[biodiv] Editor Decision

Dari: Agustina Putri (smujo.id@gmail.com)

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Tanggal: Rabu, 16 September 2020 21.38 WIB

Murni Dwiati; Agus Susanto; Lucky Prayoga:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Intergeneric Hybrids of Phalaenopsis 2166 x Vanda 'saint valentine' Showing Maternal Inheritance: Genetic Analysis based on ndhE Partial Gene".

Our decision is: Revisions Required

Agustina Putri
sectioneditor4@smujo.id

Reviewer A:

Dear author,

The manuscript 'Intergeneric hybrids of *Phalaenopsis* 2166 x *Vanda* 'saint valentine' showing maternal inheritance: Genetic analysis based on *ndhE* partial gene' has been reviewed. This current research is valuable, but some revisions are necessary before accepting this manuscript for publication. My suggestions for each section of the manuscript as follows:

1. This manuscript needs to improve English quality as some sentences are not understandable completely at present. Please ask to your native speaker colleagues to check the manuscript or send to professional translator.
2. Abstract: Please rewrite. It should be contained research background/research problem, research aims, methods, and novelty/research findings.
3. Introduction: This part is about 400-600 words, covering the background and aims of the research. Please delete some unrelevant statements to the current research, recheck how to write hybrid's name, and add information of *ndhE*
4. Materials and Methods: Please add source of *Phalaenopsis* 2166 and *Vanda* 'saint valentine' and all sequences obtained in the current research should be submitted in NCBI GenBank.
5. Results and Discussion: This part is not written properly, please rewrite. I recommend to divide into two subtitles: Molecular characteristics and Leaves morphology. Present summary of DNA sequence data in a table and Figure 1, 2, and 3 should be presented in one Figure.
6. References: Please follow author guideline.
7. All comments are presented in the manuscript, please read carefully, and make appropriate revisions.

Good luck.

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

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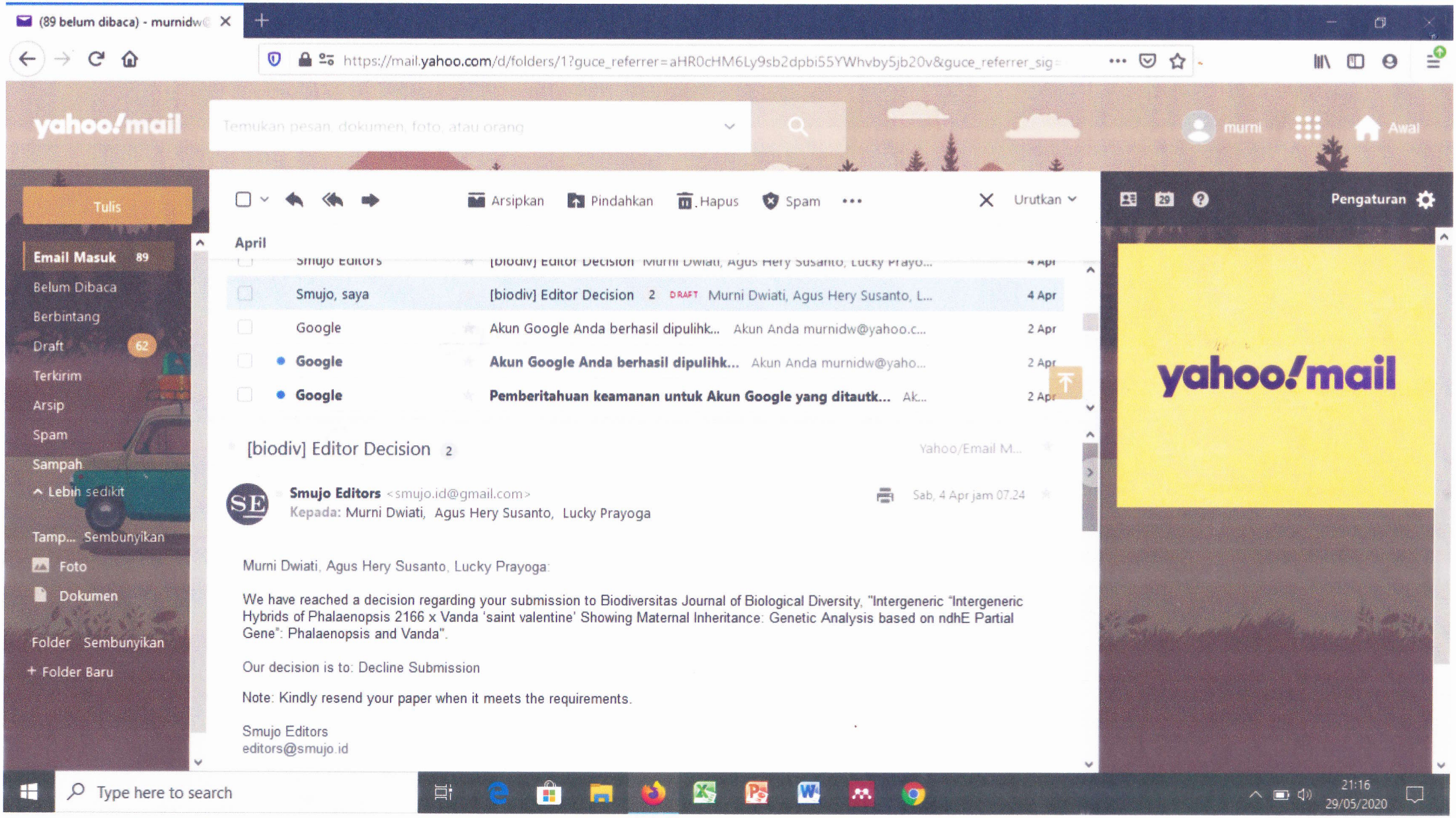
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We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Intergeneric "Intergeneric Hybrids of Phalaenopsis 2166 x Vanda 'saint valentine' Showing Maternal Inheritance: Genetic Analysis based on ndhE Partial Gene": Phalaenopsis and Vanda".
Our decision is to: Decline Submission
Note: Kindly resend your paper when it meets the requirements.
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Intergeneric hybrids of *Phalaenopsis* 2166 x *Vanda* 'saint valentine' showing maternal inheritance: Genetic analysis based on *ndhE* partial gene

Abstract. The inheritance pattern of intergeneric hybridization in orchids can be analyzed using genetic markers from chloroplast genome, e.g. ndhE gene which has been showing highly variable sequences among Oncidiinae, a subtribe of the family Orchidaceae. 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 colours of the hybrid seedlings. In general, these traits tend to resemble those of the female parent. The aim of this study is 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 sequence from those of the other hybrids in that some base substitutions are observed. In general, it can be said that the ndhE partial sequences of the hybrids are maternally inherited.

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

Running title: Intergeneric hybrids of *Phalaenopsis* 2166 x *Vanda* 'saint vaentine'

INTRODUCTION

Intergeneric hybridizations in orchids are basically carried out to obtain hybrids with flowers of better performance in compare 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 of hybrid characterization, both phenotypically and genetically, in compare to their parents once the hybrid seedlings are produced (Hsiao et al. 2011).

Several intergeneric hybridizations in orchids have successfully produced hybrids of favourable phenotypic traits. For instance, it has been reported those between *Dactylorhiza praetermissa* and *Gymnadenia borealis* (Bateman et al. 2017), *Phalaenopsis* sp. and *Vanda tricolor* (Hartati 2010), *Sedirea japonica* and *Neofinitea falcata* (Been et al. 2014; Kim et al. 2015), *Oncidium* Sweet Sugar and *Ionopsis utricularoides* (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. Thus, genetic charaterization of the hybids should necessarily be performed. Appropriate genetic markers need to be developed for more accurate identification of orchids (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 of structure in compare 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 bacterials (Singh et al. 2017).

To characterize orchid hybrids, several cpDNA markers have been employed, e.g. *ndhE* encoding gene, which proves to have highly variable pattern among Oncidiinae, 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 sunkiss, *O.* Lemon Heart and *O.* Sweet Sugar 'Million coin'. On the other hands, 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 *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

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Please delete some un relevant statements to the current research.
Please recheck how to write hybrid's name.

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Please add male and female parents of those species.

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Comment [F5]: It means that those previous studies present all maternal inheritances? How about leaves? It is important to present, since your research is mainly based on leaves characters, but not flowers.
Please give some examples paternal inheritances in orchids.

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It is better: DNA sequences from chloroplast genome, such as ….. are widely used in plants, especially in orchids.

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well, deletion in *ndhE* sequence occurs in *Odontocidium* Golden Gate, *O.* Wildcat 'Garfield' and *Degarmoara* Flying High (Wu et al., 2010).

Intergeneric hybridization between *Phalaenopsis* 2166 possessing specific pattern of flowers as the female parent and *Vanda* 'saint valentine' of flashy red flowers as the male parent has been successfully carried out resulting in several hybrid seedlings. These hybrid seedlings show various shapes and colours in leaves, which in general tend to resemble those of *Phalaenopsis* 2166 assuming maternal inheritance to occur. On the other hands, the partial *ndhE* sequences of both *Phalaenopsis* 2166 (NCBI accession number MH646649) and *Vanda* 'saint valentine' (NCBI accession number MH646650) have been aligned showing the similarity of only 53% (Dwiati et al. unpublished). 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.

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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. These have been described regarding their leaf morphology (Table 1).

Comment [F13]: Please add source of those orchids.

Table 1. Some leaf morphological traits of hybrid seedlings of intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda* 'saint valentine'

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Parent	Leaf Morphology		
	shape	colour	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

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~~Genomic DNA extraction~~Procedure

Genomic DNAs of the hybrid seedlings were extracted following CTAB method (Abdel-Latif and Osman 2017). Approximately 0.1 g of leaf pieces were put into a mortar, after which 800 µL CTAB solution previously incubated in a waterbath at 65ºC for 30 mins was added. This was then homogenized using a pestle and moved into a 1.5 µL microtube for incubation in the waterbath at 65ºC for 1 h, during which the microtube was turned upside down gently in every 10 mins. After this, the mixture was allowed to cool down at room temperature for 2 mins and then was added with 500 µL CIAA solution. This was mixed gently, vortexed for 5 mins and then centrifuged at 12,000 rpm for 15 mins. The supernatant was moved carefully into a new microtube, where 3M sodium acetate of 1/10 supernatant volume was added and mixed gently. Then, cold isopropanol of 2/3 total volume was added and mixed gently by flipping the tube. The mixture was stored in the freezer for 24 hs before centrifugation at 12,000 rpm for 10 mins. The supernatant was removed and the DNA pellet was added with 500 µL 70% ethanol while the microtube was flipped gently. The DNA solution was centrifuged at 12,000 rpm for 5 mins, after which the supernatant was removed and the DNA pellet was air dried. The extracted DNAs were dissolved in 100 µl TE buffer and were stored at 4^{o}C. Quantification of the DNAs was performed using genequant.

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It is not necessary to present.

~~Amplification and sequencing of *ndhE* partial sequences~~

The genomic DNAs were used as PCR templates to amplify *ndhE* partial sequences of approximately 200 bp employing universal 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 *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 (maker?), 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 mins, proceeded by 35 reaction cycles consisting of denaturation at 94°C for 30 secs, primer annealing at 50°C for 30 secs, primer extension at 72°C for 90 secs, and terminated by a final extension at 72°C for 3 mins. 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 mins. Fluorosave DNA stain was used to visualize the PCR products on a UV transiluminator.

The PCR products of approximately 200 bp were purified using 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. The respective sequence was registered to NCBI GenBank for accession number.

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 (Fig. 1?). The highest similarity is observed with those of *Ravenea hilde~~r~~brandtii* (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.

Multiple sequence alignment among *ndhE* sequences of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'saint valentine' is depicted in Figure 1, while those including *Phalaenopsis* 2166 and *Vanda* 'saint valentine' are presented in Figure 2 and 3 respectively. Overall, it is shown that higher homology is observed between hybrids and *Phalaenopsis* 2166 in compare to that between hybrids and *Vanda* 'saint valentine'. Relatively larger deletions in *Vanda* 'saint valentine' than those in *Phalaenopsis* 2166 are observed (Figure 3).

Figure 1. Multiple sequence alignment among *ndhE* sequences of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'saint valentine'

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.

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I recommend to submit all sequences obtained in NCBI GenBank.

Comment [F19]: Results and Discussion is not written properly, please rewrite.
It is better to divide into two subtitles:
Molecular characteristics
Leaves morphology

Comment [F20]: It is better to present summary of DNA sequences data in a table

Comment [F21]: How many % of similarity?

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Comment [F23]: Please place this data in the beginning of the first paragraph. Please rewrite: how long complete sequence of *ndhE* in common plants, then in orchids, then mention partial sequences number … to ….

Comment [F24]: You should present Fig. 1, Fig. 2, and Fig. 3 into one Figure, please add sequence number in the above DNA sequence data.
Name of hybrids should be written consistently

Comment [F25]: Please point, which one?

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All hybrid's name presented in table 1, Fig. 1, Fig. 2, and Fig. 3 should be similar

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If based your own analysis, any Figure or Table should be presented?

Figure 2. Multiple sequence alignment among *ndhE* sequences of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'saint valentine' including that of *Phalaenopsis* 2166 as female parent

The higher similarity of *ndhE* sequences of the hybrids with that of *Phalaenopsis* 2166 in compare to that of *Vanda* 'saint valentine' apparently indicates the occurrence of maternal inheritance in the intergeneric hybridization. This corresponds to what 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 to 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 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.*

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Name of hybrids should be written consistently

Comment [F29]: References cited?

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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 truely 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).

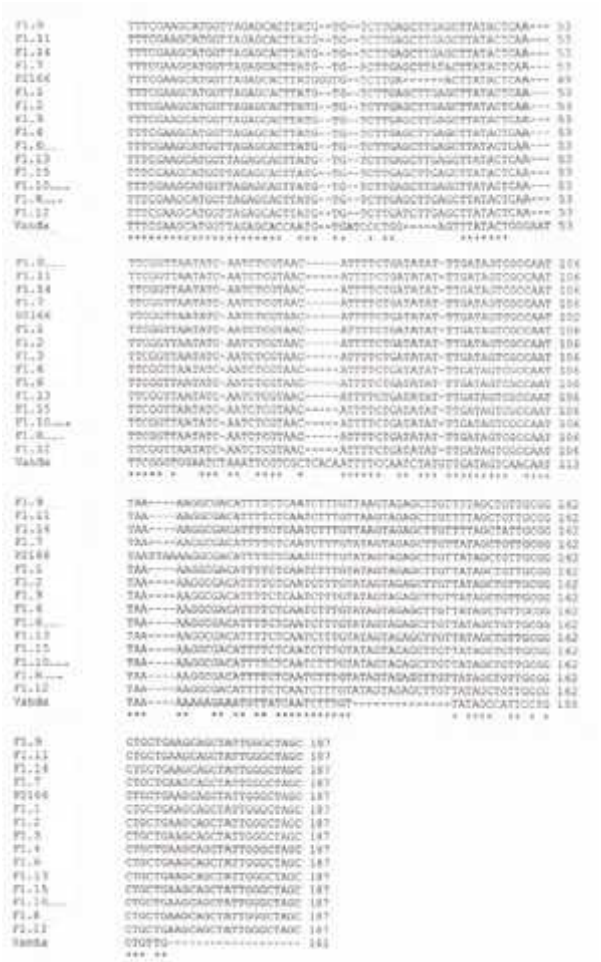

Figure 3. Multiple sequence alignment among *ndhE* sequences of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'saint valentine' including those of both parents

In the case of intergeneric hybridization between *Phalaenopsis* 2166 and *Vanda* 'saint valentine', no barrier with respect of 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),* where of 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* species 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

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Name of hybrids should be written consistently

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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. 2015). 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 vigour, 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) and *Vanda tricolor*. Although pods were formed in all crossing combinations showing 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. were 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* and *Doriteanopsis* were originally tropical or thermophilic floral plants, while the wind orchids which 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 were 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 to those of *Ionopsis* (Cardoso 2017).

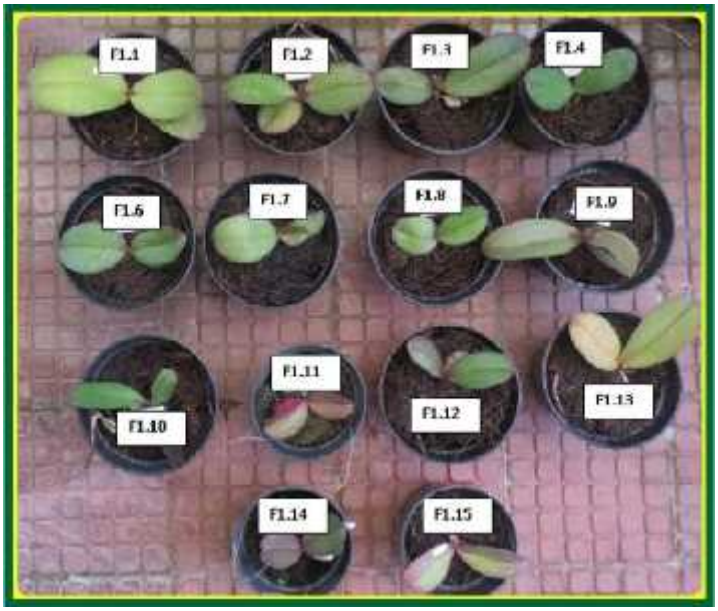

Figure 4. Leaf morphology of seedlings of the hybrids of *Phalaenopsis* 2166 x *Vanda* 'saint valentine'

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Comment [F36]: Mention species name

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Figure 5. Leaf morphology of *Phalaenopsis* 2166 (left) and *Vanda* 'saint valentine' (right)

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 4 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 5).

The *ndhE* gene is the only one which 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 compare 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 actually 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 nuclear genome, with the exception of *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 photoautothropic 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 seen in Figure 1 that some base substitutions are observed in the *ndhE* partial sequences of the hybrids F1.9, F1.11 and F1.14 in compare to those of the other eleven. 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 with respect to the percentage, the *ndhE* partial sequences of the three hybrids are registered with a different accession number, i.e. MH646651.

Based on the results, 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 supports the assumption of maternal inheritance of some phenotypic traits in the intergeneric hybrids resulting from the two parents.

ACKNOWLEDGEMENTS

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