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Short Communication: *Fusarium* as endophyte of some terrestrial orchid from Papua, Indonesia

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Abstract. *Sufaati S, Agustini V, Suharno. 2016. Fusarium as endophyte of some terrestrial orchid from Papua, Indonesia. Biodiversitas 17: xxx.* The aim of the study was to identify endophytic fungi associated with the roots of terrestrial orchid *Phaius tankervilleae* (Banks) Blume, *Dendrobium lancifolium* A. Rich var. *papuanum* and *Calanthe triplicata* (Willem) Ames from Papua, Indonesia. The endophytic fungi were isolated from the transversal section of the orchid roots. Identification of the endophytes was carried out based on the morphological characters. The phylogenetic analysis of nucleotide sequences generated from ITS rDNA region of the endophytic fungi isolated from *P. tankervilleae* showed that those isolates were determined as *Fusarium solani*. This is the first report of *F. solani* found as endophyte of *P. tankervilleae* in Papua. While the ITS rDNA of *Fusarium* isolated from *C. triplicata* need to be sequenced for further identification.

Keywords: Endophyte, *Fusarium*, ITS rDNA, terrestrial orchid, Papua

INTRODUCTION

Endophytic fungi are one of fungal group that grow in the intracellular tissue healthy plants (Cabezas et al. 2012). Fungal endophyte is defined as all or part of their life cycle colonizing inter- and/or intra-cellular of healthy tissue of the host plant, usually does not cause symptoms (Zhao et al. 2010). The linkage between endophytic fungi and plant host system enables systematic work involving a variety of metabolic processes in plant cells. In addition, endophytic fungi also source of various antibacterial metabolites (Sugijanto et al. 2009; Sinaga et al. 2009), various types of enzymes (Kumala et al. 2007), anti-tumor/cancer (Tabudrayu and Jaspars 2005; Kumala et al. 2008; Tejesvi and Pirttila 2011), and other bioactive compounds for various purposes (Zhao et al. 2010), including biological control (Zimmerman and Vitousek 2012). It also increases resistance of plants to herbivores, pathogens and various abiotic stress (Diaz et al. 2012).

Environmental conditions affect the existence of endophytic fungi, in association with various types of host plant as phycobiont symbiotic (Zimmerman and Vitousek 2012; Aschehoug et al. 2012). Endophytic fungi association is not only symbiotic mutualism, but also parasitism depending on the condition of their host. Several fungi have shown this type of association, such as *Fusarium* spp. and *Colletotrichum* spp. (Redman et al. 2001). Several endophytic fungi are also capable to shift their lifestyle to saprobes once their host decayed, such as *Xylaria* spp. and *Diaporthe* spp. The remaining endophytic fungi are true endophytes, including many *mycelia sterilia* fungi (Redman et al. 2001; Aschehoug et al. 2012). Therefore, determination of the endophytic fungal identity is very important in studying their diversity and ecology.

However, several problematic in endophytic fungal studies were reported; include limitation in isolation method and accurate identification method to species level. The identification based on morphological characters can perform only to the level of genus of sporulating fungi. The identification to species level is still needed technical or other aids; given that the fungi have a high morphological similarity among them. Thus, the molecular identification is expected to provide more accurate information about the species name of endophytic fungi (Faeth and Fagan 2002). The nucleotide sequences providing virtually unlimited character for phylogenetic analysis (Diaz et al. 2010). An area that can be used to detect sequences of fungi is Internal Transcribed Spacer region (ITS) (Gomes et al. 2002). This area has a high variability nucleotide sequences for studies molecular systematic of fungi at the species level. Therefore, ITS sequences can also be used for studying and determining endophytic fungi because it shows high sequence heterogeneity.

Endophytic fungi also found in some Orchidaceae in several area of the world (Tupac and Otero 2006, Gezgin and Eltem 2009), both epiphytic (Ovando et al. 2005; Yuan et al. 2009) and terrestrial orchid (Chutima 2010). In Indonesia, however, little is known about the endophytic fungi associated with orchid. Suciatmih (2008) was able to isolate and identify the endophytic fungus from *Dendrobium crumenatum* Sw.

Papua is home of about 512 species of *Dendrobium* (Millar 1978). The terrestrial orchid *Phaius tankervilleae*, *Dendrobium lancifolium* A. Rich var. *Papuanum* and *Calanthe triplicata* (Willem) Ames are also common found in Papua (Agustini et al. 2013; Agustini and Sufaati 2014).

In addition, forming mycorrhizal symbiosis with fungi such as *Rhizoctonia*, they are also associated with a group of other fungi, such as endophyte. So far, there is few data on the presence of the endophytic fungi in the Papuan orchid (Agustini et al. 2009). Therefore, this study was done to identify endophytic fungi associated with the roots of terrestrial orchid *P. tankervilleae, D. lancifolium* var. *Papuanum* and *C. triplicata* from Papua, Indonesia.

MATERIALS AND METHODS

Collection site

Roots of *P. tankervilleae* were collected at Papua Province of Indonesia, namely: Ifar Gunung, Sentani, Jayapura District (August 2014), *D. lancifolium* var. *Papuanum* from Biak District (April 2015) and *C. triplicata* from Keerom District (April 2015) (Figure 1).

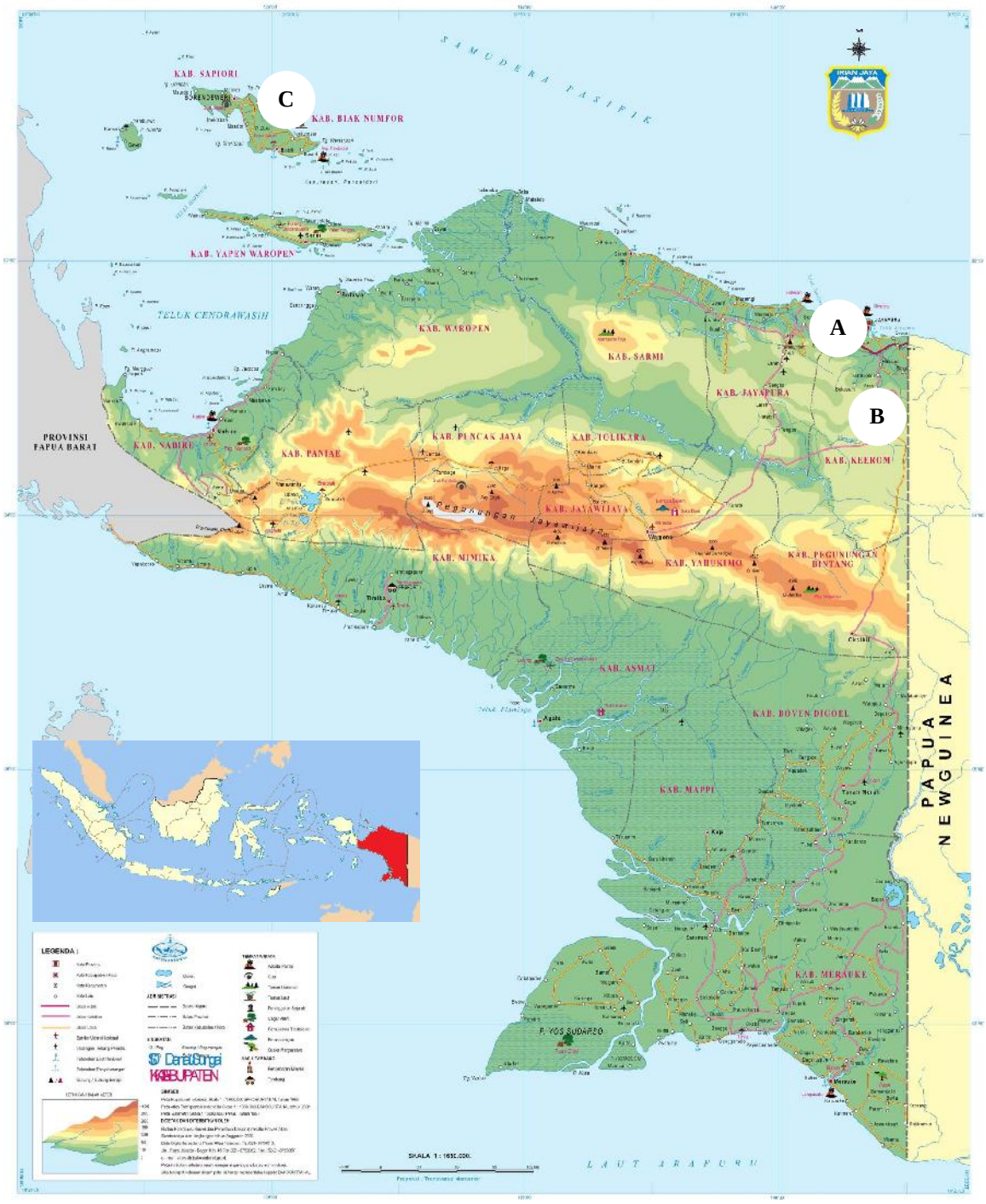

Figure 1. Location of root collection of *P. tankervilleae*: A. Ifar Gunung, Sentani, Jayapura, Papua Indonesia (2º55'69.78"S, 140º54'02.87"E), B. *Calanthe triplicata* (Willem) Ames from Keerom (2º50'03.60"S, 140º43'54.94"E) and C. *Dendrobium lancifolium* A. Rich var. *papuanum* from Biak (01º09'94.0"S, 136º10'95.5"E)

B

Isolation of endophytic fungi from plant roots

Isolation of fungi was done using a technique developed by Manoch and Lohsomboon (1991) with modification. Roots was washed to remove soil debris, and then cut ± 1 cm, followed by surface sterilization with 10% Clorox for 30 seconds, 70% alcohol for 1 min, and rinsed with distilled water three times. Sterilized root pieces were thinly sliced (200-300 µm) using a razor blade in a laminar air flow (LAF). About 1-3 pieces of the sliced roots were placed on the Fungal Isolating Medium (FIM) and incubated at 28°C in dark condition. Mycelia emerged after 1-2 days. Mycelia tips grow from the roots tissue were cut and transferred to another Potato Dextrose Agar (PDA) medium for purification. The morphological characteristics include color, diameter colonies, form (shape) and pattern of colony growth, and microscopic features such as hypha, spores or conidia were observed.

DNA isolation

Fungal mycelia from liquid medium (PDB) was vacuum filtered using a 0.22 µm sterile filter. Mycelia (wet weight ± 20 mg) was then transferred to the mortar and crushed using liquid nitrogen. Extraction process was conducted using CTAB method (Roger and Bendich 1994).

DNA amplification

DNA amplification of ITS rDNA region was done by Polymerase Chain Reaction (PCR) using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990; Diaz et al. 2012). PCR reaction was performed as follow: initial denaturation at a temperature of 95°C for 120 seconds, 35 cycles of denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds, extension at 72°C for 60 seconds, a final elongation of 72°C for 600 seconds. The quality of the PCR products was checked by agarose gel electrophoresis (1% agarose).

DNA sequencing

The amplicons were sent to 1st BASE, Malaysia for sequencing process. Sequences were compared with the homologous nucleotides sequence in GenBank database (NCBI) (www.ncbi.nlm.nih.gov/guide/sequence-analysis/). The most homologous sequences were retrieved from the GenBank for phylogenetic analysis.

Phylogenetic analysis

Multiple alignments were done using CLUSTALW in MEGA 6. Phylogenetic analysis was conducted using Neighbor Joining (NJ) method PAUP 4.0 (Swofford 1999). Clade robustness was assessed using bootstrap analysis using 1000 replications. The phylogenetic tree was refined using Tree Graph 2 software.

RESULTS AND DISCUSSION

Result

Endophytic fungi were successfully isolated from the root of terrestrial orchid *P. tankervilleae*, *D. lancifolium*, and *C. triplicata*. The orchid habitat of *P. tankervilleae* and *C. triplicata* is generally found in a protected area, is quite moist and not far from water source, while *D. lancifolium* was found in dry area in the road side (Figure 2).

Three isolates (IPAR2B, IPAR2C, and IPAR3) fungi isolated from the roots of *P. tankervilleae* show slightly different characteristics in morphology of colony, but they have a similar pattern of growth. The isolates grow rapidly, hyphae reach a diameter of 9 cm within 7 to 10 days. One isolate from *D. lancifolium* has hyaline mycelia that grew covering the edge of 9 cm diameter petridish in 8 days. The hyphae show distinctive septate under 1000x magnification. Fungi that isolated from *C. triplicata* (CalFus 1 and CalFus 4) also grow rapidly. The colony reach 9 cm diameter in day 10. The thin hyaline hyphae

Figure 2. Habitat and the morphology of *P. tankervilleae* (A-C), *D. lancifolium* (D-E), and *C. triplicata* (F).

Table 1. *Fusarium* isolates of *P. tankervilleae* and *D. lancifolium*, and *C. triplicata* from Papua

Orchid species	Location	Time collection	Isolate code	GenBank accession code	Closest species matches	Accession code	Sequence identity/ similarity (%)
Phaius tankervilleae	Jayapura	May 2014	IPAR2B*	KU842423	*Fusarium solani*	NRRL 28579T	100
			IPAR2C*	KU842422		MAFF 238538	
			IPAR3*	KU842424		CBS 132898	
Dendrobium lancifolium	Biak Island	April 2015	SIIID2C (1)**	KU842428	*Fusarium solani*	JF436948.1	99
Calanthe triplicata	Keerom	April 2015	CalFus 1***		na	na	na
			CalFus 4***		na	na	na

Note: * : DNA were sequence to construct the phylogenetic tree ; ** : DNA were sequence and submitted to a similarity search using BLASTn software ; ***: DNA was not isolated yet

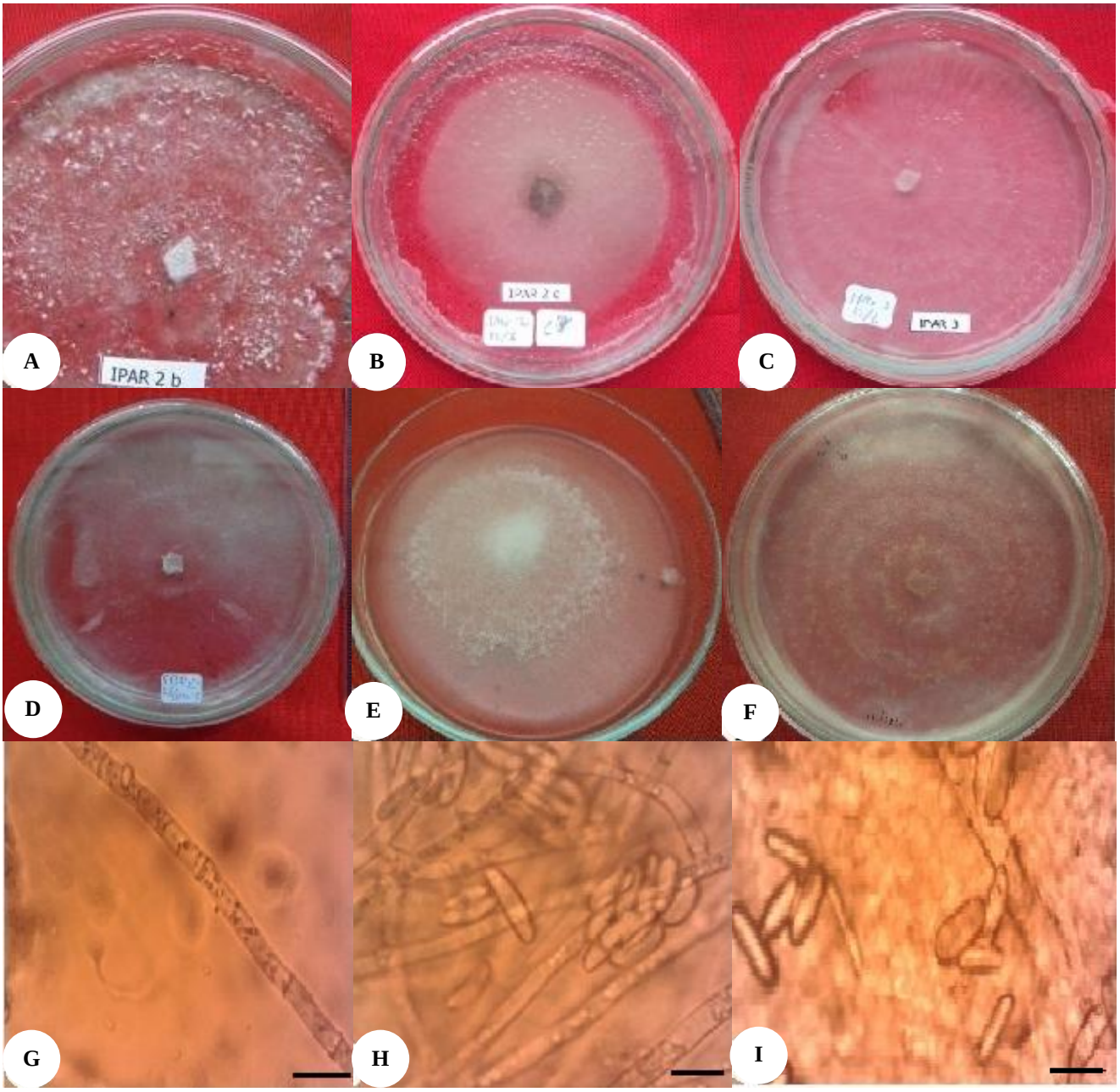

Figure 3. Morphology of *Fusarium* isolated from *P. tankervilleae* (A, B and C), *D. lancifolium* (D and G), and *C. triplicata* (E, F, H, I) (scale bar = 20 µm)

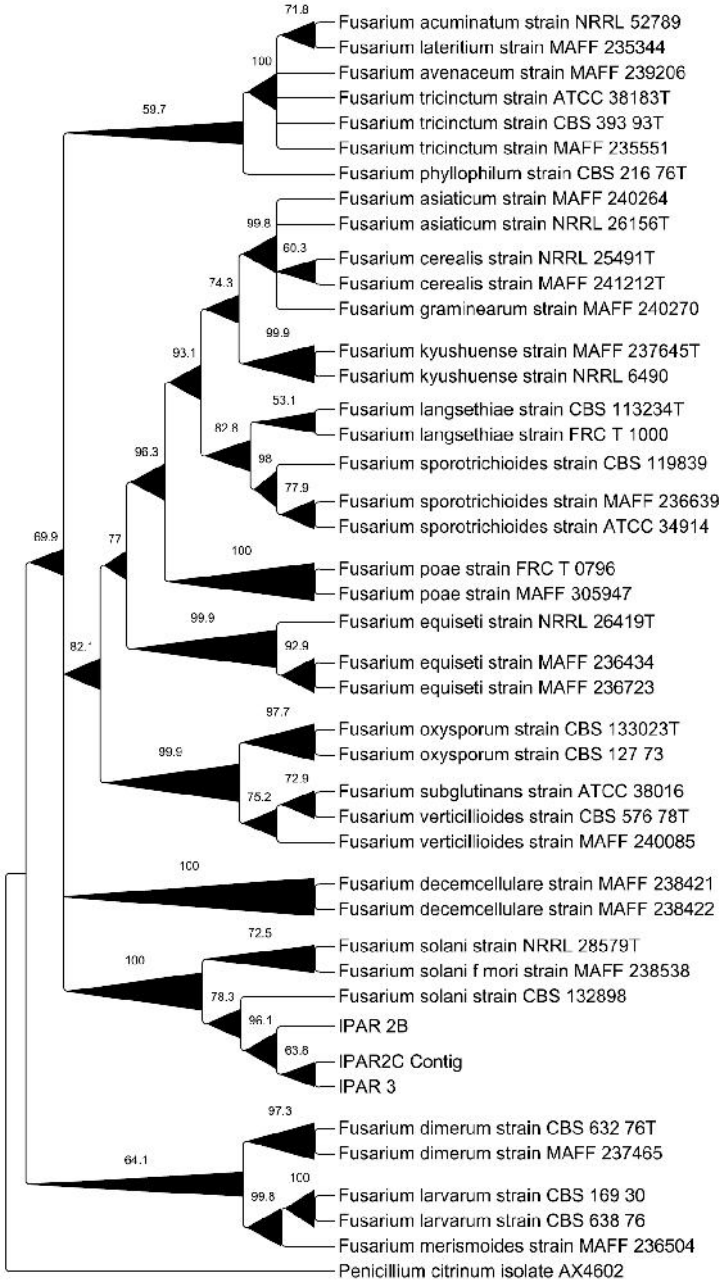

Figure 4. Phylogenetic tree of fungal endophyte of *P. tankervilleae* generated from NJ analysis with 1000 bootstrap replication

show concentric zone. Under the low power of the microscope, the curve shape conidia were able to observe (Figure 3).

Discussion

Identification based on colony and microscopic structure characteristics showed that the endophytic fungi isolated from the root of terrestrial orchid *P. tankervilleae*, *D. lancifolium*, and *C. triplicata* belong to *Fusarium* spp. These fungi is commonly found as endophytes in orchids and other hosts (Ma et al. 2015). Spore morphology is the important character of fusaria. Conidia are fusoid or curved with 0-1 septate. However, many isolation of *Fusarium* tend to grow with abundant mycelium without forming spores (Booth 1971). It is why the morphological identification make difficult to be done. Therefore, to determine the species of *Fusarium*, then we did molecular identification using the DNA sequence, especially for the isolate from *P. tankervilleae* and *D. lancifolium* that we could not find the conidia in the culture.

Based on the BLAST identification of the ITS nrDNA sequence, the *Fusarium* isolates from *P. tankervilleae* and *D. lancifolium* was confirmed as *Fusarium solani* (GenBank accession no NRRL 28579T, MAFF 238538, CBS 132898 and JF436948.1) with 99-100 % similarity (Table 1). Furthermore, the phylogenetic tree that was constructed using closely related species sequences generated from GenBank and *Penicillium citrinum* isolate

AX4602 as an outgroup showed that the sequences of fungal endophytes IPAR 2C, IPAR 3 and IPAR 2B which were isolated from *P. tankervilleae* are nested in the same clade with *Fusarium solani* strain CBS 132898, *Fusarium solani* f. *mori* strain MAFF 238538 and *F. solani* NRRL 28579T (Figure 3).

F. solani is one type of fungus that associated with many species of orchids, including the *Phalaenopsis* sp (Chung et al. 2011; Su et al. 2012). This fungus caused leaf yellowing on *Phalaenopsis* (Chung et al. 2011). The teleomorphic state of this fungus is *Nectria haematococca*, the causal agent of root rot disease in *Phalaenopsis* spp (Chung et al. 2011; Benyon et al. 1996). Besides *F. solani*, *F. oxysporum* and *F. proliferatum* also cause the same disease in *Dendrobium* (Latiffah et al. 2009).

F. solani may affect of 30–60 % seedling growth in *Phalaenopsis*. Infected seedling shows small leaves and yellow with black rotten spots or dots (Su et al. 2012). This fungus was also found in *Cymbidium* spp., *Oncidium* sp., *Dendrobium* sp. and *Cattleya* sp., but did not shows the symptom as in *Phalaenopsis* sp. (Chung et al. 2011). *Fusarium solani* are often associated with orchids and well-known as a virulent species, under optimal growth conditions, tend to asymptomatic endophyte rather than pathogens (Ma et al. 2015). Endophytic *Fusarium* promoted seed germination in *Cypripedium* and *Platanthera* orchid (Ma et al. 2015).

This preliminary study is the first report on the presence of *Fusarium* as endophyte in terrestrial orchid from Papua. Therefore, further study on re-inoculation test of *F. solani* in *P. tankervilleae*, *D. lancifolium*, and *C. triplicata* is necessary to investigate whether it has effect on both seed germination in axenic condition and seedling growth of that orchid. It is just the beginning steps in conserving the beautiful natural orchid in the New Guinea Island.

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