Intergeneric hybrids of Phalaenopsis 2166 x Vanda ‘Saint Valentine’ showing maternal inheritance: Genetic analysis based on ndhE partial gene

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Abstract. Dwiatil M, Susanto AH, Prayoga L. 2020. Intergeneric hybrids of Phalaenopsis 2166 x Vanda ‘Saint Valentine’ showing maternal inheritance: Genetic analysis based on ndhE partial gene. Biodiversitas 21: 5138–5145. Genetic characterization in the intergeneric hybridization of orchids employing a particular molecular marker, such as ndhE gene, is needed to avoid phenotypic plasticity. The hybridization between Phalaenopsis 2166 as a female parent and Vanda ‘Saint Valentine’ as a male parent has been successfully made to produce various leaf shapes and colors of the hybrid seedlings, which tend to resemble those of the female parent. This study aims to assess whether the maternally phenotypic traits of the hybrids of Phalaenopsis 2166 x Vanda ‘Saint Valentine’ are congruent with the inheritance pattern of ndhE partial sequences. The result reveals that the ndhE partial sequences of the hybrids are seemingly similar to that of Phalaenopsis 2166 as the female parent rather than to that of Vanda ‘Saint Valentine’. It is also found that three hybrids, i.e. F1.9, F1.11, and F1.14 show slightly different ndhE partial sequences from those of the other hybrids in that some base substitutions are observed. In general, the ndhE partial sequences of the hybrids are maternally inherited. This finding provides a fact that maternally phenotypic traits of the hybrids of Phalaenopsis 2166 x Vanda ‘Saint Valentine’ have strong genetic background rather than environmental involvement.

Keywords: Intergeneric hybridization, ndhE partial sequence, Phalaenopsis 2166, Vanda ‘Saint Valentine’

INTRODUCTION

Intergeneric hybridizations in orchids are carried out to obtain hybrids with flowers of better performance in comparison to those of both parents. Orchid cultivars of high economic values are usually hybrids of relatively remote genetic sources, such as those resulting from intergeneric hybridization. They are named after their parental background despite the seemingly inconsistent nomenclature. Therefore, it is sometimes difficult to know the origin of orchid hybrids, especially when some of the parental information has been lost. This leads to the need for hybrid characterization, both phenotypically and genetically, in comparison to their parents once the hybrid seedlings are produced (Hsiao et al. 2011).

Several intergeneric hybridizations in orchids have successfully produced hybrids of favorable phenotypic traits. For instance, it has been reported those between Phalaenopsis sp. and Vanda tricolor (Hartati 2010), Sedirea japonica and Neofinetia falcata (Been et al. 2014; Kim et al. 2015b), Dactylorhiza praetermissa and Gymnadenia borealis (Bateman et al. 2017), Oncidium Sweet Sugar and Ionopsis utricularioides (Cardoso 2017). Mostly, maternal inheritances of the flower traits of the hybrids were observed.

The main problem with phenotypic traits, is however the involvement of environmental factors that may result in phenotypic plasticity. For instance, floral volatile emissions to attract pollinators in some plant species are influenced by soil moisture (Campbell et al. 2019). Thus, genetic characterization of plant individuals should necessarily be performed. Appropriate genetic markers need to be developed for more accurate identification of plant species, especially with respect to hybrids (Siew et al. 2018).

Genetic or molecular markers from chloroplast genome (cpDNA) are widely used in plants, especially in angiosperms, because they are relatively simple and stable with respect to structure in comparison to those of nuclear DNA (Dong et al. 2012; Ong et al. 2012). Another advantage of using cpDNA markers in plant genetic analysis is the absence of contamination with DNAs of other organisms having no cpDNA such as fungi and bacterial (Singh et al. 2017).

To characterize orchid hybrids, several cpDNA markers have been employed, such as ndhE encoding gene, which proves to have a highly variable pattern among Oncidinae, a subtribe of the family Orchidaceae. The ndhE gene is found to encode a functional protein in four Oncidium cultivars, i.e. Oncidium Grower Ramsey, O. Grower Ramsey ‘Sunkist’, O. Lemon Heart, and O. Sweet Sugar ‘Million Coin’. On the other hand, this gene is truncated in three Beallara cultivars, i.e. Beallara Euro Star, B. Peggy Ruth Carpenter ‘Morning Joy’, B. Maritch’ Howard Dream’, while no PCR product is obtained from B. Tahama Glacier ‘Sugar Sweet’ and B. Smile Eri. Similarly, no PCR product results from Zelenkoncidium Little Angle ‘Black Star’. The ndhE gene of Odontoglossum Margerette Holm encodes a functional protein, but that of O. Violetta von
Holm undergoes frameshift mutation, where some nucleotide deletion is observed. As well, deletion in ndhE sequence occurs in Odontocidium Golden Gate, *O. Wildcat ‘Garfield’ and Degarmoara Flying High (Wu et al. 2010). Several plant species, e.g. *Passiflora ciliata* (accession number JX664634.1), *Pera bumelilofia* (accession number JX664635.1), *Phyllanthus urinaria* (accession number JX664536.1), and *Rhizophora mangle* (accession number JX664642.1), have ndhE genes of approximately 300 bp in length.

In our previous study, we have been successfully carrying out intergeneric hybridization between *Phalaenopsis* 2166 possessing a specific pattern of flowers as the female parent and *Vanda ‘Saint Valentine’* of flashy red flowers as the male parent resulting in several hybrid seedlings. These hybrid seedlings show various shapes and colors in leaves, which in general tend to resemble those of *Phalaenopsis* 2166 assuming maternal inheritance to occur. On the other hand, the partial ndhE sequences of both *Phalaenopsis* 2166 (accession number MH646649; 187 bp long) and *Vanda ‘Saint Valentine’* (accession number MH646650; 161 bp long) have been aligned showing the similarity of only 53%. To confirm the phenotypic traits observed in the hybrids, molecular characterization by the use of ndhE partial sequence is necessarily performed.

This study aims to assess the congruency of phenotypic traits maternally inherited in the intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda ‘Saint Valentine’* with the inheritance pattern of ndhE partial sequences. In other words, we compare the ndhE partial sequences of the intergeneric hybrids with those of both parents.

**MATERIALS AND METHODS**

**Plant materials**

Fourteen seedlings resulting from intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda ‘Saint Valentine’* were used as samples to study the inheritance mode of ndhE partial sequences. The parent plants were purchased from Taman Anggrek Indonesia Permai (TAIP) Jakarta. All the hybrid seedlings and both parent plants have been described regarding their leaf morphology (Table 1). The leaf morphological features can also be seen in Figures 1 and 2.

**Procedures**

Genomic DNAs of the hybrid seedlings were extracted following CTAB method (Abdel-Latif and Osman 2017). The genomic DNAs were used as PCR templates to amplify ndhE partial sequences of approximately 200 bp employing primers we have designed, i.e. 5’-GCTAGCCCAATAGCTGCTTC-3’ (forward primer) and 5’-TCGAAGCATGGTTAGAGCAGC-3’ (reverse primer). These primers were designed using Primer 3 software based on conserved areas of ndhE sequences of some orchid species of the Oncidinae subtribe available in the NCBI database. The reaction was carried out in a total volume of 10 µl containing 5 µl Gotaq green master mix (Promega), 2.25 µl nuclease-free water, 2.5 µl genomic DNA, and 0.25 µl primers. The PCR condition was as follows: pre-denaturation at 94°C for 3 minutes, proceeded by 35 reaction cycles consisting of denaturation at 94°C for 30 seconds, primer annealing at 50°C for 30 seconds, primer extension at 72°C for 90 seconds, and terminated by a final extension at 72°C for 3 minutes. The reaction mixture was then stored at 4°C. The PCR products were visualized in a 1.5% agarose gel electrophoresis using TBE buffer. The electrophoresis was run in 100 V and 400 mA for 40 minutes. Fluoresave DNA stain was used to visualize the PCR products on a UV transilluminator.

The PCR products of approximately 200 bp were purified using the QIAquick kit. These were then sent to Firstbase Malaysia for sequencing using terminator dye Sanger method.

**Sequence editing and analysis**

The ndhE sequences were edited using Bioedit version 7.0.4.1 and were checked manually. Blasting was performed to see the sequence similarities with those available in the NCBI database. Then, sequence alignment was carried out using Clustal W. All sequences were registered to NCBI GenBank for accession numbers.

**Table 1.** Some leaf morphological traits of hybrid seedlings of intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda ‘Saint Valentine’*

| Parent            | Shape  | Leaf morphology   | Tip      |
|-------------------|--------|-------------------|----------|
|                   |        | Color             |          |
| *Phalaenopsis* 2166 | Oval   | Purplish green    | Obtuse   |
| *Vanda ‘Saint Valentine’* | Linear | Yellowish green   | Retuse   |
| Hybrid seedling   |        |                   |          |
| F1                | Big oval| Bright green      | Obtuse   |
| F2                | Round  | Purplish green    | Obtuse   |
| F3                | Oblong | Purplish green    | Obtuse   |
| F4                | Oval   | Purplish green    | Obtuse   |
| F6                | Round  | Purplish green    | Obtuse   |
| F7                | Oval   | Purplish green    | Obtuse   |
| F8                | Oval   | Purplish green    | Retuse   |
| F9                | Oblong | Purplish green    | Retuse   |
| F10               | Oblong | Yellowish green   | Retuse   |
| F11               | Oblong | Purplish green    | Obtuse   |
| F12               | Oblong | Reddish green     | Retuse   |
| F13               | Oblong | Purplish green    | Obtuse   |
| F14               | Round  | Purplish green    | Retuse   |
| F15               | Oblong | Reddish spotted   | Retuse   |
RESULTS AND DISCUSSION

Blasting of the sequences of all the PCR products shows similarities ranging from 94% to 99% with ndhE sequences available in the NCBI database. The highest similarity is observed with those of Ravenea hildebranii (Arecaceae, accession number HQ181094.1) and Chamaedorea seifrizii (Arecaceae, accession number HQ181067.1), while the lowest similarity is noticed with those of numerous plant species, none of which is of the family Orchidaceae. Nevertheless, this indicates that all the PCR products of 187 bp length are undoubtedly ndhE partial sequences. The length of total ndhE sequences in several plant species is about 300 bp.

Multiple sequence alignment among ndhE sequences of the hybrids of Phalaenopsis 2166 x Vanda ‘Saint Valentine’
Valentine’ is depicted in Figure 3, while those including Phalaenopsis 2166 and Vanda ‘Saint Valentine’ are presented in Figure 4 and 5 respectively. Overall, it is shown that higher homology is observed between hybrids and Phalaenopsis 2166 in comparison to that between hybrids and Vanda ‘Saint Valentine’. Relatively larger deletions in Vanda ‘Saint Valentine’ than those in Phalaenopsis 2166 are observed (Figure 5).

Some base substitutions are observed in the ndhE partial sequences of the hybrids F1.9, F1.11, and F1.14 in comparison to those of the other eleven (Figure 3). In this case, T and A are in replace of A and T in base numbers 135 and 136 respectively. As well, in base number 150 there is T instead of A. Though the substitutions are seemingly too small concerning the percentage, the ndhE partial sequences of the three hybrids are registered with a different accession number, i.e. MH646651.

Although no ndhE sequence of the hybrids shows similarity with those of Orchidaceae species, a relatively high similarity between that of Phalaenopsis 2166 (MH646649) as the female parent and those of some Orchidaceae species is observed. For instance, 92% similarities with ndhE sequences of both Oncidium cultivar Grower Ramsey ‘Sunkist’ and O. cultivar Sweet Sugar ‘Million Coin’ are found. Likewise, a slightly lower similarity between that of Vanda ‘Saint Valentine’ (MH646650) as the male parent and those of some Orchidaceae species is observed, e.g. 90% similarities are found with ndhE sequences of O. cultivar Grower Ramsey ‘Sunkist’ and O. cultivar Sweet Sugar ‘Million Coin’. This makes sense because the primers used in this study are based on the conserved areas of ndhE sequences of some Orchidaceae species, especially those belonging to subtribe Oncidiinae.

The higher similarity of ndhE sequences of the hybrids with that of Phalaenopsis 2166 in comparison to that of Vanda ‘Saint Valentine’ apparently indicates the occurrence of maternal inheritance in the intergeneric hybridization. This corresponds to what is observed in the intergeneric crosses between Renanthera imschootiana as the female parent and Vanda coerulea as the male parent. The hybrids produced, i.e. Renanthera Kebisna Shija, showed an EcoRI restriction pattern of trnL-F which looked like that of R. imschootiana more than that of V. coerulea. Conversely, the reciprocal crosses between V. testacea as the female parent and R. imschootiana as the male parent resulted in hybrids, i.e. Renanthera Prof. GJ Sharma, possessing an EcoRI restriction pattern of trnL-F which resembles that of V. testacea in compare to that of R. imschootiana. Another molecular marker, i.e. RAPD employing primer OPA1, also revealed maternal inheritance in the intergeneric crosses, where the RAPD profiles of the hybrids were likely to be similar to that of the female parent regardless of the genera used in the intergeneric crosses. Even based on a nuclear marker, i.e. nrITS digested with MspI, maternal inheritance seemed to occur (Kishor and Sharma 2010).

Strong maternal dominance was also reported in the naturally intergeneric hybridization between Dactylodelhiza praetermissa and Gymnadenia borealis. The hybrid produced, which was named as Dactylodelhiza lacerta, showed much higher homology in trn-L-F partial sequence to that of D. praetermissa as the female parent rather than to that of G. borealis as the male parent. In this case, sequence alignment was performed by the use of trn-L-F sequences of both parents from GenBank. A nuclear marker, i.e. ITS, was also employed revealing that D. lacerta was truly an intergeneric hybrid between both species (Bateman et al. 2017). Confirmation of intergeneric hybrids should involve the use of nuclear markers, since they are biparentally inherited. For instance, PCR-RFLP analysis on ETS region has demonstrated the intergeneric hybrids resulted from crosses between Ascocenda John De Biase ‘Blue’ as female parent and Phalaenopsis Chih Shang’s Stripe as male parent (Liu et al. 2016).

Figure 3. Multiple sequence alignment among ndhE sequences of the hybrids of Phalaenopsis 2166 x Vanda ‘Saint Valentine’
Figure 4. Multiple sequence alignment among ndhE sequences of the hybrids of *Phalaenopsis* 2166 x *Vanda* ‘Saint Valentine’ including that of *Phalaenopsis* 2166 as female parent
Figure 5. Multiple sequence alignment among ndhE sequences of the hybrids of Phalaenopsis 2166 x Vanda ‘Saint Valentine’ including those of both parents.
In our previous study, we found that intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda* ‘Saint Valentine’ showed no barrier with respect to the difference in flowering period occurs. Yet, only two hybrid pods are formed among four crosses (50%), which then produce a number of viable seeds. Such a low success was also found in the intergeneric hybridization between some lepidopteron orchids (*Dorisit pulcherrima* and *Phalaenopsis eustress*) and wild wind orchids (*Neoefinetia falcata* and *Sedirea japonica*), where one hundred and sixty cross combinations carried out, only two selected hybrid lines were successfully produced, i.e. those resulted from crosses between *D. pulcherrima* and *S. japonica*. Most failures in the hybridization were due to cross incompatibility leading to the absence of pod formation and premature pod dropping (Kim et al. 2019). A slightly higher percentage of pod formation was reported in the intergeneric crosses between *Phalaenopsis* alliances as the female parent and *S. japonica* as the male parent, where 34 pods bearing some viable seeds were produced from 65 crosses. The relatively low percentage of pod formation causing less hybrid plants to develop in the orchid intergeneric hybridization is in general due to both pre- and post-fertilization problems, e.g. morphological incompatibility between pollen and stigma, failure of pollen germination and pollen tube growth, degeneration or abnormal development of embryo (Kim et al. 2015a). The pollen-stigma interaction may be influenced by the presence of the so-called allergens, which are proteins collectively found in the pollen-grain surface. Pollen viability in several genera of Mediterranean orchids positively correlates with pollination systems which could, in turn, have an influence on various types of reproductive barriers (Belluscio et al. 2010). Other factors, such as genetic incompatibilities in terms of the difference in chromosome number, experimental mishandling, and reduced plant vigor, may also lead to the failure of intergeneric hybridization in orchids. Even in the interspecific hybridizations among *Phalaenopsis* orchids, breeding barriers arise mainly due to difference in chromosome number (Hsu et al. 2010), although this is not apparently the case in the interspecific hybridization between *Epidendrum fulgens* and *E. paniculatum*, where difference in chromosome number remains to enable interspecific gene flow among natural populations (Pinheiro et al. 2010).

The low rate of success was also reported in the intergeneric hybridization between *Phalaenopsis* sp. (three cultivars, i.e. ‘Joane Kileup June’, ‘Pinlong Cinderella’, ‘Fortune Budha x Princess Kaiulani’) and *Vanda tricolor*. Although pods were formed in all crossing combinations showing an absolutely high level of compatibility between both genera, only a very small number of pods ready to harvest was obtained in most crosses. As a whole, the percentage of pods ready to harvest was relatively higher when *Phalaenopsis* sp. was used as male parents rather than in the case of their reciprocal combinations (Hartati 2010). In general, both intergeneric and interspecific hybridizations in orchids are known to occur readily due to the relatively low genetic incompatibility related to recent radiations. Nevertheless, orchids often show considerably specific habitats and pollination systems which can in turn restrict hybridization among species (Johnson 2018).

Regardless of the difficulties in the intergeneric hybridization, intermediate phenotypic and cytogenetic traits were observed in the hybrids resulting from intergeneric crosses between moth orchids and wind orchids. The moth orchids which were hybrids between *Phalaenopsis equestris* and *Doritaeanopsis pulcherrima* were originally tropical or thermophilic floral plants, while the wind orchids were hybrids between *N. falcata* and *S. japonicum* were psychrophilic, so that they persisted during the winter season in nature. Hence, the hybrids exhibited both cold-tolerant and summer-flowering traits (Been et al. 2014). Instead of intermediate traits, a combination of female and male characteristics was observed in *Ionnopis*, an intergeneric hybrid between *Oncidium* Sweet Sugar as the female parent and *Ionnopis utricularioides* as the male parent. The vegetative and flower characteristics were similar to *Oncidium*, while the number of branches in inflorescence and the number of flowers resembled those of *Ionnopis* (Cardoso 2017).

The maternal inheritance of partial *ndhE* sequence in the intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda* ‘Saint Valentine’ supports those of phenotypic traits shown in the hybrid leaves (Figure 2 and Table 1). Most of the leaf morphological traits of the hybrids resemble those of *Phalaenopsis* 2166 as the female parent rather than those of *Vanda* ‘Saint Valentine’ (Figure 1). The *ndhE* gene is the only one that encodes functional protein among the other ten *ndh* genes in 15 varieties of Oncidinae. Even some of them can not be found in most of the varieties, so that in comparison to the other *ndh* genes, *ndhE* seems to be the most suitable molecular marker to be used in analyzing orchid variability (Wu et al. 2010). Though *ndh* genes are required for encoding protein complexes involved in photosynthetic functions, loss of them has been reported in an aquatic species of angiosperm, i.e. *Najas flexilis*, shown adaptable to a submersed environment where limited light penetration occurs (Peredo et al. 2013). The complete loss of all functional *ndh* genes from the chloroplast genomes of *Phalaenopsis equestris*, *Dendrobium officinale*, and *D. catenatum* occurs, while only *ndhB* and *ndhE* remain intact in both *Dendrobium* species (Lin et al. 2017). Relocated *ndh* genes from cpDNA into the nuclear genome, except for *ndhG* and *ndhE*, were reported in some gymnosperm species (Ranade et al. 2016). The loss of most *ndh* genes is strongly assumed as related to the conversion of photoautotrophic plants into carnivorous plants (Nevill et al. 2019). It was speculated that either lost or impaired *ndh* genes in cpDNA had interrelationship to sunlight-intolerance in *Allium paradoxum* (Omelchenko et al. 2019).

It can be concluded that *ndhE* partial sequence is maternally inherited in the intergeneric hybridization between *Phalaenopsis* 2166 as the female parent and *Vanda* ‘Saint Valentine’ as the male parent. This provides evidence that maternal inheritance of some phenotypic traits in the intergeneric hybrids has a strong genetic background.
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