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REVIEW

The role of cytogenetic tools in orchid breeding

Samantha Sevilleno Sevilleno[1], Raisa Aone Cabahug-Braza[2], Hye Ryun An[3], Ki-Byung Lim[4], Yoon-Jung Hwang[1,2,*]

[1]Department of Convergence Science, Sahmyook University, Seoul 01795, Korea
[2]Plant Genetics and Breeding Institute, Sahmyook University, Seoul 01795, Korea
[3]Floriculture Research Division, National Institute of Horticultural & Herbal Science, Rural Development Administration, Wanju 55365, Korea
[4]Department of Horticulture Science, Kyungpook National University, Daegu 41566, Korea

[*]Corresponding author: hyj@syu.ac.kr

Abstract

Orchidaceae species account for one-tenth of all angiosperms including more than 30,000 species having significant ecological, evolutionary, and economic importance. Despite Orchidaceae being one of the largest families among flowering plants, crucial cytogenetic information for studying species diversification, inferring phylogenetic relationships, and designing efficient breeding strategies is lacking, except for 10% or less of orchid species cases involving mostly chromosome number or karyotype analysis. Also, only approximately 1.5% of the identified orchid species from less than a hundred genera have genome size data that provide crucial information for breeders and molecular geneticists. Various molecular cytogenetic techniques, such as fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH), have been developed for determining ploidy levels, analyzing karyotypes, and evaluating hybridity, in several ornamental crops including orchids. The estimation of genome size and the determination of nuclear DNA content using flow cytometry have also been employed in some Orchidaceae subfamilies. These different techniques have played an important role in supplementing beneficial knowledge for effective plant breeding programs and other related plant research. This review focused on orchid breeding summarizes the status of current cytogenetic tools in terms of background, advancements, different techniques, significant findings, and research challenges. Principal roles and applications of cytogenetics in orchid breeding as well as different ploidy level determination methods crucial for breeding are also discussed.

Key words: breeding, chromosome, cytogenetics, FISH (fluorescence *in situ* hybridization), orchids

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Introduction

Orchidaceae, consisting of about 30,000 species belonging to 880 genera, is one of the largest families of flowering plants (Hsiao et al., 2011). Orchids are predominantly grown as ornamentals, consisting of a million-dollar cut-flower industry in most Asian countries with the most popular hybrids belonging to the genera *Phalaenopsis, Cymbidium, Cattleya,* and *Oncidium* (Chugh et al., 2009).

Orchids represent a highly advanced and terminal line of evolution in angiosperms with a unique and eccentric floral diversity and a large ploidy level disparity (Hsiao et al., 2011). Orchid breeding aims to produce hybrids with optimum floral quantity and morphology, blooming periods, and improved tolerance to stress (Pamarthi et al., 2019). Driven by the economic benefits, scientists are engaged in orchid biology, cytogenetics, and genomics research which is essential for successful breeding (Tsai et al., 2017).

Cytogenetic information, i.e., ploidy level, chromosome number, morphology, length, symmetry, and karyotype, are basic yet essential for understanding species differentiation and diversification (Raven, 1975), as well as for elucidating phylogenetic relationships between wild and cultivated species (Stace, 2000). Furthermore, mitotic and meiotic cytogenetic studies are crucial in checking the viability and stability of orchid hybrids (Kiihl et al., 2011). However, cytogenetic information is available in only 10% of orchids because of their high diversity in chromosome number and considerably small size (Sharma and Mukai, 2015). Lack of cytogenetic information and varying ploidy levels between species represent an additional persistent difficulty in transferring favorable genes of distantly related diploid wild species to commercial orchid hybrids (Chen et al., 2010). Thus, an enhanced understanding of orchid genomic makeup and polyploidy nature is necessary for designing an efficient breeding strategy (Jauhar, 2006).

This review highlights the role, significance, and applications of cytogenetic tools while discussing common challenges and technologies applied in orchid breeding.

Brief background of orchid breeding

Challenges in orchid breeding

Natural or induced polyploidization has provided the means for improving orchid species in terms of increased organ size, genome buffering, heterozygosity, and hybrid vigor which led to an increased level of fertility and stress tolerance (Hwang et al., 2019). However, orchid breeding is challenged by: 1) Inherently slow growth of species requiring approximately 2 - 3 years to reach maturity (Kao et al., 2007); 2) Difficulties in species and interspecific hybrids identification using traditional methods (Vu et al., 2017); 3) Extensive intra- and inter-specific differences in ploidy levels creating genetic obstacles in introducing desirable traits; 4) Scarcity of polyploid wildtype germplasms, restricting polyploidization; and 5) Sterility of progenies resulting from hybridization of parents with different chromosome sizes or ploidy levels (Chen et al., 2010).

The first two aforementioned challenges have already been addressed in multiple reviews, while this paper aims to discuss the techniques used to resolve the remaining three.

Technologies applied in orchid breeding

Micropropagation

Orchids were the first plants to be propagated *in vitro*, both from seeds or through tissue culture methods of vegetative parts. Chugh et al. (2009) have provided a detailed review of the different explants used in orchid micropropagation.

Molecular analysis for species identification

One of the most commonly used DNA marker techniques for cultivar identification analysis in orchids is the Random Amplified Polymorphic DNA (RAPD) (Kurniawati et al., 2019) because it is quick, cheap, does not require prior DNA sequencing, and provides robust estimates of intra-specific genetic diversity (Borowsky, 2001). Microsatellite markers are also considered useful for genotype characterization such as genotype analysis, population genetic analysis, and genetic mapping (Lee and Eo, 2016). A detailed summary of the available molecular markers and measurements for orchid identification is provided by Kumar et al. (2018) and Vu et al. (2017).

Next-Generation Sequencing (NGS) technologies

In many plant species, NGS technologies have augmented genomics research. Evolutionary studies on the genomic structure through sequencing provide knowledge on understanding relationships among different species (Choi et al., 2016). To date, four orchid genomes have been sequenced. Significant results were found for the following species: 1. *Phalaenopsis equestris*, expanded and diversified families of genes were found which are reported to possibly contribute to highly specialized orchid flower morphologies (Cai et al., 2015); 2. *Dendrobium catenatum*, the expansion of many resistance-related genes, as well as extensive duplication of genes involved in glucomannan synthase activities, were observed (Zhang et al., 2016); 3. *Dendrobium officinale*, an important medicinal plant, the assembled genome is 1.23 Gb long with contig N50 of 1.44 Mb (Yan et al., 2015); and 4. *Apostasia shenzhenica,* among the four orchid species, its genome sequencing revealed clear evidence of an ancient whole-genome duplication (WGD) shared by all orchids (Zhang et al., 2017). NGS technologies were also applied to orchids and were used to investigate their transcriptomes and systematically analyze their small RNAs, and these were summarized in a review published by Tsai et al. (2017). The gathered data in these genome sequencing analyses have provided a reference for studying orchid genome diversity and evolution.

Genome editing technologies

At present, gene editing techniques such as the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system have been used to considerably accelerate and improve orchid breeding. In *Phalaenopsis amabilis*, CRISPR/Cas9 successfully mutated the Phytoene desaturase (PDS3) gene encoding the enzyme involved in the carotenoid biosynthesis pathway. The PDS3 mutants showed an albino phenotype in the leaf tissues (Semiarti et al., 2020). This technology was also able to produce a null mutant of the *MADS* gene in *P. equestris*, which is often highly expressed in floral organs and may have significance in flower initiation and development (Tong et al., 2020). In *D. officinale*, a 100% success rate was reported in knocking out the expression of several lignocellulose biosynthesis genes (Kui et al., 2017). These studies laid the groundwork for the breeding of new orchid varieties with desirable trait genes via precise and speedy genome editing.

Cytogenetics in breeding

Although the aforementioned technologies significantly contributed to the advancement of orchid research, no technique enabling direct observation of orchid chromosomes is available. Conventional and molecular cytogenetic tools have played a pivotal role in accelerating crop improvement, including the production of new cultivars by enhancing the crossing efficiency between valuable plant varieties (Hwang et al., 2019). Knowledge of chromosome pairing, genomic relationships between and within plant species, polyploidy, aneuploidy, and other chromosome-related properties have profoundly assisted the breeding of major flowering ornamental plants including orchids (Jauhar, 2006). Studies on chromosome meiotic and mitotic behavior in crossbred plants can aid the genetic viability and stability assessment of plants (Kiihl et al., 2011). Cytogenetic studies can also provide tools for the identification and differentiation of wild and domesticated species. In-depth analysis of genome size and ploidy levels in orchids using these tools can aid breeders establish breeding strategies including interspecific and interploidy hybridization (Lin et al., 2001).

In addition, artificial polyploidy induction using antimitotic agents has been used to produce improved hybrid orchid cultivars with unique phenotypic traits, higher content of medicinal ingredients, and greater adaptability and resistance (de Chandra et al., 2019). These chemicals, which are applied *in vitro*, interfere during cell division, generating chromosome duplication in plant cells (Germanà, 2012). The efficacy in producing artificial polyploid orchids depends on the type, concentration, and exposure time to the antimitotic agent, explant type and age, and *in vitro* induction protocol (Dhooghe et al., 2011; Vilcherrez-Atoche et al., 2022). Cytogenetic approaches are crucial in confirming the chromosomal duplication in these plants.

Ploidy level determination in orchids

Chromosomal content and ploidy levels should be determined to effectively cross two species and ensure that true hybrids with confirmed ploidy levels are produced. Among methods that have been employed in ploidy level determination, cytogenetic analysis is the most accurate (Sattler et al., 2016). The above mentioned methods may be direct or indirect based on whether actual observation of chromosomes is conducted. Direct methods involve the observation of chromosomes using microscopy techniques, whereas indirect methods do not require any chromosome analysis (Maluszynska, 2003). Comparisons and examples of these methods are provided in Table 1.

Chromosome counting for ploidy validation

The most common and established method used in determining ploidy levels in plants is chromosome counting, which has been proven to be effective and reliable (Hwang et al., 2019). Preparation of metaphase chromosomes for counting includes three basic steps: (1) sample collection and pre-treatment, (2) fixation, and (3) chromosome spreading and staining (Ochatt, 2008). Root pre-treatment is a critical step during which the spindle fiber formation is blocked and chromosome metaphase is arrested facilitated by an increase in the cytoplasm viscosity, which is crucial for chromosome movement (Singh, 2016). The most commonly used microtubule inhibitors in orchids are 8-hydroxyquinoline and α–bromonaphthalene (Maluszynska, 2003; Begum et al., 2009; Daviña et al., 2009; Lee et al., 2020).

Table 1. Methods used in determining ploidy level in plants.

Method		Ploidy level determination	Necessary equipment	Advantages	Disadvantages	References
Direct	Chromosome counting	Comparison of chromosome base number	Fluorescence microscope	Data can be obtained in two days	Labor-intensive, occasionally misleading	Maluszynska (2003); Yuan et al. (2009)
Indirect	Flow cytometry	Comparison of sample fluorescence density of previously known diploid or tetraploid standards	Flow cytometer	Data can be obtained within a day	Expensive equipment, special techniques required to operate the machine and interpret data	Yuan et al. (2009)
	Stomatal size	Higher stomata sizes = higher ploidy level	Light microscope	Ease of use	Cytotype, physiology, and ecology should be considered	He et al. (2018)
	Chloroplast number in guard cells	Chloroplast number per single stoma: < 10 (haploid) 11 - 15 (diploids) > 15 (polyploids)	Light microscope	Ease of use	No defined number of chloroplasts to distinguish between ploidy levels	Yuan et al. (2009); He et al. (2018)
	Morphological observation	Bigger flowers; delayed and/or prolonged flowering; altered length/width ratio of leaves; darker green coloration of leaves; thicker leaves and stems = polyploid	Digital caliper, color meter	No expensive equipment is required	Growing plants until flowering is time-consuming	Sakiroglu and Kaya (2012); He et al. (2018)

Chromosome number, size, morphology, and karyotypes vary in orchids even within the same genus. Mitotic chromosome preparation in orchids is challenging because: a) their cells have a low mitotic index and usually display early or late stages of cell division and b) their root tips are covered with multiple layers of velamen, a tissue, which plays an important role in water and nutrient uptake of epiphytic orchids (Sharma and Mukai, 2015). Chromosome numbers in orchids vary from 2n = 10 to 240. Although high chromosome numbers are common, the most frequent is n = 19 or 20 (Daviña et al., 2009). Despite the difficulties in preparing metaphase chromosomes, classical cytogenetic methods have provided key information on the genetic variations and chromosome characterization among orchid species.

Indirect methods of ploidy estimation

Recently, advanced ploidy level determining techniques other than chromosome counting have been reported in some plant species. These techniques include flow cytometric analysis, stomatal size and density measurement, counting the number of chloroplasts in guard cells, and morphological observations (Maluszynska, 2003).

Flow cytometry is a powerful and accurate tool for verifying ploidy levels. However, in plants having a wide range of ploidy levels such as orchids, this method has several disadvantages such as a long time for generating samples (Doležel and Bartoš, 2005) and poor reproducibility as reported in previous flow cytometric studies in certain orchid species (Hwang et al., 2019).

The stomatal assay method has been used to evaluate the ploidy levels in orchids such as *Cymbidium* (Russell, 2004) and *Phalaenopsis* (Chen et al., 2010). Measuring stomatal length and counting chloroplast numbers within the stoma typically conducted in this method, are accurate and inexpensive procedures but time-consuming (Beck et al., 2005).

Since ploidy level determination using morphological or anatomical assays alone has limitations, chromosome counting of root mitotic cells and flow cytometry should be applied in combination with other indirect methods to further validate ploidy levels (Vanstechelman et al., 2010).

Molecular cytogenetic tools used in orchids

Although useful, conventional karyotyping methods have certain limitations in polyploid plants and are challenged by their variable physical characteristics of homologues, morphological similarities, relatively small-sized chromosomes, and the requirement for labor-intensive procedures (Dutrillaux et al., 2009). The development of advanced cytogenetic techniques using radioisotope labeled probes such as *in situ* hybridization (ISH), paved the way for the transition from the classical to modern molecular cytogenetics era (Jiang and Gill, 2006). In molecular cytogenetic techniques, DNA sequences are used as probes to locate specific target sequences on the chromosomes of a species of interest and visualize them using microscopy. The ISH methods evolved from using environmentally dangerous radioisotope-labeled probes, into fluorescence *in situ* hybridization (FISH) methods using fluorescence-based labeled probes. This technique is referred to as genomic *in situ* hybridization (GISH) in cases where whole genomes are used as probes. Overall, modern molecular cytogenetic tools enable a more precise identification and mapping of chromosomes (Devi et al., 2005). Moreover, in mutation breeding, molecular cytogenetic approaches play an important role as a quick assessment of the first genetic effects after mutagenic treatment (Vilcherrez-Atoche et al., 2022).

Fluorescence *in situ* hybridization

FISH provides basic information on ploidy level, chromosome characteristics, the parental origin of hybrids, etc. which are prerequisites of crossbreeding methods (Hwang et al., 2019). It has been extensively used to locate complementary DNA sequences in nuclei with the use of chromosome-specific probes labeled with fluorescent dyes (Hwang et al., 2011). Based on the material or procedure utilized, probe labeling can be direct or indirect when fluorochromes or haptens are used, respectively. The use of fluorochrome-labeled probes in direct labeling is more efficient due to shorter time requirements and clearer signals produced in highly repetitive sequences (Perumal et al., 2017). In indirect labeling, a hapten (typically biotin or digoxigenin)-labeled DNA probe forming an antibody-fluorochrome conjugate is used (Bishop, 2010). This method is suitable for the amplification of FISH signals from short DNA targets. FISH is a powerful tool for identifying karyotypes as well as physically mapping specific DNA sequences in chromosomes. The most commonly used probes for FISH karyotyping are the 5S and 45S ribosomal DNAs (rDNAs) consisting of conserved among plants tandem repeats. The number, size, and characteristic positions of rDNAs along chromosomes consist of useful markers for chromosome analysis (Heslop-Harrison, 2000). Basic FISH karyotype analyses using these probes have been performed in several orchid species listed in Table 2, revealing the complex genome organization of orchids. Aside from the different number of chromosomes, the number of 5S and 45S rDNA sites also varied in some wild orchids in Italy (D'emerico et al., 2001), and in some species belonging to the genera *Maxillaria* (Cabral et al., 2006), *Cephalanthera* (Moscone et al., 2007), and *Paphiopedilum* (Lee and Chung, 2008). In contrast, the number of chromosomes was common (2n = 40) in eight horticulturally important *Cymbidium* species from Northern-East India, and a single pair of 45S rDNA was found in all analyzed species, although three *Cymbidium* species showed a decondensed, dispersed, and extended form of rDNA FISH signal (Sharma et al., 2012).

Table 2. List of orchid species with chromosome number and rDNA fluorescence *in situ* hybridization (FISH) data. (continued)

Genus	Species name	Chromosome number (2n)	No. of rDNA loci		Reference
			5S	45S	
Anacamptis	*A. collina* (Banks & Sol. ex Russell) R.M. Bateman, Pridgeon & M.W. Chase	36	2	2	D'emerico et al. (2001)
	A. gennarii (Rchb. f.) H. Kretzschmar, Eccarius & H. Dietr.	34	3	3	
	A. morio (L.) R.M. Bateman, Pridgeon & M.W. Chase	36	2	4	
	A. papilionacea (L.) R.M. Bateman, Pridgeon & M.W. Chase	32	4	2	
Barlis	*B. robertiana (*Loisel.) Greuter	36	2	2	
Cephalantera	*C. damasonium* (Mill.) Druce	36	4	2	Moscone et al. (2007)
	C. longifolia (L.) Fritsch.	32	6	2	
	C. rubra (L.) Rich.	44	2	4	
Cymbidium	*C. aloifolium* Wall.	40	-	2	Sharma et al. (2012)
	C. cyperifolium Wall. ex Lindl.	40	-	2	
	C. elegans Lindl.	40	-	2	
	C. hookerianum Rchb. f.	40	-	2	
	C. iridioides D. Don	40	-	2	
	C. mastersii Griff. ex Lindl.	40	-	2	
	C. tigrinum Parish ex Hook. f.	40	-	2	
	C. tracyanum L. Castle.	40	-	2	
Dendrobium	*D. aggregatum* Roxb.	38	2	2	Begum et al. (2009)
	D. aphyllum (Roxb.) Fisch	38	2	4	
	D. moschatum (Buch.-Ham.) Sw.	40	2	6	
Heterotaxis	*H. brasiliensis* (Brieger & Illg) F. Barros	42	2	4	Moraes et al. (2016)
	H. equitans (Brieger & Illg) F. Barros	42	4	4	
	H. superflua (Rchb. f.) F. Barros	42	2	4	
	H. valenzuelana (A. Rich.) Ojeda & Carnevali	40	2	2	
	H. villosa (Barb. Rodr.) F. Barros	42	2	4	
	H. violaceopunctata (Rchb. f.) F. Barros	42	2	4	
Mapinguari	*M. desvauxiana* (Rchb. f.) Carnevali & R.B. Singer	40	4	2	
Maxillaria	*M. acicularis* Herb. ex Lindl.	38	4	4	Cabral et al. (2006)
	M. discolor Rchb.	42	2	4	
	M. notylioglossa Rchb. f.	38	4	2	
Paphiopedilum	*P. acmodontum* M.W. Wood	38	4	2	Lan and Albert (2011)
	P. adductum Asher	26	32	9	
	P. armeniacum S.C. Chen & F.Y. Liu	26	2	4	
	P. bellatulum Pfitzer	26	2	2	
	P. curtisii Pfitzer	36	2	2	
Paphiopedilum	*P. dayanum* Pfitzer	36	10	2	Lan and Albert (2011)
	P. delenatii Guillaumin	26	2	2	
	P. dianthum Tang & F.T. Wang	26	32	2	
	P. druryi Pfitzer	30	20	2	
	P. emersonii Koop. & P.J. Cribb	26	2	4	
	P. fairrieanum (Lindl.) Stein	26	16	2	
	P. gigantifolium Braem. M.L. Baker & C.O. Baker	26	38	6	
	P. glanduliferum Pfitzer	26	30	4	
	P. hangianum Perner & O. Gruss	26	2	4	
	P. haynaldianum Pfitzer	26	12	4	

Table 2. List of orchid species with chromosome number and rDNA fluorescence *in situ* hybridization (FISH) data.

Genus	Species name	Chromosome number (2n)	No. of rDNA loci		Reference
			5S	45S	
	P. hennisianum (M.W. Wood) Fowlie	34	8	2	
	P. henryanum Braem	26	19	2	
	P. hirsutissimum Pfitzer	26	27	2	
	P. liemianum (Fowlie) K. Karas. & K. Saito	32	26	2	
	P. lowii Pfitzer	26	32	6	
	P. malipoense S.C. Chen & Z.H. Tsi	26	2	2	
	P. micranthum Tang & F.T. Wang	26	2	4	
	P. moquettianum	34	24	2	
	P. niveum Pfitzer	26	2	2	
	P. parishii Pfitzer	26	38	4	
	P. primulinum M.W. Wood & P. Taylor	32	29	2	
	P. purpuratum Pfitzer	40	12	2	
	P. randsii Fowlie	26	34	4	
	P. sanderianum Pfitzer	26	20	2	
	P. sangii Braem	38	22	2	
	P. stonei Pfitzer	26	29	2	
	P. sukhakulii Schoser & Senghas	40	15	2	
	P. supardii Braem & U.W.A. Löb.	26	30	9	
	P. tigrinum Koop. & N. Haseg.	26	23	2	
	P. venustum (Wall. ex Sims) Pfitzer	40	12	2	
	P. victoria-regina (Sander) M.W. Wood	34	28	2	
	P. wardii Summerh.	42	4	2	
Phalaenopsis	*P. aphrodite* Rchb. f.	38	4	4	Kuo et al. (2016)
	P. equestris (Schauer) Rchb. f.	38	2	0	
Vanda	*Vanda* sp.	38	4	2	Sharma and Mukai (2015)

Retrieved from https://www.plantrdnadatabase.com/.

BAC-FISH, using genomic DNA cloned in large-insert vectors such as bacterial artificial chromosomes (BACs) in combination with FISH, has been shown to be an efficient technique for physically mapping specific DNA sequences and identifying individual chromosomes and chromosome-specific markers in large genome plants such as orchids (Sharma and Mukai, 2015). Two BAC libraries constructed for *P. equestris,* provided insights into the complex genome of the above species in terms of guanine-cytosine (GC) content, transposable elements presence, protein-coding regions, SSRs, and potential microsynteny between *Phalaenopsis* and other plant species (Hsu et al., 2011). BAC-FISH analysis conducted by Matsuba et al. (2015) in two orchid species, *Neofinetia falcata,* and *Rhynchostylis coelestis,* mapped the distribution of certain repetitive sequences in orchid chromosomes and provided a chromosome-based comparison of specific regions between the two species and their hybrids.

In other plant species, FISH has also been used in giving a comprehensive evaluation of the effects of various mutagens on the plant genome that are observed as chromosomal aberrations, including micronuclei. Knowledge on the composition and genetic activity of the chromatin that is involved in micronuclei can be revealed by FISH. This information is important as it could be related to the ability of this chromatin to exert proper DNA expression and DNA repair (Kwasniewska and Bara, 2022). In *Brachypodium distachyon*, the use of centromeric and telomeric FISH probes provided information regarding the

origin of the micronucleus that were induced by X-radiation (Kus et al., 2017). Chromosome rearrangements are usually observed in meiocytes of presumptive orchid allopolyploids, along with micronuclei in tetrads (Bolanos-Villegas et al., 2008). Although fluorescent methods, such as DAPI (4',6-diamidino-2-phenylindole) or acridine orange staining have already been used for micronuclei detection (Dias et al., 2005), to date, FISH has not yet been used investigating micronuclei present in both wild and cultivated orchids.

Genomic *in situ* hybridization

GISH follows the same principle as FISH except that it uses (1) the total genomic DNA of a genitor involved in the hybrid formation and (2) unlabeled DNA from another genitor (i.e., the blocking DNA) as probes (Silva and Souza, 2013). For hybrids derived from closely related, high homologous species, increasing the concentration of blocking DNA is necessary to avoid indiscriminate genome labeling of both parents (Brammer et al., 2009). GISH allows the characterization of the genome and chromosomes of hybrid plants, allopolyploid and interspecific introgression lines and thus, the deciphering of ancestry in hybrid and polyploid species (Devi et al., 2005).

GISH has aided the elucidation of the genome organization and the relationships of seven interspecific hybrids of *Phalaenopsis* with varying genome sizes. However, the strength and distribution of GISH hybridization signals were indistinguishable in hybrids whose parents had similar genomes. Furthermore, all large genome species had chromosomes that produced strong hybridization signals which indicates that such species contain abundant repetitive sequences (Lin et al., 2001). GISH was also able to provide a clear distinction between the parental genomes and the resulting interspecific hybrids, e.g., *Paphiopedilum delenatti* × *Paphiopedilum glaucophyllum* (Lee and Chung, 2008). Furthermore, Lee et al. (2011) reported the successful use of GISH for the differentiation and visualization of the chromosome pairing affinities between parental genomes in interspecific F_1 hybrids of *Paphiopedilum*, allowing the determination of the phylogenetic distances among these species. In harlequin and novel cultivars of *Phalaenopsis* possessing large and/or asymmetrical chromosomes, utilizing GISH is necessary for detecting the differential introgression of larger chromosomes and/or their segments and tracing valuable for future breeding horticultural traits associated with the remaining large chromosomes (Lee et al., 2020).

Interspecific hybrids have been developed for the improvement of ornamental plants like orchids, introducing traits that can enhance crop performance such as resistance to pests and diseases, better flower shape and color, etc. (Hwang et al., 2019). Species intercrossing between subgenera may result in sterile or low fertility progeny due to irregular chromosome pairing. GISH can contribute to understanding and tackling the problems that may occur during breeding program crosses through the visualization of meiosis in hybrids, revealing whether pairing only occurs between homologous or heterologous chromosomes (Silva and Souza, 2013).

Flow cytogenetics

Plant genome size information is crucial in breeding programs and provides insights into a genus which is helpful in improving breeding strategies (Doležel and Bartoš, 2005). Several methods have been employed to estimate plant genome sizes and currently, the most widely recommended is flow cytometry (FCM) due to a number of advantages such as high data throughput, ease of sample preparation, low quantity requirements, and high accuracy (Trávníček et al., 2015). The precise determination of genome size is important since it provides essential information for breeders and molecular geneticists. Moreover, comparative analysis of nuclear DNA content is valuable in conducting cytotaxonomic and evolutionary studies

(Lin et al., 2001).

Despite being one of the largest and most diverse angiosperm families, genome size data is available in only approximately 1.5% of the identified orchid species from less than a hundred genera. To date, all five Orchidaceae subfamilies (i.e., Apostasioideae, Vanillodeae, Cypripediodeae, Orchidoideae, and Epidendroideae) have been characterized in regard to genome size (Chen et al., 2013). Among these, Epidendroideae has the most diverse genome size with values varying over 60-fold, although the majority of its species possess small genomes. Moreover, aside from displaying a wide variation in chromosome numbers, Orchidaceae shows a remarkable variation in 2C DNA content, almost 170-fold ranging from 1C = 0.33 pg in *Trichocentrum maduroi* to 55.4 pg in *Pogonia ophioglossoides* (Sharma and Mukai, 2015).

An organ-specific degree of endopolyploidy has been observed in several orchid species and hybrids (Chen et al., 2010). This fact in combination with the lack of 2C nuclei in different plant organs, as well as the occurrence of both conventional endopolyploidy and progressively partial endoreplication (PPE) in orchids, may cause incorrect FCM estimates. Trávníček et al. (2015) highlighted the challenges in estimating nuclear genome size using flow cytometry in orchids and proposed a method to avoid misinterpretation of DNA content histograms. However, due to the various genome sizes of orchids, predicting the ploidy of the species based only on the C-value measured by flow cytometry is difficult thus, additional confirmation of the results with the conventional cytogenetic technique of chromosome counting using root tip cells arrested in metaphase is required (Ochatt, 2008).

Conclusion

Considering all the conducted orchid chromosome studies, it can be concluded that great potential exists in investigating these species at the cytogenetic level. Although conventional methods of chromosome research allow the identification and observation of the ploidy level, numerical and structural variations in chromosomes, and meiotic behavior of chromosomes, deeper cytogenetic and genomic information are required in order to develop efficient breeding strategies for improving orchid cultivars (Sharma and Mukai, 2015). Molecular cytogenetic techniques such as FISH, GISH, and flow cytometry have played an essential role in providing supplemental information to be used for efficient plant breeding programs and other related research. The contribution of these techniques in obtaining the chromosomal characteristics of plants in a more accurate and efficient way is undisputed. The continuous advancement of cytogenetic tools leading to innovations in chromosome engineering will be of immense importance in the development of meaningful breeding programs, such as the applications of haploid induction, conversion of meiosis into mitosis to produce diploid gametes that are clones of the parent plant, and new homologous recombination technologies (Chan, 2010), for the improvement of orchids. Overall, cytogenetic data in conjunction with molecular markers should aid in the accurate study of genetic diversity and provide new insights into the genome architecture of orchids.

Conflict of Interests

No potential conflict of interest relevant to this article was reported.

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Authors Information

Samantha S. Sevillen, https://orcid.org/0000-0001-9906-4992

Raisa Aone M. Cabahug, https://orcid.org/0000-0003-0863-4721

Hye Ryun An, https://orcid.org/0000-0002-3606-9195

Ki-Byung Lim, https://orcid.org/0000-0003-2342-4052

Yoon-Jung Hwang, https://orcid.org/0000-0002-5984-8968

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