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Short Communication

Intergeneric Hybridization between *Phalaenopsis* 2166 and *Vanda* 'saint valentine': Characterization of Parents Using *ndhE* cpDNA Partial Sequence

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ABSTRACT
An intergeneric cross between *Phalaenopsis* 2166 and *Vanda* 'saint valentine' has successfully produced protocorms that will be grown further to form seedlings. The present study aims to genetically characterize both parents by using *ndhE* partial gene as its sequence is shown polymorphic among five orchid genera of the subtribe Oncidiinae. The results reveal that the *ndhE* partial sequences of *Phalaenopsis* 2166 and *Vanda* 'saint valentine' are considerably homologous with those of *Oncidium*. However, alignment of *ndhE* partial sequences between both parents shows only 58% similarity, leading to the conclusion that a relatively high genetic difference between them may occur.

Keywords: intergeneric hybridization, *Phalaenopsis* 2166, *Vanda* 'saint valentine', *ndhE* partial gene

Parent selection is an important initial step that determines the success of hybridization in orchids. This must involve knowledge of parental traits, particularly those of economic values such as flower size and shape of the hybrids will be produced (Widyastoety et al. 2010). For successful hybridization, maternal parents of strong and long-lasting flower buds with short gynostemium enabling pollen tubes to reach embryo sacs more readily should be selected (Hartati 2010).

Intergeneric hybridization between genus *Phalaenopsis* and *Vanda* has been reported to successfully produce seedlings with a number of characteristics seemingly showing maternal inheritance (Hartati 2010). Several species of *Phalaenopsis* and *Vanda* are commercially exploited to produce hybrids of high economic and aesthetic values (Sharma et al. 2013). *Phalaenopsis* is an epiphytic monopodial orchid with long leaves and various flower patterns, while *Vanda* is also epiphytic monopodial with flowers of sharp colour. Some *Phalaenopsis* species show long-lasting flowers with a blooming period of up to three months. On the other hand, *Vanda* flowers blossom commonly for only three weeks.

Monopodial orchids have stems of continual apical growth with little or no axillary sprouting (Johnson & Kane 2007). From an evolutionary point of view, monopodials are the most diverse developing orchids by means of

anagenesis. The members of this group show potentials in horticultural practices because they have vigorous stems and long-lasting flowers blooming for 15 – 30 days (Sharma et al. 2013).

More specifically, a cross between *Phalaenopsis* 2166 of pink flower with red spots as the female parent and *Vanda* 'saint valentine' of plain red as the male parent has also been performed resulting in hybrid fruits brought in *Phalaenopsis*. The seeds of this intergeneric cross have been grown *in vitro* and successfully produced protocorms or zygotic embryos. Then, these intergeneric protocorms will be grown to form seedlings using several *in vitro* culture media.

To predict whether the phenotypical traits of the hybrid seedlings are prone to correspond to those of *Phalaenopsis* 2166 as their female parent or not, characterization of both *Phalaenopsis* 2166 and *Vanda* 'saint valentine' genotypes using a particular marker from cpDNA is required. The *ndhE* gene is one of the sequences in the cpDNA suitable to be used as a molecular marker in orchids since the sequence is polymorphic among five orchid genera of the subtribe Oncidiinae (Wu et al. 2010). Hence, it is assumed that the sequence also shows polymorphism between *Phalaenopsis* 2166 and *Vanda* 'saint valentine', so that both genera can be genetically characterized using *ndhE* as a molecular marker. This study aims to characterize *Phalaenopsis* 2166 and *Vanda* 'saint valentine' as parents of the intergeneric hybridization using *ndhE* partial sequence.

In our study genomic DNAs were isolated from the completely developed second leaves of *Phalaenopsis* 2166 and *Vanda* 'saint valentine' following the CTAB method (Doyle & Doyle 1990). We used *Phalaenopsis* 2166 of four years old and *Vanda* 'saint valentine' of six years old. The quality and quantity of the isolated DNAs were measured using a genequant. Amplification of *ndhE* partial sequences was performed using universal primers, i.e. 5' – GCTAGCCCAATAGCTGCTTC – 3' as forward primer and 5' – TCGAAGCATGGTTAGAGCAC – 3' as reverse primer. This pair of primers have been designed using Primer 3 on the basis of *ndhE* conserved areas of three *Oncidium* hybrid cultivars available at the NCBI database, i.e. Grower Ramsey (acc. no. GU175400.1), Grower Ramsey 'Sunkist' (acc. no. GU175389.1) and Sweet Sugar 'Million Coin' (acc. no. GU175397.1). A total volume of 10 µl PCR mixture containing 2.5 µl template DNA, 5 µl Gotaq green, 2.25 µl nuclease-free water (NFW), and 0.25 µl of individual primer was subjected to PCR condition as follows: pre-denaturation at 94˚C for 3 mins, proceeded by 35 cycles of denaturation at 94˚C for 30 secs, primer annealing at 50˚C for 30 secs, primer elongation at 72˚C for 90 secs, and terminated by final elongation at 72˚C for 10 mins prior to storage at 4˚C. The PCR products were visualized in a 1.5% agarose gel using TBE buffer. These were then sent to Firstbase Malaysia for sequencing after (Sanger & Nicklen 1977) automated with terminator labelling.

Data of sequences were edited using Bioedit version 7.0.4.1 (Hall 1999) and were checked manually. The edited sequences were then examined for similarity with *ndhE* sequences available at the NCBI database using BLAST analysis. Sequence alignment was carried out with ClustalW (Thompson et al. 1994), which was also implemented in Bioedit version 7.0.4.1 (Hall 1999).

An amplicon resulting from *Vanda* 'saint valentine' sample is well visualized as an electrophoretic band of approximately 160 bp. BLAST analysis on the sequence of the amplicon shows 91% similarity with that of cpDNA of an orchid species, i.e., *Neofinetia falcata* (acc. no. KT726909.1). In addition, they also show 90% similarity with *ndhE* gene sequences of three *Oncidium* hybrid cultivars, i.e., Grower Ramsey, Grower Ramsey 'Sunkist' and Sweet Sugar 'Million Coin'. This certainly makes sense, because the *ndhE* sequences of the *Oncidium* hybrid cultivars are from which the PCR primers

used in this study have been designed. Thus, the amplicon obtained from *Vanda* 'saint valentine' is undoubtedly a *ndhE* partial sequence.

As in the case of *Vanda* 'saint valentine', the amplicon obtained from *Phalaenopsis* 2166 has also about 160 bp in length. Its sequence shows 92% similarity with *ndhE* gene sequences of the three *Oncidium* hybrid cultivars. The similarity to these sequences is even slightly higher than that of *Vanda* 'saint valentine' (i.e., 90% similarity). Thus, the amplicon of *Phalaenopsis* 2166 sample is also obviously a *ndhE* partial sequence like that of *Vanda* 'saint valentine'.

The partial *ndhE* sequences of both *Phalaenopsis* 2166 and *Vanda* 'saint valentine' have now available at the NCBI database with accession numbers of MH646649 and MH646650 respectively. The alignment of both sequences, however, shows only 58% similarity (Figure 1).

Intergeneric hybridization in plants, either naturally or artificially, are very important to evolution and speciation. It has been proven to provide beneficial significance including increased genetic diversity, improved environmental adaptation, and reproductive isolation breaking. On the other hand, it has also some detrimental effects such as genetic domination which may result in the potential extinction of original species. To assess intergeneric hybridization in plants, molecular markers could be employed along with morphological and chemical properties (Akita et al. 2021).

CpDNA constitutes a source of molecular markers commonly used to assess intergeneric hybridization in plants. Some of them are microsatellites or simple sequence repeats (SSR), the application of which in the pine family has been reviewed revealing their potentials as molecular markers in evolutionary biology of the plant family (Filiz & Koc 2014; Niu et al. 2017). Another marker widely used is *ndhE* gene, which is a member of *ndh* genes. The sequences have been proven to be polymorphic among five orchid genera of the family Oncidiinae, i.e. *Oncidium*, *Beallara*, *Odontoglossum*, *Odontocidium,* and *Zelenkocidium* (Wu et al. 2010).

The *ndh* genes are assumed to increase photosynthetic performance in fluctuating terrestrial conditions (Peredo et al. 2013). As many as 11 subunits of *ndh* genes, i.e., from *ndhA* to *ndhK*, are encoded in cpDNA of higher plants. In addition, three cyanobacterial nuclear-encoded subunits genes, i.e., *ndhM*, *ndhN,* and *ndhO*, are found in cpDNA. This indicates that nuclear *ndh* genes originated in cyanobacteria and were transferred from cpDNA to the nuclear genome during evolution. In *Oncidium* hybrid cultivar Grower Ramsey, however, all the 11 subunits of *ndh* genes are encoded in cpDNA, and it is merely *ndhE* that is translated into a functional protein. Genes that

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P2166new        TTTCGAAGCATGGTTAGAGCACTTATG-GGTGTCTTGAACTTATACTCAA---TTCGGTT
Vanda           TTTCGAAGCATGGTTAGAGCACCAATGTGATCCCTGGAGTTTATACTGGGAATTTCGGGT
                **********************  *** * *  ** **  *******      ***** *

P2166new        AATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAATTAATTAA
Vanda           GGAATCTAAATTCGTCGCTCACAATTTTCCAATCTATGTTGATAGTCAACAATTAAAAAA
                   *** **  ****  *     ******  ** *** *********  *******  **

P2166new        AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGA
Vanda           A----GAAATGTTATCAATCTTTGT----TATAGCC-ATTCCTGCTGTTG----------
                *    ** ** ** **********    ** ***   **   *******

P2166new        AGCAGCTATTGGGCTAGC
Vanda           ------------------
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Figure 1. Sequence alignment of *ndh*E partial genes of *Phalaenopsis* 2166 and *Vanda* 'saint valentine'.

do not encode functional protein have usually a high evolution rate causing high polymorphism. This is also the case in *ndh* genes of *Phalaenopsis aphrodite* (Wu et al. 2010; Kim et al. 2020). It is reported that *ndhE* genes in *P. aphrodite* and *P. equestris* are not in complete condition, while *ndhA*, *ndhE,* and *ndhI* genes in *Erycina* and two varieties of *Oncidium* have the same pattern (Luo et al. 2014; Smidt et al. 2020).

Complex *ndh* genes are responsible for encoding NADH dehydrogenase, which serves as transferring electrons from NADH to plastoquinone in the cyclic electron cycle. NADH should be reduced into NADPH in the non-cyclic electron cycle in photosystem I, but when NADPH is not needed, electron is not caught by NADH. In this case, cyclic electron cycle occurs, where electron from photosystem I is caught by pheredoxin, and then is passed to cytochrome B6 complex, cytochrome f, and back to photosystem I. From this cyclic electron cycle, only ATP is produced. All the other photosynthetic elements are encoded by cpDNA genes (Wu et al. 2010; Peredo et al. 2013; Wang et al. 2013; Kim & Chase 2017; Dong et al. 2018; Zhu et al. 2019). The loss of *ndh* genes or the presence of pseudogenes has been elucidated in the genome of monocotyl plants. Degenerative genes in cpDNA of photosynthetic orchids result from the change in *ndh* gene structure. Non-functional *ndh* genes controlled by cpDNA are observed in C3 and CAM plants (Pan et al. 2012; Kim et al. 2020). The loss of genes encoded in cpDNA has no effect on the plant life cycle (Luo et al. 2014).

The absence of *ndh* genes is found in monocotyledon plants other than orchid species of *Najas flexilis* and *Petrosavia stellaris*. This loss of *ndh* genes is caused by adaptation to submerge environments. In orchids, particular types of *ndh* genes sometimes disappear, but *ndhE* can be found in some orchid species of *Oncidium*, *Cymbidium,* and *Cattleya*. Specifically, in *Dendrobium*, *ndhE* gene is found but with a deletion of 21 bp (Kim et al. 2015).

In *Phalaenopsis aphrodite ndhA*, *ndhF,* and *ndhH* genes are not found in the cpDNA, while *ndhB*, *ndhC*, *ndhD*, *ndhE*, *ndhG*, *ndh I*, *ndhJ,* and *ndhK* genes show variation (Wu et al. 2010; Sui et al. 2018; Kim et al. 2020). Then, in *P. aphrodite*, which is the ancestor of all orchids, a change in *ndh* gene control into the nuclear genome occurs. It is confirmed that *ndhE* gene is observed in *P. aphrodite* (Kim et al. 2015). In *Oncidium*, *ndhE* gene is subjected to truncation and becomes pseudogene, while in *P. equestris*, the loss of *ndhA*, *ndhE*, *ndhF,* and *ndhH* genes is reported (Jheng et al. 2012; Kim et al. 2020).

Vanda and *Phalaenopsis* are of two different genera despite the same subtribe, i.e. Aeridinae. *Vanda* is, however, known capable of hybridization with other monopodial genera, e.g. *Rhyncostylis*, *Aerides*, *Ascocentrum,* and *Phalaenopsis* (Sharma et al. 2013; Johnson & Kane 2007). Nevertheless, some intergeneric hybridizations show reciprocally different results. For instance, the relative success of intergeneric hybridization between *Renanthera imschootiana* as the female parent and *V. coerulea* as the male parent is reported, while the reciprocal crosses remain failed although both genera are monopodials (Kishor & Sharma 2009). The use of *V. testacea* as the female parent instead of *V. coerulea* shows better results (Kishor & Sharma 2008). Similarly, the percentage of pods ready to harvest is relatively higher when *Phalaenopsis* sp. are used as male parents in the intergeneric crosses of *Phalaenopsis* x *Vanda* rather than in the case of the reciprocal combinations (Hartati 2010). On the contrary, we found in our study that no pod at all was produced when *Vanda* 'saint valentine' was used as the female parent and *Phalaenopsis* 2166 served as the male parent. The occurrence of maternal inheritance was demonstrated in the case between *Phalaenopsis* and *Vanda* previously reported (Hartati 2010) so that it will be very interesting to further study the *ndhE* partial sequences of the intergeneric hybrid seedlings that will

be produced.

The relatively low similarity between *ndhE* partial sequences of *Vanda* 'saint valentine' and *Phalaenopsis* 2166 leads to the conclusion that a relatively long genetic distance between both genera may exist. This supports their taxonomical status as belonging to two different genera, implying the possibility of a heterosis phenomenon in the intergeneric hybrids that will be produced.

AUTHORS CONTRIBUTION

M.D. designed, analysed the research data, and supervised all the processes, A.H.S. collected the data and wrote the manuscript.

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CONFLICT OF INTEREST

There is no conflict of interest regarding the research or the research funding.

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