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Short Communication: *Rhizoctonia*-like fungi isolated from roots of *Dendrobium lancifolium* var. *papuanum* and *Calanthe triplicata* in Papua, Indonesia

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Abstract. *Agustini V, Sufaati S, Suharno, Suwannasai N. 2016.* Rhizoctonia-*like fungi isolated from roots of* Dendrobium lancifolium *var*. papuanum *and* Calanthe triplicata *in Papua, Indonesia*. *Biodiversitas 17: 377-383.* The aim of this study was to isolate and identify *Rhizoctonia*-like fungi associated with the roots of the terrestrial orchids *Dendrobium lancifolium* A. Rich var. *papuanum* and *Calanthe triplicata* (Willem) Ames in Papua. The fungi were isolated from the transversal section of the orchid roots. Two isolates have been morphologically identified as genus *Rhizoctonia*. Further identification was carried out based on the analysis of nucleotide sequences generated from ITS and 28S rDNA. The results revealed that both isolates closed to *Ceratobasidium* sp. and the phylogenetic analysis confirmed that they were determined as *Rhizoctonia*-like fungi.

Key words: *Calanthe triplicata*, *Dendrobium lancifolium*, ITS, 28S rDNA, Papua, *Rhizoctonia*-like fungi

INTRODUCTION

Tropical orchids constitute the greater part of orchid diversity, than can be found in anywhere in the world (Atala et al. 2015). Orchid is one of the world's largest plant families and contains over 25000 species (Berga-Pana 2005; Tao et al. 2013). *Dendrobium* is orchid that commonly found in the eastern part of Indonesia such as Papua and Maluku (Agustini et al. 2013). Two biggest genera in Papua are *Bulbophyllum* (569 species) and *Dendrobium* (512 species) (Millar 1978). *Dendrobium lancifolium* A. Rich var. *papuanum* as well as *Calanthe triplicata* (Willem) Ames (Agustini et al. 2013) spread widely in Papua.

Mycorrhizal fungi have a unique role in the life cycle of orchids (Pandey et al. 2013; Perotto et al. 2014). In nature, orchid associated with mycorrhizal fungi has become very essential in seed germination because of their lack of endosperm and seedling growth that require nutrients from the outside (Ding et al. 2014; Perotto et al. 2014). Orchid mycorrhiza has a significant effect on the growth of plantlets life, vegetative and reproductive growth (Cheng et al. 2012; Perotto et al. 2014; Wang and Liu 2013).

Fungi associated with photosynthetic orchids are generally included in the subdivision Basidiomycota class Hymenomycetes, genus *Rhizoctonia*. Nine species of orchids that grow in Puerto Rico found it has association with as many as 108 *Rhizoctonia*-like fungi that includes *Tulasnella*, *Ceratobasidium* and *Thanatephorus* (Otero et al. 2002; Ding et al. 2014). As Taylor and Bruns (1997) and Taylor et al. (2004) showed, 17–22 species of fungi associated with orchid are family Russulaceae. Studies on *Limodorum abortivum*, common orchid growing in Mediterranean regions, showed that it associated with fungi of the family Russulaceae (Gurlanda et al. 2006).

Identification of the orchid mycorrhizal fungi is very crucial in studying that association. Morphological identification of mycorrhizal pure isolates using the characteristics like the color, form and pattern of colonies, can only reach the genus level, whereas to the species level is more appropriate with the aid of molecular techniques. This molecular approach is simple because it only requires a DNA sequence that is not too long. The short sequence likes ITS (internal transcribed spacer) between the small subunit (SSU) and large subunit (LSU) rDNA can be used for identification to the species level (Kristiansen et al. 2001; Diaz et al. 2012).

Agustini et al. (2009) reported that several species of orchids in Papua, including the orchid *Calanthe* sp., have symbiosis with mycorrhizal fungi. However, some of the mycorrhizal fungi are still unidentified yet. In order to know fungal identity, this study was conducted to identify *Rhizoctonia*-like fungi associated with the root of terrestrial orchid *Dendrobium lancifolium* var. *papuanum* and *C. triplicata* in Papua using classical morphological identification together with molecular approach.

MATERIALS AND METHODS

Collection site

Root samples of *D. lancifolium* were collected from Biak Island while *C. triplicata* from Keerom, Papua, Indonesia (Figure 1) in April, 2015.

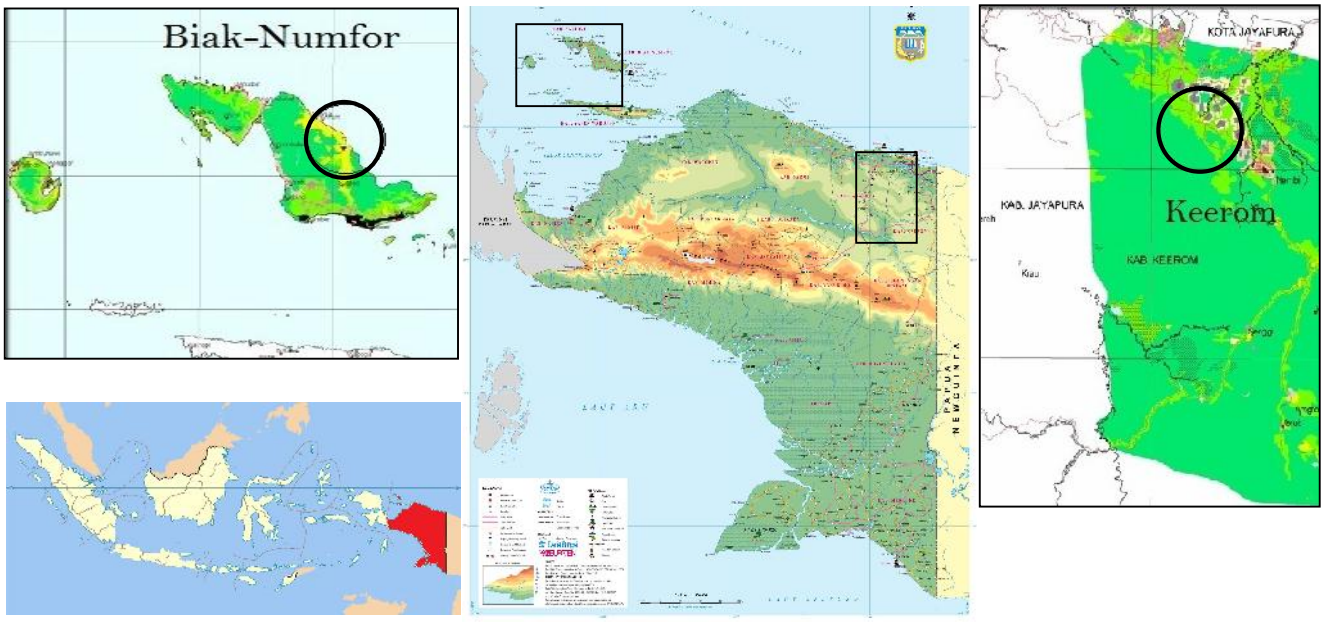

Figure 1. Locations of root collections of *D. lancifolium* var. *papuanum* from Biak (01º09'94.0"S, 136º10'95.5"E) and *C. triplicata* from Keerom (2º50'03.60"S, 140º43'54.94"E)

Fungal isolation

Isolation of fungi was conducted following Chutima et al. (2011) with modification. Orchid plant roots were cut at the root tip (about 2–5 cm from the root tip) and then sliced through the cross section. Roots were washed and cut ± 1 cm and sterilized with 70 % ethanol for 30 seconds, then with a solution of 95% alcohol : 5.25 % chlorox : distilled water (1:1:1 v/v/v) for 30 seconds, and rinsed with sterile distilled water three times. The sterilized roots were cut in thin slices ± 200-300 µm or 3-4 pieces in 1 mm. The presence of peloton (coil hyphae) in the root cortical cell was observed under the compound microscope at a magnification of 400 to 1000 x. The root pieces with peloton (hyphae coil) were grown on PDA and then incubated at a temperature of 28 ^{o}C on dark condition.

Morphological identification

Morphological identification was based mainly on the colony, the branching hyphae, conidia and other structures formed by fungi. Special characteristics formed by orchid mycorrhiza are the monilioid cell (Athipunyakom et al. 2004).

Molecular identification-DNA Sequence analysis

Genomic DNA was extracted from fresh mycelium by using DNA extraction kit (Favorgen). The ITS regions and partial ribosomal RNA gene of 28S were amplified by using ITS1/ITS4 (White et al. 1990) and LR0R/LR5 (Vilgalys and Hester 1990) primers respectively. The amplification was carried out in a BioRad thermocycler (USA) in a 50 µL reaction mixture containing 100 nM of template DNA, 0.5 mM of each primer, 10 mM of dNTP, 1.25 Unit of Top *Taq* DNA polymerase (Qiagen) and 1 x PCR buffer. The PCR cycles were run following the protocol: 94°C for 5 min initial denaturation; 35 cycles of 94°C for 1 min, 52°C (for ITS) or 55°C (for 28S rDNA) for 1 min, 72°C for 1 min; and 72°C for 10 min final extension (Suwannasai et al. 2013). The PCR products were purified following the QIAquick PCR Purification Kit protocol (Qiagen) and then sequenced at the 1st BASE laboratories Sdn Bhd (Malaysia). The sequences obtained were manually checked by using BioEdit program (Hall 1999) before analyzed by using BLASTN program to GenBank database (www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analysis

DNA sequences of orchid mycorrhizal fungi were aligned to closely related sequences obtained from GenBank database by using MUSCLE program (Edgar 2004). The phylogenetic tree was analyzed based on maximum likelihood and Bayesian analysis using Mr. Bayes version 3.2.6 (Ronquist et al. 2012) with two independent runs of Markov Chain Monte Carlo chains with 1,000,000 generations sampling trees every 100th generations. A final standard deviation of <0.01 for the split frequency was interpreted to reflect convergence.

RESULTS AND DISCUSSION

Results

Two *Rhizoctonia*-like isolates, SIID3B2 and Cal8, were successfully cultured from roots of two terrestrial orchids, *D. lancifolium* and *C. triplicata* respectively (Table 1). The colonies of both isolates were white at first and became to pale yellow with flat or leathery in appearance. Both cultures were absent sporulation. The rapidly growing isolates reach a diameter of 9 cm within 7 to 10 days with concentric zone, hyaline mycelia has septate. Under 1000x magnification of the compound microscope show the presence of monilioid cells (Figure 2).

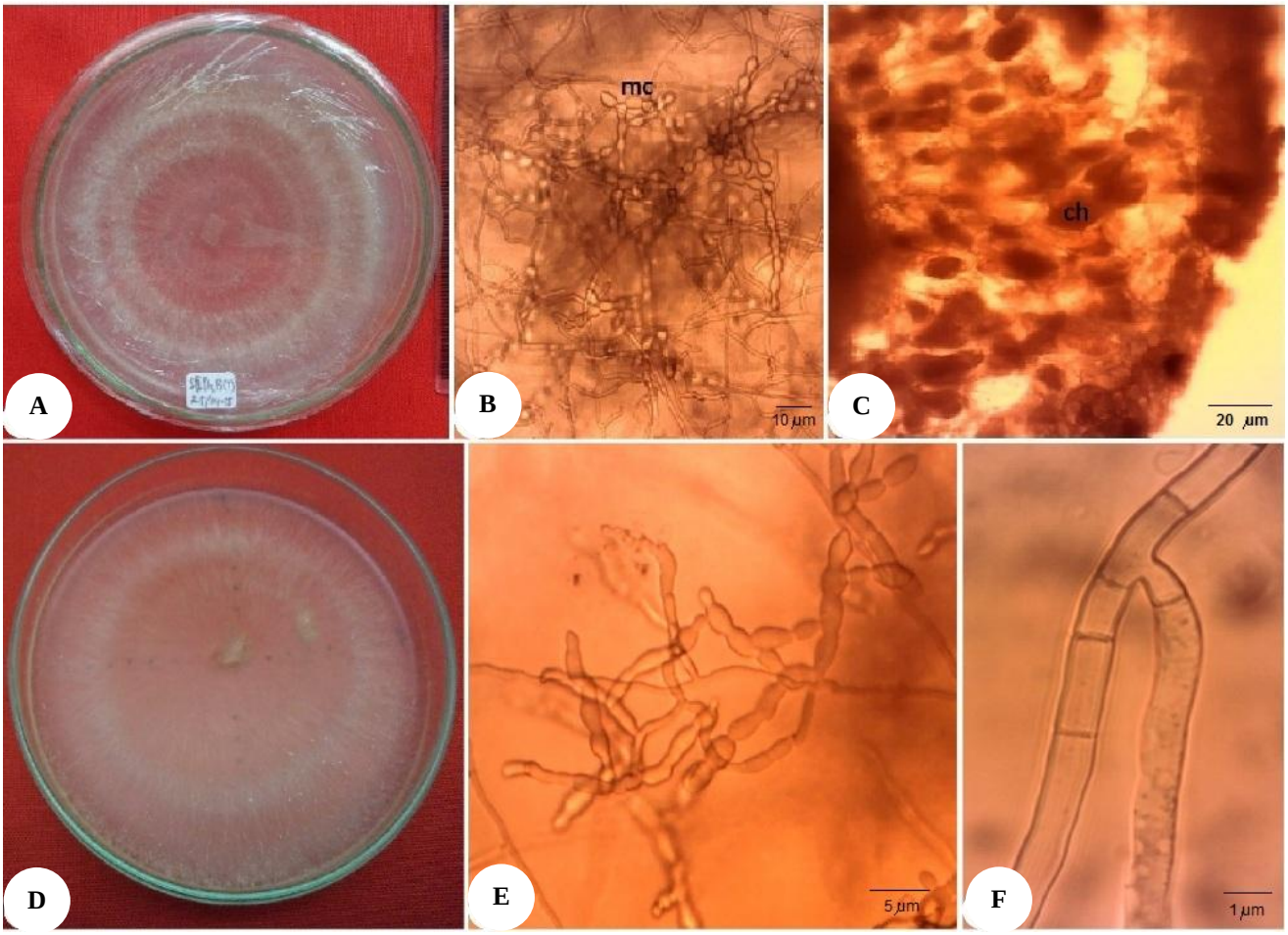

Figure 2. Isolate of *Rhizoctonia*-like fungi. a-c for the isolate SIID3B2. *D. lancifolium* on PDA, b. monilioid cells, c. hyphae-coil in the transversal root section of *D. lancifolium*. d–f for the isolate Cal 8 from *C. triplicata*: d. colony on PDA, e. monilioid cell and f. hyphae with sepatae; *mc*: monilioid cell, *h*: hyphae, *sh*: septate hyphae, *ch*: coil hyphae/peloton

Table 1. Orchid mycorrhiza associate with *Dendrobium lancifolium* and *Calanthe triplicata* from Papua, Indonesia

Orchid source	Location	Time collection	Isolate code	Cultural characteristics	Microscopic characteristics
Dendrobium lancifolium	Biak Island	April 2015	SIID3B2	Diameter of fungal colony reached 9 cm in day 8, concentric growth, white, aerial hyphae	Septate hyphae, globouse monilioid cells appear in day 14
Calanthe triplicata	Keerom	April 2015	Cal 8	Diameter of fungal colony is 9 cm in d 9, concentric growth, white, hyphae	Septate hyphae, irregular monilioid cells appear in day 7

Table 2. ITS and 28S rDNA sequences analysis of orchid mycorrhizal fungal isolates aerial SIID3B2 and Cal8 using BLAST program

Isolate code	BLAST results of ITS			BLAST results of 28S rDNA		
	Taxon	Accession no.	% similarity	Taxon	Accession no.	% similarity
SIID3B2	*Ceratobasidium* sp.	JX913817	90	*Ceratobasidium* sp.	AF354094	99
Cal8	*Ceratobasidium* sp.	JX913817	91	*Ceratobasidium* sp.	AF354094	99

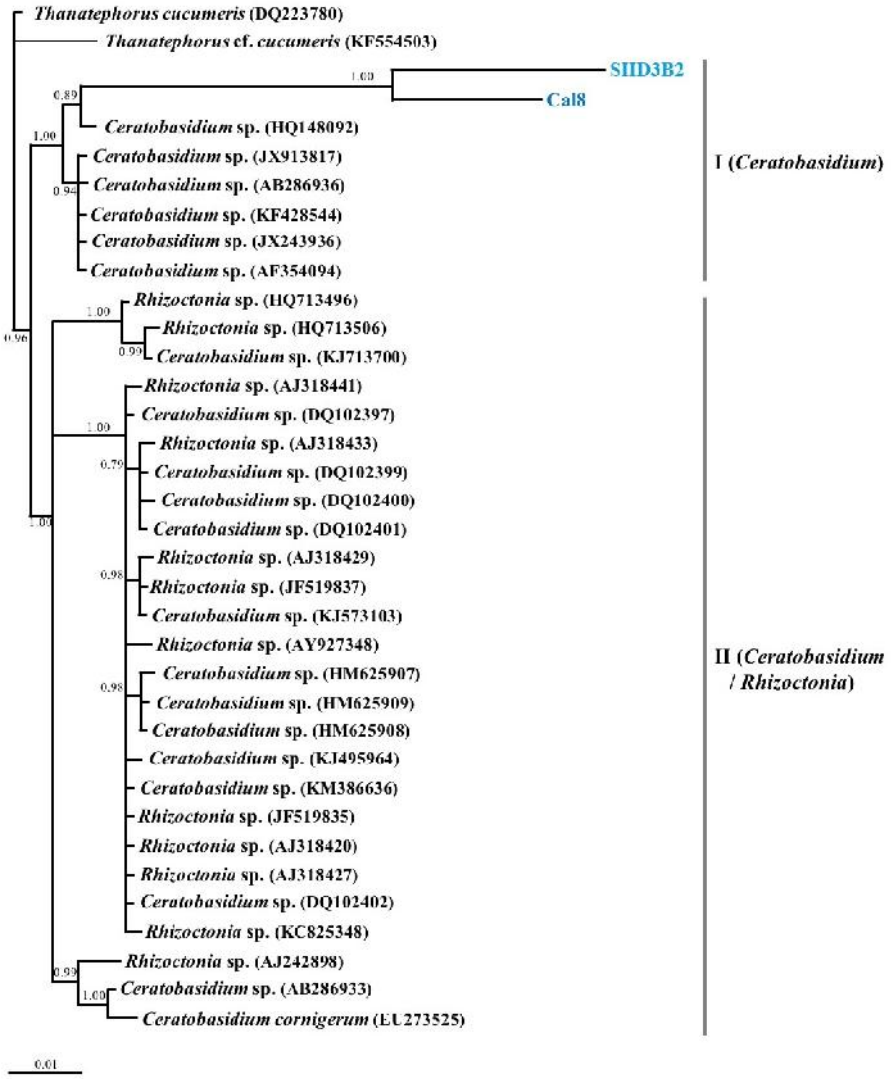

Figure 3. Phylogenetic tree of orchid mycorrhizal fungal isolates SIID3B2 and Cal8 and related species based on ITS rDNA sequences using Bayesian program. Number above branches represent their Bayesian posterior probabilities (500,000 generations). *Thanatephorus cucumeris* (DQ223780) and *T.* cf. *cucumeris* (KF554503) are out groups

ITS rDNA sequences of *Rhizoctonia*-like fungi, SIID3B2 and Cal8, were amplified and analyzed with BLAST against GenBank database. ITS amplicon sizes were 604 and 608 bp respectively. BLAST results of SIID3B2 and Cal8 revealed low similarity against nucleotide sequences from GenBank database (Table 2). They closed to *Ceratobasidium* sp. (JX913817) with 90% similarity for SIID3B2 and 91% similarity for Cal8. Due to the low similarity of ITS sequences, 28S rDNA sequences were then analyzed to confirm the genus and species identification. The amplified fragments of SIID3B2 and Cal8 resulted in 869 bp. BLAST identification of both isolates revealed 99% similarity to *Ceratobasidium* sp. (AF354094) (Table 2). The phylogenetic trees of both ITS and 28S rDNA sequences were then constructed with closely related species obtained from GenBank database (Figure 3, 4). ITS phylogenetic analysis was divided into two major clades. Clade I consisted of our orchid mycorrhizal isolates, SIIB3D2 and Cal8. They were grouped together with *Ceratobasidium* species with high bootstrap support. Although SIIB3D2 and Cal8 were placed in the same cluster, the pairwise comparison among them showed 93% similarity. This indicated that both isolates were different in species level. Clade II was a large group consisted of *Ceratobasidium* and *Rhizoctonia* species. The results obtained exhibited that SIIB3D2 and Cal8 isolates belong to *Ceratobasidium* sp. having *Rhizoctonia*-like anamorph. For 28S rDNA phylogenetic analysis, it contained three major clades, clade I (*Gloeophyllum* and *Griseoporia*), clade II (*Ceratobasidium* and *Thanatephorus*) and clade III (*Ceriporia*). The orchid mycorrhizal isolates of SIIB3D2 and Cal8 were placed in clade IIb which contained *Ceratobasidium* species. The results of both ITS and 28S rDNA analysis confirmed that SIIB3D2 and Cal8 belong to the same genus *Ceratobasidium* but they are different in species.

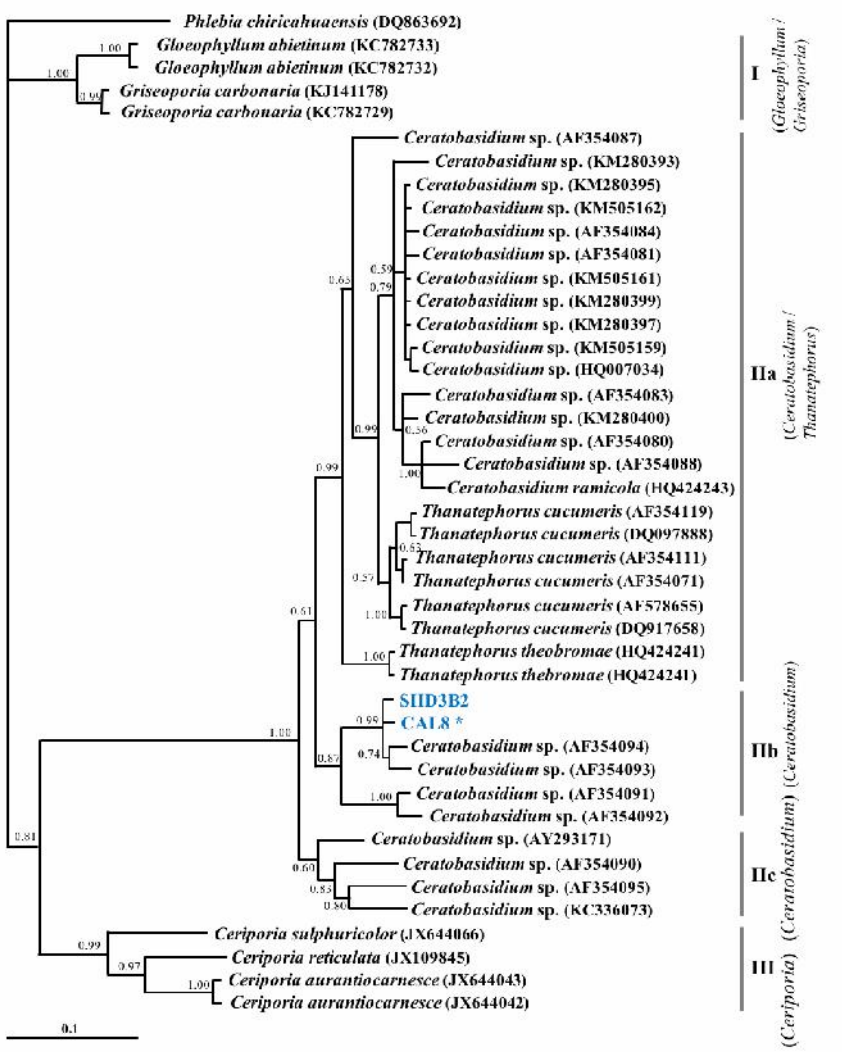

Figure 4. Phylogenetic tree of orchid mycorrhizal fungal isolates SIID3B2 and Cal8 and related species based on 28S rDNA sequences using Bayesian program. Number above branches represents their Bayesian posterior probabilities (500,000 generations). *Phlebia chiricahuaensis* (DQ863692) is an outgroup

Discussion

For morphological identification, mycelial color, number of nuclei per young vegetative hyphal cells, and the morphology of teleomorph can be used to differentiate the species of *Rhizoctonia*. The morphological character of the fungal isolates, such as the presence of monilioid cells (Figure 2), showed that they belong to *Rhizoctonia*-like fungi (Athipunyakom et al. 2004). The teleomorph of *Rhizoctonia* spp. belongs to class Hymenomycetes, subdivision Basidiomycota (Yang and Li 2012). On the other hand, the anamorph of *Rhizoctonia* are heterogeneous, for example, anamorph of *Ceratobasidium* was placed in new genus *Ceratorhiza* (Moore 1987), but many publications still used the name of *Rhizoctonia* (Yang and Li 2012).

The molecular technique using DNA sequences was used to confirm the *Rhizoctonia* that was isolated. Firstly, we used ITS rDNA sequences to analyze and resulted closely related to *Ceratobasidium* sp. (JX913817) with 90-91% similarity, which belong to Basidiomycota. The 28S rDNA sequences were then analyzed to confirm the identification of these orchid mycorrhizal fungi because of the low similarity of ITS sequences. BLAST results of 28S rDNA analysis were 99% similarity to *Ceratobasidium* sp. (AF354094) (Table 1). The phylogenetic trees of both ITS and 28S rDNA sequences analyzed in corporate representative sequences of various *Ceratobasidium* species supported fungal identification inferred from BLAST at the generic level. Although 28S rDNA sequences of both isolates were similar, their pairwise analyses were 93% similarity. Therefore, the orchid mycorrhizal isolates SIID3B2 and Cal8 belong to the same genus *Ceratobasidium* sp., but they are different in species. *Ceratobasidium* is a major genus of orchid mycorrhizal fungi which was originally incorporated in to the anamorphic form genus *Rhizoctonia* (Roberts 1999). Even though both isolates come from geographically different

area, they are similar in genus level. Cal 8 was isolated from *C. triplicate* at the forest in Keerom, New Guinea mainland, while SIID3B2 was isolated from *D. lancifolium* in Biak, the small island in Pacific Ocean. Pereira et al. (2005) reported that some orchid from rain forest in Atlantic have association with *Rhizoctonia*-like fungi including genus *Ceratorhiza* and *Epulorhiza*. Zettler and Piskin (2011) found *Ceratorhiza* from protocorm, seedling and adult *Platanthera leucophaea*.

One interesting thing that is *D. lancifolium* was able to grow in hot (31^{o}C) and dry habitat (50%) along the road side. Presumably, this mycorrhizal association contributes to the survival of this orchid in this area. Mycorrhizal fungi may also be a key source of water for orchids. Water content in both the terrestrial orchid *Platanthera integrilabia* (Correll) Luer and the epiphytic *Epidendrum conopseum* R.Br. was higher for mycorrhizal seedlings than uncolonized controls (Yoder et al. 2000). Alexander et al. (1984) found that mycorrhizal *Goodyera repens* acquired 100 times more P than non-mycorrhizal controls. P and N (as glycine) transfer from fungus to plant was confirmed in radio-labeling experiments (Dearnaley 2007).

This study showed that molecular approach help in determining the orchid mycorrhizal fungi isolated from *D. lancifolium* and *C. tripicata* in Papua. For the future work, we still have to elucidate about the role of *Rhizoctonia* spp. in the growth-promoting of those terrestrial orchid, especially *D. lancifolium* for in situ conservation purpose.

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