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Characterization of Genomic Inheritance of Interspecific Hybrids between *Vanda ampullacea* var. auranticum and *Vanda testacea* by GISH

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Abstract
The interspecific hybrids between *Vanda ampullacea* var. auranticum and *Vanda testacea* have been generated to introduce the pale yellow color into the Vanda germplasm in prior study. In order to confirm the inheritance in hybrid progenies, genomic in situ hybridization (GISH) analysis was conducted to confirm the interspecific hybridization status. GISH analysis showed the presence of both maternal and paternal chromosomes in the cells of the hybrids indicating that the hybrid seedlings were interspecific hybrids of the two parents. GISH analysis is an effective detection technology to identify the interspecific hybridization status of hybrids.
Keywords: Genomic in situ hybridization (GISH), orchid, Vanda

1. Introduction

Comprising approximately 35,000 species, Orchidaceae is considered the largest family of flowering plants in the world [1]. Vandaceous orchids include Vanda, Ascocenda, Phalaenopsis, Renanthera, Rhynchostylis and Aerides, which are characterized by a monopodial growth habit and are mainly found in tropical Asia [2]. *Vanda testacea* is an endangered orchid with highly medicational value, ornamental and epiphytic forest orchid. *Vanda ampullacea* var. *auranticum* is an endemic orchid of Manipur with highly ornamental values. *Vanda* PB Mazumder is a hybrid orchid synthesized from *Vanda testacea* × *Vanda ampullacea* var *auranticum* with pale yellow flower.
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 [3]. 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 [4]. 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 indicate that such species contain abundant repetitive sequences [5]. GISH was also able to provide a clear distinction between the parental genomes and the resulting interspecific hybrids, e.g., *Paphiopedilum delenatti* ×

Paphiopedilum glaucophyllum [6]. Furthermore, Lee et al. (2011) [7] reported the successful use of GISH for the differentiation and visualization of the chromosome pairing affinities between parental genomes in interspecific F1 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 [8]. 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. [9]. 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 [10].

2. Materials and Method

The plant materials used in this study consisted of F1 interspecific hybrid seedlings derived from *Vanda ampullacea* var. auranticum and *Vanda testacea*. The interspecific hybrids were obtained via in vitro tissue culture. All plants were cultivated in poly green house. For chromosome preparation, we follow the protocol of Sri et al., 2017 [11] in which chromosome analysis of the parent orchid and their hybrid was taken using the root tips. Pieces of roots are soaked in a solution of 0.002 M 8 - Hydroxyquinoline at ca 4°C for 3-5 hours and then fixed in 45 % acetic acid for 10 minutes. They were macerated in a mixture of 1 N HCl and 45 % acetic acid (1:3) at 60°C for 1-5 minutes and then stained with 2 % aceto-orcein by the usual squash method. After that piece meristem pressed on object glass and then observed under a microscope with a magnification x 1000 for the calculation of the number of chromosomes. The plates were considered for the karyotype analysis in each species in the present investigation. GISH technique is performed by extracting the genomic DNA of both the parents as well as hybrids by following CTAB method with slight modifications. 20 samples were examined by GISH analysis. Genomic DNA was extracted from young leaves of the two parents and their hybrids using the CTAB method. DNA was then labeled with DIG-11-dUTP (Roche Ltd.) via nick translation and post-fixed in 4% paraformaldehyde for 10 min. The chromosomal DNA was denatured in 70% formamide in 2× SSC at 70°C for 2.5 min and dehydrated through an ethanol series at 4°C. The hybridization mixture consisted of 50% formamide, 10% dextran sulfate, 2× SSC, 0.1% SDS, and 50 ng/μl of DNA probe. Hybridization was performed at 37°C overnight. The slides were washed in 20% formamide in 0.1× SSC at 42°C for 10 min, in 2× SSC at 42°C for 10 min, and in 2× SSC at room temperature for 5 min. The labeled probe was detected with fluorescein-conjugated antibodies (Roche Molecular Biochemicals), and the chromosomes were counterstained with propidium iodide (PI). The prepared materials were observed by confocal fluorescence microscopy by following Liu et al., 2016 [12] with slight medication of using blocking DNA our GISH experiment is performed.

3. Result and Discussion

GISH has proven to be a useful technique to distinguish genetically recombined genomes and assess the genomic relationships between different hybrid plants [12,13,14]. In GISH analysis, the addition of a large amount of blocking DNA can increase the probe specificity and is usually required for hybrids derived from two parents that have a close relationship, such as has been described in Triticeae species [15] and Phalaenopsis species [16].
Aceto- orecein staining showed that *Vanda ampullacea* var. auranticum and *Vanda testacea* have 2n =38 (Figure 1 and 2) number of chromosome and their hybrid also have 2n= 38 (Figure 3) chromosome. GISH analysis showed that chromosome derived from both the female and male parents could be found in the hybrid seedling (Figure 4). These results demonstrate that the assayed hybrid seelings were interspecific hybrids derived from the hybridization of *Vanda ampullacea* var. auranticum and *Vanda testacea*. In addition, GISH analysis showed chromosome recombination in the hybrid. GISH analysis showed that chromosomes derived from both the female and male parents could be found in the hybrid seedlings. In addition, in the intergeneric hybrids GISH analysis showed no obvious homologous chromosome recombination, likely resulting from the low sequence similarity of the homologous chromosomes of both parents [17]. Compared with intergeneric hybrids, in interspecific hybrids, homologous chromosome recombination usually occurs at a higher frequency [18].

4. Conclusions

GISH technique analyses are useful for identifying interspecific hybrids derived from *Vanda ampullacea* var. auranticum and *Vanda testacea* and evaluating the genetic inheritance of these hybrids. The results showed that the GISH method can effectively identify all F1 hybrid seedlings are interspecific hybrids. Cytological studies of orchid is very important to study and support of orchid's plant breeding however, very rare. Variations in the numbers, size, banding patterns, shape, position of chromosomes and order which are used in distinction of normal or mutated chromosomes.

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Author Contributions
JH carried out as well as planned the experiment analysis. PBM lays out the complete analysis as the major patron to the current work.
Availability of Data
This manuscript provides all the data.
Declarations
Ethics Approval and Consent to Participate
Not applicable.
Generative AI in Scientific Writing

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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Figure 1: Chromosome of *Vanda ampullacea* var. auranticum (2n=38)

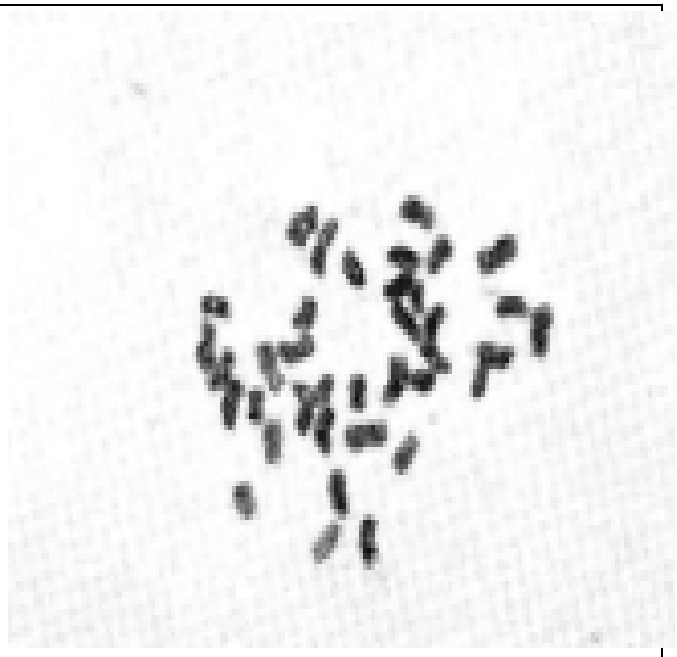

Figure 2 : Chromosome of *Vanda testacea* (2n=38)

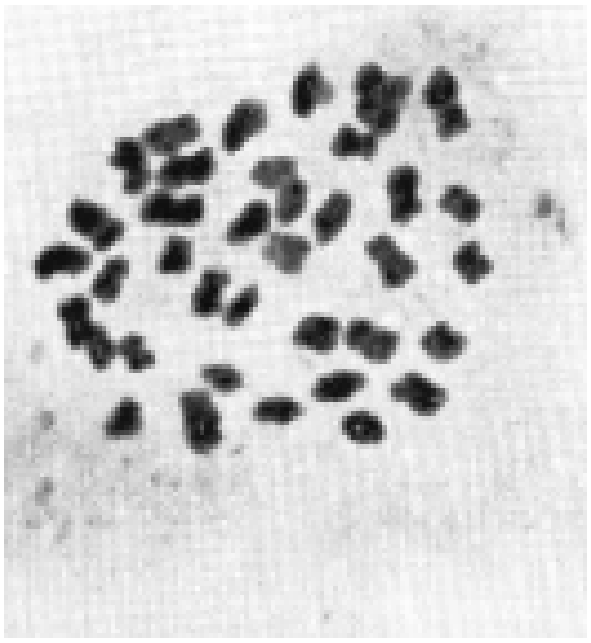

Figure 3: Chromosome of Vanda PB Mazumder (2n=38)

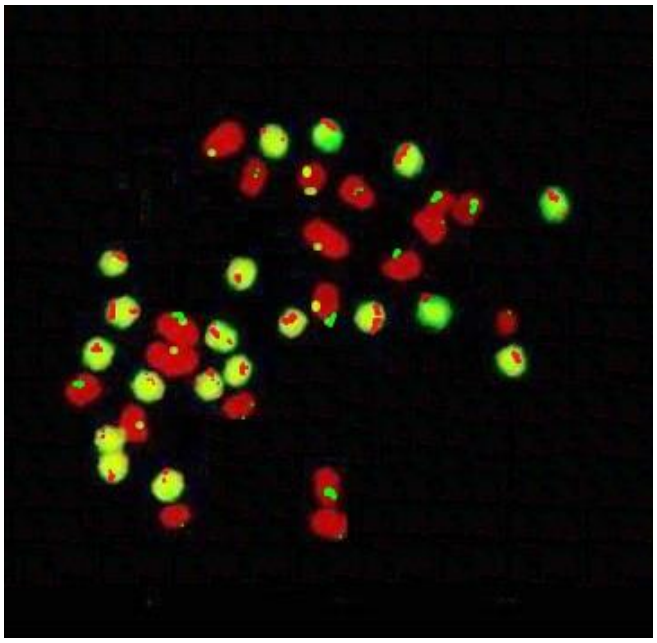

Figure 4: Chromosome of GISH analysis of Vanda PB Mazumder (red = *Vanda ampullacea* var. auranticum , green = *Vanda testacea*)