



Isolation of endophytic fungi from tropical forest in Indonesia

Ahdiar Fikri Maulana^{1,2} · Maman Turjaman³ · Takumi Sato¹ · Yasushi Hashimoto⁴ · Weiguo Cheng¹ · Keitaro Tawaraya¹

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Abstract

Endophytic fungi (EPF) are an important contributor to fungal diversity. It is surmised that EPF colonizing plant roots have high diversity. This study aimed to alleviate the scarcity of information regarding EPF in tropical forests, by isolating and identifying EPF from a tropical forests in Indonesia. Soils were collected from five forests: (1) *Tectona grandis* monoculture; (2) *Swietenia macrophylla* monoculture; (3) *Gmelina* sp., *Artocarpus champeden*, Dipterocarp mixed; (4) Dipterocarp primary; (5) *Macaranga* sp. secondary. Four trees (*Calliandra calothyrsus*, *Paraserianthes falcataria*, *Sesbania grandiflora*, and *Cassia siamea*) and three crops (*Sorghum bicolor*, *Allium fistulosum*, and *Trifolium repens*) were grown in the forest soils to trap EPF. EPF were isolated from roots and isolation rates were calculated. Based on the isolation rates, *P. falcataria* and *S. bicolor* were chosen and grown again in forest soils. EPF were isolated and identified by their rDNA ITS1 region. Twelve and 21 EPF were isolated from 250 roots of *P. falcataria* and 300 roots of *S. bicolor*, respectively. Identified EPF were from genera *Acrocalymma*, *Fusarium*, *Tolypocladium*, *Penicillium*, *Talaromyces*, *Exophiala*, *Dictyosporium*, *Pseudochaetosphaeronea*, *Mariannaea*, *Trichoderma*, and *Mycocleptodiscus*. *Acrocalymma*, *Tolypocladium*, *Penicillium*, *Exophiala*, *Pseudochaetosphaeronea*, *Mariannaea*, and *Mycocleptodiscus* spp. were isolated from only one forest. *Fusarium*, *Talaromyces*, and *Trichoderma* spp. were isolated from more than one forest. The numbers of EPF isolated from *Gmelina* sp., *Artocarpus champeden*, Dipterocarp mixed forest, and *Macaranga* sp. secondary forest were higher than those from other forests, suggesting that different plant species in forests affect the root EPF community.

Keywords Endophytic fungi · *Paraserianthes falcataria* · *Sorghum bicolor* · Tropical forest

1 Introduction

Endophytic fungi (EPF) colonize plant tissue without causing any visible disease symptoms at any particular moment

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✉ Keitaro Tawaraya
tawaraya@tds1.tr.yamagata-u.ac.jp

¹ Faculty of Agriculture, Yamagata University, Tsuruoka 997-8555, Japan

² The United Graduate School of Agricultural Sciences, Iwate University, Morioka 020-8550, Japan

³ Forest Microbiology Research Group, Forest Research and Development Centre, Environment and Forestry Research, Development and Innovation Agency (FORDA), Ministry of Environment and Forestry, Jalan Raya Gunung Batu No. 5, Bogor 16610, Indonesia

⁴ Agro-environmental Science, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

(Schulz and Boyle 2005). Practically, EPF colonize almost any plant tissue, including leaf, stem, and root (Rodriguez et al. 2009). Since their discovery, EPF have been studied in many types of plants, including non-vascular, such as mosses (e.g., Schulz et al. 1993) and algae (e.g., Zuccaro et al. 2008), and vascular, such as shrubs (e.g., Schulz et al. 1993) and trees (e.g., Arnold and Lutzoni 2007). Most of the isolated EPF belong to the Ascomycota or Basidiomycota (Schulz and Boyle 2005).

A conservative estimate of worldwide fungal diversity has been proposed as 1.5 million species, which has been accepted as the working hypothesis and basis for the discovery of more fungal species (Hawksworth 2001). With only around 72,000 described species known so far, more than 1 million species are waiting to be found. EPF have been found in many plant species and are considered an important component of fungal diversity (Rodriguez et al. 2009). Despite the increasing number of studies of EPF in many countries, those based in the tropics are still lacking.

Arnold et al. (2000) isolated 418 EPF morphospecies colonizing the leaves of two understory tree species in a tropical forest in Panama, 59% of which were represented by single isolates. Cannon and Simmons (2002) isolated 64 EPF morphospecies colonizing the leaves of 12 tree species in a tropical forest in Guyana, 29 of which were from single leaf samples. These two studies reflect the high diversity of EPF colonizing tree leaves in tropical forests.

The different environmental factors in forests are expected to influence fungal diversity (Saikkonen 2007). However, most studies of the diversity of EPF colonizing leaves in tropical forests are limited to one forest site (e.g., Arnold et al. 2000, 2001; Cannon and Simmons 2002). Suryanarayanan et al. (2011) compared the EPF communities of 75 dicotyledonous trees belonging to 33 families from three tropical forest types in Southern India, namely, tropical dry thorn forest, dry deciduous forest, and montane evergreen forest. The type of forest appeared to have a larger effect on shaping the EPF community than the taxonomy of the host.

Existing studies of EPF in tropical forests are limited to EPF that colonize the above-ground part of plant, particularly leaf. EPF colonizing roots, further termed as root EPF, of tropical forest trees are rarely studied. Rodriguez et al. (2009) classified root EPF into two groups: class 2 EPF, which colonize the shoot, root, and rhizome, and class 4 EPF, also termed dark septate endophyte (DSE), which colonize the root only. In a review by Jumpponen and Trappe (1998), they noted that DSEs colonized approximately 600 plant species, representing 320 genera and 114 families, highlighting the abundance of DSE. However, DSE is not the only group of root EPF, indicating the possibility of an even higher abundance of root EPF in nature.

Root EPF are considered to play an important role in plant growth, similar to mycorrhizal fungi (Jumpponen and Trappe 1998). Researchers have recorded evidence about the growth promoting ability of several class 4 EPF. *Exophiala pisciphila* improved tolerance of maize (*Zea mays* L.) to cadmium toxicity (Wang et al. 2016). *Leptodontidium orchidicola* and *Phialophora mustea* improved growth of Tasmanian blue gum (*Eucalyptus globulus*) and birch (*Betula pendula*) (Berthelot et al. 2016). *Veronaeopsis simplex* suppressed *Fusarium* disease in Chinese cabbage (*Brassica campestris*) (Khastini et al. 2012). *Heteroconium chaetospora* promoted plant growth by increasing nitrogen uptake (Usuki and Narisawa 2007). *Fusarium* sp. improved plant resistance against fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Maciá-Vicente et al. 2008). There have also been studies that recorded evidence about the growth promoting ability of class 2 EPF. *Penicillium citrinum* improved resistance of banana to a fungal pathogen, *Fusarium oxysporum* (Ting et al. 2012). *Fusarium* sp. improved resistance of eggplant to Verticillium wilt (Narisawa et al. 2002). Meta-analysis of data from temperate and boreal areas showed that root EPF

colonization could have a negative, neutral, or positive effect on plant growth (Mandyam et al. 2013; Mayerhofer et al. 2013; Newsham 2011). Evidence of the positive effects of root EPF, along with the expectation of high EPF abundance in tropical forests, has underscored the necessity to conduct more studies of root EPF in tropical forests. However, studies of root EPF in tropical area remain a rarity (Mandyam and Jumpponen 2005).

The trap culture method is rarely used in the study of EPF, but is common in study of arbuscular mycorrhizal fungi (AMF). EPF and AMF have been found in the same plant community (Lingfei et al. 2005). Thus, utilization of the same plant species in trap culture for isolation of EPF is highly possible. *Sorghum bicolor*, *Trifolium repens*, and *Allium fistulosum* are crop species commonly used to isolate AMF (Del Val et al. 1999), thus can also be used to isolate EPF. Tree species are also a possible candidate to isolate EPF. *Paraserianthes falcataria*, *Calliandra calothyrsus*, *Cassia siamea*, and *Sesbania grandiflora* are common leguminous tree species in the tropics, particularly Indonesia. These tree species are fast-growing and are also candidate species for reforestation efforts (Otsamo et al. 1997, Wulandari et al. 2016). Considering the importance of the future utilization of EPF in reforestation, these tree species can also be used as host plants to isolate EPF. The objectives of present study were to (1) isolate root EPF from five forests soils, and identify them based on their rDNA ITS region, and (2) compare EPF community among different forests.

2 Materials and methods

2.1 Collection of forest soil

Forest soils were collected from five forests in Indonesia, with five replications in September 2012 (Table 1). Soils between 0 and 10 cm depth and within 0–5 cm distance from the root of representative seedlings, or around the main stem of representative tree species, were collected after removing the organic layer. Two kilograms of soil were collected for each replication. The distance between replications in one forest was ≥ 20 m. The latitude coordinates of each forest were recorded.

2.2 Chemical analysis of soil

Soil pH was measured at the soil:solution ratio of 1:2.5 in deionized water and 1 M KCl solution (Table 2). Soil available phosphate was determined following the Truog method (Truog 1930). Briefly, air-dried soil was suspended in 0.001 M sulfuric acid solution, stirred by a mechanical shaker, and filtered immediately using a filter paper (Advantec Toyo No. 6, Japan). Color development solution (Olsen and Sommers 1982) was added to the filtrate, and absorbance

Table 1 Characteristics of forest sites used in this study

Forest No	Forest	Location	Latitude	Remarks
1	Teak (<i>Tectona grandis</i> Linn. f.) monoculture forest	East Java, Java Island	07° 20.891' S, 111° 19.981' E	Planted in 1968 as a seed production area (SPA) of Perum Perhutani, a state-owned company
2	Mahogany (<i>Swietenia macrophylla</i> King) monoculture forest	Special Region of Yogyakarta, Java Island	07° 54.578' S, 110° 31.088' E	Planted in 1969 as a reforestation effort in Karst landscape
3	<i>Gmelina</i> sp., <i>Artocarpus champeden</i> , and Dipterocarp mixed forest	East Kalimantan, Kalimantan Island	00° 59.050' S, 116° 55.228' E	Dipterocarp forest enriched in non-Dipterocarp species. Research forest of Wanariset Tropical Forest Research Station
4	Dipterocarp primary forest	East Kalimantan, Kalimantan Island	00° 59.363' S, 116° 56.792' E	Research forest of Wanariset Tropical Forest Research Station
5	<i>Macaranga</i> sp. secondary forest	East Kalimantan, Kalimantan Island	00° 59.519' S, 116° 57.241' E	<i>Macaranga</i> sp. grew naturally in Dipterocarp forest after forest fire. Research forest of Wanariset Tropical Forest Research Station

was measured with a spectrophotometer (U-2900, Hitachi, Japan) at 880 nm.

Cation exchange capacity (CEC) was determined by the semi-micro Schollenberger method, which is based on the displacement of soil cations by leaching soil with an excess of 1 M neutral (pH 7.0) $\text{NH}_4\text{CH}_3\text{COO}$ solution, as described by the United States of Agriculture (USDA) (Soil survey staff 1992). Briefly, the soil was leached with $\text{NH}_4\text{CH}_3\text{COO}$ solution, allowing NH_4^+ to replace soil cations. The soil was leached again with 1.35 M KCl solution to replace NH_4^+ . By titration with formaldehyde and thymol blue, the amount of NH_4^+ was measured and CEC was calculated. Leached exchangeable cations (Ca, Mg, Na, and K) from the soil were measured by an atomic absorption spectrophotometer (Z-5000, Hitachi, Japan).

2.3 Experiment 1. Isolation of EPF from tree and crop species

Sand was acidified and sterilized by autoclaving at 80 °C for 45 min. Forty grams of sterilized sand was mixed with 40 g of forest soil, then used as growth medium. Seeds of *Calliandra calothyrsus*, *Paraserianthes falcataria*, *Sesbania grandiflora*, and *Cassia siamea* were sown on sterilized sand and incubated in a growth chamber (Biotron LPH-350S, NK System, Japan) at 27 °C with a 16-h photoperiod. One of two-leaf-stage seedlings of *C. calothyrsus*, *P. falcataria*, *S. grandiflora*, and *C. siamea* was transplanted onto the medium in a 50 ml syringe pot. Three, five, or 20 seeds of *Sorghum bicolor*, *Allium fistulosum*, or *Trifolium repens*, respectively, were sown onto the same medium. Ten grams of sterilized sand was further added into the syringe pot to cover the root system of seedlings or seeds. All plants were grown in the growth chamber for 90 days at 27 °C, with a 16-h photoperiod. Five to ten milliliters of 1 mg P L⁻¹ nutrient solution

(based on Wagatsuma et al. 1988) was applied once every two days. Twenty-five pots were prepared for each plant species.

C. calothyrsus, *P. falcataria*, *S. grandiflora*, *C. siamea*, *S. bicolor*, *A. fistulosum*, and *T. repens* were harvested 90 days after transplanting or sowing. Fresh roots were extracted from each soil and washed under running tap water. The roots were surface-sterilized following the method of Verma et al. (2012) by dipping in 90% EtOH (1 min), 5% NaClO (5 min), and 90% EtOH (10 s) and rinsing three times with sterilized de-ionized water. The roots were dried with sterilized Kimtowels™ and left to air dry. The air-dried roots were cut into 5 mm. Five pieces were plated on half-strength malt extract agar (MEA) containing 100 µg mL⁻¹ Penicillin-Streptomycin (Lonza Biowhitaker, Penicillin-Streptomycin Mixture) (modified from Verma et al. 2012) and water agar. Five replication plates were made for each medium, for roots from one pot, for a total of 50 plates per pot. The plates were sealed with Parafilm™ and incubated in the dark at 25 °C. Emerging colonies from roots were subcultured on new half-strength MEA medium. EPF isolated from this experiment were used to calculate the isolation rate. Isolation rates were calculated by dividing the number of isolates by the number of initial plates.

2.4 Experiment 2. Isolation of EPF from *P. falcataria* and *S. bicolor*

EPF were isolated again from *P. falcataria* and *S. bicolor*. *P. falcataria* was chosen because this species would be used as the target species in future studies and the EPF isolation rates for the four tree species were not markedly different (Table 3). This species is not only a candidate for reforestation, but also economically profitable in a mixed plantation with crop species, or as single-species plantation (Siregar et al. 2007; Krisnawati et al. 2011), and a candidate for energy

Table 2 Chemical properties of forest soils collected from Indonesia

Forest site	pH (H ₂ O)	pH (KCl)	Available phosphate (mg P 100 g dry soil ⁻¹)	CEC (cmol kg ⁻¹)	Ca (cmol kg ⁻¹)	Mg (cmol kg ⁻¹)	Na (cmol kg ⁻¹)	K (cmol kg ⁻¹)
<i>Tectona grandis</i> monoculture forest	7.02 ± 0.04 a	5.99 ± 0.08 a	1.00 ± 0.08 a	59.66 ± 5.47 b	34.82 ± 1.01 a	2.89 ± 0.75 a	0.38 ± 0.02 b	1.59 ± 0.09 a
<i>Swietenia macrophylla</i> monoculture forest	6.74 ± 0.07 a	5.78 ± 0.12 a	0.99 ± 0.08 a	75.39 ± 1.88 a	37.51 ± 1.32 a	3.07 ± 0.40 a	0.57 ± 0.09 a	1.02 ± 0.03 b
<i>Gmelina</i> sp., <i>Artocarpus champeden</i> , and Dipterocarpaceae mixed forest	4.82 ± 0.10 b	3.74 ± 0.07 b	0.32 ± 0.03 b	11.88 ± 0.86 c	2.33 ± 0.62 b	0.74 ± 0.11 b	0.12 ± 0.01 c	0.26 ± 0.01 c
Dipterocarpaceae primary forest	3.87 ± 0.08 c	3.26 ± 0.06 c	0.26 ± 0.02 b	13.03 ± 1.41 c	0.26 ± 0.12 b	0.31 ± 0.11 b	0.12 ± 0.01 c	0.32 ± 0.04 c
<i>Macaranga</i> sp. secondary forest	4.55 ± 0.17 b	3.76 ± 0.13 b	0.25 ± 0.01 b	9.34 ± 1.21 c	1.32 ± 0.77 b	0.49 ± 0.28 b	0.09 ± 0.01 c	0.28 ± 0.07 c

production (Amirta et al. 2016). *S. bicolor* was chosen because it had the highest EPF isolation rate among crop species, and also among all species.

EPF were isolated from *P. falcataria* and *S. bicolor* using the same method described above (Experiment 1). However, the nutrient solution for watering was changed to tap water to minimize nutrient input, mimicking the conditions in the forest. Seedling number per pot, plant height, leaf number, and symptoms of nutrient deficiency, on leaves of *P. falcataria* and *S. bicolor* were recorded before harvest (Tables S1 and S2). Plants showing good growth were harvested and EPF were isolated from the roots.

2.5 DNA extraction and amplification

EPF isolated in experiment 2 were subcultured on half-strength MEA for 1–3 weeks depending on the growth rate of each isolate. Hyphae of each isolate were collected using forceps and placed on the lid of a plastic tube containing 20 µL of InstaGene™ Matrix (Bio-Rad, USA). Hyphae were crushed with a pipet tip having a blunt end, further mixed with 180 µL of InstaGene™ Matrix, and vortexed. Next, rDNA was extracted following the manufacturer's protocol for InstaGene™ Matrix. The extracted DNA was stored at –20 °C until use.

The internal transcribed spacer (ITS) region of the fungi was amplified using universal primers, ITS1F (5'-GTAACAAGGTTTCCGT-3') and ITS1R (5'-CGTTCTTC ATCGATG-3') (Fujita et al. 2010), with an Expand High Fidelity^{PLUS} PCR system (Roche, Germany) at the following composition: 4 µL of 5× buffer with MgCl₂, 0.2 µL of DNA polymerase, 2 µL of 2.0 mM dNTP, 0.4 µL of ITS1F, 0.4 µL of ITS1R, 11 µL of Milli-Q water, and 2 µL of DNA template. The reaction was performed in a Takara PCR Thermal Cycler Dice (Model TP600, Takara Bio, Japan) under the following conditions: initial denaturation at 94 °C for 120 s; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s; and final extension at 72 °C for 420 s. The PCR products were separated on 1.0% agarose gel (D1 Agarose Low EEO, Conda, Spain) in 1× Tris-borate-EDTA buffer, stained with SYBR® Safe DNA Gel Stain (Invitrogen, USA), and viewed under blue light (470 nm, MBP-LED, Bio-Pyramid, USA). PCR-amplified fragments were purified using a MonoFas DNA Purification Kit (GL Science, Japan) following the manufacturer's protocol. Purified DNA was ligated into pT7Blue T-Vector (Novagen, USA) using a DNA Ligation Kit Ver 1 (Takara Bio, Japan). Twenty microliters of IPTG (Takara Bio, Japan) and 35 µL of X-Gal (Takara Bio, Japan) were applied to Luria Bertani (LB) medium containing 100 mg L⁻¹ ampicillin. T-Vector containing DNA was transformed into *Escherichia coli* JM109 (Takara Bio, Japan) by plating onto this LB medium. Plates with *E. coli* were incubated at 37 °C for 16 h.

Table 3 Numbers of isolates and isolation rates of endophytic fungi from seven plant species

Forest	Number of EPF isolates based on host plant							Total	Number of initial plates	Isolation rate (%)
	<i>Calliandra calothyrsus</i>	<i>Paraserianthes falcataria</i>	<i>Sesbania grandiflora</i>	<i>Cassia siamea</i>	<i>Sorghum bicolor</i>	<i>Allium fistulosum</i>	<i>Trifolium repens</i>			
<i>Tectona grandis</i> monoculture	2	3	3	5	19	0	20	52	350	15
<i>Swietenia macrophylla</i> monoculture	5	4	3	5	9	0	29	55	350	16
<i>Gmelina</i> sp., <i>Artocarpus champeden</i> , and Dipterocarp mixed	1	2	9	7	29	3	2	53	350	15
Dipterocarp primary	1	0	1	1	8	0	2	13	350	4
<i>Macaranga</i> sp. secondary	4	0	1	3	10	0	6	24	350	7
Total	13	9	17	21	75	3	59	197		
Number of initial plates	250	250	250	250	250	250	250		1750	
Isolation rate (%)	5	4	7	8	30	1	24			11

Single colonies of *E. coli* were collected and DNA was amplified using primers T7 (5'-TAATACGACTCACT ATAG-3') and U19 (5'-GTTTTCCCAGTCACGACT-3') (Ikenaga et al. 2016) with GoTaq® DNA Polymerase (Promega, USA) at the following composition: 2 µL of 5 x reaction buffer, 0.05 µL of DNA polymerase, 0.8 µL of 2.0 mM dNTP, 0.2 µL of T7, 0.2 µL of U19, 6.75 µL of Milli-Q water, and a single colony of *E. coli*. The reaction was performed in a Takara PCR Thermal Cycler Dice (Model TP600, Takara Bio, Japan) under the following conditions: initial denaturation at 94 °C for 120 s; 35 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 60 s, and extension at 72 °C for 80 s; and final extension at 72 °C for 600 s. The PCR products were separated on 1.0% agarose gel in 1× Tris-borate-EDTA buffer, stained with SYBR® Safe DNA Gel Stain, and viewed under blue light. The PCR products were used for sequencing.

Sequencing reactions were performed in a Bio-Rad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using an ABI BigDye™ Terminator v3.1 Cycle Sequencing Kit with AmpliTaq DNA Polymerase (FS enzyme, Applied Biosystems, Japan) following the manufacturer's protocol. Single pass sequencing was performed on each DNA template using a T7 promoter. Fluorescent-labeled fragments were purified from the unincorporated terminators by adopting an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, Japan).

2.6 Phylogenetic analyses

Sequences of EPF isolates were submitted for BLAST analysis (Altschul et al. 1990). The sequences and their corresponding BLAST top hits were aligned by Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh et al. 2002)

through <http://guidance.tau.ac.il>. Maximum parsimony method was performed by MEGA 7 (www.megasoftware.net) with 1000 replications of bootstrap analysis (Fig. 1).

3 Results

3.1 Soil chemical properties

Soil pH (H₂O) ranged from 3.87 to 7.02 and soil pH (KCl), from 3.26 to 5.99 (Table 2). Based on USDA classification, soil pH (H₂O) was neutral in both *Tectona grandis* and *Swietenia macrophylla* monoculture. Soil pH (H₂O) was extremely acidic to very strongly acidic in *Gmelina* sp., *Artocarpus champeden*, and Dipterocarp mixed, Dipterocarp primary, and *Macaranga* sp. secondary forests. Available P concentration was 1.00 mg 100 g dry soil⁻¹ in *T. grandis* monoculture and 0.99 mg 100 g dry soil⁻¹ in *S. macrophylla* monoculture, and both values were higher than those in *Gmelina* sp., *A. champeden*, and Dipterocarp mixed, Dipterocarp primary and *Macaranga* sp. secondary forests. CEC ranged from 9.34 to 75.39 cmol kg⁻¹; it was higher in *T. grandis* monoculture and *S. macrophylla* monoculture than in *Gmelina* sp., *A. champeden*, and Dipterocarp mixed, Dipterocarp primary or *Macaranga* sp. secondary forest. The same tendency as soil CEC was noted for exchangeable Ca, Mg, Na, and K.

3.2 Isolation of EPF

A total of 197 EPF were isolated from the roots of the seven host plant species grown in the five forest soils (Table 3). Among the 197 EPF, 13, 9, 17, 21, 75, 3, and 59 EPF were isolated from the roots of *C. calothyrsus*, *P. falcataria*, *S. grandiflora*, *C. siamea*, *S. bicolor*, *A. fistulosum*, and

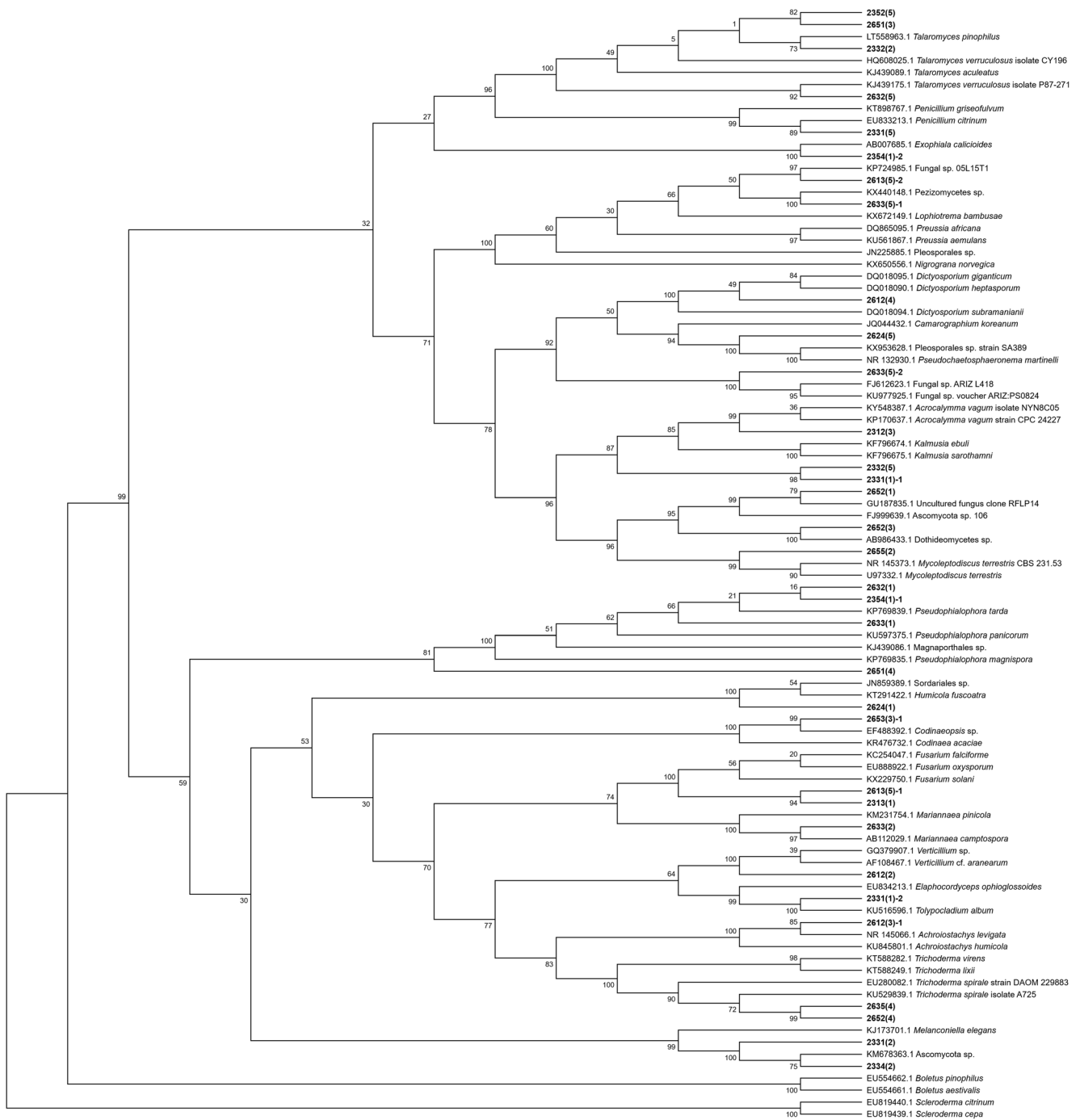


Fig. 1 Maximum parsimony analysis of ITS1 region sequences of endophytic fungi. Values from bootstrap analysis (1000 replications) are shown

T. repens, respectively. *S. bicolor* and *T. repens* had higher EPF isolation rates than the other plant species. Moreover, among these, 197 EPF, 52, 55, 53, 13, and 24 EPF were isolated from *T. grandis* monoculture, *S. macrophylla* monoculture, *Gmelina* sp., *A. champeden*, and Dipterocarp mixed, Dipterocarp primary, and *Macaranga* sp. secondary forest, respectively. Soils of *T. grandis* monoculture, *S. macrophylla* monoculture, and *Gmelina* sp.,

A. champeden, and Dipterocarp mixed forest had higher EPF isolation rates than the other forest soils.

Leaf necrosis was detected in some seedlings of *S. bicolor*. Seedlings with higher plant height and leaf number, and less leaf necrosis, in 12 of a total 25 pots, were selected and used for isolation of EPF (Table S1). Twenty-one EPF were isolated from the roots of *S. bicolor* (Tables 4 and S1). Among the 21 EPF, 5, 2, 7, and 7 were isolated from soils of *T. grandis*

Table 4 EPF isolated from roots of *Paraserianthes falcataria* and *Sorghum bicolor* grown in forest soils of Indonesia

No	Host plant	Forest	EPF isolate	Accession number	Closest relative based on sequence homology (Genbank accession)	Order	Family	Similarity
1	<i>Paraserianthes falcataria</i>	<i>Tectona grandis</i> monoculture	2312(3)	LC334061	<i>Acrocalymma vagum</i> strain CPC 24227 (KPI170637.1)	Pleosporales	Morosphaeriaceae	100%
2			2313(1)	LC334062	<i>Fusarium solani</i> isolate FJAT-30854 (KX229750.1)	Hypocreales	Nectriaceae	99%
3		<i>Gmelina arborea</i> , <i>Artocarpus champeden</i> and Dipterocarp mixed	2331(1)-1	LC334063	Uncultured fungus clone T2949 (KF742561.1)	—	—	84%
4			2331(1)-2	LC334064	<i>Tohypocladium album</i> isolate 3601b14 (KU516596.1)	Hypocreales	Ophiocordycipitaceae	99%
5			2331(2)	LC334065	<i>Ascomycota</i> sp. Glum291 (KM678363.1)	—	—	98%
6			2331(5)	LC334066	<i>Penicillium citrinum</i> strain P1.21 (EU833213.1)	Eurotiales	Trichocomaceae	100%
7			2332(2)	LC334067	<i>Talaromyces verruculosus</i> isolate AT281 (HQ607919.1)	Eurotiales	Trichocomaceae	100%
8			2332(5)	LC334068	Uncultured fungus clone T2949 (KF742561.1)	—	—	85%
9			2334(2)	LC334069	Uncultured Sordariomyces clone OTU68 (KU144662.1)	—	—	99%
10		<i>Macaranga</i> sp. secondary	2352(5)	LC334070	<i>Talaromyces aculeatus</i> C36-375 (KJ439089.1)	Eurotiales	Trichocomaceae	99%
11			2354(1)-1	LC334071	Uncultured <i>Ascomycota</i> clone 736 (HM162169.1)	—	—	99%
12			2354(1)-2	LC334072	<i>Exophiala calcicoides</i> strain JCM9764 (AB007685.1)	Chaetothyriales	Herpotrichiellaceae	84%
13	<i>Sorghum bicolor</i>	<i>Tectona grandis</i> monoculture	2612(2)	LC334073	<i>Clavicipitaceae</i> sp. MEXU 26354 (JQ811555.1)	Hypocreales	Clavicipitaceae	99%
14			2612(3)-1	LC334074	Uncultured fungus clone 109A74984 (JX386729.1)	—	—	99%
15			2612(4)-4	LC334075	<i>Dictyosporium heptasporum</i> CBS 396.59 (DQ018090.1)	Pleosporales	Dictyosporiaceae	98%
16			2613(5)-1	LC334076	<i>Fusarium solani</i> isolate FJAT-30854 (KX229750.1)	Hypocreales	Nectriaceae	99%
17			2613(5)-2	LC334077	Uncultured organism clone ciidir1004 C.11 (JN660770.1)	—	—	92%
18		<i>Swietenia mahogany</i> monoculture	2624(1)-2	LC334078	<i>Sordariales</i> sp. REF169 (JN859389.1)	Sordariales	—	95%
19			2624(5)	LC334079	<i>Pseudochaetosphaeronema martinelli</i> CBS 135986 (NR_132930.1)	Pleosporales	Pleosporales incertae sedis	95%
20		<i>Gmelina arborea</i> , <i>Artocarpus champeden</i> and Dipterocarp mixed	2632(1)	LC334080	Uncultured <i>Ascomycota</i> clone 736 (HM162169.1)	—	—	99%
21			2632(5)	LC334081	<i>Talaromyces verruculosus</i> isolate P87-271 (KJ439175.1)	Eurotiales	Trichocomaceae	100%
22			2633(1)	LC334082	Uncultured <i>Ascomycota</i> clone 736 (HM162169.1)	—	—	98%
23			2633(2)	LC334083	<i>Mariannaea camptospora</i> (AB112029.1)	Hypocreales	Nectriaceae	99%
24			2633(5)-1	LC334084	Uncultured <i>Ascomycota</i> clone OMG C15 (GU174012.1)	—	—	97%
25			2633(5)-2	LC334085	Fungal sp. voucher ARIZ:PS0824 (KU977925.1)	—	—	96%
26			2635(4)	LC334086	<i>Trichoderma spirale</i> A725 (KU529839.1)	Hypocreales	Hypocreaceae	99%
27		<i>Macaranga</i> sp. secondary	2651(3)	LC334087	<i>Talaromyces aculeatus</i> C36-375 (KJ439089.1)	Eurotiales	Trichocomaceae	99%
28			2651(4)	LC334088	Uncultured fungus clone 034A4289 (JX321968.1)	—	—	87%
29			2652(1)	LC334089	Uncultured fungus clone RFLP14 (GU187835.1)	—	—	96%
30			2652(3)	LC334090	Dothideomyces sp. KO-groupG 2014 (AB986433.1)	—	—	99%
31			2652(4)	LC334091	<i>Trichoderma spirale</i> isolate A725 (KU529839.1)	Hypocreales	Hypocreaceae	99%
32			2653(3)-1	LC334092	<i>Fusarium</i> sp. isolate E-179.5 (KU059845.1)	Hypocreales	Nectriaceae	99%
33			2655(2)	LC334093	<i>Mycocleptodiscus terrestris</i> CBS 231.53 (NR_145373.1)	Magnaporthales	Magnaporthaceae	94%

monoculture, *S. macrophylla* monoculture, *Gmelina* sp., *A. champeden*, and Dipterocarp mixed, and *Macaranga* sp. secondary forest, respectively. EPF isolation rate for *S. bicolor* was 35%.

Leaf necrosis was not detected in all seedlings of *P. falcataria*. Seedlings with higher plant height and leaf number in 10 of a total 25 pots, were selected and used for isolation of EPF (Table S2). Twelve EPF were isolated from the roots of *P. falcataria* (Tables 4 and S2). Among the 12 EPF, 2, 7, and 3, were isolated from soils of *T. grandis* monoculture, *Gmelina* sp., *A. champeden*, and Dipterocarp mixed, and *Macaranga* sp. secondary forest, respectively. The EPF isolation rate for *P. falcataria* was 24%, which was higher than that of the first isolation of EPF.

3.3 Identification of EPF

The sequences of the ITS regions of all isolates were submitted to BLAST including uncultured or environmental samples. The similarity scores between isolated EPF and their closest relatives in GenBank were between 84 and 100% (Table 4). All sequences of isolated EPFs were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC334061–LC334093.

Seven genotypes of EPF isolated from the roots of *P. falcataria* closely matched fungi, identified to the species level (Table 4). One genotype closely matched a fungus identified to the phylum level. The remaining four genotypes closely matched uncultured fungi. Based on the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov>) database, the identified fungi were classified in orders Pleosporales, Hypocreales, Eurotiales, and Chaetothyriales.

Nine genotypes of EPF isolated from the roots of *S. bicolor* closely matched fungi identified to the species level (Table 4). One genotype closely matched a fungus identified to the genus level (*Fusarium*). One genotype closely matched a fungus identified to the family level (Clavicipitaceae). One genotype closely matched a fungus identified to the order level (Sordariales). One genotype closely matched a fungus identified to the class level (Dothideomycetes). There was one genotype that closely matched unidentified fungus (Fungal sp. voucher). The remaining seven genotypes closely matched uncultured fungi. Based on the NCBI database, the identified fungi were classified in the orders Pleosporales, Hypocreales, Eurotiales, and Magnaporthales.

4 Discussion

4.1 Specificity of EPF for host plants and forest sites

EPF from the orders Pleosporales, Hypocreales, Eurotiales, Magnaporthales, and Chaetothyriales were isolated in the

present study (Table 4). EPF from orders Pleosporales, Hypocreales, and Eurotiales were isolated from both host plants *P. falcataria* and *S. bicolor*, whereas EPF from order Magnaporthales and Chaetothyriales were specifically isolated from *P. falcataria* and *S. bicolor*, respectively. EPF in these five orders were also isolated from the roots of subtropical *Hordeum murinum* in Ireland (Murphy et al. 2015). EPFs from two orders Pleosporales and Hypocreales were also isolated from the roots of plants growing in places with different environmental conditions, such as desert grass *Bouteloua gracilis* in New Mexico (Porrás-Alfaro et al. 2008) and tropical shrub *Sophora tonkinensis* in China (Yao et al. 2017), showing the wide distribution of EPF from these two orders in nature.

EPF from the genera *Acrocalymma*, *Fusarium*, *Tolypocladium*, *Penicillium*, *Talaromyces*, *Exophiala*, *Dictyosporium*, *Pseudochaetosphaerionema*, *Mariannaea*, *Trichoderma*, and *Mycoleptodiscus*, have also been reported in other studies. Those EPFs were reported in the studies by Jin et al. (2017) (*Acrocalymma vagum*), Kwasna et al. (2016) *Talaromyces verruculosus* and *Trichoderma spirale*, Lin et al. 2007 (*Dictyosporium* sp.), Shubin et al. 2014 (*Mycoleptodiscus* sp. and *Penicillium* sp.), Yao et al. 2017 (*Fusarium solani*), Zhang et al. 2017a (*Exophiala piscipila*), Waipara et al. 1996 (*Mariannaea* sp.), Zhang et al. 2017b (*Pseudochaetosphaerionema larense*), and Sánchez Márquez et al. 2010 (*Tolypocladium cylindrosporium*). However, to our knowledge, the present study is the first to isolate EPF in these genera from the roots of *P. falcataria*. Amin (2013) isolated EPF belonging to a different genus, *Nigrospora* sp. (order Trichosphaeriales), from the roots of *P. falcataria*.

Sixteen of the 33 isolates had the closest match to fungi identified to the species level (Table 4). Ninety-seven percent similarity is widely used as the cut-off point (O'Brien et al. 2005) to determine whether the isolates are identical at the species level, or not. Thirteen of the 16 isolates were considered to be of the same species with the closest match. The remaining three isolates, 2354(1)-2, 2624(5), and 2655(2), were similar to *Exophiala calicioides* (84% similarity), *Pseudochaetosphaerionema martinelli* (95% similarity), and *Mycoleptodiscus terrestris* (94% similarity), respectively. These three isolates may be categorized in the same genera with the closest match.

Among the 16 isolates, 3 were specific to certain forest sites shared by *P. falcataria* and *S. bicolor*: *Fusarium solani* in *T. grandis* monoculture, *Talaromyces verruculosus* in *Gmelina* sp., *A. champeden* and Dipterocarp mixed, and *Talaromyces aculeatus* in *Macaranga* sp. secondary forest (Table 4). In addition, some of the isolates were specific to certain forest sites, but were not shared by the two host plants, examples of which are *Dictyosporium heptasporum* in *T. grandis* monoculture, *Mariannaea camptospora* in *Gmelina* sp., *A. champeden*, and Dipterocarp mixed, and *Mycoleptodiscus* sp. in *Macaranga* sp. secondary forest.

These results indicated that EPF had low or high specificity for host plants, as well as forest sites. This phenomenon was also observed by Kernaghan and Patriquin (2011) in their study in a Boreal area. Kernaghan and Patriquin (2011) isolated root EPF from *Betula papyrifera*, *Abies balsamea*, and *Picea glauca* from two different sites and identified them by a molecular method. They revealed that the EPF communities were different among host trees in one site but not in another site. They also observed that some EPF were found only on certain host, showing the specificity of EPF to host tree species. Jumpponen and Trappe (1998) and Mandyam and Jumpponen (2005) clarified that root EPF, specifically DSE, colonized 587 plants, representing 320 genera and 114 families. Further inoculation experiments under natural and experimental conditions confirmed that DSE species had low host specificity.

The numbers of isolates in *Gmelina* sp., *A. champeden*, and Dipterocarp mixed forest and *Macaranga* sp. secondary forests were higher than those in the other forest sites (Table 4). The dominant species in *Gmelina* sp., *A. champeden*, and Dipterocarp mixed forest were *Gmelina* sp., *A. champeden*, and Dipterocarp sp. The dominant species in *T. grandis* monoculture forest and *S. macrophylla* monoculture forests were only one species each, *T. grandis* and *S. macrophylla*, respectively. The number of plant species in each forest might be the reason why the number of isolates was higher in *Gmelina* sp., *A. champeden*, and Dipterocarp mixed forest than the other forest sites. The dominant species in *Macaranga* sp. secondary forest was also one species: *Macaranga* sp. The reason why the number of isolates was larger in *Macaranga* sp. secondary forest than *T. grandis* monoculture forest and *S. macrophylla* monoculture forest is not known.

Our results demonstrate that the root EPF community differed among forest sites. Utilization of the trap culture method with the same host plant, *P. falcataria* or *S. bicolor*, still yielded different EPF among the five forests. Thus, differences in the root EPF community were mainly owing to differences in forest sites, involving the plant community and environmental factors, that resulted in the specific conditions in each forest.

4.2 Role of EPF from the same genera

Some of the root EPF isolated in the present study have also been isolated and studied for their importance, especially in relation with plant growth. Some were proven to promote plant growth under biotic and abiotic stress. *Trichoderma* sp. improved plant resistance against fungal pathogens (Vinale et al. 2008; Mukherjee et al. 2012), plant tolerance to salt stress (Brotman et al. 2013), and plant tolerance to drought (Bae et al. 2009). *Exophiala* sp. improved plant tolerance to heat stress (Khan et al. 2012) and cadmium toxicity (Wang et al. 2016). *Talaromyces pinophilus* promoted the growth of

rice seedlings by gibberellin production (Khalmuratova et al. 2015). *Penicillium citrinum* improved plant resistance against fungal pathogens (Ting et al. 2012) and promoted growth by gibberellin production (Khan et al. 2008). Conversely, *Mycoleptodiscus* sp. was recorded to be a pathogen of Eurasian watermilfoil (Shearer et al. 2011). There is no information available regarding other remaining genera.

4.3 EPF isolation rate

The isolation of root colonizing microbes by trap culture is widely used in microbiological studies. However, the isolation from field-collected plants is a more common method in EPF studies, especially in studies that aim to isolate organic compounds for biotechnological applications (Strobel 2003). The trap culture method is common for studies that aim to clarify the role of EPF in protecting plants against soil pathogens and promoting plant growth (Amin 2013; Narisawa et al. 2002, 2007) or to clarify the existence of certain EPF in the field (Ahlich et al. 1998).

Studies of root EPF by some isolation methods might underestimate the number of species compared to studies that directly assess root samples by using molecular methods. However, the isolation method has one advantage, namely, the availability of culture for further studies. Brock et al. (2009) emphasized the importance of a herbarium or a fungal culture for the detailed assessment of unknown fungi.

In the present study, the trap culture method was adopted, and EPF were isolated from seven plant species grown on five forest soils. There was no difference in the EPF isolation rate among the four tree species (Table 3), but there was a difference among crop species, with *S. bicolor* showing the highest isolation rate. Among the seven plant species, *S. bicolor* showed the highest EPF isolation rate. Narisawa et al. (2002) isolated EPF by the trap culture method using eggplant, Chinese cabbage, tomato, melon, and strawberry as host plants. Among these five plant species, eggplant showed the highest EPF isolation rate. In our study, only representatives of EPF having the same morphology which emerged from root segments were isolated. In contrast, in the study of Narisawa et al. (2002), all emerging EPF were isolated. Thus, the EPF isolation rate in our study might be very low compared to that of Narisawa et al. (2002). In addition, there were also studies that isolated EPF from plant species that were taxonomically close to plant species of the present study: *Sesbania bispinosa* (Sreelalitha and Sridhar 2015), *P. falcataria* (Amin 2013), and *Trifolium subterraneum* (Mugerwa et al. 2013).

P. falcataria and *S. bicolor* were not originally part of the plant community in the five forest sites. Nevertheless, EPF were still isolated from their roots. Moreover, different EPF were isolated from different forests, indicating that the trap culture method can be used to evaluate differences in EPF

community among forests. Considering that both host plants were not part of the plant community in the five forest sites, the effect of original plant species on the identity of isolated EPF was minimal. Therefore, differences in EPF isolates among the forest sites reflect the natural differences in the EPF community among the forest sites.

4.4 Relationship between soil chemical properties and EPF community

Soil chemical properties differ between forests in Java Island and Kalimantan Island. Different isolates were found in the two islands: *Fusarium solani* in Java Island and *Talaromyces* sp. in Kalimantan Island. There were specific isolates in each forest, that were not separated by the difference of soil chemical properties of two islands, such as *F. solani* and *Talaromyces* sp. Soil chemical properties may not be an important factor determining EPF community.

Root EPF were isolated from forest soil with pH (H₂O) ranging from 3.87–4.82. The present study is not the only one to find EPF in soil with low pH. Hakim et al. (2015) isolated EPF from the roots of *Shorea leprosula* and *Shorea selanica* grown in reddish Latosol, a type of acid soil in the tropics. Göransson et al. (2008) observed EPF colonization in the roots of four woodland grasses (*Elymus caninus*, *Poa nemoralis*, *Deschampsia cespitosa*, and *Deschampsia flexuosa*) grown in subtropical forest soil with pH (H₂O) ranging from 4.20 to 6.00. These findings emphasize the fact that EPF are commonly found in subtropical or tropical forest soils with low pH.

Root EPF were isolated from soil with available P lower than 1.00 mg 100 g soil⁻¹. This value indicated low P availability for plant growth, according to USDA classification and Brady and Weil (2002). Della Monica et al. (2015) inoculated *T. repens* with four EPF isolates and cultivated the plant in soil:perlite (2:1 v:v) containing 3.42 mg 100 g⁻¹ total P. One of the four EPF, *Phialocephala glacialis*, increased both P concentration in soil solution and shoot P content in *T. repens*. Hiruma et al. (2016) inoculated *Arabidopsis thaliana* with *Colletotrichum tofieldiae* and cultivated the plant in half-strength Murashige and Skoog medium, containing 0.68 mg 100 g⁻¹ total P. *C. tofieldiae* increased the shoot fresh weight of *A. thaliana*. Root EPF isolated in the present study may have the potential to mineralize P and promote plant growth.

5 Conclusion

EPF were isolated from the roots *C. calothyrsus*, *P. falcataria*, *S. grandiflora*, *C. siamea*, *S. bicolor*, *A. fistulosum*, and *T. repens*, grown in five different forest soils in Indonesia. EPF isolation rates were not markedly different among the

tree species. *S. bicolor* exhibited the highest EPF isolation rate among crop species, and also among all species used in the present study. Some EPF were isolated from more than one forest, and some specifically from only one forest. In addition, the number of isolated EPF from *Gmelina* sp., *A. champeden*, and Dipterocarp mixed were larger than that from other forests. These results indicated that EPF had specificity to the plant species. Future studies including EPF inoculation of plants is necessary to clarify the effect of EPF on plant growth.

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