.6 ± 0.6
	$p<0.05$		*	*					*	
Standard deviation	Normal	15.5	3.4	0.5	1.8	20.4	1.5	0.7	2.4	1.1
	High	10.6	3.7	0.3	2.5	19.3	1.0	1.0	2.4	0.4

± indicate standerd error

Table5 Flower characteristics of NP and HP plants. F_1 progeny of Crosses of 'Towaakane' × 'Fujitsubo'

	Ploidy (No. of plants)	Flower				Flower color			Flowering day
		Length (mm)	Width (mm)	Area (mm^2)	Thickness*z (mm)	L	a	b	
Average	Normal (n = 9)	28.7 ± 1.0	30.4 ± 1.5	472.0 ± 32.2	0.52 ± 0.02	54.8 ± 2.4	32.6 ± 2.3	-7.8 ± 3.5	2020/04/21 ± 9.1
	High (n = 18)	29.9 ± 0.8	32.6 ± 0.8	560.8 ± 18.6	0.54 ± 0.02	61.3 ± 0.9	26.1 ± 0.9	-10.5 ± 1.0	2020/03/29 ± 6.3
	p<0.05			*		*	*		
Standard deviation	Normal	3.1	4.5	96.7	0.05	7.1	6.8	10.6	27.4
	High	3.2	3.5	78.7	0.07	3.9	3.7	4.3	26.6

*z Thickness of flower were analysed on 2021 (Normal Ploidy; n = 7, High Ploidy; n = 9)
± indicate standerd error

Table6 Flower characteristics of NP and HP plants. F_1 progeny of Crosses of 'Fujitsubo' × 'Towaakane'

	Ploidy (No. of plants)	Flower				Flower color			Flowering day
		Length (mm)	Width (mm)	Area (mm^2)	Thickness*z (mm)	L	a	b	
Average	Normal (n = 8)	27.6 ± 1.0	28.4 ± 0.9	413.6 ± 23.2	0.50 ± 0.05	54.3 ± 3.4	31.9 ± 3.2	-7.4 ± 3.6	2020/03/30 ± 10.9
	High (n = 11)	27.7 ± 1.0	29.0 ± 1.2	483.4 ± 38.8	0.58 ± 0.07	51.0 ± 1.7	35.4 ± 1.6	-3.8 ± 2.4	2020/04/16 ± 7.2
	p<0.05								
Standard deviation	Normal	2.8	2.6	65.7	0.05	9.6	9.0	10.3	30.8
	High	3.4	4.1	128.8	0.07	5.7	5.4	8.0	23.8

*z Thickness of flower were analysed on 2021 (Normal Ploidy; n = 5, High Ploidy; n = 7)
± indicate standerd error

General Discussion

Polyploidy of Orchidaceae

Along with the Asteraceae, Orchidaceae are one of the two largest families of flowering plants. Orchids are the most diverse family of angiosperms, with over 25000 species (Christenhusz and Byng, 2016). Furthermore, orchids have a very wide variety of lifestyle, flower morphologies and polyploidy, and have been successful colonisers of a wide variety of different habitats (Leitch *et al.,* 2009; Felix and Guerra., 2010; Givnish *et al.,* 2015). Why is the Orchidaceae so diverse? Polyploidy contributes greatly to plant genome evolution via genetic variability (Alix *et al.,* 2017). Thus, some species of orchids could have specific traits of 2*n* gametes production for adaptation and evolution (Givnish *et al.,* 2015). In present study, *Dendrobium* and *Epidendrum* showed presence of 2*n* gametes. Furthermore, unreduced 2*n* gametes should be involved the breeding of *Phalaenopsis* cultivars of Orchidaceae because majority of phalaenopsis cultivars consist of triploid and tetraploid (Lee *et al.,* 2020). Thus, 2*n* gametes would have been contribute to evolution and speciation as well as domestication and breeding in Orchidaceae. Furthermore, in *Epidendrum*, unreduced 2*n* gametes may be involved the breeding more than once in breeding because the cultivar of *Epidendrum* consist of wider and higher polyploids than phalaenopsis. The genus *Epidendrum*, which are second largest genera in Orchidaceae, is a neotropical genus comprising approximately 1500 species with a distribution ranging from south-eastern United States to northern Argentina. The genus shows wide

morphological diversification and inhabits various climatic zones, from dry tropical jungles to humid forests. This research is the first reports of multiple pathways of formation of 2*n* gametes in *Epidendrum* which is not restricted in Orchidacease, but as far as I know in the whole plant kingdom. Furthermore, my thesis showed other three unique characters of *Epidendrum*: (i) higher capacity of 2*n* gametes production of *Epidendrum* than those of other plant species. (ii) increase in production of 2*n* gametes in response to environmental stress. (iii) *Epidendrum* shows vigorous growth in progenies with high polyploid levels and less prone to evolutionary dead end. I revealed that the capacity of 2*n* gametes production contributes to high plant diversity. The characters of *Epidendrum* even more unique among Orchidaceae, in respect to polyploidy, would be appropriated as materials for revealing plant evolution related to polyploidization and formation of 2*n* gametes. This study provides a new model plant for future ploidy research.

Polyploid breeding via 2n gametes

Polyploids arise in nature through somatic doubling and unreduced 2*n* reproductive cells, and unreduced 2*n* reproductive cells predominantly lead to polyploidy in plants (De Storme & Geelen, 2013a; Sattler *et al.,* 2016; Loginova & Silkova, 2017). Polyploids are also useful in horticulture because polyploid individuals exhibit larger organs and deeper flower color (Takamura & Miyajima, 1996; Sattler *et al.,* 2016). Artificial chromosome doubling has been usually conducted by the

application of antimitotic reagent, such as colchicine, oryzalin, trifluralin and amiprophos-methyl (APM) (Dhooghe et al. 2011). The protocol of APM treatment established in the present study could be also applied for the chromosome doubling of not only other *Dendrobium* genotypes but also wide range of orchid species and genera. Sexual polyploidization, namely polyploidization via 2*n* gametes is an efficient way to produce polyploids (Ramanna & Jacobsen, 2003). Triploid induction via reduced and 2*n* gametes is the most efficient method because it can be obtained via manipulation of one generation, namely hybridization although the frequencies of 2*n* gametes are relatively low in other plants. However, the frequencies of 2*n* gametes of *Epidendrum* both wild species and hybrids were higher than other plants. Additively, unreduced 2*n* pollen formation in genus *Epidendrum* also influenced by seasonal effect. Thus, sufficient frequencies of 2*n* gametes for polyploid breeding are obtained by induction of 2*n* gametes formation via environmental control. HP progeny of *Epidendrum* exhibited vigour plant growth and larger area of flower. Larger flowers could have novel marketability as cut flower and potted flower. Furthermore, differences in color between NP and HP progeny are useful for primary selection by ploidy analysis using FCM in the *Epidendrum* breeding. Although artificial chromosome doubling has been usually conducted by the application of antimitotic reagent, present study provided possibility of efficient polyploid breeding via 2*n* gametes.

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Summary

Polyploids arise in nature through somatic doubling and unreduced reproductive cells. Polyploids are also useful in horticulture because polyploid individuals exhibit larger organs and deeper flower. Artificial chromosome doubling has been usually conducted by the application of antimitotic reagent. In present study, I focused on the polyploidy of orchid, and two pathways of polyploidization namely, artificial chromosome doubling and sexual polyploidization. The protocol of APM treatment established in the present study could be also applied for the chromosome doubling of not only other *Dendrobium* genotypes but also wide range of orchid species and genera. *Dendrobium* and *Epidendrum* showed presence of 2*n* gametes. This research is the first reports of multiple pathways of formation of 2*n* gametes in *Epidendrum* which is not restricted in Orchidacease, but as far as I know in the whole plant kingdom. Furthermore, my thesis showed other three unique characters of *Epidendrum*: (i) higher capacity of 2*n* gametes production of *Epidendrum* than those of other plant species. (ii) increase in production of 2*n* gametes in response to environmental stress. (iii) *Epidendrum* shows vigorous growth in progenies with high polyploid levels and less prone to evolutionary dead end. The characters of *Epidendrum* even more unique among Orchidaceae, in respect to polyploidy, would be appropriated as materials for revealing plant evolution related to polyploidization and formation of 2*n* gametes. This study provides a new model plant for future ploidy research. Furthermore, I showed possibility of efficient polyploid breeding via 2*n* gametes.

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