

REVIEW

The role of cytogenetic tools in orchid breeding

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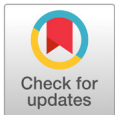
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Abstract

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 antimetabolic 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 antimetabolic 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	
<i>Anacamptis</i>	<i>A. collina</i> (Banks & Sol. ex Russell) R.M. Bateman, Pridgeon & M.W. Chase	36	2	2	D'emerico et al. (2001)
	<i>A. gennarii</i> (Rchb. f.) H. Kretzschmar, Eccarius & H. Dietr.	34	3	3	
	<i>A. morio</i> (L.) R.M. Bateman, Pridgeon & M.W. Chase	36	2	4	
	<i>A. papilionacea</i> (L.) R.M. Bateman, Pridgeon & M.W. Chase	32	4	2	
<i>Barlis</i>	<i>B. robertiana</i> (Loisel.) Greuter	36	2	2	
<i>Cephalanthera</i>	<i>C. damasonium</i> (Mill.) Druce	36	4	2	Moscone et al. (2007)
	<i>C. longifolia</i> (L.) Fritsch.	32	6	2	
	<i>C. rubra</i> (L.) Rich.	44	2	4	
<i>Cymbidium</i>	<i>C. aloifolium</i> Wall.	40	-	2	Sharma et al. (2012)
	<i>C. cyperifolium</i> Wall. ex Lindl.	40	-	2	
	<i>C. elegans</i> Lindl.	40	-	2	
	<i>C. hookerianum</i> Rchb. f.	40	-	2	
	<i>C. iridioides</i> D. Don	40	-	2	
	<i>C. mastersii</i> Griff. ex Lindl.	40	-	2	
	<i>C. tigrinum</i> Parish ex Hook. f.	40	-	2	
	<i>C. tracyanum</i> L. Castle.	40	-	2	
<i>Dendrobium</i>	<i>D. aggregatum</i> Roxb.	38	2	2	Begum et al. (2009)
	<i>D. aphyllum</i> (Roxb.) Fisch	38	2	4	
	<i>D. moschatum</i> (Buch.-Ham.) Sw.	40	2	6	
<i>Heterotaxis</i>	<i>H. brasiliensis</i> (Brieger & Illg) F. Barros	42	2	4	Moraes et al. (2016)
	<i>H. equitans</i> (Brieger & Illg) F. Barros	42	4	4	
	<i>H. superflua</i> (Rchb. f.) F. Barros	42	2	4	
	<i>H. valenzuelana</i> (A. Rich.) Ojeda & Camevali	40	2	2	
	<i>H. villosa</i> (Barb. Rodr.) F. Barros	42	2	4	
	<i>H. violaceopunctata</i> (Rchb. f.) F. Barros	42	2	4	
<i>Mapinguari</i>	<i>M. desvauxiana</i> (Rchb. f.) Camevali & R.B. Singer	40	4	2	
<i>Maxillaria</i>	<i>M. acicularis</i> Herb. ex Lindl.	38	4	4	Cabral et al. (2006)
	<i>M. discolor</i> Rchb.	42	2	4	
	<i>M. notylioglossa</i> Rchb. f.	38	4	2	
<i>Paphiopedilum</i>	<i>P. acmodontum</i> M.W. Wood	38	4	2	Lan and Albert (2011)
	<i>P. adductum</i> Asher	26	32	9	
	<i>P. armeniacum</i> S.C. Chen & F.Y. Liu	26	2	4	
	<i>P. bellatulum</i> Pfitzer	26	2	2	
	<i>P. curtisii</i> Pfitzer	36	2	2	
	<i>P. dayanum</i> Pfitzer	36	10	2	
	<i>P. delenatii</i> Guillaumin	26	2	2	
	<i>P. dianthum</i> Tang & F.T. Wang	26	32	2	
	<i>P. druryi</i> Pfitzer	30	20	2	
	<i>P. emersonii</i> Koop. & P.J. Cribb	26	2	4	
	<i>P. fairieanum</i> (Lindl.) Stein	26	16	2	
	<i>P. gigantifolium</i> Braem. M.L. Baker & C.O. Baker	26	38	6	
	<i>P. glanduliferum</i> Pfitzer	26	30	4	
	<i>P. hangianum</i> Perner & O. Gruss	26	2	4	
	<i>P. haynaldianum</i> 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	
	<i>P. hennisianum</i> (M.W. Wood) Fowlie	34	8	2	
	<i>P. henryanum</i> Braem	26	19	2	
	<i>P. hirsutissimum</i> Pfitzer	26	27	2	
	<i>P. liemianum</i> (Fowlie) K. Karas. & K. Saito	32	26	2	
	<i>P. lowii</i> Pfitzer	26	32	6	
	<i>P. malipoense</i> S.C. Chen & Z.H. Tsi	26	2	2	
	<i>P. micranthum</i> Tang & F.T. Wang	26	2	4	
	<i>P. moquettianum</i>	34	24	2	
	<i>P. niveum</i> Pfitzer	26	2	2	
	<i>P. parishii</i> Pfitzer	26	38	4	
	<i>P. primulinum</i> M.W. Wood & P. Taylor	32	29	2	
	<i>P. purpuratum</i> Pfitzer	40	12	2	
	<i>P. randsii</i> Fowlie	26	34	4	
	<i>P. sanderianum</i> Pfitzer	26	20	2	
	<i>P. sangii</i> Braem	38	22	2	
	<i>P. stonei</i> Pfitzer	26	29	2	
	<i>P. sukhakulii</i> Schoser & Senghas	40	15	2	
	<i>P. supardii</i> Braem & U.W.A. Löb.	26	30	9	
	<i>P. tigrinum</i> Koop. & N. Haseg.	26	23	2	
	<i>P. venustum</i> (Wall. ex Sims) Pfitzer	40	12	2	
	<i>P. victoria-regina</i> (Sander) M.W. Wood	34	28	2	
	<i>P. wardii</i> Summerh.	42	4	2	
<i>Phalaenopsis</i>	<i>P. aphrodite</i> Rchb. f.	38	4	4	Kuo et al. (2016)
	<i>P. equestris</i> (Schauer) Rchb. f.	38	2	0	
<i>Vanda</i>	<i>Vanda</i> 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, Vanilloideae, Cyripedioideae, 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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References

- Beck SL, Visser G, Dunlop RW. 2005. A comparison of direct (flow cytometry) and indirect (stomatal guard cell lengths and chloroplast numbers) techniques as a measure of ploidy in black wattle, *Acacia mearnsii* (de Wild). *South African Journal of Botany* 71:354-358.
- Begum R, Alam SS, Menzel G, Schmidt T. 2009. Comparative molecular cytogenetics of major repetitive sequence families of three *Dendrobium* species (Orchidaceae) from Bangladesh. *Annals of Botany* 104:863-872.
- Bishop R. 2010. Applications of fluorescence *in situ* hybridization (FISH) in detecting genetic aberrations of medical significance. *Bioscience Horizons* 3:85-95.
- Bolanos-Villegas P, Chin SW, Chen FC. 2008. Meiotic chromosome behavior and capsule setting in *Doritaenopsis* hybrids. *Journal of the American Society for Horticultural Science* 133:107-116.
- Borowsky RL. 2001. Estimating nucleotide diversity from random amplified polymorphic DNA and amplified fragment length polymorphism data. *Molecular Phylogenetics and Evolution* 18:143-148.
- Brammer SP, Poersch LB, de Oliveira AR, Vasconcelos S, Brasileiro-Vidal AC. 2009. Genomic *in situ* hybridization in *Triticeae*: A methodological approach. *Embrapa Trigo-Comunicado Técnico (INFOTECA-E)* 270:15. (in Portuguese)
- Cabral JS, Felix LP, Guerra M. 2006. Heterochromatin diversity and its co-localization with 5S and 45S rDNA sites in chromosomes of four *Maxillaria* species (Orchidaceae). *Genetics and Molecular Biology* 29:659-664.
- Cai J, Liu X, Vanneste K, Proost S, Tsai WC, Liu KW, Chen LJ, He Y, Xu Q, Bian C, et al. 2015. The genome sequence of the orchid *Phalaenopsis equestris*. *Nature Genetics* 47:65-72.
- Chan SW. 2010. Chromosome engineering: Power tools for plant genetics. *Trends in Biotechnology* 28:605-610.
- Chen WH, Kao YL, Tang CY, Tsai CC, Lin TY. 2013. Estimating nuclear DNA content within 50 species of the genus *Phalaenopsis* Blume (Orchidaceae). *Scientia Horticulturae* 161:70-75.
- Chen WH, Tang CY, Kao YL. 2010. Polyploidy and variety improvement of *Phalaenopsis* orchids. *Acta Horticulture* 878:133-138.
- Choi SC, Chung YS, Kim C. 2016. Perspectives on the genomics research of important crops in the tribe Andropogoneae: Focusing on the Saccharum complex. *Korean Journal of Agricultural Science* 43:1-13.
- Chugh S, Guha S, Rao IU. 2009. Micropropagation of orchids: A review on the potential of different explants. *Scientia Horticulturae* 122:507-520.
- Daviña JR, Grabiele M, Cerutti JC, Hojsgaard DH, Almada RD, Insaurralde IS, Honfi AI. 2009. Chromosome studies in Orchidaceae from Argentina. *Genetics and Molecular Biology* 32:811-821.
- D'Emérico S, Galasso I, Pignone D, Scrugli A. 2001. Localization of rDNA loci by fluorescent *in situ* hybridization in some wild orchids from Italy (Orchidaceae). *Caryologia* 54:31-36.

- De Chandra L, Pathak P, Rao AN, Rajeevan PK. 2019. Breeding approaches for improved genotypes. p. 300. In Commercial Orchids. De Gruyter Open Ltd., Berlin, Germany.
- Devi J, Ko JM, Seo BB. 2005. FISH and GISH: Modern cytogenetic techniques. *Indian Journal of Biotechnology* 4:307-315.
- Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J. 2011. Mitotic chromosome doubling of plant tissues *in vitro*. *Plant Cell, Tissue and Organ Culture (PCTOC)* 104:359-373.
- Dias VM, Manelli-Oliveira R, Machado-Santelli GM. 2005. Using fluorescence for improvement of the quantitative analysis of micronucleus in cell culture. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 565:173-179.
- Doležel J, Bartoš J. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany* 95:99-110.
- Dutrillaux AM, Lemonnier-Darcemont M, Darcemont C, Krpač V, Fouchet P, Dutrillaux B. 2009. Origin of the complex karyotype of the polyploid parthenogenetic grasshopper *Saga pedo* (Orthoptera: Tettigoniidae). *European Journal of Entomology* 106:477-483.
- Germanà MA. 2012. Use of irradiated pollen to induce parthenogenesis and haploid production in fruit crops. *Plant mutation breeding and biotechnology*. pp. 411-421. CABI, Wallingford, UK.
- He P, Li L, Cheng L, Wang H, Chang Y. 2018. Variation in ploidy level and morphological traits in the progeny of the triploid apple variety Jonagold. *Czech Journal of Genetics and Plant Breeding* 54:135-142.
- Heslop-Harrison JS. 2000. Comparative genome organization in plants: From sequence and markers to chromatin and chromosomes. *Plant Cell* 12:617-635.
- Hsiao YY, Pan ZJ, Hsu CC, Yang YP, Hsu YC, Chuang YC, Shih HH, Chen WH, Tsai WC, Chen HH. 2011. Research on orchid biology and biotechnology. *Plant Cell Physiol* 52:1467-1486.
- Hsu CC, Chung YL, Chen TC, Lee YL, Kuo YT, Tsai WC, Hsiao YY, Chen YW, Wu WL, Chen HH. 2011. An overview of the *Phalaenopsis* orchid genome through BAC end sequence analysis. *BMC Plant Biology* 11:3.
- Hwang YJ, Cabahug RA, Mancía FH, Lim KB. 2019. Molecular cytogenetics and its application to major flowering ornamental crops. *Horticulture, Environment, and Biotechnology* 61:1-9.
- Hwang YJ, Kim HH, Kim JB, Lim KB. 2011. Karyotype analysis of *Lilium tigrinum* by FISH. *Horticulture, Environment, and Biotechnology* 52:292-297.
- Jauhar PP. 2006. Modern biotechnology as an integral supplement to conventional plant breeding: The prospects and challenges. *Crop Science* 46:1841-1859.
- Jiang J, Gill BS. 2006. Current status and the future of fluorescence *in situ* hybridization (FISH) in plant genome research. *Genome* 49:1057-1068.
- Kao YY, Lin CC, Huang CH, Li YH. 2007. The cytogenetics of *Phalaenopsis* orchids. In *Orchid Biotechnology* edited by Chen WH, Chen HH. pp. 115-134. World Scientific Publishing Co., Ltd., Singapore, Singapore.
- Kiihl PRP, Pereira ARA, Godoy SM de, Stenzel NMC, Risso-Pascotto C. 2011. Chromosome stickiness during meiotic behavior analysis of *Passiflora serrato-digitata* L. (Passifloraceae). *Ciência Rural* 41:1018-1023.
- Kui L, Chen H, Zhang W, He S, Xiong Z, Zhang Y, Yan L, Zhong C, He F, Chen J, et al. 2017. Building a genetic manipulation toolbox for orchid biology: Identification of constitutive promoters and application of CRISPR/Cas9 in the orchid, *Dendrobium officinale*. *Frontiers in Plant Science* 7:2036.
- Kumar M, Chaudhary V, Sharma VR, Sirohi U, Singh J. 2018. Advances in biochemical and molecular marker techniques and their applications in genetic studies of orchid: A review. *International Journal of Chemical Studies* 6:806-822.
- Kuo YT, Hsu HL, Yeh CH, Chang SB. 2016. Application of a modified drop method for high-resolution pachytene chromosome spreads in two *Phalaenopsis* species. *Molecular Cytogenetics* 9:44.
- Kurniawati J, Sugiyarto L, Yulianti E, Nurcahyo H, Mercuriani IS. 2019. Molecular identification of several orchid species based on OPA10 and OPA18 RAPD marker. *International Journal of Chemical Studies* 1397:12042.
- Kus A, Kwasniewska J, Hasterok R. 2017. *Brachypodium distachyon*—a useful model in the qualification of mutagen-induced micronuclei using multicolor FISH. *PLoS One* 12:e0170618.

- Kwasniewska J, Bara AW. 2022. Plant cytogenetics in the micronuclei investigation—the past, current status, and perspectives. *International Journal of Molecular Sciences* 23:1306.
- Lan T, Albert VA. 2011. Dynamic distribution patterns of ribosomal DNA and chromosomal evolution in *Paphiopedilum*, a lady's slipper orchid. *BMC Plant Biology* 11:126.
- Lee BJ, Eo SH. 2016. Current trends in forest science research using microsatellite markers in Korean national journals. *Korean Journal of Agricultural Science* 43:221-231. [in Korean]
- Lee YI, Chang FC, Chung MC. 2011. Chromosome pairing affinities in interspecific hybrids reflect phylogenetic distances among lady's slipper orchids (*Paphiopedilum*). *Annals of Botany* 108:113-121.
- Lee YI, Chung MC. 2008. Identification of genome relationships among *Paphiopedilum* species by genomic and fluorescent *in situ* hybridization. *Acta Horticulturae* 766:331-334.
- Lee YI, Tseng YF, Lee YC, Chung MC. 2020. Chromosome constitution and nuclear DNA content of *Phalaenopsis* hybrids. *Scientia Horticulturae* 262:10908.
- Lin S, Lee HC, Chen WH, Chen CC, Kao YY, Fu YM, Chen YH, Lin TY. 2001. Nuclear DNA contents of *Phalaenopsis sp.* and *Doritis pulcherrima*. *Journal of the American Society for Horticultural Science* 126:195-199.
- Maluszynska J. 2003. Cytogenetic tests for ploidy level analyses—chromosome counting. In *Doubled Haploid Production in Crop Plants* edited by Maluszynski M, Kasha KJ, Forster BP, Szarejko I. pp. 391-395. Springer, Dordrecht, Netherlands.
- Matsuba A, Fujii M, Lee SS, Suzuki G, Yamamoto M, Mukai Y. 2015. Molecular cytogenetic use of BAC clones in *Neofinetia falcata* and *Rhynchostylis coelestis*. *Nucleus* 58:207-210.
- Moraes AP, Olmos SA, Ojeda ADI, De Barros F, Forni-Martins ER. 2016. Detecting mechanisms of karyotype evolution in *Heterotaxis* (Orchidaceae). *PLoS One* 11:e0165960.
- Moscone EA, Samuel R, Schwarzacher T, Schweizer D, Pedrosa-Harand A. 2007. Complex rearrangements are involved in *Cephalanthera* (Orchidaceae) chromosome evolution. *Chromosome Research* 15:931-943.
- Ochatt SJ. 2008. Flow cytometry in plant breeding. *Journal of Quantitative Cell Science* 73:581-598.
- Pamarthi RK, Devadas R, Kumar R, Rai D, Kiran Babu P, Meitei AL, De LC, Chakrabarthy S, Barman D, Singh DR. 2019. PGR diversity and economic utilization of orchids. *International Journal of Current Microbiology and Applied Science* 8:1865-1887.
- Perumal S, Waminal NE, Lee J, Lee J, Choi BS, Kim HH, Grandbastien MA, Yang TJ. 2017. Elucidating the major hidden genomic components of the A, C, and AC genomes and their influence on *Brassica* evolution. *Science Reports* 7:17986.
- Raven PH. 1975. The bases of angiosperm phylogeny: Cytology. *Annals of the Missouri Botanical Garden* 1:724-764.
- Russell G. 2004. Stomatal guard cell measurements using leaf prints. *Certified Senior Advisor Journal* 4:137-139.
- Sakiroglu M, Kaya MM. 2012. Estimating genome size and confirming ploidy levels of wild tetraploid alfalfa accessions (*Medicago sativa* subsp. *X varia*) using flow cytometry. *Turkish Journal of Field Crops* 17:151-156.
- Sattler MC, Carvalho CR, Clarindo WR. 2016. The polyploidy and its key role in plant breeding. *Planta* 243:281-296.
- Semiarti E, Nopitasari S, Setiawati Y, Lawrie MD, Purwantoro A, Widada J, Ninomiya K, Asano Y, Matsumoto S, Yoshioka Y. 2020. Application of CRISPR/Cas9 genome editing system for molecular breeding of orchids. *Indonesian Journal of Biotechnology* 25:61-68.
- Sharma SK, Mehra P, Kumari J, Kumar S, Kumaria S, Tandon P, Rao SR. 2012. Physical localization and probable transcriptional activity of 18S–5.8 S–26S rRNA gene loci in some Asiatic *Cymbidiums* (Orchidaceae) from north-east India. *Genes* 499:362-366.
- Sharma SK, Mukai Y. 2015. Chromosome research in orchids: Current status and future prospects with special emphasis from molecular and epigenetic perspective. *Nucleus (India)* 58:173-184.
- Silva GS, Souza MM. 2013. Genomic *in situ* hybridization in plants. *Genetics and Molecular Research* 12:2953-2965.
- Singh RJ. 2016. *Plant cytogenetics*. CRC Press, London, UK.
- Stace CA. 2000. Cytology and cytogenetics as a fundamental taxonomic resource for the 20th and 21st centuries. *Taxonomy* 49:451-477.

- Tong CG, Wu FH, Yuan YH, Chen YR, Lin CS. 2020. High-efficiency CRISPR/Cas-based editing of *Phalaenopsis* orchid MADS genes. *Plant Biotechnology Journal* 18:889-891.
- Trávníček P, Ponert J, Urfus T, Jersáková J, Vrána J, Hřibová E, Doležel J, Suda J. 2015. Challenges of flow-cytometric estimation of nuclear genome size in orchids, a plant group with both whole-genome and progressively partial endoreplication. *Cytometry Part A* 87:958-966.
- Tsai WC, Dievart A, Hsu CC, Hsiao YY, Chiou SY, Huang H, Chen HH. 2017. Post genomics era for orchid research. *Botanical Studies* 58:1-22.
- Vanstechelman I, Eeckhaut T, Van Huylbroeck J, Van Labeke MC. 2010. Histogenic analysis of chemically induced mixoploids in *Spathiphyllum wallisii*. *Euphytica* 174:61-72.
- Vilcherrez-Atoche JA, Iiyama CM, Cardoso JC. 2022. Polyploidization in orchids: From cellular changes to breeding applications. *Plants* 11:469.
- Vu THT, Le TL, Nguyen TK, Tran DD, Tran HD. 2017. Review on molecular markers for identification of Orchids. *Vietnam Journal of Science, Technology and Engineering* 59:62-75.
- Yan L, Wang X, Liu H, Tian Y, Lian J, Yang R, Hao S, Wang X, Yang S, Li Q, et al. 2015. The genome of *Dendrobium officinale* illuminates the biology of the important traditional Chinese orchid herb. *Molecular Plant* 8:922-934.
- Yuan S, Liu Y, Fang Z, Yang L, Zhuang M, Zhang Y, Sun P. 2009. Relationship between the ploidy level of microspore-derived plants and the number of chloroplast in stomatal guard cells in *Brassica oleracea*. *Scientia Agricultura Sinica* 42:189-197.
- Zhang GQ, Liu KW, Li Z, Lohaus R, Hsiao YY, Niu SC, Wang JY, Lin YC, Xu Q, Chen LJ, et al. 2017. The *Apostasia* genome and the evolution of orchids. *Nature* 549:379-383.
- Zhang GQ, Xu Q, Bian C, Tsai WC, Yeh CM, Liu KW, Yoshida K, Zhang LS, Chang SB, Chen F, et al. 2016. The *Dendrobium catenatum* Lindl. genome sequence provides insights into polysaccharide synthase, floral development and adaptive evolution. *Scientific Reports* 6:19029.