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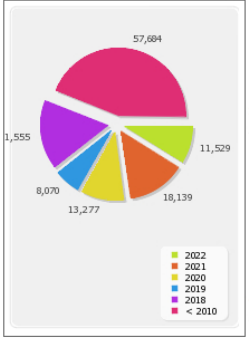

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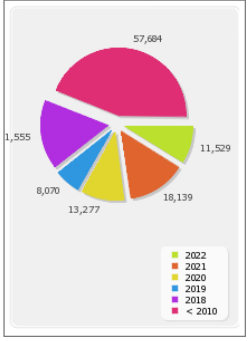

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Molecular phylogeny of orchids mycorrhiza isolated from native tropical orchids in Indonesia

Mahfut[1], Ari Indrianto[2], Susamto Somowiyarjo[3], Budi Setiadi Daryono[4].

Malaysian Journal of Microbiology (Volume 16, No. 1, February 2020, Pages 68 to 72)
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Aims: Mycorrhiza has an important role as a biocontrol agent. Its association with Phalaenopsis amabilis was molecularly identified through rDNA-ITS sequence analysis. The aims of the study were to identify molecular of orchids mycorrhiza isolate from native tropical orchids in Indonesia, conducted as one of native orchid conservation efforts in Indonesia. Methodology and results: One group of Ceratobasidium were isolated from the root of orchid plant in Yogyakarta based on morphological and microscopical analysis. The results of molecular analysis showed 600-750 bp of DNA products located on the ITS1-5.8S-ITS4 region. The sequenced products showed insertion and substitution occurances, which may result in strain diversity and possible variation. Reconstruction of phylogenetic trees using Maximum Parsimony and Bootstrap-1000 approach showed showed the Indonesian isolate is at the basal clade and already far apart from the other isolates. Conclusion, significance and impact of study: Isolate Ceratobasidium from Yogyakarta, Indonesia successfully isolated based on identification of rDNA-ITS sequences. Results of this study were expected to become the basic information in an effort of native orchid cultivation and protection against infectious diseases in Indonesia. The study was the first to report regarding Ceratobasidium isolated from native tropical orchids in Indonesia.

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Molecular phylogeny of orchids mycorrhiza isolated from native tropical orchids in Indonesia

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ABSTRACT

Aims: Mycorrhiza has an important role as a biocontrol agent. Its association with *Phalaenopsis amabilis* was molecularly identified through rDNA-ITS sequence analysis. The aims of the study were to identified molecular of orchids mycorrhiza isolate from native tropical orchids in Indonesia, conducted as one of native orchid conservation efforts in Indonesia.
Methodology and results: One group of *Ceratobasidium* were isolated from the root of orchid plant in Yogyakarta based on morphological and microscopical analysis. The results of molecular analysis showed 600-750 bp of DNA products located on the ITS1-5.8S-ITS4 region. The sequenced products showed insertion and substitution occurances, which may result in strain diversity and possible variation. Reconstruction of phylogenetic trees using *Maximum Parsimony* and *Bootstrap*-1000 approach showed showed the Indonesian isolate is at the basal clade and already far apart from the other isolates.
Conclusion, significance and impact of study: Isolate *Ceratobasidium* from Yogyakarta, Indonesia successfully isolated based on identification of rDNA-ITS sequences. Results of this study were expected to become the basic information in an effort of native orchid cultivation and protection against infectious diseases in Indonesia. The study was the first to report regarding *Ceratobasidium* isolated from native tropical orchids in Indonesia.

Keywords: Ceratobasidium, rDNA-ITS, *Phalaenopsis*, Indonesia

INTRODUCTION

Phalaenopsis is a genus of orchids, which some of its members have important role as parent crosses. Approximately 30 of the total 62 species are spread throughout Indonesia. The presence of this genus in its native habitat (nature) has been reported to have greatly diminished, even some of the members have been recorded to the IUCN red list version 2013.2 due to excessive exploration and forest degradation. Thus, it is very necessary to conserve the existence of native *Phalaenopsis* orchids in Indonesia through the efforts of preservation and protection of plants.

Infections by bacteria, fungi, and viruses are still major obstacles in conducting cultivation and development of native orchids in Indonesia (Kumalawati *et al*., 2011; Mahfut and Daryono, 2014; Mahfut *et al*., 2016a; Mahfut *et al*., 2016b; Mahfut *et al*., 2017; Mahfut et al., 2019b). Cultivating and protecting *Phalaenopsis* sp. against diseases in Indonesia could be done through the induction of endophytic microorganisms (Tanawy, 2009), which includes the Orchid Mycorrhizal Fungi (OMF). Endophytic microorganisms are biological agents that can prevent and reduce the severity of disease caused by infectious pathogens by producing enzymes and secondary metabolites that are antagonistic (Harish *et al*., 2008), which in addition can also help the growth and development of plants. One type of OMF which have been isolated and identified is *Ceratobasidium*.

Ceratobasidium could induce the resistance of *Phalaenopsis* sp. against infections by *Erwinia chrysanthemi* causing soft root disease (Wu *et al*., 2011), fungal infection by *Fusarium* sp. causing rotten stems, leaves, and shoots, as well as inhibit the replication *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CymMV). In addition for playing a role as biological control agents in crop protection (Otero *et al*., 2013), OMF has a role in stimulating the germination of orchid seeds and helping the establishment of more buds and flowers (Andersen *et al*., 1996; Moreno *et al*., 2000;

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Wu *et al*., 2011; Mahfut et al., 2019a). In this research, the identification of *Ceratobasidium* was molecularly conducted through analysis of rDNA-ITS sequence isolated from *P. amabilis* grown in Indonesia. This research was expected to become the basic information on the development of cultivation and protection of nature orchids and where possible, on the prevention of the occurence of the mentioned diseases in Indonesia.

MATERIALS AND METHODS

Plants materials

The sampling of healthy roots of *P. amabilis* was conducted at four different locations: orchid garden in Condong Catur and Parakan (Yogyakarta), Balikpapan Botanical Garden (East Kalimantan), and Sultan Adam Forest (South Kalimantan).

Molecular analysis

Genomic DNA isolation was performed using techniques modified from *cetyltrimethylammonium bromide* (CTAB) method (Doyle and Doyle, 1987; Weiland, 1997) on samples of pure cultures of isolated mycorrhizal endophyte *Ceratobasidium*. Genomic DNA was PCR amplified according to the manual instructions of *GoTaq® Green PCR mix (Promega)*. Predenaturation reaction and amplification was carried out using methods by Nadarajah *et al*. (2014), with a pair of universal primers, rDNA-ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and rDNA-ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR products were visualized using electrophoresis on 2% agarose gel stained with ethidium bromide and 100 bp *Vivantis DNA ladder* was used as marker. The visualized DNA bands indicated the length of the targeted base pairs of rDNA-ITS, which were subjected to sequencing.

Phylogenetic analysis

Sequencing results were analyzed using *Sequence Scanner* software; the nucleotide sequences were combined using *EditSeq* and *SeqMan* of the *Software Suite for Sequence Analysis DNASTAR Lasergene DM Version 3.0.25*. *BLAST* software was used to determine and to compare the sequence homology with the data contained in the DDBJ database. Comparation between sequences of isolates was carried out using *Algorithm Multiple Alignment Parameters DNA* with *Kimura-2 Parameters*, relationship and phylogenetic analysis using the *Neighbour Joining* of *MEGA 5 Beta* program. Statistical analysis on internal branch was done using *the bootstrap* value with 1000 replication.

RESULTS

Sample collection

A total of 12 samples of healthy roots of *P. amabilis* were isolated from 4 different locations, such as orchid garden in Condong Catur and Parakan (Yogyakarta), Balikpapan Botanical Garden (East Kalimantan), and Sultan Adam Forest (South Kalimantan). One positive sample of *Ceratobasidium* was found from orchid garden in Parakan (Figure 1), which had colony characteristic traits i.e. yellowish to white color, colony appearance like a cotton, 90^{o} branching hyphae shape, binucleate, with colony growth rate of 0.72 mm/hour, referring to Currah and Zelmer (1992) and Shan *et al*. (2002).

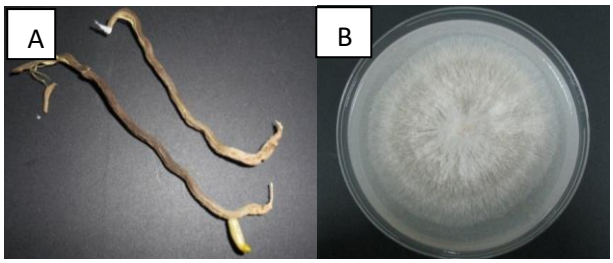

Figure 1: Sample collections of root of *P. amabilis* orchid (A) and *Ceratobasidium* isolate (B) from Parakan (Yogyakarta). Bar size 1 cm.

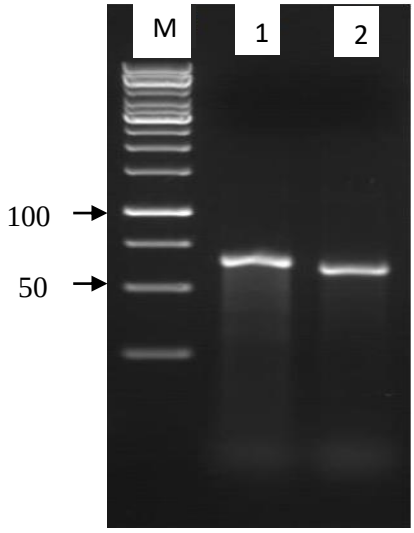

Figure 2: Results of ITS rDNA amplification on endophyte mycorrhizae *Ceratobasidium*; M = Marker (1 kb), 1 and 2 = Mychorrizae Parakan (MP)

Molecular analysis

ITS rDNA amplification results showed a specific band with a size between 600 and 750 bp (Figure 2) similar to that reported by Johansson *et al*. (1998), Nadarajah *et al*. (2014) and Pannecoucque *et al*. (2008). Internal transcribed spacer (ITS) is an area of the nuclear ribosomal DNA (nrDNA), which has the role of providing important information on the reconstruction of phylogenetic trees at different taxonomic levels (Bayer *et al*., 1996), as well as similarity at the level of intrageneric (Lee *et al*., 2010).

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1	100														
2	99.4	100													
3	99.5	99.5	100												
4	99.7	99.7	99.8	100											
5	99.7	99.4	99.5	99.7	100										
6	100.0	99.5	99.7	99.8	99.8	100									
7	99.5	99.2	99.7	99.7	99.5	99.7	100								
8	96.6	99.4	99.5	99.6	96.3	99.8	96.5	100							
9	99.8	99.7	99.8	100.0	99.7	99.8	99.8	98.7	100						
10	99.7	99.7	99.8	100.0	99.7	99.8	99.8	96.6	100.0	100					
11	99.5	99.5	99.7	99.9	99.5	99.7	99.6	96.5	99.8	99.8	100				
12	99.5	99.5	99.7	99.9	99.5	99.7	99.6	96.5	99.8	99.8	99.7	100			
13	99.8	99.2	99.7	99.9	99.8	100.0	99.3	97.2	99.8	99.8	99.4	99.7	100		
14	99.8	99.7	99.8	100.0	99.7	99.8	99.8	98.7	100.0	100.0	99.8	99.8	99.8	100	
15	46.7	46.5	46.9	46.7	46.8	46.6	45.2	46.1	46.7	45.7	46.5	46.9	45.3	46.7	100

Figure 3: Matrix of similarity level percentage of the obtained *Ceratobasidium* sp. nucleotide sequences analyzed using *DNASTAR Lasergene program DM Version 3.0.25*. %: Percentage of Identity; 1: Singapore2; 2: Spain; 3: Taiwan; 4: America; 5: Argentina; 6: Netherland; 7: China; 8: Cuba; 9 : India; 10: Singapore; 11: Iran; 12: Israel; 13: Italy; 14: Japan; 15: Indonesia.

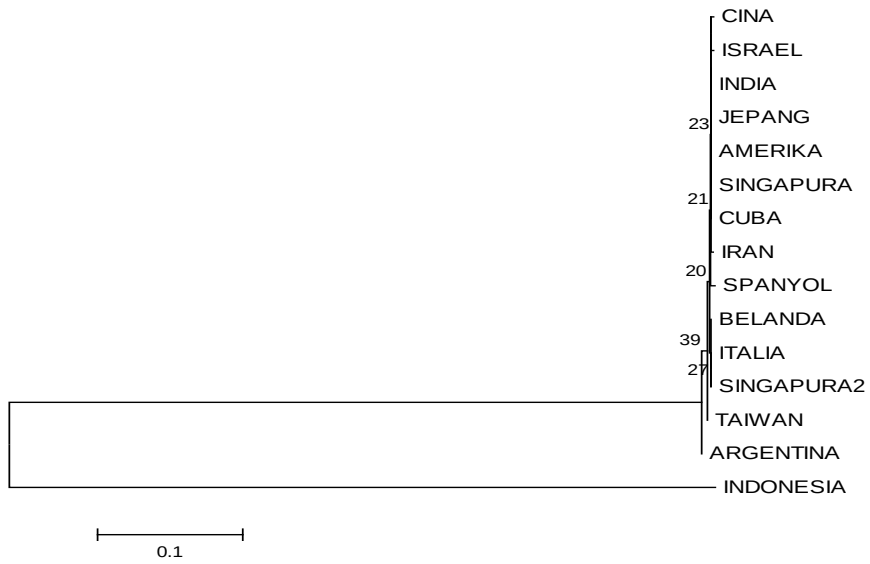

Figure 4: Reconstruction of phylogenetic trees of *Ceratobasidium* sp. isolates based on nucleotide sequences with *Maximum Parsimony* method with *Bootstrap-1000* approach.

Sequencing results were analyzed using *DNASTAR Lasergene DM Version 3.0.25*. Total number of nucleotides of MP isolates which succesfully scanned was 661 with 41.4% GC content. Analysis results were obtained by incorporating sequences to http://blast.ncbi.nlm.nih.gov/ site and confirmed that MP isolates were *Ceratobasidium*. Sharon *et al*. (2008) explained that mychorriza in the anamorphic classification is divided into 3 main categories: multinucleate (teleomorphs: *Thanatephorus* and *Waitea*), binucleate (teleomorphs: *Ceratobasidium* and *Tulasnella*), and uninucleate (teleomorph: *Ceratobasidium*). Based on microscopic observation, MP isolates were found to be binucleate (data not shown). Furthermore, analysis search of homologous sequences carried out using BLAST on NCBI site resulted in 47 sequences indicating a close relationship with high level of similarity of 99%. This result showed that the MP sequences are representative of *Ceratobasidium* sp. isolates, a non-pathogenic (endophyte) associating with the root of orchid (OMF).

All isolates were chosen and compared from various countries in Asia (Israel, China, Taiwan, Japan, Iran, Singapore and India), America (US, Cuba, and Argentina), and Europe (Italy, the Netherlands, and Spain). Some isolates of *Ceratobasidium*, was collected

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from the roots of *Vanda* miss Joaquim (AJ318420) and Brite Ng (AJ318429) orchids, and also from the roots of other plants such as *Rosa hybrida* cv. Linda (KC825348), strawberry *Fragaria x ananassa* (AY927319) and apples (EU002945.1). Total bases amplified from each isolate ranged from 560 to 725 bp, except from DQ097889.1 isolate with a total amplified base of 1500 bp. Maximum *BLAST* score between isolates ranged from 1005 to 1186 with homology (ident.) 99% and query cover ranged from 84 to 99%.

Analysis on 14 other *Ceratobasidium* sp. isolates selected based on the distribution area of each different country, showed that isolates from Indonesia were highly different from those of other countries with similiarity index (IS) ranging from 45.2 to 49.9% (Figure 3). It was clear that isolates from Indonesia has been undergoing speciation and are different from isolates from other countries.

Alignment result of nucleotide sequences of 15 *Ceratobasidium* sp. isolates showed high occurrence of point mutations in isolates from Indonesian, which were largely insertions and substitutions. Of the 606 total bases aligned, there were 44 bases having substitution consisting of 21 transition and 23 transversion bases.

Each of these mutations had an influence on changes in amino acids formed. Of the total 194 amino acids belonging to isolates from Indonesia, some of which had very different percentage from those of other isolates. Some amino acids of isolates from Indonesia had increased their number dramatically compared to those of other isolates, i.e. Ala, Glu, Val, Gln, Lys, and Tyr by 0.7%, 1.76%, 1.8%, 2.3%, 3.1%, and 4.73%, respectively, of total average. Several other amino acids had also decreased in number, i.e. Asp, Arg, Trp, Thr, and Leu by 0.11%, 1.53%, 2.07%, 2.25%, and 4.32%, respectively, of total average.

It appeared that the resulted amino acid changes had enormous influence in the process of adaptation to the environment in Indonesia. Results of the relationship analysis between isolates through the reconstruction of phylogenetic tree showed that isolates from Indonesia were on separated branches and far apart from 14 other isolates (Figure 4).

DISCUSSION

Maximum Parsimony method with *Bootstrap-1000* approach classify the data based on specific characters (discrete character states) on which nucleotide sequences are used to obtain information about evolution through evolutionary data changes. The analysis result showed that Indonesia isolates were on the basal clade. Indonesia isolate showed high occurrence of point mutations, which were largely insertions and substitutions. It appeared that the resulted amino acid changes had enormous influence in the process of adaptation to the environment in Indonesia. Indonesia isolate was on separated branches and far apart from 14 other isolates. A lengthy branch, which well separating between the two groups, indicated that isolates from Indonesia had considerably evolved and it led to speciation events. Such changes in sequence through mutation was supported by the ability of the mycorrhizal itself to adapt to environmental changes as well as to its host range. The adaptability of mycorrhiza is strongly influenced by the presence of amino acid changes due to mutations that alter the function of genes which they arrange.

The reconstruction of phylogenetic tree, in addition to explaining relationship based on geographic regions, also determine the origin and history of distribution of the isolates. Trading activities of plants, fruits, and seedlings from several countries which are reported to have been infected by *Ceratobasidium* sp. is most likely the cause of its spreading. Indonesia is known as an importer country of orchid plant seedlings from Asian countries such as Thailand, Singapore and Taiwan (Mahfut *et al*., 2016a), and possibly have imported seedling and fruit of apple and strawberry plants from other countries such as India and Italy that had been reported infected by *Ceratobasidium* sp. isolates. Proximity with Singapore in terms of geographical location is also very possible to allow distribution activities of orchids between the two countries.

This study results proved that nucleotide sequences and amino acid of *Ceratobasidium* sp. isolates from Indonesia were different with other isolates from another country (Asia, Europe, and America). This is thought to be a form of adaptation to the natural environment in Indonesia. Another possible result of mutations is that *Ceratobasidium* sp. could have the ability to infect plants while showing pathogenic characters or otherwise become mycorrhizal endophytic.

This study results clarified the MP sequence as OMF *Ceratobasidium* sp. isolates from Indonesia based on identification of its rDNA-ITS sequences. Furthermore, this study was expected to be the basic information beneficial for the improvement of cultivation effort as well as for the development of biocontrol agents through natural orchid protection against pathogens and diseases.

CONCLUSION

Molecular analysis based on rDNA-ITS resulted in isolate from the root of orchid plant in Yogyakarta being *Ceratobasidium*. These isolates showed 600-750 bp in length DNA products located on the ITS1-5.8S-ITS4 region. The sequence products showed insertion and substitution occurances, which may result in strain diversity and possible variation in severity. Reconstruction of phylogenetic tree using *Maximum Parsimony* and *Bootstrap*-1000 approach showed that Indonesian isolates have undergone speciation and have been positioned in the cluster, which are already far apart from the other isolates.

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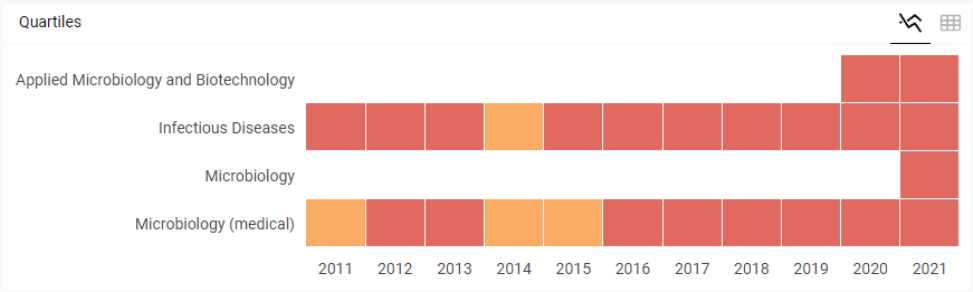

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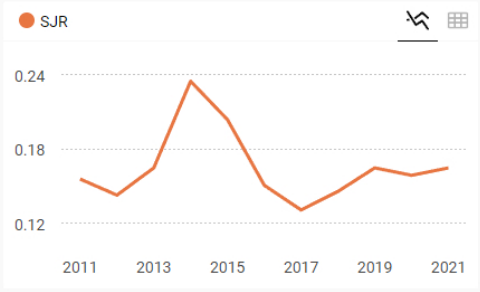

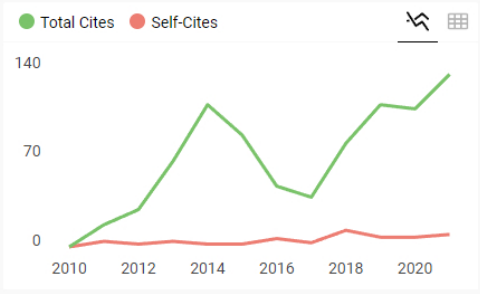

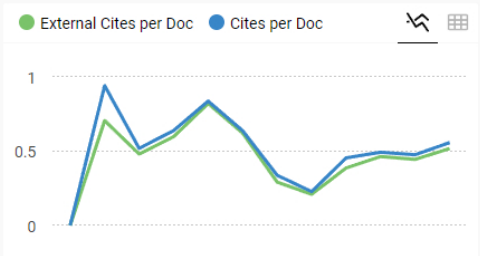

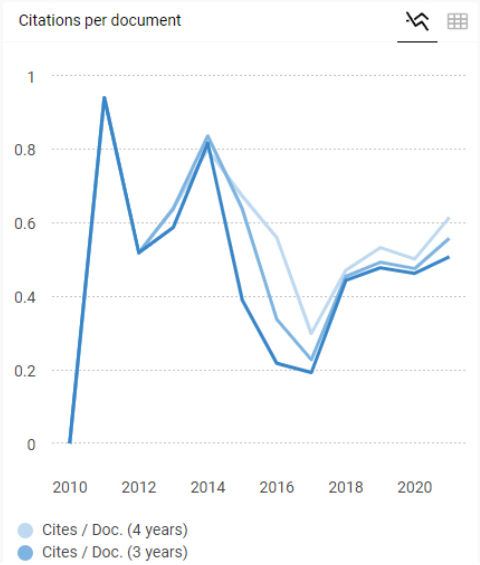

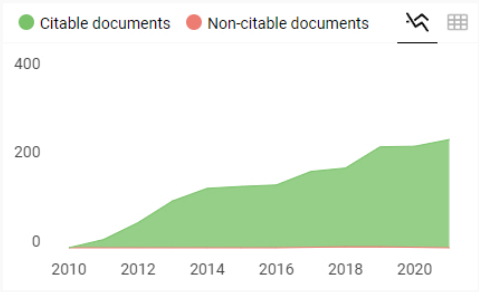

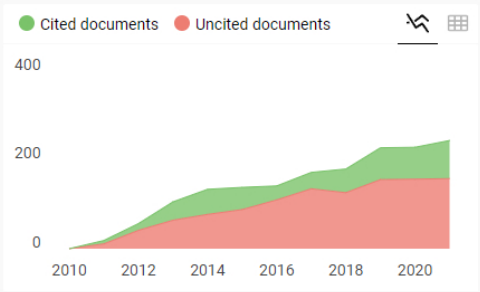

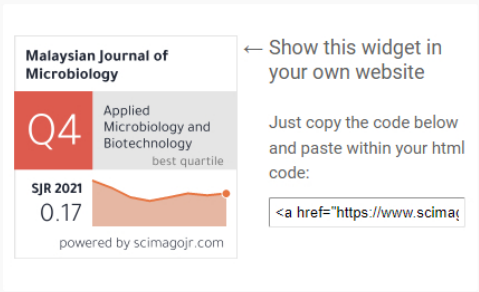

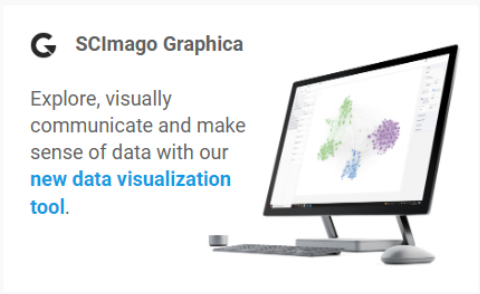

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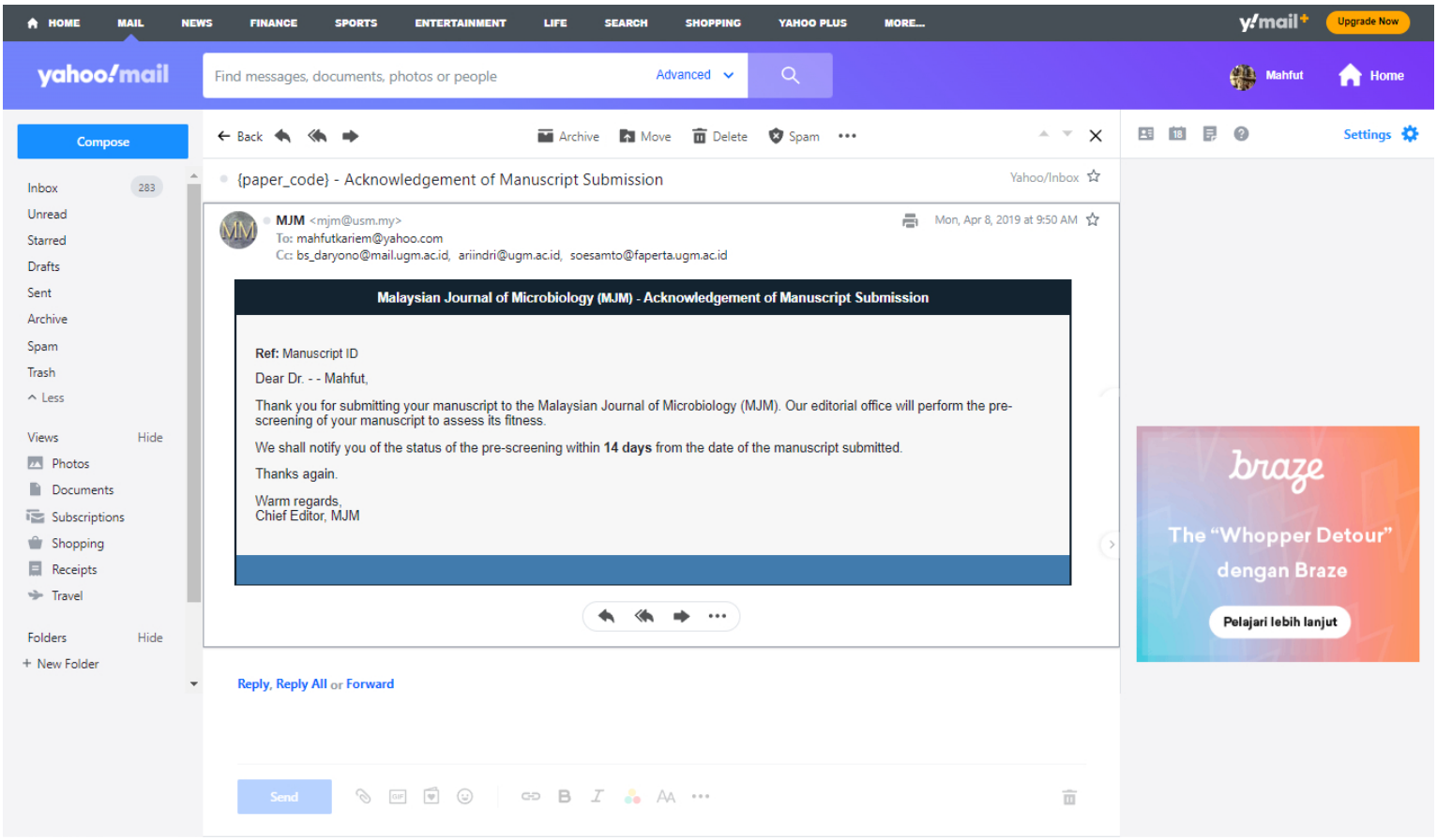
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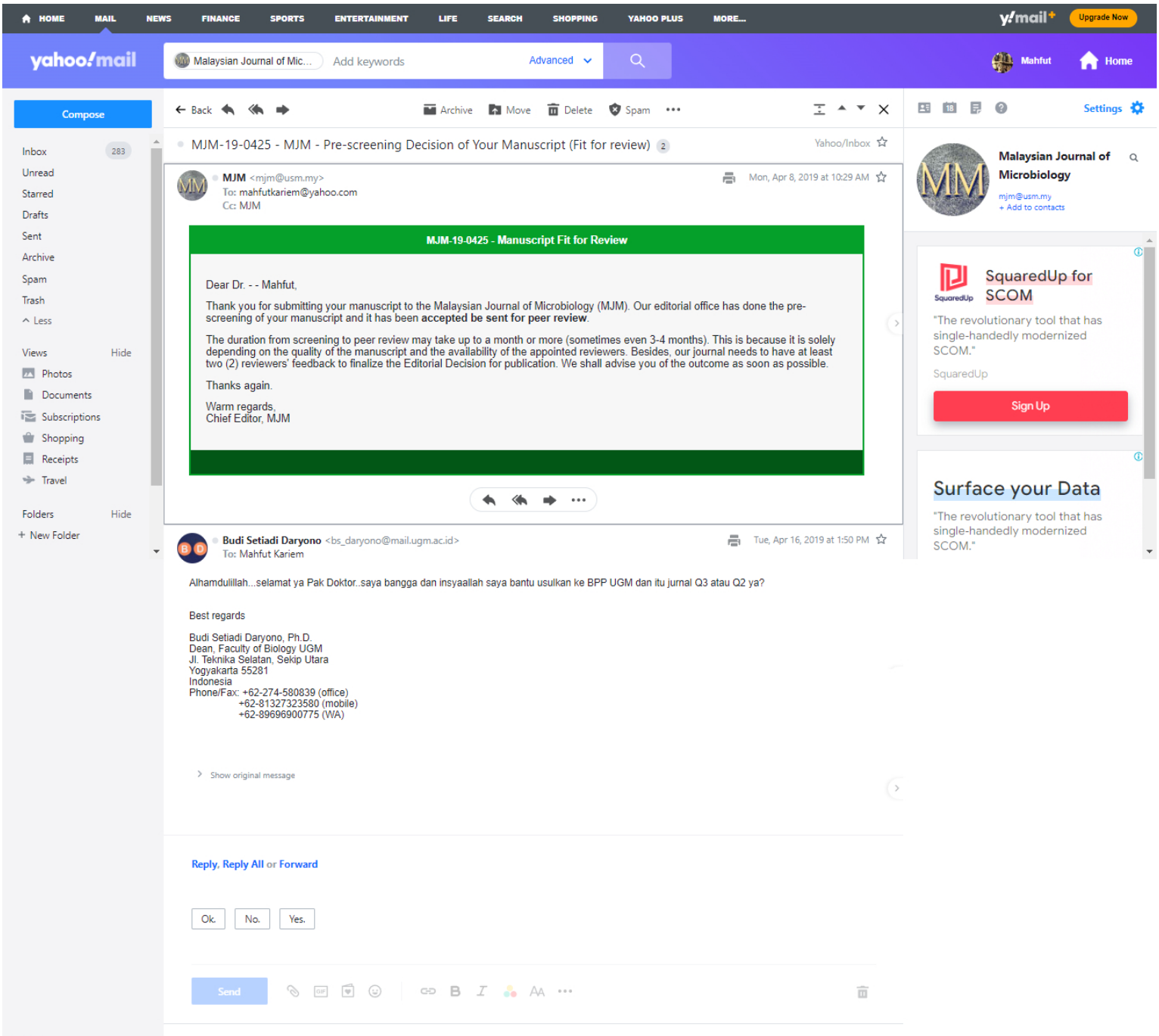

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Budi Setiadi Daryono, Ph.D.
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MOLECULAR PHYLOGENY OF ORCHIDS MYCORRHIZA ISOLATED FROM NATIVE TROPICAL ORCHIDS IN INDONESIA

Mahfut[1], Budi Setiadi Daryono[2]*, Ari Indrianto[2], and Susamto Somawiyarjo[3]

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[2]Department of Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia;
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ABSTRACT

Aims: Mycorrhiza has an important role as a biocontrol agent. Its association with *Phalaenopsis amabilis* was molecularly identified through rDNA-ITS sequence analysis. The aims of the study were to identify isolated molecular of orchids mycorrhiza from native tropical orchids in Indonesia, conducted as one of native orchid conservation efforts in Indonesia.

Methodology and results: One group of *Ceratobasidium* were isolated from the root of orchid plant in Yogyakarta based on morphological and microscopical analysis. Verification analysis molecular of these isolates resulted in 600-750 bp DNA products located on the ITS1-5.8S-ITS4 region. The sequenced products showed insertion and substitution occurances, which may result in strain diversity and possible variation in severity. Reconstruction of phylogenetic trees using *Maximum Parsimony* and *Bootstrap*-1000 approach showed that Indonesian isolates have undergone speciation and have been positioned in the cluster, which are already far apart from the other isolates.

Conclusion, significance and impact of study: Isolate *Ceratobasidium* from Yogyakarta, Indonesia successfully isolated based on identification of RNA-ITS sequences. Results of this study were expected to become the basic information in an effort of native orchid cultivation and protection against infectious diseases in Indonesia. The study was the first to report regarding *Ceratobasidium* isolated from native tropical orchids in Indonesia.

Keywords: *Ceratobasidium*; rDNA-ITS; *Phalaenopsis*; Indonesia

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INTRODUCTION

Phalaenopsis is a genus of orchids, which some of its members have important role as parent crosses. Approximately 30 of the total 62 species are spread throughout Indonesia. The presence of this genus in its native habitat (nature) has been reported to have greatly diminished, even some of the members have been recorded to the IUCN red list version 2013.2 due to excessive exploration and forest degradation. Thus, it is very necessary to conserve the existence of native *Phalaenopsis* orchids in Indonesia through the efforts of preservation and protection of plants.

Infections by bacteria, fungi, and viruses are still major obstacles in conducting cultivation and development of native orchids in Indonesia (Kumalawati *et al*., 2011; Mahfut and Daryono, 2014; Mahfut *et al*., 2016[a]; Mahfut *et al*., 2016[b]; Mahfut *et al*., 2017). Cultivating and protecting *Phalaenopsis* sp. against diseases in Indonesia could be done through the induction of endophytic microorganisms (Tanawy, 2009), which includes the Orchid Mycorrhizal Fungi (OMF). Endophytic microorganisms are biological agents that can prevent and reduce the severity of disease caused by infectious pathogens by producing enzymes and secondary metabolites that are antagonistic (Harish *et al*., 2008), which in addition can also help the growth and development of plants. One type of OMF which have been isolated and identified is *Ceratobasidium*.

Ceratobasidium could induce the resistance of *Phalaenopsis* sp. against infections by *Erwinia chrysanthemi* causing soft root disease (Wu *et al*., 2011), fungal infection by *Fusarium* sp. causing rotten stems, leaves, and shoots, as well as inhibit the replication *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CymMV). In addition for playing a role as biological control agents in crop protection (Otero *et al*., 2013), in general OMF has a role in stimulating the germination of orchid seeds (Andersen *et al*., 1996), in supporting the provision of nutrients for growth and development of plantlets (Moreno *et al*., 2000; Wu *et al*., 2011), and

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in helping the establishment of more buds and flowers (Wu *et al*., 2011). In this research, the identification of *Ceratobasidium* was molecularly conducted through analysis of rDNA-ITS sequence isolated from *Phalaenopsis amabilis* grown in Indonesia. This research was expected to become the basic information on the development of cultivation and protection of nature orchids and where possible, on the prevention of the occurence of the mentioned diseases in Indonesia.

MATERIALS AND METHODS

Plants Materials

The sampling of healthy roots of *Phalaenopsis amabilis* was conducted at four different locations: orchid garden in Condong Catur and Parakan (Yogyakarta), Balikpapan Botanical Garden (East Kalimantan), and Sultan Adam Forest (South Kalimantan). On the separated study, isolation results showed positive samples of mycorrhizal endophyte *Ceratobasidium* sp. based on morphological and microscopical analysis.

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Molecular Analysis

Genomic DNA isolation was performed using techniques modified from *cetyltrimethylammonium bromide* (CTAB) method (Doyle and Doyle, 1987; Weiland, 1997) on samples of pure cultures of isolated mycorrhizal endophyte *Ceratobasidium*. Genomic DNA was PCR amplified according to the manual instructions of *GoTaq® Green PCR mix (Promega)*. Predenaturation reaction and amplification was carried out using methods by Nadarajah *et al*. (2014), with a pair of universal primers, rDNA-ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and rDNA-ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR products were visualized using electrophoresis on 2% agarose gel stained with ethidium bromide and 100 bp *Vivantis DNA*

ladder was used as marker. The visualized DNA bands indicated the length of the targeted base pairs of rDNA-ITS, which were subjected to sequencing.

Phylogenetic Analysis

Sequencing results were analyzed using *Sequence Scanner* software; the nucleotide sequences were combined using *EditSeq* and *SeqMan* of the *Software Suite for Sequence Analysis DNASTAR Lasergene DM Version 3.0.25*. *BLAST* software was used to determine and to compare the sequence homology with the data contained in the DDBJ database. Comparation between sequences of isolates was carried out using *Algorithm Multiple Alignment Parameters DNA* with *Kimura-2 Parameters*, relationship and phylogenetic analysis using the *Neighbour Joining* of *MEGA 5 Beta* program. Statistical analysis on internal branch was done using *the bootstrap* value with 1000 replication.

RESULTS

Sample Collection

A total of 12 samples of healthy roots of *Phalaenopsis amabilis* were isolated from 4 different locations, such as orchid garden in Condong Catur and Parakan (Yogyakarta), Balikpapan Botanical Garden (East Kalimantan), and Sultan Adam Forest (South Kalimantan). Positive result of *Ceratobasidium* sp. was found from orchid garden in Parakan (Figure 1), which had colony characteristic traits i.e. yellowish to white color, colony appearance like a cotton, 90° branching hyphae shape, binucleate, with colony growth rate of 0.72 mm/hour, referring to Currah and Zelmer (1992) and Shan *et al*. (2002).

Commented [FYKS9]: Please provide the observation from this study.

Morphology and microscopic observation is based on how many replicates?

How many isolates successfully obtained?

Molecular Analysis

ITS rDNA amplification results showed a specific band with a size between 600 and 750 bp (Figure 2) similar to that reported by Johansson *et al*. (1998), Nadarajah *et al*. (2014), and Pannecoucque *et al*. (2008). Internal transcribed spacer (ITS) is an area of the nuclear ribosomal DNA (nrDNA), which has the role of providing important information on the reconstruction of phylogenetic trees at different taxonomic levels (Bayer *et al*., 1996), as well as similarity at the level of intrageneric (Lee *et al*., 2010).

Sequencing results were combined and analyzed using *DNASTAR Lasergene DM Version 3.0.25*. Total number of nucleotide of MP isolates which succesfully scanned was 661 with 41.4% GC content. Analysis results were obtained by incorporating sequences to http://blast.ncbi.nlm.nih.gov/ site and confirmed that MP isolates were *Ceratobasidium*. Sharon *et al*. (2008) explained that mychorriza in the anamorphic classification is divided into 3 main category: multinucleate (teleomorphs: *Thanatephorus* and *Waitea*), binucleate (teleomorphs: *Ceratobasidium* and *Tulasnella*), and uninucleate (teleomorph: *Ceratobasidium*). Based on microscopic observation, MP isolates were found to be binucleate (data not shown). Furthermore, search analysis of homologous sequences carried out using BLAST on NCBI site resulted in 47 sequences indicating a close relationship with high level of similarity of 99%. This result showed that the MP sequences are representative of *Ceratobasidium* sp. isolates, a non-pathogenic (endophyte) associating with the root of orchid (OMF).

The whole isolates originated from various countries in Asia (Israel, China, Taiwan, Japan, Iran, Singapore, and India), America (USA, Cuba, and Argentina), and Europe (Italy, Netherlands and Spain). Some isolates of *Ceratobasidium*, was collected from the roots of *Vanda* miss Joaquim (AJ318420) and Brite Ng (AJ318429) orchids, and also from the roots of other

Commented [FYKS10]: Combine what?

Commented [FYKS11]: Meaning? How many sequence per country?

126 plants such as *Rosa hybrida* cv. Linda (KC825348), strawberry *Fragaria x ananassa*
127 (AY927319) and apples (EU002945.1). Total bases amplified from each isolate ranged from 560
128 to 725 bp, except from DQ097889.1 isolate with a total amplified base of 1500 bp. Maximum
129 *BLAST* score between isolates ranged from 1005 to 1186 with homology (ident.) 99% and query
130 cover ranged from 84 to 99%.

131 Analysis on 14 other *Ceratobasidium* sp. isolates selected based on the distribution area of
132 each different country, showed that isolates from Indonesia were highly different from those of
133 other countries with similiarity index (IS) ranging from 45.2 to 49.9% (Figure 3). It was clear
134 that isolates from Indonesia has been undergoing speciation and are different from isolates from
135 other countries.

136 Alignment result of nucleotide sequences of 15 *Ceratobasidium* sp. isolates showed high
137 occurrence of point mutations in isolates from Indonesian, which were largely insertions and
138 substitutions. Of the 606 total bases aligned, there were 44 bases having substitution consisting
139 of 21 transition and 23 transversion bases.

140 Each of these mutations had an influence on changes in amino acids formed. Of the total
141 194 amino acids belonging to isolates from Indonesia, some of which had very different
142 percentage from those of other isolates. Some amino acids of isolates from Indonesia had
143 increased their number dramatically compared to those of other isolates, i.e. Ala, Glu, Val, Gln,
144 Lys, and Tyr by 0.7%, 1.76%, 1.8%, 2.3%, 3, 1%, and 4.73%, respectively, of total average.
145 Several other amino acids had also decreased in number, i.e. Asp, Arg, Trp, Thr, and Leu by
146 0.11%, 1.53%, 2.07%, 2.25%, and 4.32%, respectively, of total average.

147 It appeared that the resulted amino acid changes had enormous influence in the process of
148 adaptation to the environment in Indonesia. Results of the relationship analysis between isolates

149 through the reconstruction of phylogenetic tree showed that isolates from Indonesia were on
150 separated branches and far apart from 14 other isolates (Figure 4).
151
152 **DISCUSSION**
153 *Maximum Parsimony* method with *Bootstrap-1000* approach classify the data based on specific
154 characters (discrete character states) on which nucleotide sequences are used to obtain
155 information about evolution through evolutionary data changes. The results of analysis were
156 classified based on distribution on the geographical areas. A lengthy branch, which well
157 separating between the two groups, indicated that isolates from Indonesia had considerably
158 evolved and it led to speciation events. Such changes in sequence through mutation was
159 supported by the ability of the mycorrhizal itself to adapt to environmental changes as well as to
160 its host range. The adaptability of mycorrhiza is strongly influenced by the presence of amino
161 acid changes due to mutations that alter the function of genes which they arrange.
162 The reconstruction of phylogenetic tree, in addition to explaining relationship based on
163 geographic regions, also determine the origin and history of distribution of the isolates. Trading
164 activities of plants, fruits, and seedlings from several countries which are reported to have been
165 infected by *Ceratobasidium* sp. is most likely the cause of its spreading. Indonesia is known as
166 an importer country of orchid plant seedlings from Asian countries such as Thailand, Singapore
167 and Taiwan (Mahfut *et al*., 2016[a]), and possibly have imported seedling and fruit of apple and
168 strawberry plants from other countries such as India and Italy that had been reported infected by
169 *Ceratobasidium* sp. isolates. Proximity with Singapore in terms of geographical location is also
170 very possible to allow distribution activities of orchids between the two countries.

Commented [FYKS12]: Then why Indonesian isolates were distinctly placed at the basal clade on its own?

This study results proved that nucleotide sequences and amino acid of *Ceratobasidium* sp. isolates from Indonesia were different with other isolates from another country (Asia, Europe, and America) due to adaptation to various environmental conditions. Furthermore, the mutations are capable of causing the *Ceratobasidium* sp. to lose its ability to infect and to associate with host plants. Another possible result of mutations is that *Ceratobasidium* sp. could have the ability to infect plants while showing pathogenic characters or otherwise become mycorrhizal endophytic.

One way to control pathogens of orchids is by using biological control, such as the mechanisms of mycorrhizal resistance induction. *Ceratobasidium* as OMF has a role as a biological agent in disease control (Otero *et al*., 2013) including infection of ORSV. This study results clarified the MP sequence as OMF *Ceratobasidium* sp. isolates from Indonesia based on identification of its rDNA-ITS sequences. Furthermore, this study was expected to be the basic information beneficial for the improvement of cultivation effort as well as for the development of biocontrol agents through natural orchid protection against pathogens and diseases.

Commented [FYKS13]: Evidence?

Commented [FYKS14]: Any evidence suggesting mutation in ITS wil cause this?

Commented [FYKS15]: irrelevant

CONCLUSION

Molecular analysis based on rDNA-ITS resulted in isolate from the root of orchid plant in Yogyakarta being *Ceratobasidium*. These isolates showed 600-750 bp in length DNA products located on the ITS1-5.8S-ITS4 region. The sequence products showed insertion and substitution occurances, which may result in strain diversity and possible variation in severity. Reconstruction of phylogenetic tree using *Maximum Parsimony* and *Bootstrap*-1000 approach showed that Indonesian isolates have undergone speciation and have been positioned in the cluster, which are already far apart from the other isolates.

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Figure 1. Sample collections of root of *Phalaenopsis amabilis* orchid (A) and *Ceratobasidium* isolate (B) from Parakan (Yogyakarta). Bar size 1 cm.

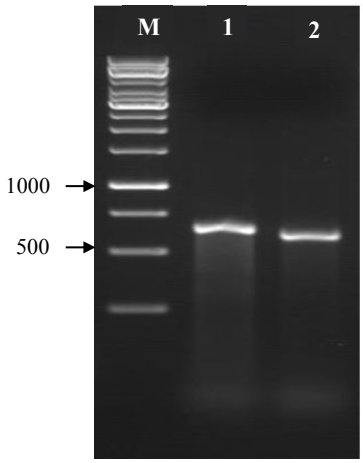
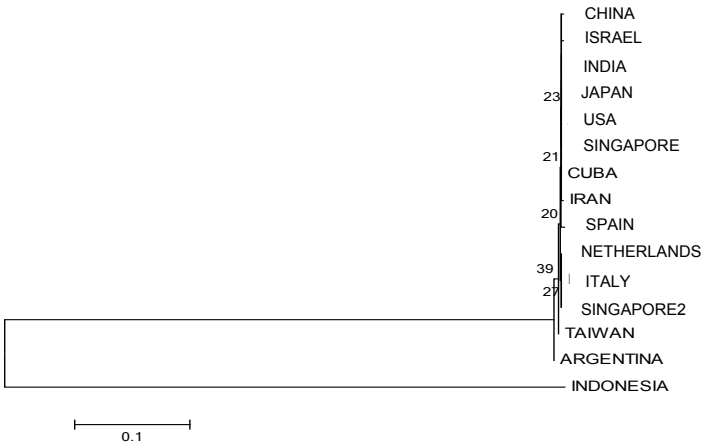


Figure 2. Results of ITS rDNA amplification on endophyte mycorrhizae *Ceratobasidium*; M = Marker (1 kb), 1 and 2 = Mychorrizae Parakan (MP)

%	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	100														
2	99,4	100													
3	99,5	99,5	100												
4	99,7	99,7	99,8	100											
5	99,7	99,4	99,5	99,7	100										
6	100,0	99,5	99,7	99,8	99,8	100									
7	99,5	99,2	99,7	99,7	99,5	99,7	100								
8	96,6	99,4	99,5	99,6	96,3	99,8	96,5	100							
9	99,8	99,7	99,8	100,0	99,7	99,8	99,8	98,7	100						
10	99,7	99,7	99,8	100,0	99,7	99,8	99,8	96,6	100,0	100					
11	99,5	99,5	99,7	99,9	99,5	99,7	99,6	96,5	99,8	99,8	100				
12	99,5	99,5	99,7	99,9	99,5	99,7	99,6	96,5	99,8	99,8	99,7	100			
13	99,8	99,2	99,7	99,9	99,8	100,0	99,3	97,2	99.8	99,8	99,4	99,7	100		
14	99,8	99,7	99,8	100,0	99,7	99,8	99,8	98,7	100,0	100,0	99,8	99,8	99,8	100	
15	46,7	46,5	46,9	46,7	46,8	46,6	45,2	46,1	46,7	45,7	46,5	46,9	45,3	46,7	100

Figure 3. Matrix of similarity level percentage of the obtained *Ceratobasidium* sp. nucleotide sequences analyzed using *DNASTAR Lasergene program DM Version 3.0.25*. %: Percentage of Identity; 1: Singapore2; 2: Spain; 3: Taiwan; 4: America; 5: Argentina; 6: Netherland; 7: China; 8: Cuba; 9 : India; 10: Singapore; 11: Iran; 12: Israel; 13: Italy; 14: Japan; 15: Indonesia.



290

291 **Figure 4.** Reconstruction of phylogenetic trees of *Ceratobasidium* sp. isolates based on

292 nucleotide sequences with *Maximum Parsimony* method with *Bootstrap-1000*

293 approach

294

Commented [FYKS16]: not clear.

8.

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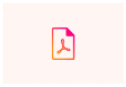

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Molecular phylogeny of orchids mycorrhiza isolated from native tropical orchids in Indonesia

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ABSTRACT

Aims: Mycorrhiza has an important role as a biocontrol agent. Its association with *Phalaenopsis amabilis* was molecularly identified through rDNA-ITS sequence analysis. The aims of the study were to identify molecular of orchids mycorrhiza isolate from native tropical orchids in Indonesia, conducted as one of native orchid conservation efforts in Indonesia.
Methodology and results: One group of *Ceratobasidium* were isolated from the root of orchid plant in Yogyakarta based on morphological and microscopical analysis. The results of molecular analysis showed 600-750 bp of DNA products located on the ITS1-5.8S-ITS4 region. The sequenced products showed insertion and substitution occurances, which may result in strain diversity and possible variation. Reconstruction of phylogenetic trees using *Maximum Parsimony* and *Bootstrap*-1000 approach showed showed the Indonesian isolate is at the basal clade and already far apart from the other isolates.
Conclusion, significance and impact of study: Isolate *Ceratobasidium* from Yogyakarta, Indonesia successfully isolated based on identification of rDNA-ITS sequences. Results of this study were expected to become the basic information in an effort of native orchid cultivation and protection against infectious diseases in Indonesia. The study was the first to report regarding *Ceratobasidium* isolated from native tropical orchids in Indonesia.

Keywords: Ceratobasidium, rDNA-ITS, *Phalaenopsis*, Indonesia

INTRODUCTION

Phalaenopsis is a genus of orchids, which some of its members have important role as parent crosses. Approximately 30 of the total 62 species are spread throughout Indonesia. The presence of this genus in its native habitat (nature) has been reported to have greatly diminished, even some of the members have been recorded to the IUCN red list version 2013.2 due to excessive exploration and forest degradation. Thus, it is very necessary to conserve the existence of native *Phalaenopsis* orchids in Indonesia through the efforts of preservation and protection of plants.

Infections by bacteria, fungi, and viruses are still major obstacles in conducting cultivation and development of native orchids in Indonesia (Kumalawati *et al*., 2011; Mahfut and Daryono, 2014; Mahfut *et al*., 2016[a]; Mahfut *et al*., 2016[b]; Mahfut *et al*., 2017; Mahfut et al., 2019[b]). Cultivating and protecting *Phalaenopsis* sp. against diseases in Indonesia could be done through the induction of endophytic microorganisms (Tanawy, 2009), which includes the Orchid Mycorrhizal Fungi (OMF). Endophytic microorganisms are biological agents that can prevent and reduce the severity of disease caused by infectious pathogens by producing enzymes and secondary metabolites that are antagonistic (Harish *et al*., 2008), which in addition can also help the growth and development of plants. One type of OMF which have been isolated and identified is *Ceratobasidium*.

Ceratobasidium could induce the resistance of *Phalaenopsis* sp. against infections by *Erwinia chrysanthemi* causing soft root disease (Wu *et al*., 2011), fungal infection by *Fusarium* sp. causing rotten stems, leaves, and shoots, as well as inhibit the replication *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CymMV). In addition for playing a role as biological control agents in crop protection (Otero *et al*., 2013), OMF has a role in stimulating the germination of orchid seeds and helping the establishment of more buds and flowers (Andersen *et al*., 1996; Moreno *et al*., 2000;

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Wu *et al*., 2011; Mahfut et al., 2019[a]). In this research, the identification of *Ceratobasidium* was molecularly conducted through analysis of rDNA-ITS sequence isolated from *P. amabilis* grown in Indonesia. This research was expected to become the basic information on the development of cultivation and protection of nature orchids and where possible, on the prevention of the occurence of the mentioned diseases in Indonesia.

MATERIALS AND METHODS

Plants materials

The sampling of healthy roots of *P. amabilis* was conducted at four different locations: orchid garden in Condong Catur and Parakan (Yogyakarta), Balikpapan Botanical Garden (East Kalimantan), and Sultan Adam Forest (South Kalimantan).

Molecular analysis

Genomic DNA isolation was performed using techniques modified from *cetyltrimethylammonium bromide* (CTAB) method (Doyle and Doyle, 1987; Weiland, 1997) on samples of pure cultures of isolated mycorrhizal endophyte *Ceratobasidium*. Genomic DNA was PCR amplified according to the manual instructions of *GoTaq® Green PCR mix (Promega)*. Predenaturation reaction and amplification was carried out using methods by Nadarajah *et al*. (2014), with a pair of universal primers, rDNA-ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and rDNA-ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR products were visualized using electrophoresis on 2% agarose gel stained with ethidium bromide and 100 bp *Vivantis DNA ladder* was used as marker. The visualized DNA bands indicated the length of the targeted base pairs of rDNA-ITS, which were subjected to sequencing.

Phylogenetic analysis

Sequencing results were analyzed using *Sequence Scanner* software; the nucleotide sequences were combined using *EditSeq* and *SeqMan* of the *Software Suite for Sequence Analysis DNASTAR Lasergene DM Version 3.0.25*. *BLAST* software was used to determine and to compare the sequence homology with the data contained in the DDBJ database. Comparation between sequences of isolates was carried out using *Algorithm Multiple Alignment Parameters DNA* with *Kimura-2 Parameters*, relationship and phylogenetic analysis using the *Neighbour Joining* of *MEGA 5 Beta* program. Statistical analysis on internal branch was done using *the bootstrap* value with 1000 replication.

RESULTS

Sample collection

A total of 12 samples of healthy roots of *P. amabilis* were isolated from 4 different locations, such as orchid garden in Condong Catur and Parakan (Yogyakarta), Balikpapan Botanical Garden (East Kalimantan), and Sultan Adam Forest (South Kalimantan). One positive sample of *Ceratobasidium* was found from orchid garden in Parakan (Figure 1), which had colony characteristic traits i.e. yellowish to white color, colony appearance like a cotton, 90^{o} branching hyphae shape, binucleate, with colony growth rate of 0.72 mm/hour, referring to Currah and Zelmer (1992) and Shan *et al*. (2002).

Figure 1: Sample collections of root of *P. amabilis* orchid (A) and *Ceratobasidium* isolate (B) from Parakan (Yogyakarta). Bar size 1 cm.

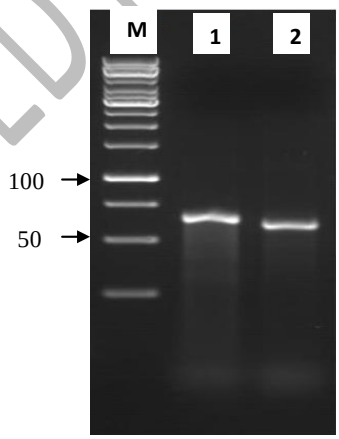

Figure 2: Results of ITS rDNA amplification on endophyte mycorrhizae *Ceratobasidium*; M = Marker (1 kb), 1 and 2 = Mychorrizae Parakan (MP).

Molecular analysis

ITS rDNA amplification results showed a specific band with a size between 600 and 750 bp (Figure 2) similar to that reported by Johansson *et al*. (1998), Nadarajah *et al*. (2014) and Pannecoucque *et al*. (2008). Internal transcribed spacer (ITS) is an area of the nuclear ribosomal DNA (nrDNA), which has the role of providing important information on the reconstruction of phylogenetic trees at different taxonomic levels (Bayer *et al*., 1996), as well as similarity at the level of intrageneric (Lee *et al*., 2010).

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%	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	100														
2	99.4	100													
3	99.5	99.5	100												
4	99.7	99.7	99.8	100											
5	99.7	99.4	99.5	99.7	100										
6	100.0	99.5	99.7	99.8	99.8	100									
7	99.5	99.2	99.7	99.7	99.5	99.7	100								
8	96.6	99.4	99.5	99.6	96.3	99.8	96.5	100							
9	99.8	99.7	99.8	100.0	99.7	99.8	99.8	98.7	100						
10	99.7	99.7	99.8	100.0	99.7	99.8	99.8	96.6	100.0	100					
11	99.5	99.5	99.7	99.9	99.5	99.7	99.6	96.5	99.8	99.8	100				
12	99.5	99.5	99.7	99.9	99.5	99.7	99.6	96.5	99.8	99.8	99.7	100			
13	99.8	99.2	99.7	99.9	99.8	100.0	99.3	97.2	99.8	99.8	99.4	99.7	100		
14	99.8	99.7	99.8	100.0	99.7	99.8	99.8	98.7	100.0	100.0	99.8	99.8	99.8	100	
15	46.7	46.5	46.9	46.7	46.8	46.6	45.2	46.1	46.7	45.7	46.5	46.9	45.3	46.7	100

Figure 3: Matrix of similarity level percentage of the obtained *Ceratobasidium* sp. nucleotide sequences analyzed using *DNASTAR Lasergene program DM Version 3.0.25*. %: Percentage of Identity; 1: Singapore2; 2: Spain; 3: Taiwan; 4: America; 5: Argentina; 6: Netherland; 7: China; 8: Cuba; 9 : India; 10: Singapore; 11: Iran; 12: Israel; 13: Italy; 14: Japan; 15: Indonesia.

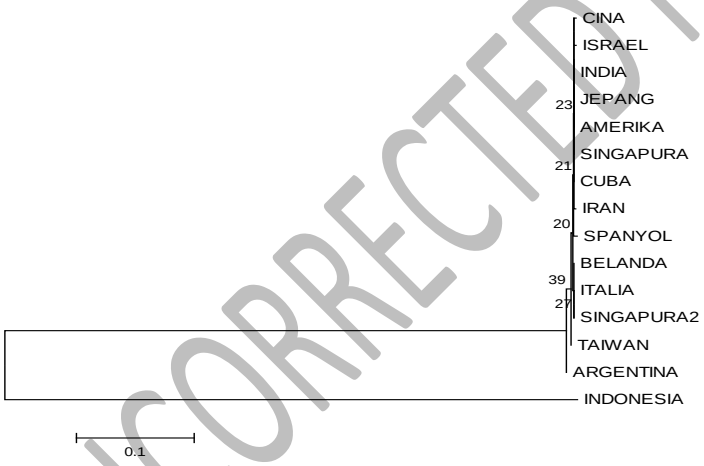

Figure 4: Reconstruction of phylogenetic trees of *Ceratobasidium* sp. isolates based on nucleotide sequences with *Maximum Parsimony* method with *Bootstrap-1000* approach.

Sequencing results were analyzed using *DNASTAR Lasergene DM Version 3.0.25*. Total number of nucleotides of MP isolates which succesfully scanned was 661 with 41.4% GC content. Analysis results were obtained by incorporating sequences to http://blast.ncbi.nlm.nih.gov/ site and confirmed that MP isolates were *Ceratobasidium*. Sharon *et al*. (2008) explained that mychorriza in the anamorphic classification is divided into 3 main categories: multinucleate (teleomorphs: *Thanatephorus* and *Waitea*), binucleate (teleomorphs: *Ceratobasidium* and *Tulasnella*), and uninucleate (teleomorph: *Ceratobasidium*). Based on microscopic observation, MP isolates were found to be binucleate (data not shown). Furthermore, analysis search of homologous sequences carried out using BLAST on NCBI site resulted in 47 sequences indicating a close relationship with high level of similarity of 99%. This result showed that the MP sequences are representative of *Ceratobasidium* sp. isolates, a non-pathogenic (endophyte) associating with the root of orchid (OMF).

All isolates were chosen and compared from various countries in Asia (Israel, China, Taiwan, Japan, Iran, Singapore and India), America (US, Cuba, and

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Argentina), and Europe (Italy, the Netherlands, and Spain). Some isolates of *Ceratobasidium*, was collected from the roots of *Vanda* miss Joaquim (AJ318420) and Brite Ng (AJ318429) orchids, and also from the roots of other plants such as *Rosa hybrida* cv. Linda (KC825348), strawberry *Fragaria x ananassa* (AY927319) and apples (EU002945.1). Total bases amplified from each isolate ranged from 560 to 725 bp, except from DQ097889.1 isolate with a total amplified base of 1500 bp. Maximum *BLAST* score between isolates ranged from 1005 to 1186 with homology (ident.) 99% and query cover ranged from 84 to 99%.

Analysis on 14 other *Ceratobasidium* sp. isolates selected based on the distribution area of each different country, showed that isolates from Indonesia were highly different from those of other countries with similiarity index (IS) ranging from 45.2 to 49.9% (Figure 3). It was clear that isolates from Indonesia has been undergoing speciation and are different from isolates from other countries.

Alignment result of nucleotide sequences of 15 *Ceratobasidium* sp. isolates showed high occurrence of point mutations in isolates from Indonesian, which were largely insertions and substitutions. Of the 606 total bases aligned, there were 44 bases having substitution consisting of 21 transition and 23 transversion bases.

Each of these mutations had an influence on changes in amino acids formed. Of the total 194 amino acids belonging to isolates from Indonesia, some of which had very different percentage from those of other isolates. Some amino acids of isolates from Indonesia had increased their number dramatically compared to those of other isolates, i.e. Ala, Glu, Val, Gln, Lys, and Tyr by 0.7%, 1.76%, 1.8%, 2.3%, 3.1%, and 4.73%, respectively, of total average. Several other amino acids had also decreased in number, i.e. Asp, Arg, Trp, Thr, and Leu by 0.11%, 1.53%, 2.07%, 2.25%, and 4.32%, respectively, of total average.

It appeared that the resulted amino acid changes had enormous influence in the process of adaptation to the environment in Indonesia. Results of the relationship analysis between isolates through the reconstruction of phylogenetic tree showed that isolates from Indonesia were on separated branches and far apart from 14 other isolates (Figure 4).

DISCUSSION

Maximum Parsimony method with *Bootstrap-1000* approach classify the data based on specific characters (discrete character states) on which nucleotide sequences are used to obtain information about evolution through evolutionary data changes. The analysis result showed that Indonesia isolates were on the basal clade. Indonesia isolate showed high occurrence of point mutations, which were largely insertions and substitutions. It appeared that the resulted amino acid changes had enormous influence in the process of adaptation to the environment in Indonesia. Indonesia isolate was on separated branches and far apart from 14 other isolates. A lengthy branch, which well separating between the two groups, indicated that isolates from Indonesia had considerably evolved and it led to speciation events. Such changes in sequence through mutation was supported by the ability of the mycorrhizal itself to adapt to environmental changes as well as to its host range. The adaptability of mycorrhiza is strongly influenced by the presence of amino acid changes due to mutations that alter the function of genes which they arrange.

The reconstruction of phylogenetic tree, in addition to explaining relationship based on geographic regions, also determine the origin and history of distribution of the isolates. Trading activities of plants, fruits, and seedlings from several countries which are reported to have been infected by *Ceratobasidium* sp. is most likely the cause of its spreading. Indonesia is known as an importer country of orchid plant seedlings from Asian countries such as Thailand, Singapore and Taiwan (Mahfut *et al*., 2016[a]), and possibly have imported seedling and fruit of apple and strawberry plants from other countries such as India and Italy that had been reported infected by *Ceratobasidium* sp. isolates. Proximity with Singapore in terms of geographical location is also very possible to allow distribution activities of orchids between the two countries.

This study results proved that nucleotide sequences and amino acid of *Ceratobasidium* sp. isolates from Indonesia were different with other isolates from another country (Asia, Europe, and America). This is thought to be a form of adaptation to the natural environment in Indonesia. Another possible result of mutations is that *Ceratobasidium* sp. could have the ability to infect plants while showing pathogenic characters or otherwise become mycorrhizal endophytic.

This study results clarified the MP sequence as OMF *Ceratobasidium* sp. isolates from Indonesia based on identification of its rDNA-ITS sequences. Furthermore, this study was expected to be the basic information beneficial for the improvement of cultivation effort as well as for the development of biocontrol agents through natural orchid protection against pathogens and diseases.

CONCLUSION

Molecular analysis based on rDNA-ITS resulted in isolate from the root of orchid plant in Yogyakarta being *Ceratobasidium*. These isolates showed 600-750 bp in length DNA products located on the ITS1-5.8S-ITS4 region. The sequence products showed insertion and substitution occurances, which may result in strain diversity and possible variation in severity. Reconstruction of phylogenetic tree using *Maximum Parsimony* and *Bootstrap*-1000 approach showed that Indonesian isolates have undergone speciation and have been positioned in the cluster, which are already far apart from the other isolates.

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