ISSN: 1412-033X
E-ISSN: 2085-4722

BIODIVERSITAS

Journal of Biological Diversity

Volume 21 - Number 11 - November 2020

BIODIVERSITAS

Journal of Biological Diversity
Volume 21 – Number 11 – November 2020

ISSN/E-ISSN:
1412-033X (printed edition), 2085-4722 (electronic)

PUBLISHER:
The Society for Indonesian Biodiversity

CO-PUBLISHER:
Department of Biology, Faculty of Mathematics and Natural Sciences, Sebelas Maret University, Surakarta

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Jl. Ir. Sutami 36A Surakarta 57126. Tel. +62-271-7994097, Tel. & Fax.: +62-271-663375, email: editors@smujo.id

ONLINE:
biodiversitas.mipa.uns.ac.id; smujo.id/biodiv

Society for Indonesia Biodiversity

Sebelas Maret University Surakarta

Published by Smujo International for The Society for Indonesian Biodiversity and Sebelas Maret University Surakarta

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Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com

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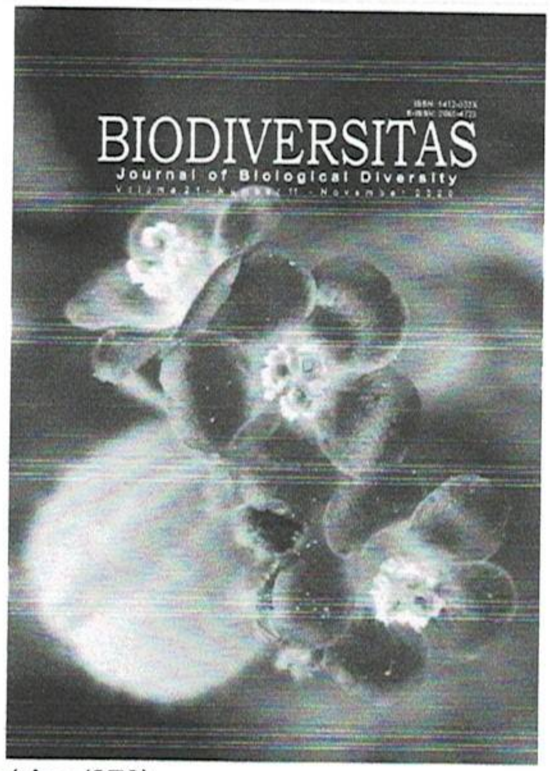

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BIODIVERSITAS
Volume 21, Number 11, November 2020
Pages: 5138-5145

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d211119

Intergeneric hybrids of *Phalaenopsis* 2166 x *Vanda* 'Saint Valentine' showing maternal inheritance: Genetic analysis based on *ndh*E partial gene

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Manuscript received: 1 August 2020. Revision accepted: 13 October 2020.

Abstract. *Dwiati M, Susanto AH, Prayoga L. 2020. Intergeneric hybrids of* Phalaenopsis *2166 x* Vanda *'Saint Valentine' showing maternal inheritance: Genetic analysis based on* ndh*E partial gene. Biodiversitas 21: 5138-5145.* Genetic characterization in the intergeneric hybridization of orchids employing a particular molecular marker, such as *ndh*E 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 *ndh*E partial sequences. The result reveals that the *ndh*E 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 *ndh*E partial sequences from those of the other hybrids in that some base substitutions are observed. In general, the *ndh*E 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, *ndh*E 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 *Neofinitea 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 *ndh*E encoding gene, which proves to have a highly variable pattern among Oncidiinae, a subtribe of the family Orchidaceae. The *ndh*E 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.* Marfitch' Howard Dream', while no PCR product is obtained from *B.* Tahoma Glacier 'Sugar Sweet' and *B.* Smile Eri. Similarly, no PCR product results from *Zelenkoncidium* Little Angle 'Black Star'. The *ndh*E 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 *ndh*E 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 bumeliifolia* (accession number JX664635.1), *Phyllanthus urinaria* (accession number JX664536.1), and *Rhizophora mangle* (accession number JX664642.1), have *ndh*E 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 *ndh*E 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 *ndh*E 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 *ndh*E partial sequences. In other words, we compare the *ndh*E 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 *ndh*E 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 *ndh*E partial sequences of approximately 200 bp employing primers we have designed, i.e. 5'-GCTAGCCCAATAGCTGCTTC-3' (forward primer) and 5'-TCGAAGCATGGTTAGAGCAC-3' (reverse primer). These primers were designed using Primer 3 software based on conserved areas of *ndh*E 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. Fluorosave 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 *ndh*E 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	Leaf morphology		
	Shape	**Color**	**Tip**
Phalaenopsis 2166	Oval	Purplish green	Obtuse
Vanda 'Saint Valentine'	Linear	Yellowish green	Retuse
Hybrid seedling			
F1	Big oval	Bright green	Obtuse
F2	Round	Yellowish 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

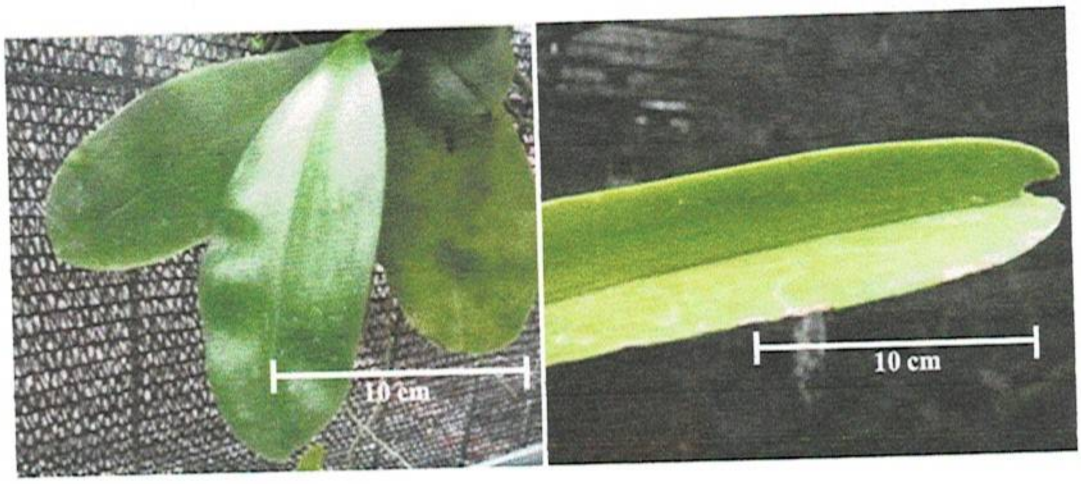

Figure 1. Leaf morphology of *Phalaenopsis* 2166 (*left*) and *Vanda* 'Saint Valentine' (*right*)

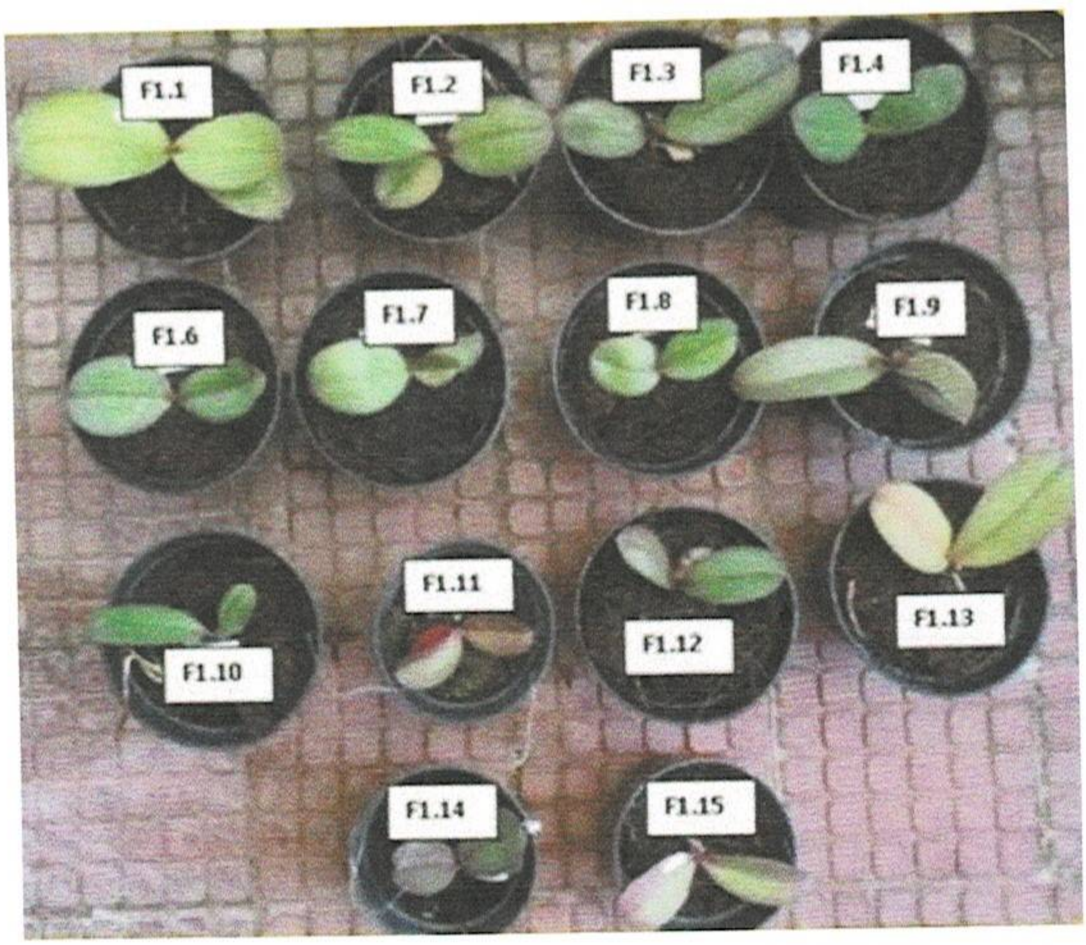

Figure 2. Leaf morphology of seedlings of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'Saint Valentine'

RESULTS AND DISCUSSION

Blasting of the sequences of all the PCR products shows similarities ranging from 94% to 99% with *ndh*E sequences available in the NCBI database. The highest similarity is observed with those of *Ravenea hildebrandtii* (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 *ndh*E partial sequences. The length of total *ndh*E sequences in several plant species is about 300 bp.

Multiple sequence alignment among *ndh*E sequences of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'Saint

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 *ndh*E 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 *ndh*E partial sequences of the three hybrids are registered with a different accession number, i.e. MH646651.

Although no *ndh*E 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 *ndh*E 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 *ndh*E 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 *ndh*E sequences of some Orchidaceae species, especially those belonging to subtribe Oncidiinae.

The higher similarity of *ndh*E 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. *Renantanda* Kebisana Shija, showed an *Eco*RI restriction pattern of *trn*L-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. *Renantanda* Prof GJ Sharma, possessing an *Eco*RI restriction pattern of *trn*L-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 *Msp*I, maternal inheritance seemed to occur (Kishor and Sharma 2010).

Strong maternal dominance was also reported in the naturally intergeneric hybridization between *Dactylorhiza praetermissa* and *Gymnadenia borealis.* The hybrid produced, which was named as *Dactylodenia 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).

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F1.12      TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.15      TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.13      TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.10      TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.8       TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.6       TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.4       TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.3       TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.2       TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.1       TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.9       TTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.11      TTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
F1.14      TTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTATTGCGGCTGCTGAAGCAGCTATTG 180
F1.7       TTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCTATTG 180
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Figure 3. Multiple sequence alignment among *ndh*E sequences of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'Saint Valentine'

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F1.9      TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.11     TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.14     TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.15     TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.13     TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.10     TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.8      TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.6      TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.4      TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.3      TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.2      TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.1      TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGAGCTTGAGCTTATACTCAATTCGGTT 60
F1.12     TTTCGAAGCATGGTTAGAGCACTTATGTGTCTTGATCTTGAGCTTATACTCAATTCGGTT 60
F1.7      TTTCGAAGCATGGTTAGAGCACTTATGTGACTTGAGCTTATACTTATACTCAATTCGGTT 60
P2166     TTTCGAAGCATGGTTAGAGCACTTATGGGTGT----CTTGAACTTATACTCAATTCGGTT 56
          *************************** *  *    ***   ******************

F1.9      AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.11     AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.14     AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.15     AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.13     AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.10     AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.8      AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.6      AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.4      AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.3      AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.2      AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.1      AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.12     AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
F1.7      AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAA----AAGGCGA 116
P2166     AATATCAATCTCGTAACATTTTCTGATATATTTGATAGTCGCCAATTAATTAAAAGGCGA 116
          *************************************************    *******

F1.9      CATTTTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.11     CATTTTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.14     CATTTTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTATTGCGGCTGCTGAAGCAGCT 176
F1.15     CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.13     CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.10     CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.8      CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.6      CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.4      CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.3      CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.2      CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.1      CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.12     CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
F1.7      CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
P2166     CATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGGCTGCTGAAGCAGCT 176
          ******************  ************* ***** ********************

F1.9      ATTGGGCTAGC 187
F1.11     ATTGGGCTAGC 187
F1.14     ATTGGGCTAGC 187
F1.15     ATTGGGCTAGC 187
F1.13     ATTGGGCTAGC 187
F1.10     ATTGGGCTAGC 187
F1.8      ATTGGGCTAGC 187
F1.6      ATTGGGCTAGC 187
F1.4      ATTGGGCTAGC 187
F1.3      ATTGGGCTAGC 187
F1.2      ATTGGGCTAGC 187
F1.1      ATTGGGCTAGC 187
F1.12     ATTGGGCTAGC 187
F1.7      ATTGGGCTAGC 187
P2166     ATTGGGCTAGC 187
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Figure 4. Multiple sequence alignment among *ndh*E sequences of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'Saint Valentine' including that of *Phalaenopsis* 2166 as female parent

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F1.9     TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.11    TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.14    TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.7     TTTCGAAGCATGGTTAGAGCACTTATG--TG--ACTTGAGCTTATACTTATACTCAA--- 53
P2166    TTTCGAAGCATGGTTAGAGCACTTATGGGTG--TCTTGA------ACTTATACTCAA--- 49
F1.1     TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.2     TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.3     TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.4     TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.6     TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.13    TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.15    TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.10    TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.8     TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGAGCTTGAGCTTATACTCAA--- 53
F1.12    TTTCGAAGCATGGTTAGAGCACTTATG--TG--TCTTGATCTTGAGCTTATACTCAA--- 53
Vanda    TTTCGAAGCATGGTTAGAGCACCAATG--TGATCCCTGG-----AGTTTATACTGGGAAT 53
         **********************  ***  **   * **         *******

F1.9     TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.11    TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.14    TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.7     TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
P2166    TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 102
F1.1     TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.2     TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.3     TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.4     TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.6     TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.13    TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.15    TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.10    TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.8     TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
F1.12    TTCGGTTAATATC-AATCTCGTAAC-----ATTTTCTGATATAT-TTGATAGTCGCCAAT 106
Vanda    TTCGGGTGGAATCTAAATTCGTCGCTCACAATTTTCCAATCTATGTTGATAGTCAACAAT 113
         ***** *   *** **  ****   *     ******  ** *** *********  ****

F1.9     TAA----AAGGCGACATTTTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTGTTGCGG 162
F1.11    TAA----AAGGCGACATTTTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTGTTGCGG 162
F1.14    TAA----AAGGCGACATTTTCTCAATCTTTGTTAAGTAGAGCTTGTTTTAGCTATTGCGG 162
F1.7     TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
P2166    TAATTAAAAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.1     TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.2     TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.3     TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.4     TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.6     TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.13    TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.15    TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.10    TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.8     TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
F1.12    TAA----AAGGCGACATTTTCTCAATCTTTGTATAGTAGAGCTTGTTATAGCTGTTGCGG 162
Vanda    TAA----AAAAAGAAATGTTATCAATCTTTGT--------------TATAGCCATTCCTG 155
         ***    **   ** ** ** ***********              * ****  ** * *

F1.9     CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.11    CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.14    CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.7     CTGCTGAAGCAGCTATTGGGCTAGC 187
P2166    CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.1     CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.2     CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.3     CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.4     CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.6     CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.13    CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.15    CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.10    CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.8     CTGCTGAAGCAGCTATTGGGCTAGC 187
F1.12    CTGCTGAAGCAGCTATTGGGCTAGC 187
Vanda    CTGTTG------------------- 161
         *** **
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Figure 5. Multiple sequence alignment among *ndh*E 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 *(Doritis pulcherrima* and *Phalaenopsis eustress)* and wild wind orchids *(Neofinetia falcata* and *Sedirea japonica)*, whereof 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 (Bellusci 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. puniceoluteum*, 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 *Doriteanopsis 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 *Ionocidium*, an intergeneric hybrid between *Oncidium* Sweet Sugar as the female parent and *Ionopsis 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 *Ionopsis* (Cardoso 2017).

The maternal inheritance of partial *ndh*E 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 *ndh*E gene is the only one that encodes functional protein among the other ten *ndh* genes in 15 varieties of Oncidiinae. Even some of them can not be found in most of the varieties, so that in comparison to the other *ndh* genes, *ndh*E 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 *ndh*B and *ndh*E remain intact in both *Dendrobium* species (Lin et al. 2017). Relocated *ndh* genes from cpDNA into the nuclear genome, except for *ndh*G and *ndh*E, 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 *ndh*E 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.

ACKNOWLEDGEMENTS

The authors are very grateful to University Research Grant of Universitas Jenderal Soedirman, Indonesia of the financial year 2018 with contract number of 2350/UN23.14/PN.01.00/2018 for funding this work. High appreciation is also addressed to Yeni Fatmawati for her valuable assistance in the laboratory work.

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