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Antagonistic Fungal Endophytes Colonizing Rhizome of *Amomum centrocephalum* A.D. Poulsen from North Sumatera, Indonesia

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***Abstract*— Endophytic fungi were successfully isolated from healthy rhizomes of the wild ginger *Amomum centrocephalum* A.D. Poulsen from Hutan Sibayak, North Sumatera, Indonesia. This study aimed to evaluate the antagonistic properties of isolated endophytic fungi against selected pathogenic bacteria and phytopathogenic fungi assessed by dual culture plate assay. Methanolic extract of *A. centrocephalum* rhizome was also tested as a control. Nine morphotypes of endophytic fungi were differentiated morphologically with further molecular identification using ITS rDNA sequencing for potential antagonistic strains. Most isolates showed considerable inhibitory activities towards microbial indicators than MeOH extract of *A. centrocephalum*. All strains showed distinct inhibitory activities against bacterial pathogens. The strongest activity was shown against gram-positive, *Staphylococcus aureus* ATCC 29213™ followed by gram-negative, *Escherichia coli* ATCC 25922™ while there was no evidence of inhibition against clinical yeast strain, *Candida albicans*. All strains were known as prominent antagonists of phytopathogens against *Fusarium oxysporum, Ganoderma boninense,* and *Rigidoporus ligneous*. The five potential strains were identified as *Aspergillus aculeatus, Clonostachys rosea, Daldinia caldariorum*, and two strains of *Trichoderma*. Hence, we reported the newly found species of endophytic fungi colonizing rhizomes of Sumatran Zingiberacean species with prospects upon finding novel metabolite compounds combating bacterial infection and plant diseases.**

***Keywords*— *Amomum centrocephalu;* antagonism assay; endophytic fungi; medicinal plants; zingiberaceae.**

I. INTRODUCTION

Emerging microbial infections have caused demands for new antibiotics findings. Finding chemotherapeutic agents combating those infections is currently ongoing [1]. Discoveries in the field of biogenic drugs or natural compounds as antibiotics from various biological sources are currently appreciated due to its minor effects, contributing to lower incidence of microbial resistance [2], [3]. Medicinal plants and their fungal associates are reported as a potential warehouse of bioactive metabolites [4]–[6].

Endophytes, in specific of endophytic fungi, are one of the microbial inhabitants of plant tissues. They colonize either the intercellular or intracellular site of plant tissues within healthy hosts. The symbiosis between endophytes and hosts is considered as a mutualistic relationship conferring many benefits for both partners, by promoting growth, suppressing phytopathogens and expressing resistance to host plants under environmental stressors [7]–[9]. Endophytes are microorganisms that are currently becoming a promising source of novel bioactive compounds [1], [10].

Endophytic fungi may be isolated from well-known medicinal plants exerting the similar metabolites or biological properties from the host. The rationale upon selecting those plants was evidential through their uses in tribal medicine or traditional remedies [4]. Members of the Zingiberaceae family are widely cultivated medicinal plants in Indonesia with historical uses such as ethnobotanical formulations to treat many health disorders and ailments [11]–[13]. Medicinal properties exerted from these plants were reported due to their occurring natural products stored in various plant organs, i.e., foliar, stems, inflorescences, and rhizomes. Many members of Zingiberaceae have been reported to exert biological properties through laboratory studies, exhibiting properties as anti allergics, anthelmintics, anti-inflammations, antimicrobials, antioxidants, cytotoxic, chemopreventive and larvicidal [14]–[17].

Amomum centrocephalum A.D. Poulsen is a species in the Zingiberaceae family with fewer studies on its bioprospection and microbial properties. Due to its relevant taxonomic relationship among species in Zingiberaceae, *A. centrocephalum* is assumed to host a diverse fungal strain within the organs, especially the healthy rhizome. A previous study has reported an assemblage of endophytic fungi comprising 7 Ascomycetes and 26 mitosporic fungi from *Amomum siamense* in Thailand. Dominant genera found in that study were *Colletotrichum, Glomerella,* and *Phomopsis* spp. [18]. This study reported the existence of

fungal endophytes in rhizome of *A. centrocephalum* that were antagonists to selected pathogenic bacteria and phytopathogenic fungi. The results will be used as our preliminary investigation to assess the production of antimicrobial metabolites by potential strains in the future.

II. Materials and Method

A. Plant Material

Samples of wild Zingiberaceae were collected during exploration in Hutan Sibayak, located in Deli Serdang District, North Sumatera. Sampling was conducted incidentally without considering any climate and spatial factors. Plants anchoring to soils were dug up and cut to separate its shoots and roots. The root or rhizome was wrapped with paper and stored in plastic bags. Duplicate samples were collected separately to be authenticated by the Herbarium Medanese, Universitas Sumatera Utara for identification. In the laboratory, rhizomes were later cut into smaller segments and composites were made by pooling segments into one bulk sample for each species of Zingiberaceae. The samples were then used in the isolation step.

B. Isolation of Endophytic Fungi

The isolation of fungal endophytes were based on steps from a previous report [19]. Bulk samples from each species of Zingiberaceae were washed with tap water to remove remaining soil and dirt. The samples were surface-sterilized by dipping in 75% ethanol for 2 min, 5.3% NaOCl for 5 min, and 75% ethanol for 30 secs. The pieces were again washed several times with sterile distilled water to remove remaining solutions. Samples were dried on Whatman filter paper and cut into 1–2 smaller pieces. The pieces were placed on top of Potato Dextrose Agar (Oxoid™) supplemented with chloramphenicol. Plates were incubated in ambient condition for 3 days. Any visible fungal growth from each piece was then sub-cultured onto new medium to preserve the strains. Each fungal strain was visibly differentiated from their colony.

C. Antagonism assay against Pathogenic Bacteria

Pathogenic bacterial strains used in this study were: *Staphylococcus aureus* ATCC® 29213™, *Escherichia coli* ATCC® 25922™ and a clinical strain of *Candida albicans* from the Hospital of Universitas Sumatera Utara, Medan, Indonesia. Both *S. aureus* and *E. coli* were first grown in Nutrient Agar (NA) while *C. albicans* was grown in Potato Dextrose Agar (PDA) prior to antagonisms assay. Antagonisms assay was performed based on the agar plug method in dual culture plate assay [20].

Direct colony suspensions from each pathogenic strains were made by swabbing colonies into a sterile physiological saline solution (0.95% NaCl) to obtain OD_{600} = 0.5. One mL of cell suspension was mixed with 15 mL of molten PDA (45^{o}C), supplemented with 1% (w/v) yeast extracts for bacteria and 1% peptone (w/v) for *C. albicans*. The molten agar medium was then plated to obtain microbial lawns. Three plugs of aerial mycelium from each fungal endophytes were placed on top of medium as triplicates. The plates were incubated for 2 days in ambient condition. Clear zones indicating inhibitory activities around mycelial plugs indicating antagonism were measured using standard caliper in millimetre unit (mm). Each inhibition zone (IZ) was categorized into four arbitary levels of antagonisms: Very Strong (++++) IZ > 30 mm, Strong (+++) 30 ≥ IZ > 20 mm, Mild (++) 20 ≥ IZ > 10 mm, Weak (+) 10 ≥ IZ > 0 mm, and None (–) IZ = 0 mm.

D. Antagonism assay against Phytopathogenic Fungi

Phytopathogenic fungal species used in this study were: *Fusarium oxysporum*, *Ganoderma boninense* and *Rigidoporus lignosus*, collections from the Laboratory of Microbiology, Department of Biology, Universitas Sumatera Utara, Medan, Indonesia. Phytopathogens were first grown in PDA to obtain fresh mycelial plugs. Mycelial plugs of phytopathogens were then planted three days in advance at the center of a new PDA medium, followed by agar plugs of endophytic fungi in antagonism assay. Assay was performed in triplicates. Plates were incubated for 7 days at a room temperature. Colony Growth Inhibition (CGI) of phytopathogenic fungi was calculated using the formula [21]:

$$CGI\ (\%) = \frac{(R_1 - R_2)}{R_1} x100$$

Where, CGI is the percentage (%) of colony growth inhibition. R_1 represents the diameter of phytopathogens colony growth in the absence of fungal antagonist and R_2 represents the diameter of phytopathogens from the direction of the antagonist. Each CGI result was again categorized into four arbitary levels of antagonism: Very Strong (++++) CGI > 75 %, Strong (+++) 75 ≥ CGI > 50 %, Mild (++) 50 ≥ CGI > 25 mm, Weak (+) 25 ≥ CGI > 0 %, and None (–) CGI = 0 %.

E. Extraction of Rhizome Metabolite

The extraction procedure performed in this experiment was maceration. Ten grams of shade-dried rhizome of *A. centrocephalum* were placed into 250-mL flask prior addition of 100 mL (1:10, w/v) of MeOH. The flask was agitated on an orbital shaker at 120 rpm for 5 days. After agitation, macerates were filtered through cotton wrapped muslin cloth to separate extracts and debris. The extracts were then passed through a filter paper (Whatman No.1). The extracts were further concentrated in a rotary-evaporator under reduced pressure at 45^{o}C. The crude extracts were obtained and dissolved 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 under similar levels of antagonism as previously described.

F. Molecular Identification of Potential Endophytic Fungi

Extraction procedure of fungal DNA genome is based on Wizard® Genomic DNA Purification Kit Protocol (United States). Dried mycelium were crushed and 0.5g dissolved in SDS Tris-HCl buffer pH 8.0 (600 µL) and Phenol: Chloroform (600 µL). Mixtures were centrifuged at 10,000xg for 45 min at 4^{o}C. Chloroform and cold iso-

propanol were added as solvents followed with further centrifugation. Pellets containing DNA genome were dissolved in TE buffer (100 µL). The DNA quality was assessed by observing the ratio of DNA: protein content ($A_{260/280}$ and $A_{260/230}$) in a spectrophotometer.

The ITS-DNA region was amplified using ITS-1F (5'-CTTGGTCATTTAGAGGAAGTAA-3) and ITS-4R (5'-TCCTCCGCTTATTGATATGC-3') primer [22] with a 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 in an Eppendorf tube with a total volume of 40 µL. Specification of PCR reaction using thermal cycler: Pre-denaturation at 95°C for 3 min, Denaturation at 95°C for 45 sec, Annealing at 55°C for 45 sec, Elongation at 72°C for 45 sec, and Final extension at 72°C for 7 min with 35 cycles. ITS-DNA amplicons were assessed using UV visualization on agarose electrophoresis within gel-doc. The ITS-DNA amplicons were sequenced commercially to Macrogen, Inc. Korea.

G. Bioinformatics Analysis

Molecular identity from each ITS-DNA sequences of five isolates was 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 [23], [24]. Phylogenetic tree is constructed based on neighbor-joining method with bootstrap replication 1000x [25], [26].

III. Results and Discussion

A. Endophytic Fungi Isolates from Amomum centrocephalum Rhizome

Wild specimens of *A. centrocephalum* were sampled from Sibayak forest, as one of representative natural habitat in North Sumatera (Fig.1). The total of forest area is around 7,030 ha and is known to be inhabited by several plant families, i.e., Araceae, Balsaminaceae, Begoniaceae, Gesneriaceae, Pandanaceae, and Zingiberaceae. The number of Zingiberacean species previously reported from this area comprised of 23 species, grouped into members of *Amomum*, *Etlingera*, *Geocharis*, *Geostachys*, *Globba*, *Hedychium*, *Hornstedtia*, and *Zingiber* [27]. However, the *A. centrocephalum* species was not found during the previous report, indicating the need for re-exploring the forest area to find possible new records of species.

Zingiberaceae is the plant family widely distributed in Southeast Asia and is known for their resistances towards both biotic and abiotic stressors [28]. *Amomum centrocephalum* is not commonly known in Indonesia, contradicting from its relative, *Amomum compactum* or *kapulaga Jawa* which commonly used as spices. The chemical composition and essential oils from other species of *Amomum* have been extensively studied and reported to contain terpenoid compounds while still limited information upon *A. centrocephalum* both for its phytochemical and endophytic studies [29]–[31].

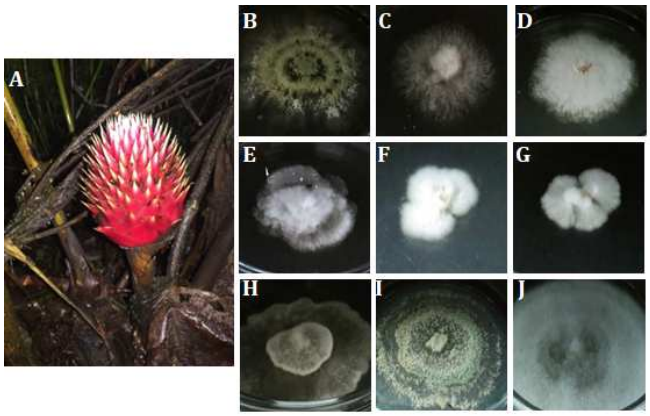

Fig. 1. (A) Wild *Amomum centrocephalum* rhizome sampled from Hutan Sibayak, North Sumatera, Indonesia; Endophytic fungi from *A. centrocephalum* cultured on PDA plates: (B) Am01SU, (C) Am02SU, (D) Am03SU, (E) Am04SU, (F) Am05SU, (G) Am06SU, (H) Am07SU, (I) Am08SU, (J) Am09SU

Nine morphotypes of endophytic fungi were successfully recovered from the healthy rhizome of *Amomum centrocephalum.* The most utilized part of Zingiberacean species is rhizome in the context of raw consumption as medicine and spice [15], [17], [32], [33]. However, the existence of endophytic fungi in the rhizome part of Zingiberaceae family may be different across members and cultivars. Different metabolite profiles of endophytic fungi were reported through the study of four *Zingiber officinale* cultivars collected from Rio de Janeiro and India, explaining varieties of host genotypes [34]. Assemblages of fungal community were also reported to be different among plant parts in one species. In *Hedychium coronarium*, the rhizome part exposed the greatest diversity of culturable endophytic fungi [35].

B. Antagonism Activity of Endophytic Fungal Isolates

From the screening result, five endophytic fungal isolates namely Am01SU, Am02SU, Am05SU, Am06SU and Am08SU were designated as potential bacterial antagonists against *S. aureus* while one isolate, Am05SU was known to inhibit the growth of *E. coli.* Isolate Am01SU and Am05SU displayed the strongest inhibitions against *S. aureus.* Generally, endophytic fungal isolates were able to inhibit phytopathogens. All strains were able to display mild to very strong inhibitory levels against *F. oxsporum*, followed by *G. boninense* and *R. lignosusi* (Fig.2). In comparison, the MeOH extracts of rhizome, were not able to exhibit strong inhibitory activities against pathogenic bacteria and phytopathogenic fungi. Categorization of antagonism levels from tested subjects is presented in Table 1.

TABLE I
RESULT OF ANTAGONISM ASSAY SHOWN BY ENDOPHYTIC FUNGI AND METHANOLIC EXTRACT (MEOH) OF *AMOMUM CENTROCEPHALUM* RHIZOME

Endophytic fungi	Antagonism levels					
	S. aureus[a)]	*E. coli*[a)]	*C. albicans*[a)]	*F. oxysporum*[b)]	*G. boninense*[b)]	*R. lignosus*[b)]
Am01SU	+++	–	–	+++	+++	+++
Am02SU	++	–	–	+++	+++	+++
Am03SU	–	–	–	+++	–	+++
Am04SU	–	–	–	++++	+	+++
Am05SU	+++	++	–	+++	++++	+++
Am06SU	+	–	–	+++	++++	–
Am07SU	–	–	–	+++	++	+++
Am08SU	++	–	–	+++	+++	+++
Am09SU	–	–	–	+++	–	+
MeOH	++	–	–	++	++	++

[a)]Antagonism levels based on Inhibition Zones (IZ) (mm): Very Strong (++++) IZ > 30 mm, Strong (+++) 30 ≥ IZ > 20 mm, Mild (++) 20 ≥ IZ > 10 mm, Weak (+) 10 ≥ IZ > 0 mm, and None (–) IZ = 0 mm
[b)]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 %.

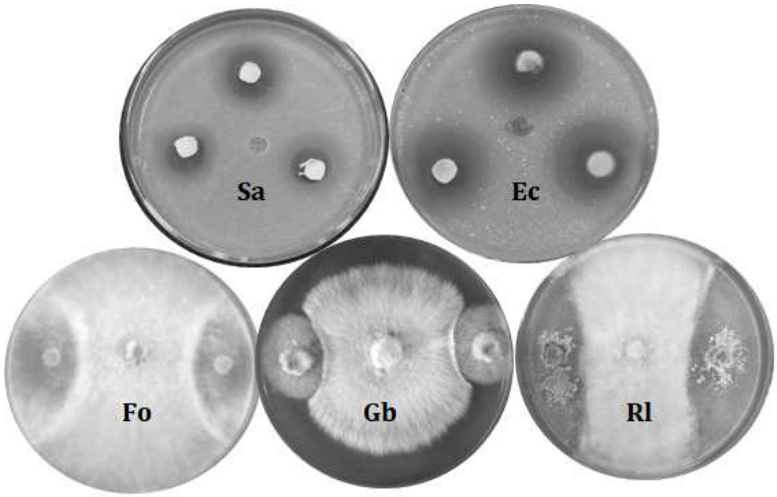

Fig. 2. Representative images of antagonism assay: Isolate Am05SU against *Staphylococcus aureus* ATCC® 29213™, Sa; and *Escherichia coli* ATCC® 25922™ Ec; Isolate Am01SU against *Fusarium oxysporum*, Fo; Isolate Am06SU against Ganoderma boninense, Gb; Isolate Am08SU against *Rigidoporus lignosus*, Rl.

Endophytic fungi is recently considered as a reservoir of antibacterials in emerging microbial infection [36]. Many antagonistic fungal endophytes have been studied extensively from Zingiberacean sources. Endophytic fungi isolated from *Hedychium acuminatum* were reported to produce antibacterial activities against gram-positive bacteria, *S.aureu,* and *B.subtilis* [37]. Secondary metabolites produced by an endophytic fungus, *Arthrinium* sp. MFLUCC16-1053 isolated from *Zingiber cassumunar* was reported to be effective against both gram-positive and gram negative pathogens with minimum inhibitory concentrations of 31.25 µg/mL against *S.aureus* [38]. Endophytic fungi isolated from red ginger (*Zingiber officinale*) were able to display inhibitory activities against *F.oxysporum* ranging from 1.4 to 68.8% of CGI [32].

The less inhibitory activities of the methanolic extract against *E.coli* and *C.albicans* also explained the ineffectiveness of most fungal isolates against similar pathogens. It is assumed that endophytic fungi may synthesize the similar compounds as host plants, which supports our ineffective results [5]. However, inhibitory activities of the methanolic extracts towards phytopathogens are somehow improved as shown by the results from endophytic fungi. This implies that exploration and further development of fungal isolates will improve the value of host species regarding the discovery of novel phytochemical compounds as antibiotics, in specific to *A. centrocephalum* along with any Zingiberacean species in the future.

C. Molecular Identities of Potential Endophytic Fungi based on ITS–DNA region

The amplified ITS-DNA fragments from each fungal isolates were visualized on 2% agarose gel electrophoresis. Amplification of DNA samples using ITS1-ITS4 primers revealed gene fragments of an approximately 600 bp (Fig.3**)**. Five potential fungal isolates based on their antagonistic performances were further identified using the ITS-DNA region. A dendrogram of the phylogenetic relationship was then constructed to show precise identity among species (Fig.4). The bootstrap value generated through 1000

replications for each isolate was varied from 70 to 100% and was then considered as correspondent to the real situation [39]. The identities of potential isolates were *Aspergillus aculeatus*, *Clonostachys rosea*, *Daldinia caldiriorum* and two strains of *Trichoderma*.

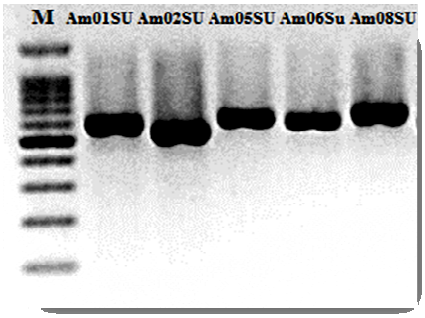

Fig. 3. Agarose gel electrophoresis (0.2% w/v) image of PCR products for ITS-DNA gene fragment. Lanes showing different fungal isolates with M: DNA marker size of 100 bp. Amplicons are estimated within 600 bp.

Aspergillus aculeatus was reported as rhizospheric fungi isolated from gram (*Cicer ariatenum*) [40]. The isolates were reported as rock phosphate solubilizers that maximize the absorption of nutrients in soil by plants. This may reflect future investigation upon its properties as phosphate solubilizing fungi that may also explain the adamant nature of Zingiberaceae in the nature.

Clonostachys rosea was previously isolated as endophytic fungi promoting the growth performance of certain crops. Improved crop performances of miniature roses, geraniums and cucumbers were observed through the inoculant use of *C. rosea* strain 88-710 [41]. Further study on this species revealed its use as biocontrol agents against plant nematode and phytopathogens. The anthelminthic properties of *C. rosea* have proven effective in controlling veterinarian parasite, *Haemonchus contortus* in sheep [42].

In another study, *C.rosea* was also reported to decrease the infection rate caused by *Fusarium graminearum* in soybean plant, showing a mycoparasitic habit (Pan et al., 2013). Integration of pesticide with *C. rosea* and *Trichoderma* spp were also reported to effectively control wheat foot rot caused by *Fusarium cumorum* [43].

Daldinia caldariorum is a species from Ascomycota, Xylariaceae. The genus, *Daldinia*, is known as prominent secondary metabolite producers as nematicidal and antimicrobials. Chemical profiles of potential metabolites were reported as promising agrochemicals from this fungi (Stadler et al., 2014). However, the *D. caldariorum* species is still limited in scientific information regarding the bioprospection study as well as endophytic associates in Zingiberaceae.

Trichoderma spp are common rhizospheric fungi that have been reviewed numerously under many scientific studies. The members are known as potential and effective bio-fungicide and notable phytopathogens [44]. Information on their existence as Zingiberacean endophytic associates have also been reported. *Trichoderma* isolated from *Hedychium coronarium* as one of the medicinal plants in the Western Ghats, India, was reported as a potential cellulase producer that may be used in industrial application [35]. *Trichoderma* was also reported to harbor rhizome of *Amomum siamense* cultivated in Thailand during dry and wet season [18].

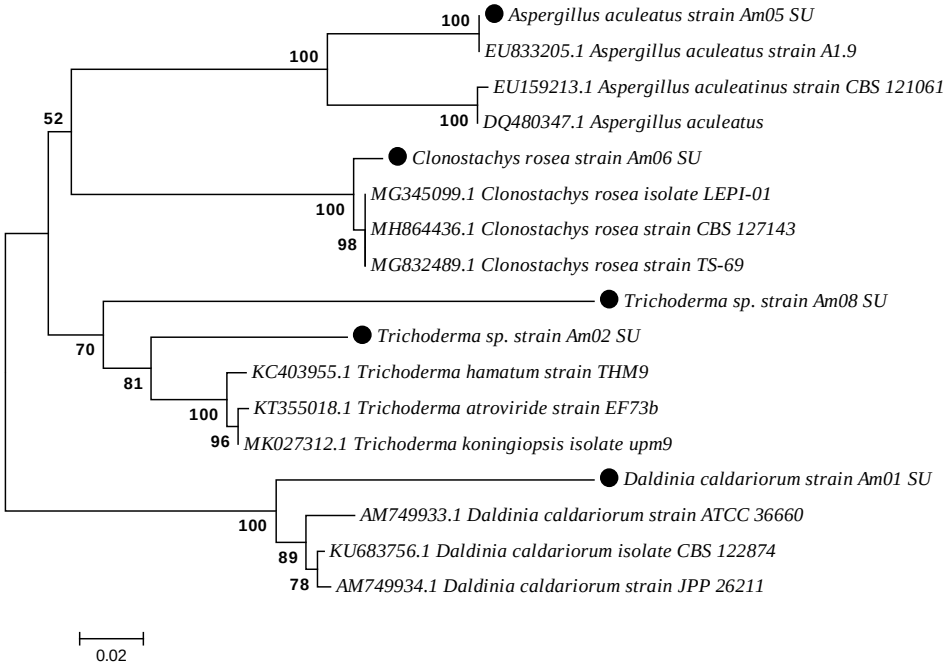

Fig. 4. Phylogenetic relationship among endophytic fungi isolated from *Amomum centrocephalum* A. D. Poulsen rhizome based on the ITS-DNA region compared to databases retrieved from BLASTn. Databases are shown in the form of accession number followed by species names and strains. The neighbor-joining tree (NJ) was constructed using MEGA6.0. Bootstrap value (BV) through 1000 replications supports on the nodes represent ML ≥ 50%. Five newly generated sequences in this study are presented by black-filled dots (•)

IV. CONCLUSIONS

Preliminary investigation upon endophytic fungi residing in the rhizome of *Amomum centrocephalum* has discovered five antagonistic fungal isolates identified as *Aspergillus aculeatus*, *Clonostachys rosea*, *Daldinia caldariorium*, and two *Trichoderma* sp. The potential isolates showed considerable inhibitory activities against gram-positive and gram-negative and phytopathogens. Through literature studies, many prospects and developments are needed in exploring the biological properties from each isolate in future studies. To our understanding, this is the first report on culturable endophytic fungi isolated from the rhizome of *A. centrocephalum* from North Sumatera, Indonesia.

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