

## Molecular identification of endophytic fungi from torch ginger (*Etlingera elatior*) antagonist to phytopathogenic fungi

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**Abstract.** Lutfia A, Munir E, Yurnaliza. 2020. Molecular identification of endophytic fungi from torch ginger (*Etlingera elatior*) antagonist to phytopathogenic fungi. *Biodiversitas* 21: 2681-2689. Medicinal plants are valuable sources in exploration of endophytic microorganisms. The utilization of culturable endophytes, particularly endophytic fungi in pharmacological aspects has been reported in many literature. Zingiberaceae, being known as one of medicinal plant families, is commonly used in tropical countries, e.g Indonesia to treat health disorders and ailments. However, little information has been reported upon their associative endophytic fungi that may be used as biocontrol agents against phytopathogenic fungi. Efforts to elaborate on the symbiotic relationship between endophytic fungi and host to explain the antifungal ability from endophytes are presented in this study. Six fungal morphotypes were recovered from the rhizome fragment of torch ginger (*Etlingera elatior* (Jack) R.M. Smith) sampled from Sibayak forest, North Sumatra, Indonesia. All isolates displayed potential antifungal activities against *Fusarium oxysporum*, *Ganoderma boninense*, and *Rigidoporus lignosus* as representative phytopathogens in a dual culture plate assay. All fungal morphotypes were identified based on their 18S rDNA-ITS regions. Molecular identification revealed that most isolates were related to *Trichoderma* spp. while one isolate was identified as *Pestalotiopsis* sp. The endophytic assemblage of mostly *Trichoderma* strains in our study may reveal a mutual relationship of these soil fungi as root colonizer displaying biocontrol properties against phytopathogenic fungi to host.

**Keywords:** Endophytic fungi, *Etlingera elatior*, rDNA-ITS, *Pestalotiopsis*, *Trichoderma*

### INTRODUCTION

Endophytic fungi are fungal community residing inter- or intracellular part of a plant exhibiting no apparent disease sign to a healthy plant. Recently, this form of interaction has brought an alternative way of discovering novel metabolites as the identification of plant secondary metabolites has been conducted progressively (Strobel and Daisy 2003; Kaul et al. 2012). The bioprospective study upon these microorganisms is based on the way that endophytic microorganisms may synthesize or even mimic the similar compound produced by their host plants hence increasing the probability of finding new compounds (Venieraki et al. 2017).

The rationale upon isolating potential endophytes is by choosing a plant with a historical background in traditional or ethnobotanical usage as practical medicine (Strobel 2003). Zingiberaceae is known as a multi-purpose plant family that already being utilized as spices and medicines in several countries of Southeast Asia. In Indonesia, Zingiberaceae has been reported as economical and medicinal plants in most ethnobotanical studies (Fernando et al. 2008; Auliani et al. 2014; Hartanto et al. 2014).

Torch ginger or *kecombrang* (*Etlingera elatior* (Jack) R.M. Smith) is a species in Zingiberaceae, widely cultivated and traditionally used as medicinal plants in Indonesia (Siregar and Pasaribu 2008). In North Sumatra, the species is commonly utilized as an ingredient in ethnobotanical medicine to treat cough and fever while also

being added as spice or *cekala* by the Batak Karo sub-ethnic, in traditional cuisine of *arsik* and *terites* (Silalahi et al. 2015; Silalahi and Nisyawati 2018). The ginger species is also well-studied for its biological properties such as anti-microbial, anti-oxidant, anti-tumor, anti-hyperglycemic, anti-hyperuricemic, anti-inflammatory, anti-larvae, anti-aging and functional properties as a topical medicine and containing phytochemicals, i.e. flavonoids, glycosides, phenols, saponins, steroids, tannins, and terpenoids (Juwita et al. 2018). Although the species has been comprehensively reported for its pharmacological properties, information regarding bioprospective study of their endophytic microorganisms is still limited.

Preliminary investigations have revealed the occurrence of endophytic fungi isolated from some unreported Zingiberaceous species in North Sumatra, such as *Amomum centrocephalum*, *Etlingera littoralis*, *Hedychium coronarium*, and *Globba pendula*, with major functional roles as biocontrol agents to pathogenic bacteria and phytopathogenic fungi (Lutfia et al. 2019a; Lutfia et al. 2019b; Lutfia et al. 2019c; Lutfia et al. 2019d). Based on the reports, we assume that endophytic fungi of torch ginger may also be effective as antagonistic agents, especially against phytopathogenic fungi. This research is then aimed to evaluate the antagonistic properties of culturable endophytic fungi isolated from the rhizome of *E. elatior* medicinal plants and to assign their identity based on molecular identification.

## MATERIALS AND METHODS

### Plant materials

Plant sample, *Etilingera elatior* (Jack.) R. M. Smith was collected during exploration in the Sibayak forest, located in Deli Serdang District, North Sumatra. Sampling was conducted purposively without considering any climate and spatial factors. Plants anchoring to soils were dug up and cut to separate its shoots and roots. The root parts or rhizome were wrapped with paper and stored in plastic bags. Duplicate samples were collected separately to be identified at Herbarium Medanese, Universitas Sumatera Utara. In the laboratory, rhizomes were later cut into smaller pieces, and composites were made by pooling pieces into one bulk sample for each species of Zingiberaceae. The samples were then used in fungal isolation step.

### Isolation of endophytic fungi

Step in isolating fungal endophytes was based on a previous report (Yurnaliza et al. 2014). Bulk samples from each species of Zingiberaceae were cleansed with mineral water to remove the remaining soil and dirt. The sample's surface was sterilized by dipping the samples into following sterilizing agents: 75% EtOH (2 min), 5.3% NaOCl (5 min), 75% EtOH (30 s). The pieces were again washed several times with sterile deionized water to remove remaining solutions. Samples were blotted using Whatman filter paper and let to dry following cut into 1–2 minor pieces. The pieces were inoculated on Potato Dextrose Agar (Oxoid™) as isolation medium pre-added with standard antibiotic, chloramphenicol. Plates were incubated in ambient conditions for 3 days. Any visible fungal growth from each piece was then sub-cultured onto a new medium to preserve the strains. Each fungal strain was differentiated from their colony appearances.

### Antagonistic assay against phytopathogenic fungi

Phytopathogenic fungal species used in this study were: *Fusarium oxysporum*, *Ganoderma boninense*, and *Rigidoporus lignosus*, collections of Laboratory of Microbiology, Department of Biology, Universitas Sumatera Utara, Medan, Indonesia. Phytopathogens were first grown in the PDA medium to obtain fresh mycelial plugs. Mycelial plugs of phytopathogens were then planted three days in advance at the center of the new PDA medium, followed by agar plugs of endophytic fungi in antagonism assay. Plates were incubated for 7 days at room temperature. The assay was conducted in triplicates. Colony Growth Inhibition (CGI) of phytopathogenic fungi was then calculated (Bivi et al. 2010):

$$CGI (\%) = \frac{(R_1 - R_2)}{R_1} \times 100$$

Where, CGI is percentage (%) of colony growth inhibition.  $R_1$  represents the diameter of phytopathogens colony growth in the absence of fungal antagonist,  $R_2$  represents the diameter of phytopathogens from antagonist direction. Each CGI results represented four arbitrary levels

of antagonism: Very Strong (++++ CGI > 75 %, Strong (+++) 75 ≥ CGI > 50 %, Mild (++) 50 ≥ CGI > 25 mm, Weak (+) 25 ≥ CGI > 0 %, and None (–) CGI = 0 %.

### Methanolic extraction of dried rhizome

The extraction procedure performed in this experiment was maceration. Ten grams of the shade-dried rhizome of *Etilingera elatior* were placed into a 250-mL flask prior addition of 100 mL (1:10, w/v) of MeOH. The flask was shaken at 120 rpm for 5 days. After agitation, macerates were filtered through muslin cloth to separate extracts and debris. The extracts were then filtered (Whatman No.1) and further concentrated in a rotary evaporator under low pressure at 45 °C. The crude extracts in the form of paste were diluted in a sufficient amount of Dimethyl Sulfoxide (DMSO) (w/v). Antimicrobial assays against pathogenic bacteria and phytopathogenic fungi using MeOH extract were similar as previously performed by replacing the use of agar plugs to sterile blank discs (Oxoid™). The antimicrobial activities were categorized based as previously described.

### Fungal genomic DNA extraction

Fungal genomic DNA extraction was performed following the technical procedure provided by Wizard® Genomic DNA Purification Kit Protocol (United States). 0.5 g of fresh mycelium from each isolate was picked and dissolved into a mixture of 600 µL SDS Tris-HCl pH 8.0, and 600 µL phenol-chloroform. Homogenates were centrifuged at 4°C, 10000×g for 45 min. Pellets were further dissolved in 100 µL Tris-EDTA. The DNA quality was assessed qualitatively ( $A_{260/280}$ ,  $A_{260/230}$ ).

### PCR amplification

The rDNA-ITS region was amplified using the universal primer for fungi: ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Manter and Vivanco 2007) with reaction mixture composed of: 12 µL nuclease-free water (NFW), 20 µL GoTaq DNA Polymerase solution, 2 µL ITS-1F primer solution, 2 µL ITS-4 primer solution, 4 µL DNA template solution with total volume of 40 µL in Eppendorf tube. The PCR was conditioned as: 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 sec, 55 °C for 45 sec, 72 °C for 45 sec, and final extension at 72 °C for 7 min.

### Electrophoresis and DNA sequencing

The result of DNA extraction or PCR products were visually checked on 1% agarose gels stained with Ethidium Bromide (EtBr) The gel was processed by immersing in a solution of Tris Acetate EDTA (TAE) buffer 1× following the run of device at 110 V. After electrophoresis, the gel was examined under UV illumination inside a gel-doc uv transilluminator. The PCR products were sequenced at Macrogen, Inc (Singapore). The acquired ITS sequences were submitted to NCBI Genbank database to obtain the accession numbers: MT053420, MT053421, MT053422, MT053423, MT053424, and MT053425.

### Bioinformatics study

Sequence pools were qualitatively assembled and trimmed based on the quality of electropherograms provided by the service agent using BioEdit ver. 7.2 (Hall 1999). The sequences were subjected to BLASTn search in National Center for Biotechnology Information (NCBI) database as compare with other fungal ITS sequences retrieved from database (Altschul et al 1990; Altschul et al 1997). Additional sequence identification was performed in TrichOKEY ver. 2.0 by the International Subcommittee on *Trichoderma* and *Hypocrea* Taxonomy (ISTH) to confirm the presence of unique multiple loci in *Trichoderma* species (Druzhinina et al. 2005; Bissett et al. 2015). Multiple sequence alignment was performed to trimmed sequences using MUSCLE feature provided in MEGA ver. 7.0 (Edgar 2004; Kumar et al 2016). Phylogenetic tree of rDNA-ITS sequence was constructed based on the maximum likelihood fits of nucleotide substitution models which produced the lowest Bayesian Information Criterion (BIC) in order to select the appropriate statistical model, along with 1000× bootstrapping for reliability test of constructed trees (Felsenstein 1985; Nei and Kumar 2000).

## RESULTS AND DISCUSSION

### Antagonistic endophytic fungi from *Etlingera elatior*

Seven isolates of endophytic fungi were successfully recovered from rhizome fragments of *E. elatior*. The result of antagonism assay between isolated fungi and phytopathogenic fungi is presented in Table 1. A survey on Zingiberaceae species has been reported in which 9 species were found inhabiting the Sibayak forest. The species were *E. elatior*, *E. fimbriobracteata*, *E. punicea*, and 6 unknown species of *Etlingera* (Siregar and Pasaribu 2008). Sibayak forest is then considered as a representative natural habitat for a diversity of Zingiberaceae species.

It can be seen that most isolates produced antifungal activities, CGI > 50 % against three phytopathogenic fungi. Only one isolate, Ee03 did not show antifungal activity against *F. oxysporum* and less potential against the other two phytopathogenic fungi. Meanwhile, the methanolic

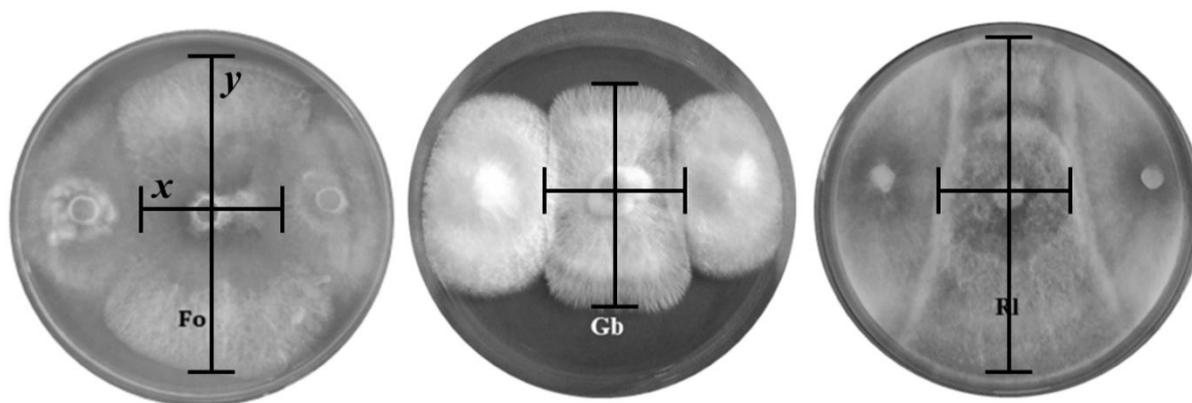
extract of *E. elatior* rhizome was only able to display antifungal activities, CGI < 50% (Figure 1). Hence, five isolates excluding Ee03 were subjected to molecular identification using the ITS-rDNA region.

Mechanisms of antifungal activities from *E. elatior* phytochemical compounds and their derivatives have been suspected through interaction with the cell wall, cell membrane, and intracellular enzymatic inhibition (Ma et al. 2010; Sung and Lee 2010; Cheah et al. 2014). Floral extract of *E. elatior* was able to inhibit the colony growth of *Candida albicans* and *Aspergillus niger* (Lachumy et al. 2010). Furthermore, the floral extract of *E. elatior* was tested against anthracnose pathogen, *Colletotrichum gloeosporioides* which displayed potential antifungal activity towards the phytopathogen (Punnawich et al. 2009). Currently, only one report on endophytic bacterial isolates from *E. elatior* which displayed antifungal activities through assessment of their antagonistic interactions and antifungal crude metabolites. Solid-state fermentation of each bacterial culture was subjected to maceration within organic solvents. The difference in antifungal activities was observed in two results: hexane extract of one isolate, IAK3 was more effective against *F. oxysporum* and *S. rolfii* while ethyl acetate extract of IAK9 was more effective against *R. solani*, *R. microsporus*, *Curvularia*, and *Saprolegnia* (Suryanto et al. 2016).

**Table 1.** Antagonistic properties by endophytic fungi isolated from *Etlingera elatior*

Isolate	Colony growth inhibition		
	<i>Fusarium oxysporum</i>	<i>Ganoderma boninense</i>	<i>Rigidoporus lignosus</i>
Ee01	+++	+++	+++
Ee02	+++	++++	+++
Ee03	–	++	+
Ee04	+++	+++	+++
Ee05	+++	++++	+++
Ee06	+++	+++	+++
Ee07	+++	+++	+++
MeOH	++	++	++

Note: Antagonism levels based on Colony Growth Inhibition (%): Very Strong (++++ CGI > 75 %, Strong (+++) 75 ≥ CGI > 50 %, Mild (++) 50 ≥ CGI > 25 mm, Weak (+) 25 ≥ CGI > 0 %, and None (–) CGI = 0 %



**Figure 1.** The inhibitory activities by endophytic fungi against phytopathogenic fungi (center) as revealed from its horizontal (x) apparent to its vertical colony growth (y). Fo: *Fusarium oxysporum*; Gb: *Ganoderma boninense*; Rl: *Rigidoporus lignosus*

While other reports have revealed the potency of endophytic fungi as antifungal agents from other species of Zingiberaceae, little is known from *E. elatior*. Varied inhibitory activities were produced by endophytic fungi of common ginger (*Zingiber officinale*). Three isolates identified as *Curvularia affinis*, *Fusarium solani*, and *Glomerella cingulata* were able to produce high antagonistic activity with CGI > 65% against *F. oxysporum* in dual culture assay (Ginting et al. 2013). In addition, studies on testing endophytic fungi against phytopathogenic fungi such as *G. boninense* and *R. lignosus* are still limited.

#### Electrophoresis image of rDNA-ITS amplicons

DNA extracts from all endophytic fungal isolates were obtained from 5-days old mycelium. The active-growing mycelia were sampled and crushed in the absence of spores which may affect the extraction process. The presence of a single band from each well indicated the results of PCR amplification using ITS1 and ITS4 primers (Figure 2). A 1 kbp (Promega Corp., USA) was used as molecular marker in this study.

#### Endophytic fungi identification based on BLASTn

Sequence analysis of six isolates was done to reveal the identity of endophytic fungi to the genus/species level. Oligonucleotide fragment of ITS-rDNA sequences was compared with their reference sequences. Molecular identification of selected five endophytic fungi revealed that 5 of them were relatively closed to *Trichoderma* (Tables 2, 4-7), while Ee02 was identified as *Pestalotiopsis*, each of them was aligned with the ITS-rDNA sequence in GenBank database (Table 3). Based on nucleotide database pairing in GeneBank, there are numerous suggestions on identification results as indicated by the list of species ranked by their scores, query covers, and maximum identities. In this study, however, majority of results showed no distinction in overall scores although the identification was placed in the same genus. Here we report the 10 species listed from the BLASTn results. Isolate Ee01 was closely related to *Trichoderma* sp. originating from Colombia, followed by the other species i.e. *T. neotropicale* and *T. harzianum* and *Hypocrea* sp., within the score of 1086 and similarities of 100%. In short, isolate Ee02 and Ee05 were identified as *Pseudopestalotiopsis theae* (100%) and *T. caribbaeum* (100%), respectively, while isolate Ee04, Ee06, and Ee07 were similarly designated as an unidentified species of *Trichoderma* sp.

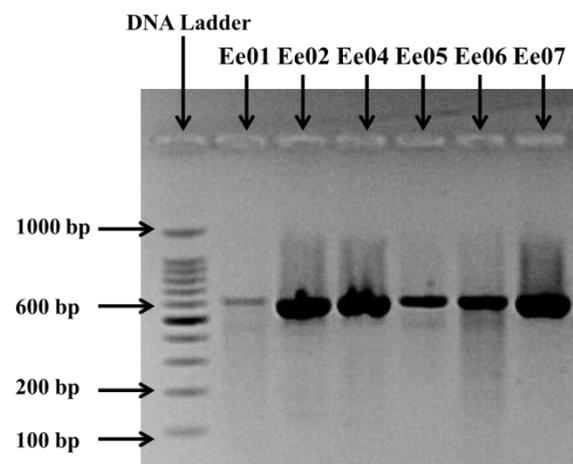
#### *Trichoderma* identification based on TrichOKEY

Molecular identification of these endophytic fungi, especially in the genus *Trichoderma*, may be explored thoroughly by using the online-based identification or TrichOKEY (<http://www.isth.info/tools/molkey/>). The diagnostic was promoted due to the findings on unique oligonucleotide barcodes (hallmarks) residing within the internal transcribed spacer 1 and 2 (ITS1 and 2)

(Druzhinina et al. 2005). The genus-specific hallmarks (GSH) from each sequence were found to be present in different nucleotide sites (anchors) as presented in Table 7. Based on this identification, isolate Ee01 was designated as *Hypocrea lixii* or *T. harzianum* in section I of Lixii-Captotron clade, while three remaining sequences namely Ee04, Ee05, and Ee07 were designated as *T. koningiopsis* in section XII of Rufa clade. Only isolate Ee06 which was designated as unidentified *Trichoderma* in the section of the similar XII of Rufa clade. In addition to TrichOKEY, is the web-based TrichoBLAST, by integrating the GenBank rDNA-ITS or multilocus database which have also been helping in the *Trichoderma*-related study (Kopchinsky et al. 2005). Although the sequence database (TrichOKEY) has been upgraded to additional *Trichoderma* species, there are still differences in identified species which must be confirmed through evolutionary analysis (Bissett et al. 2015). Phylogenetic trees were then constructed to discriminatively assign the identities to all endophytic fungal isolates.

#### Phylogenetic analysis

The maximum likelihood statistical method and Kimura-2 parameter model were used to construct the phylogenetic trees of the endophytic fungal isolates from *Etlingera elatior*. Here, we constructed two phylogenetic trees to divide between *Trichoderma* and *Pestalotiopsis* members (Figures 3 and 4). Based on the evolutionary distance and bootstrap value (BV)  $\geq 60\%$ , each isolate was reliably assigned to the species level of identification. Therefore, the isolate Ee01 was identified as *Trichoderma neotropicale* with an accession code: MT053420; isolate Ee02 as *Pestalotiopsis* sp (MT053421); the isolates Ee04 and Ee07, both identified as *T. koningiopsis* (MT053422, MT053425); isolate Ee05 as *T. caribbaeum* (MT053423); and isolate Ee06 as *T. gamsii* (MT053424).



**Figure 2.** Electrophoresis product of ITS-rDNA amplicons from antagonistic fungal isolates with product size  $\pm 600$  bp.

**Table 2.** BLASTn result of isolate Ee01 identification

Species	Isolate/strain	Accession code	Score	Query cover	Max identity	Source
<i>Trichoderma</i> sp.	GH40	MN602871.1	1086	100%	100.00%	Colombia
<i>Trichoderma netropicale</i>	CBS 130633	MH865818.1	1086	100%	100.00%	Peru <sup>a</sup>
<i>Trichoderma harzianum</i>	42315bDRJ	MF782822.1	1086	100%	100.00%	Poland
<i>Trichoderma</i> sp.	S259	MF076625.1	1086	100%	100.00%	Brazil
<i>Trichoderma harzianum</i>	Th.12	KJ010951.1	1086	100%	100.00%	Algeria
<i>Trichoderma</i> sp.	AM140-P1T5R	KT264464.1	1086	100%	100.00%	USA
<i>Hypocrea</i> sp.	AE-2013	KF746146.1	1086	100%	100.00%	Panama <sup>b</sup>
<i>Hypocrea</i> sp.	AE-2013	KF746100.1	1086	100%	100.00%	Panama <sup>b</sup>
<i>Trichoderma harzianum</i>	RSPG_28	KC478545.1	1086	100%	100.00%	Thailand
<i>Trichoderma harzianum</i>	CKP01	KC330218.1	1086	100%	100.00%	India

Note: <sup>a</sup> Vu et al (2019), <sup>b</sup> Higginbotham et al (2014)**Table 3.** BLASTn result of isolate Ee02 identification

Species	Isolate/strain	Accession code	Score	Query cover	Max identity	Source
<i>Pseudopestalotiopsis theae</i>	AXLKY2019015	MN202256.1	1090	100.00%	100.00%	China
<i>Pestalotiopsis</i> sp.	TAP18N011	LC427147.1	1090	100.00%	100.00%	Japan
<i>Pestalotiopsis kenyana</i>	BM4	MK910059.1	1090	100.00%	100.00%	China
<i>Pestalotiopsis kenyana</i>	PC4	MK910055.1	1090	100.00%	100.00%	China
<i>Pestalotiopsis</i> sp.	CQ1A2	KU252298.1	1090	100.00%	100.00%	China
<i>Pestalotiopsis</i> sp.	CQ1A1	KU252297.1	1090	100.00%	100.00%	China
<i>Pestalotiopsis</i> sp.	HUN1A4	KU252296.1	1090	100.00%	100.00%	China
<i>Pestalotiopsis</i> sp.	ZJ2A7	KU252295.1	1090	100.00%	100.00%	China
<i>Pestalotiopsis</i> sp.	HB2A5	KU252294.1	1090	100.00%	100.00%	China
<i>Pestalotiopsis</i> sp.	HB2A2	KU252291.1	1090	100.00%	100.00%	China

**Table 4.** BLASTn result of isolate Ee04 identification

Species	Isolate/strain	Accession code	Score	Query cover	Max identity	Source
<i>Trichoderma</i> sp.	SDAS203143	MK870992.1	1094	100%	100.00%	China
<i>Trichoderma</i> sp.	HZA8	MH624143.1	1094	100%	100.00%	China
<i>Trichoderma</i> sp.	HZA5	MH624140.1	1094	100%	100.00%	China
<i>Trichoderma dorotheae</i>	CBS 119089	MH863050.1	1094	100%	100.00%	New Zealand <sup>a</sup>
<i>Trichoderma koningiopsis</i>	CRM-RCH169	MK396898.1	1094	100%	100.00%	Mexico
<i>Trichoderma</i> sp.	CM-CNRG444	MK311190.1	1094	100%	100.00%	Mexico
<i>Trichoderma</i> sp.	yi1481_1	MH284844.1	1094	100%	100.00%	China
<i>Trichoderma</i> sp.	yi1440_1	MH284799.1	1094	100%	100.00%	China
<i>Trichoderma</i> sp.	Cg05	MF101380.1	1094	100%	100.00%	South Korea
<i>Trichoderma koningiopsis</i>	UFSMQ40	KX496883.1	1094	100%	100.00%	Brazil

Note: <sup>a</sup> Vu et al (2019)**Table 4.** BLASTn result of isolate Ee05 identification

Species	Isolate/strain	Accession code	Score	Query cover	Max identity	Source
<i>Trichoderma caribbaeum</i>	CBS 119093	MH863051.1	1335	100%	100.00%	Guadeloupe <sup>a</sup>
<i>Trichoderma</i> sp.	16 BRO-2013	KF367557.1	1322	100%	99.59%	Portugal <sup>b</sup>
<i>Trichoderma</i> sp.	15 BRO-2013	KF367555.1	1322	100%	99.59%	Portugal <sup>b</sup>
<i>Hypocrea koningii</i>	ATCC 64262	AJ301990.1	1322	100%	99.59%	Germany
<i>Trichoderma gamsii</i>	Clone 2	KM491887.1	1320	100%	99.59%	Portugal
<i>Trichoderma gamsii</i>	Clone 1	KM491886.1	1318	99%	99.59%	Portugal
<i>Trichoderma atroviride</i>	NG_13	HQ115671.1	1313	100%	99.32%	Austria <sup>c</sup>
<i>Trichoderma atroviride</i>	-	EF417482.1	1307	100%	99.19%	Iran
<i>Trichoderma koningii</i>	APSAC 01	KY886134.1	1303	100%	99.19%	India
<i>Trichoderma koningii</i>	S54	MF076590.1	1287	98%	99.18%	Brazil

Note: <sup>a</sup> Vu et al (2019), <sup>b</sup> Oliveira et al (2013), <sup>c</sup> Gorfer et al (2011)

**Table 5.** BLASTn result of isolate Ee06 identification

Species	Isolate/strain	Accession code	Score	Query cover	Max identity	Source
<i>Trichoderma</i> sp.	HSrS3-7-MR	MK793785.1	1094	100%	100.00%	Mexico
<i>Trichoderma</i> sp.	RS3-4-MA	MK793784.1	1094	100%	100.00%	Mexico
<i>Trichoderma</i> sp.	RS3-3-MA	MK793783.1	1094	100%	100.00%	Mexico
<i>Trichoderma gamsii</i>	TRS125	KP009326.1	1094	100%	100.00%	Poland
<i>Trichoderma gamsii</i>	Clone 2	KM491887.1	1094	100%	100.00%	Portugal
<i>Trichoderma gamsii</i>	Clone 1	KM491886.1	1094	100%	100.00%	Portugal
<i>Trichoderma gamsii</i>	TJG4	KJ652466.1	1094	100%	100.00%	China
<i>Trichoderma gamsii</i>	CQBN3005	JQ040342.1	1094	100%	100.00%	China
<i>Trichoderma gamsii</i>	ICC080	GQ351597.1	1094	100%	100.00%	Spain
<i>Trichoderma gamsii</i>	MIAE00029	HM176559.1	1094	100%	100.00%	France <sup>a</sup>

Note: <sup>a</sup> Anees et al (2016)

**Table 6.** BLASTn result of isolate Ee07 identification

Species	Isolate/strain	Accession code	Score	Query cover	Max identity	Source
<i>Trichoderma</i> sp.	SDAS203143	MK870992.1	1094	100%	100.00%	China <sup>a</sup>
<i>Trichoderma</i> sp.	AA-2019	MH624143.1	1094	100%	100.00%	China
<i>Trichoderma</i> sp.	AA-2019	MH624140.1	1094	100%	100.00%	China
<i>Trichoderma dorotheae</i>	CBS 119089	MH863050.1	1094	100%	100.00%	New Zealand <sup>b</sup>
<i>Trichoderma koningiopsis</i>	CRM-RCH169	MK396898.1	1094	100%	100.00%	Mexico
<i>Trichoderma</i> sp.	CM-CNRG444	MK311190.1	1094	100%	100.00%	Mexico
<i>Trichoderma</i> sp.	yi1481_1	MH284844.1	1094	100%	100.00%	China
<i>Trichoderma</i> sp.	yi1440_1	MH284799.1	1094	100%	100.00%	China
<i>Trichoderma</i> sp.	Cg05	MF101380.1	1094	100%	100.00%	South Korea
<i>Trichoderma koningiopsis</i>	UFSMQ40	KX496883.1	1094	100%	100.00%	Brazil

Note: <sup>a</sup> Hu et al (2019), <sup>b</sup> Vu et al (2019)

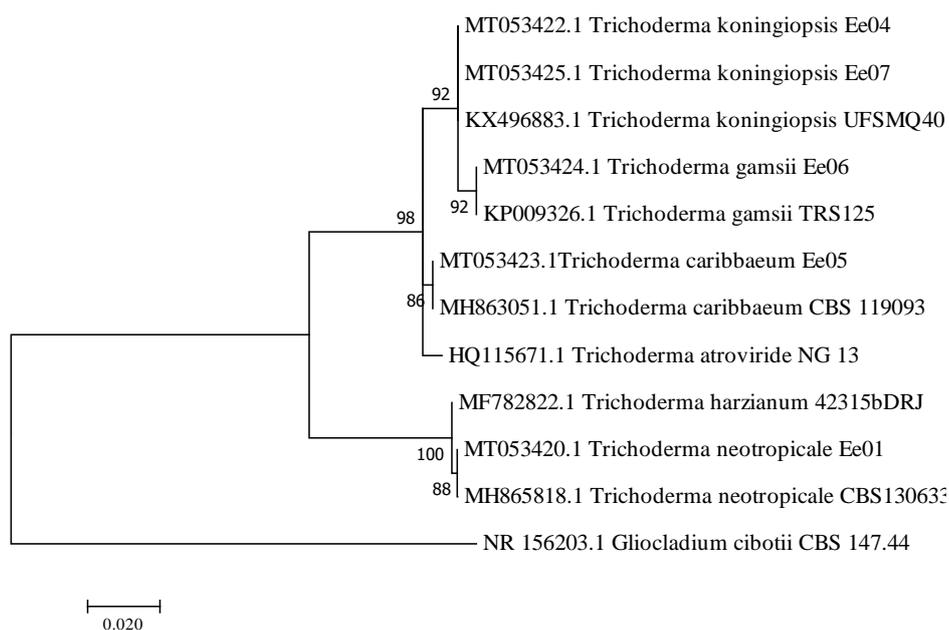
**Table 7.** TrichoKEY result of *Trichoderma* isolates identification

Isolate	Species	Genus-Specific Hallmarks (GSH) position				
		1 <sup>st</sup> Anchor	2 <sup>nd</sup> Anchor	3 <sup>rd</sup> Anchor	4 <sup>th</sup> Anchor	5 <sup>th</sup> Anchor
Ee01	<i>Hypocrea lixii</i> / <i>Trichoderma harzianum</i>	71	93	254	412	512
Ee04	<i>Trichoderma koningiopsis</i>	86	107	253	411	513
Ee05	<i>Trichoderma koningiopsis</i>	87	108	254	412	514
Ee06	Unidentified/ <i>Trichoderma</i> sp.	83	104	251	409	511
Ee07	<i>Trichoderma koningiopsis</i>	86	107	253	411	513

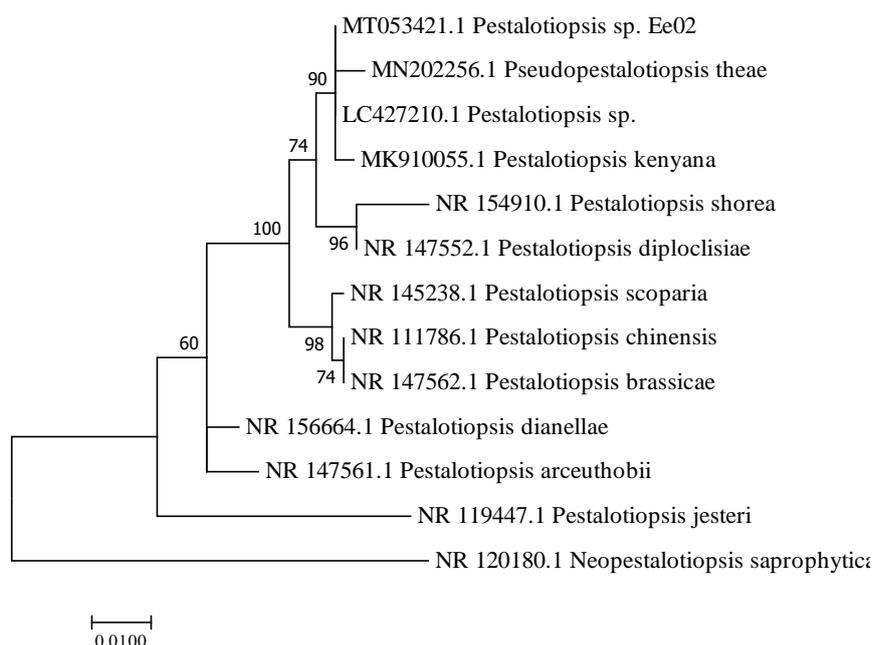
Isolation of *Trichoderma* isolates/strains from several medicinal plants has been reported from various plant host species. *Trichoderma* strains were known to colonize several parts of *Tinospora cordifolia*, *Piper nigrum*, and *Hedychium coronarium*, some representatives of medicinal plants used in Western Ghats, India. The isolated strains secreted extracellular enzymes, i.e. asparaginase and cellulase that might lead to industrial bioprospecting study in the future (Uzma et al. 2016). *Trichoderma piluliferum* isolated from a medicinal plant, *Bauhinia forficata* from Brazil was also known to secrete other extracellular enzymes, i.e. xylanase and lipase (Jadson et al. 2015). *Trichoderma koningiopsis* isolated from *Artemisia argyi* from China was known to synthesize and secreted novel fungal polyketides in the culture extracts. The polyketide compounds exhibited antibacterial activity against human pathogen, *Escherichia coli*, and antifungal activities against agro-pathogenic fungi (Shi et al., 2017). *Trichoderma atroviride* PG87 isolated from *Panax ginseng* significantly

reduced the disease incidence by the ginseng pathogenic fungi, the *Botrytis cinerea*, and *Cylindrocarpon destructans*, (Park et al. 2018).

A similar potential strain of *Trichoderma* was also obtained from soil samples of Andaman and Nicobar Islands, India. All isolates produced significant inhibitions (CGI>50 %) to mycelium of *Sclerotium rolfsii*, a root and foliar phytopathogen in a dual culture plate assay (Kumar et al. 2012). The assemblage of dominantly *Trichoderma* in the rhizome of *E. elatior* was considered logical since the lifestyle of these fungi as common soil inhabitants with high population in nature. A comprehensive review has stated upon their use as a biological control agent in suppressing phytopathogenic fungi either in laboratory or field study. Members of *Trichoderma* are known as prominent Biological Control Agent (BCA) through several mechanisms: plant growth promotion, systemic-acquired resistances, fast colonization, antifungal production, and mycoparasitism (Mukesh et al. 2016).



**Figure 3.** Phylogenetic tree of *Trichoderma* isolates and its members based on rDNA-ITS sequence similarity



**Figure 4.** Phylogenetic tree of *Pestalotiopsis* isolate Ee02 and its based on rDNA-ITS sequence similarity

A study reported the laboratory test of *Trichoderma* sp. strain T11 isolated from the rhizosphere of *Zingiber officinale*. The mechanism proposed in explaining the mycelium inhibition of *F. oxysporum* is through the synthesis of chemo-attractants by *Trichoderma* followed by rapid colonization or mycoparasitism towards *F. oxysporum* colony (Sharma 2011). In addition, the dominantly isolated *Trichoderma* spp. were also reported from *Elettaria* sp. from North Sumatrar revealing their high

population as soil mycoflora (Munir et al. 2019). Our finding on their colonization in *E. elatior* is a new report that may reveal further aspects in supporting the hardy nature of Zingiberacean species to exist in the disturbed habitat (Habsah et al. 2000).

The genus *Pestalotiopsis* exists as a dominant endophytic fungus in the world's rainforest with potential producer of a diverse chemical compounds, mostly known as antibacterial compounds (Deshmukh et al. 2014). The

assemblage of endophytic *Pestalotiopsis* species from plants of Podocarpaceae, Theaceae, and Taxaceae was documented in Southern China. The study concluded that *Pestalotiopsis* species was not generally specific to host plants explaining their diversity in temporal and spatial scale (Wei et al. 2007).

Both genera, *Pestalotiopsis* and *Trichoderma* obtained from our study has been reported as potential BCA against phytopathogenic fungi. A field study has been conducted to assess the potential application of *Pestalotiopsis* and *Trichoderma* isolated from cacao and cupuac trees. The strains successfully colonized the axenic culture of cacao plantlets and produced a lesser diseased plants in comparison to a positive control (Hanada et al. 2010). A key feature in successful *Trichoderma* application as BCA is influenced by its greatest quantity or former presence in soils. Control of damping-off in tobacco plants caused by *Rhizoctonia solani* is reported the best by applying the highest inoculum (log 4 CFU/g) of *Trichoderma* strains with sowing (Gveroska 2013). Hence, a field study in proving the effectiveness of our isolated strains as BCA is needed to assess their efficacies.

The relationship among specific strains with their hosts may be revealed through comprehensive studies. Exploration of specific interaction of *Trichoderma* species so far has been reported with Cupressaceae. The *Trichoderma* strains demonstrated significant bioactivities leading to novel agrochemical/drug discovery and as potential BCAs in the future (Hosseyini-Moghaddam and Soltani 2014). This part of our study will also further evaluate and reveal the maximum potency of the diverse endophytic fungi of Zingiberaceae as a potential biocontrol agent for the application in agriculture.

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