Records of Culturable Endophytic Fungi Inhabiting Rhizome of *Elettaria* in Hutan Sibayak, North Sumatera

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Records of Culturable Endophytic Fungi Inhabiting Rhizome of *Elettaria* in Hutan Sibayak, North Sumatera

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Abstract. Endophytic microorganisms are microbial associates living in various part of host, yet expressing benefits to healthy plants. Existence of species-specific endophytes, endophytic fungi is still poorly studied especially in Zingiberaceae. In this study, we reported several endophytic fungal species isolated from rhizome of *Elettaria* through isolative efforts and molecular evidence. Host plant, *Elettaria* sp. was sampled from representative natural area, i.e. Hutan Sibayak which is known as biodiversity spot for Zingiberaceae in North Sumatera. Molecular identification revealed the identity of five isolated fungal strains collected from rhizomes of *Elettaria*, namely *Trichoderma atroviride, Curvularia lunata, Schizophyllum commune, Trichoderma harzianum* and *Pholiota multicingulata*. Phylogenetic tree is constructed based on Neighbor-joining method in a bootstrap test (1000x replication) with outgroup and database retrieved from NCBI GenBank. From our perspective, this is the first report on finding fungal endophytes from rhizomes of *Elettaria* sp. yet further investigation is needed to evaluate their future bioprospectives.

1. Introduction

Existence of fungal associates in host plant or endophytes have drawn many attention to researchers recently. Many studies reported the beneficial use of endophytes in producing valuable natural compounds similar to host while contributing to the ease of extraction and bulk sample preparation. [1,2]. Zingiberaceae is one of medicinal plants in Indonesia with many health benefits and prospects of development. Due to its long history of ethnobotanical medicine, it is worth to study the fungal endophytic associates residing within the most utilized parts that is rhizome [3,4]. In North Sumatera, Hutan Sibayak is reported to have the most diverse of Zingiberacean species [5].

A number of investigations have reported the occurrence of culturable endophytic microorganisms from Zingiberaceaen species, mostly from fungi. All parts of *Zingiber officinale, Hedychium flavescens* and *H.coronarium* were known to be a habitat for fungal endophytic genera such as: *Aspergillus, Bipolaris, Cladosporium, Alternaria, Curvularia, Mucor, Penicillium, Nigrospora, Colletotrichum,* and *Pithomyces.* Each fungal endophytes were screened for their extracellular enzyme activities yet some of them were potential enzyme producers [6]. Nine fungal endophytes, i.e. *Fusarium oxysporum, Colletotrichum alienum, C.aotearoa, C.ti, C.coccodes, C.gloeosporoides* and *Aspergillus parasiticus* were isolated from *Hedychium acuminatum* producing antimicrobial activities [7]. Other study reported culturable fungal endophytes from Indonesian Red Ginger (*Zingiber officinale*), i.e. *Acremonium, Cochliobolus, Curvularia, Fusarium, Glomerella, Lecanicillium,*

Leiosphaerella, Myrothecium, Neonectria, Periconia, Rhizopycnis and *Talaromyces* to be differently colonizing plant parts. Majority of isolates were potential antagonists against *F.oxysporum* [8].

Furthermore, four cultivars of *Zingber officinale* cultivated from Rio de Janeiro and Vellayanikkara were known to harbor different fungal endophytes intraspecifically: *Acremonium*, *Gliocladiopsis*, *Fusarium*, *Colletotrichum*, *Aspergillus*, *Phlebia*, *Earliella*, and *Psuedolagarobasidium*. The pattern showed an evidence of spatial or cultivar-specific endophytic assemblages in same host [9].

A preliminary study has been conducted to evaluate the antagonistic fungal endophytes from rhizomes of Zingiberaceae. Five culturable isolates were recovered from rhizome parts of *Elettaria*. By our understanding, the genus *Elettaria* is still limited in information regarding their use as medicine and its fungal associates. In this study, we reported the existence of five species according to molecular evidence that may lead the further investigation upon their biological application.

2. Materials and Methods

2.1. Isolation of culturable fungal endophytes from rhizome of Elettaria

The plant materials used in this study were specimens of *Elettaria* rhizome collected accidentally from Hutan Sibayak. Standard surface-sterilization methodology is used in isolation step [10]. Subsequent sterilization was performed in following solutions: 75% ethanol (2 min), 5.3% NaOCl (5 min) and 75% ethanol (30 secs). Rhizome parts were plated on top of Potato Dextrose Agar (Oxoid™) supplemented with 0.1% commercial chloramphenicol to prevent bacterial contamination. Plates were incubated in room temperature for 7 d. Observation of growing mycelial was conducted daily and each mycelial parts were sub-cultured into new PDA to make a pure colony.

2.2. Molecular identification of fungal endophytes

Extraction procedure of fungal DNA genome is based on Wizard® Genomic DNA Purification Kit Protocol (United States). Dried mycelium were crushed and 0.5 g dissolved in SDS Tris-HCl buffer pH 8.0 (600 µL) and phenol:chloroform (600 µL). Mixtures were centrifuged at 10.000 ×g for 45 min at 4 ^{o}C. Series of solvents were added: chloroform and cold iso-propanol followed with further centrifugation. Pellets containing DNA genome were dissolved in TE buffer (100 µL). DNA quality was assessed ratio of DNA : protein content ($A_{260/280}$ and $A_{260/230}$) in spectrophotometer.

Amplification of ITS-DNA region using primer ITS-1F (5'-CTTGGTCATTTAGAGGAAGTAA-3) and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') [11] with reaction mixture composed of: 10× PCR buffer 5 µL, 1 µL 10 mM ddNTP, 10 µL Q solution, 2,5 µL 0,1–0,5 µM primer, 0,25 portion of 1,5 unit Taq DNA polymerase, 2 µL DNA template with total volume of 50 µL in eppendorf tube. Specification of PCR reaction using thermal cycler: Pre-denaturation at 93°C for 3 min, Denaturation at 94°C for 30 sec, Annealing at 53°C for 45 sec, Elongation at 72°C for 10 min, and Final extension at 72°C for 10 menit with 35 cycles. Visualization of ITS-DNA amplicons were assessed under UV visualization on agarose electrophoresis within gel-doc. The ITS-DNA amplicons were sequenced commercially to Macrogen, Inc. Korea.

2.3. Bioinformatics study

Molecular identity from each ITS-DNA sequences of five isolates were analyzed. DNA sequences were compared with other fungal ITS sequences retrieved from *National Centre for Biotechnology Information* (NCBI) databases. The sequences were checked using *Basic Local Alignment Search Tool* for nucleotide (BLASTn) Sequence pools were aligned using MUSCLE in software MEGA6.0 [12,13]. Phylogenetic tree is constructed based on neighbor-joining method with bootstrap replication 1000x [14,15].

3. Results and Discussions

We successfully amplified the ITS-DNA region of five fungal endophytes isolated from *Elettaria.* The five isolates namely JRS1A, JRS1B, JRS1C, JRS2A, JRS2B were potential antagonists assayed in

other study (data not shown). The ITS-DNA bands are estimated to size ± 500 bp (Figure 1). Visualization under UV illumination on agarose electrophoresis revealed the quality of clear and compact bands from each lanes. The ITS-DNA extracts were then sequenced commercially.

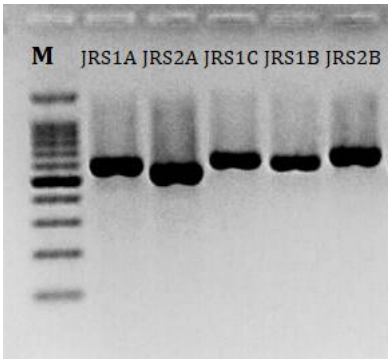

Figure 1 Visualisation of ITS-DNA bands under UV illumination (M = Marker, 1000 bp)

Bioinformatics analysis among inter-related species was performed using the NCBI database on fungal ITS region. List of fungal ITS region along with their taxa retrieved from NCBI database is presented in Table 1. The sequence of *Fusarium nurragi* and *Penicillium oxalicum* were used as outgroup in the phylogenetic constructions. The phylogenetic relationship among isolates and databases is presented in Figure 2. The phylogenetic trees is considered representative based on overall bootstrap confidence value ≥ 70% as suggested from previous study.

Two clades namely *Curvularia-Schizophyllum-Pholiota* and *Trichoderma* groups are formed based on the phylogenetic construction. The placement of each isolates indicated a new identity in the level of strains. Therefore, isolate JRS1A is designated as *Trichoderma atroviride* strain JRS1A; Isolate JRS1B is designated as *Curvularia lunata* strain JRS1B; Isolate JRS1C is designated as *Schizophyllum commune* strain JRS1C; JRS2A is designated as *Trichoderma harzianum* strain JRS2A; and isolate JRS2B is designated as *Pholiota multicingulata* strain JRS2B.

Table 1. List of taxa and GenBank accession numbers of ITS sequences from NCBI database

No	Taxa	Accession Number
1	*Curvularia geniculata* strain P1227	KU23939.1
2	*Curvularia geniculata* strain P1224	KU23938.1
3	*Curvularia geniculata* strain P1194	KU23937.1
4	*Curvularia palmicola* isolate MFLUCC 14-044	MF621582.1
5	*Curvularia lunata* isolate FR17	KP689195.1
6	*Curvularia lunata* isolate PSU-ES195	JN116704.1
7	*Curvularia verruculosa* strain PSU-ES60	JN116620.1
8	*Fusarium nurragi* strain ATCC 200255	AB586921.1
9	*Penicillium oxalicum* strain IODB-F5	KM972407.1
10	*Pholiota spumosa* strain Ps1311013	AB985276.1
11	*Pholiota multicingulata* strain NY108	KM216337.1
12	*Pholiota multicingulata* strain	KX773884.1
13	*Schizophyllum commune* strain E25F	KY425733.1
14	*Schizophyllum commune* isolate HBM2	KU726504.1
15	*Trichoderma atroviride* strain ACCC32867	MF871524.1
16	*Trichoderma atroviride* strain HZA13	MH624148.1
17	*Trichoderma atroviride* strain CBS 110086	MH862849.1
18	*Trichoderma eijii* strain TUFC 100002	JX238476.1

19	*Trichoderma harzianum* strain 11392	KX379189.1
20	*Trichoderma harzianum* strain LIPIMC5064	KC847177.1
21	*Trichoderma nothescens* strain GJS 99-142	DQ315427.1

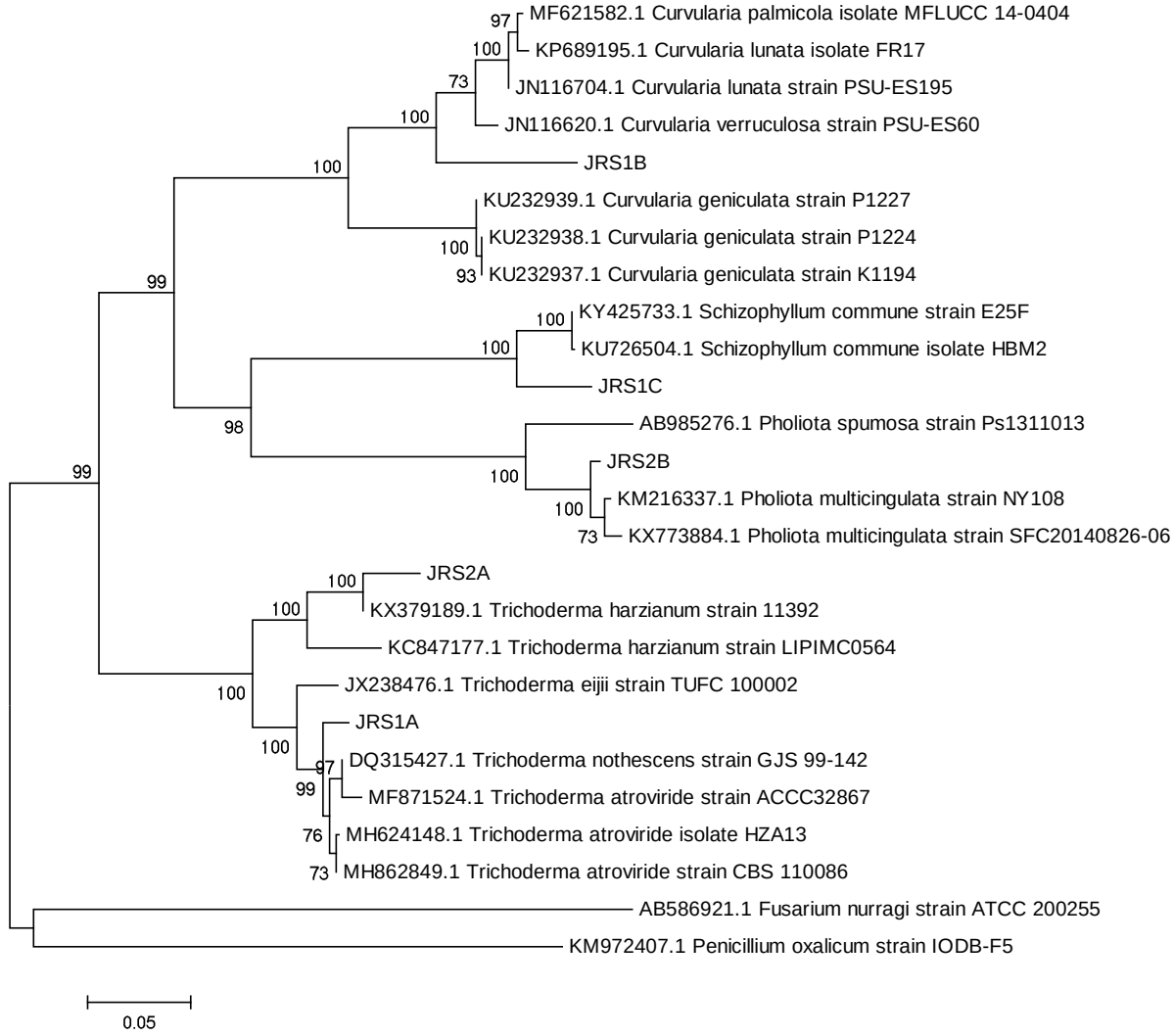

Figure 2. Phylogenetic relationship among fungal endophytes of *Elettaria* in Hutan Sibayak

The genus *Trichoderma* is commonly soil organisms that colonize plant roots, yet contributing a symbiotic relationship with plants. Both *T.harzianum* and *T.atroviride* are known as potential rhizospheric fungi exhibiting many mechanisms of antagonisms i.e. antifungal enzyme production, mycoparasitism and competitive exclusion [16]. Entry passage from roots may indicate for their presence into rhizomes of *Elettaria*.

The genus *Schizophyllum* is a basiodiomycetous fungi reported to harbor plant organs as well as endophyte and producing secondary metabolites related to host. Isolated *S.commune* from *Panax* ginseng was able to enhance the biomass and content of ginsenoside through synthesis of intermediates. The strain formed a stable relationship within hairy roots in parenchyma cells of *Panax* [17]. A preliminary study also revealed its potential as proteolytic enzyme producers namely *S.commune* strain JF766994 isolated from *Piper hispidum* [18]. However, evidence also showed that the species may confirm for its identity as emerging human pathogens in recent findings [19]. Further characterization is needed to confirm the safety use of our strain in future study.

The species, *Curvularia lunata* is mostly known as phytopathogen. *Curvularia lunata* greatly caused low productivity of maize cultivation and production worldwide. The disease is called Curvularia leaf spot and many studies are conducted to combat the infection [20]. Despite on its pathogenicity, a study also revealed for its potency in producing bioactive metabolites from its endophytic association with host. A study reported the antimicrobial properties of broth extract of *C.lunata* isolated from *Cymbopogon caesius.* The broth extracts are highly effective against *Staphylococcus aureus* and *Candida albicans* [21]. The species is also reported to produce bioinsecticide compound against *Spodoptera litura,* one of the economically important pest [22]. Bioprospective study upon our *C.lunata* strain may be seen as promising step in future study.

The genus, *Pholiota* is a basiodiomycetous fungi with limited information upon its symbiotic relationship as endophytes, especially on *Pholiota multicingulata*. However, reports on its bioactive metabolites with benefit to health perspectives are numerous. Most studies reported the utilization of polysaccharides extracted from biomass of certain *Pholiota* species. Polysaccharides extracted from *P.dinghuensis* mycelium exhibited a high antiproliferative activity against human gastric cancer cells *in vitro*. In addition, the extracts was known as anti-tumor agent [23]. Polysaccharides extracted from *P.nameko* mycelium exhibited a hypolipidemic effect toward tested hyperlipidemic wistar rats. Yet, the extracts also enhance the synthesis of antioxidative enzymes (SOD, CAT and GSH-Px) leading to lower incidence of obesity [24]. Another study also reported the 60% methanolic extract of *P.adiposa* exhibiting antimicrobial activity against *Pseudomonas aeruginosa* and *Salmonella typhimurium* while no activity reported against yeasts [25]. Further investigations are needed to evaluate our strain regarding the production of bioactive metabolites in the future.

4. Conclusions

Five culturable fungal endophytes namely *Trichoderma atroviride, T.harzianum, Curcularia lunata,Pholiota multicingulata* and *Schizophyllum commune* are reported to harbor the rhizome of *Elettaria* cultivated in Hutan Sibayak, North Sumatera. The fungal endophytes may be exploited in many aspects for further investigation

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